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The Stress Physiology of Enteromorpha flexuosa (Wulfen) and Enteromorpha intestinalis (Linnaeus) (Chlorophyta) as an Indicator of Environmental Stress in the Intertidal Zones.

By:

Reema PRAKASH

A thesis submitted as a requirement for the fulfillment of a degree of Master of Science in Biology at the University of the South Pacific.

Division of Biology
School of Biological, Chemical and Environmental Sciences.
Faculty of Science and Technology
University of the South Pacific

2008
DECLARATION

“I, Reema Prakash, Student ID number S99007253, hereby declare that the work presented in this thesis has not been previously submitted for a degree at any university. To the best of my knowledge and belief, this thesis contains material that is original and has not been previously published or written by another author except where due reference is made.”

Reema Prakash

Date

I hereby declare that the work contained in this thesis is the work of Reema Prakash unless stated otherwise.

Dr. Anjeela Jokhan
Senior Lecturer in Plant Physiology
Principal Supervisor

Date
ABSTRACT

*Enteromorpha flexuosa* and *Enteromorpha intestinalis* are filamentous green algae that usually grow attached to surfaces in the intertidal zones. Algae are rapid responders to water quality changes, hence considerable information concerning the environmental condition of an aquatic habitat can be obtained from their physiological analyses. This thesis examined the effects of changes on environmental conditions such as temperature, pH, nitrogen levels and presence of herbicide on the physiology of *E. flexuosa* and *E. intestinalis*. The possibility of the algae’s physiological conditions to act as a bioindicator of the intertidal environment was investigated. The algae were cultured in the artificial seawater medium under laboratory conditions and exposed to variable temperature, pH, nitrogen (ammonium and nitrate) and herbicide levels. The germling growth rates, photosynthetic rates, total chlorophyll content, total soluble proteins and carbohydrates within the algae were then determined. Similar physiological studies were done on *E. flexuosa* and *E. intestinalis* collected from Nasese, Lami and Laucala Beach area around Suva. The results obtained showed that *E. flexuosa* and *E. intestinalis* were affected by the variations in their abiotic environment. Increase in temperature and herbicide levels had an adverse effect on algal growth and survival while increase in pH and high nitrogen levels advanced algal growth and survival. The findings indicate that *Enteromorpha* are quite sensitive to temperature and herbicide. They are a little more tolerant to varying pH. These algae are excellent bio-indicators for high nitrogen levels (especially ammonium) as their presence in the intertidal zone indicates high nitrogen levels.
ACKNOWLEDGMENTS

My sincere thanks to my supervisor, Dr Anjeela Jokhan, for her continuous encouragement and guidance throughout my study.

I also thank Abhineshwar Prasad for helping me setup the aeration system and the basics for the experimentation. I greatly appreciate the technical support and contributions received from the Biology department technical staff including Arvindra Rishi Prasad, Shiva Padiyachi, Dinesh Kumar, Ragni Mudaliar, Babita Narayan, Vere and Amit Singh. I appreciate the help of Vinay Narayan and Amit Sukal for helping me with my sample collection. I also thank the Biology postgraduate students who have helped me during this study. I am indebted to Sunil Prasad and Romika Chandra for their constant help and support and encouragement throughout the writing and editing of the thesis. I greatly appreciate Avinesh Prasad from Mathematics Division, Ravinesh Ram, Mere Tabudravu and Sujlesh Sharma for helping me with the statistical analysis of the results. I am grateful to my husband Rinal Deo and his family for their continuous support and considerable patience during the study.

Finally I would like to acknowledge the NZ Aid and University of the South Pacific for funding this research.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DECLARATION</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS AND SYMBOLS</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xvi</td>
</tr>
<tr>
<td>LIST OF PLATES</td>
<td>xvi</td>
</tr>
<tr>
<td>LIST OF APPENDICES</td>
<td>xvii</td>
</tr>
<tr>
<td>CHAPTER 1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Background to research</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Literature review</td>
<td>4</td>
</tr>
<tr>
<td>1.2.1 Habitat and distribution</td>
<td>5</td>
</tr>
<tr>
<td>1.2.2 Biology and ecology</td>
<td>6</td>
</tr>
<tr>
<td>1.2.3 Life history</td>
<td>6</td>
</tr>
<tr>
<td>1.2.4 Photosynthesis in <em>Enteromorpha</em></td>
<td>9</td>
</tr>
<tr>
<td>1.2.5 Factors affecting the distribution of <em>Enteromorpha</em></td>
<td>10</td>
</tr>
<tr>
<td>1.2.5.1 Temperature</td>
<td>10</td>
</tr>
<tr>
<td>1.2.5.2 pH</td>
<td>11</td>
</tr>
<tr>
<td>1.2.5.2.1 Inorganic carbon sources and uptake in <em>Enteromorpha</em></td>
<td>11</td>
</tr>
<tr>
<td>1.2.5.3 Salinity</td>
<td>14</td>
</tr>
<tr>
<td>1.2.5.4 Nutrients</td>
<td>14</td>
</tr>
<tr>
<td>1.2.5.5 Other factors that limit the species distribution</td>
<td>16</td>
</tr>
<tr>
<td>1.2.6 The importance of these species</td>
<td>18</td>
</tr>
<tr>
<td>1.2.6.1 Economic importance</td>
<td>18</td>
</tr>
<tr>
<td>1.2.6.2 Environmental importance</td>
<td>19</td>
</tr>
<tr>
<td>1.2.7 Physiological response to environmental stresses</td>
<td>21</td>
</tr>
<tr>
<td>1.3 Significance of this thesis</td>
<td>24</td>
</tr>
<tr>
<td>1.4 Objectives</td>
<td>26</td>
</tr>
</tbody>
</table>
CHAPTER 2  METHOD AND MATERIALS

2.1 Algal Sample Collection
2.2 Laboratory experimental methods
2.3 Exposure of algae to abiotic stress conditions
  2.3.1 pH
  2.3.2 Temperature
  2.3.3 Nitrogen
  2.3.4 Herbicide
2.4 Experimental procedure
  2.4.1 Growth Experiment
    2.4.1.1 Seeding (Propagule collection)
    2.4.1.2 Measuring growth rate
  2.4.2 Exposure of adult thalli to stress
2.5 Analysis of stress response
  2.5.1 Photosynthesis
  2.5.2 Chlorophyll content
  2.5.3 Total soluble protein
  2.5.4 Total soluble carbohydrates
  2.5.5 Sporulation
2.6 Statistical analysis of results

CHAPTER 3  EFFECTS OF pH ON Enteromorpha flexuosa AND Enteromorpha intestinalis: RESULTS AND DISCUSSION

3.1 Results
  3.1.1 Growth
  3.1.2 Photosynthesis
  3.1.3 Chlorophyll content
  3.1.4 Total soluble protein
  3.1.5 Total soluble carbohydrates
3.2 Discussion
CHAPTER 4  EFFECTS OF TEMPERATURE ON Enteromorpha flexuosa AND Enteromorpha intestinalis: RESULTS AND DISCUSSION..............................53
4.1 Results..........................................................53
  4.1.1 Growth......................................................53
  4.1.2 Photosynthesis.............................................54
  4.1.3 Chlorophyll content.................................55
  4.1.4 Total soluble protein.............................55
  4.1.5 Total soluble carbohydrates.......................57
4.2 Discussion..........................................................57

CHAPTER 5  EFFECTS OF AMMONIUM (NH₄⁺) AND NITRATE (NO₃⁻) ON Enteromorpha flexuosa AND Enteromorpha intestinalis: RESULTS AND DISCUSSION..........................................................64
5.1 Results illustrating the effects of Ammonium (NH₄⁺)...............64
  5.1.1 Growth......................................................64
  5.1.2 Photosynthesis.............................................64
  5.1.3 Chlorophyll content.................................66
  5.1.4 Total soluble protein.............................66
  5.1.5 Total soluble carbohydrates.......................68
5.2 Results illustrating the effects of Nitrate (NO₃⁻)..................68
  5.2.1 Growth......................................................68
  5.2.2 Photosynthesis.............................................70
  5.2.3 Chlorophyll content.................................70
  5.2.4 Total soluble protein.............................71
  5.2.5 Total soluble carbohydrates.......................71
5.3 Discussion..........................................................72

CHAPTER 6  EFFECTS OF DIURON 80 (HERBICIDE) ON Enteromorpha flexuosa AND Enteromorpha intestinalis: RESULTS AND DISCUSSION.................78
6.1 Results..................................................................78
  6.1.1 Growth......................................................80
  6.1.2 Photosynthesis.............................................80
  6.1.3 Chlorophyll content.......................................82
6.1.4 Total soluble protein................................................................. 82
6.1.5 Total soluble carbohydrates...................................................... 83

6.2 Discussion.................................................................................... 83

CHAPTER 7 ANALYSIS OF Enteromorpha flexuosa AND Enteromorpha intestinalis FROM 3 SITES AROUND THE SUVA AREA (NASESE, LAMI AND LAUCALA BEACH): RESULTS AND DISCUSSION ............................................. 88

7.1 Results.......................................................................................... 88
7.1.1 Photosynthesis......................................................................... 89
7.1.2 Chlorophyll content................................................................. 90
7.1.3 Total Soluble Protein............................................................... 91
7.1.4 Total soluble carbohydrates.................................................... 91

7.2 Discussion...................................................................................... 92

CHAPTER 8 SIGNIFICANCE OF THE RESEARCH, CONCLUSION AND RECOMMENDATIONS ............................................................................. 98

8.1 Research significance and conclusion........................................... 98
8.2 Future work recommendations..................................................... 101

REFERENCES.................................................................................... 102

APPENDIX.......................................................................................... 116
LIST OF ABBREVIATIONS AND SYMBOLS

%  percentage
Abs  absorbance
ADP  adenosine diphosphate
AE  anion exchange mechanism
Ag  silver
AHLs  N-acylhomoserine lactones
aq  aqueous
ASW  artificial seawater
ATP  adenosine triphosphate
C₂OH₃₉COO—  long fatty acid tail
CA  carbonic acid
CaCl₂. 2H₂O  calcium chloride
CC  core complex
CO₂  carbon dioxide
CP47 and CP43  membrane-bound chlorophyll a binding proteins
Cu  copper
Cyt f  cytochrome f
Cytb6  cytochrome b6
D1  polypeptide (PS II-A)
D2  polypeptide (PS II-D)
DIC  dissolved inorganic carbon
DIN  dissolved inorganic nitrogen
DMS  Dimethylsulfide
DMSP  dimethylsulfoniopropionate
DON  dissolved organic nitrogen
dwt  dry weight
e.g.  example
et. al  and others
Fe  iron
FeS  iron-sulphur
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl</td>
<td>ammonium chloride</td>
</tr>
<tr>
<td>Ni</td>
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<tr>
<td>NO₂⁻</td>
<td>nitrite ion</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>nitrate ion</td>
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<tr>
<td>O</td>
<td>oxygen</td>
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<tr>
<td>O₂</td>
<td>oxygen</td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<tr>
<td>OEC</td>
<td>oxygen evolving complex</td>
</tr>
<tr>
<td>OH⁻</td>
<td>hydroxide</td>
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<td>Pb</td>
<td>lead</td>
</tr>
<tr>
<td>PC</td>
<td>plastocyanin</td>
</tr>
<tr>
<td>pH</td>
<td>power of hydrogen</td>
</tr>
<tr>
<td>PQ</td>
<td>plastoquinone</td>
</tr>
<tr>
<td>PS I</td>
<td>photosystem one</td>
</tr>
<tr>
<td>PS II</td>
<td>photosystem two</td>
</tr>
<tr>
<td>QA</td>
<td>quinone</td>
</tr>
<tr>
<td>RC</td>
<td>reaction centre</td>
</tr>
<tr>
<td>RUBISCO</td>
<td>ribulose 1,5 biphosphate oxygenase</td>
</tr>
<tr>
<td>RuBP</td>
<td>ribulose biphosphate</td>
</tr>
<tr>
<td>S</td>
<td>sulphur</td>
</tr>
<tr>
<td>spp</td>
<td>species</td>
</tr>
<tr>
<td>TCA</td>
<td>tricarboxylic acid</td>
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<td>TSC</td>
<td>total soluble carbohydrates</td>
</tr>
<tr>
<td>TSP</td>
<td>total soluble proteins</td>
</tr>
<tr>
<td>Zn</td>
<td>zinc</td>
</tr>
<tr>
<td>µg</td>
<td>micrograms</td>
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<tr>
<td>µg/ml</td>
<td>micrograms per milliliter</td>
</tr>
<tr>
<td>µl</td>
<td>microlitre</td>
</tr>
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<td>µm</td>
<td>micrometres</td>
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<td>µmoles</td>
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<tr>
<td>µmoles/L</td>
<td>micromoles per litre</td>
</tr>
<tr>
<td>µmoles/min/ g fwt</td>
<td>micromoles per minute per gram fresh weight</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Percentage distributions of different forms of inorganic carbon in seawater as a function of pH in three different salinities (Source: Lobban and Harrison, 1994)</td>
<td>11</td>
</tr>
<tr>
<td>1.2</td>
<td>Diagram showing two concurrent mechanisms of bicarbonate use by the macroalgae <em>Ulva lactua</em> (Axelsson <em>et al.</em>, 1999)</td>
<td>13</td>
</tr>
<tr>
<td>1.3</td>
<td>Schematic representation of the nitrogen cycle of the sea. PON- particulate organic nitrogen, DON- dissolved organic nitrogen, DIN- dissolved inorganic nitrogen. (Turpin, 1980)</td>
<td>15</td>
</tr>
<tr>
<td>3.1</td>
<td>The growth rates of <em>E. flexuosa</em> and <em>E. intestinalis</em> (together) at different levels of pH in Artificial Seawater Medium</td>
<td>43</td>
</tr>
<tr>
<td>3.2</td>
<td>Photosynthesis rates of <em>E. flexuosa</em> and <em>E. intestinalis</em> at different pH levels over 10 days</td>
<td>43</td>
</tr>
<tr>
<td>3.3</td>
<td>The total chlorophyll content in <em>E. flexuosa</em> and <em>E. intestinalis</em> at different pH levels in Artificial Seawater Medium</td>
<td>44</td>
</tr>
</tbody>
</table>
Figure 3.4  The amount of total soluble protein in *E. flexuosa* and *E. intestinalis* after treatment at different levels of pH in Artificial Seawater Medium………………..45

Figure 3.5  The amount of total soluble carbohydrates in *E. flexuosa* and *E. intestinalis* after treatment at different levels of pH in Artificial Seawater Medium………………..45

Figure 3.6  The effects of pH on percentage of H$_2$CO$_3$, HCO$_3^-$, and CO$_3^{2-}$ to total dissolved inorganic carbon (DIC)…………………………………..……………………..47

Figure 3.7  The carbonate equilibrium. (Lobban and Harrison, 1994)……………………..48

Figure 4.1  The growth rates of *E. flexuosa* and *E. intestinalis* (together) at different temperatures in Artificial Seawater Medium ………………………………………53

Figure 4.2  Photosynthesis rates of *E. flexuosa* and *E. intestinalis* at different temperatures over 10 days……………………………………………………………..54

Figure 4.3  The total chlorophyll content in *E. flexuosa* and *E. intestinalis* at different temperatures in Artificial Seawater Medium …………………………………….55

Figure 4.4  The amount of total soluble protein in *E. flexuosa* and *E. intestinalis* after treatment at different temperatures in Artificial Seawater Medium ………….56
Figure 4.5 The amount of total soluble carbohydrates in *E. flexuosa* and *E. intestinalis* at different temperatures in Artificial Seawater Medium ........................................56

Figure 5.1 The growth rates of *E. flexuosa* and *E. intestinalis* (together) at different levels of NH$_4^+$ in Artificial Seawater Medium.................................................................65

Figure 5.2 Photosynthesis rates of *E. flexuosa* and *E. intestinalis* at different NH$_4^+$ levels over 10 days........................................................................................................65

Figure 5.3 The total chlorophyll content in *E. flexuosa* and *E. intestinalis* at different levels of NH$_4^+$ in Artificial Seawater Medium.................................................................66

Figure 5.4 The amount of total soluble protein in *E. flexuosa* and *E. intestinalis* after the treatment at different concentrations of NH$_4^+$ in Artificial Seawater Medium........................................................................................................67

Figure 5.5 The amount of total soluble carbohydrates *E. flexuosa* and *E. intestinalis* after the treatment at different concentrations of NH$_4^+$ in Artificial Seawater Medium........................................................................................................67

Figure 5.6 The growth rates of *E. flexuosa* and *E. intestinalis* (together) at different levels of NO$_3^-$ in Artificial Seawater Medium .................................................................69
Figure 5.7  Photosynthesis rates of *E. flexuosa* and *E. intestinalis* at different NO₃⁻ levels over 10 days.................................................................69

Figure 5.8  The total chlorophyll content in *E. flexuosa* and *E. intestinalis* at different levels of NO₃⁻ in Artificial Seawater Medium.........................................................70

Figure 5.9  The amount of total soluble protein in *E. flexuosa* and *E. intestinalis* after the treatment at different concentrations of NO₃⁻ in Artificial Seawater Medium...........................................................................71

Figure 5.10  The amount of total soluble carbohydrates in *E. flexuosa* and *E. intestinalis* after the treatment at different concentrations of NO₃⁻ in Artificial Seawater Medium...........................................................................72

Figure 5.11  Showing main features of nitrogen uptake and assimilation in a eukaryotic algal cell.  (Lobban and Harrison, 1994).................................................................74

Figure 6.1  The growth rates of *E. flexuosa* and *E. intestinalis* (together) at different concentrations of Diuron 80 in Artificial Seawater Medium .........................79

Figure 6.2  Photosynthesis rates of *E. flexuosa* and *E. intestinalis* at different concentrations of Diuron 80 over 10 days.................................................................79
Figure 6.3  The total chlorophyll content in *E. flexuosa* and *E. intestinalis* at different concentrations of Diuron 80 in Artificial Seawater Medium…………………………81

Figure 6.4  The total soluble protein content in *E. flexuosa* and *E. intestinalis* after the treatment at different concentrations of Diuron 80 in Artificial Seawater Medium…………………………………………………………...81

Figure 6.5  The total soluble carbohydrates content in *E. flexuosa* and *E. intestinalis* after treatment at different concentrations of Diuron 80 in Artificial Seawater Medium…………………………………………………………………………83

Figure 7.1  The photosynthesis rates of *E. flexuosa* and *E. intestinalis* (together) from Nasese, Lami and Laucala…………………………………………………………………………………89

Figure 7.2  The total chlorophyll content in *E. flexuosa* and *E. intestinalis* from Nasese, Lami and Laucala Beach area measured randomly over a 12 month period…………………………………………………………………………………………..90

Figure 7.3  The total soluble protein content in *E. flexuosa* and *E. intestinalis* from Nasese, Lami and Laucala Beach area measured randomly over a 12 month period…………………………………………………………………………………………..91
Figure 8.4  The total soluble carbohydrate content in *E. flexuosa* and *E. intestinalis* from Nasese, Lami and Laucala Beach area measured randomly over a 12 month period.................................................................92

**LIST OF TABLES**

Table 7.1 Averages of temperature, pH, NH$_4^+$ and NO$_3^-$ in Lami, Laucala Beach and Nasese area measured randomly.................................................................88

**LIST OF PLATES**

Plate 1.1  A coastal habitat showing extensive growth of *Enteromorpha* which includes *E. intestinalis* and *E. flexuosa*.................................................................2

Plate 2.1 Locations of the Nasese, Lami and Laucala Beach sampling sites.................31

Plate 2.2  The growth experimental setup tiles containing the germlings placed in Artificial Seawater Medium in the beaker.........................................................36

Plate 2.3  Adult algae exposed to stress conditions (in a similar setup as the growth experiment) (Plate 2.1).................................................................37

Plate 2.4  Adult algae in beaker with Artificial Seawater Medium with spores stuck on the sides of the beaker (which is evidence for sporulation).................................41
# LIST OF APPENDICES

<table>
<thead>
<tr>
<th>Appendix</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1.1</td>
<td>Preparation of Artificial Seawater Medium (Brand, 1984)</td>
<td>116</td>
</tr>
<tr>
<td>Table A 1.1</td>
<td>Functions and compounds of the essential elements in seaweeds</td>
<td>117</td>
</tr>
<tr>
<td>Table A 1.2</td>
<td>Concentrations of some essential elements in seawater and in seaweeds</td>
<td>118</td>
</tr>
<tr>
<td>Plate A 1.1</td>
<td><em>Enteromorpha flexuosa</em> and <em>Enteromorpha intestinalis</em> associations growing near a drainage outfall at Nasese, Suva</td>
<td>119</td>
</tr>
<tr>
<td>Plate A 1.2</td>
<td>Adult algae exposed to stress in a beaker with Artificial Seawater Medium. (a) Healthy algae with sporulation (spores stuck and germinating on the sides of the beaker). (b) Unhealthy algae with no sporulation (no spores on the sides of the beaker)</td>
<td>119</td>
</tr>
</tbody>
</table>
CHAPTER 1
INTRODUCTION

1.2 Background to Research

Enteromorpha are filamentous green alga that belong to the phylum Chlorophyta and class Ulvophyceae. They usually grow attached to surfaces in the intertidal zones (Plate 1.1). Species within the genus Enteromorpha are very difficult to identify, as differences between species are small and hard to spot with naked eyes. They are bright green seaweeds, with tubular and elongate fronds that may be branched, flattened or inflated. Occasionally they appear bleached (white in colour). They are attached to the substrate with their minute disc-like holdfasts. The fronds of species from this genus may vary in appearance due to changes in environmental conditions, which sometimes confuses their identification, and microscopic cell details are often required to identify certain species (Graham and Wilcox, 2000). The two species used in this research are Enteromorpha flexuosa and Enteromorpha intestinalis.

