University of Plymouth

PEARL **https://pearl.plymouth.ac.uk**

04 University of Plymouth Research Theses 01 Research Theses Main Collection

2018

The effects of selected herbicides and booster biocides on the brown seaweed Ectocarpus siliculosus

Bin Hashim, Mohd Akmal

http://hdl.handle.net/10026.1/11298

http://dx.doi.org/10.24382/509
University of Plymouth

All content in PEARL is protected by copyright law. Author manuscripts are made available in accordance with publisher policies. Please cite only the published version using the details provided on the item record or document. In the absence of an open licence (e.g. Creative Commons), permissions for further reuse of content should be sought from the publisher or author.

The effects of selected herbicides and booster biocides on the brown seaweed *Ectocarpus siliculosus*

By

Mohd Akmal Bin Hashim

A thesis submitted to the University of Plymouth in partial fulfilment for the degree of

Doctor of Philosophy

School of Biological and Marine Sciences

December 2016

STATEMENT OF COPYRIGHT

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without the author's prior consent.

The effects of selected herbicides and booster biocides on the brown seaweed *Ectocarpus siliculosus*, by Mohd Akmal Bin Hashim

ABSTRACT

In the present study, two strains of *Ectocarpus siliculosus* with different pollution histories, LIA4 and Es524, were exposed to three PSII inhibitor herbicides, diuron (DIU), terbuthylazine (TBA) and isoproturon (IPU). Evaluation of their effects on growth and photosynthetic efficiency (Fv/Fm, ΦPSII, qP) have demonstrated negative impacts of all herbicides on both strains. With reference to the 7 d RGR EC_{50} values; DIU (9.9 - 25 µg L^{-1}); TBA (18 – 28 µg L^{-1}); IPU (257 – 315 µg L^{-1}), the reported environmental concentrations for DIU were higher than the values that exert negative effects to *E. siliculosus*. However, as for TBA and IPU, the environmental concentrations were below the values which caused detrimental effects to *E. siliculosus*. Strain Es524, which originated from a Cu-polluted site in Chile, was found to exhibit greater resistance to the herbicides, with higher EC_{50} values recorded, compared to LIA4. To further elucidate the factors contributing to the relative tolerances of the two strains, responses associated with reactive oxygen species (ROS) were investigated. Measurements of H_2O_2 concentrations and lipid peroxidation showed significant differences between the strains, with increases in both parameters recorded at lower concentrations in LIA4 than Es524. Activities of antioxidant enzymes (CAT, APX and GR) were significantly ($P < 0.05$) greater in Es524 than LIA4, and total phenolic content and DPPH scavenging activity were also greater in the more tolerant strain. The rank order of toxicity of the three herbicides was diuron > terbuthylazine > isoproturon in both strains of *E. siliculosus*. In addition to exposure to individual herbicides, the interactions between binary mixtures were also investigated in both strains using physiological and biochemical biomarkers. All three ways of action (synergistic,

additive and antagonistic) were exhibited through different endpoints applied in the present study. Significant differences ($P < 0.05$) between LIA4 and Es524 were observed for the DIU+TBA and TBA+IPU mixtures, while higher impacts were recorded in LIA4 strain compared to Es524. Further studies showed synergistic interactions were observed in Es524 for the aforementioned mixtures (DIU+TBA, TBA+IPU) on the antioxidative enzyme activities while in LIA4 different interactions were exhibited, which probably contribute to the higher tolerance of Es524 to the mixtures. The presence of TBA together with the phenylureas DIU/IPU was also observed to increase the stimulation of antioxidative enzymes (CAT, APX, GR) in both strains of *E. siliculosus*. This investigation provides new information on the abiotic stress metabolism in brown algae, and HPLC analysis demonstrates the important role of polyphenols in overcoming the impact of oxidative stress. In conclusion, exposure to the herbicides, singly and in mixtures, caused significant ($P < 0.05$) changes in the growth, photosynthetic efficiency, and ROM of both strains of *E. siliculosus*. Strain Es524 was found to be more tolerant than LIA4.

TABLE OF CONTENTS

TITLE PAGE…….. i

STATEMENT OF COPYRIGHT ……. ii

ABSTRACT…….…. iii

TABLE OF CONTENTS……..……. v

LIST OF TABLES…….….... xii

LIST OF FIGURES…….….… xv

ACKNOWLEDGEMENTS…… xxi

AUTHOR'S DECLARATION……...… xxii

LIST OF ABBREVIATIONS……..….. xxiii

Chapter 1: Introduction and literature review…… 1

1.1 Marine pollution: a global issue 2

1.2 Herbicides: a need that needs to be evaluated 3

1.2.1 Phenylurea: Isoproturon (IPU) 5

1.2.2 Triazine: Terbuthylazine (TBA) 16

1.3 Antifouling (AF) booster biocides 26

1.3.1 Diuron (DIU) 28

1.4 Marine macroalgae as an important entity in the environment 37

1.4.1 *Ectocarpus siliculosus* (brown algae) 39

1.4.2 The life cycle of *E. siliculosus* 43

1.4.3 Responses to biotic and abiotic stresses 44

1.5 Objectives of the study 45

Chapter 2: General Materials and Methods ……..…46

2.1 Prerequisite preparation for culturing *Ectocarpus siliculosus* 47

2.1.1 Preparation of Provasoli nutrient enrichment solution 47

2.1.2 Materials preparation 48

2.2 Culture conditions of *Ectocarpus siliculosus* 48

2.3 Handling and manipulation of *Ectocarpus siliculosus* cultures 49

2.3.1 Transferring cultures: 50

2.3.2 Reminders: 50

2.4 Test chemicals and solutions 51

2.5 Algal test species and culture conditions 52

2.6 Evaluation of growth and photosynthetic efficiency responses 53

2.6.1 Relative growth rate, (RGR) 53

2.6.2 Photosynthetic activity (Chlorophyll *a* fluorescence) 54

2.7 Preparation of protein extract for antioxidative enzyme assays..... 57

Chapter 3: Growth and physiological responses of *Ectocarpus siliculosus* to herbicide exposure ……..…. 59

3.1 Introduction..... 60

3.2 Materials and methods 61

3.2.1 Culture conditions of *Ectocarpus siliculosus*..... 61

3.2.2 Test chemicals and solutions..... 62

3.2.3. Determination of relative growth rate 63

3.2.4 Chlorophyll fluorescence 63

3.2.5 Statistical analysis 63

3.3 Results 64

3.3.1 Effect of diuron (DIU) on *E. siliculosus*..... 64

3.3.2 Effect of terbuthylazine (TBA) on *E. siliculosus*..... 71

3.3.3 Effects of isoproturon (IPU) on *E. siliculosus* 78

3.4 Discussion 88

3.4.1 *E. siliculosus* response to diuron (DIU) 88

3.4.2 *E. siliculosus* response to terbuthylazine (TBA)..... 92

3.4.3 *E. siliculosus* response to isoproturon (IPU) 95

3.5. Conclusion 99

Chapter 4: Biochemical response of *Ectocarpus siliculosus* strains with different polution history to diuron, terbuthylazine and isoproturon ……..… 102

4.1 Introduction 103

4.2 Materials and methods 104

4.2.1 Algal material and culture conditions 104

4.2.2 Diuron, terbuthylazine and isoproturon treatments 104

4.3 Evaluation of oxidative stress: 104

4.4 Antioxidative enzymes analysis: 106

4.4.1 Preparation of protein extracts 106

4.4.2 Catalase (CAT) 107

4.4.3 Ascorbate peroxidase (APX) 107

4.4.4 Glutathione reductase (GR) 107

4.5 Determination of total phenolic content 108

4.6 Evaluation of antioxidant activity 108

4.7 Analysis of polyphenols 109

4.8 Statistical analyses ……..……111

4.9 Results 112

4.9.1 The effects of DIU, TBA and IPU on H_2O_2 and lipid peroxidation levels in LIA4 and Es524 112

4.9.2 Effects of DIU, TBA and IPU on different antioxidant enzymes in LIA4 and Es524 118

4.9.3 Total phenolic content (TPC) and DPPH scavenging activity of LIA4 and Es524 in response to DIU, TBA and IPU exposure 127

4.9.4 Polyphenols response in *E. siliculosus* strains to DIU, TBA and IPU exposure 133

4.10 Discussion 142

4.10.1 Oxidative stress and antioxidative enzyme responses of *E. siliculosus* strains to diuron (DIU) 142

4.10.2 Oxidative stress and antioxidative enzyme responses of *E. siliculosus* strains to terbuthylazine (TBA) 146

4.10.3 Oxidative stress and antioxidative enzyme responses of *E. siliculosus* strains to isoproturon (IPU) 149

4.10.4 Effects of DIU, TBA and IPU on non-enzymatic antioxidant activity 151

4.11 Conclusion 154

Chapter 5: The effects of herbicide singly and in combinations on physiological and biochemical responses of *Ectocarpus siliculosus* ……..……. 156

5.1 Introduction 157

5.2 Materials and methods 158

5.2.1. Biological material 158

5.2.2. Preparation of single herbicide solutions 158

5.2.3 Combination studies 158

5.2.4 Individual and mixture effects of herbicides on growth 159

5.2.5 The effects of herbicides singly and in mixtures on photosynthetic efficiency of *E. siliculosus* 159

5.2.6 The effects of herbicides singly and in mixtures on oxidative stress of *E. siliculosus* 160

5.2.7 The effects of herbicides singly and in mixtures on antioxidative enzyme activities of *E. siliculosus* 160

5.2.8 The effects of herbicides singly and in mixtures on non-enzymatic antioxidant activities of *E. siliculosus* 160

5.2.9 Statistical analyses 160

5.3 Results 161

5.3.1 Individual and combined effects of herbicides on growth and photosynthetic efficiency of *E. siliculosus* 161

5.3.2. Effects of DIU, TBA and IPU singly and in mixtures on oxidative stress of *E. siliculosus* strains 175

5.3.3 Effects of DIU, TBA and IPU singly and in mixtures on antioxidative defense mechanisms (enzymatic and non-enzymatic) of *E. siliculosus* strains 179

5.5 Discussion 187

5.6 Conclusion 194

Chapter 6: General discussion and conclusions ……. 196

6.1 Risks posed to *E. siliculosus* from herbicide environmental concentrations ……...197

6.2 Intra-specific responses of *E. siliculosus* strains to herbicides (DIU, TBA and IPU) …200

6.3 Future works 207

References..... 209

LIST OF TABLES

Table 1.1 The effects of isoproturon (IPU) on macrophytes and algae ……..……. 7

Table 1.2 The effects of triazines (TBA and atrazine) on photosynthetic organisms 18

Table 1.3 The effects of diuron on photosynthetic organisms …….……. 30

Table 2.1 Preparation of Provasoli solution …….. 47

Table 2.2 List of herbicides and booster biocide …….……. 52

Table 3.1 Photosynthetic efficiency of *E. siliculosus* exposed to different concentrations of diuron……..……...…… 71

Table 3.2 Photosynthetic efficiency of *E. siliculosus* exposed to different concentrations of terbuthylazine…… 78

Table 3.3 Photosynthetic efficiency of *E. siliculosus* exposed to different concentrations of isoproturon…….…… 85

Table 3.4 Toxicity of diuron (DIU), terbuthylazine (TBA) and isoproturon (IPU) to LIA4 and Es524……...…….…….. 87

Table 4.1: Detection of standard polyphenol compounds……...……...…… 133

Table 4.2: The effects of diuron (DIU) on polyphenolic compounds in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* …… 136

Table 4.3: The effects of terbuthylazine (TBA) on polyphenolic compounds in two strains (Es524 and LIA4) of *Ectocarpus siliculosus*…….....…… 138

Table 4.4: The effects of isoproturon (IPU) on polyphenolic compounds in two strains (Es524 and LIA4) of *Ectocarpus siliculosus*…….…… 140

Table 5.1: Percentage of reductions of relative growth rate (RGR) in LIA4 and Es524 in response to the binary combination of the herbicides…..... 162

Table 5.2: Percentage of inhibitions of Fv/Fm in LIA4 and Es524 in response to the binary combination of the herbicides……..……...…….…...….. 164

Table 5.3: Percentage of inhibitions of ΦPSII in LIA4 and Es524 in response to the binary combination of the herbicides…….…… 166

Table 5.4: Percentage of inhibitions of qP in LIA4 and Es524 in response to the binary combination of the herbicides……. 168

Table 5.5: Percentage of increases of qN in LIA4 and Es524 in response to the binary combination of the herbicides ……....……. 170

Table 5.6: Photosynthetic efficiency of *E. siliculosus* exposed to different concentrations of herbicides singly and in mixtures ..……... 173

Table 5.7: Percentage of inhibitions of $rETR_{max}$ and α in LIA4 and Es524 in response to the binary combination of the herbicides ……...…….. 174

Table 5.8: Percentage of increases of H_2O_2 in LIA4 and Es524 in response to the binary combination of the herbicides ……...……...……. 176

Table 5.9: Percentage of increases of lipid peroxidation in LIA4 and Es524 in response to the binary combination of the herbicides ……...…....…. 178

Table 5.10: Percentage of changes of catalase (CAT) activity in LIA4 and Es524 in response to the binary combination of the herbicides ……...…… 180

Table 5.11: Percentage of changes of ascorbate peroxidase (APX) activity in LIA4 and Es524 in response to the binary combination of the herbicides ……...…… 182

Table 5.12: Percentage of changes of glutathione reductase (GR) activity in LIA4 and Es524 in response to the binary combination of the herbicides …….…… 184

Table 5.13: The effects of DIU, TBA and IPU singly and in mixtures on polyphenolic compounds in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* ……..……...……. 186

Table 6.1: The effects of diuron, terbuthylazine and isoproturon in relation to the reported environmental concentrations ……...…. 198

LIST OF FIGURES

Figure 1.1 Schematic illustration of the behaviour of a biocide-based antifouling system exposed to sea water ……...…….. 27

Figure 1.2 *Ectocarpus siliculosus* …….. 39

Figure 1.3 Phylogeny of brown algae and Ectocarpales……...42

Figure 1.4 Life cycle of *E. siliculosus* …….……...…... 43

Figure 2.1 Handling and manipulation of *Ectocarpus siliculosus* ……. 49

Figure 2.2 Culture of *Ectocarpus siliculosus* in 10 L bottles …… 51

Figure 2.3 The Wintrobe tube …… 54

Figure 2.4 The FMS-1, Hansatech Ltd., England …….……..… 56

Figure 2.5 Mini PAM (Walz, Germany) ……...…… 57

Figure 3.1: The effects of DIU on RGR in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (4/7-11/7/13)…….……..… 65

Figure 3.2: The effects of DIU on Fv/Fm in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (25/7-1/8/13)……..…… 66

Figure 3.3: The effects of DIU on ΦPSII in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (25/7-1/8/13)……...…… 67

Figure 3.4: The effects of DIU on qP in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (25/7-1/8/13)……..…….. 67

Figure 3.5: The effects of DIU on qN in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (25/7-1/8/13)……...…….….. 68

Figure 3.6: Relative electron transport rate (rETR) of LIA4 exposed to diuron (12/2-19/2/14)…… 69

Figure 3.7: Relative electron transport rate (rETR) of Es524 exposed to diuron (12/2-19/2/14)…… 70

Figure 3.8: The effects of TBA on RGR in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (4/9-11/9/13)…… 72

Figure 3.9: The effects of TBA on Fv/Fm in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (25/9-2/10/13)…… 73

Figure 3.10: The effects of TBA on ΦPSII in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (25/9-2/10/13)…… 74

Figure 3.11: The effects of TBA on qP in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (25/9-2/10/13)…… 74

Figure 3.12: The effects of TBA on qN in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (25/9-2/10/13)…… 75

Figure 3.13: Relative electron transport rate (rETR) of LIA4 exposed to terbuthylazine (TBA) (15/4-22/4/14)…… 76

Figure 3.14: Relative electron transport rate (rETR) of Es524 exposed to terbuthylazine (TBA) (15/4-22/4/14)…… 77

Figure 3.15: The effects of IPU on RGR in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (4/11-11/11/13)…… 79

Figure 3.16: The effects of IPU on Fv/Fm in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (25/11-2/12/13)…… 80

Figure 3.17: The effects of IPU on ΦPSII in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (25/11-2/12/13)…… 81

Figure 3.18: The effects of IPU on qP in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (25/11-2/12/13)…… 82

Figure 3.19: The effects of IPU on qN in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (25/11-2/12/13)…… 83

Figure 3.20: Relative electron transport rate (rETR) of LIA4 exposed to isoproturon (IPU) (17/6-24/6/14)…… 84

Figure 3.21: Relative electron transport rate (rETR) of Es524 exposed to isoproturon (IPU) (17/6-24/6/14)…… 84

Figure 4.1: Sample preparations for HPLC analysis …… 110

Figure 4.2: HPLC systems …… 111

Figure 4.3: The effects of DIU on H_2O_2 levels in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (16/9-23/9/14)…… 112

Figure 4.4: The effects of TBA on H_2O_2 levels in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (7/10-14/10/14)…… 113

Figure 4.5: The effects of IPU on H_2O_2 levels in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (28/10-4/11/14)…… 114

Figure 4.6: The effects of DIU on lipid peroxidation in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (18/11-25/11/14)…… 115

Figure 4.7: The effects of TBA on lipid peroxidation in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (9/12-16/12/14)…… 116

Figure 4.8: The effects of IPU on lipid peroxidation in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (30/12/14-6/1/15)…… 117

Figure 4.9: The effects of DIU on CAT activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (18/3-25/3/15)……...……. 118

Figure 4.10: The effects of TBA on CAT activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (10/6-17/6/15)……..…….. 119

Figure 4.11: The effects of IPU on CAT activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (2/9-9/9/15)……...……..…….. 120

Figure 4.12: The effects of DIU on APX activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (18/3-25/3/15)…….……..……. 121

Figure 4.13: The effects of TBA on APX activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (10/6-17/6/15)……..……. 122

Figure 4.14: The effects of IPU on APX activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (2/9-9/9/15)……..……. 123

Figure 4.15: The effects of DIU on GR activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (18/3-25/3/15)……....…….. 124

Figure 4.16: The effects of TBA on GR activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (10/6-17/6/15)……...…....… 125

Figure 4.17: The effects of IPU on GR activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (2/9-9/9/15)……...……. 126

Figure 4.18: The effects of DIU on total phenolic content (TPC) in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (4/11-11/11/15)……. 127

Figure 4.19: The effects of TBA on total phenolic content (TPC) in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (18/11-25/11/15)..……. 128

Figure 4.20: The effects of IPU on total phenolic content (TPC) in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (2/12-9/12/15)……..…. 129

Figure 4.21: The effects of DIU on DPPH scavenging activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (19/1-26/1/16)...…..…. 130

Figure 4.22: The effects of TBA on DPPH scavenging activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (2/2-9/2/16)..……... 131

Figure 4.23: The effects of IPU on DPPH scavenging activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (16/2-23/2/16)…….. 132

Figure 4.24: HPLC analysis of mixed polyphenol standards (6/4/16)……..… 134

Figure 4.25: HPLC chromatogram at 254 nm of the *E. siliculosus* exposed to different herbicides……...……..……. 141

Figure 5.1: The effects of individual and binary combinations of herbicides on RGR in two strains (Es524 and LIA4) of *E. siliculosus* (3/5-10/5/16) …….…….…. 161

Figure 5.2: The effects of individual and binary combinations of herbicides on Fv/Fm in two strains (Es524 and LIA4) of *E. siliculosus* (24/5-31/5/16) ……..… 163

Figure 5.3: The effects of individual and binary combinations of herbicides on ΦPSII in two strains (Es524 and LIA4) of *E. siliculosus* (24/5-31/5/16)……... 165

Figure 5.4: The effects of individual and binary combinations of herbicides on qP in two strains (Es524 and LIA4) of *E. siliculosus* (24/5-31/5/16)…….. 167

Figure 5.5: The effects of individual and binary combinations of herbicides on qN in two strains (Es524 and LIA4) of *E. siliculosus* (24/5-31/5/16)…….……. 169

Figure 5.6: Relative electron transport rate (rETR) of LIA4 exposed to mixtures of herbicide (14/6-21/6/16)…….…….…….. 171

Figure 5.7: Relative electron transport rate (rETR) of Es524 exposed to mixtures of herbicide (14/6-21/6/16)…….…… 171

Figure 5.8: The effects of individual and binary combinations of herbicides on H_2O_2 in two strains (Es524 and LIA4) of *E. siliculosus* (5/7-12/7/16)……...……..… 175

Figure 5.9: The effects of individual and binary combinations of herbicides on lipid peroxidation in two strains (Es524 and LIA4) of *E. siliculosus* (19/7-26/7/16)……..……. 177

Figure 5.10: The effects of individual and binary combinations of herbicides on catalase activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (9/8-16/8/16)…….….…….. 179

Figure 5.11: The effects of individual and binary combinations of herbicides on ascorbate peroxidase activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (9/8-16/8/16)……...…….. 181

Figure 5.12: The effects of individual and binary combinations of herbicides on glutathione reductase activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* (9/8-16/8/16)……...……. 183

ACKNOWLEDGEMENTS

Thanks to merciful God for without His graces and blessings, this study would not have been possible.

Immeasurable appreciation and deepest gratitude to my Director of Studies (DOS) Associate Professor Dr. Murray Brown. Without his continuous optimisms concerning this work, enthusiasm, encouragement and support this study would hardly have been completed. I also express my warmest gratitude to my second supervisor Associate Professor Dr. John Moody for his unwavering support, collegiality, and mentorship throughout this project.

My appreciation also extends to my laboratory colleagues; Claudio Saez, and Alex Santillan for their mentoring and encouragement have been especially valuable, and their insights launched the greater part of this thesis. Thanks also go to Angela Harrop and William Vevers, who enlivened the labs with good ideas and good humor.

Not to forget, I am indebted to my family - my parents Hashim and Rosnani; my brothers Azmer, Ashraf and Farhan.

And finally, I also would like to express my gratitude for all the help, support, and services provided by all the people mentioned and not mentioned in helping me to complete this studies.

Mohd Akmal bin Hashim

AUTHOR'S DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee. Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

Word count of main body of thesis: 48,268

Signed ……..

Date 11/04/2018 ……..