Enteromorpha flexuosa is a long (usually up to 150 mm), filamentous, light green algae. However, it can grow up to 200 - 300 mm in length (Hill, 2001). Its thallus consists of hollow tubes that are made up of walls one cell thick and axes 1 - 7 mm wide (N’Yeurt, 2001). The cells of this species are angular or sub angular in shape, up to 25 x 35 μm in size and are arranged in distinct longitudinal rows, where each cell usually has 3 pyrenoids (N’Yeurt, 2001). E. flexuosa is commonly known as the hollow weed.

Enteromorpha intestinalis, similarly, is light green in colour and consists of tubular thallus. Its thallus is coarse and twisted; filaments are irregularly and radially branched near its
narrow holdfast. The cells of this species are angular to sub rectangular 7 - 20 μm x 7 - 13 μm and are arranged in an irregularly disposed manner with cells having 2, rarely 3 pyrenoids per cell (N’Yeurt, 2001). A common name for *E. intestinalis* is gut weed.

Plate 1.1 A coastal habitat (in Nasese, Viti Levu) showing extensive growth of *Enteromorpha* which includes *E. intestinalis* and *E. flexuosa*.

*Enteromorpha* species in general are found at all levels of the shore and usually they are found in large quantities in areas where fresh-water runoffs occur. They are often referred to as fouling algae as they develop abundantly in coastal zones affected by pollution from municipal or industrial discharge (Hill, 2001). They can also occur as ‘green tides’ in the form of accumulations of unattached green algae in most polluted and eutrophicated marine environments.
Algae respond rapidly to water quality changes hence considerable information concerning the environmental condition of an aquatic habitat can be obtained from their analysis (Lewis and Wang, 1997). Abiotic environmental stresses (such as variations in temperature, salinity, pH and nutrient availability) limit algal distribution and abundance. Because of their trophic level and rapid growth, algae are rapid responders to environmental stresses within an ecosystem. A variety of physiological, morphological and community parameters of algae can be used to monitor the health of an ecosystem (Lewis and Wang, 1997). Decrease or increase in the diversity of algae that indicates the environmental quality of a system has been mostly studied at the community level. This is done on a structural basis that includes measurement of algal biomass and non-taxonomic measures for example the presence, abundance or absence of a population or species composition (Lewis and Wang, 1997). In Fiji, Tabudravu, (1998) and Tabudravu et. al, (2002) carried out chemical analysis of E. flexuosa and confirmed the algae to be an excellent bioindicator for heavy metals.

Investigating physiological parameters which include functional measures (such as the rates of productivity, photosynthesis, nutrient flux and cellular activity) require a large amount of time and work hence indications of environmental stresses are not preferably studied at physiological levels (Carins and McCormick, 1997). However, physiological parameters and processes provide an accurate account of environmental stress response since the physiological responses of most species reveal the mechanisms by which the organisms adjust to cope with the environment. Once these mechanisms and processes are understood, the information can be applied towards understanding the distribution and abundance of a species in an area (Nilsen and Orcutt, 1996).
1.2 Literature review

This review is about the biology, ecology and physiology of the *Enteromorpha* species, factors that affect its distribution in general together with its abiotic environmental interactions. *E. flexuosa* and *E. intestinalis* are believed to be highly adapted to grow in the intertidal zones and coastal areas especially where there is pollution. The algae are highly associated with macroalgal blooms since they possess life-history characteristics that enhance their response to the increased nutrient supplies. In addition the algae are very good bio-accumulators which enables them to accumulate certain pollutants such as heavy metals (Tabudravu *et. al*, 2002). Hence the species of these genera have excellent qualities to act as bio-indicators of coastal environmental quality. In Fiji they are commonly found along the coastal areas adjacent to settlements and estuaries.

*Enteromorpha* are included in the order Ulvales and family Ulvaceae (Graham and Wilcox, 2000). When this alga was discovered it was initially included in the genera called *Ulva*. The genus *Ulva* in general included a variety of unrelated algae and was first named by Linnaeus (1753) cited in Hayden *et. al*, (2003). Later in the nineteenth century, the algal members of this genus were split into several genera. The genus *Ulva* contained only the seaweeds with distromatic blades, and the tubular forms of green algae were placed in new genera called *Enteromorpha* (Hayden *et. al*, 2003). Both genera remained closely related. The term *Enteromorpha* literally means ‘intestine shape’ which describes the tube like filaments of these species. Generally *Enteromorpha* thalli consist of elongated tubes, sometimes with constrictions that are attached to substrate by rhizoidal branches that form an attachment disk. Some species exist as an intermediate form of the two genera, for example *Enteromorpha linza* that has *Enteromorpha flexuosa* like tubular base, and distromatic blades...
like *Ulva*. Several studies carried out on the two genera report close similarities (Hayden *et. al.*, 2003). *Ulva* and *Enteromorpha* were not two separate genera according to the molecular data obtained by Hayden *et. al.*, (2003) and therefore *Enteromorpha* could be referred to as *Ulva* and vice-versa.

### 1.2.1 Habitat and Distribution

Both species *E. flexuosa* and *E. intestinalis* have a worldwide distribution in shallow brackish or marine habitats (Hill, 2001; Graham and Wilson, 2000). The species are generally found at all zones of the shore, ranging from high intertidal zone up to 5 m below the surface of the seawater (Hill, 2001). Both species grow in clusters on suitable substrates such as rocks, mud, sand, mangrove roots and wood. They are also common epiphytes on other algae, shells and organisms. For example, they commonly grow on limpet shells. This becomes an ideal place for them to grow since limpets cannot turn around and graze them (Thomas, 2002). They are excellent pioneer species, that is, they are able to colonize newly available substrata at all times during the year (Hill, 2001). They are usually referred to as an opportunistic species, they are very successful under the right conditions. However, both species are considered invasive and problem seaweeds as they periodically densely cover the mudflats and clog up sheltered bays and shallow waters. They are often washed up on shores where they lay rotting and produce foul odor (Thomas, 2002). They are also members of the group of algae that cause harmful green tides in marine coastal areas. The green macro-algal biomasses occur excessively (up to 27 kg wet mass/ m$^2$) during green tides that drift in shallow water. *E. intestinalis* has been identified as the major species in most of the ‘green tide’ studies, where it has been observed to be occurring as unattached masses (Back *et. al.*, 2000; Nelson *et. al.*, 2003; Blomster *et. al.*, 2002).
1.2.2 Biology and Ecology

Both algae, like any other Enteromorpha species, are bleached and decay at the end of the season (Budd and Pizzola, 2002). They are fast growing species under suitable conditions. Maximum growth and increase in biomass of E. flexuosa was recorded at depths less than 400 mm and it can grow up to 200 – 300 mm long if left ungrazed by fish and invertebrates (Hill, 2001). Similar conditions have been reported for E. intestinalis. Its maximum habitual growth is up to 300 – 400 mm but it can grow up to 1 m in length at a speed of 1.5 – 2.5 mm/ day if left ungrazed under optimum conditions (Budd and Pizzola, 2002). Both species are opportunistic; they have an r-type strategy for survival thus posses high growth rate and high reproductive rates (Budd and Pizzola, 2002). Both species can continue growing in floating masses at the water surface if they become detached from the substratum (Fish and Fish, 1989), since they can reproduce very quickly through both asexual and sexual reproduction.

1.2.3 Life history

Asexual reproduction of Enteromorpha is by vegetative propagation (by transversal scission) and the production of zoospores. Sexual reproduction involves the production of gametes by the gametophytes, which join together to produce a sporophyte. Enteromorpha is dioecious, that is, having separate sexes, it can be isogamous (reproduction involving gametes of identical size and shape) or anisogamous (reproduction involving gametes (reproduction with gametes that are not identical in size and shape). However, it is difficult to differentiate male or female gametophytes because of their similar morphology. Any of the cells in a frond however, is capable of producing gametes Hayden et. al., (2003). The high reproductive potential of Enteromorpha is due to the release of many thousands of motile spores (propagules or zoospores), which contribute to its high ecological success.
Propagule release by means of both meiotic gametes and mitotic spores occur on a daily basis in lower altitudes (Hill, 2001). Tidal and lunar rhythms generally drive the release of propagules (Budd and Pizzola, 2002). Maximum release of gametes occurs a few days prior to the highest tide in a lunar cycle (Graham and Wilcox, 2000). The zoospores first sense the surface they attach on, they then adhere on to that surface on a temporary basis. If the conditions during the temporary attachment are not favourable, the propagules get detached and continue to find other suitable attachment sites (Patel et. al., 2003). Once a favourable site is detected, it releases a glycoprotein adhesive, which attaches it permanently to the substrate (Patel et. al., 2003; Callow and Callow 2002; Joint et. al., 2002). These permanently attached propagules then grow and differentiate into new plants. The settling of zoospores however, is affected by factors such as negative phototaxis, thigmotaxis, chemotaxis (Callow and Callow, 2000), surface chemistry and wettability (Callow et. al., 2000) and surface topography (Callow et. al., 2002). These factors contribute in finding a suitable surface for propagule settling. A very important factor that contributes towards zoospore settling is the presence of bacterial biofilms (Patel et. al., 2003). Bacterial biofilms are generally present on all submerged surfaces in the marine environment; they change the surface properties and produce signals that attract the zoospores to those surfaces (Patel et. al., 2003). The presence of biofilms enhances the settlement of Enteromorpha zoospores (Dillion et. al., 1989). Joint et. al. (2002), observed the positive co-association between the bacterial biofilms and Enteromorpha zoospore number on glass slides in natural seawater. In addition, Joint et. al. (2002) also found that the zoospores attached to only certain bacteria present in the biofilm. Hence they suggested that this attachment was due to the zoospores’ ability to detect the chemical signal emitted by the bacteria that involves the process of cell-to-cell signalling across the bacterial cell and the algal cell. The chemical signal molecules responsible are N-
acylhomoserine lactones (AHLs), which are involved in cell signalling in Gram-negative bacteria (Joint et. al., 2002). Zoospores were found not being attracted to bacterial biofilm in which the AHLs were destroyed (Joint et. al., 2002). Patel et. al. (2003) obtained a total of 99 bacterial isolates from the biofilms of rocks and Enteromorpha, where all isolates were found to be Gram-negative and rod shaped. Some genera include Pseudoalteromonas, Vibrio, Shewanella, Halomonas and Pseudomonas.

In addition, the propagules of these algae along with other marine algae can form banks of dormant propagules similar to dormancy in higher land plants during unfavourable conditions. They can remain dormant for a few months until the environmental conditions become favourable (Worm et. al., 2001). The propagule banks of Enteromorpha are common in winter seasons. The propagules and germlings however, are delicate in structure and are vulnerable to physical and biological stresses since they lack the protection or resistance mechanisms as in adults. In addition, propagule banks are exposed to some biotic interactions such as herbivore grazing and competition. They respond to changes in the environment in different ways when compared with the adults (Lotze et. al., 2001). Herbivory and competition with other algal species have been considered as a means of controlling the algal boom especially where Enteromorpha is involved. Herbivore grazing naturally decreases blooms but increase in nutrients in the water can overcome this control mechanism (Lotze et. al., 2000).

The propagules released have the ability to photosynthesize as soon as they are released into the water (Budd and Pizzola, 2002; Beach et. al., 1995; Hill, 2001). Amsler and Searles (1980) confirmed that the propagules of Enteromorpha along with other pioneer genera
*Blidirlia* and *Ulothrix* can stay long enough in the planktonic phase to reach a distance of about 10 kilometres. The photosynthetic rate for gametes and zoospores of *E. flexuosa* including *E. intestinalis* which fall under pioneering species are significantly higher when compared with the reproductive cells of the latter successional genera. *Enteromorpha* was recorded to have the highest net photosynthesis rate of 11 mg/day/g dwt/hr (Luning, 1990). The species have increased ecological success in settlement and mobilization and this is largely due to their highly motile characteristics. Their ecological success is also greatly enhanced due to gas filled thallus, which keeps the algae in an upright position, optimising photosynthesis (Luning, 1990).

### 1.2.4 Photosynthesis in *Enteromorpha*

Photosynthesis in marine algae is similar to that in higher plants. Some factors however, make photosynthesis a little different from higher plants. These factors include their habitat (aquatic environment), diversity of photosynthetic pigments in marine algae, diversity of climates in the oceans, the type of carbon supply in the habitat and in the diversity of photosynthetic products in different types of algae (Lobban and Harrison, 1994).

Photosynthesis generally (both in algae and higher plants) involves two major groups of reactions involving capturing of light energy, and chemical reactions involving conversion of light energy to chemical energy in the form of ATP and NADPH. Light dependent reactions involve the trapping of light and the conversion of photon energy into chemical energy via an electron transport chain. The electron transport chain is powered by reducing equivalents extracted from water. During the light reaction, the excited electrons from the chlorophyll in the reaction centre enter the electron transport chain. These electrons arise from the splitting
of water molecules, which produces oxygen molecules and hydrogen ions. Electrons from this reaction are used together with the hydrogen ions to produce NADPH from NADP⁺ while some hydrogen ions are used to produce ATP from ADP (Salisbury and Ross, 1992). The NAPDH and ATP from the light reactions are used in a second group of carbon fixation reactions (Calvin Cycle or the light-independent reactions) for the reduction and fixing of carbon dioxide (CO₂) to produce sugars and starch.

1.2.5 Factors affecting the distribution of Enteromorpha

Spatial and temporal distribution of these species is limited by factors such as temperature, pH, salinity and nutrient availability.

1.2.5.1 Temperature

The ability of various seaweeds to tolerate the water temperature up to a certain level is partly responsible for the patterns of geographic distribution of adult plants. Enteromorpha species can tolerate temperatures up to 30°C (Graham and Wilcox, 2000). My preliminary field studies done in 2003 in Nasese, Suva area showed that both species were successfully growing at a pH of 7 and at an average temperature of 28°C. Hill (2001) noted a favorable growth of E. flexuosa at temperatures ranging from 15°C - 30°C and adverse effects on growth together with bleaching at temperatures of 33°C and above. He also reported E. flexuosa to have optimum reproduction in waters with temperature under 30°C and a pH of 8.2 (Hill, 2001). The species’ temperature tolerance up to 30°C is an extra advantage as it may help benefit indirectly through the removal of other competing algae at 27 - 30°C (Budd and Pizzola, 2002).
1.2.5.2 pH

pH of seawater generally is around 8.1 but can range from 7.5 - 8.5 (Lobban and Harrison, 1994). These algae prefer high pH since they use inorganic carbon (Ci) as their major source of carbon for photosynthesis similar to all other algae. The relative forms of inorganic carbon are dependent on the pH and the salinity of the seawater (Fig 1.1).

Figure 1.1 Percentage distributions of different forms of inorganic carbon in seawater as a function of pH in three different salinities (source: Lobban and Harrison, 1994).

1.2.5.2.1 Inorganic carbon sources and uptake in Enteromorpha

Algae of the genus Enteromorpha and Ulva possess limited ability to take up and use organic carbon sources such as glucose, acetate and leucine together with inorganic carbon if they are available (Mrakager and Sand-Jenson, 1990).

Inorganic carbon properties in seawater are different when compared to that in the air or fresh water, since the pH and salinity are high. Carbon dioxide concentration in seawater is
basically the same as in the air but its diffusion into plants in water is about 104 times slower than in air (Lobban and Harrison, 1994). In seawater, CO$_2$ is a component of the carbonate buffer system and inorganic carbon is available as bicarbonate (HCO$_3^-$). In seawater of pH 8 and 35% salinity, about 90% of the inorganic carbon occurs as HCO$_3^-$ (Lobban and Harrison, 1994), which is about 200 times higher than the CO$_2$ concentration in seawater (Sand-Jensen and Gordon, 1984).

Green algae can carry out photosynthesis at high pH using HCO$_3^-$ since they have an inducible mechanism to do so (Carlberg et. al., 1990). Bojork et. al. (1993) described two principle ways (Fig 1.2) in which green algae utilize HCO$_3^-$; one way is when the surface bound carbonic anhydrase (CA) dehydrates HCO$_3^-$ extracellularly to form CO$_2$ that is then taken up by the cells. The other way is when HCO$_3^-$ is taken up directly for example in the Ulva spp (Giordano and Maberly, 1989; Drechsler and Beer, 1991). In addition, Drechsler et. al. (1993 and 1994) found that a mechanism similar to the anion exchanger (AE) of the red blood cells could allow uptake of HCO$_3^-$ in Ulva spp.
In both cases, the enzyme carbonic anhydrase (CA) greatly speeds up the equilibrium between HCO$_3^-$ and CO$_2$ (Lobban and Harrison, 1994). In algae, the enzyme can be located extracellularly, in the cytoplasm and or possibly in the chloroplast (Lobban and Harrison, 1994).

In addition both *Enteromorpha* spp. and the *Ulva* spp., like other intertidal seaweeds, can carry out photosynthesis almost at equal rates either when emerged or submerged in water (Kremer and Schmitz, 1973 cited in Lobban and Harrison, 1994). This means that they have the ability to take up CO$_2$ directly as well. This is because the bicarbonate that remains in the capillary layer (diffusion boundary layer) of the water remaining on the plant surface is quickly exhausted (Kerby and Raven, 1985 cited in Lobban and Harrison, 1994).
1.2.5.3 Salinity

Like temperature and pH, there are optimum salinities for the metabolic processes and growth for algae. *Enteromorpha* grows well at normal salinity. Some species can even occur at hypersaline conditions (Graham and Wilcox, 2000). *Enteromorpha flexuosa* however, grows well in eurysaline conditions (Hill, 2001). However, both species are found to be widely tolerant of salinity, Irene *et. al.* (1998) showed that *E. intestinalis* growth was highly affected by varying salinity and its abundance decreased at low salinity.

1.2.5.4 Nutrients

About 21 elements are required for the main metabolic processes in plants (see appendix for Table A 1.1). In seaweeds, more than double that number are present. However, the presence and the amount of an element in seaweed does not mean that the element is an essential one or is relatively important (Lobban and Harrison, 1994). Vital elements are usually accumulated in algal tissues in amounts above their concentrations in seawater resulting in concentration factors of up to $10^3$ (Phillips, 1991 cited in Lobban and Harrison, 1994).

The nutrient elements necessary for macroalgal growth include nitrate, ammonium and phosphate. Nitrate, nitrite, ammonium and phosphate concentrations vary from 0 to 30, 1, 3 and 2 μM respectively for most of the pristine temperate areas (Lobban and Harrison, 1994). Nitrogen is the limiting nutrient for algal growth in the sea. The important features of nitrogen cycle and the processes, which bring nitrogen into the sea, are illustrated in Fig 1.3.
Nitrogen, in particular, greatly increases the growth of *E. intestinalis* and *Ulva expansa* (Kamer and Fong, 2001). Kamer et al. (2002) showed that *E. intestinalis* and *Ulva expansa* were growing excessively in the upper New Port Bay, California and had very little or no affinity for phosphorous while it had high affinity for nitrogen. Nutrient rich water enhanced the growth of both species. The genera, as a whole has always been associated with the algal bloom caused by nutrient-rich water. *Enteromorpha* can also successfully grow in low nutrient water. The species can acquire the dissolved nutrients from numerous sources such as the water column or estuarine sediments that have dissolved nutrient flux from pore waters (Kamer et al., 2002). Dissolved nutrients regulate the biomass of these algae and this makes the species a useful and a very good indicator of the nutrient enrichment. Excessive growth of *E. intestinalis* is quite common near sewage outfalls. Cohen, (2002) found that when ammonium and nitrate were present in equal amounts, *E. intestinalis* preferred to uptake (transport across the plasmalemma) and assimilate (sequence of reactions in which inorganic
ions are incorporated into organic cellular components) ammonium. In addition, Cohen, (2002) determined that *E. intestinalis* was able to take up and store nutrients for future growth even in times of environmental stresses due to short-term reduction in salinity and light availability. He found that tissue nutrients increased with increased nutrient supply regardless of variations in salinity or light availability.

1.1.5.5 Other factors that limit the species distribution

Other factors that limit the species distribution include substrate loss, smothering, sediment suspension, desiccation, water flow rate, wave exposure, and contamination (Budd and Pizzola, 2002). The algae are intolerant to substrate loss, as they require it for development even though they may continue to grow in mats once they become displaced from the substrate. They are highly vulnerable to smothering due to increased suspended sediments in water. Smothering reduces light and hence may interfere with the photosynthesis and within a month the algae would rot. In shore areas where the water current is reduced, sediments are likely to increase and the spores, germlings and juveniles are highly intolerant of smothering which leads to high mortality. However, if returned to earlier conditions the species is expected to recolonise the available substrate.

Desiccation stress is another primary factor that controls the distribution of these algae on high shore areas such as areas above the tidal limits of the shore. The species can live for several weeks in completely dried pools while becoming entirely bleached on the uppermost areas but remaining moist below the bleached fronds. *Enteromorpha intestinalis* has a unique ability to survive without water for long, making it an ideal shelter for copepods in supralittoral rock pools (Budd and Pizzola, 2002).
The water flow rate affects the algae to some extent since the algae have no structural support in the thalli. The fronds conform to the direction of water flow and increased flows can cause tearing of fronds and dislodgement of the holdfast. Yet the recovery of the species is high since flow rate does not affect the settlement of the propagules. Houghton et. al. (1973) observed that the propagules of *Ulva* were able to settle on surfaces exposed up to 10.7 knots of water flow speed. The species has different ranges of tolerance to different chemical pollution.

When considering exposure to herbicide pollution, *E. intestinalis* is intolerant to synthetic compound pollution at an intermediate level (Budd and Pizzola, 2002). Herbicides are not directly used in the marine environment but may enter the estuary areas through river discharge and runoffs from the terrestrial environment. Moss and Woodhead, (1975) investigated the effects of two commercial herbicides (Paraquat and 3AT) on settlement, germination and growth of *Enteromorpha*. They observed that zygotes germinated into filaments in the presence of Paraquat at 7 mg/l but stopped growing when the level was increased. They also observed that the zygotes were highly resistant when they settled in clumps on the substratum. The adult thalli however, were more vulnerable to the herbicides than the un-germinated zygotes. Some synthetic chemicals can be directly introduced into the marine environment such as chemicals that are used as the antifouling agents. Scarlett et. al. (1997) studied the occurrence of the marine antifouling agent s-triazine herbicide Irgarol 1051 (an ingredient of antifouling paints used on boats and ships) within the Plymouth Sound area in UK and its effect on the *E. intestinalis*. The authors found out that the highest concentration of Irgarol 1050 occurred in areas close to where there were high numbers of boats, and the highest concentration detected (120 ng/ l) caused a significant decrease in the growth of *E.*
*intestinalis* under laboratory conditions. However, smaller concentrations like 22 ng/l had no effect on the species. Higher concentrations also inhibited photosynthetic efficiency.

Heavy metal toxicity in algae varies with different species but normally the order of toxicity is Hg>Cu>Ag>Pb>Zn (Rice *et. al*, 1973 and Rai *et. al*, 1981). These species have been studied mostly to find out the effect of copper since copper is used in antifouling paints (Budd and Pizzola, 2002).

1.2.6 The importance of these species

1.2.6.1 Economic importance

Generally *Enteromorpha* algae are consumed as food and other uses include animal feed, fertilizers, industrial applications, medicines, food processing agents and manufacturing of personal care products (McHugh, 2003). A great variety of essential amino acids have been found to be present in *E. intestinalis* (Haroon *et. al*, 2000). In addition, it was found that the vitamin-B group content was higher in *E. intestinalis* than most vegetables and it was also a good source of vitamin A (McHugh, 2003).

Methanisation tests showed that *Enteromorpha* had a good capacity for fermentation (Haroon *et. al* 2000). Research has also been carried out using *Enteromorpha* species to generate methane through anaerobic digestion (Haroon *et. al.*, 2000). In addition, *Enteromorpha* have also been used as a source of bioactive compounds similar to those that inhibit the bacterium *Xanthomonas oryzae* which causes leaf blight disease in paddy (Manimala and Rengasamy, 1993 cited in Haroon *et. al.*, 2000). *Enteromorpha flexuosa* was found to have antibacterial activity against *Mycobacterium tuberculosis* (Hill, 2001).
1.2.6.2 Environmental importance

Like any other seaweed in the marine ecosystem, these species also acts as a food source and shelter for several organisms in the ecosystem. They also produce oxygen and fix carbon in the water. They are particularly important as a food source for some ‘grazing’ organisms such as *Littorina* snails (Fish and Fish, 1989). In addition, they are important in holding of sediments in environments they exist in.

*Enteromorpha* are often referred to as the fouling algae as they develop abundantly in zones directly affected by pollution discharges (Hill, 2001). In most pollution affected areas and eutrophicated marine environments, they occur as ‘green tides’ in the form of accumulations of unattached green algae.

Since these species can bio-accumulate, they have proved to be suitable bio-indicators (Tabudravu, 1998; Philips and Segar, 1986; Hill, 2001). In fact the *Enteromorpha* genus is used as heavy metal indicators in waters world-wide (Ratkevicius *et. al.*, 2003). Tabudravu (1998) and Tabudravu *et. al.* (2002) specifically studied the potential of *E. flexuosa* for monitoring heavy metals such as copper, zinc, and lead concentration in the coastal and estuary area and they revealed a perfect correlation between the heavy metal content in water and the algae. In 2001, Vallares *et. al.* studied *Ulva* and *Enteromorpha* species as the indicators of heavy metal contamination in the north coast of Spain. They evaluated external contamination whereby fine particles adhered to the algal thalli and they found out that the external contamination was high and evident in *Enteromorpha* species. Tabudravu (1998) and Philip and Segar (1986) also used *E. intestinalis* with other green, brown and red algae to access the heavy metal content in sediment and water from the Bulgarian Black Sea coast.
and found the similar results. *E. compressa* was studied by Ratkevicious *et. al.* (2003) in order to identify the biochemical mechanisms responsible for buffering the effect of heavy metals, especially copper. He suggested that the species bio-accumulated copper, triggered ascorbate peroxidase, synthesized ascorbate and consumed glutathione and water-soluble phenolic compounds in order to tolerate the copper enriched environment and the accompanying oxidative stress. The algae were also found to express heat shock proteins (HSPs) in response to a variety of stresses such as temperature stress including heavy metal stress as their cellular response to stress (Lewis *et. al.*, 1998). Lewis *et. al.* (1998) investigated the effect of copper exposure and heat shock on the physiology at cellular level and found that HSP in *E. intestinalis* was at Stress-70 level and that Stress-70 in *E. intestinalis* was a better indicator of copper exposure. These studies suggest that *E. intestinalis* is a reliable indicator of heavy metals.

Moreover, *Enteromorpha* like any other marine algae contributes to atmospheric sulphur by producing Dimethylsulfide (DMS) (Van Alstyne *et. al.*, 2003). Sulphur compounds such as DMS quickly form sulphur oxides that function as cloud nuclei; hence the release of DMS is likely to affect the global climate (Van Alstyne *et. al.*, 2003). DMS is produced during the cleavage of dimethylsulfiniopropionate (DMSP) by the enzyme DMSPlyase hence resulting in DMS and acrylic acid (Van Alstyne *et. al.*, 2003). Evidence suggests that the production of DMSP is part of an activated anti-herbivore chemical defence system where DMS is the by-product during the production of the defensive compound acrylic acid (Van Alstyne *et. al.*, 2001). Van Alstyne *et. al.* (2003) showed that *Enteromorpha* and *Ulva* produced DMS when macro-invertebrates grazed on them suggesting that DMS may function as a feeding inhibitor. In addition, they observed that DMSP containing
Enteromorpha and Ulva together with Polysiphonia were avoided by macro-invertebrates during grazing when they were offered a choice between these species and other species which did not contain DMS in their defence system. It is not so clear why Enteromorpha with many other different taxa of algae occurring in different environments produce DMS. However, DMSP can function as an osmoregulator (Edwards et. al., 1987 and 1988) and as osmoprotectants (compatible solutes) (McNeil et. al., 1999). Osmoprotectants function by increasing the osmotic pressure in cytoplasm and can also serve as protein and membrane stabilizers when salt and temperature levels are unfavourable. Apart from DMS, Enteromorpha also produces volatile hydrocarbons (which have ozone depleting potential) during oxidative stress involving H₂O₂ and algal peroxidases (Abrahamsson et. al., 2003).

1.2.7 Physiological response to environmental stresses.

Very little has been done on the laboratory analysis of the physiological responses of E. flexuosa and E. intestinalis to pollution. According to Davison and Pearson (1996) laboratory physiological studies are very important in establishing correlations between stress tolerance and the distribution of seaweeds in a particular area. Most of the physiological work that has been done on macro-algae to establish this correlation has been on photosynthesis metabolism and growth studies.

Villares and Carballeria (2004) studied photosynthesis and growth rate in Ulva and Enteromorpha from the coastal embayment in Galicia (NW Spain) in response to the nutrient limitations. They found out that the physiological activity of Enteromorpha species was not affected by the low amount of nutrients in the water. Similarly, Chapman et. al. (1978) studied the effects of nitrate concentration on Laminaria saccharina using
photosynthesis and growth as the physiological parameters under laboratory conditions and found an increase in photosynthesis, chlorophyll content and growth of the algae with increasing nitrate concentration. Photosynthetic performance of a number of green and brown algae were studied under natural conditions (*in-situ*) by Ramus and Rosenberg (1980) who showed that photosynthesis was a satisfactory measure of determining the total net diurnal productivity. In addition, Wheeler (1980) quantified the photosynthetic rates and pigment content of the giant kelp *Macrocystis pyriformia* to study the unique distribution and adaptation of the species to a very large gradient of irradiances.

Cabello-Pasini *et. al.* (2003) studied the distribution and survival of some competing marine macrophytes in the Gulf of California under fluctuating irradiance and temperature. They studied photosynthesis, growth, together with nitrogen uptake and found that increase in photosynthesis is reflected in an increase of growth rates which in turn may regulate the abundance and survival of different species in field. Abrahamsson *et. al.* (2003) studied the effects of temperature on the production of hydrogen peroxide and volatile halocarbons in *E. flexuosa* and *E. intestinalis* with other brackish water algae. They suggested that the formation of these compounds were related to oxidative stress. They concluded that the production of certain halocarbons in these species may increase with temperature and the amount and composition of the volatile halocarbons released by the algae are more affected by temperature-associated species shift.

Biochemical composition of nitrogen, phosphorous and carbon content has also been used to study the effects of abiotic stresses. Israel *et. al.* (1999) studied photosynthesis, growth rate together with carbon and nitrogen content and soluble protein content to see the effect
of salinity and pH on growth and agar yield of *Gracillaria tenuistipitata* (a red alga) in laboratory and outdoor culture. He found that photosynthesis responses represented growth responses. Pinchetti *et. al.* (1998) investigated photosynthesis and biochemical composition in terms of fatty acids, dietary fibre content, carbon and nitrogen content and ash, and caloric contents to see the influence of variable amounts of nitrogen availability in *Ulva rigida*. They found an increase in all the variables with an increase in the available nitrogen. Similarly, the effects of temperature and nitrogen availability were studied in terms of photosynthetic performance by Rivers and Peckol (1995) to assess the summer decline of *Ulva lactuca* in a eutrophic embayment and found out that the summer decline was because of the low photosynthetic performance due to high temperature and low nitrogen availability. Lewis *et. al.* (2001) investigated the potential of using stress protein HSP70 in *E. intestinalis* as a biomarker of contaminant (thermal stress, copper, variable nutrient availability and herbicides) exposure to the species. They proved it to be an insensitive biomarker and stated that growth measurements were more reliable indicators of stress.

The studies above have been carried out to investigate the effects of various factors such as temperature, pH, salinity, nutrient availability, UV light and irradiance on algae. All studies overall have shown a decrease in photosynthesis rate, growth rate and tissue constituents when the tested abiotic variables were altered from the optimum range of tolerance of the investigated species. A few studies on this aspect have been on *E. intestinalis* but physiological studies of this kind are yet to be carried out on *E. flexuosa*. However, preliminary studies done by the author in 2003, where *E. flexuosa* was exposed to different pH and temperature for 3 days under the laboratory conditions, photosynthesis measured in terms of dissolved oxygen in the medium showed a marked decrease in treatments that
varied from the control. In addition, some degree of plasmolysis of cells was also observed except for the set-ups with optimum conditions. Bleaching of alga was also observed at extreme temperatures and pH. Bleaching in Ulvaceae however can be caused by number of factors other than stress. Rao et al., (2006) showed that bleaching in *Ulva australis* was associated with the epiphytic bacteria that form biofilms (see section 1.2.3). They found that algae without any bacterial association bleached and died easily. Specific bacterial colonies are located on specific parts of the thallus. Stress probably affects bacteria as well, which explains the bleaching of only part (part housing specific bacteria) of the algal thallus sometimes.

Some physiological studies on *E. flexuosa* and *E. intestinalis* done to examine the stress response when exposed to variable temperatures, pH, nutrient (nitrogen) and herbicide over a longer period of time are investigated in this research.

### 1.3 Significance of this thesis

1. The results of this study will provide an account of stress response of *E. intestinalis* and *E. flexuosa* to environmental stresses in terms of physiological parameters. This would then provide better information about the distribution of the algae in various coastal areas both polluted and unpolluted.

2. The physiological stress response of the algae could be efficiently used as a bioindicator to monitor environmental quality of coastal areas.
(3) Studying algae at community level does not always provide a definite indication of the level of stress and pollution impact in an area because algae are bioaccumulators and they keep on accumulating toxins up to the level they can tolerate. This does not necessarily show up as a physical character. Studying algae at physiological level would give an account of stress at an early stage of pollution. Hence the pollution source and the impact could be identified and managed before any major harm is done to the environment.

(4) This research would allow and form a baseline for the proper management of pollution and discharges in the intertidal zone before the health of the ecosystem, including people, is affected severely. It can also be used to formulate strategies to control pollution and the risks associated with it. The research will be highly beneficial to the Pacific region since people in the region are dependent upon food items, especially seaweeds they collect from the intertidal zones and people could be at risk of consuming items that are polluted. For example in Fiji, Gracillaria species (a red algae) is collected for consumption and since this species grows together with the Enteromorpha species within the intertidal zone it will also be affected by the degrading environmental quality.

(4) This is the first study in Fiji Islands on aspects of E. intestinalis and E. flexuosa stress physiology.
1.4 Objectives

The detection of the physiological stress responses of *E. flexuosa* and *E. intestinalis* depends on understanding of how selected abiotic environmental factors (i.e. temperature variation, pH levels and nutrition (nitrogen) and herbicide levels) limit algal distribution and abundance by affecting physiological parameters. Thus, the objectives of this research are:

(1) To study the stress response of *E. flexuosa* and *E. intestinalis* at different temperatures, pH, variable amounts of nitrogen and exposure to an herbicide (Diuron 80) for 1 month and analyze them in terms of growth rate, photosynthesis rate, chlorophyll content, total soluble protein and total carbohydrates.

(2) To study the stress response of *E. flexuosa* and *E. intestinalis* collected from sites with different pollution rates and analysed in terms of growth rate, photosynthesis rate, chlorophyll content, total soluble protein and total carbohydrates.

(3) To test the hypothesis that pH, temperature and nutrients, higher or lower than those of natural seawater have a significant effect on the algae’s growth rate, photosynthesis rate, chlorophyll content, total soluble protein and total carbohydrates. Also that Diuron has adverse effect on algae hence reduces growth rate, photosynthesis rate, chlorophyll content, total soluble protein and total carbohydrates.

(4) To show that the stress physiology of *E. flexuosa* and *E. intestinalis* can be used as a reliable biological indicator of environmental stresses resulting through pollution in the intertidal zones.
Based on these objectives, Chapter 2 states the methodology used for the study. Chapter 3 examines the stress response of the two species when exposed to different pH levels while Chapter 4 looks at the response to temperature. Further, Chapter 5 looks at the response to different levels of Nitrogen availability while Chapter 6 looks at the response to Diuron 80 levels. Chapter 7 examines the stress responses of algae in some polluted and unpolluted sites and Chapter 8 states conclusions and outlines future work that can be done on this aspect.
CHAPTER 2

METHOD AND MATERIALS

2.1 Algal Sample Collection

The experiments were done over 18 months, from April, 2005 to September, 2006. Samples of *E. flexuosa* and *E. intestinalis* were collected from intertidal flats at low tide along Nasese, Lami and Laucala Beach area in Suva, Fiji. All three sites were used for comparative studies (see chapter 7 and section 2.4.3 below). Nasese samples, due to its accessibility and location were used for lab experiments. The Lami and Laucala Beach sites are within close proximity of anthropogenic pollution sources hence were not used for lab experiments. See below for a detailed description of the sites and plate 2.1 for a map of the sample sites.

*Nasese area*

The intertidal zone is adjacent to the main road connecting the residential and recreational areas. The shoreline consists of reef platform rather than rubble and beach sand. The area has a small estuary and several drainage outfalls that carry drainage water from the connecting residential and developing areas.

*Enteromorpha* were found growing mainly on the rocky platforms, rocks, on the sides of the seawall and on some foreign items that are the result of dumping of rubbish such as on pieces of clothes, bamboos, coconut shells etc. *Enteromorpha* here were mainly associations of *E. intestinalis* and *E. flexuosa* that were about 5 – 10 cm in length, brightly green colored forms. The thallus appeared bright green and slightly transparent and about 2-3 mm in width.
Possible means of pollution in the area could be small amount of sewage discharge and runoffs from drainage outfalls, soap and detergent contaminated water from household drainage, possible thermal pollution resulting from drainage outfalls, herbicide runoffs from wayside sediment runoffs from nearby developing sites. There is also a slight possibility of oil pollution from the small outboard boats which may be insignificant to a certain extent.