LIST OF ABBREVIATIONS

Abbreviation	Meaning
ABS/RC	Energy transfer efficiency from absorbed light toPSII reaction center
ADME	Absorption, Distribution, Metabolism, and Excretion
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
a.i.	active ingredient
CAT	Catalase
Conc.	Concentration
DIU	Diuron
DIU + IPU	Mixture of diuron and isoproturon
DIU + TBA	Mixture of diuron and terbuthylazine
DOC	Dissolved organic carbon
DPPH	1,1-diphenyl-2-picrylhydrazyl
EC_{50}	Effective concentration (50%)
ET/RC	Electron transport per reaction center
ETR_{max}	Maximum electron transport rate
FW	Fresh weight

Fv/Fm	Maximum efficiency of photosystem II
ΦPSII	Effective quantum yield of photosystem II
GST	Glutathione S-transferase
GR	Glutathione reductase
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
IPU	Isoproturon
ISO	International Association for Standardization
LOEC	Lowest observed effect concentration
MDA	Malondialdehyde
NOEC	No observed effect concentration
NSW	Natural seawater
OECD	Organization for Economic Co-operation and Development
PAM	Pulse-amplitude modulation
PAR	Photosynthetically active radiation
PES	Provasoli enriched solution
POP	Photosynthetic O_2 production
Ppt	Parts per thousand

PSII	Photosystem II
qP	Photochemical quenching
$qP_{(rel)}$	Relative photochemical quenching
qN	Non-photochemical quenching
rETR	Relative electron transport rate
RGR	Relative growth rate
ROM	Reactive oxygen metabolism
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBA	Terbuthylazine
TBARS	Thiobarbituric acid reactive substances
TBA + IPU	Mixture of terbuthylazine and isoproturon
$UQF_{(rel)}$	Relative unquenched fluorescence
α	Photosynthetic efficiency
95% C.I.	95% confidence interval

Chapter 1

Introduction and Literature review

1.1 Marine pollution: a global issue

In the 21st century, various anthropogenic activities such as agriculture, mining, shipping and heavy industry continue to release chemicals that ultimately result in environmental pollution. The variety and quantities of chemicals released from diverse anthropogenic sources are known to have wide-ranging adverse effects that are capable of disrupting the integrity of terrestrial and aquatic ecosystems (Hela *et al*., 2005). For instance, intensive agricultural activities serve as major diffuse sources of pesticides that contaminate streams and rivers. This contamination is ultimately transported downstream and enters estuaries and coastal waters (Schwarzenbach *et al*., 2006). Moreover, due to the rapid growth in global trading, much of which is transported by ships, there has been an increase in the use of antifouling biocides to counter the impacts of the fouling of ships' hulls (Jacobson and Willingham, 2000; Terlizzi *et al*., 2001).

The oceans were once considered to be a vast reservoir for the safe disposal of contaminants. However, it is now recognised that chemicals such as herbicides, organochlorine compounds, petroleum products and metals have adverse effects on marine biota, even at low concentrations (Haynes and Johnson, 2000; Pinto *et al*., 2003). It has been estimated that over 80% of marine pollution originates from industrial, agricultural and urban activities (UNEP, 2013). Riverine inputs contribute largely to the occurrence of pesticides, and especially herbicides, in estuarine and coastal systems (Haynes *et al*., 2000; Shaw and Mueller, 2005; Lewis *et al*., 2009).

Despite widespread attempts at implementing legislation that would reduce the quantities of chemicals being released, environmental pollution remains a significant global issue. The application of chemicals to meet the needs of human life continues to result in habitat degradation and destruction, and ultimately the instability of entire

ecosystems (Duke *et al*., 2004; Mitchell *et al*., 2005). To address this issue, several of the most commonly used herbicides or booster biocides in antifouling paints have been chosen for further evaluation.

1.2 Herbicides: a need that needs to be evaluated

A major challenge facing mankind today is to produce sufficient quantities of food to sustain an acceptable standard of living for all. Driven by this goal, intensive agriculture was introduced, which requires judicious use of agrochemicals and pesticides. Among the thousands of pesticides on the market, herbicides are the most widely utilized, accounting for 48% of worldwide pesticide use in 2005 (Zhang *et al*., 2011). Indeed, approximately 50% of approved pesticides in the UK are herbicides, according to the UK Ministry of Agriculture, Fisheries and Food (Kirby and Sheahan, 1994).

Herbicides or weedkillers are one group of crop protection agents vital for preventing competition for space, water, nutrients and light by weeds. They are used to kill or reduce the growth of plants by interfering with photosynthetic electron transfer, resulting in the disruption of the energy supply or by irreversible damage to thylakoid membranes within the chloroplast. For example, herbicides of the urea and triazine classes function biochemically by displacing a plastoquinone (Q_B) from its binding site in the D1 protein of photosystem II (Singh *et al*., 1997). While the agricultural sector has benefited from their use, it has not been without problems.

Often, in order to compensate for losses caused by the transport and degradation processes, pesticides applicators exceed limits on labels which greatly exceed those required for control of the target organisms (Tejada *et al*., 2013). The abundance of these foreign chemicals can exceed the buffering function of soil compartments, resulting in their detection in rivers, estuaries, and coastal waters draining agricultural

watersheds (Dorigo and Leboulanger, 2001). As a consequence, the migration of herbicides from farmland, either by runoff to surface water or by leaching to groundwater, may lead to toxic effects on non-target organisms (Arias *et al*., 2008; Hildebrandt *et al*., 2008; Dalton *et al*., 2010).

The degree of herbicides transport in the environment depends on several factors such as application rate, herbicide persistence and mobility, rainfall, topography, and climate (Lin *et al*., 1999). During rain events, herbicides can be transported from their point of application to surface waters, where they may harm aquatic organisms (Doppler *et al*., 2012). In addition, herbicides in soil are subject to sorption as well as several biological and chemical degradation mechanisms including, microbial and photodecomposition, which cause decreased in their concentrations. Besides, herbicides may also be transported to different parts of the environment by volatilization, wind, erosion, runoff and leaching, with the latter two pathways considered major causes of contamination of surface and ground water (Rivard, 2003). For instance, inshore marine habitats are prone to contamination by herbicides, via runoff, leaching, spray drift and accidental spills (Luna-Acosta *et al*., 2012). Although concentrations may not necessarily exceed the levels of acute toxicity to marine organisms, they lead to changes in coastal communities.

Structural parameters, e.g. biomass, species composition (Peres *et al*., 1996), or pigment profiles (Eser, 2001), as well as functional parameters, e.g. photosynthesis capacity or primary production (Dorigo and Leboulanger, 2001) have been used to demonstrate community changes after exposure to herbicides. For instance, Chl *a* fluorescence measurement of PSII is a unique, rapid, non-intrusive and universal technique that reveals information on plant performance and protective responses (Kumar *et al*., 2014). This intriguing tool could be applied in most studies that address

photosynthetic responses of plants and algae in the environment (Maxwell and Johnson, 2000; Murchie and Lawson, 2013). Moreover, Rutherford and Krieger-Liszkay (2001) stated that some herbicides (PSII inhibitors) are hypothesized to cause oxidative stress in plants due to the formation of reactive oxygen species (ROS, e.g. $^1O_2$). Therefore, evaluation of biochemical markers such as pigments and antioxidative enzymes has been widely used as the reliable indicator for assessing the toxicity of herbicides (Kumar *et al*., 2010; Bi *et al*., 2012; Fiori *et al*., 2013).

While there is a need for selective weed control, and herbicides have proven efficiency on targeted organisms, they also generate non-specific phytotoxicity (Saladin *et al*., 2005). Indeed, less than 0.1% of the pesticides applied in the field actually reach their target, with the remainder available to cause adverse effects in non-target organisms (El-Dib *et al*., 1989; Carafa *et al*., 2007; Dalton and Boutin, 2010; Kumar *et al*., 2014). It is, therefore, essential that their impacts against non-target organisms are evaluated so that deterioration to ecosystems can be minimised. Thus, in the present study, the effects of the widely-used herbicides diuron, terbuthylazine and isoproturon will be investigated on a non-target organism using the brown filamentous alga, *Ectocarpus siliculosus* as a model.

1.2.1 Phenylurea: Isoproturon (IPU)

Isoproturon (IPU) is a selective systemic herbicide widely used to control annual grasses as well as broad-leaved weeds in wheat, rye and barley crops. It is one of the most commercially significant herbicides used in conventional European agriculture (Federal Environmental Protection Agency, 2000). It is absorbed by the roots, then rapidly transported through the xylem to the leaves (Pietsch *et al*., 2006). IPU is moderately soluble in water (70 mg L^{-1}), and persistent with a half dissociation time (DT50) ranging from 12 to 33 days (Palacios *et al*., 2010). The average half-life of IPU

in the water column ranges from approximately 50 to 60 days (Peres *et al*., 1996), with an earlier study by Linders *et al*., (1994), stated that the half-life was much longer, ranging from 140 to 365 days at pH 5 to 9.

IPU mainly enters the environment during its application to the soil before plants emerge and is added to soils around growing crops (Noyrod *et al*., 2014). As a result of its extensive application and its properties of moderate persistence and relatively low adsorption, IPU has been detected in ground and surface waters in Europe at levels which exceed the European Commission drinking water limit of 0.1 µg L^{-1} (Spliid and Koppen, 1998). According to INRA (Institut national de la recherche agronomique / National Institute of Agronomic Research, 2004) and SIABAVE (Syndicat mixte Intercommunal d'aménagement du BAssin de la VEsle / Inter-Union Joint watershed management Vesle, 2002), IPU is one of the herbicides most likely to be found in surface waters. For example, in Germany, IPU accounts for about 2 tonnes of the approximately 30 tonnes of total pesticides entering surface waters each year (Pietsch *et al*., 2006). The levels measured by Muller *et al*., (2002) in the Zwester Ohm Catchment, Germany, ranged between 0.05 and 23.18 µg L^{-1}, with a median at 0.21 µg L^{-1}. Likewise, in the River Vannetin, France concentrations of up to 2.6 µg L^{-1} have been recorded (Irace–Guigand *et al*., 2004). Further, Kirby and Sheahan (1994) have reported short-lived values of up to 17 µg L^{-1} in agricultural run-off waters. A maximum concentration of 500 µg L^{-1} was recorded by Johnson *et al*., (1996) for drain water from a crop (cereals/oilseed) field after rainfall event.

As a phenylurea herbicide, IPU targets the photosynthetic apparatus of plants (Pietsch *et al*., 2006). Its mode of action is inhibiting photosynthetic electron transport due to specific binding to the D1 protein, a 32-kDa protein of photosystem II within the thylakoid membranes (Berger and Heitefuss, 1991; Grouselle *et al*., 1995). However, to

date, evaluation on the effect of IPU on non-target photosynthetic organisms remains limited, with most studies focused on rooted macrophytes. With reference to Table 1.1, a few studies on the effect of IPU on macrophytes (*Elodea densa*, *Ludwigia natans*) (Grouselle *et al*., 1995; Feurtet-Mazel *et al*., 1996; Grollier *et al*., 1997) and algae (Bi *et al*., 2012; Lazar and Lazar 2001; Kirby *et al*., 1994) have been highlighted.

Table 1.1: The effects of isoproturon (IPU) on photosynthetic organisms

Photosynthetic organisms	End-points measured	Duration	Conc. of IPU tested	Effect parameter	Reference
Chlamydomonas reinhardtii (FW algae)	Growth, chlorophyll fluorescence, Chl. content, oxidative stress, antioxidant enzymes, gene expression	72 hours	0, 5, 15, 25, 35 and 50 µg L^{-1}	Decreased in cell number: 72 h EC_{50} = 43.25 µg L^{-1} NOEC = 5 µg L^{-1} LOEC = 15 µg L^{-1} Reduction of chlorophyll content Reduction in Fv/Fm Reduction in ΦPSII Increase TBARS content Increase of SOD, CAT, APX and GST activity Upregulation of gene expression coding for SOD, CAT and APX	Bi *et al*., (2012)

Photosynthetic organisms	End-points measured	Duration	Conc. of IPU tested	Effect parameter	Reference
FW macrophytes: *Elodea Canadensis* (EC), *Myriophyllum spicatum* (MS), *Potamogeton lucens* (PL)	Growth, photosynthetic efficiency	35 days	14 µg L^{-1}	EC =ΦPSII not significantly affected, RGR not affected MS = ΦPSII was significantly reduced, RGR not affected PL =ΦPSII not significantly affected	Knauert *et al*., (2010)
Ceratophyllum demersum L. (FW macrophyte)	Photosynthetic oxygen production (POP)	48 hours	0.2, 2, 20 and 200 µg L^{-1}	0.2 µg L^{-1} = Significant reduction of POP 2.0 µg L^{-1} = Non-significant reduction of POP 20 µg L^{-1} = Significant reduction of POP 200 µg L^{-1} = Significant reduction of POP	Pietsch *et al*., (2006)
FW macrophytes: *Elodea densa* (ED), *Ludwigia natans* (LN)	Growth: fresh weight and stem lengths	21 days	0, 30 and 60 µg L^{-1}	ED = Increase of fresh weight after exposure to 30 and 60 µg L^{-1} of IPU between 12-28 ºC,	Grollier *et al*., (1997)

Photosynthetic organisms	End-points measured	Duration	Conc. of IPU tested	Effect parameter	Reference
				but decrease at 12 and 28ºC. 43% decrease of stem length at 28ºC in response to 60 µg L^{-1} of IPU. LN = Increase of FW between 12-28 ºC with no significant effect of the two IPU conc. No inhibition effect on stem length	Grollier *et al.*, (1997)
FW macrophytes: *Elodea densa* (ED) *Ludwigia natans* (LN)	Growth (weight, stem and lateral branch length	21 days	0 to 1000 µg L^{-1}	ED = Reduction in weight, stem and lateral branch length LN = No significant effect on weight	Feurtet-Mazel *et al.*, (1996)

Photosynthetic organisms	End-points measured	Duration	Conc. of IPU tested	Effect parameter	Reference
FW macrophytes: *Eichhornia crassipes* (EC) *Ipomoea aquatic* (IA)	Growth and chlorophyll content	9 days	2500 µg L^{-1}	EC = Decrease in density, leaf area, green area and chlorophyll a content IA = Increase in density, leaf area, green area and chlorophyll a content	Rana and Kumar (1995)
Botryococcus braunii (FW algae)	Growth, pigment contents, photosynthesis rate	21 days	8.24 – 618 µg L^{-1}	Stimulation of growth at low concentration (8.24 µg L^{-1}), but decrease at higher concentrations: 2 d EC_{50} = 1157 µg L^{-1} 7 d EC_{50} = 196 µg L^{-1} 14 d EC_{50} = 164 µg L^{-1} 21 d EC_{50} = 170 µg L^{-1} Decrease in pigment content, photosynthesis and respiration rate	Lazar and Lazar (2001)

Photosynthetic organisms	End-points measured	Duration	Conc. of IPU tested	Effect parameter	Reference
Phalaris minor 5 biotypes: KR-1 H-4 K-2 H-2 J-1	Growth (weight), membrane leakage, chlorophyll fluorescence (Fv/Fm)	14, 21 days	0 – 16 kg a.i. ha^{-1} (a.i. ha^{-1} = active ingredient per hectare)	Reduction in dry weight: 14 d EC_{50}: KR-1 = 5.95 kg a.i. ha^{-1} H-2 and J-1 = 0.33 kg a.i. ha^{-1} K-2 = 0.79 kg a.i. ha^{-1} H-4 =1.35 kg a.i. ha^{-1} Significant membrane leakage: KR-1 = at 8 and 16 kg a.i. ha^{-1} H-2 and J-1 = at 1 kg a.i. ha^{-1} Reduction in Fv/Fm in response to 5150 (0.025 mM) and 10300 (0.05 mM) µg L^{-1} of IPU.	Singh *et al.*, (1997)
FW macrophyte: *Lemna minor* (LM) FW algae: *Scenedesmus subspicatus* (SS)	Chlorophyll *a*, fresh weight, frond/ cell production	10, 4 days	28, 56, 112, 224 and 448 µg L^{-1}	LM : 10 d EC_{50} (µg L^{-1}): Frond number = 40 Fresh weight = 33	Kirby and Sheahan (1994)

Photosynthetic organisms	End-points measured	Duration	Conc. of IPU tested	Effect parameter	Reference
				Chl. a content = 31 SS : 96 h EC_{50} Cell production = 21 µg L^{-1}	Kirby and Sheahan (1994)
Scenedesmus obliquus (FW algae)	Growth (cell density), chlorophyll *a* fluorescence	24 hours	7, 15, 30, 60, 125, 250 and 500 µg L^{-1}	EC_{50} for cells density and fluorescence parameters: (µg L^{-1}) Cells density = 103 ΦPSII = 30 qP = 85 $qP_{(rel)}$ = 144 ABS/RC = 165 $UQF_{(rel)}$ = 8	Dewez *et al*., (2008)
Chlorella vulgaris (FW algae)	Growth, dry weight, photosynthetic pigments, cell volume, protein content	96 hours	0, 0.05, 0.10, 0.25, 0.50 µM (0, 10.3, 20.6, 51.5, 103 µg L^{-1})	Growth inhibition : 96 h= 41µg L^{-1} EC_{50} Increase in cellular volume and dry weight Pigment and protein content were stimulated at higher conc. (51.5 and 103 µg L^{-1})	Rioboo *et al*., (2002)

FW = freshwater, MA = marine

With reference to Table 1.1, IPU was found to exert different effects on growth of various photosynthetic organisms. Feurtet-Mazel *et al*., (1996) has reported that *Elodea densa* is more sensitive to IPU than *Ludwigia natans* based on the marked effect on growth as determined by fresh weight and stem length. A 40 % reduction in ponderal growth (weight) of *E. densa* at 100 µg L^{-1} IPU was reported as compared to the control, while for *L. natans,* the effects were barely detectable. Significant differences in stem length of both macrophytes were also observed between the control plants compared to 30 and 60 µg L^{-1} IPU. In contrast, a study by Grollier *et al*., (1997) reported an increase in the biomass of *E. densa* by 34% and 45% after exposure to 30 and 60 µg L^{-1} of IPU respectively, whereas stem length declined by 43% at 60 µg L^{-1} compared to the control. No inhibitory effects were recorded in *L. natans.* Moreover, Rana and Kumar (1995) observed that *Eichhornia crassipes* is far more sensitive to high doses of IPU than *Ipomoea aquatica* and the composition of phytoplankton was changed by exposure to this herbicide. It was observed that at 2.5 mg L^{-1} (2500 µg L^{-1}) of IPU, the density of *E. crassipes* decreased by 34, 46 and 78% after three, six and nine days of exposure respectively. The density of *I. aquatica,* however, remained unaffected until it increased to 46% on ninth day. Further, Knauert *et al*., (2010) reported that neither growth of the dicot *Myriophyllum spicatum* nor of the monocot *Elodea canadensis* was significantly reduced after exposure to 14 µg L^{-1} of IPU compared to the control. Kirby and Sheahan, (1994) otherwise have recorded a 50% inhibition in fresh weight and frond number of *Lemna minor* after being exposed to IPU at 33 and 40 µg L^{-1}, respectively. Similar effects (50% reduction) were recorded by Singh *et al*., (1997) in the dry weight of different biotypes of *Phalaris minor* after treatment with IPU in the ranges of 0.33 to 5.95 kg a.i. ha^{-1}.

Furthermore, the effects of IPU on algae growth have also been investigated by several researchers. According to Dewez *et al*., (2008), at low concentrations (7 µg L^{-1}),

the cell density of *Scenedesmus obliquus* increased by 50% while at concentrations higher than 15 µg L^{-1}, the cells density was diminished compared to the control. Subsequent exposure at 30 and 500 µg L^{-1} decreased cell density by 25% and 75% respectively when compared to control. Kirby and Sheahan (1994) also observed 50% inhibition in the growth (cell production) of *Scenedesmus subspicatus* at 21 µg L^{-1} of IPU. Moreover, the green microalgae *Botryococcus braunii* by Lazar and Lazar (2001) also showed an inhibition of growth by 50% when exposed to IPU at 0.196 mg L^{-1} for 7 days. This is in agreement with a recent study on the green alga *Chlamydomonas reinhardtii* by Bi *et al*., (2012) who reported that compared to the control, growth (expressed as cell number) at 50 µg L^{-1} IPU decreased by 44%. In fact, at 25 µg L^{-1} IPU, a significant decreased in growth was also observed after three days exposure. For freshwater microalga *Chlorella vulgaris*, Rioboo *et al*., (2002) discovered that at 10.315 µg L^{-1}, growth was not significantly different from the control while at higher concentrations, growth decreased and at 103.15 µg L^{-1}, the growth rate was negative.

In relation to the physiological aspects, the effects of IPU on photosynthesis activity of aquatic plants have been evaluated by several researchers. For instance, at 14 µg L^{-1}, the photosynthetic efficiency (Fm'-F / Fm') of *E. canadensis* and *P. lucens* was not significantly affected (Knauert *et al*., 2010). On the other hand, Singh *et al*., (1997) reported that the fluorescence yield (Fv/Fm) of *P. minor* significantly decreased after exposure for 4 hours to IPU at 5.158 and 10.315 mg L^{-1}. Pietsch *et al*., (2006), who investigated the effects of IPU on *C. demersum* also reported that the photosynthetic oxygen production was significantly inhibited after 48 hours exposure to 0.2 µg L^{-1} IPU compared to control. Plus, exposure to 2, 20 and 200 µg L^{-1} of IPU for 48 hours also lead to a reduction of the photosynthetic oxygen release by 54, 66 and 70 %, respectively, compared to the control.

Meanwhile in algae, a study by Laviale *et al*., (2011) combined the use of two fluorescence parameters, namely the effective and the optimal or maximum quantum yield of PSII photochemistry (ΦPSII and Fv/Fm), as reliable biomarkers to assess the toxicity of IPU on biofilms. Both parameters (ΦPSII and Fv/Fm) used in the biofilms treated with IPU within the ranges of 20.6 to 20.6 X 10^3 µg L^{-1} were significantly affected after 1 to 7 hours of exposure. This was in line with a study by Dewez *et al*., (2008) on *S. obliquus* where the variable fluorescence (Fv/Fm) has decreased as the IPU concentrations increased. Lazar and Lazar (2001) also reported that IPU at 8.25 to 371.34 µg L^{-1} affected the photosynthetic rate (based on the gas exchange rate) of *Botryococcus braunii* after 21 days exposure, a response similar to that on *C. reinhardtii*, in which exposure 50 µg L^{-1} decreased Fv/Fm ratio by 33.33% compared to untreated cells (Bi *et al*., 2012).

Additionally, Bi *et al*., (2012) discovered that IPU can induce oxidative stress and activities of several major antioxidant enzymes (e.g. superoxide dismutase (SOD), Glutathione-S-transferase (GST), ascorbate peroxidase (APX) and catalase (CAT) in green algae, *Chlamydomonas reinhardtii*. Using thiobarbituric acid reactive substances (TBARS) method, they have found that the TBARS content was enhanced with the increasing IPU concentrations. Furthermore, at 50 µg L^{-1}, the maximum TBARS accumulation was observed by 3.43-fold higher than the control. In terms of antioxidant enzymes activities, at 5–25 µg L^{-1}, the SOD activity was progressively increased with IPU concentrations. However, there was a slight decline in activity when the concentration of IPU was increased to 35 and 50 µg L^{-1}. Moreover, the activity of CAT enzyme always increased in the cells with increasing IPU concentrations of 0–50 µg L^{-1}. At 50 µg L^{-1}, the CAT activity increased by 1.73-fold compared to the control. Plus, the pattern of APX activities was also reported to be similar to that CAT in algae exposed to IPU. In fact, they also found that the GST activity in IPU-treated cells (*C.*

reinhardtii) increased, and top activity was observed at 15–25 µg $L^{-1}$of IPU with further increasing IPU supply resulted in the decline of GST activity below the basal level.

Due to the wide impacts of IPU, this herbicide was listed as a priority substance in the Water Framework Directive of the EU (article 16; Appendix X, WFD, EC, 2000). However, up to now, few studies have reported on the effects of IPU on marine macroalgae, in particular the brown seaweeds. This further indicates that information on macroalgae remain scarce. Therefore, despite increasing information about the effects of herbicides on terrestrial and aquatic organisms, there is paucity of knowledge about the effects of IPU on macroalgae such as seaweeds. The present study will elucidate the impacts of the herbicide, plus the phytotoxicity and adaptive mechanisms to IPU in macroalgae, which remain largely unclear and merit further investigations.

1.2.2 Triazine: Terbuthylazine (TBA)

Triazine herbicides are largely used in agriculture worldwide for selective and non-selective control of broadleaf and small seeded grass weeds in diverse crops (Tejada *et al*., 2013). Terbuthylazine (TBA) which belongs to this group (triazine) has been replacing the better known and longer used and studied atrazine in several regions of the world (Dobsikova *et al*., 2012). TBA is taken up through roots and leaves and distributed throughout the plant. For this reason, it has been widely used for decades in crop farming as weed control, mainly in the early spring on maize, sorghum, vines, citrus, coffee, potatoes, legumes, and forestry (Roberts *et al*., 1998).

The half-life of TBA was measured in river, ground and seawater samples from Murcia (South-East of Spain), incubated under different lab conditions, by Navarro *et al*. (2004), who found a range between 76 and 331 days. It is stable in neutral, weakly acid and weakly alkaline media (Dobsikova *et al*., 2012). In addition, TBA was

unaffected by microbial or hydrolytic degradation processes (Gamble *et al*., 1983; Marchini *et al*., 1988) making the chances of this herbicide to persist in the environment are huge. Due to its physicochemical properties, which have relatively long persistence, there have been numerous reports of their presence in surface or ground waters (Blanchoud *et al*., 2007; Delgado-Moreno *et al*., 2009).

Sbrilli *et al*., (2005) has reported that TBA is one of the most commonly detected herbicides in Italian rivers and ground waters. A maximum TBA concentration of 694.32 ng L^{-1} and 234.5 ng L^{-1} was reported by Carafa *et al*., (2007) in the Sacca di Goro Lagoon and Adriatic Sea, respectively, both located off Italy. In other cases, about 107 tonnes of TBA were used in Czech Republic in 2010, according to the Czech State Phytosanitary Administration (Stepanova *et al*., 2012). Furthermore, TBA was also reported to be present in about 50% of water samples taken from Czech rivers between 2005 and 2009. The highest environmental concentrations reached 0.1µg L^{-1} in 2005 to 2.8 µg L^{-1} in 2006, and slightly decreased in 2009 to 2.6µg L^{-1} (Kodes *et al*., 2010). Besides that, various concentrations ranging from less than detection limits up to 1.27 µg L^{-1} were reported in surface and groundwater in northern Spain (Hildebrandt *et al*., 2008). A 3-year survey-study by Noppe *et al*., (2007) in the Scheldt Estuary, France showed that TBA was detected at 13 to 261 ng L^{-1}. Therefore, based on the reports that have been mentioned, it is certain that TBA is widely used within the European Union (EU). Due to its toxicological properties, together with its frequent detection in surface and groundwater, TBA may pose a risk both for human and environmental health. The European Food Safety Authority (EFSA) has reported that TBA poses high long-term risks for mammals, aquatic organisms, non-target plants, and earthworms (EFSA 2011) and also can lead to genotoxic effects (Mladinic *et al*., 2012). The fact that it has been recently (16 August 2011) re-evaluated and its placing in the EU market approved until

2021 by Commission Implementing Regulation 820/2011 makes its environmental occurrence a risk both for the environment and human health (Grenni *et al*., 2012).

As a selective systemic herbicide, triazine herbicides act as a photosynthesis inhibitor (Lamoureux *et al*., 1998). However, to date, the studies on the effect of TBA on non-target photosynthetic organisms remain scarce. Table 1.2 contains previous studies that focused on the effect of triazines (TBA and Atrazine) on several photosynthetic organisms.

Table 1.2: The effects of triazines (TBA and atrazine) on photosynthetic organisms

Photosynthetic organisms	End-points measured	Duration	Conc. of triazines tested	Effect parameter	Reference
Marine (MA) flagellates: *Gonyaulax spinifera, Fibrocapsa japonica, Lingulodinium polyedrum, Prorocentrum genus, Prorocentrum minimum*	Growth, photosynthetic activity, chlorophyll content	20, 24 days	TBA: 1, 5, 10, 15, 25, 30 and 50 µg L^{-1}	Significant reduction of ΦPSII in all species except *P. minimum* to 25 and 50 µg L^{-1} TBA Reduction in growth rate of *P. minimum* and *G. spinifera* with the highest effect on growth rate at 25ºC. Chlorophyll *a* content : No significant change in *P. minimum (*30 µg L^{-1} TBA)	Fiori *et al*., (2013)

Photosynthetic organisms	End-points measured	Duration	Conc. of triazines tested	Effect parameter	Reference
				Increase in chlorophyll *a* of *G. spinifera* exposed to 5 µg L^{-1} TBA	Fiori *et al.*, (2013)
Skeletonema marinoi (MA diatom)	Growth, photosynthetic efficiency, chlorophyll content	9, 24 days	TBA: 1, 5, 10, 15, 20 and 30 µg L^{-1}	Inhibition of cell growth and PSII efficiency Increase in chlorophyll content in response to: 10 µg L^{-1} (at 15 and 20ºC) 5 µg L^{-1} (at 25ºC) 20 µg L^{-1} (at 25ºC) 30 µg L^{-1} (at 15 and 20ºC)	Fiori and Pistocchi, (2014)
Olive (*Olea europaea*)	Photosynthetic activity	24 hours, 15 days, 60 days	TBA 3 kg ha^{-1}	Significant reduction in Fv/Fm and ΦPSII after 60 days of TBA treatment but not at 24 hours or 15 days. Reduction in qN after 60 days, but not significantly different compared to the control	Canero *et al.*, (2011)

Photosynthetic organisms	End-points measured	Duration	Conc. of triazines tested	Effect parameter	Reference
12 different strains of *Scenedesmus subspicatus* (FW algae): Hegewald 1971-67 Hegewald 1973-374 Hegewald 1975-272 Hegewald 1980-24 Hegewald 1981-23 Hegewald 1981-42 Hegewald 1988-30 Hegewald 1988-54 Payer 1971-124 Bai 1971-41 Felföldy 5640 SAG 86.81	Growth, photosynthetic activity	30 min (500 µg L^{-1}) 60 days (1,5, 20 µg L^{-1})	Atrazine (ATZ): 0, 1, 5, 20 and 500 µg L^{-1}	Differences in growth rate and photosynthetic activity were observed between the strains No significant effect on growth rate and photosynthetic activity to 1, 5, and 20 µg L^{-1} ATZ Significant interaction between strain and ATZ concentration was observed (the strains were affected differently by ATZ)	Behra *et al.*, (1999)
Scenedesmus obliquus (FW algae), *Navicula pelliculosa* (MA/FW diatom) and *Microcystis aeruginosa* (FW cyanobacteria)	Growth, photosynthetic yields, pigment content	72 hours	Atrazine (0–0.15 µM) / [0 – 32.4 µg L^{-1}]	***S. obliquus*** : No significant effect on growth rate, Fv/Fm and ΦPSII, but reduction of NPQ was significantly different compared to the control at 10 and 15ºc	Chalifour and Juneau (2011)

Photosynthetic organisms	End-points measured	Duration	Conc. of triazines tested	Effect parameter	Reference
				ΦPSII-EC_{50}: (µg L^{-1}) 27.65 (10ºC) 26.57 (15ºC) 49.25 (25ºC) ***N. pelliculosa*:** No significant effect on growth rate, Fv/Fm and ΦPSII ΦPSII-EC_{50}: (µg L^{-1}) 77.76 (10ºC) 77.76 (15ºC) 84.24 (25ºC) ***M. aeruginosa* CPCC632** No significant effect on growth rate, Fv/Fm, ΦPSII and NPQ ΦPSII-EC_{50} : (µg L^{-1}) n.d. (10ºC) 41.04 (15ºC) 58.32 (25ºC)	Chalifour and Juneau (2011)

Photosynthetic organisms	End-points measured	Duration	Conc. of triazines tested	Effect parameter	Reference
				M. aeruginosa **CPCC299** No significant effect on growth rate, Fv/Fm, ΦPSII and NPQ ΦPSII-EC_{50} : (µg L^{-1}) n.d. (10ºC) 21.6 (15ºC) 49.68 (25ºC)	Chalifour and Juneau (2011)
Estuarine microalgal species: *Dunaliella tertiolecta,* *Ankistrodesmus sp.,* *Storeatula major,* *Amphidinium operculatum*	Growth rate, cell density, primary productivity, total biovolume, chlorophyll-a, total protein, total lipid	96 hours	Atrazine: 12.5, 25, 50, 100, 200 µg L^{-1}	***D. tertiolecta*** EC_{50} (µg L^{-1}) Cell density = 66.35 Growth rate = 69.44 Primary productivity = 66.81 Total biovolume = 68.66 Chl. *a* = 65.0 Total protein= n.d. Total lipid = 47.85	De Lorenzo *et al.*, (2004)

Photosynthetic organisms	End-points measured	Duration	Conc. of triazines tested	Effect parameter	Reference
				Ankistrodesmus sp. EC_{50} (µg L^{-1}) Cell density = 32.50 Growth rate = 55.7 Primary productivity = 37.07 Total biovolume = 32.36 Chl. *a* = 11.87 Total protein= n.d. Total lipid = n.d. ***Storeatula major*** EC_{50} (µg L^{-1}) Cell density = 49.16 Growth rate = 89.97 Primary productivity = 22.17 Total biovolume = 48.45 Chl. *a* = 45.81 Total protein= 93.65 Total lipid = 174.87	De Lorenzo *et al.*, (2004)

Photosynthetic organisms	End-points measured	Duration	Conc. of triazines tested	Effect parameter	Reference
				Amphidinium operculatum EC_{50} (µg L^{-1}) Cell density = 74.18 Growth rate = 132.11 Primary productivity = 33.07 Total biovolume = 17.19 Chl. *a* = 146.7 Total protein= n.d. Total lipid = 11.53	De Lorenzo *et al*., (2004)

FW = freshwater, MA = marine, n.d.= not determined, NPQ = non-photochemical quenching

Various results were observed in relation to the effects of triazines (TBA and atrazine) on growth of various photosynthetic organisms. A recent study on *Gonyaulax spinifera* and *Prorocentrum minimum* (flagellates) showed that no significant inhibitory effects was observed on *P. minimum* at 30 µg L^{-1}, but for *G. spinifera*, a pronounced effect on the growth was recorded at 5 µg L^{-1} (Fiori *et al*., 2013). Moreover, at similar concentration of TBA (5 µg L^{-1}), the growth of *Skeletonema marinoi* was significantly affected and exacerbated at higher concentrations (10, 20 and 30 µg L^{-1}) (Fiori and Pistocchi, 2014). In another study by Chalifour and Juneau (2011), atrazine at 0.1 µM (21.6 µg L^{-1}) had no effect on the growth of algae (*Scenedesmus obliquus*) and

cyanobacteria (*Microcystis aeruginosa*) at 25°C, but not for *Navicula pelliculosa.* Besides, evaluation on different strains of *S. subspicatus* to 1, 5, or 20 µg L^{-1} of atrazine by Behra *et al*., (1999) also reported that the herbicide did not significantly affect the growth rate of the algae. On the other hand, De Lorenzo *et al*., (2004) found that atrazine significantly decreased the cell density, productivity rate, biomass and biovolume in all algal populations tested at atrazine concentrations ≥12.5 µg L^{-1}.

In addition, due to the specific effect of triazine on the photosystem II, evaluation on the photosynthetic efficiency has been used in order to determine the toxicity of the herbicide on different photosynthetic organisms. For example, TBA at 25 and 50 µg L^{-1} have significantly affect the photosynthetic efficiency (ΦPSII) of a group of marine flagellates of the Northern Adriatic Sea except to the *Prorocentrum minimum*. The percentages of PSII inhibition were recorded between 18 and 95% compared to the control. In fact, significant differences were also detected between the effect of two different concentrations of TBA (25 and 50 µg L^{-1}). Subsequent study using the most sensitive flagellate, *G. spinifera* also revealed that at 1 µg L^{-1} of TBA, 23 ± 1% of photoinhibition was recorded compared to the control (Fiori *et al*., 2013). Moreover, TBA was also found to cause significant decline in PSII efficiency from the concentration of 5 µg L^{-1} up to 30 µg L^{-1} as compared to the control in *S. marinoi* (Fiori and Pistocchi, 2014). In another study using olive plants (*Olea europaea*) by Canero *et al*., (2011), no difference between treatments were recorded for Fv/Fm values after 24 hours or 15 days exposure to TBA (3 kg ha^{-1}), but after 60 days, the plants have recorded the lowest Fv/Fm values. Additionally, olives that have been treated with TBA (3 kg ha^{-1}) also showed lower ΦPSII values, 15 days after the herbicide treatment but were not statistically significant. Furthermore, atrazine at 21.57 µg L^{-1} was reported to cause significant reductions of the operational photosystem II (PSII) quantum yield (ΦPSII) and the electron transport rate in the active reaction center (ET/RC) in

Scenedesmus obliquus, *Navicula pelliculosa* and *Microcystis aeruginosa* (Chalifour and Juneau, 2011). However, Behra *et al*., (1999) found that exposure of different strains of *S. subspicatus* to 1, 5, or 20 µg L^{-1} of atrazine did not significantly affect their photosynthetic activity but not at 500 µg L^{-1}.

Recent study has demonstrated that exposure to triazine herbicides affects the antioxidant defence system in non-photosynthetic organisms (e.g. TBA on fish (*Cyprinus carpio*) (Velisek *et al*., 2014) causing an imbalance between reactive oxidative system production and elimination and resulting in oxidative stress and organ damage. However, to date, there is still lack of information on algae in terms of adaptive mechanisms such as antioxidative responses (enzymatic and non-enzymatic) as a result of TBA exposure, which makes studies into those particular responses desirable. In fact, although there have been several studies that have evaluated the effect of TBA on different model of organisms such as fish (Perez *et al*., 2013, Stepanova *et al*., 2012, Dezfuli *et al*., 2006), flagellates (Fiori *et al*., 2013), earthworms (Tejada *et al*., 2013) and terrestrial plants (Canero *et al*., 2011), almost no studies have been carried out regarding the effects of this herbicide on macroalgal species.

1.3 Antifouling (AF) booster biocides

In addition to herbicides, the extensive use of antifouling (AF) booster biocides on ship hulls and other submerged surfaces is also responsible for the contamination of water and sediments in the environment. Guardiola *et al*., (2012) have defined biocides as the chemical substances that can deter or kill the organisms responsible for biofouling. These organic booster biocides were introduced as a replacement for the most widely used active ingredients in paint formulations, tributyltin (TBT), which has been regulated internationally since 1990 due to its severe impact on the aquatic

ecosystem (Fent, 1996). Chlorothalonil, dichlofluanid, Seanine 211, diuron, Irgarol 1051, zinc pyrithione (ZPT) and zineb are some examples of booster biocides that commonly incorporated with antifouling paints and also the most frequently used in many countries (Parks *et al*., 2010). As a brief explanation, Figure 1.1 depicts the process of biocides release from antifouling paints, where the sea water will penetrate into the paint, dissolve such biocides, and diffuse out into the environment.

Figure 1.1 has been removed due to Copyright restrictions

Figure 1.1**:** Schematic illustration of the behaviour of a biocide-based antifouling system exposed to sea water (Retrieved from Yebra *et al*., 2004).

As a consequence, significant coastal concentrations of biocides have been found in areas of high yachting activity, particularly in marinas and sportive harbours. However, studies have shown that two of the most popular biocides in use, Irgarol 1051 and diuron, persist in surface waters, whilst other biocides, such as Sea-nine 211, dichlofluanid, zinc pyrithione and chlorothalonil, disappear quickly (Thomas *et al*., 2003). Despite assumed as environmentally sound, the booster biocides' effects are poorly understood. Lambert *et al.* (2006), have stressed that the presence of booster biocides in water bodies caused a wide range of calculable risk to aquatic plants. This in turn also determines the primary productivity and biodiversity of an aquatic ecosystem.

Evidence shows that 'booster' biocides can reduce the germination and growth of non-target species such as algae, *Hormosira banksii* (e.g., diuron, zineb, DCOIT and zinc pyrithione, Myers *et al*., 2006), and seagrasses such as *Zostera marina* (e.g., Irgarol 1051 and diuron, Chesworth *et al*., 2004). Moreover, it was also found to reduce the photosynthetic efficiency of symbiotic algae in corals (e.g., Irgarol 1051; Carbery *et al*., 2006) and is toxic to sea urchin eggs and embryos (e.g., DCOIT, zinc and copper pyrithione, Kobayashi and Okamura, 2002). In the most severe case, the risk that can be linked to the use of booster biocides in antifouling paints may involve humans and other predators that ingest the marine organisms (e.g. fish, shellfish, seaweeds) that have accumulated these biocides. Therefore, in this study, *E. siliculosus* will be exposed to diuron, one of the most persistent and widely used AF booster biocides in the environment. Toxicity will be determined from physiological (growth and photosynthetic efficiency) and biochemical (antioxidative activities) responses.

1.3.1 Diuron (DIU)

Diuron or DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] is a colourless crystalline compound in its pure form (Sheikh *et al*., 2012). It has been used widely as an antifouling (AF) agent in paints. After the ban of the tributyltin-based biocides, diuron has become one of the most popular used antifouling paint biocides on the market, used in a number of countries such as the United Kingdom, Sweden, Spain, the Netherlands, Portugal and Japan (Konstantinou and Albanis, 2004; Silkina *et al*., 2009).

It is a non-ionic compound, with a moderate water solubility of 42 mg L^{-1} at 20°C (Sheikh *et al*., 2012). Although it is degradable, it is still considered to be relatively persistent in seawater (Callow and Willingham, 1996). Giocomazzi and Cochet (2004) stated that diuron can remain from one month to up to one year in a given ecosystem. It is strongly hydrophilic molecule and is not volatile (Gramatica and

Di Guardo, 2002). These properties increase the potential of toxicity and bioaccumulation of diuron due to high environmental concentrations.

Various concentrations of diuron (6.74 – 238.4 µg L^{-1}) have been detected in different aquatic environments at concentrations up to some hundreds µg L^{-1} (Revitt *et al*., 2002; Thomas *et al*., 2001(b); Jones, 2005; Mitchell *et al*., 2005). For instances, high levels of diuron have been reported in UK (up to 6,742 ng L^{-1}), Spain (up to 2,000 ng L^{-1}), and Japan (up to 3,054 ng L^{-1}) previously (review by Kumar *et al*., 2010). Besides, alarming concentrations of DIU were also recorded in French (10 µg L^{-1}) (Blanchoud *et al*., 2004) and North American (30 µg L^{-1}) (Field, 2003) surface waters. Early study by Dahl and Blanck (1996), also observed the presence of diuron in Swedish marinas of between 10-100 ng L^{-1}. Moreover, Lamoree *et al*., in 2002, also evidenced higher than maximum permitted levels (430 ng L^{-1}) of diuron in the Dutch coastal waters and marinas during the yachting season. In fact, a recent survey by Loos *et al*., (2009) reported that diuron was detected in 70% of European river samples.

Due to the growing awareness of the environmental issues associated with antifouling paints, the UK Health and Safety Executive (HSE) imposed a ban on the use of diuron as antifouling agent in November 2002 (Silkina *et al*., 2009). Although antifouling paints containing diuron are no longer permitted for use in the United Kingdom and most European countries, the risk assessment approach undertaken in Australia (July 2011 environmental assessment), using a very conservative model, concluded that diuron antifouling use patterns in Australia did not present risks to aquatic organisms. Besides, the Australian Pesticides and Veterinary Medicine Authority (APVMA) also concluded that the use of diuron for algal control in aquariums and ponds did not pose risks to aquatic organisms, given the low concentrations and application rates allowed in Australia (July 2011 environ. assess.)

(APVMA, 2012). Therefore, the risks from diuron usage are still exist even though it is banned in certain regions.

In addition, diuron has been found to be a potent inhibitor of photosynthesis, through blocking of electrons transfer in the photosystem II (Giacomazzi and Cochet, 2004). This blockage of electrons flow will result in the formation of singlet oxygen ($^1O_2$) leading to lipid peroxidation (Fuerst and Norman, 1991). This condition may severely affect aquatic ecosystems due to its ability to exert selective pressure and alter phototrophic species assemblages (Silkina *et al*., 2009). Consequently, deleterious impacts on seagrasses or other autotrophic organisms and reductions of photosynthetic activity in micro- and macroalgae in the environments can be expected. However, the toxicity of diuron on non-target organisms is not thoroughly understood. Several studies (Table 1.3), have evaluated the use of diuron as a representative of organic booster biocides in replacing tributyltin (TBT)-based antifouling products in different photosynthetic organisms.