**Lami area**

The intertidal area consists of sand and rubble and is located near the main highway and a connecting industrial area. The area is reclaimed, has an estuary that runs through the industrial area. In addition, it has a rubbish dump (no longer in operation) in its vicinity as well as the main harbour (Suva harbour with Kings Wharf). The intertidal zone has several old metal scraps, which may have been washed up from the nearby dumpsite and also is a result of on site dumping.

*Enteromorpha* was found growing mainly on few of the rocks found in the area, on metal scraps, driftwood and tires etc. This area had mostly *E. flexuosa*. It was dark green in color, thinner and the growth forms were smaller (2 – 4 cm in length and 1 - 1.5 mm width).

Possible means of pollution in this area include thermal discharge (from factories operating in the area) to a certain extent, industrial discharge via the estuary, heavy metals, leaching from the former rubbish dump, factory effluent, antifouling compounds and oil pollution which could be coming from Suva harbour which has several big ships passing through everyday.
**Laucala Beach**

The shoreline is rocky and made up of a rocky reef platform. The area is reclaimed, is situated away from the main highway but is connected to a residential area. It also has an industrial area in its vicinity. In addition, it is close to a sewage treatment plant.

Like Lami, the site has several pieces of scrap metals, tires and other rubbish on which *Enteromorpha* was found growing. The algae appeared very dark green in colour, had a width ranging from 1 – 2 mm and were of smaller lengths (2 – 5 cm).

Sewage discharge could be the main source of pollution in the area, but moderate industrial discharge from the nearby industrial area, heavy metals and runoffs from the residential area could also be included.
Plate 2.1 Locations of the Nasese, Lami and Laucala Beach sampling sites. Samples from the three sites were used for the comparative study while, Nasese samples were used for lab experiment.
2.2 Laboratory experimental methods

The algae were washed in sterile seawater in a series of 10 washes (2 litres per wash) to remove debris and as many diatoms and other attached epiphytic algae before they were placed in Artificial Seawater Medium (ASM) (Brand, 1984).

Artificial Seawater Medium was used because the concentrations of materials in the medium were known and the purity of the medium is maintained unlike seawater, where the contents are unknown with anything extra such as trace metals etc. Any bioaccumulation by the algae in its habitat was ignored. Artificial Seawater Medium was further altered to establish the stress conditions investigated. The algae were exposed to different temperatures, pH, variable amounts of nitrogen and Diuron 80 (herbicide).

2.3 Exposure of algae to abiotic stress conditions.

2.3.1 pH

The algae were exposed to pH ranging from pH 4 – pH 10 (pH 4, 5, 6, 7, 8, 9 and 10). The pH of the ASM was altered using sodium hydroxide (NaOH) and hydrochloric acid (HCl) (Pederson and Henson, 2003). 1 M sodium hydroxide was added to the ASM for pH 9 and 10, and 1 M Hydrochloric acid was added to establish pH 4, 5, 6, 7 and 8. During the pH alteration, pH was measured by the Ecoscan hand-held pH meter (Eutech Instruments Pte Ltd, Singapore). pH 8 was used as control since this was found to be the pH of natural seawater.

2.3.2 Temperature

The algae were exposed to five different temperatures ranging from 20°C to 35°C (20°C, 25°C, 28°C, 30°C, 35°C). For 20°C, temperature controlled (air-conditioned) room was used
and for 25°C to 35°C water baths were used. This range of temperature used is similar to
temperature variations which can occur in the field. 28°C was used as control as it was the
average temperature in the field and was also the room temperature most of the time.

2.3.3 Nitrogen

The algae were exposed to variable amounts of ammonium (NH₄⁺) ranging from 0 – 2.5
µmoles/L (0, 0.25, 0.5, 1.0, 1.5, 2.0 and 2.5 µmoles/L) and nitrate (NO₃⁻) in the range 0 – 7.0
µmoles/L (0, 0.7, 1.4, 2.8, 4.2, 5.6 and 7.0 µmoles/L). The concentrations used are higher and
lower than those of ASM. Variable amounts of nitrogen levels were achieved by varying the
amount of ammonium chloride (NH₄Cl) or sodium nitrate (NaNO₃) added to the ASM. 0.5
µmoles/L was the control concentration for NH₄⁺ while for NO₃⁻, 1.4 µmoles/L was used
since these were the concentration in the ASM.

2.3.4 Herbicide

Herbicide Durion 80 was introduced into the ASM in concentrations ranging from 0 - 1 mg/ l
(0.0001, 0.001, 0.01, 0.1 and 1 mg/L). 0 mg/ l was used as the control concentration. These
concentrations were chosen because it has been reported that several herbicides are phytotoxic
at concentrations of less than 0.001 mg/ l. Diuron is phytotoxic at 0.004 mg/ l (Lewis and
Wang, 1997).

2.4 Experimental procedure

The experiment (exposure of algae to different abiotic conditions) was carried out in two
parts, the growth experiment which measured the growth rate of germlings when subjected to
different stresses while the exposure of adult thalli to stress was measured in the second part.
2.4.1 Growth Experiment

The propagules were collected and germinated in ASM and the growth rate was measured as an increase in fresh weight for 4 weeks.

2.4.1.1 Seeding (Propagule collection)

Following the washing as stated in part 2, about 200 g fwt thalli were swabbed with sterile cotton wool. This was done to remove as many diatoms and attached epiphytic algae (blue green algae). The algal thalli were then cut into pieces 10 mm in length, placed between moist paper towels and placed in darkness at 10°C for 24 hours. This treatment enables the thalli to produce a large number of swarmer cells (spores and gametes) (McManus et. al., 2004; Tabudravu, 1998).

After 24 hours the cut thalli were removed from the paper towels and placed in 4 containers (glass troughs) containing sterile filtered natural seawater and 50 mm x 50 mm unglazed (rough surface) ceramic tiles (8 - 9 tiles in each container). 50 g fwt algal thalli were placed in each of the 4 containers. The ceramic tiles that were placed at the bottom of the glass container were used as a substrate to collect the propagules released by the algal thalli. The containers with the algae and the ceramic tiles were placed under 100 μmol m⁻² s⁻¹ light for 16 hours and 8 hours dark cycle for 3 days with aeration for sporulation (release of spores).

After 3 days the tiles containing the propagules were placed in another clean glass trough containing ASM. After 2 days, the germlings (germinating propagules) were highly visible. The tiles containing germlings were then removed from the trough, a tile each was weighed and was placed in a beaker containing 500 ml of ASM. The ASM was altered before hand to
create the abiotic conditions (see section 3.3). The wet weight of the tile with germlings was recorded as the initial weight.

Four beakers with a tile each (4 replicate cultures) were used for each treatment set up (example, 4 culture beakers for pH 5). The setup was placed under 100 \( \mu \text{mol m}^{-2}\text{s}^{-2} \) light for 16 hours and 8 hours dark for a month. 16 hours of light was used to facilitate the germination process and the ASM medium was changed every 5 days. Light and dark hours were controlled using a light timer. The beakers containing the algal cultures were aerated using aeration tubes and aerator (aquarium aerators).

2.4.1.2 Measuring growth rate

Growth rate was measured by taking the fresh weight that is the weight of the germinating propagules (germlings) on the tiles. The tiles with germlings were removed from the beaker and placed between the paper towels to remove excess water and then weighed using a top pan balance. To reduce uncertainty in water removal via paper towels, each tile was dried 4 times between fresh paper towels before it was placed on the balance. The fresh weights of the germlings were taken every 5 days.
2.4.2 Exposure of adult thalli to stress.

For this part of the experiment, about 200 g of fresh algal samples were collected from Naese area. In the laboratory, the algal thalli were washed off debris and visible invertebrates were removed and it was placed in one large glass trough with ASM for 24 hours. This was done to acclimatize the algae in ASM. After 24 hours, the algae were removed and exposed to different abiotic conditions. For each set up, approximately 20 g fwt of thalli (with no excess water removal) was placed in 1000 ml Kimax beakers containing 800 ml of ASM with altered conditions. Four replicates (4 beakers with 20 g algae and 800 ml of ASM in each) were used.

The set up was placed at room temperature (except for where temperature was a variable) with continuous aeration under the illumination of cool daylight fluorescence of 100 μmol m\(^{-2}\) s\(^{-2}\) and a photoperiod of 12 hours daylight and 12 hours of darkness for 10 days. Since the algae were already in an adult state, 12 hours of light was used as the photoperiod. 16 hours of
light in this case would promote fast sporulation of the algae. The stress response was analysed (section 3.5) after 10 days.

2.4.3 Analysis of stress response of algal sample from 3 sites around the Suva area.

*Enteromorpha flexuosa* and *E intestinalis* were collected randomly from Nasese, Lami and Laucala Beach area around Suva and analyzed in terms of photosynthesis, total chlorophyll, total soluble protein and total soluble carbohydrates over a 12 month period. The samples were taken whenever the algae were available simultaneously at all 3 sites. Samples of seawater taken concurrently from the 3 sites were sent to the Institute of Applied Science, University of South Pacific for determination of inorganic nitrogen content (NH₄⁺ and NO₃⁻).

2.5 Analysis of stress response

2.5.1 Photosynthesis

During the exposure to variable conditions, photosynthesis rate was measured every 2 days. The rate was determined by measuring oxygen production using a Clark-type oxygen
electrode fitted with a DW 2 chamber (Hansatech, Kings Lynn, Norfolk, England). The water jacket of the DW electrode was connected to the tap so that the temperature of the system was maintained around 25°C. Algal thalli of 100 mg fresh weight was held with a thin wire hook and suspended into the 5 ml cylindrical reaction chamber that was filled with 2 ml of ASM. Photosynthesis evolution was initiated by illumination using the standard high intensity light source LS2 (Hansatech, Kings Lynn, Norfolk, England).

Photosynthesis was recorded on a pen chart recorder as change in voltage that was displayed on the CB1-D control box connected to the DW electrode. Photosynthesis was recorded for 3 minutes starting from when there was a change in the voltage displayed on CB1-D control box. Light intensity was measured using Quantitherm light meter (Hansatech, Kings Lynn, Norfolk, England). The O₂ electrode system was calibrated daily with N₂ gas that is referred to as N₂ line and O₂ saturated de-ionised water as described by Walker, (1990).

Since a small amount (100 mg) of algae was used as sample for each photosynthetic measurement, 3 measurements were made from each of the 4 replicates. Hence in total 12 replicate measurements were done for each treatment. For analysis of algae from the field (Chapter 7 and section 2.4.3), 8 replicate measurements were made depending on the availability of algae at the 3 sites.

2.5.2 Chlorophyll content

Chlorophyll was measured (once for each of the 4 replicates) at the end of each experiment (after 10 days) (see section 2.4.2). Chlorophyll extraction was done using the method described by Kreast and Grant (1976).
5 g fwt of algal thalli was initially submerged in 50 ml of 100 % acetone for a few seconds. It was removed and ground under dim light with 0.5 g of MgCO₃ per sample in fresh 10 ml of 100 % acetone. The mixture was centrifuged at 800 revolutions per minute for 15 minutes at 5°C. The supernatant (chlorophyll extract) was transferred to a clean test tube with lid. The absorbance of the extract was read at 647 nm and 664 nm using a UV-Visible Spectrophotometer (Shimadzu Corporation Analytical Instruments Division, Kyoto, Japan). Using the absorbance read at the 2 wavelengths, the total chlorophyll content was calculated using the following equations by Jeffery and Humphrey (1975):

\[
\text{Total chlorophyll (µg/ml)} = 7.04(\text{Abs 664}) + 20.27 (\text{Abs 647})
\]

Where:

Abs 664 = absorbance at 664 nm wavelength

Abs 647 = absorbance at 647 nm wavelength

2.5.3 Total soluble protein

A comparative test for total soluble protein (TSP) determination was done using Bradford Method and Biuret Method. Both methods gave similar results. Bradford’s method was chosen for measuring total soluble protein content. Total soluble protein was measured (once for each of the 4 replicates) at the end of each experiment (after 10 days) (see section 2.4.2)

1 g fwt of the algal thalli was homogenized into powder in liquid nitrogen and further ground with extraction buffer (Phosphate buffer pH 7.0) in the ratio of plant material: extraction buffer = 1 g:5 ml. The extraction was transferred to 10 ml centrifuge tubes and centrifuged at 2000 revolutions per minute for 20 minutes. Total soluble protein (TSP) was determined according to the Bradford dye binding Method (Bradford, 1976) using the Bradford reagent. Bradford reagent was made by dissolving 100 mg Coomassie Brilliant Blue G-250 in 50 ml
95% ethanol to which 100 ml 85% phosphoric acid was added. When the dye was completely dissolved, it was diluted to 1 liter and filtered through a Whatman #1 paper before use.

After centrifugation, 500 μl of supernant was taken in a test tube and made up to 1000 μl. 1 ml Bradfords reagent was added to the each of the samples and mixed thoroughly by vortexing. Bovine serum albumin was used as the standard. The samples were incubated for 5 minutes after the addition of Bradfords reagent and absorbance was read at 595 nm within 60 minutes using UV-Visible Spectrophotometer (Shimadzu Corporation Analytical Instruments Division, Kyoto, Japan).

2.5.4 Total soluble carbohydrates

Total soluble carbohydrate (TSC) was measured (once for each of the 4 replicates) at the end of each experiment (after 10 days) (see section 2.4.2).

1 g fwt of algal thalli was homogenized into powder in liquid nitrogen and further ground with 3 ml of trichloroacetic acid for extracting soluble carbohydrates. 1 ml of the extraction was transferred to an eppendorf tube and centrifuged at 20,000 revolutions per minute for 20 minutes. 50 μl of supernant was taken in a test tube and made up to 100 μl with distilled water. Total soluble carbohydrates were determined by Phenol-sulphuric method (Dubois et. al., 1956). 50 μl of 80 % phenol solution was added and mixed thoroughly by vortexing. 2 ml of 95 % sulphuric acid was then added in a stream. Glucose was used as the standard. The samples were incubated for 10 minutes at room temperature. Absorbance was read at 490 nm using a calorimeter. Total soluble carbohydrate was measured in replicates of 4 for each treatment.
2.5.5 Sporulation

Sporulation was detected through observation of the treatment beakers. Any germlings seen stuck and growing on the sides of the beakers was accounted for as sporulation. Sporulation showed that the algae were actively reproducing.

Plate 2.4 Adult algae in beaker with ASM. Spores are stuck on the sides of the beaker which shows sporulation has taken place.

2.6 Statistical analysis of results

For all the experiments done, results were analysed using Graph Pad Prism version 4. Data presented on the graphs was produced as means of the number of replicated used; error bars were used to indicate the range of results obtained from the replicates. Differences between treatments were tested for significance using ANOVA. One way ANOVA was used to test the differences between results obtained for total chlorophyll, total soluble protein content and total soluble carbohydrates while the growth and photosynthesis rates were tested with 2 ways ANOVA. For both types of testing, Bonferroni’s Test was used as a post test for multiple comparisons of means. A significance level of p < 0.05 was adopted.
CHAPTER 3

EFFECTS OF pH ON Enteromorpha flexuosa AND Enteromorpha intestinalis :

RESULTS AND DISCUSSION

3.1 Results

3.1.1 Growth

Growth rate (Fig 3.1) under control pH (pH 8) increased slightly for the first 10 days and then a sharp increase from about 0.03 g/ day to approximately 1 g/ day in the next 15 days was noted. Growth rate at a pH of 9 showed a similar pattern and did not vary from pH 8 throughout the experiment (p > 0.05). There was significant increase in growth rate at pH 8 and 9 (p < 0.0001 i.e p < 0.05). The growth rate of the algae at pH 10, 7 and 6 were significantly lower (p < 0.0001 i.e p < 0.05) throughout the experimental period, ranging from about 0.013 g/ day to 0.030 g/ day. At pH 5 and 4 the algae did not grow. At pH 4 while there was no increase in weight the plants remained green. In pH 5 medium there was a very small amount of growth initially but after 10 days the growth rate declined. The algae remained green in this case as well.

3.1.2 Photosynthesis

Similar to the growth rates, pH overall had a significant effect on the rates of photosynthesis (p < 0.0001 i.e. p < 0.05). The rates of photosynthesis (Fig 3.2) at pH 8 and 9 were the highest with no significant difference between the two (p > 0.05). The rate of photosynthesis at pH 10 increased on day 3 and then decreased there after. At pH 7 there was a constant increase in the rate of photosynthesis from 1.1 μmoles/ min/ g fwt to 2.1 μmoles/ min/ g fwt over the 8 days. The increase was not as significant as compared to pH 8 (p > 0.05). The rate of photosynthesis at pH 6 fluctuated between 1.1 μmoles/ min/ g fwt and 1.2 μmoles/ min/ g fwt.
over the experimental period. Similar to the response in growth rates, the rates of photosynthesis at pH 4 and 5 decreased over the 10 days and the growth rates were similar to pH 10 (p > 0.05).

![Figure 3.1](image1.png)

**Figure 3.1** The growth rates of *E. flexuosa* and *E. intestinalis* (together) at different levels of pH in ASM. Error bars represent the range of growth rates at each pH treatment. Growth rates were measured as fresh weights of 4 replicates at 5 day intervals over 20 days. The days were counted from the time the germlings became visible on the tiles (after 4 days).

![Figure 3.2](image2.png)

**Figure 3.2** Photosynthesis rates of *E. flexuosa* and *E. intestinalis* at different pH levels over 10 days. Data presented is the mean of 12 replicate measurements. Error bars represent the range of photosynthesis rates at each pH treatment. Photosynthesis was measured using a Clark-type oxygen electrode fitted with a DW 2 chamber (Hansatech, Kings Lynn, Norfolk, England) at room temperature and light at 100 μmol m⁻² s⁻¹.
3.1.3 Chlorophyll content

Total chlorophyll content (Fig 3.3) was high in pH 4, pH 5 and pH 6 with no significant difference (p > 0.05). From pH 7 to pH 10, total chlorophyll content decreased with increasing pH but there was no significant difference in chlorophyll content of the three treatments (p > 0.05). pH 10 had significantly lower chlorophyll content than algae at pH 4, 5 and and 6 (p < 0.01 i.e. p < 0.05).

![Figure 3.3](image.png)

Figure 3.3 The amount of total chlorophyll content in *E. flexuosa* and *E. intestinalis* at different pH levels in ASM. Data presented is the mean of 4 replicates. Error bars represent the range of total chlorophyll at each pH treatment. Total chlorophyll was measured using spectrophotometry after extraction with acetone on the 10th day immediately after photosynthesis measurements were completed.

3.1.4 Total soluble protein

Overall pH had a significant effect on the TSP (p < 0.0001 i.e. p <0.05). Protein content (Fig 3.4) was lowest at pH 4 when compared with other pH treatments (p < 0.0001 i.e. p <0.05). pH 10 and pH 6 had slightly higher protein content (9.1 mg/g fwt at pH 10 and 9.6 mg/g fwt at pH 6) than pH 5. The highest protein content was measured for pH 7 (10.8 mg/g fwt),
followed by 8 (10.6 mg/g fwt) and 9 (10.1 mg/g fwt) basically within the same range (p > 0.05).

Figure 3.4 The amount of total soluble protein in *E. flexuosa* and *E. intestinalis* after treatment at different levels of pH in ASM. Data presented is the mean of 4 replicates. Error bars represent the range of TSP at each pH treatment. The protein content was measured after 10 days of treatment using the Bradford method (see Bradford, 1976).