Table 1.3: The effects of diuron on photosynthetic organisms

Photosynthetic organisms	End-points measured	Duration	Conc. of diuron tested	Effect parameter	Reference
Hormosira banksii (MA macroalgae)	Growth and germination	48, 72 hours	0, 0.5, 1, 2.5, 5, 7.5, 10 mg L^{-1}	EC_{50} (mg L^{-1}): Germination: 48 h = 6.29 72 h = 6.82 Rhizoid growth: 48 h = 6.75 72 h = 7.33	Myers *et al*., (2006)

Photosynthetic organisms	End-points measured	Duration	Conc. of diuron tested	Effect parameter	Reference
Saccharina japonica (MA macroalgae)	Growth, photosynthetic activity, pigment content, antioxidant activity	15 days	0, 0.00625, 0.025, 0.1, 0.4 mg L^{-1}	Reduction in fresh weight: (15 d EC_{50} = 87.8 µg L^{-1}) Reduction in Fv/Fm: (15 d EC_{50} = 10.9 µg L^{-1}) Reduction in photosynthetic pigments (Chl. *a* and carotenoid) Reduction in NPQ Decline in ETR_{max} Decrease in antioxidant activity	Kumar *et al*., (2010)
Lemna gibba (FW plant)	Photosynthetic activity	5, 24, 48 hours	1 - 100 µg L^{-1}	Increase in fluorescence toxicity index Reduction in Fv/Fm, ΦPSII, qP	Dewez *et al*., (2002)

Photosynthetic organisms	End-points measured	Duration	Conc. of diuron tested	Effect parameter	Reference
Seagrass: *Cymodocea serrulata, Halophila ovalis, Zostera capricorni*	Photosynthetic efficiency	10 days	0.1, 1, 10 and 100 µg L^{-1}	Reduction in ΦPSII of all species after 2 h of exposure to 10 and 100 µg L^{-1} DIU Reduction in ΦPSII of *H. ovalis* to 0.1 and 1.0 µg L^{-1} DIU after 24 h of exposure Significant reduction of ΦPSII in *H. ovalis* and *Z. Capricorni* to all DIU conc. (0.1-100 µgL^{-1}) after 5 days exposure, whereas ΦPSII in *C. serrulata* was only significantly lower in plants exposed to highest DIU conc. (10 and 100 µg L^{-1})	Haynes *et al*., (2000)
Seagrass (MA) *Zostera marina*	Growth, chlorophyll fluorescence	10 days	0.5 – 25 µg L^{-1}	Reduction in Fv/Fm: LOEC = 1.0 µg L^{-1} 10 day EC_{50} = 3.2 µg L^{-1} Significant reduction in growth at 5 µg L^{-1} of DIU	Chesworth *et al*., (2004)

Photosynthetic organisms	End-points measured	Duration	Conc. of diuron tested	Effect parameter	Reference
Coral (MA): *Galaxea fascicularis*	Photosynthesis and calcification rate	96 hours	0, 1, and 10 µg L^{-1}	Significant reduction in photosynthesis rate to 10 µg L^{-1} DIU Dropped in calcification rate to 67.3% less than the control at 10 µg L^{-1} DIU.	Sheikh *et al.*, (2012)
Lemna minor (FW macrophyte)	Growth, chlorophyll content	7 days	5 – 100 µg L^{-1}	Inhibition of growth : IC_{50} = 25 µgL^{-1} IC_{90} = 60 µgL^{-1} LOEC= 5 µgL^{-1} Significant increase in total chlorophyll content at lower conc. of DIU (10 and 20 µg L^{-1}) but decrease at higher conc.	Teisseire *et al.*, (1999)
FW cyanobacteria: *Synechococcus sp*. PCC7942	Growth, lipid peroxidation, antioxidase activities	48, 72 and 96 hours	0.01, 0.03, 0.06, 0.09 and 0.12 µmol L^{-1} [2.33, 6.99, 13.98, 20.97 and 27.96 µg L^{-1}]	EC_{50} Growth (µg L^{-1}): 48 h EC_{50} = 17.24 72 h EC_{50} = 22.6 96 h EC_{50} = 22.6	Deng *et al.*, (2012)

Photosynthetic organisms	End-points measured	Duration	Conc. of diuron tested	Effect parameter	Reference
				Increase the soluble protein content and catalase activity at low concentrations, but inhibited them at high concentrations Increase in lipid peroxidation (MDA content).	Deng *et al*., (2012)
FW Macrophytes: *Elodea canadensis,* *Myriophyllum spicatum,* *Potamogeton lucens*	Growth, photosynthetic efficiency	35 days	5 µg L^{-1}	Growth of exposed macrophytes was not significantly affected Significant reduction in ΦPSII	Knauert *et al*., (2010)

FW = freshwater, MA = marine, NPQ = non-photochemical quenching,
IC = inhibition concentration

Investigations on the effect of diuron on growth of several photosynthetic organisms have been carried out by several researchers. According to Myers *et al*., (2006) diuron at 5-10 mg L^{-1} has been observed to cause significant declines in germination and growth of marine macroalgae, *Hormosira banksii*. EC_{50} (72h) values of 6.82 and 7.33 mg L^{-1} were recorded for germination and rhizoid growth respectively. In another study, significant reductions in relative growth rate (RGR) of fresh weight and area of *Saccharina japonica* (seaweed) were observed at 0.025 mg $L^{-1}$and 0.00625 mg

L^{-1} respectively (Kumar *et al*., 2010). In fact, an increment in diuron concentration (0.4 mg L^{-1}) caused a 72.91% reduction in fresh weight which was also indicated by a slight shrinkage of the disks. Besides, diuron at concentrations as low as 5 µg L^{-1} was observed to induce inhibition on *Lemna minor* growth with further inhibition as much as 50% was recorded at 25 µg L^{-1} of the herbicide after 7 days of exposure (Teisseire *et al*., 1999). Moreover, a study on the growth of seagrass (*Z. marina*) by Chesworth *et al*., (2004) also discovered that diuron at 5 µg L^{-1} had caused a significant reduction with further significant decrease observed at 10 µg L^{-1}. Likewise, in a study done by Deng *et al*., (2012) diuron at 0.06, 0.09, and 0.12 µmol L^{-1} also significantly inhibited the microalgal growth (*Synechococcus*) relative to the controls.

Meanwhile, another significant end-point that has commonly been evaluated in response to diuron effect is the photosynthetic activity. Conrad *et al*., (1993) clearly described the significance of measuring chlorophyll fluorescence yield in order to assess the toxic effect of diuron. Evaluation of photosynthetic efficiency (measured as effective quantum yield, ΔF/Fm') on three different seagrasses by Haynes *et al*., (2000) had observed that diuron at 10 and 100 µg L^{-1} reduced the effective quantum yield in all three tested species by 50-75% after a 5-day exposure period. In fact, at lower concentrations of diuron (0.1 and 1.0 µg L^{-1}), the effective quantum yield reduced by 10% and 30% in *H. ovalis* and *Z. capricorni*, respectively, whereas *C. serrulata* was essentially unaffected by the same concentrations of diuron. Another study by Knauert *et al*., (2010) found that diuron at 5 µg L^{-1} significantly ($P < 0.05$) reduced the photosynthetic efficiency of *E. canadensis* on days 2 and 5 by 57 and 80% relative to control respectively, whereas on *P. lucens* the photosynthetic efficiency was significantly ($P < 0.05$) affected only at day 5 with a 55% reduction relative to control. Furthermore, a severe damage to the photosystem II (PSII) of *S. japonica* due to diuron exposure was evident by the EC_{50} value of 0.0109 (0.0026-0.0218, 95% C.I.) mg L^{-1}

recorded for the maximum efficiency of PSII (Fv/Fm) (Kumar *et al*., 2010). A different study using the aquatic plant (*Lemna gibba*) by Dewez *et al*., (2002) also has reported that the effective quantum yield of PSII (ΦPSII) decreased by 22 and 90% as compared to the control at 2 and 50 µg L^{-1} diuron, respectively, after 48 hours of exposure. Moreover, diuron at 10 µg L^{-1} was also observed to significantly reduce the photosynthesis rate in coral (*Galaxea fascicularis*) after 96 hours of exposure (Sheikh *et al*., 2012). Similarly, a decrease in photosynthetic efficiency (represented as Fv/Fm) of *Z. marina* was also discovered by Chesworth *et al*., (2004). They found that after 10 days of exposure to 25 µg L^{-1} of diuron, the seagrass recorded the lowest value of Fv/Fm (40% reduction), while a significant decrease in Fv/Fm was seen at 1 µg L^{-1} of the herbicide.

In terms of biochemical activities, diuron has been reported to cause increment of intracellular reactive oxygen species (ROS) in certain strains of marine green algae (*Tetraselmis suecica*) (Stachowski-Haberkorn *et al*., 2013). Apart from that, analysis on cyanobacteria (*Synechococcus*) by Deng *et al*., (2012) using lipid peroxidation approach (MDA content), observed that the MDA content in the diuron treatments showed a certain increase with increasing diuron concentrations (0.01-0.12 µmol L^{-1}). In fact, as compared to the untreated group, the highest MDA levels in the diuron treatments were recorded at 0.06 and 0.12 µmol L^{-1}, which increased by 39.2% and 34.5%, respectively. However, a study by Kumar *et al*., (2010) on non-enzymatic antioxidant activity of *S. japonica* using DPPH scavenging assay showed decreasing antioxidant activity with increasing concentrations of diuron. A significant reduction as much as 35.56% was observed in the scavenging activity at 0.4 mg L^{-1} of diuron.

Evaluations on the antioxidative enzymes activities were also significant in order to elucidate the adaptive responses taken by living organisms to cope with

different stressors. Exposure of diuron on cyanobacteria (*Synechococcus*) by Deng *et al*., (2012) have discovered that at concentrations less than 0.09 µmol L^{-1}, no significant changes in the catalase (CAT) activity were recorded with respect to the untreated group. However, once the bacteria were exposed to 0.09 µmol L^{-1} of diuron, the highest CAT activity (8.32 U g^{-1} protein) was recorded, which decreased sharply to a level slightly higher than that in the untreated group (0.77 U g^{-1} protein) at 0.12 µmol L^{-1}. On the other hand, inhibition of enzyme activities (e.g. superoxide dismutase, phenoloxidase) in Pacific oyster (*Crassostrea gigas*) were observed by Luna-Acosta *et al*., (2012), after being exposed to 1 µg L^{-1} diuron.

To date, evaluations on antioxidative defence mechanisms, either enzymatic (e.g. catalase, ascorbate peroxidase, glutathione reductase) or non-enzymatic (antioxidant compounds) in response to diuron exposure remain scarce. Almost no studies have been done specifically on seaweed. Therefore, investigations into that particular aspect may further extend understanding of the effect of diuron.

1.4 Marine macroalgae as an important entity in the environment

Marine macroalgae (or seaweeds), are an assemblage of diverse groups of phototrophic marine plants and form the base of the marine trophic pyramid. They are sessile multicellular photosynthetic eukaryotes that are differentiated from plants by their lack of specialized tissues (e.g. root system and vascular structures) (Graham & Wilcox, 1999). Moreover, seaweeds are fast-growing, highly photosynthetically efficient, and live in most seas and oceans (Van Hal *et al*., 2014). Macroalgae can be classified as brown (Phaeophyta), green (Chlorophyta), or red (Rhodophyta) seaweeds based on the composition of their photosynthetic pigments (Kraan, 2013).

Brown seaweeds (kelps) were used in World War I to produce the acetone needed for the production of cordite-based gun and artillery shells (Neusheul, 1989). In addition, the minerals from seaweed were recycled as fertilizer and also have been investigated as a raw material for digestive production of biogas in the US (Chynoweth, 2002). The seaweed industry has an annual global value of USD 5.5-6 billion and is mainly used for food (USD 5 billion), phycocolloids (hydrocolloids), fertiliser, animal feed additives, cosmetics and medicines (Phang, 2010). Recent efforts in seaweed biorefining have focused on the production of biofuels such as ethanol (Wargacki *et al*., 2012), butanol (Wal *et al*., 2012), and biogas (Langlois *et al*., 2012).

Apart from that, in marine ecosystems, macroalgae communities are ecologically and biologically important. They provide nutrition, reproduction, and an accommodating environment for other living organisms (McClanahan *et al*., 2002, Wilson 2002). They make a major contribution to primary productivity and determine the physical structure of the habitat (Schiel & Foster, 2006). They allow for the maintenance of local biodiversity (Schiel, 2006; Schiel & Lilley, 2007) and act as nurseries and protective shelter for many invertebrate species. They also provide essential space for epibionts ranging from bacteria to macroinvertebrates (Wilson *et al*., 1990; Bulleri *et al*., 2002). In addition, they form the basis of the species-rich communities that dominate shallow coastal areas, where marine outfalls are located. These communities are a valuable ecological and economic resource that may be adversely affected by exposure to discharges (e.g. herbicides and AF paints) (Anderson and Hunt, 1988; Burridge *et al*., 1999; Eklund and Kautsky, 2003).

The importance of macroalgae as the key components of ecosystems in coastal waters and estuaries (Lam and Harder, 2007) makes it essential to study the impacts of

contaminants derived from anthropogenic activities have on them, as any deleterious effects on these photoautotorphs can have knock-on effects at higher trophic levels.

1.4.1 *Ectocarpus siliculosus* (brown algae)

Research on *Ectocarpus* began in the 19th century with a description of species and investigation of their taxonomic positions (Dillwyn, 1809). Subsequent studies were aimed at investigating the life cycle and the ultrastructure of the organism at different stages of the life cycle (Müller, 1972). Later investigation by Boland *et al*. (1995) looked into the identification of the sexual pheromone and its role in gamete recognition, while characterization of the *Ectocarpus* virus EsV-1 was carried out by Delaroque *et al*., (2001). These advances, including the most notable one where its genome have been fully sequenced (Cock *et al*., 2010), bring *Ectocarpus* to be a model organism for investigating responses to environmental stresses in brown algae more generally (Charrier *et al*., 2008).

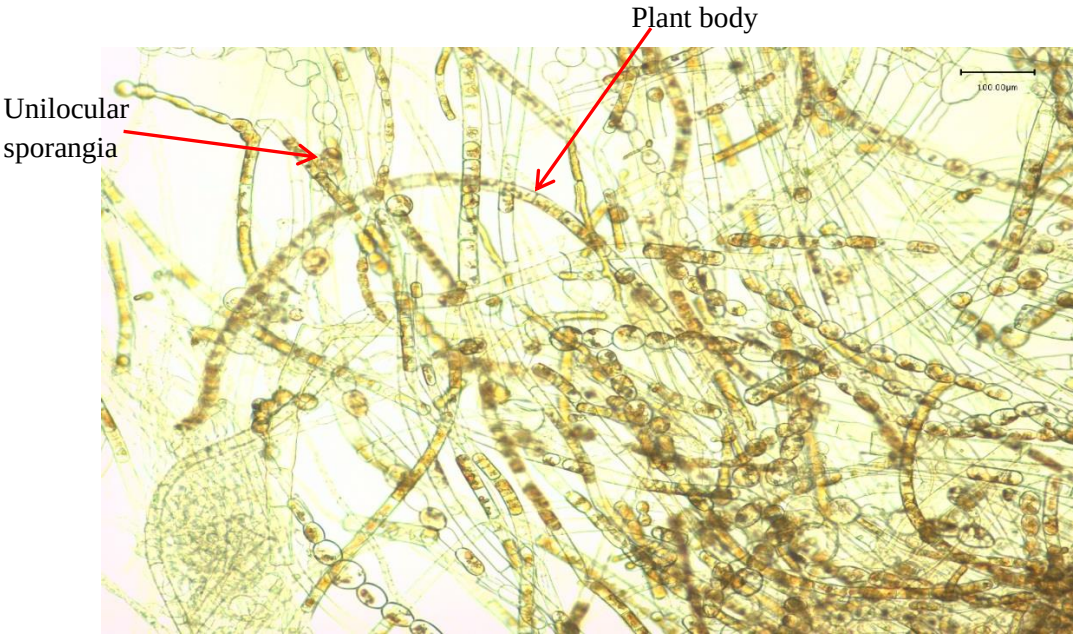

Figure 1.2: *Ectocarpus siliculosus*.

E. siliculosus (Figure 1.2) is a small filamentous brown alga. It has been extensively studied over the last two centuries for its complex life cycle and its physiological features (Charrier *et al*., 2008). One of the unique features of brown algae is that it shares several obvious features with land plants, such as the presence of a cell wall, although with a different composition (Kloareg and Quatrano, 1988), and similar growth metabolism and response (i.e. photosynthesis and phototropism). Plus, they also share subcellular features with animal cells, such as the presence of centrosomes (Katsaros *et al*., 2006), and certain aspects of their metabolism (production of eicosanoid oxylipins; Ritter *et al*., 2008). Moreover, it is widely distributed geographically, from the Arctic (Lee, 1980) to warm temperate regions such as the coast of Texas, USA (Edwards and Kapraun, 1973).

E. siliculosus belongs to *Ectocarpus* genus and are found to grow on rocky substrates or epiphytically on other algae and seagrass (Coelho *et al*., 2012,b). Many of them are subject to frequent changes in their local environment, because they are uncovered at low tide, and are thus exposed to desiccation and variations in osmotic pressure due to rain or evaporation (Le Bail *et al*., 2008). Since most brown algae grow on rocky shore habitats which indeed are stressful environments, these organisms (brown algae) are subjected to both biotic aggression from grazers and pathogens and various abiotic stresses including large variations in temperature, immersion, light irradiation and mechanical forces. In fact, pollution of the coasts, due to various anthropogenic activities, constitutes an additional source of abiotic stress against which they must develop adaptive mechanisms. In consequence, the brown algae represent a novel model system for several aspects of responses to biotic and abiotic stresses, including innate immunity (Potin *et al.*, 2002), viral infection (Müller *et al.*, 1998; Delaroque *et al.*, 2001), novel pathosystems (Maier *et al.*, 2000) and osmotic stress (Coelho *et al.*, 2002).

In addition to their suitability for use as research model organisms, brown algae in general have economic importance in some areas of the globe, with certain groups in Asia considering them as a central part of their diet (wakame, kombu) and Europe using them as a source of fertilizers, cosmetics, pharmaceutical products, and defence elicitors (Klarzynski *et al*., 2000; Abad *et al*., 2008; Holtkamp *et al*., 2009).

The rationale for selecting *E. siliculosus* as the model species was based on several characteristics. These includes its small size, the fact that the entire life cycle can be completed in Petri dishes in the laboratory (Müller *et al.*, 1998), its high fertility and rapid growth (the life cycle can be completed in 2-3 months), the ease with which genetic crosses can be carried out (Peters *et al.*, 2004) and the relatively small size of the genome. Due to these factors, this species was selected ~11 years ago as a genetic and genomic model organism for brown algae (Peters *et al.*, 2004). Moreover, Ectocarpales are closely related to the most economically important brown algal group, the Laminariales (Draisma *et al.*, 2003), making evaluations of this species desirable. Figure 1.3 indicates the phylogeny of brown algae and Ectocarpales.

Figure 1.3 has been removed due to Copyright restrictions

Figure 1.3: Phylogeny of brown algae and Ectocarpales (retrieved from Charrier *et al*., 2008). (a) Position of brown algae within the eukaryotes (adapted from Baldauf, 2003). Brown algae belong to the heterokont phylum, which is phylogenetically distant from land plants and the green and red algae. Photosynthetic organisms are framed. (b) Position of the Ectocarpales (in bold) within the brown algae (adapted from Kawai *et al*., 2007).

1.4.2 The life cycle of *E. siliculosus*

Ectocarpus has a haploid–diploid life cycle involving alternation between two multicellular generations, the sporophyte and the gametophyte (Arun *et al*., 2013). Diploid sporophytes produce haploid meiospores in unilocular sporangia. Following release, the meiospores germinate to give the haploid gametophyte generation. The dioecious gametophytes produced male and female gametes, which fuse to produce a zygote, which is the initial cell of the sporophyte generation (Arun *et al*., 2013). In certain condition in which the gametes unable to fuse with a gamete of the opposite sex, they can germinate parthenogenetically to produce haploid partheno-sporophytes (Arun *et al*., 2013). Figure 1.4 depicts the life cycle of *E. siliculosus*.

Figure 1.4 has been removed due to Copyright restrictions

Figure 1.4: Life cycle of *E. siliculosus*
(retrieved from Charrier *et al*., 2008)

1.4.3 Responses to biotic and abiotic stresses

Coastal ecosystems are harsh environments and therefore *Ectocarpus* is not only prone to infection by a wide range of pathogens, including viruses, oomycetes, chytrids, hyphochytrids, and parasites related to Plasmodiophorea (Müller *et al*. 1998; Maier *et al*. 2000; Gachon *et al*. 2009). They are also subject to considerable abiotic stress because of continuous variations in temperature, salinity, and light intensity. In relation to abiotic stressor such as heavy metals, a previous study by Russell and Morris (1972) demonstrated that copper tolerance is associated with the transport of ship fouling algae such as *Ectocarpus siliculosus*. A subsequent study by Morris (1974) on two different strains of Ectocarpus*; E. fasciculatus and E. siliculosus* revealed that interspecific variations in copper tolerance have been observed with the latter being more tolerant but yet there is no clear explanation for the interspecific variations between the strains. Moreover, Hall (1980) also observed that a copper-tolerant population of *E. siliculosus* confer co-tolerance to other metals such as cobalt and zinc.

In addition, salinity ecotypes within *E. siliculosus* have been recorded by Russell and Bolton (1975). Due to variations in salinity, Thomas & Kirst, (1991a,b) also have found that *E. siliculosus* isolates from different geographic locations showed huge differences in photosynthesis, accumulation of osmotically active compounds (mannitol; Davis et al., 2003) and vitality. Moreover, changes such as down-regulation of the synthesis and metabolism of amino acids were observed by Dittami *et al*., (2009) under hyposaline and hypersaline stresses. In fact, the salinity stress has affected the genes involved in the synthesis of valine, leucine, and isoleucine as well as the aromatic amino acids (phenylalanine, tyrosine, tryptophan), and arginine and proline metabolism. In another study by De Franco *et al*., (2009), significant biochemical change in *E. siliculosus* (increment of GST activity) was observed after 6 hours of exposure to herbicide (glyphosate formulated).

A recent study on metal stress (Cu) indicated different response between *E. siliculosus* strains isolated from different locations (Saez *et al*., 2015). However, there is no study has been done on the effects of organic anthropogenic discharges as described previously (e.g. diuron, terbuthylazine and isoproturon) on the species, which are commonly coexist with the metal wastes (Sandrin and Maier, 2003; Olaniran *et al*., 2013). Therefore, the underlying adaptive mechanisms of *E. siliculosus* to the herbicides remain questionable and interesting to be explored. In order to fill this knowledge gap, toxicological studies encompassing the metabolic adaptation of *E. siliculosus* strains to the herbicides singly and in mixtures were carried out. Effects of the herbicides at different level (0-500 µg L^{-1}) on growth, photosynthetic efficiency, oxidative stress, and antioxidant response were investigated in order to elucidate the homeostasis mechanisms of brown algae to pollution and environmental changes.

1.5 Objectives of the study

1.5.1 To evaluate the effects of diuron, terbuthylazine and isoproturon on the growth and photosynthetic efficiency of *Ectocarpus siliculosus* strains (LIA4 and Es524)

1.5.2 To investigate the biochemical response exhibited by both strains of *Ectocarpus siliculosus* to the different herbicides / booster biocides

1.5.3 To evaluate the effects of the mixture of the herbicides at physiological and biochemical levels in both strains of *Ectocarpus siliculosus*

Chapter 2

General Materials and Methods

2.1 Prerequisite preparation for culturing *Ectocarpus siliculosus*

2.1.1 Preparation of Provasoli nutrient enrichment solution

The method was originally adopted from Provasoli and Carlucci (1974). The protocol was later modified from Coelho *et al*., (2012a). The solutions are prepared separately, autoclaved and conserved at 4°C in glass amber bottles.

Solution 1 – 10X (for 1 L) :	**Quantity for 1 L**	**Final concentration**
H_3BO_3 (MW=61.83)	1.9 g	30.7 mM
$FeCl_3$ (MW= 162.21)	0.05 g	0.3 mM
$MnSO_4(H_2O)$ (MW=169.02)	0.273 g	1.6 mM
$ZnSO_4(7H_2O)$ (MW=287.54)	0.0367 g	0.127 mM
$CoSO_4(7H_2O)$ (MW=281.1)	0.008 g	28 µM
0.5 M EDTA pH8 (MW=292.24)	11.4 ml	5.7mM

Solution 2 – 100X	**Quantity for 0.5 L**	**Concentration**
Vitamin B12 (Cyanocobalamine)	3.35 mg	0.0067 g/litre
Vitamin B1(Thiamine hydrochloride) (MW= 337.27)	165 mg	0.33
Biotine $C_{10}H_{16}N_2O_3S$ (MW=244.31)	1.65 mg	0.0033
TRIS = Trisma base $C_4H_{11}NO_3$ (MW= 121.14)	166.5 mg	333.0

Solution 3 – 10X	**Quantity for 1 L**	**Concentration**
$(NH_4)_2Fe(SO_4)_2(6H_2O)$ (MW= 392.14)	1.17 g	3mM
EDTA 0.5 M pH8 (MW= 292.24)	6.8 ml	3.4mM

Solution 4 (10X)	**Quantity for 1 L**	**Concentration**
$NaNO_3$ (MW= 84.99)	23 g	270 mM

Solution 5 (10X)	**Quantity for 1 L**	**Concentration**
Glycérophosphate $C_3H_7Na_2O_6P$ $(5H_2O)$ (MW = 216.04)	3.33 g	15.4 mM

Table 2.1 Preparation of Provasoli solution

Provasoli solution is prepared according to need. Dark bottle is used for solution 2 since it contains light sensitive vitamins. For 1 litre of Provasoli solution, 100 ml each of solution 1, 3, 4, and 5 plus 10 ml of solution 2 were added to milliQ water (starting pH should be between 9.6 and 9.8). The pH was adjusted to 7.8 with concentrated HCl (37%) and the volume was made up to 1 litre with milliQ water. Finally, the solution was aliquoted into small glass bottles (20, 50, 100 or 200 ml), autoclaved, and stored at 4°C. Usage amount of Provasoli: 10 or 20 ml L^{-1} of seawater.

2.1.2 Materials preparation

Prior to experiments, all glassware (petri dishes, measuring cylinders, volumetric flasks) and plasticwares used to prepare and store either medium or stock solutions are first washed in HCl (5 %) for at least 24 hours to reduce metal contamination. Then the containers were rinsed with milliQ water and dried.

2.2 Culture conditions of *Ectocarpus siliculosus*

Two strains of *E. siliculosus (*LIA4 and Es524) originating from a pristine area of Scotland (Lon Liath/LIA4, 56º56´N: 5º51´W) and a copper polluted site in Chile (Palito la Boca, Chanaral, 26º15´S: 69º34´W), respectively, were obtained from the Marine Biological Association of the United Kingdom culture collection. Both strains were cultivated in clear, acid wash, 10 L polycarbonate flasks in controlled environment chambers (CEC, Sanyo MLR-350/HT) at 15°C using filtered and autoclaved natural seawater enriched with Provasoli medium (Dubber and Harder, 2008). The medium was changed every 2 weeks. Light was provided by cool white fluorescence tubes (Philips) with a photon fluence rate of 40 μmol m^{-2} s^{-1} for 14 h per day. Cultures were bubbled with filtered (0.22 μm) compressed air to avoid CO_2 depletion and maintain the seaweed in suspension (Starr and Zeikus, 1993).

2.3 Handling and manipulation of *Ectocarpus siliculosus* cultures

The methods used in the handling and manipulation of *E. siliculosus* cultures were adapted from those provided by Station Biologique of Roscoff, France (Figure 2.1). To avoid contamination by pathogens and microorganisms, *E. siliculosus* was manipulated under sterile conditions within a laminar flow cabinet (LFC). The LFC was first cleaned with ethanol (70% ethanol + 30 % distilled water) before being switched on and left for 10 min prior to use. All required materials (culture medium, petri dishes, tweezers, parafilm, 100% ethanol, pipettes, Bunsen burner, matches, scissors, gloves) were placed inside the LFC during the manipulation procedure.

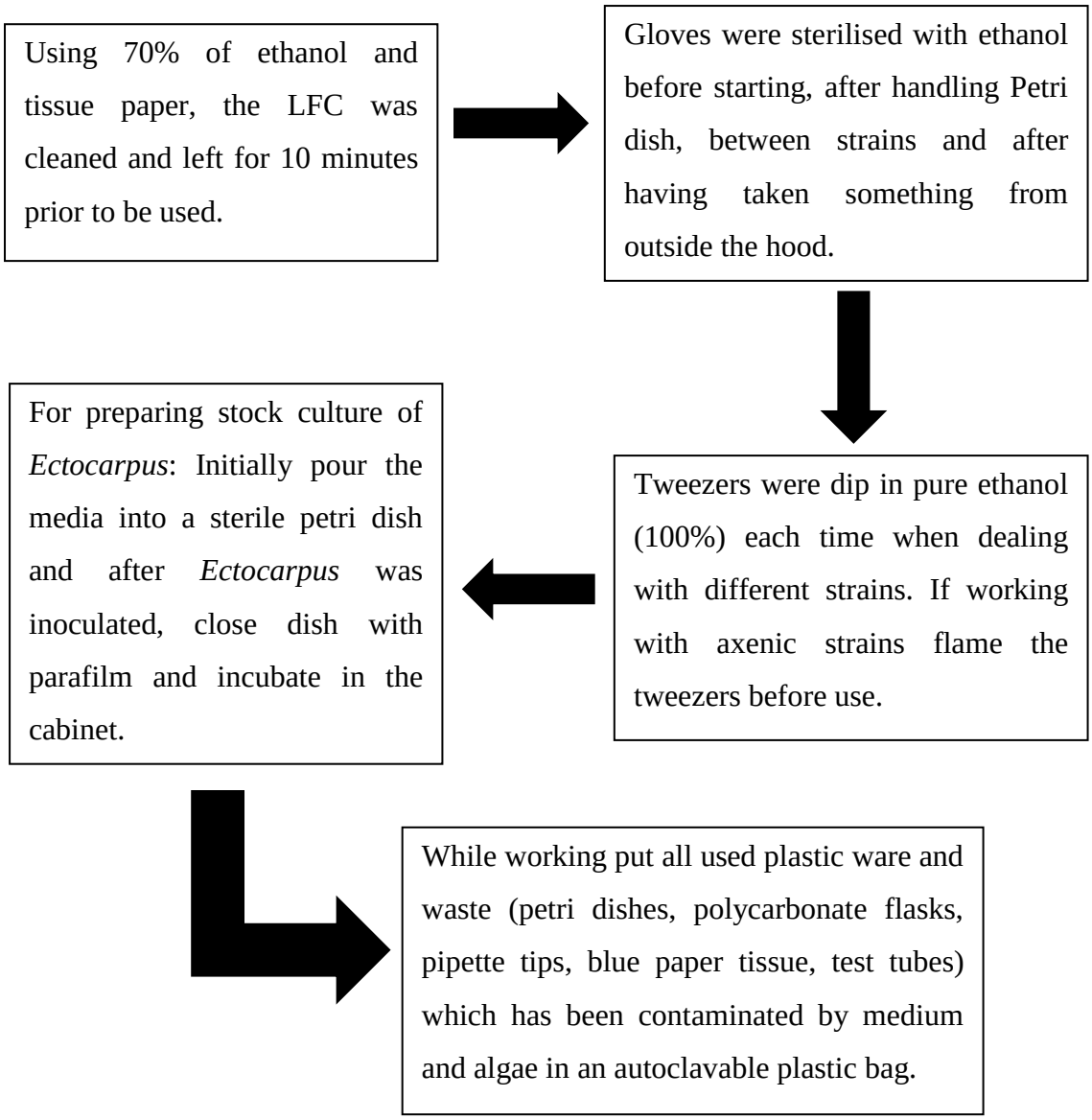

Figure 2.1: Handling and manipulation of *Ectocarpus*

2.3.1 Transferring cultures:

To stimulate growth, a transfer of material was required every 1 to 3 weeks, depending on the strain and experimental design. Sterile petri dishes used for the transfers were filled in with the desired media: 10 ml of Provasoli enriched solution (PES) in 55 mm diameter petri dishes, 30 ml PES in 90 mm diameter petri dishes, or 100 ml PES in 140 mm diameter petri dishes. Then, the biological material was transferred onto a clean Petri dish and sealed with parafilms before being incubated at 15°C to enhance sporophyte production. The fluorescent light was set at 20-30 µmol m^{-2} s^{-1} photon irradiance rate, with a 14:10 hour light:dark cycle.

2.3.2 Reminders:

In situation where the biological material was stuck at the bottom of the Petri dish, firstly, the old medium was pour to waste. Then, the cover (lid) of the Petri dish was used to pour fresh PES into the Petri dish. In addition, if a large biomass was required, the 10 L bottles (carboys) [Figure 2.2] were used as follows. The bottles should be autoclaved before being used. Prior to autoclave, the bottles (10 L) were filled in with NSW and fitted with two aeration tubes. Later, these were equipped with sterile filters (0.22 µm). After autoclave, they were left to cool at room temperature. Later, Provasoli and the strain (*E. siliculosus*) to be used were added. Then, the lid was tightened under the hood and connections to the tap of air source were made inside the growth chamber. Finally, the culture environmental conditions were set according to the conditions mentioned previously.

Figure 2.2: Culture of *Ectocarpus siliculosus* in 10 L bottles

2.4 Test chemicals and solutions

Diuron (DIU), terbuthylazine (TBA) and isoproturon (IPU) (PESTANAL grade) (Table 2.2) were supplied by Sigma-Aldrich Gillingham Dorset, UK. Stock solutions of each chemical were prepared with analytical grade DMSO (dimethyl sulfoxide, 99% purity) and the concentration of DMSO remained <0.005% in all studies of herbicide solutions. All working solutions were made immediately prior to use. Chemical analysis using sophisticated equipment such as HPLC and Mass Spectrometry was not carried out for several reasons, including the complex procedures for sample preparation, the

need for expensive chemicals and equipment, and potential interference from secondary pollutants during analysis (Park *et al*., 2012). This purely chemical approach does not provide ecologically significant information on temporal changes in exposure or the interactive effects of pollutants (Kumar and Han, 2010). Therefore, the concentrations of herbicides in the present study were reported in terms of nominal concentration. Biological assays were developed and employed to assess pollutant-induced ecological risks.

Chemicals	Molecular weight	EC-No	Prepared in:
Diuron	233.09 g mol^{-1}	206-354-4	DMSO
Terbuthylazine	229.71 g mol^{-1}	227-637-9	DMSO
Isoproturon	206.28 g mol^{-1}	251-835-4	DMSO

Table 2.2 List of herbicides and booster biocide used in the present study.

2.5 Algal test species and culture conditions

E. siliculosus strains (LIA4 and Es524) were exposed to six concentrations (1, 5, 10, 50, 100 and 500 µg L^{-1}) of DIU and TBA, while IPU at 10, 50, 100 and 500 µg L^{-1}. The experiments were conducted using 4 replicates, unless specified otherwise, under constant light intensity (40 µmol photons $m^{-2}s^{-1}$) and temperature (15 ± 1ºC) conditions. The pH and salinity of test solutions were 7.4 ± 0.1 and 33 ± 1 ppt respectively. Negative (NSW) and carrier control were also run simultaneously.

2.6 Evaluation of growth and photosynthetic efficiency responses

In present study, the responses of *E. siliculosus* towards different herbicides were assessed through various biomarkers at different levels of biological organization. According to Peakall and Walker, (1994), biomarker is defined as quantitative measures of changes in the biological system that can be related to exposure to the toxic effects of environmental chemicals.

2.6.1 Relative growth rate, (RGR)

The relative growth rate (RGR) was determined by estimating the changes in tissue volume (V) over time (t) using the method described by Russell and Morris (1970). Known volumes of algae material (100 ± 20 mm^3) were measured by means of slow centrifugation (4000 rpm) for 5 minutes in 'Wintrobe' blood sedimentation tubes (Figure 2.3) and used as inoculum in successive media containing different concentrations of herbicides. The cultures were grown at 15°C under fluorescent light set at 40 μmol m^{-2} s^{-1} photon irradiance rate, with 14:10 hours on a light:dark cycle. Volume assessments of the total algal material were made after 7 days of exposure and the percentage increase or decrease in volume was noted. The RGR was calculated using the formula (Hunt, 1982) stated below:

$$\text{RGR (\% per day)} = \ln (V_{Final}) - \ln (V_{Initial}) / \Delta t * 100\%$$

Figure 2.3 has been removed due to Copyright restrictions

Figure 2.3: The Wintrobe tube used for relative growth rate measurements (retrieved from: www.hecht-assistent.de)

2.6.2 Photosynthetic activity (Chlorophyll *a* fluorescence)

Photosynthetic activity in *E.siliculosus* was evaluated based on the chlorophyll fluorescence measurements. It is a non-invasive technique that provides valuable information on the physiological status of plants and algae (Maxwell and Johnson 2000). Light (ΦPSII) and dark-adapted (Fv/Fm) fluorescence parameters were chosen as to determine whether the herbicides and booster biocide affected the photosynthetic activity of *E. siliculosus*. Fv/Fm is a useful parameter, as it indicates the efficiency of the PS II photosystem, which is directly targeted by herbicides, and shows a high degree of correlation with the quantum yield of net photosynthesis (Hall and Rao, 1995).

Prior to the dark adapted or maximum efficiency of PSII (Fv/Fm) measurements, *E. siliculosus* was adapted to darkness for 30 minutes (Dewez *et al*., 2005) in order to induce an equilibrium state of the photosynthetic electron transport and also to allow all reaction centres to open and minimize fluorescence associated with the energization of the thylakoid membrane (Krause *et al*., 1984). This was done by orienting the samples across the centre of the leaf-clips. A Hansatech® fluorometer

(Fluorescence Monitoring System, FMS-1, Hansatech Ltd., England) was used to analyse the fluorescences.

The minimal fluorescence level in the dark-adapted state (F_o) was measured using a modulated pulse (<0.05 µmol m^{-2} s^{-1} for 1.8 µs) which was too small to induce significant physiological changes in the samples. Maximal fluorescence in this state (F_m) was measured after applying a saturating actinic light pulse of 1500 µmol m^{-2} s^{-1} for 0.7 s. Values of the variable fluorescence ($Fv = Fm – F_o$) and maximum efficiency of photosystem II (Fv/Fm) were calculated from F_o and F_m (Bolhar-Nordenkampf *et al*., 1989).

The same areas of the samples for each replicate were also used to measure the light-adapted parameters or also known as effective quantum yield of PSII (ΦPSII). Steady state fluorescence yield (F_s) was recorded after adapting the samples to ambient light conditions for 30 min. A saturating actinic light pulse of 1500 µmol m^{-2} s^{-1} for 0.7 s was then used to produce the maximum fluorescence yield (F_m') by temporarily inhibiting PSII photochemistry. At a steady state of variable fluorescence (F_s) induced by actinic light, the effective quantum yield of PSII (ΦPSII) was evaluated by the ratio $ΦPSII = (F_m' - F_s) / F_m'$, where F_m' represents the maximum fluorescence yield induced by a saturating flash for a light-adapted plant (partial closure of reaction centres). The photochemical (qP = (Fm'- Fs) / (Fm'- Fo')) and non-photochemical (qN = 1- ((Fm'- Fo') / (Fm - Fo)) quenching were determined as in Schreiber *et al*., (1986).

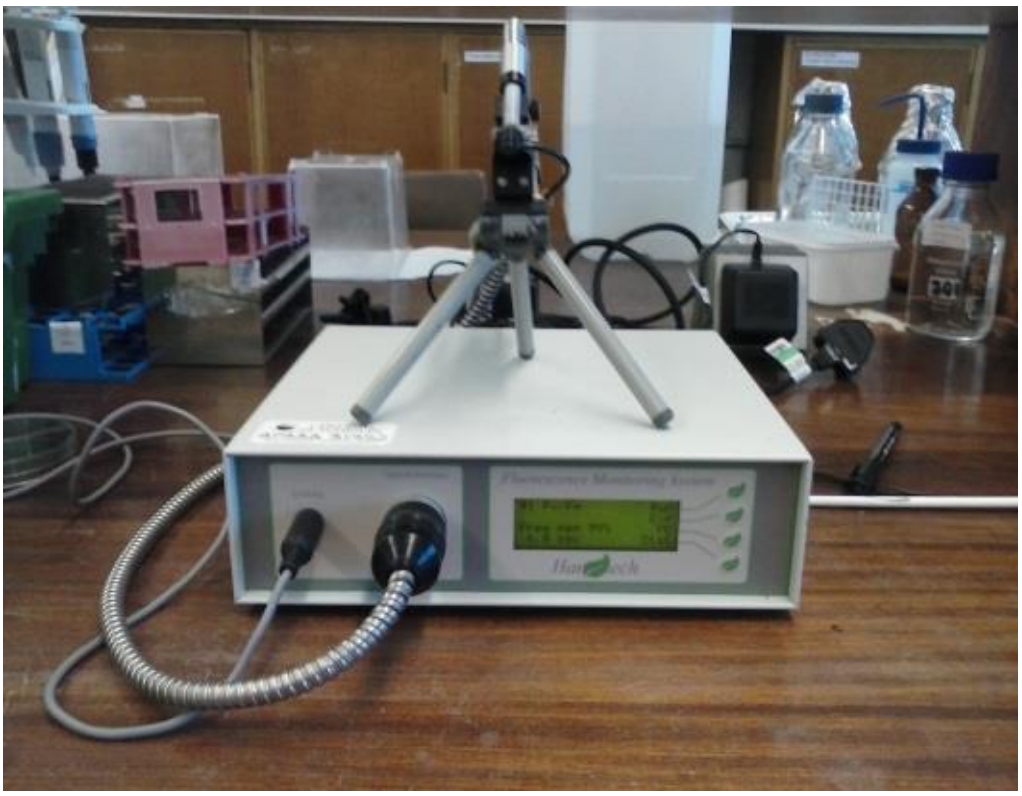

Figure 2.4: The FMS-1, Hansatech Ltd., England

Rapid light curves (RLC) were measured after 7 days of herbicide exposure on each LIA4 and Es524 strain (*n* = 4 replicates per strain per treatment) using a Mini-PAM fluorometer. The RLC were constructed by exposing the samples to nine increasing actinic light levels covering 0–2059 µmol photons m^{-2} s^{-1} and by calculating the relative electron transport rate (rETR) according to equation:

$$\text{rETR} : \frac{\Delta F}{F'\text{m}} \text{ X PAR}$$

which is the product of the effective quantum yield by the actinic irradiance (photosynthetically active radiation, PAR) from the internal halogen lamp of the Mini-PAM (Negri *et al*., 2015). This is considered a relative ETR (rETR), since the seaweed absorptance was not directly measured. rETR was plotted against irradiance, and maximum relative electron transport rate ($rETR_{max}$) and photosynthetic efficiency (α) were determined using a single exponential function: $f(x)= a(1-e^{-bx})$ (Rascher *et al*., 2000).

Figure 2.5: Mini PAM (Walz, Germany)

2.7 Preparation of protein extract for antioxidative enzyme assays

The method by Ratkevicius *et al*., (2003) was adopted with minor modifications to prepare the protein extracts. (1.5 ± 0.2 g per replicate) of frozen *Ectocarpus* biomass (treated and control samples) were grinded in a mortar with liquid nitrogen (-196°C) to powder. Solution containing 0.1 M potassium phosphate buffer (pH 7) with 5mM 2-mercaptoethanol (extraction buffer) was added in a ratio of 1 g : 3 ml. The homogenate was filtered through Miracloth (Calbiochem) and centrifuged at 13,000 rpm for 10 min at 4°C (BioFuge 22R, Heraeus Sepatech GmBH, Germany). In order to precipitate the proteins, the supernatant was transferred to a new tube (50ml) and 0.5 grams per millilitres (ml) of ammonium sulphate were added; the mixture was vortexed at 400 rpm for 2 hours at 4°C. The mixture was then centrifuged at 13,000 rpm for 30 min at 4°C,

and the pellet was re-suspended in 0.1 M potassium phosphate buffer (pH7), containing 2mM 2-mercaptoethanol and 10% glycerol. Protein extracts were adjusted to a final concentration of 1 mg ml^{-1} using Bradford method and bovine serum albumin (BSA) as standard (Bradford, 1976). Extracts were stored at -80°C for further enzymatic activity analyses.

Chapter 3

Growth and physiological responses of

Ectocarpus siliculosus to herbicide exposure

3.1 Introduction

Primary producers such as macroalgae (seaweed) are extremely important components of the food chain because they function as food for a variety of organisms in marine and freshwater environments and also provide habitat and nursery grounds for fish and invertebrates (Misheer *et al*., 2006; Han *et al*., 2008). In the present study, two different strains of brown seaweed, *E. siliculosus*; LIA4 and Es524 were used in order to assess the effects of herbicides exposure. *Ectocarpus siliculosus* has been positioned as a model for the study of brown algae, and that the genome has been recently codified (Cock *et al*., 2010), new research lines are ahead for the study of these ecologically important organisms, including the field of ecotoxicology. In recent years, the extensive use of pesticides in agriculture has resulted in concentrations exceeding the buffering capacity of soil compartments causing contamination of surface and ground-water (Dalton *et al*., 2010). In fact, many researchers such as Galassi *et al*., (1992); Ahel *et al*., (1992); Battaglin *et al*., (2000); Arias-Estevez *et al*., (2008) have highlighted the increasing frequency of pesticide pollution in lakes, rivers, estuaries and coastal waters, with a high predominance of herbicides. Although these chemicals exhibit potent effects on target organisms, they can generate non-specific phytotoxicity (affect the non-target plants and causing damage) (Saladin *et al*., 2005). Moreover, booster biocides in antifouling paint formulations such as diuron have gained popularity since the ban of tributyltin (TBT). It is estimated to be found in 50% of all antifouling paints sold in the UK (Environment Agency, 1998) and commonly found in coastal waters and sediments in various parts of the world (Thomas *et al*., 2001a). Although the UK Health and Safety Executive (HSE) imposed a ban on the use of diuron as an antifouling agent in November 2002, unfortunately, the ban is not applicable to other regions (e.g. Australia). In fact, due to the wide usage and long persistence of diuron, it may still pose a threat to the marine environment (Chesworth *et al*., 2004; Kumar *et al*., 2010).