Figure 3.5 The amount of total soluble carbohydrates in *E. flexuosa* and *E. intestinalis* after treatment at different levels of pH in ASM. Data presented is the mean of 4 replicates. Error bars represent the range of TSC at each pH treatment. The TSC was measured after 10 days of treatment using the Phenol-Sulphuric method (Dubois *et al.*, 1956).
3.1.5 Total soluble carbohydrates

Total soluble carbohydrates content (Fig 3.5) was slightly low at pH 4, 5 followed by pH 10, which was slightly higher. TSC content at pH 6 was slightly higher than pH 10. High TSC content was measured at pH 7 and pH 8 and algae treated at pH 9 had the highest TSC content. Overall there were no significant differences in the TSC content between all the pH treatments (p > 0.05).

3.2 Discussion

The growth experiments (Fig 3.1) showed optimum growth of Enteromorpha at high pH levels below pH 10. This pattern of growth was similar to the observed photosynthetic rate of the adult thalli (Fig. 3.2), where algae at pH 7, 8, 9 had high photosynthetic rates compared to algae at pH 4, 5 and 6. Similar patterns of photosynthesis and growth rates indicate that growth in this case was a direct result of photosynthesis. The similar pattern of growth rates and photosynthesis confirms that pH has a significant effect on growth (p < 0.05) and photosynthesis (p < 0.05). Enteromorpha cannot exist in low pH conditions whereas high pH actually favours the algae.

The effect of environmental pH or the external pH on the algae is a result of the regulation of intracellular pH and the speciation of the inorganic carbon species in the medium (Raven and Geider, 2003). Inorganic carbon is the sole carbon source for seaweeds (Lobban and Harrison, 1994) and in seawater where there is a high pH, 90% of inorganic carbon occurs as bicarbonate (HCO$_3^-$).
Inorganic carbon in the medium is often a major determinant of pH (Raven and Geider, 2003). *Enteromorpha* and *Ulva* (similar genus) both utilize HCO$_3^-$ as described earlier (in chapter 2) either by extracellular carbonic anhydrase (CA) mediated dehydration resulting in CO$_2$ which then diffuses into the cells; or directly taken up via facilitated transportation using a general anion-exchange protein (Axelsson *et. al.*, 1995). However, *Enteromorpha* and *Ulva* can also effectively use CO$_2$ directly to carry out photosynthesis in addition to utilizing HCO$_3^-$ as Beer and Shragge (1997) found out that CO$_2$ added to plants already saturated with HCO$_3^-$ increased their photosynthetic rate. According to Bidwell and Mclachlan (1985), intertidal seaweed species can photosynthesize at equal rates when emerged or submerged. Kerby and Raven (1985) (cited in Lobban and Harrison, 1994) stated that intertidal seaweed species must use CO$_2$ because the bicarbonate in the capillary layer of water that remains on the plant surface becomes quickly exhausted. The forms of inorganic carbon depend on pH and salinity (Fig 1.1) (Lobban and Harrison, 1994). At low pH levels (4 to 6), CO$_2$ is freely available while at high pH level (up to 10) HCO$_3^-$ is dominant (see Fig 3.6).

![Figure 3.6 The effects of pH on percentage of H$_2$CO$_3$ (---), HCO$_3^-$ (-----) and CO$_3^-$ (-----) to total dissolved inorganic carbon (DIC). (H$_2$CO$_3$ = CO$_2$ aq + H$_2$CO$_3$. Source: (Lobban and Harrison, 1997).](image)
Carbon dioxide (CO₂) reacts with water (H₂O) to form carbonic acid (H₂CO₃) which dissociates into bicarbonate ions (HCO₃⁻) (Fig 3.7) which in turn affects the pH and alkalinity. Therefore equilibrium shifts to the right with increasing pH (Lobban and Harrison, 1994).

![Chemical reaction diagram]

**Figure 3.7 The carbonate equilibrium (source: Lobban and Harrison, 1994).**

Hence both growth (Fig 3.1) and photosynthesis (Fig 3.2) observed are higher in algae at high pH due to adequate supply and utilization of HCO₃⁻ for photosynthesis. However, at pH 10, both growth and photosynthesis were lower than at other pH levels (pH 7, 8, and 9). This is due to a further increase in pH level (more than 10 or 11) at which free CO₂ and HCO₃⁻ were depleted (Fig 3.6). In addition it could be due to other toxic effects on enzyme activity and membrane permeability of the algal cells brought about by increasing pH.

Biocarbonate users raise the pH around their thalli while utilizing the HCO₃⁻, some algae raise the pH to over 10.5 (Maberly, 1990). *E. intestinalis* was found to raise the pH of its surrounding seawater to more than 10 under closed conditions (Larsson et. al., 1997). The ability to raise the pH is due to its capacity to transport HCO₃⁻ across the plasma membrane into the cells through an anion exchange protein. Carbon dioxide concentrating mechanism inside the cell has to work against the leakiness of the plasmalemma to avoid a back flux. The electrochemical potential across the membrane is affected once the HCO₃⁻ is exchanged for another anion and OH⁻ is released when HCO⁻ is dissociated (Raven and Lucas, 1985).
Plants in aquatic environment can also get CO₂ of similar concentration as the plants in air but the diffusion rate of CO₂ is 104 time slower than air (Lobban and Harrison, 1994). At low pH photosynthesis is fairly low even though free CO₂ is readily available (which *Enteromorpha* also has the ability to utilize) and it can easily diffuse across cells and chloroplast membranes. This is mainly due to a very low concentration of CO₂ present in the medium as compared to HCO₃⁻, hence the amount of CO₂ incorporated into Rubisco is relatively lower than the CO₂ that could be obtained from HCO₃⁻ in the same medium. A low concentration of this CO₂ is evident in this case. According to Beer and Shragge, (1987), increasing CO₂ addition to HCO₃⁻ saturated *Enteromorpha* plants increases the photosynthetic rate and this indicates that bicarbonate does not fully compensate for the CO₂ limitation in *Enteromorpha* and that CO₂ is significant in regulating the photosynthesis.

Furthermore, environmental surrounding (external) pH affects the intracellular pH. Coleman and Coleman (1981) found a drop in the intracellular pH of a blue green alga *Coccolithus peniocystis* in response to a drop in the external (medium) pH. In addition the blue green alga was not able to maintain its intracellular pH when placed in an acidic medium hence its photosynthetic capacity decreased by a direct effect on the principal CO₂ fixing enzyme Rubisco.

The level of enzyme activities effectively controls photosynthesis. The enzymes of the Calvin cycle are sensitive to the proton concentration of the chloroplast stromal compartment. The activity of Rubisco is optimum at around pH 8. The proton pump during the light absorption moves protons from stroma to the lumenal side to the thylakoid membrane that increases the stromal pH. This activates the Rubisco and hence the Calvin cycle. Moreover, the key
enzymes of Calvin cycle, fructose biphosphate and sedoheptulose biphosphate are sensitive to pH changes (McDonald, 2003). Coleman and Coleman, (1981) found that at a low external (medium) pH of 5.25, the intracellular pH of Coccochloris was lowered to an extent where the activity of RuBP-caboxylase was minimum and photosynthesis was inhibited. Raising the external pH raised the intracellular pH, hence increasing the optimal pH up to 7.8 at which there is an optimum activity of Rubisco. Woodraw et. al. (1984) cited in Velilova and Yordanov (1996) also stated that input of excess protons into stroma could lower the rates of enzymatic reaction of Calvin cycle and further increase the sensitivity of the photosynthetic apparatus to photo damage.

Chlorophyll content in pH 4, 5, 6 was higher than in pH 7, 8, 9 (Fig 3.3). The results obtained shows that pH has a significant effect (p < 0.05) on total chlorophyll. Lower pigment content at these pH levels was due to sporulation and the release of zoospores from the cells. Germlings were found growing robustly on the walls of the glass beaker. Germlings were not observed in beakers with pH 4, 5 and pH 6 which had very little growth. This indicates that at pH 7, 8 and 9, the conditions were most favourable for the algae to survive and thrive on while at low pH the conditions were not so favourable. Lowest chlorophyll content was found at pH 10 due to the disintegration of the algae. On the other hand, during the growth experiment, the growing germlings were lighter in colour at pH 4 and pH 5 when compared to the other pH treatments. The lighter colour of the algal thalli could be due to low pH affecting the key enzymes that are involved in the formation and biosynthesis of chlorophyll molecules. Low pH on the other hand leads to loss of Magnesium ions from the chlorophyll molecule which leads to breakdown of the whole chlorophyll structure (Lewis and Wang,
However, this case is not evident in this experiment since there is a considerable amount of chlorophyll at pH 4 and pH 5 (Fig 3.3).

The total soluble protein (TSP) content was significantly affected by pH ($p < 0.05$). TSP was lower at low pH levels with lowest TSP content in pH 4 (Fig 3.4) and higher at high pH levels from pH 7 – 9. The high TSP in pH 7 – 9 is an indication of the high photosynthesis rates at these pH as well the high growth rates (except at pH 7). The low protein content at low pH levels is due to pH severely affecting the biochemical compositions (proteins and enzymes). pH of the medium influences enzyme activity in various ways since all enzymes function best at certain optimum pH. The optimum pH for enzyme activity is usually between pH 6 – 8 but can vary for some enzymes (Salisbury and Ross, 1991). Extreme pH causes denaturing of enzymes which can be seen in algae exposed at pH 4. In addition, pH can influence the enzyme reaction rates. The enzyme activity depends on the presence of free amino or carboxyl groups. These groups can occur as charged or uncharged depending on the enzyme, with only one form being active in a given case. The pH optimum for enzymes are high if an uncharged amino group is required for reaction and for neutral carboxyl groups, a low pH optimum is needed (Salisbury and Ross, 1991). Hence, it can be said that enzymes in *Enteromorpha* require an uncharged amino group for active reactions. Moreover, the ionization of many substrates (which must be ionized for the reaction to continue) is controlled by pH (Salisbury and Ross, 1991). Apart from the enzymes and enzymatic reactions of the Calvin cycle, increased acidity in chloroplasts can also cause damage to the chlorophyll-proteins particulary those that are involved in PS II (Siefermann-Harms, 1992 cited in Velilova and Yordanov, 1996).
Similar to TSP, the total soluble carbohydrates (TSC) was lower in algae at low pH levels and higher at high pH levels (p < 0.05). At pH 10 the TSC content was equivalent to the TSC levels at pH 4 and 5 (Fig 3.5). The TSC content emulates the pattern of photosynthesis and growth of Enteromorpha at various pH levels. Hence the pattern can be simply explained by the effects of pH on photosynthesis as stated earlier. Apart from photosynthesis, pH also affects the membrane proteins and transport processes (Pedersen and Hansen, 2003) which affect the sites at which the various cell wall and storage polysaccharides are synthesized.

Overall, it can be said that pH changes in the surrounding environment affects photosynthesis, membrane transport processes, biochemical composition, enzyme activity jointly with protein and carbohydrates which in turn affects the growth of Enteromorpha. However when looking at the growth and survival of Enteromorpha, the most important factor affected by the pH is the inorganic carbon content, which, in turn affects the photosynthesis. The experiment showed that Enteromorpha was able to grow and carry out photosynthesis over a wider pH range (pH 6 – 9) hence showing the algae’s ability to grow in and tolerate a variable pH environment.
CHAPTER 4
EFFECTS OF TEMPERATURE ON *Enteromorpha flexuosa* AND *Enteromorpha intestinalis*:

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Growth

Temperature in general has a significant effect on growth rates (p < 0.0001 i.e. p < 0.05) Growth rates (Fig 4.1) of germlings at temperatures 20°C, 25°C and 28°C increased gradually over the 20 days. The highest growth rate however was recorded at 20°C followed by 25°C when compared with growth at other temperatures (p < 0.001 i.e. p < 0.05). Growth rate at 28°C which was the control temperature remained lower than at 20°C and 25°C over the 20 days (p < 0.01 i.e. p < 0.05). Germlings kept at 30°C had a gradual increase in growth rate until the 10th day after which it became stable and there was no further increase. Germlings at 35°C showed absolutely no increase in the growth rate from the 1st day and were assumed to be dead, as they had lost their green colour as well.

Figure 4.1 The growth rates of *E. flexuosa* and *E. intestinalis* (together) at different temperatures in ASM. Error bars represent the range of growth rates at each temperature. Growth rates were measured as fresh weights of 4 replicates at 5 day intervals over 20 days. The days were counted
from the time the germlings became visible on the tiles (3 days). 28°C was the control temperature.

4.1.2 Photosynthesis

The photosynthesis rates (Fig 4.2) of *Enteromorpha* decreased slightly for all temperature treatments for the first 2 days after which it increased significantly (p < 0.0001 i.e. p < 0.05) for all the treatments except for algae at 35°C. Similar to the growth rates, the photosynthesis rate was highest at 20°C and 25°C. Photosynthesis of algae at 20°C was higher than algae at 25°C. However the difference between the photosynthetic rates from 20°C to 28°C was not that significant (p > 0.05). Algae at 28°C (control) showed similar pattern till day 7 after which it decreased slightly. Algae at 30°C had significantly lower photosynthetic rate than algae at 28°C though on the 9th day the photosynthesis rate of 28°C was the same as 30°C on the 10th day (p > 0.05). Algae at 35°C had the lowest photosynthesis rate as it decreased dramatically from the first day of treatment.

Figure 4.2 Photosynthesis rates of *E. flexuosa* and *E. intestinalis* at different temperatures over 10 days. Data presented is the mean of 12 replicate measurements. Error bars represent the range of photosynthetic rates at each temperature. Photosynthesis was measured using a Clark-type oxygen electrode fitted with a DW 2 chamber (Hansatech, Kings Lynn, Norfolk, England) at room temperature and light at 100 μmol m⁻² s⁻².
4.1.3 Chlorophyll content

There was a significant decrease in the total chlorophyll content (Fig 4.3) in algae at 35 °C (p < 0.0001 i.e. p < 0.05). Algae at 35°C had the lowest chlorophyll content while algae at 20°C had the highest chlorophyll content. Algae at 25°C, 28°C (control temperature) and 30°C had similar (p > 0.05) total chlorophyll content (in the range of 115 – 125 μg/ mg/ g fwt).

![Figure 4.3 The total chlorophyll content in E. flexuosa and E. intestinalis at different temperatures in ASM. Data presented is the mean of 4 replicates. Error bars represent the range of total chlorophyll at each temperature. Chlorophyll was measured using spectrophotometry after extraction with acetone on the 10th day immediately after photosynthesis measurements were completed.](image)

4.1.4 Total Soluble protein

Total soluble protein content (TSP) (Fig 4.4) significantly decreased with increasing temperature (p < 0.0001 i.e. p < 0.05). TSP was highest in algae at 20°C. Algae at 25°C, 28°C (control temperature), 30°C and 35°C had significantly lower TSP than 20°C (p < 0.0001 i.e. p < 0.05) but there was no significant difference in TSP from 25°C to 35°C. 35°C treatment had the lowest TSP content.
Figure 4.4 The amount of total soluble protein in *E. flexuosa* and *E. intestinalis* after treatment at different temperatures in ASM. Data presented is the mean of 4 replicates. Error bars represent the range of TSP at each temperature. The protein content was measured after 10 days of treatment using the Bradford method (see Bradford, 1976).

Figure 4.5 The amount of total soluble carbohydrates (TSC) in *E. flexuosa* and *E. intestinalis* at different temperatures in ASM. Data presented is the mean of 4 replicates. Error bars represent the range of TSC at each temperature. The TSC was measured after 10 days of treatment using the Phenol-Sulphuric method (Dubois *et. al.*, 1956).
4.1.5 Total soluble carbohydrates.

The total soluble carbohydrates (Fig 5.5) decreased significantly with increasing temperatures (p < 0.0001 i.e. p < 0.05). The highest TSC (5.5 mg/g fwt) was measured for algae at 20°C. Algae at 25°C and 28°C (control temperature) had the TSC in the similar range of (4 – 4.3 mg/g fwt) (p > 0.05). Algae at 30°C had fairly low TSC (3.3 mg/g fwt) similar (p > 0.05) to algae at 35°C, which had the lowest (2.7 mg/g fwt) TSC content.

4.2 Discussion

Highest growth of the germlings of the two Enteromorpha species was recorded at 20°C (Fig 4.1) and lowest growth was recorded at 30°C while no growth occurred at 35°C. The pattern observed is similar to the recorded photosynthesis rate of the adult thalli (Fig 4.2), total soluble protein (Fig 4.4) and total soluble carbohydrates. However, photosynthesis rates between 20°C and 30°C were not significantly different (p > 0.05). These analogous patterns show that Enteromorpha can exist over a wide temperature range although it appears not to accumulate soluble protein and soluble carbohydrate as temperature increases. The results also illustrate that high temperature above its tolerance range (35°C) has adverse effects on the algae.

Hill (2001) noted a favourable growth of E. flexuosa at temperatures ranging from 15°C -30°C and adverse effects on growth together with bleaching at temperatures of 33°C and above. This research shows that while photosynthesis and chlorophyll content do not appear to be adversely affected between 20 – 30°C, TSP and TSC contents significantly (p < 0.05) decrease with temperature.
Generally, algae that are permanently submerged are less tolerant to high temperatures than the intertidal algae that are exposed to air during low tide. Submerged algae are usually not faced with high temperatures within their habitat but intertidal algae such as *Enteromorpha* may face considerable amount of heat due to sun radiation especially during low tides when they are fully exposed to air. However, rise in temperature up to 30°C and above shows detrimental effects on the algae at least in terms of overall growth, TSP and TSC.

The low growth can be partly due to the depletion of reserve foods and an increase in the respiration, more light is then required to carry out photosynthesis to keep up with the respiration (Mohsen *et. al.*, 1973). Temperatures in general have fundamental effects on the chemical reaction rates that are embodied in the concept of $Q_{10}$ (Lobban and Harrison, 1994). The $Q_{10}$ temperature coefficient is a measure of the rate of change of a biological or chemical system as a consequence of increasing the temperature by 10°C. For typical chemical reactions, $Q_{10}$ values are ~2 meaning the rate doubles over a 10°C rise in temperature, but rates can be much higher (Lobban and Harrison, 1994). This explains the decrease in TSC and TSP.

The effect of temperature is much higher on catalysed reactions when compared with the unanalysed ones. The rate of an enzyme-catalysed reaction varies with changes in temperature but is also affected by pH. Denaturing of enzymes takes place when the temperature rises above a critical temperature level. After cooling, when the temperature is below the critical level, enzymes may regain their original active conformation or it may be permanently damaged (Lobban and Harrison, 1994). In the experiment, denaturing of enzymes was
assumed to be due to permanent damage to their original active conformation. This is evident to some extent when looking at the TSP content (Fig. 4.4).

When measuring reaction rates versus temperatures in complex reactions such as photosynthesis and respiration, the overall rate is the total of all individual reaction rates. Hence the effects of a given temperature change is not the same for all the metabolic processes since different temperature sensitivities exist for different enzymes in different processes and in addition they are influenced by other factors such as light, pH, and nutrients. The rate is always determined by the rate limiting steps.

High temperatures may induce lethal effect possibly by shifting the equilibrium between the reaction sequences with different $Q_{10}$ values (Marre, 1962). This is evident in algae at 35°C. Inhibition of both photosynthesis and respiration occur at high temperatures, but during the increase in temperature, photosynthesis rates decrease before respiration rates in algae at 35°C. Respiration has higher $Q_{10}$ value than photosynthesis hence when temperature is increased photosynthesis decreased due to denaturing of enzymes and hence the depletion of food due to respiration is must faster than the rate of food manufacturing and storage. In a given time, the temperature at which the amount of CO$_2$ is fixed via photosynthesis is equal to the amount of CO$_2$ released via respiration is known as the compensation point. Any rise in temperature above this does not allow photosynthesis to replace the carbon that is used in respiration as a substrate. The carbohydrate reserves decrease consequently (Taiz and Zeiger, 1998). This is evident when looking at the low TSC of Enteromoprha at high temperatures (Fig 4.5).
The total chlorophyll content (Fig 4.3) was slightly lower in algae at higher temperatures (Fig 4.3), bleaching of thalli was observed. However bleaching of fronds was incomplete, meaning entire frond did not get bleached; part of the thalli was green in color, while part appeared white. This may be because some cells become resistant to heat. Mohsen et. al. (1973) observed incomplete bleaching in cells of *Ulva fasciata* during his study of the effects of temperature variations on *U. fasciata*. They found the resistant cells of *U. fasciata* were growing on nylon threads and rope, indicating that such cells were associated with regeneration under drastic conditions. Bleached cells were assumed to be dead since all the parameters measured became lower with an increase in the amount of bleached algae during the experiment. In addition there was no germlings observed in the beakers at 30°C and 35°C, which confirms the death of the algal cells. Little sporulation was observed at 20°C while at 25°C and 28°C, considerable amount of sporulation was observed. Considerable sporulation at these two temperatures indicates favorable temperatures for reproduction. Extreme temperature changes the quantity of pigments, organization of thylakoids, the primary photochemistry and carbon dioxide fixing ability of the chloroplasts (Biswal, 2005). In contrast to this, the behavior of adult thalli to varying temperatures was different. Adult thalli exhibited a greater degree of tolerance to a wider temperature range.

Photosynthesis and respiration decrease largely since they are highly dependent on the activity of membrane associated electron carriers and enzymes (Tiaz and Zeiger, 1998). PS II is more sensitive to high temperatures than PS I (Berry and Bjorkmann, 1980). Havaux et. al. (1991) found that that at high temperatures, PS II activity in pea leaves was inhibited while the PS I activity was stimulated. Photosynthetic oxygen evolution was inhibited and the photochemical energy storage decreased and these were correlated with a significant loss of
variable PS II chlorophyll fluorescence emission while the capacity of cyclic electron flow around PS I increased (Havaux et al., 1991). Inactivation of PS II at high temperature is due to extraction of divalent ions Ca$^{2+}$ and Mn$^{2+}$ from the oxygen-evolving complex of PS II. In addition dissociation of the 32kDa extrinsic polypeptide that is involved in Mn-cluster stabilization occurs (Carpentier, 1999). Bjorkman et al. (1980) (cited in Tiaz and Zeiger, 1998) found that the temperature at which the enzymes involved in photosynthesis began to denature and lose activity was much higher than the temperatures at which the photosynthesis began to decline hence they suggested that the early stages of heat stress to photosynthesis is directly related to changes in membrane properties and to uncoupling of energy transfer mechanisms in the chloroplast than to general denaturing of enzymes and proteins. This explains the lowering of the photosynthesis rate as the temperature increases from 20$^\circ$C to 30$^\circ$C (Fig 4.2).

In addition, the cause of death by high temperatures is generally explained to be the result of denaturing of proteins (Daniell et al., 1969). The TSP recorded is lower at high temperatures (Fig 4.4). High temperatures affect the stability of various cellular membranes. The loss of physiological functions is often correlated with the excessive fluidity of the membrane lipids at high temperature. Exposure to high temperatures decreases the strength of the hydrogen bonds and the electrostatic interactions between the polar groups of proteins within the aqueous phase of the membrane allowing the integral membrane proteins to associate more strongly with the lipid phase. The membrane composition and structure is modified which may lead to leakage of ions. Hence membrane fluidity is increased, leading to peroxidation and lateral diffusion of membrane lipids. Increased membrane permeability causes a decrease in proton gradient formation across the thylakoid membrane (Berry and Bjorkmann, 1980).
According to Crafts-Brandner et. al. (2000) high temperature also decreases the level and activation state of the carboxylating enzyme Rubisco. Photosynthesis (Fig 4.2) in the experiment does not noticeably indicate the Rubisco deactivation and death except at 35°C. Further investigation on Rubisco deactivation is needed to confirm this.

Denaturing of various enzymes and proteins may also lead to a lower TSP content in Enteromorpha at high temperatures (28°C, 30°C and 35°C) (Fig. 4.4). Mohsen (1973) also found within the temperature range from 20°C to 25°C, most of the amino acids in Ulva fasciata increased greatly while it decreased below the initial content at 30°C.

The low soluble sugar content in Enteromoprha at high temperatures (30°C and 35°C) can also be accounted by the denaturing and degradation of enzymes responsible for the cleavage of several glucosides of various polysaccharides hence inhibiting the synthesis of polysaccharides (Mohsen et. al., 1973). The TSC content in algae also follows the pattern of photosynthetic rate in Enteromoprha, low photosynthesis hence low sugars are synthesized.

Total soluble protein (Fig 4.4) and TSC (Fig 4.5) seem to be direct results of photosynthesis as at 20°C, photosynthesis is high and so is TSP and TSC. However, at higher temperature while photosynthesis occurs, respiration demands appear to decrease TSC and TSP content. While increase in temperature is normally expected to increase membrane fluidity resulting in the loss of metabolic activity, this would be unlikely in the case of Enteromorpha in the experiment since the photosynthesis structures appear to be intact and functioning well between 25°C – 30°C.
Overall, the response of *E. flexuosa* and *E. intestinalis* show that this alga is able to carry out photosynthesis over a wide temperature range and remain viable. The germlings of algae are much more sensitive to temperature than the adult thalli. Extremely high temperature (35°C in this experiment) has adverse effects (p <0.05) on the algae.
CHAPTER 5

EFFECTS OF AMMONIUM (NH$_4^+$) AND NITRATE (NO$_3^-$) ON Enteromorpha flexuosa AND Enteromorpha intestinalis:

RESULTS AND DISCUSSION

5.1 Results illustrating the effects of Ammonium (NH$_4^+$).

5.1.1 Growth

There was an overall increase in the growth rates of germlings (Fig 5.1) of the algae in all the treatments, increasing as the concentration of NH$_4^+$ increased (p = 0.0062 i.e. p < 0.05). The highest overall growth was seen in the algae with 2.5 μmoles of NH$_4^+$, which after 10 days gradually increased from 0.04 g/ day to 0.08 g/ day. Algae at 0.5 μmoles/L (control concentration) had similar growth rate at 1.0 μmoles/L (p > 0.05) much lower growth rate than 1.5 μmoles/L, and 2.0 μmoles/L (p < 0.01). The growth pattern at 0 μmoles/L was similar to that at 0.25 μmoles/L (p > 0.05), which was lower than the control levels. There was still some N present in the medium even though no NH$_4^+$ was added.

5.1.2 Photosynthesis

Overall all the treatments showed an increase in the photosynthesis rate (Fig 5.2) from the 1st day till the 10th day regardless of the fluctuations. However there was no significant differences between the photosynthetic rates obtained at different ammonium levels (p > 0.05). On the last day all the treatments had the photosynthesis rate in the range of 0.9 μmoles/ min/ g fwt to 1.0 μmoles/ min/ g fwt (all rates seemed to even out in the end) (p > 0.05).
Figure 5.1 The growth rates of *E. flexuosa* and *E. intestinalis* (together) at different levels of NH$_4^+$ in ASM. Error bars represent the range of growth rates at each NH$_4^+$ concentration. Growth rates were measured as fresh weights of 4 replicates at 5 day intervals over 20 days. The days were counted from the time the germlings became visible on the tiles (4 days).

Figure 6.2 Photosynthesis rates of *E. flexuosa* and *E. intestinalis* at different NH$_4^+$ levels over 10 days. Data presented is the mean of 12 replicate measurements. Error bars indicate the range of photosynthetic rates at each NH$_4^+$ concentration. Photosynthesis was measured using a Clark-type oxygen electrode fitted with a DW 2 chamber (Hansatech, Kings Lynn, Norfolk, England) at room temperature and fluoresce light at 100 μmol photons m$^{-2}$ s$^{-1}$. 
5.1.3 Chlorophyll content

There was no significant difference in the total chlorophyll content (Fig 5.3) in all treatments (p = 0.09 i.e. p > 0.05) including the treatment with zero ammonium. The values ranged between 125 – 150 μg/ ml/ g fwt. The lowest chlorophyll content however was seen in the treatment with the highest amount of NH₄⁺ (2.5 μmoles/L).

![Figure 5.3 The total chlorophyll content in E. flexuosa and E. intestinalis at different levels of NH₄⁺ in ASM. Data presented is the mean of 4 replicates. Error bars indicate the range of total chlorophyll at each NH₄⁺ treatment. Total chlorophyll was measured using Spectrophotometry after extraction with acetone on the 10th day immediately after photosynthesis measurements were completed.]

5.1.4 Total soluble protein

Generally there was an increase in the total soluble protein (TSP) content (Fig 5.4) in the algae with increasing NH₄⁺ (p = 0.045 i.e. p < 0.05). While the difference between TSP content was significant between the lowest (0 μmoles/L) and highest concentration (2.5 μmoles/L), there was generally no significant difference between the consecutive concentrations (p > 0.05).
Figure 5.4 The amount of total soluble protein in *E. flexuosa* and *E. intestinalis* after the treatment at different concentrations of NH$_4^+$ in ASM. Data presented is the mean of 4 replicates. Error bars indicate the range of TSP at each NH$_4^+$ concentration. The protein content was measured after 10 days of treatment using the Bradford method (see Bradford, 1976).

Figure 5.5 The amount of total soluble carbohydrates in *E. flexuosa* and *E. intestinalis* after the treatment at different concentrations of NH$_4^+$ in ASM. Data presented is the mean of 4 replicates. Error bars indicate the range of TSC at each NH$_4^+$ concentration. The TSC was measured after 10 days of treatment using the Phenol-Sulphuric method (see Dubois *et al.*, 1956).
5.1.5 Total soluble carbohydrates

The total soluble carbohydrate content (Fig 5.5) at all 7 concentrations was basically the same ranging from 1.88 mg/g fwt to 2.02 mg/g fwt (p = 0.0997 i.e. p > 0.05). Algae in the control concentration of ammonia (0.5 μmoles/L) showed the lowest TSC content and at zero concentration the TSC content was the highest. However, there was no significant difference in the TSC content in all 7 concentrations (p > 0.05).

5.2 Results illustrating the effects of Nitrate (NO₃⁻)

5.2.1 Growth

There was an overall increase in growth rate (Fig 5.6) of germlings of algae in all the treatments, increasing as NO₃⁻ concentration in the growth media increased (p = 0.0004 i.e. p < 0.05). The highest growth rate was observed for 7.0 μmoles/L treatment which after the 10th day increased rapidly from 0.040 g/ day to 0.07 g/ day. The 4.2 μmoles/L and 5.6 μmoles/L treatments showed a similar trend of gradual increase in the growth rates from approximately 0.03 g/ day up to 0.055 g/ day while 2.8 μmoles/L treatments showed lower growth rates (an increase from 0.025 g/ day to 0.055 g/day). The 1.4 μmoles/L treatment, which was the control, together with 0.7 μmoles/L showed lower growth rates (an approximate increase from 0.025 g/ day to about 0.047 g/ day). The lowest rate was recorded for the germlings with no NO₃⁻ (p < 0.01).
Figure 5.6 The growth rates of *E. flexuosa* and *E. intestinalis* (together) at different levels of NO$_3^-$ in ASM. Error bars indicate the range of growth rates at each NO$_3^-$ concentration. Growth rates were measured as fresh weights of 4 replicates at 5 day intervals over 20 days. The days were counted from the time the germlings became visible on the tiles (5 days).

Figure 5.7 Photosynthesis rates of *E. flexuosa* and *E. intestinalis* at different NO$_3^-$ levels over 10 days. Data presented is the mean of 12 replicate measurements. Error bars indicate the range of photosynthetic rates at each NO$_3^-$ concentration. Photosynthesis was measured using a Clark-type oxygen electrode fitted with a DW 2 chamber (Hansatech, Kings Lynn, Norfolk, England) at room temperature and fluorescent light at 100 μmol photons m$^{-2}$ s$^{-1}$.
5.2.2 Photosynthesis

Generally there was a significant increase in the photosynthetic rates (Fig 5.7) in all treatments over the 10 days ($p = 0.016$ i.e $p < 0.05$). The lowest photosynthesis rate was in algae with no NO$_3^-$ while in all other treatments, the rate was within similar range ($1.88 - 1.97$ $\mu$moles/ min/ g fwt) with no significant difference ($p > 0.05$). Overall there was no significant difference between the photosynthetic rates measured except for the algae with no NO$_3^-$. 

5.2.3 Chlorophyll

There is no significant difference in the measured total chlorophyll content (Fig 5.8) between algae in all treatments ($p = 0.061$ i.e. $p < 0.05$).

![Figure 5.8](image-url)

Figure 5.8 The concentrations of total chlorophyll in *E. flexuosa* and *E. intestinalis* at different levels of NO$_3^-$ in ASM. Data presented is the mean of 4 replicates. Error bars indicate the range of total chlorophyll at each NO$_3^-$ concentration. Total chlorophyll content was measured using Spectrophotometry after extraction with acetone on the 10th day immediately after photosynthesis measurements were completed.
5.2.4 Total soluble protein

Generally higher total soluble protein content (Fig 5.9) was measured for algae with high NO$_3^-$ in 4.2 μmoles/L, 5.6 μmoles/L, 7.0 μmoles/L treatments where the TSP was around 7 mg/ g fwt with no significant difference (p > 0.05). Algae at 1.4 μmoles/L and 2.8 μmoles/L treatments that had TSP around 6.5 mg/g fwt. Algae in 0.7 μmoles/L treatment had slightly lower TSP content (6.2 mg/g fwt). The lowest TSP (5.3 mg/g fwt) was measured for algae with no NO$_3^-$ . TSP in algae with no NO$_3^-$ was significantly lower when compared with 4.2 μmoles/L, 5.6 μmoles/L and 7.0 μmoles/L (p < 0.01) but did not differ significantly from TSP content in 1.4 μmoles/L and 0.7 μmoles/L (p > 0.05).

![Graph showing total soluble protein content](image)

Figure 5.9 The amount of total soluble protein content in *E. flexuosa* and *E. intestinalis* after the treatment at different concentrations of NO$_3^-$ in ASM. Data presented is the mean of 4 replicates. Error bars indicate the range of TSP at each NO$_3^-$ concentration. The protein content was measured after 10 days of treatment using the Bradford method (see Bradford, 1976).

5.2.5 Total soluble carbohydrates

A significant increase in the total soluble carbohydrate content (Fig 5.10) was recorded as the amount of NO$_3^-$ in the medium increased (p < 0.0001 i.e p < 0.05). Highest TSC was
measured for algae in 7 μmoles/L NO$_3^-$ (54 mg/g fwt). Algae at 1.4 μmoles/L, 2.8 μmoles/L, 4.2 μmoles/L NO$_3^-$ treatments which had TSC in the range of 4.32 – 4.97 mg/g fwt. Algae at 0.7 μmoles/L NO$_3^-$ had slightly lower TSC content while the lowest TSC content was measured for algae with no NO$_3^-$.

![Figure 5.10](image)

**Figure 5.10** The amount of total soluble carbohydrates in *E. flexuosa* and *E. intestinalis* after the treatment at different concentrations of NO$_3^-$ in ASM. Data presented is the mean of 4 replicates. Error bars indicate the range of TSC at each NO$_3^-$ concentration. The TSC was measured after 10 days of treatment using the Phenol-Sulphuric method (see Dubois *et. al.*, 1956).

### 5.3 Discussion

The growth experiments both in NH$_4^+$ treatments (Fig 5.1) and in NO$_3^-$ treatments (Fig 5.6) showed an increase in the growth of *Enteromorpha* with an increase in the concentration of nitrogen contents in the medium which means that the growth rates are directly proportional to the variations in the nitrogen supply. Similarly an increase in the photosynthetic rates of *Enteromorpha* in both the treatments was observed with an increase in the nitrogen concentrations. However there was no significant difference between the rates observed. This
suggests that photosynthesis in *Enteromorpha* in presence of nitrogen within the ranges used in the experiment is not as vastly affected by the variable nitrogen content in seawater as was the growth, when the other factors were not limiting. This can be verified by looking at the chlorophyll levels in both treatments (Fig 5.3 and Fig 5.8), where there was no significant difference in the chlorophyll levels. However, both growth and photosynthesis were affected when there was absence of adequate amount of nitrogen. Algae with no NO$_3^-$ showed significantly lower growth rates (Fig 5.6) while there was no significant difference in growth rates with no NH$_4^+$ and low NH$_4^+$. This is because nitrogen is a constituent of many cell components including amino acids, purines, pyrimidines, amino sugars and amines and chlorophyll (Tiaz and Zeiger, 1998; Lobban and Harrison, 1994) and NH$_4^+$ alone in the growth medium (growth media without NO$_3^-$) would not provide the adequate amount of nitrogen for these cell components since it is present in the growth media in lesser amounts than the NO$_3^-$. 

According to Cohen (2002) both species uptake and assimilate NH$_4^+$ in preference to NO$_3^-$. Cohen (2002) found that when ammonium and nitrate were present in equal amounts, *E. intestinalis* preferred to uptake and assimilate ammonium. Ammonium is already in reduced form and can be directly incorporated into amino acids while nitrate has to be reduced intracellularly to ammonium before it can be incorporated. Since no enzymatic reductions are required for ammonium assimilation, the uptake and assimilation rates for ammonium are usually higher than for nitrates.

Nitrate is first reduced to nitrite via \( \text{NO}_3^- + \text{NAD(P)H} + \text{H}^+ \rightarrow \text{NO}_2^- + \text{NAD(P)}^+ + \text{H}_2\text{O} \) the reaction is catalyzed by Nitrate reductase (NR). According to Solomonson and
Barber (1990) cited in Lobban and Harrison (1994) Nitrate reductase may be associated with chloroplast membranes. Nitrate reduction to ammonium requires 8 electrons (8 ferrodoxins) and the reduction is catalyzed by the enzyme nitrate reductase (Davison and Stewart, 1984 cited in Lobban and Harrison, 1994). After formation of nitrite (by reduction of nitrate), it is transported to the chloroplasts for reduction to ammonium via the reaction: 

\[ \text{NO}_2^- + 6\text{Fe}_{\text{red}} + 8\text{H}^+ \rightarrow \text{NH}_4^+ 6\text{Fe}_{\text{ox}} + 2\text{H}_2\text{O} \]

where \( \text{Fe}_{\text{red}} \) and \( \text{Fe}_{\text{ox}} \) are the reduced and oxidized forms of ferrodoxins respectively) that is catalysed by nitrite reductase (NiR). NiR is also thought to be associated with the chloroplast.

![Figure 5.11 showing main features of nitrogen uptake and assimilation in a eukaryotic algal cell. (source: Lobban and Harrison, 1994)](image)

Once the nitrate is reduced to intermediate compounds such as ammonium and amino acids, it is then incorporated into proteins and chlorophyll while incorporation of ammonium is direct. As a result there would be an increase in protein and chlorophyll content as it would then promote cell division hence increasing algal biomass. This is however not evident in the experiment as the TSP is higher in NO\textsubscript{3}\textsuperscript{-} treatments (Fig 5.9) than in NH\textsubscript{4}\textsuperscript{+} treatments (Fig
5.4). The increase or decrease in TSP was not prominent because the algae used for the experiment were already in their adult form growing possibly in much higher nitrogen levels in their natural environment than the levels in ASM used for the experiment.

Preferential uptake of ammonium also means lower energy costs since 8 ferrodoxins is not required (Lara et. al., 1987 cited in Ruckert and Giani, 2004). Preferential uptake of ammonium can be also appreciated by the fact that the nitrate reductase and nitrite reductase are associated with the chloroplasts. It was observed that the algae lost chloroplasts in parts (matured portions usually located on the upper sides) of the thallus during sporulation but still thrived on. This is because they uptake ammonium and incorporate it directly and the process of reduction of nitrates is not required.