Therefore, in the present study, diuron (DIU), terbuthylazine (TBA) and isoproturon (IPU), were selected for investigation of their impacts on two different strains of *E. siliculosus* originated from different locations; Es524 (Cu-polluted site, Caleta Palito, Chile) and LIA4 (pristine site, Lon Liath, Scotland). By investigating the toxicity of the contaminants (DIU, TBA, IPU), we were able to test the hypothesis of different responses displayed by the two strains from two different geographical locations under laboratory conditions. Various chlorophyll fluorescence indicators such as maximum efficiency of photosystem II (Fv/Fm), effective quantum yield of photosystem II (ΦPSII), photochemical quenching (qP), non-photochemical quenching (qN), electron transport rate and photosynthetic efficiency (α), including relative growth rate (RGR), were evaluated on both strains in order to elucidate the impact of the herbicides toward growth and photosynthetic efficiency of the *E. siliculosus* strains.

3.2 Materials and methods

3.2.1 Culture conditions of *Ectocarpus siliculosus*

Stock cultures of two strains of *E. siliculosus (*LIA4 and Es524; Culture Collection of Algae and Protozoa (CCAP) accession numbers 1310/339 and 1310/333, respectively), originating from a pristine area in Scotland (Lon Liath) and a copper-polluted site in Chile (Caleta Palito), respectively, were cultured in acid washed, 10 L polycarbonate carboys containing filtered and autoclaved natural seawater enriched with Provasoli medium (Saez *et al*., 2015) in a controlled environment chamber (CEC, Sanyo MLR-350/HT) at 15 ± 1°C. Lighting was provided by cool white fluorescence tubes (Philips) with a photon fluence rate (PFR) of 40 μmol m^{-2} s^{-1} on a 14:10 h light:dark cycle. Cultures were bubbled with filtered (0.22 μm) compressed air to avoid CO_2 depletion and to maintain the seaweed in suspension (Starr and Zeikus, 1993). The culture medium was changed every two weeks.

3.2.2 Test chemicals and solutions

Stock solutions of the herbicides were prepared according to Manzo *et al*., (2008) with dimethyl sulfoxide (DMSO, ≥99.5%), due to their moderate/low solubility in water (DIU= 42 mg L^{-1} (Giacomazzi and Cochet, 2004); TBA= 8.5 mg L^{-1} (Zsolnay, 1994); IPU= 65 mg L^{-1} (Schmitt-Jansen and Altenburger, 2005)). The final concentration of DMSO in the exposure media did not exceed 0.005% (v/v). The three herbicides (diuron (CAS No: 330-54-1), terbuthylazine (CAS No: 5915-41-3) and isoproturon (CAS No: 34123-59-6) were added to filtered, enriched autoclaved natural seawater (taken from the south side of Mount Batten Pier, coordinates: 50° 21′ 34″ N, 4° 07′ 47″ W) to yield different nominal concentrations of 1, 5, 10, 50, 100 and 500 µg L^{-1}. The pH, salinity and temperature of the test solutions were 7.4 ± 0.1, 33 ± 1.0 ppt and 15 ± 1 ºC respectively. The selected concentrations were based on published data on toxicity of the chemicals on different photosynthetic organisms (Myers *et al*., 2006 [0-10 mg L^{-1} for diuron (DIU)]; Fiori *et al*., 2013 [5-100 µg L^{-1} for terbuthylazine (TBA)]; Pietsch *et al*., 2006 [0.2-200 µg L^{-1} for isoproturon (IPU)] with higher concentrations for TBA and IPU (> 100 and > 200 µg L^{-1}) respectively in order to obtain a uniform comparative effects for every chemical tested. Known volumes of algal material (100 ± 20 mm^3), measured by means of slow centrifugation (4000 rpm) for 5 min in 'Wintrobe' blood sedimentation tubes (Russell and Morris, 1970), were used as inocula for experimental treatments. All experimental material was exposed for 7 d in acid-washed glass Petri dishes containing 50 ml of test solutions with 4 replicates per treatment. Experiments were carried out in controlled environment chambers (Sanyo MLR-351H) at 15 ± 1 °C, 90% relative humidity, a PFR of 40 µmol m^{-2} s^{-1} (cool white fluorescent tubes) on a 14:10 light:dark cycle.

3.2.3. Determination of relative growth rate

Relative growth rates (RGRs) were calculated from estimations of changes in tissue volume (V) over time (t) using the following formula (Hunt, 1982):

$$RGR\ (\%\ per\ day) = \ln (V_{Final}) - \ln (V_{Initial}) / \Delta t * 100\%$$

3.2.4 Chlorophyll fluorescence

Photosynthetic activity of *E. siliculosus* was evaluated from measurements of various photosynthetic efficiency indicators using Fluorescence Monitoring System, FMS-1, Hansatech Ltd., England and Mini-PAM, Walz, Germany. The methods used for the evaluations were described earlier (Refer **2.6.2**)

3.2.5 Statistical analysis

All statistical analyses were carried out using STATGRAPHICS Centurion (Version XVI, Statpoint Technologies, Inc., USA), unless specified. Prior to analysis of variance (ANOVA), the data were tested for normality and homogeneity of variance using Shapiro-Wilk and Bartletts tests, respectively. General linear model (GLM) analysis of variance (ANOVA) was used to compare responses of the two strains (LIA4 and Es524) to the herbicides (DIU, TBA, IPU) for each measured endpoint. A *post hoc* Tukey Multiple Comparison procedure with probability of $P < 0.05$ was used to identify significant differences between individual means. Herbicide concentrations causing a 50% effect (EC_{50}), together with the 95% C.I. values in the various measured endpoints over the carrier control, were estimated using Graphpad Prism 7, (Graphpad Prism Software Inc., San Diego, California, USA). The method calculates effective concentration (EC) values together with corresponding 95% confidence interval by

optimizing the curve fit with successive iterations. The no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values were determined through ANOVA test with post-hoc Dunnett's test. The data were tested for normality and homogeneity of variance before proceeding with the ANOVA test.

3.3 Results

3.3.1 Effect of diuron (DIU) on *E. siliculosus*

Reductions in the relative growth rates (RGR) of *E. siliculosus* strains (LIA4 and Es524) were observed with increasing DIU concentrations (**Figure 3.1**). Significant decreases in both strains were recorded at 50 µg L^{-1} and above. No significant interaction was observed between the strains and treatments (S×T, $P>0.05$) which indicates similar RGR response between the strains. At 50, 100 and 500 µg L^{-1}, reductions, as much as 118, 157 and 168% were recorded in LIA4 respectively, while 62, 80 and 93%, in Es524. Negative RGR values observed in LIA4 at 50 µg L^{-1} of DIU and above may indicate shrinkage of the tissues. 7d EC_{50} RGR value for LIA4; 9.94 µg L^{-1} (6-14 µg L^{-1}) was significantly lower compared to Es524; 24.65 µg L^{-1} (19-31 µg L^{-1}) which indicates the lower tolerance of LIA4 to DIU compared to Es524. NOEC values for LIA4 and Es524 were 5 and 10 µg L^{-1} respectively.

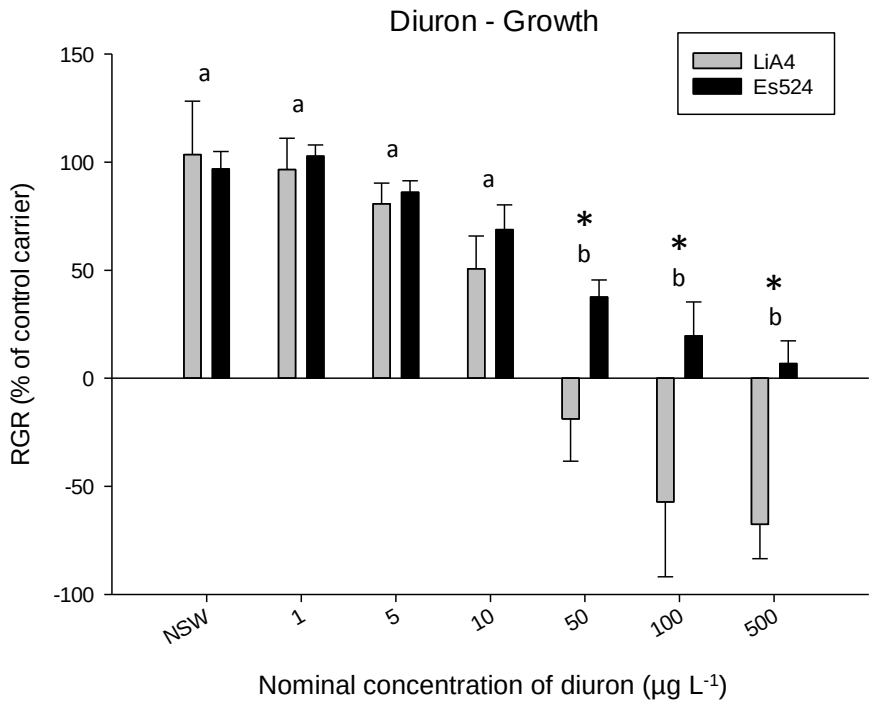

Figure 3.1: The effects of DIU on RGR in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean RGR values of the carrier control for LIA4 and Es524 were (5.36 and 7.85 % d^{-1}) respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets that are significantly different from each other are represented by different letters ($P < 0.05$).

Evaluation of the photosynthetic efficiency was carried out in two different conditions of dark and light adapted states. **Figures 3.2** and **3.3** indicate that DIU caused significant effects on the maximal quantum yield (Fv/Fm) and effective quantum yield (ΦPSII) of photosystem II, respectively. Generally, photosynthetic efficiency indicators (Fv/Fm, ΦPSII) in both strains have decreased with increasing DIU levels. Significant reductions as compared to the carrier control were observed at 5 µg L^{-1} and above for both indicators in both strains. For Fv/Fm, significant difference between LIA4 and Es524 (S×T, $P<0.05$) was observed at 10 µg L^{-1} with 33 and 49% reductions recorded in Es524 and LIA4, respectively.

While for ΦPSII, significant differences between the strains were observed at 5 and 50 µg L^{-1} with diminutions as much as 13 and 76% in Es524 respectively and 30 and 66% in LIA4. Exposure to 100 and 500 µg L^{-1} of DIU showed no significant difference between the strains for both Fv/Fm and ΦPSII parameters. The 7d EC_{50} values for Fv/Fm and ΦPSII in LIA4 were 17.76 (13-22) and 8.04 (5-9) µg L^{-1} respectively, which were significantly lower compared to Es524 with 32.75 (27-38) and 11.18 (10-14) µg L^{-1}. The higher EC_{50} values for Es524 signify better resistant to DIU compared to LIA4. The NOEC values determined from Fv/Fm and ΦPSII parameters in both strains were both 1 µg L^{-1}.

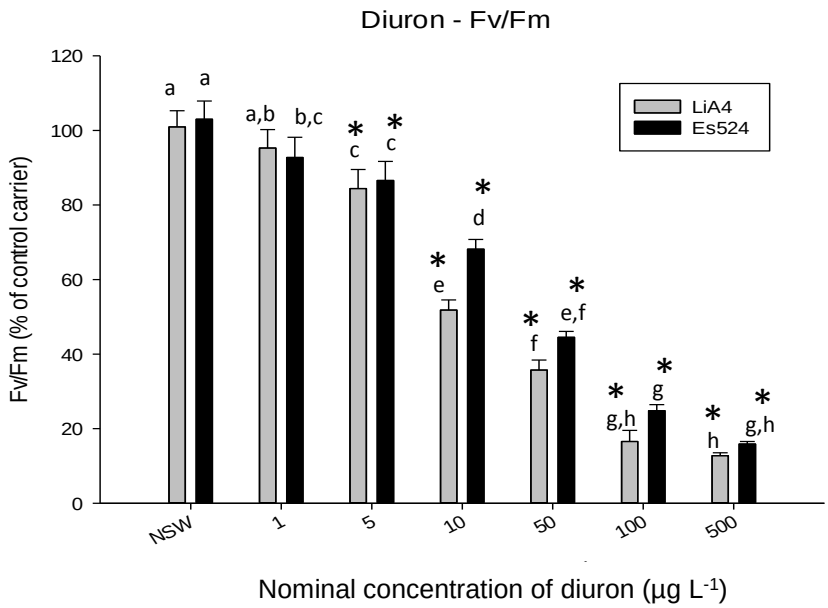

Figure 3.2: The effects of DIU on Fv/Fm in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean Fv/Fm values of the carrier control for LIA4 and Es524 were (0.720 and 0.690) respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$)

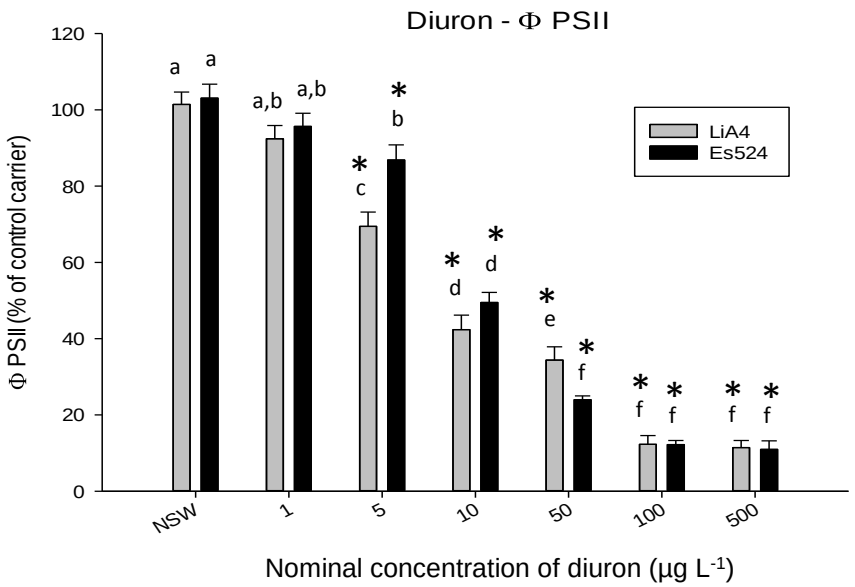

Figure 3.3: The effects of DIU on ΦPSII in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean ΦPSII values of the carrier control for LIA4 and Es524 were (0.684 and 0.647) respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$).

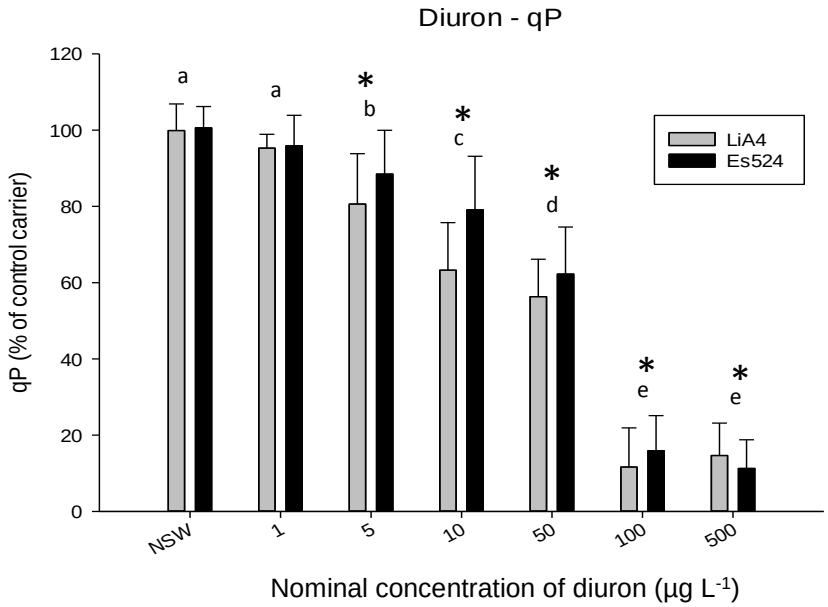

Figure 3.4: The effects of DIU on **qP** in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean qP values of the carrier control for LIA4 and Es524 were (0.664 and 0.652) respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$)

A concentration-dependent decrease of photochemical quenching (qP) response was observed in both strains after exposure to DIU (**Figure 3.4**). Significant decreases as compared to the carrier control were observed at 5 µg L^{-1} and above in both strains. No significant difference was observed between the strains (S×T, P>0.05) for the qP response within the concentrations of DIU tested. Diminutions as much as 19-85% were recorded in LIA4 between 5 to500 µg L^{-1}, while 12- 89% decreases were observed in Es524. The 7d EC_{50} qP value for Es524 and LIA4 were 62.94 (52-73) and 52.83 (45-62) µg L^{-1} respectively, which was not significantly different between the strains. The NOEC values for qP in LIA4 and Es524 were 1 and 5 µg L^{-1}, respectively.

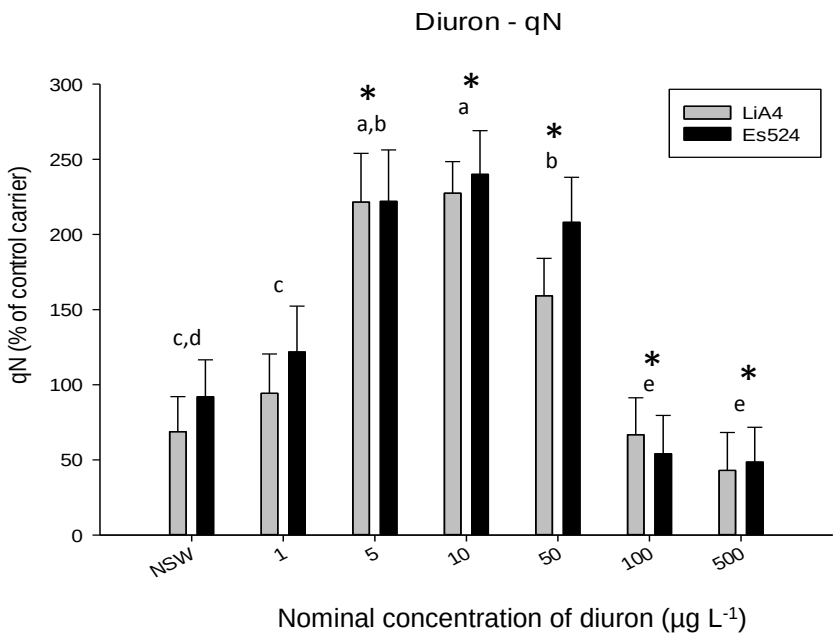

Figure 3.5: The effects of DIU on **qN** in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean qN values of the carrier control for LIA4 and Es524 were (0.068 and 0.061) respectively. Values with an asterisk are significantly different at *P* < 0.05 compared to carrier control. Data sets which are significantly different from each other are represented by different letters (*P*< 0.05)

Exposure to DIU caused increments of non-photochemical quenching response (qN) in both *E. siliculosus* strains **(Figure 3.5).** As compared to the carrier control, significant increments were observed between 5 to 50 µg L^{-1} in both strains. At 100 µg L^{-1} and above, the qN responses have significantly reduced, which may indicate damage to the photosynthetic apparatus. No significant difference was observed between the strains (S×T, P>0.05), indicating similar qN response between LIA4 and Es524 within the concentrations of DIU tested. The 7d EC_{50} qN value for Es524 and LIA4 were 1.276 (0.8-1.7) and 1.816 (1.3-2.3) µg L^{-1} respectively, which was not significantly different between the strains. The NOEC values recorded in LIA4 and Es524 for qN were similar at 1 µg L^{-1}.

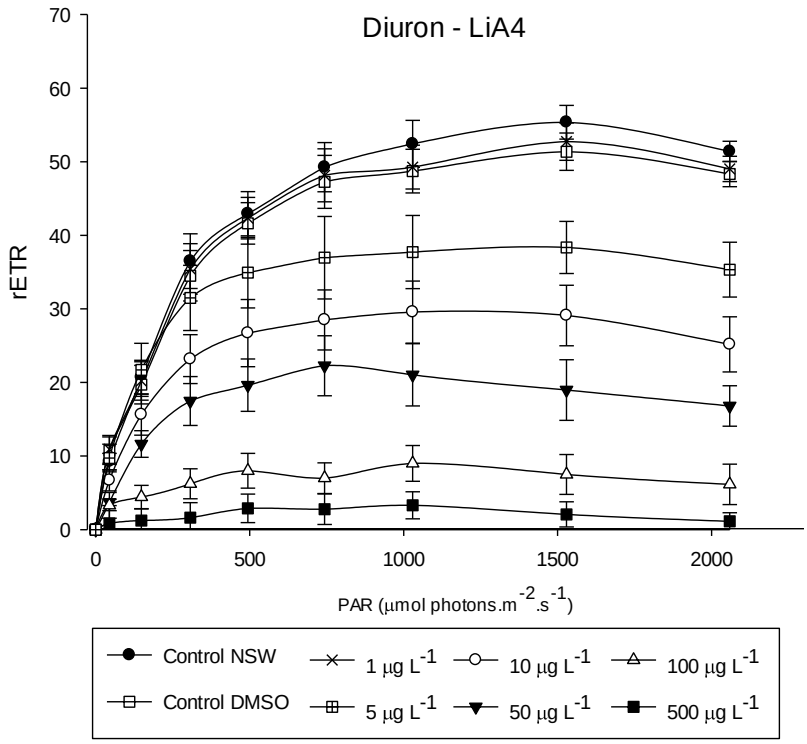

Figure 3.6 Relative electron transport rate (rETR) of LIA4 exposed to diuron for a period of 7 days. Means ± SD, n=4.

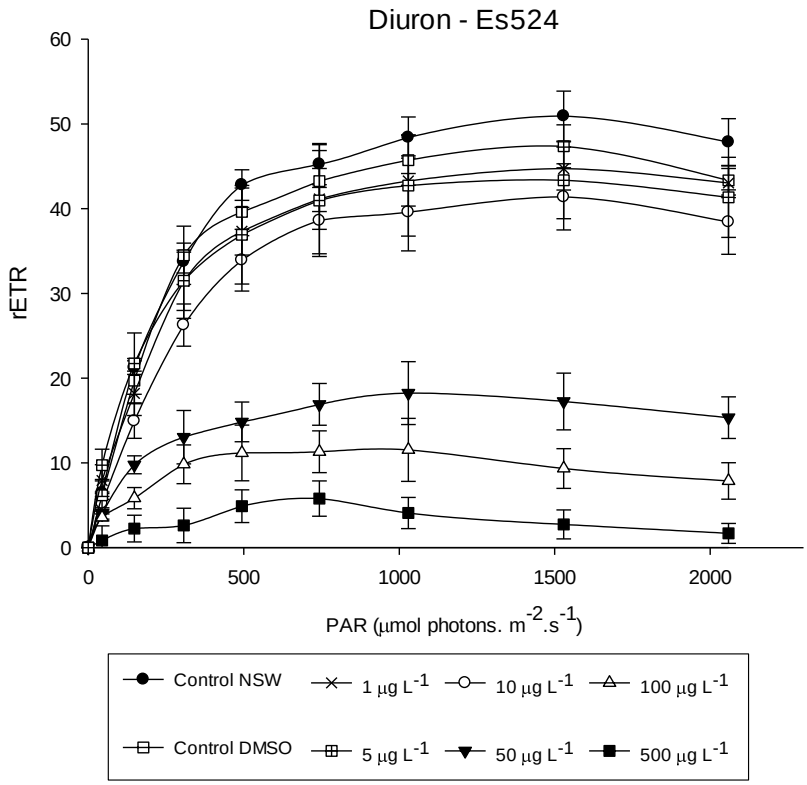

Figure 3.7 Relative electron transport rate (rETR) of Es524 exposed to diuron for a period of 7 days. Means ± SD, n=4.