There was no significant difference in the carbohydrate content of the algae in NH$_4^+$ treatments, even in the treatment where there was no NH$_4^+$. The treatments with high NH$_4^+$ content also did not show any significant changes. This could have been due to process of conversion of present nitrate to ammonium and being used up the plant, which requires more energy hence the carbohydrates are being used up subsequently to provide the energy for the conversion process. As evident from Chapter 7, the algae are exposed to much higher NH$_4^+$ levels in their natural environment. In nitrate treatments the soluble carbohydrate increased with an increase in the nitrate content. The patterns in both treatments are analogous to the pattern of photosynthesis. In nitrate treatment however ammonium was present as well hence lower energy to convert adequate amounts of nitrate to ammonia is much lower and hence the carbohydrate content is slightly higher (Fig 5.10).
Nitrogen in both higher plants and algae is a key regulator of photosynthetic carbon flow (Elrifri and Turpin, 1986). Ammonium increase in higher plants increases the flow of newly fixed carbon into tricarboxylic acid (TCA) cycle intermediates and amino acids while it decreases the flux into starch and sucrose (Bassham et. al., 1981 cited in Elrifri and Turpin, 1986). Decrease in photosynthetic carbon fixation is due to the competition of metabolites between Calvin cycle and nitrogen assimilation. During nitrogen assimilation, it is likely that the carbon skeletons are derived from the TCA intermediates and to maintain the activity of TCA cycle, triose phosphates are likely to be derived from the chloroplasts. This would result in the decrease of ribulose biphosphate regeneration and therefore result in the decrease of net photosynthetic carbon accumulation (Elrifri and Turpin, 1986). According to Elrifri and Turpin (1986) the regulation of algal carbon flow by nitrogen is significant only under the conditions of nitrogen limitation. However, this is not shown in this experiment since nitrogen is present either as NH₄⁺ or NO₃⁻ in the medium at all time. There was no significant difference in the photosynthesis and TSC content in both ammonium and nitrate treatments while there was an increase in growth and TSP, which suggests that carbon flow was regulated to some extent in this experiment and this could be another reason for no significant difference in the carbohydrate levels.

There was no significant change in the chlorophyll contents although nitrogen is an important component of chlorophyll. This suggests that the biosynthesis of chlorophyll was low or zero at the levels of nitrogen which the algae were exposed to. The initial substrate for tetrapyrrole synthesis inside plastids is the activated form of glutamate (Glu) that is GLU-tRNA^{Glu} which is also used for synthesis of protein hence it could have been that the initial substrate was used up more for protein synthesis rather than for chlorophyll synthesis.
No obvious change in the results obtained for both the treatments could be due to another reason that, algae efficiently store large amounts of nutrients and use them for growth when nutrient supplies are low. This was demonstrated by (Kamer et. al., 2002), they observed significant growth in *E. intestinalis* and *Ulva expansa* regardless of the amount and frequency of nutrient addition. This was highly likely since the algal samples were obtained from the site where the ammonia and nitrate levels in water were much higher than the levels used in the experiment.

Overall it can be said that the experiment does not distinctly show the effects of ammonia and nitrate concentrations on photosynthesis and biochemical composition with minus or plus either one (ammonium or nitrate) in the medium since one is present to cover up for the other. In general the experiment does show that an increase in nitrogen level in either form increases the growth, photosynthesis and biochemical compositions.
CHAPTER 6

EFFECTS OF THE HERBICIDE, DIURON 80, ON Enteromorpha flexuosa
AND Enteromorpha intestinalis:

RESULTS AND DISCUSSION

6.1 Results

6.1.1 Growth

The patterns of growth of algae at different Diuron 80 levels (Fig. 6.1) were quite distinct. There was a significant decrease in the growth rate of algae with an increase in the Diuron 80 concentration in the medium (p < 0.0001 i.e. p < 0.05). The highest growth rate was observed for algae that were grown in the medium containing no Diuron 80 (control), where the growth rate increased rapidly till the last day. Growth rate also increased for algae grown in the medium with minimum Diuron 80 level (0.0001 mg/l), but the increase was not as rapid as in the control. At Diuron 80 level 0.001 mg/l there was a little increase in the growth rate (about 0.01-0.04 g/day) in the first 5 days, after which it gradually decreased. For algae at 0.01 mg/l, the growth rate decreased rapidly to zero after very little increase for the first 5 days. The germlings with Diuron 80 appeared to lose colour towards the last day. Germlings at 0.1 mg/l and 1 mg/l Diuron 80 level showed no grow further growth and appeared completely bleached on the 2nd day. There was a significant difference (p < 0.01 i.e. p < 0.05) between the growth rates at different Diuron concentrations from the 15th day onwards.
Figure 6.1 The growth rates of *E. flexuosa* and *E. intestinalis* (together) at different concentrations of Diuron 80 in ASM. Error bars indicate the range of the growth rates at each concentration. Growth rates were measured as fresh weights of 4 replicates at 5-day intervals over 20 days. The days were counted from the time the germlings became visible on the tiles (4 days).

Figure 6.2 Photosynthesis rates of *E. flexuosa* and *E. intestinalis* at different concentrations of Diuron 80 over 10 days. Data presented is the mean of 12 replicate measurements. Error bars indicate the range of photosynthetic rates at each concentration. Photosynthesis was measured using a Clark-type oxygen electrode fitted with a DW 2 chamber (Hansatech, Kings Lynn, Norfolk, England) at room temperature and light at 100 μmol m$^{-2}$ s$^{-2}$. 
6.1.2 Photosynthesis

The photosynthesis rates (Fig 6.2), like growth rates were inversely proportional to the Diuron 80 levels in the medium and decreased with an increase in the Diuron 80 levels (p < 0.0001 i.e. P < 0.05). The photosynthesis rate was the highest in algae that were not exposed to any Diuron 80 in the medium (control) and showed a gradual increase until the last day. For algae at minimum Diuron 80 level (0.0001 mg/l), there was a rapid decrease in the photosynthesis rate within the first 2 days, after which the decrease became gradual. A rapid decrease in photosynthesis rate was also observed for algae at 0.001 mg/l and 0.01 mg/l until the 5th day, after which photosynthesis ceased. There was no photosynthesis observed for algae at 0.1 mg/l and 1mg/l as the rate of photosynthesis decreased sharply to zero within the first 2 days.

6.1.3 Chlorophyll content

Total chlorophyll content (Fig 6.3) decreased with increasing Diuron 80 levels (p < 0.0001 i.e. p < 0.05). Total chlorophyll was highest in algae at 0.0001 mg/l Diuron 80 which was similar to that of control (p > 0.05). Algae at concentrations (0.001 mg/l – 1 mg/l) had significantly lower chlorophyll concentration than algae at 0.0001 mg/l and the control concentration. However there was no significant difference (p > 0.05) between the lower chlorophyll concentrations obtained at these (0.001 mg/l – 1 mg/l) concentrations.
Figure 6.3 The total chlorophyll content in *E. flexuosa* and *E. intestinalis* at different concentrations of Diuron 80 in ASM. Data presented is the mean of 4 replicates. Error bars indicate the range of total chlorophyll at each concentration. Total chlorophyll was measured using Spectrophotometry after extraction with acetone on the 10th day immediately after photosynthesis measurements were completed.

Figure 6.4 The total soluble protein (TSP) content in *E. flexuosa* and *E. intestinalis* after the treatment at different concentrations of Diuron 80 in ASM. Data presented is the mean of 4 replicates. Error bars represent the range of TSP at each concentration. The protein content was measured after 10 days of treatment using the Bradford method (see Bradford, 1976).
6.1.4 Total soluble protein

The highest total soluble protein content (5.75 mg/g fwt) was measured in the control setup with no Diuron 80 (Fig 6.4). The TSP content decreased significantly with increasing levels of Diuron 80 (p < 0.0001 i.e p < 0.05). Algae at 0.0001 mg/l had about 4.5 mg/ g fwt TSP that was less than the control but more than the content in algae exposed to higher Diuron 80 levels. There was no significant difference between TSP at 0.0001 mg/l and 0 mg/l. Algae at 0.001 mg/l and 0.01 mg/l Diuron 80 had similar TSP content (3.2 – 3.4 mg/g fwt) while TSP algae at 0.1 mg/l and 1 mg/l Diuron 80 had slightly lower TSP with algae at 1 mg/l having the lowest TSP of 2.4 mg/g fwt. However there was no significant difference in TSP in algae from 0.001 mg/l – 1 mg/l (p > 0.05).

6.1.5 Total soluble carbohydrates

The total soluble carbohydrate (Fig 6.5) decreased with increasing Diuron 80 levels (p < 0.0001 i.e p < 0.05) in the medium similar to the pattern seen in TSP. The highest TSC (3.9 mg/ g fwt) was measured in algae in the control setup with no Diuron 80 followed by algae at minimum Diuron 80 level (0.0001 mg/l) that had 3.2 mg/g fwt. Algae at 0.001 mg/l, 0.01mg/l and 0.1 mg/l had significantly lower TSC than the algae at control and 0.0001 mg/l. Algae at 1 mg/l Diuron 80 level had the lowest TSC content of 2 mg/g fwt. There was no significant difference (p > 0.05) between TSC content in algae from 0 – 0.001 mg/l.
6.2 Discussion

The growth experiments (Fig 6.1) showed maximum and healthy growth of *Enteromorpha* under control conditions where there was no presence of Diuron 80. Otherwise the pattern of growth (Fig 6.1) was very distinct and inversely proportional to the Diuron 80 concentration in the artificial seawater medium. The growth pattern was similar to the observed rates of photosynthesis of the adult thalli (Fig 6.2) where the highest photosynthesis rate was recorded for algae in the medium with no Diuron 80. However, at minimum Diuron 80 level, increase in the algal growth and photosynthetic rates of the adult thalli were brought about gradually. All the figures show that the largest effect was when Diuron 80 was introduced to the algae. While at lowest concentration, the algae were able to survive, at higher concentrations growth and photosynthesis ceased and bleaching was evident.
Diuron 80 is a broad spectrum herbicide used for controlling broadleaf and grass weeds in many crops. Diuron 80 is chemically named N-(3,4-dichlorophenyl)-N,N-dimethylurea (C₆H₁₀C₁₂N₂O) (Moncada, 2004). Diuron 80 is usually used for control of pre-emergent and post-emergent control of both broadleaf and annual grassy weeds. It is widely used in agriculture on wide variety of crops. In addition, it is widely used for vegetation control in non-agricultural applications, specially industrial and wayside uses which include fence lines, pipelines, powerlines, railway lines, roads, footpaths, in timber yards and storage areas, around commercial, industrial and farm buildings, electrical substations and petroleum storage tanks (Moncada, 2004). The possibility of ‘offsite’ movement of herbicides is quite high especially in areas where there is frequent and high rainfall. Usually this ‘offsite’ movements end up contaminating the flanking aquatic environments.

Powell et al. (1996) studied off-site movement of Diuron 80 in surface water from a highway wayside application and found that the concentrations of Diuron 80 in runoff (water and sediment combined) were as high as 1770 ug/ l after one day of herbicide application. Typical reporting limits for the Diuron 80 analyses in California Department of Pesticide Regulation’s surface water database are 0.05- 0.1 ug/ l and out of the concentrations detected in the range of 0.01 –30.6 ug/ l, majority of concentrations range from 0.1 – 1 ug/ l (Monacada, 2006).

In plants, Diuron 80 is easily taken up from the soil via the root system and xylem. It is taken less rapidly through the leaves and stem, translocation from roots to shoots through xylem is rapid, and translocation via phloem is very little to nil (Hess and Warren, 2002 cited in Monacada, 2006). Diuron 80 primarily functions by inhabiting the Calvin cycle in
photosynthesis. It also limits the production of high energy compounds like adenosine triphosphate (ATP) that is used for various metabolic processes.

Ferrell et al. (2004) showed that Diuron 80 allowed normal seed germination when used on pre-emergent weeds but causes chlorophyll loss which inhibited photosynthesis and the plants died of starvation. Loss of chlorophyll occurred in algae which had Diuron 80 concentration 0.001 mg/l, 0.01 mg/l, 0.1 mg/l and 1 mg/l, with 0.1 mg/l and 1 mg/l having the greatest amount of bleached thalli on the 10th day of treatment and chlorophyll content measured was low (Fig 7.4). Destruction and loss of chlorophyll is the primary symptom of Diuron 80 phytoxicity (Ridley, 1977). Chlorosis is due to the inability of chlorophyll to dissipate its absorbed light energy which leads to irreversible photo-destruction (Heath and Packer, 1968 cited in Ridley, 1977). This photo-destruction is brought about by the breakdown or overloading of the mechanism that usually protects the chloroplasts from excessive illumination. The total chlorophyll content (Fig. 6.3) however was slightly lower in the control setup (with no Diuron 80) than algae at minimum Diuron 80 (0.0001 mg/l). Lower pigment content resulted due to sporulation and the release of zoospores from the cells. No sporulation was observed for algae with Diuron 80 treatments although the cells appeared bleached.

Inhibition of photosynthesis by Diuron 80 involves the blockage of electron transport from QA to QB (electron acceptor molecules in the electron transport chain) (Barr and Crane, 2005; Salisbury and Ross, 1992). Diuron 80 binds to the secondary electron acceptor QB-binding niche on D1 protein of the PS II complex in the thylakoid membranes. This causes a decrease in the oxidation reduction potential of QB with respect to the primary electron
acceptor QA hence it becomes hard for QA to reduce QB (Allen et. al., 1983). Studies on inhibition of photosynthesis electron transport by Diuron 80 using a cyanobacterium, *Aphanocapsa* 6714, showed that Diuron 80 acted at the same step of the electron transport in cyanobacteria and higher plants (Astier et. al., 1981) hence it can be said that Diuron 80 acts on the same step in *Enteromorpha* as well. The inhibition of electron transport further puts a stop to the CO₂ fixation and the production of ATP hence inhibiting photosynthesis hence reducing the carbohydrate content. The total soluble carbohydrate content in *Enteromorpha* (Fig 6.5) at different Diuron 80 concentration is analogous to the amount of photosynthesis at different Diuron 80 levels and the TSC is significantly lower even at the minimum Diuron 80 concentration.

The total soluble protein content as well is significantly lower in the algae that had Diuron 80 in the medium (Fig 6.4). The low protein content can be accounted on the basis of the electron transport blockage. The inability to reoxidize QA advances formation of triplet state chlorophyll which interacts with oxygen in a ground state to form singlet oxygen. Both triplet chlorophyll and singlet oxygen are capable of extracting hydrogen molecules from unsaturated lipids which produces a lipid radical and initiates a chain reaction of lipid peroxidation. Hence lipids and proteins are attacked during the electron transport blockage and oxidized resulting in chlorophyll and carotenoids loss together with leaky membranes which causes cells and cell organelles to dry out and disintegrate rapidly (Hess and Warren, 2002 cited in Monacada, 2006).
Overall, it can be said that the effect of Diuron 80 on *Enteromoprha* is drastic even at minimum concentration. It primarily affects photosynthesis, which in turn inhibits the overall growth of the algae.
CHAPTER 7

ANALYSIS OF Enteromorpha flexuosa AND Enteromorpha intestinalis FROM 3 SITES AROUND THE SUVA AREA (NASESE, LAMI AND LAUCALA BEACH):

Results and Discussion

7.1 Results

The average temperature was almost the same for all 3 sites. pH was highest in Nasese area while Lami had the lowest. There was no significant difference in the pH at Laucala Beach and Nasese area. The levels of both forms of nitrogen (NH$_4^+$ and NO$_3^-$) in the water samples obtained from the three sites were much higher. While samples from Lami area had relatively low NH$_4^+$ (1.9 µmoles/L), it was still 4 times higher than that in the ASW. Those from the other two sites were about 10 times higher. The NO$_3^-$ values in the water samples from the three areas were approximately twice the concentration found in the ASW.

Table 7.1 Randomly measured physical variable from the three sites taken during the course of the experiment.

<table>
<thead>
<tr>
<th></th>
<th>Lami</th>
<th>Laucala Beach</th>
<th>Nasese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Temperature (°C)</td>
<td>25</td>
<td>26</td>
<td>25</td>
</tr>
<tr>
<td>Average pH</td>
<td>7.7</td>
<td>8.4</td>
<td>8.6</td>
</tr>
<tr>
<td>Average NH$_4^+$ (µmoles/L)</td>
<td>1.9</td>
<td>5.1</td>
<td>4.5</td>
</tr>
<tr>
<td>Average NO$_3^-$ (µmoles/L)</td>
<td>3.7</td>
<td>2.9</td>
<td>2.9</td>
</tr>
</tbody>
</table>
7.1.1 Photosynthesis

Highest rates of photosynthesis (Fig 7.1) in *E. intestinalis* and *E. flexuosa* were observed in algae from the Laucala Beach area, the observed rates are significantly higher (p < 0.0001 i.e. p < 0.05) than the rates in Nasese or Lami area. The average recorded photosynthetic rates for all the samples were in the range of 1.05 – 1.3 μmoles/ min/ g of fwt. Algae in the Lami area had slightly lower photosynthetic rates than the algae at Nasese but the difference was not significant (p > 0.05). In two out of the five analyses done, algae at Nasese and Lami had similar rates. Overall, photosynthetic rates of algae from Lami and Nasese were similar, while that from Laucala Beach was much higher, although showed fluctuations in this high range.

Figure 7.1 The photosynthesis rates of *E. flexuosa* and *E. intestinalis* (together) from Nasese, Lami and Laucala Beach area measured randomly over a 12 months. Data presented is the mean of 8 replicates. Error bars indicate the range of photosynthetic rate for each site. The samples were taken whenever the algae were available at all 3 sites. Photosynthesis was measured using a Clark-type oxygen electrode fitted with a DW 2 chamber (Hansatech, Kings Lynn, Norfolk, England) at room temperature and light at 100 μmol m⁻² s⁻¹.
7.1.2 Chlorophyll content

The algae from Laucala Beach recorded the highest level of chlorophyll in all five analyses with some fluctuations. The total chlorophyll content is significantly higher ($p < 0.01$ i.e. $p < 0.05$) than in the Nasese and Lami areas. Algae from Nasese and Lami had the average chlorophyll in the range of 115 – 140 μg/ ml/ g fwt which was lower than that in algae from Laucala Beach. There was no significant difference between the total chlorophyll recorded in Lami and Nasese area ($p > 0.05$).

![Figure 7.2](image)

**Figure 7.2** The concentrations of total chlorophyll in *E. flexuosa* and *E. intestinalis* from Nasese, Lami and Laucala Beach area measured randomly over a 12 month period. Data presented is the mean of 4 replicates. Error bars indicate the range of total chlorophyll at each site. The samples were taken randomly whenever the algae were available at all 3 sites. Total chlorophyll was measured using spectrophotometry after extraction with acetone on the 10th day immediately after photosynthesis measurements were completed.
7.1.3 Total Soluble Protein

Total soluble protein (TSP) measured (Fig 7.3) overall was highest in algae from Laucala Beach (between 6.5 – 7.5 mg/ g fwt) followed by algae in Nasese area. The TSP recorded in both areas was not significantly different (p > 0.05). Algae from Lami showed significantly low (p < 0.001 i.e. p < 0.05) TSP contents ranging from approximately 1.5 – 2.5 mg g fwt, corresponding to the lowest photosynthesis rates.

![Figure 7.3](image)

**Figure 7.3** The total soluble protein content in *E. flexuosa* and *E. intestinalis* from Nasese, Lami and Laucala Beach area measured randomly over a 12 month period. Data presented is the mean of 4 replicates. Error bars indicate the range of TSP for each site. The samples were taken randomly whenever the algae were available at all 3 sites. The protein content was measured using the Bradford method (see Bradford, 1976).

7.1.4 Total soluble carbohydrates

That the total soluble carbohydrate (TSC) measured was highest in *Enteromorpha* at Nasese. Algae from Laucala Beach area had moderate amounts of TSC in the range of 6.7 – 7.5 mg/ g fwt. Algae collected from Lami had the lowest TSC in the range of 5.3 – 6.3 mg/ g fwt. There is a significant difference in the TSC content of algae from the 3 sites (p < 0.01 i.e. p < 0.05).
Figure 7.4 The total soluble carbohydrate content in *E. flexuosa* and *E. intestinalis* from Nasese, Lami and Laucala Beach area measured randomly over a 12 month period. Data presented is the mean of 4 replicates. Error bars indicate the range of TSC obtained for each site. The samples were taken whenever the algae were available at all 3 sites. The TSC was measured using the Phenol-Sulphuric method (see Dubois *et al.*, 1956).

### 7.2 Discussion

The results show that there is significant difference in the recorded photosynthesis rate (p < 0.0001), total chlorophyll content (p < 0.0001), TSP content (p < 0.0001) and TSC content (p < 0.0001). p < 0.05 in all the cases. The results show optimum growth of *Enteromorpha* in the Laucala Beach area. This is evident from the measured high photosynthesis (Fig 7.1), high chlorophyll (Fig 7.2) and high total soluble protein content (TSP) (Fig 7.3). Algae from Nasese area had moderate photosynthetic rate, chlorophyll and TSP but the highest levels of total soluble carbohydrates (TSC) (Fig 7.4) while algae from Lami area had low photosynthetic rate, total chlorophyll, TSP and TSC contents.
The credit of most favourable growth of *Enteromorpha* in Laucala Beach area could be given to the high nutrient (nitrogen) content (in the form of NH$_4^+$) in the area since it is situated near the sewage treatment plant. The high nitrogen content is reflected in the high TSP of algae from this area. High TSP content (Fig 7.3) results in biomass accumulation as well as an increase in proteins that takes part in photosynthesis hence increasing photosynthetic activity (Fig 7.1). However in two of the analysis, algae from Nasese have similar TSP (high content) as the algae from Laucala Beach. This is because nutrient content could be high at Nasese as well since it does have drainage outfalls that open up in its intertidal area. On an average, water from the Nasese area had less NH$_4^+$ than the Laucala Beach water, although NO$_3^-$ contents were the same.

The carbohydrate content however was highest in algae from the Nasese area, which indicates a more healthy growth than Laucala Beach and Lami. Though photosynthesis and TSP and chlorophyll were high at Laucala Beach, the carbohydrate content was lower than the Nasese area. This could be because of increased transfer of photosynthetically incorporated carbon to synthesis of amino acid skeletons at the expense of sucrose synthesis, which results during nitrate reduction and ammonia incorporation (Platt, 1977; Elrifi and Turpin, 1986). Nitrogen plays an important role in the photosynthetic carbon flow in both higher plants and algae (Elrifi and Turpin, 1986). However the nutrient levels can fluctuate on short time scales, hence it is difficult to relate the physiological changes found during the experiment in terms of nitrogen availability. *Enteromorpha* usually grows where there is high nitrogen level in the water.
A fluctuation in nutrient content at Laucala Beach was evident as *Enteromorpha* did not occur continuously over the period of research; it cropped up, flourished a few times while the site did not have any algae at other times. Similar patterns were observed at Nasese. The site at Lami remained bare (without *Enteromorpha* growth) for a longer period of time.

Algae from Lami area according to the results obtained were the unhealthiest with low photosynthesis, TSP and TSC. The chlorophyll content however was similar to algae in Laucala Beach and Nasese. This is obvious as the algal growth observed in the area was very little and whatever growth occurred was after very longer interval compared to the other two sites. Lami area also had lower nitrogen content than the other two sites which could be a factor contributing to low growth. However growth of germlings in ASW (Chapter 5, Fig 5.1) around that concentration showed considerably increased growth. Hence it can be said that apart from that the algae here could be affected due to pollution which includes, thermal pollution, industrial and factory effluents, heavy metals, antifouling compounds and oil pollution. Thermal pollution that could have resulted from factory effluents and from heating up of metal scraps lying in the area, posed temperature related stress (chapter 4) that could have resulted in low growth of algae in the area. However, there was no significant difference in the temperature in all 3 sites; hence temperature could not definitely be the factor affecting the growth. Industrial chemicals such as Polychlorinated biphenyls (PCBs) are toxic to aquatic organism (Lobban and Harrison, 1994). The PCBs can inhibit photosynthesis at a site on the electron transport chain, close to PS II (Sinclair *et. al.*, 1977). Furthermore, antifouling compound such as triphenyltin (TPT) and tributyltin (TBT) that are used as antifouling compositions on boat hulls could be posing an effect on *Enteromophrha* in the area. Antifouling compounds are highly effective against *Enteromorpha* species as the
photosynthetic apparatus of zoospores and the vegetative tissues of *Enteromorpha* are very sensitive to it (Lobban and Harrison, 1994). Antifouling compounds from the Suva harbour area provide a good reason for low growth of *Enteromorpha* in the Lami area. *Enteromorpha* and *Ulva* are usually the target species for antifouling compositions; it does however affect the other non-target organisms including fish and oysters but at low levels. This can be a matter of concern as people residing in the area consume organisms such as fish and oysters. Similarly, oil pollution from the harbour area could be another reason for low algal growth. Petroleum or crude oil consists of a mixture of hydrocarbons and some additional compounds containing O, S and N and metals such as Ni, V, Fe and Cu (Preston, 1988). Algae are affected by oil pollution in two ways: one associated with the coating of the organism which reduces CO₂ diffusion and light penetration, secondly due to the uptake of hydrocarbons which leads to disruption of cell metabolism (Lobban and Harrison, 1994). *Enteromorpha* could have been affected by oil pollution to some extent possibly due the uptake of whatever hydrocarbons were present in the water. Heavy metals in addition are one of the major sources of pollution in the Lami area. *Enteromorpha* can bioaccumulate heavy metals (Tabudravu, 1998). The order of metal toxicity in algae are Hg > Cu > Cd > Ag > Pb > Zn, but it varies with algal species and environmental conditions (Rai *et al.*, 1981). The toxic effects generally include growth cessation in extreme cases, inhibition of photosynthesis, reduction of chlorophyll content, increased cell permeability and disruption of enzyme activity (Lobban and Harrison, 1994). Presence of Cu in the area could be possibly resulting from the antifouling agents as well, which inhibits reproduction and growth of *Enteromorpha*.

When looking at the possible means of pollution in all the 3 sites, Lami area appears to be the most polluted in terms of other pollutants. It does have the highest NO₃⁻ levels compared to
the other 2 sites but much lower NH$_4^+$. *Enteromorpha* in this area complements the results obtained as its photosynthesis, TSP and TSC were lower than the other two sites. In addition the growth of algae was very low throughout the duration of the experiment and the algae did not appear healthy when compared to the other two sites. During two visits to Lami, most of the existing algae appeared bleached (whole frond bleaching). Bleaching and loss of colour was also observed in Nasese but loss of colour here could be accounted by the sporulation process, as only part of the thalli picked appeared bleached while part of it remained green and healthy.

Nasese area had significant amount of pollution via small amount of sewage discharge and runoffs from the drainage outfalls, soap and detergent contaminated water from the household drainage. Yet the algae here appeared healthy and were in larger growth forms. Significant growth in Nasese can be related to the increase in alkalinity, which probably resulted from the household drainage. The pH experiments (chapter 4) illustrated that *Enteromorpha* growth was highest at pH 9. In addition it had high nutrient supply in the form of NH$_4^+$ from the drainage outfall.

In Laucala Beach the presence and flourishing of *Enteromorpha* can be attributed to the high nitrogen content especially in the form of NH$_4^+$ resulting from sewage discharge from the near by sewage treatment plant (Table 7.1). The effects of nutrients in the area seemed to mask of the effects of other pollutants in the area.

Overall, it can be said that the algae in these three different sites are affected to some extent by their environment and the pollution within their environment. The most striking
observation is that the high levels of NH$_4^+$ influenced algal growth and photosynthesis, perhaps masking the effects of other pollutants in Laucala Beach and Nasese, while in Lami in the presence of relatively low levels of NH$_4^+$, other pollutants appeared to have an adverse effect on the growth of these algae.
CHAPTER 8

SIGNIFICANCE OF THE RESEARCH, CONCLUSIONS AND
RECOMMENDATIONS.

8.1 Research significance and conclusions.

Overall, the results obtained from the research are consistent with the hypothesis that Enteromorpha flexuosa and Enteromorpha intestinalis are affected by the variations in their abiotic environment that pose stressful conditions. From the obtained growth rate in germlings and photosynthesis rate, total soluble protein, and total soluble carbohydrates and chlorophyll content whilst exposure of Enteromorpha to variations in pH, temperature, nitrogen (nitrate and ammonium) and herbicide in seawater, it was apparent that while some stresses (temperature and herbicide) had an adverse effect on algal growth, high alkalinity and high nitrogen levels promoted the growth of algae.

From the pH experiment (chapter 3), it was evident that Enteromorpha preferred higher pH for growth and its optimal metabolic activities and growth and metabolic activities were low at unfavourable pH. The optimum pH was in the range 8 – 9. The optimum temperature for Enteromorpha was between 20 – 25°C. There was significant decrease in growth and photosynthesis in both the experiments where the range was unfavorable. When looking at herbicide experiments (chapter 6), minimal growth and an apparent decrease in photosynthesis together with chlorophyll, TSP and TSC were obtained even with the minimum level of exposure. Nitrogen experiments (chapter 5) however did not portray clear consequences except that increase in nitrogen content in either ammonia (NH₄⁺) or nitrate (NO₃⁻) form prospers growth and metabolic activity in general. Its effect is more explicit
when looking at the TSP content in this case. Growth and photosynthesis both increase significantly in relation to the level of ammonia and nitrate and are slightly low only in absence of ammonia and nitrate. The lack of clarity in the response of the algae to the two forms of nitrogen in chapter 5 is probably because of the low levels of NH$_4^+$ and NO$_3^-$ used in the experiment. As it can be seen from chapter 7, NH$_4^+$ in Laucala Beach and Nasese were at least 10 times the control levels in the ASW. The effect of NH$_4^+$ in these areas is highly evident in chapter 7.

Most of the evidence that was obtained from the laboratory experiment is complemented by Enteromorpha in the natural environment (chapter 7). However, the productivity (the measured photosynthesis rate, total chlorophyll, TSP and TSC) is lower in samples analysed from the laboratory experiments (chapter 3, 4, 5 and 6) compared to the samples from the sites (natural environment) (chapter 7). The observed difference is due to the laboratory environment conditions being dissimilar from the natural environment hence lower productivity.

Photosynthesis and TSP, TSC were low in Lami area, which was assumed to be the most pollution affected area although NH$_4^+$ levels were relatively low. In Laucala Beach area which had high nutrient content particularly nitrogen had high photosynthesis, chlorophyll and TSP content while in Nasese area, Enteromorpha had moderate photosynthetic rate, chlorophyll, TSP and TSC content.

The research shows that growth measurements, photosynthetic rates, protein contents and carbohydrate contents give a clear account of changes in functional physiology brought about
by the stress created via changes in the pH, temperature and nutrient content of the habitat that could result from point source and non-point source pollution. Chlorophyll, on the other hand does not provide clear evidence of physiological damage as does photosynthesis, TSP and TSC in stressed and non-stressed algae in the natural environment have similar chlorophyll levels (chapter 7) unless the pollution is severe as in the case of herbicide contamination in significant amount (chapter 6) and thermal pollution (chapter 4) which resulted in bleaching. *Enteromorpha* collected from Nasese area had lower chlorophyll content when compared to Lami and Laucala Beach area (chapter 7) but looked the healthiest out of the three sites. Nasese area was assumed to be the least polluted one and was used as the reference site. However, NH$_4^+$ levels were very high here. Algae from Lami area on the other hand had similar levels of chlorophyll as algae in Nasese area but appeared the unhealthiest and had low metabolic activity in terms of photosynthesis, TSP and TSC.

This research illustrates that *Enteromorpha* can be used as a bioindicator for marine pollution in Fiji with respect to high nitrogen content (especially for high NH$_4^+$ levels). *Enteromorpha* presence in any intertidal zone itself indicates high level of nitrogen. It also appears to indicate high pH levels. Moreover, these algae are adversely affected by unfavourable temperature and herbicides.

Since *Enteromorpha* is a opportunist algae, it prefers and is well adapted to grow in coastal areas where there is pollution (chapter 1, section 2.1). Hence, logically it would exhibit stress responses when the pollution level is significant to it and beyond its tolerance range, which for other existing life forms could be very much harmful. It could be more harmful than it is to *Enteromorpha*. Since, *Enteromorpha* is a fast growing alga and responds rapidly to water
quality changes, it can efficiently be used to spot and detect polluted environments before other life forms are vastly affected. Its growth rate can also be effectively used as indicators of environment health apart from its photosynthetic rates, chlorophyll, TSP and TSC since in the growth experiment carried out in lab it grew very fast under optimal condition and its growth rate was slow in unfavorable conditions. Growth rates can be easily monitored by random sampling of algae in pollution predicted areas. However to use these stress responses, a reference ‘unpolluted’ site should be chosen to compare the physiological parameters.

9.2 Future work recommendations

- The work done was of very small scale. When done on a much larger scale that is, with natural light, bigger growth area (outside tanks instead of beakers) and using more natural substrates such as rocks etc would allow obtaining more prominent and precise results.

- Work of this approach would give more precise account of stress physiology if possibly carried out in-situ.

- Detailed aspects of stress physiology could be studied in terms of enzyme activity, respiration, various metabolic pathways and biochemistry with a similar experimental approach.

- The same laboratory experiments can be done together with other macro-algae from the same habitat to compare the responses and pick out the most efficient algae to be used as bioindicators.
REFERENCES


APPENDIX

A 1.1 Preparation of Artificial Seawater Medium (Brand, 1984)

Artificial Seawater Medium was prepared using the following ingredients:

- 925 ml of glass distilled water
- 18 g NaCl
- 10 ml MgSO\(_4\).7H\(_2\)O
- 10 ml KCl
- 10 ml NaNO\(_3\)
- 10 ml CaCl\(_2\).2H\(_2\)O
- 10 ml KH\(_2\)PO\(_4\)
- 10 ml Tris buffer (Sigma Co.)
- 1 ml NH\(_4\)Cl
- 1 ml vitamin B12
- 10 ml PI metal solution
- 3 ml Chelated iron solution
Table A 1.1. Functions and compounds of the essential elements in seaweeds.

<table>
<thead>
<tr>
<th>Element</th>
<th>Probable functions</th>
<th>Examples of compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td>Major metabolic importance in compounds</td>
<td>Amino acids, purines, pyrimidines, amino sugars, amines</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>Structural, energy transfer</td>
<td>ATP, GTP, etc., nucleic acids, phospholipids, coenzymes (including coenzymes A), phosphoenolpyruvate</td>
</tr>
<tr>
<td>Potassium</td>
<td>Osmotic regulation, pH control, protein conformation and stability</td>
<td>Probably occurs predominantly in ionic form</td>
</tr>
<tr>
<td>Calcium</td>
<td>Structural, enzyme activation, cofactor in ion transport</td>
<td>Calcium alginate, calcium carbonate</td>
</tr>
<tr>
<td>Magnesium</td>
<td>Photosynthetic pigments, enzyme activation, cofactor in ion transport, ribosome stability</td>
<td>Chlorophyll</td>
</tr>
<tr>
<td>Sulphur</td>
<td>Active groups in enzymes and coenzymes, structural</td>
<td>Methionine, cystine, glutathion, agae, carrageenan, sulfolipids, coenzyme A</td>
</tr>
<tr>
<td>Iron</td>
<td>Active groups in porphyrin molecules and enzymes</td>
<td>Ferredoxin, cytochromes, nitrate reductase, nitrite reductase, catalase</td>
</tr>
<tr>
<td>Manganese</td>
<td>Electron transport in photosystem II, maintenance of chloroplast membrane structure</td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>Electron transport in photosynthesis, enzymes</td>
<td>Plastocyanin, amine oxidase</td>
</tr>
<tr>
<td>Zinc</td>
<td>Enzymes, ribosome structure (?)</td>
<td>Carbonic anhydrase</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>Nitrate reduction, ion absorption</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td>Sodium</td>
<td>Enzyme activation, water balance</td>
<td>Nitrate reductase</td>
</tr>
<tr>
<td>Chlorine</td>
<td>Photosystem II, secondary metabolites</td>
<td>Violacene</td>
</tr>
<tr>
<td>Boron</td>
<td>Regulation of carbon utilization (?), ribosome structure (?)</td>
<td></td>
</tr>
<tr>
<td>Cobalt</td>
<td>Component of vitamin B_{12}</td>
<td>B_{12}</td>
</tr>
<tr>
<td>Bromine*</td>
<td>Toxicity of antibiotic compounds (?)</td>
<td>Wide range of halogenated compounds, especially in Rhodophyceae</td>
</tr>
<tr>
<td>Iodine*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Possibly an essential element in some seaweed.

**Modified from Lobban and Harrison, (1994).**
Table A 1.2 Concentrations of some essential elements in seawater and in seaweeds.

<table>
<thead>
<tr>
<th>Element</th>
<th>Mean concentration in seawater (mmol kg⁻¹)</th>
<th>Range Concentration in dry matter μg g⁻¹</th>
<th>Ration of concentration in seawater to concentration in tissue</th>
<th>Concentration in dry matter (μg g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>105,000</td>
<td>10,500</td>
<td>49,500</td>
<td>22,000-72,000</td>
</tr>
<tr>
<td>Mg</td>
<td>53.2</td>
<td>1,293</td>
<td>7,300</td>
<td>1,900-66,000</td>
</tr>
<tr>
<td>S</td>
<td>28.2</td>
<td>904</td>
<td>19,400</td>
<td>4,500-8,200</td>
</tr>
<tr>
<td>K</td>
<td>10.2</td>
<td>399</td>
<td>41,100</td>
<td>30,000-82,000</td>
</tr>
<tr>
<td>Ca</td>
<td>10.3</td>
<td>413</td>
<td>14,300</td>
<td>2,000-360,000</td>
</tr>
<tr>
<td>C</td>
<td>2.3</td>
<td>27.6 a, b</td>
<td>274,000</td>
<td>140,000-460,000</td>
</tr>
<tr>
<td>B</td>
<td>0.42</td>
<td>4.50</td>
<td>184</td>
<td>15-910</td>
</tr>
<tr>
<td>N</td>
<td>0.03</td>
<td>0.420 a, c</td>
<td>23,00</td>
<td>500-65,000</td>
</tr>
<tr>
<td>P</td>
<td>0.002</td>
<td>0.071</td>
<td>2,800</td>
<td>300-12,000</td>
</tr>
<tr>
<td>Zn</td>
<td>6 x 10⁻⁶</td>
<td>0.0004 a</td>
<td>90</td>
<td>2-680</td>
</tr>
<tr>
<td>Fe</td>
<td>1 x 10⁻⁶</td>
<td>0.000006 a</td>
<td>300</td>
<td>90-1,500</td>
</tr>
<tr>
<td>Cu</td>
<td>4 x 10⁻⁶</td>
<td>0.0002 a</td>
<td>15</td>
<td>0.6-80</td>
</tr>
<tr>
<td>Mn</td>
<td>0.5 x 10⁻⁶</td>
<td>0.00003 a</td>
<td>50</td>
<td>4-240</td>
</tr>
</tbody>
</table>

* = Considerable variation occurs in seawater (Bruland 1983).

b = Dissolved organic carbon.

* = Combine nitrogen (dissolved organic and inorganic).

Modified from Lobban and Harrison, (1994).
Plate A 1.1 *Enteromorpha flexuosa* and *E. intestinalis* associations growing near a drainage outfall at Nasea, Suva.

Plate A 1.2 Adult algae exposed to stress in a beaker with ASM. (a) Healthy algae with sporulation (spores stuck and germinating on the sides of the beaker). (b) Unhealthy algae with no sporulation (no spores on the sides of the beaker).