Exposure of *E. siliculosus* strains (LIA4 and Es524) to DIU between 1 - 500 µg L^{-1} showed reductions of electron transport rate ($rETR_{max}$) and the efficiency of electron transport (α) (**Figure 3.6 and 3.7**). As compared to the carrier control, significant decreases of both parameters were observed in both strains at 50 µg L^{-1} of DIU and above. Although, no significant difference was observed between the strains, the derived 7d EC_{50} values for $rETR_{max}$ and α in Es524; 24.15 (21-31) and 76.38 (65-88) µg L^{-1} respectively were significantly higher compared to LIA4; 16.93 (13-20) and 53.25 (41-63) µg L^{-1}, indicating higher tolerance of Es524 to DIU compared to LIA4. The NOEC values recorded for both $rETR_{max}$ and α in LIA4 were 5 µg L^{-1} while Es524 at 10 µg L^{-1}.

Variable	Nominal concentration **Diuron** (µg L^{-1})	*Ectocarpus siliculosus* LIA4	*Ectocarpus siliculosus* Es524
Relative electron transport rate ($rETR_{max}$)	NSW	52.97 ± 7.56 [a]	48.61 ± 7.31 [ab]
	DMSO	48.98 ± 7.66 [ab]	45.70 ± 7.86 [ab]
	1	49.17 ± 11.35 [a]	43.22 ± 10.18 [ab]
	5	35.74 ± 12.74 [abcd]	42.31 ± 9.81 [ab]
	10	27.88 ± 11.06 [bcde]	39.39 ± 11.31 [abc]
	50	20.68 ± 6.86 [cdef]	16.88 ± 6.05 [def]
	100	8.8735 ± 3.27 [ef]	11.0870 ± 1.36 [ef]
	500	2.3825 ± 1.62 [f]	4.159 ± 0.59 [f]
Photosynthetic efficiency (α)	NSW	0.209 ± 0.026 [ab]	0.198 ± 0.023 [ab]
	DMSO	0.215 ± 0.031 [ab]	0.189 ± 0.019 [ab]
	1	0.219 ± 0.032 [a]	0.177 ± 0.020 [abc]
	5	0.203 ± 0.033 [ab]	0.198 ± 0.017 [ab]
	10	0.162 ± 0.031 [bcd]	0.181 ± 0.017 [abc]
	50	0.110 ± 0.020 [de]	0.128 ± 0.017 [cd]
	100	0.041 ± 0.019 [f]	0.066 ± 0.016 [ef]
	500	0.015 ± 0.011 [f]	0.028 ± 0.010 [f]

Table 3.1: Photosynthetic efficiency of *E. siliculosus* exposed to different concentrations of diuron over a period of 7 days. Data shown as means ± SD (n=4). Different letters represent significant differences between the strains (LIA4 and Es524) for each variable at $P < 0.05$.

3.3.2 Effect of terbuthylazine (TBA) on *E. siliculosus*

The increase of TBA concentrations (1 to 500 µg L^{-1}) (**Figure 3.8**) have induced a strong trend of inhibitory effect on the relative growth rate (RGR) of *E. siliculosus* strains with exception to 1 µg L^{-1}, where slight stimulations of growth were observed in both strains. Significant decreases were observed at 50 µg L^{-1} and above in both strains, with declination between 86 to 131% in LIA4 and 70 to 96% in Es524. No significant difference was observed between the strains (S×T, P>0.05), indicating similar RGR response between the strains to TBA exposure. The negative RGR values observed in LIA4 at 100 µg L^{-1} and above may indicate shrinkage of the tissues. The derived 7d EC_{50} RGR value for LIA4 was 18.11 (12-22) µg L^{-1} which was significantly lower

compared to Es524 with 28.25 (23-36) µg L^{-1}, indicating better resistant of Es524 to TBA compared to LIA4. NOEC value of 10 µg L^{-1} was the same for RGR response in both strains.

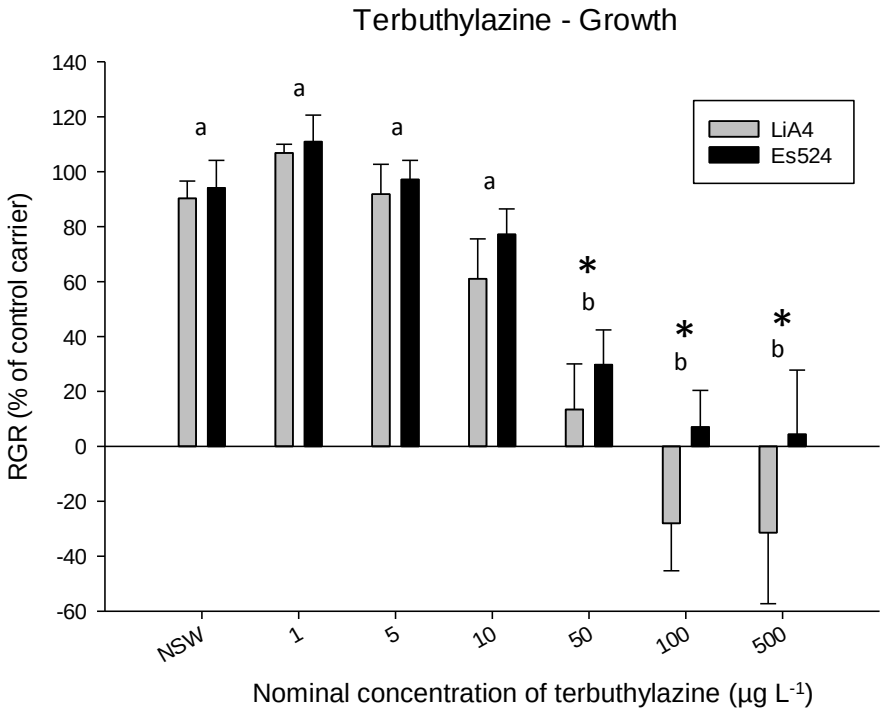

Figure 3.8: The effects of TBA on RGR in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean RGR values of the carrier control for LIA4 and Es524 were (6.15 and 7.49 $\%^{-day}$) respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$)

Photosynthetic efficiency of *E. siliculosus* as represented by the maximum efficiency of PSII (Fv/Fm) (**Figure 3.9**) and effective quantum yield (ΦPSII) (**Figure 3.10**) indicate that both strains (LIA4 and Es524) were negatively affected by TBA. For Fv/Fm, significant decreases as compared to carrier control were observed at 10 µg L^{-1} and above in both strains. Reductions as much as 27, 66 and 73% were recorded at 50, 100 and 500 µg L^{-1} in LIA4 respectively, while Es524 had 31, 70 and 76% in corresponding treatments. No significant differences were observed between the strains (S×T, P>0.05) which indicates both LIA4 and Es524 have similar Fv/Fm responses to TBA. The

derived 7d EC_{50} Fv/Fm value for Es524 and LIA4 were 80.06 (72-91) and 88.13 (77-97) µg L^{-1} respectively, which was not significantly different between the strains. The NOEC values recorded in LIA4 and Es524 for Fv/Fm were similar at 5 µg L^{-1}.

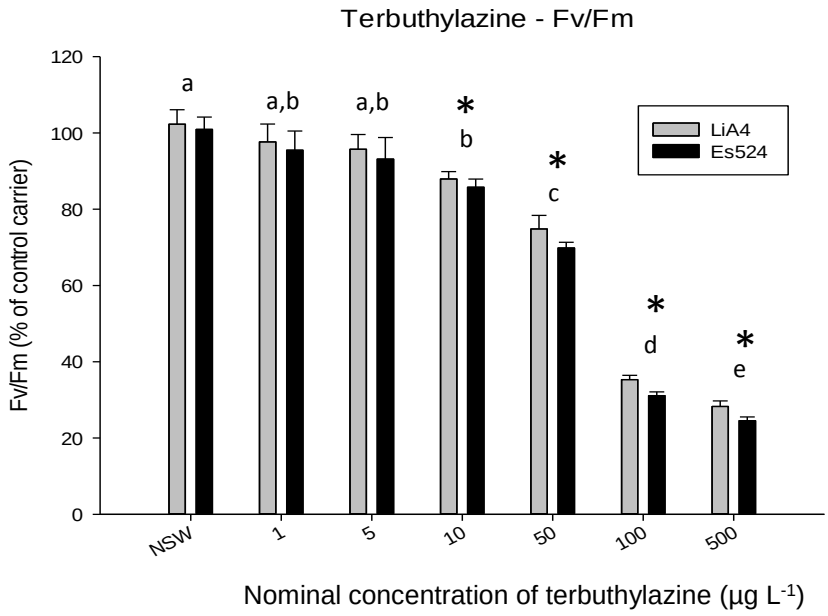

Figure 3.9: The effects of TBA on Fv/Fm in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean Fv/Fm values of the carrier control for LIA4 and Es524 were (0.711 and 0.682) respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$).

For ΦPSII (**Figure 3.10**), concentration-dependent decreases were observed in both strains in response to TBA exposure. Significant effects were observed at 10 µg L^{-1} and above, with reductions as much as 17-75% and 28-80% were recorded in LIA4 and Es524 respectively as compared to the carrier control. No significant difference was observed between the strains (S×T, P>0.05), which indicates similar ΦPSII response between LIA4 and Es524 within the concentrations of TBA tested. The derived 7d EC_{50} ΦPSII value for Es524 and LIA4 were 62.32 (55-69) and 68.17 (60-76) µg L^{-1}, respectively, with no significant differences between the strains. The NOEC values recorded in LIA4 and Es524 for ΦPSII were similar at 5 µg L^{-1}.

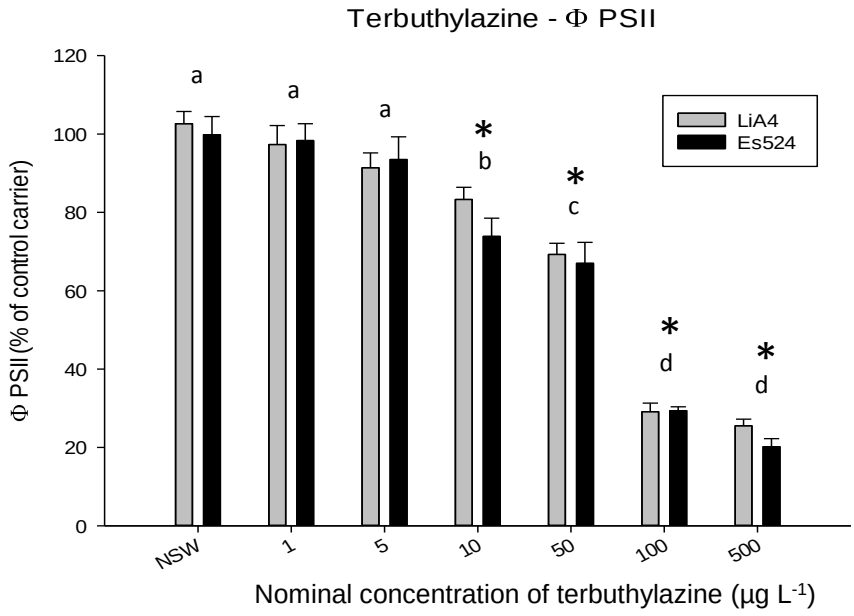

Figure 3.10: The effects of TBA on ΦPSII in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean ΦPSII values of the carrier control for LIA4 and Es524 were (0.690 and 0.649) respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$).

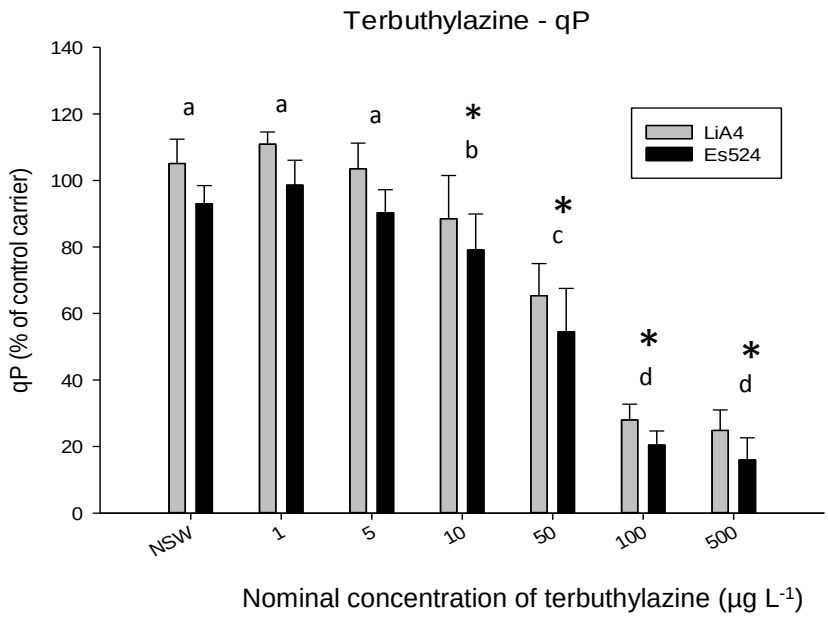

Figure 3.11: The effects of TBA on **qP** in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean **qP** values of the carrier control for LIA4 and Es524 were (0.635 and 0.672) respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control.

Data sets which are significantly different from each other are represented by different letters ($P < 0.05$).

Exposure to TBA caused a consistent decrease in qP values in both strains (**Fig. 3.11**). Significant decreases as compared to carrier control were observed at 10 µg L^{-1} and above, with diminutions as much as 11-75% and 21-84% recorded in LIA4 and Es524 respectively. No significant difference was observed between the strains (S×T, P>0.05), which indicates similar qP response between LIA4 and Es524. The derived 7d EC_{50} qP value for Es524 and LiA4 were 83.37 (71-95) and 90.16 (76-105) µg L^{-1} respectively, which was not significantly different between the strains. The NOEC values for qP in LIA4 and Es524 were 10 and 5 µg L^{-1}, respectively.

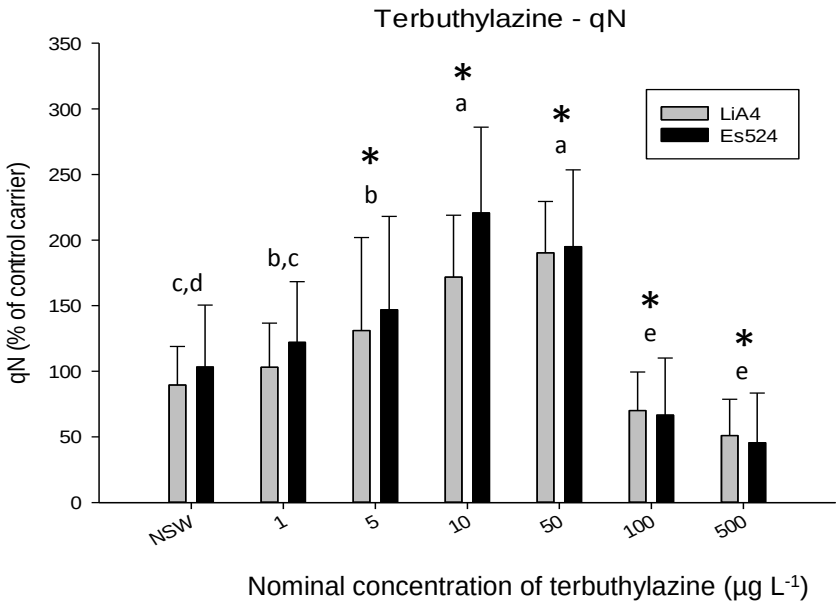

Figure 3.12: The effects of TBA on **qN** in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (n=4). Mean **qN** values of the carrier control for LIA4 and Es524 were (0.067 and 0.065) respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$).

The increases of qN values were observed in both strains up to 50 µg L^{-1} before declined (**Fig. 3.12**). Significant changes of qN values as compared to carrier control were observed at 5 µg L^{-1} and above in both strains. Increments as much as 31-90% and 47-121% were recorded between 5 to 50 µg L^{-1} in LIA4 and Es524 respectively. On the other hand, significant reductions were observed at 100 and 500 µg L^{-1}, with 30 and 49% in LIA4 respectively and 33 and 54% in Es524. No significant difference was observed between the strains (S×T, P>0.05), which indicates similar qN response between LIA4 and Es524 within the concentrations of TBA tested. The derived 7d EC_{50} qN values for Es524 and LIA4 were 5.06 (3.2-7.1) and 6.34 (4.5-8.0) µg L^{-1} respectively, which was not significantly different between the strains. The NOEC of TBA recorded in LIA4 and Es524 for qN were similar at 1 µg L^{-1}.

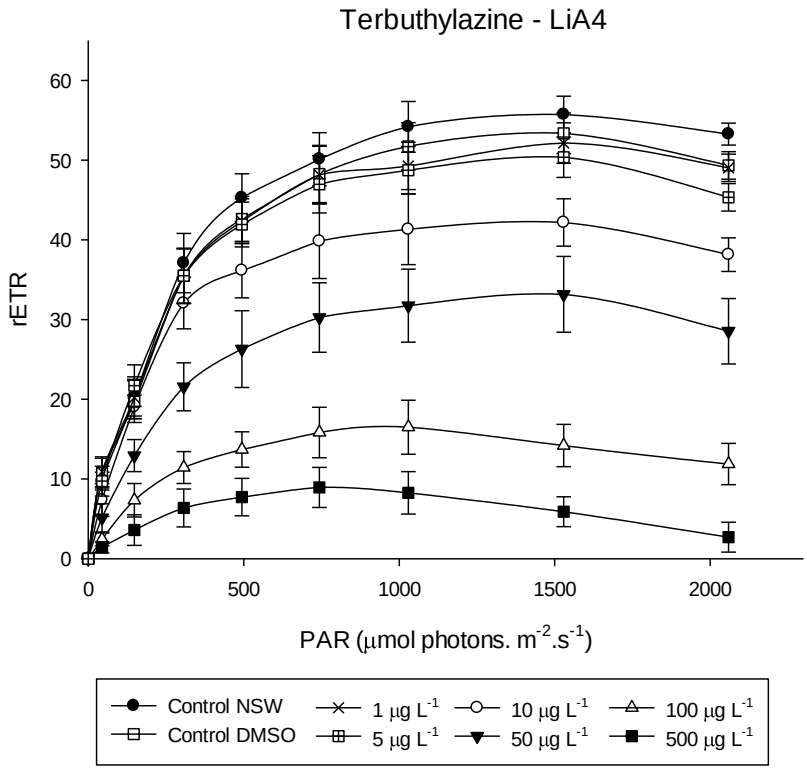

Figure 3.13 Relative electron transport rate (rETR) of LIA4 exposed to terbuthylazine for a period of 7 days. Means ± SD, n=4.

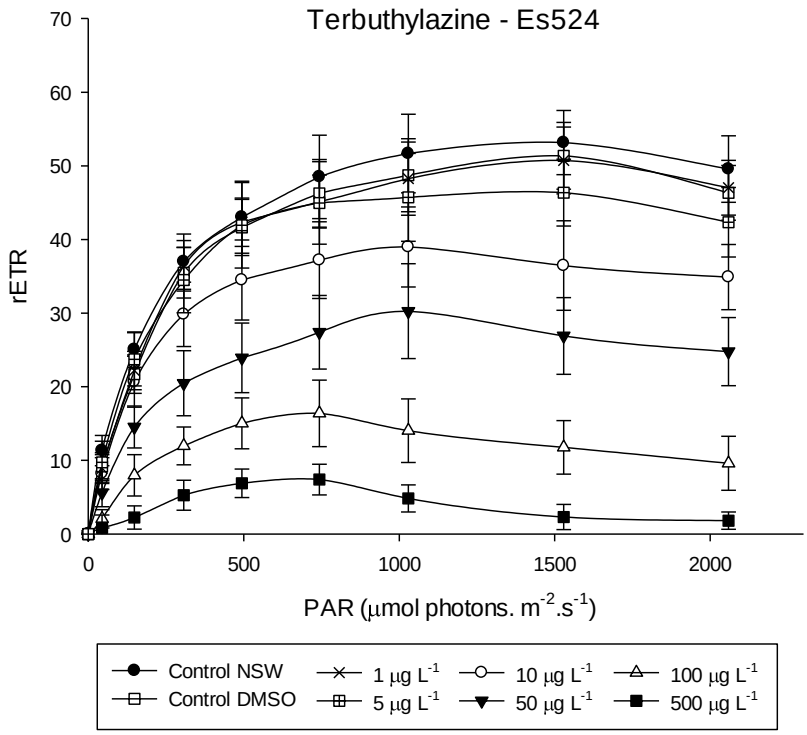

Figure 3.14 Relative electron transport rate (rETR) of Es524 exposed to terbuthylazine for a period of 7 days. Means ± SD, n=4.

Negative impacts of terbuthylazine (TBA) on photosynthetic efficiency of LIA4 and Es524 were shown in **Figure 3.13** and **3.14** respectively. The maximum electron transport rate ($rETR_{max}$) and photosynthetic efficiency (α) values were decreased in both strains after treatment with TBA. For $rETR_{max}$, significant reductions as compared to the carrier control were observed at 50 µg L^{-1} of TBA and above in both strains, while α, significant decreases were noticed at ≥ 50 µg L^{-1} in LIA4 and ≥ 100 µg L^{-1} in Es524. No significant difference was observed between the strains indicating similar response to TBA within the concentrations tested. The obtained 7d EC_{50} values of $rETR_{max}$ and α for LIA4, 67.56 (55-79), 89.89 (74-106) respectively, were not significantly different compared to Es524; 60.67 (47-73) and 95.32 (78-114) µg L^{-1}. The NOEC values recorded for both indicators in LIA4 and Es524 were similar at 10 µg L^{-1}.

Variable	Nominal concentration	*Ectocarpus siliculosus*	
	Terbuthylazine (µg L^{-1})	LIA4	Es524
Relative electron transport rate ($rETR_{max}$)	NSW	54.47 ± 8.87 [a]	50.66 ± 7.59 [a]
	DMSO	51.81 ± 8.17 [a]	48.58 ± 7.33 [ab]
	1	50.54 ± 10.08 [a]	48.41 ± 5.95 [ab]
	5	48.38 ± 7.22 [ab]	45.17 ± 6.96 [abc]
	10	40.57 ± 6.22 [abc]	36.43 ± 7.26 [abc]
	50	31.76 ± 6.84 [bcd]	27.25 ±10.20 [cd]
	100	14.68 ± 5.23 [de]	13.47 ± 5.99 [de]
	500	6.72 ± 4.09 [e]	5.16 ± 4.41 [e]
Photosynthetic efficiency (α)	NSW	0.214 ± 0.023 [a]	0.193 ± 0.017 [abc]
	DMSO	0.219 ± 0.024 [a]	0.189 ± 0.023 [abc]
	1	0.221 ± 0.031 [a]	0.194 ± 0.028 [abc]
	5	0.230 ± 0.032 [a]	0.201 ± 0.028 [ab]
	10	0.196 ± 0.019 [ab]	0.174 ± 0.025 [abc]
	50	0.142 ± 0.028 [bcd]	0.134 ± 0.022 [cd]
	100	0.073 ± 0.018 [e]	0.090 ± 0.018 [de]
	500	0.040 ± 0.014 [e]	0.047 ± 0.022 [e]

Table 3.2: Photosynthetic efficiency of *E. siliculosus* exposed to different concentrations of terbuthylazine over a period of 7 days. Data shown as means ± SD (*n*=4). Different letters represent significant differences between the strains (LIA4 and Es524) for each variable at $P < 0.05$.

3.3.3 Effects of isoproturon (IPU) on *E. siliculosus*

A concentration-dependent decrease of RGR was observed in both strains in response to IPU exposure (**Figure 3.15).** As compared to carrier control, significant reductions were observed at 100 and 500 µg L^{-1}, with diminution as much as 25 and 62% in Es524 respectively, and 26 and 71% in LIA4. No significant effect was observed at 10 and 50 µg L^{-1}, which indicate the homeostasis conditions in both strains were well balanced at these concentrations. Two-way ANOVA analysis, shows no significant difference between the strains (S×T, P>0.05), which signifies both LIA4 and Es524 have demonstrated similar RGR responses within the IPU concentrations tested.

The derived 7d EC_{50} RGR value for Es524 and LIA4 were 315.2 (281-349) and 257 (213-302) µg L^{-1} respectively, which was not significantly different between the strains. However, higher EC_{50} value recorded for Es524 might indicate better tolerance of the strain towards IPU compared to LIA4. The NOEC of IPU recorded in LIA4 and Es524 for RGR was similar at 50 µg L^{-1}.

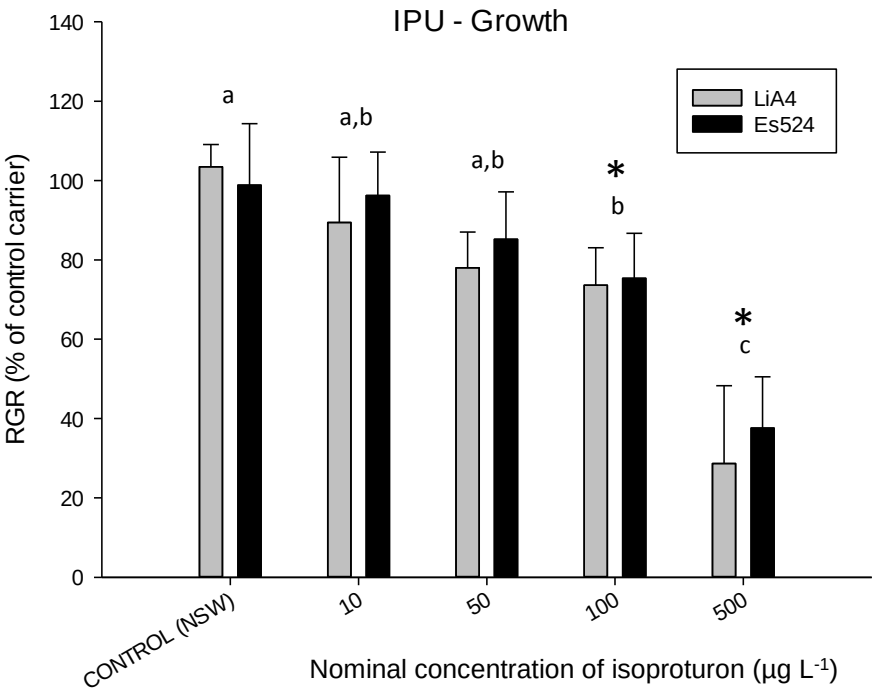

Figure 3.15: The effects of IPU on **RGR** in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean **RGR** values of the carrier control for LIA4 and Es524 were (6.49 and 7.69 $\%^{-day}$) respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$).

Exposure to IPU has caused mild impact on Fv/Fm in both strains after 7 d of exposure (**Figure 3.16**). In general, negative effects on photosynthetic activity in both strains were observed, with the Fv/Fm values decreasing with an increase in IPU concentrations. Significant reductions as compared to carrier control, were observed at 100 and 500 µg L^{-1} in LIA4 while only at 500 µg L^{-1} in Es524. No significant difference

was recorded between the treated groups in Es524, contrary to LIA4 where significant differences were observed between 500 µg L^{-1} to other IPU treatment groups. At 500 µg L^{-1} of IPU, the Fv/Fm value of Es524 was significantly higher compared to LIA4. The derived 7d EC_{50} Fv/Fm value for Es524 and LIA4 were >10 mg L^{-1} and 3.65 (3.3-4.0) mg L^{-1} respectively. The significantly lower EC_{50} value recorded for LIA4 indicates a higher sensitivity of the strain toward IPU compared to Es524. The NOEC values for Fv/Fm in LIA4 and Es524 were 50 and 100 µg L^{-1} respectively.

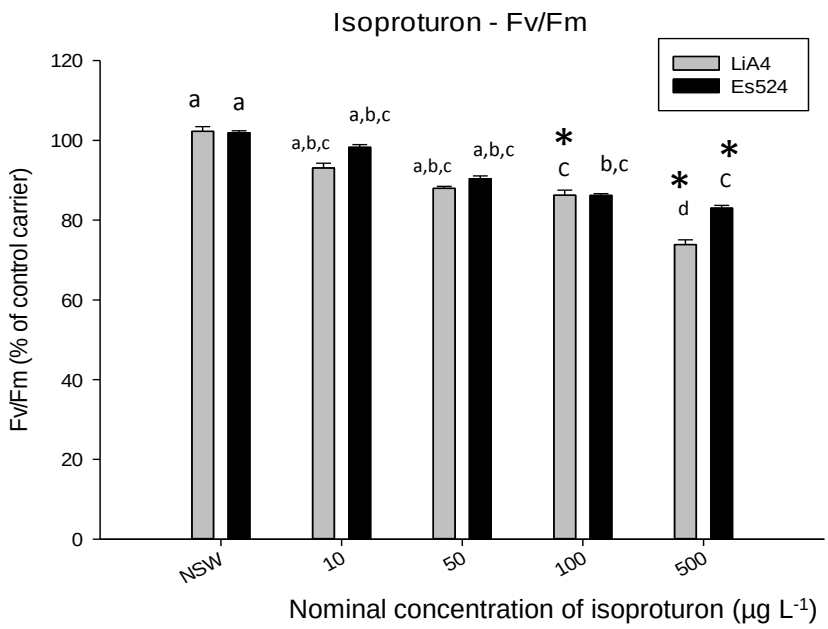

Figure 3.16: The effects of IPU on **Fv/Fm** in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean Fv/Fm values of the control carrier for LIA4 and Es524 were (0.722 and 0.712) respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$).

Figure 3.17 shows the effect of IPU on another photosynthetic efficiency indicator, the effective quantum yield of photosystem II (ΦPSII). A significant diminution as compared to carrier control was observed at every IPU concentrations used, 10 to 500

µg L^{-1} with maximum decline of 28 and 25% in LIA4 and Es524, respectively. No significant differences were observed between the strains (S×T, P>0.05), which indicate similar ΦPSII response in LIA4 and Es524 within the IPU concentrations tested. Interestingly, although the IPU-treated groups (10-500 µg L^{-1}) show significant differences as compared to the carrier control, they displayed no significant difference between each other. The derived 7d EC_{50} ΦPSII value for Es524 and LIA4 were 2.67 (2.4-2.9) mg L^{-1} and 2.072 (1.88-2.27) mg L^{-1} respectively. Again, the significantly lower EC_{50} value recorded for LIA4 indicates higher sensitivity of the strain toward IPU compared to Es524. The NOEC values in both strains for IPU-ΦPSII were less than 10 µg L^{-1}.

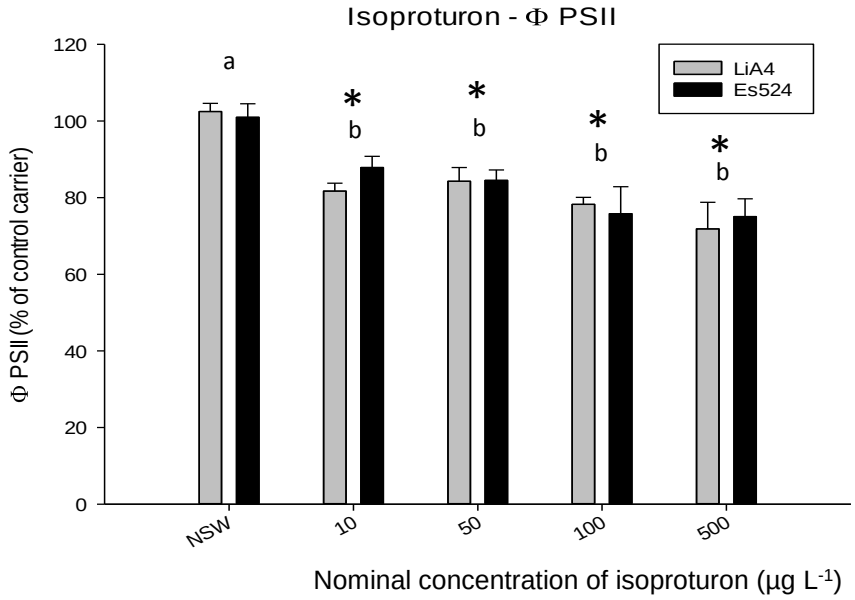

Figure 3.17: The effects of IPU on ΦPSII in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean ΦPSII values of the control carrier for LIA4 and Es524 were (0.665 and 0.656) respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$).

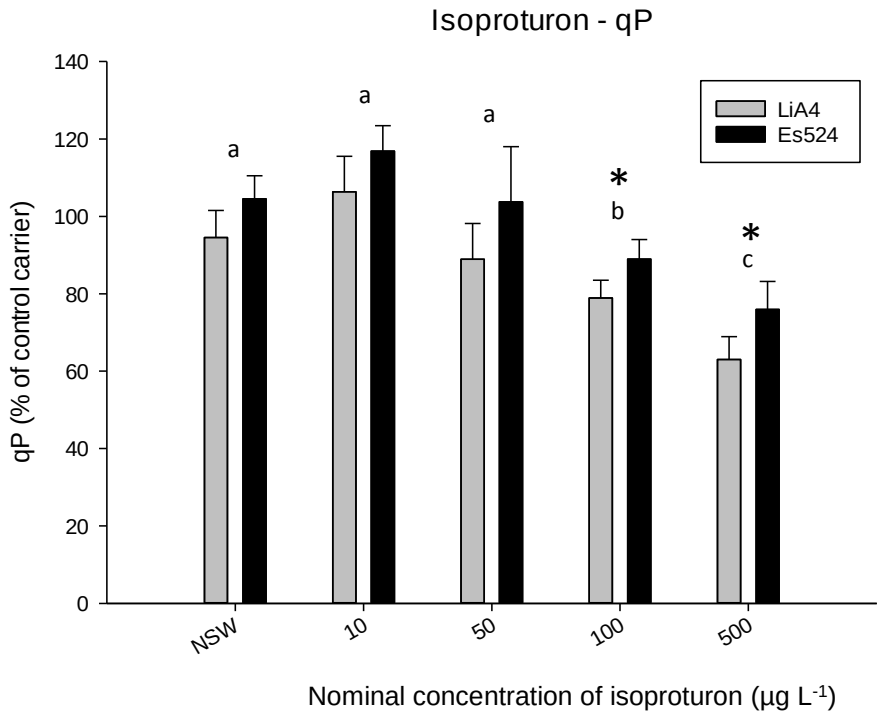

Figure 3.18: The effects of IPU on **qP** in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean qP values of the control carrier for LIA4 and Es524 were (0.660 and 0.616) respectively Values with an asterisk are significantly different at $P < 0.05$ compared to control carrier. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$).

The photochemical quenching (qP) responses of LIA4 and Es524 strains to IPU are shown in **Fig. 3.18**. A slight increment was observed at 10 µg L^{-1} before a decline. Significant decrease of qP values were observed at 100 and 500 µg L^{-1}, with 21 and 37% recorded in LIA4 respectively, while 11 and 24% in Es524. However, no significant difference was recorded between the strains (S×T, P>0.05), which indicate similar qP response in LIA4 and Es524 within the range of IPU treatments. The derived 7d EC_{50} qP value for Es524 and LIA4 were 2.704 (2.24-3.18) mg L^{-1} and 1.841 (1.59-2.11) mg L^{-1}, respectively. A significant difference of EC_{50} qP value between LIA4 and Es524 indicates a better tolerant of Es524 toward IPU which exhibits higher EC_{50} qP value. The NOEC values for qP in LIA4 and Es524 were similar at 50 µg L^{-1}.

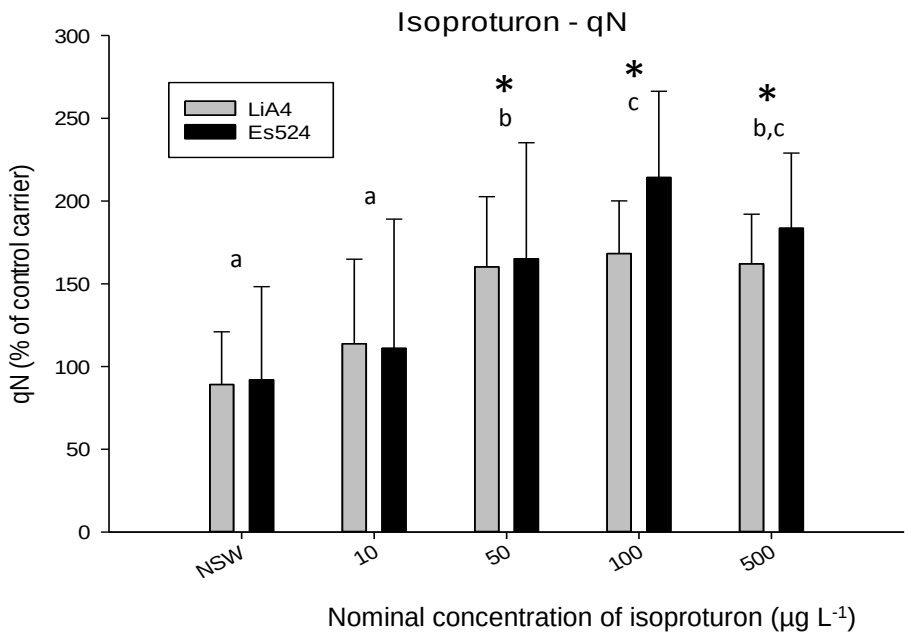

Figure 3.19: The effects of IPU on **qN** in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean qN values of the control carrier for LIA4 and Es524 were (0.063 and 0.054) respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to control carrier. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$).

Exposure to IPU has induced increments of non-photochemical quenching response in both strains (**Figure 3.19**). Significant changes as compared to the carrier control, were observed at 50 µg L^{-1} and above with increment up to 68 and 114% in LIA4 and Es524 respectively. No significant difference was observed between the strains (S×T, P>0.05), which indicates similar qN response in LIA4 and Es524 within the IPU concentrations tested. The derived 7d EC_{50} qN value for Es524 and LIA4 were 37.84 (28.1-47.3) µg L^{-1} and 29.65 (22.9-37.1) µg L^{-1} respectively, which was not significantly different between the strains. However, higher EC_{50} value recorded for Es524, might indicate better tolerance of the strain towards IPU compared to LIA4. The NOEC values recorded in LIA4 and Es524 for qN were similar at 10 µg L^{-1}.

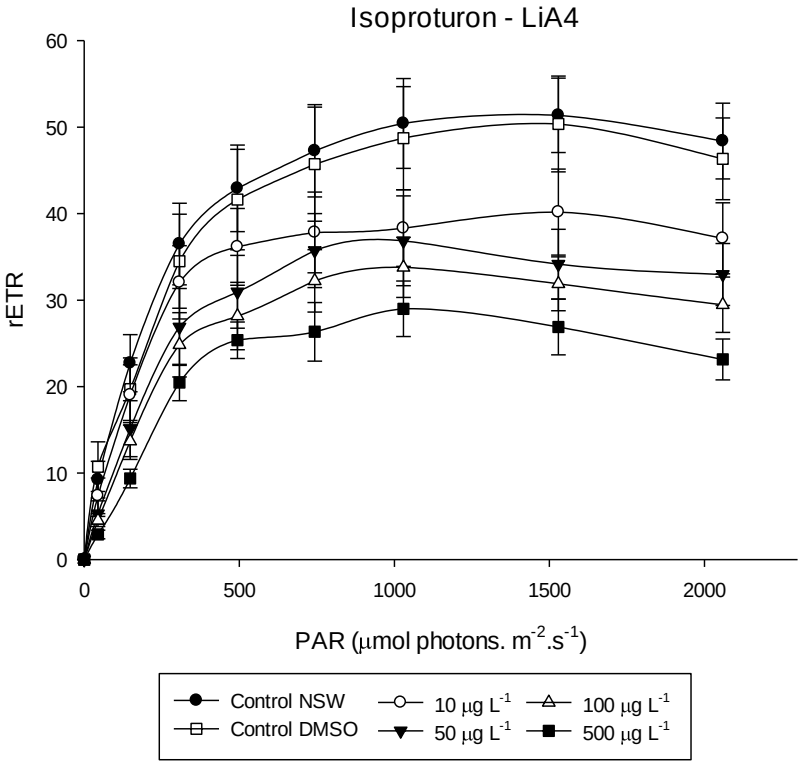

Figure 3.20 Relative electron transport rate (rETR) of LIA4 exposed to isoproturon for a period of 7 days. Means ± SD, n=4.

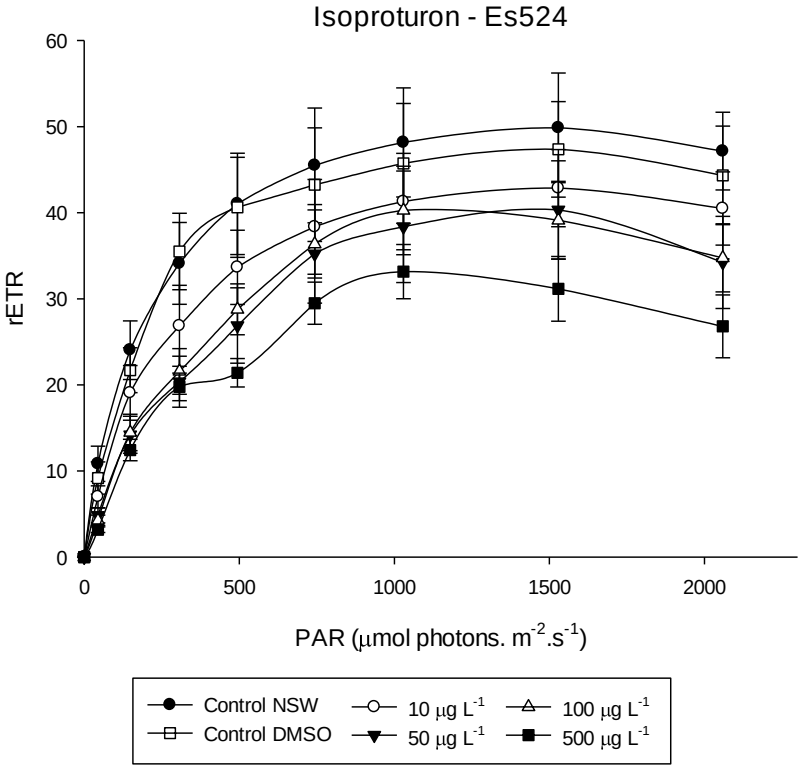

Figure 3.21 Relative electron transport rate (rETR) of Es524 exposed to isoproturon for a period of 7 days. Means ± SD, n=4.

Variable	Nominal concentration	*Ectocarpus siliculosus*	
	Isoproturon (µg L^{-1})	LIA4	Es524
Relative electron transport rate ($rETR_{max}$)	NSW	50.05 ± 7.30 [a]	47.87 ± 7.08 [a]
	DMSO	48.56 ± 8.40 [a]	45.80 ± 6.49 [ab]
	10	38.73 ± 8.05 [ab]	41.87 ± 9.61 [ab]
	50	35.19 ± 8.15 [ab]	38.42 ± 7.46 [ab]
	100	32.45 ± 9.37 [ab]	39.58 ± 10.27 [ab]
	500	27.90 ± 5.00 [b]	31.94 ± 6.65 [ab]
Photosynthetic efficiency (α)	NSW	0.217 ± 0.022 [a]	0.195 ± 0.019 [abc]
	DMSO	0.209 ± 0.020 [ab]	0.189 ± 0.016 [abc]
	10	0.198 ± 0.023 [abc]	0.177 ± 0.023 [abcd]
	50	0.155 ± 0.032 [bcd]	0.172 ± 0.024 [abcd]
	100	0.132 ± 0.024 [d]	0.165 ± 0.027 [abcd]
	500	0.124 ± 0.024 [d]	0.147 ± 0.024 [cd]

Table 3.3: Photosynthetic efficiency of *E. siliculosus* exposed to different concentrations of isoproturon over a period of 7 days. Data shown as means ± SD (*n*=4). Different letters represent significant differences among the strains (LIA4 and Es524) for each variable at $P < 0.05$.

Compared to DIU and TBA, exposure of IPU for 7 d on *E. siliculosus* strains (LIA4 and Es524) had a lower toxicity impact. For the $rETR_{max}$, no significant decrease was observed in Es524, unlike LIA4 where significant decrease was observed at 500 µg L^{-1} of IPU. This difference might indicate higher tolerance of Es524 compared to LIA4, although no significant difference was recorded between the strains. The derived 7 d EC_{50} of $rETR_{max}$ for LIA4 was 1.411 (1.2-1.6) mg L^{-1} which was slightly lower compared to Es524 with 1.567 (1.3-1.8) mg L^{-1}. For α, significant decreases were observed in LIA4 at 100 and 500 µg L^{-1} of IPU. On the other hand, no significant reduction was recorded in Es524, suggesting higher resistance of the strain compared to LIA4. 7 d EC_{50} of α for Es524; 1.715 (1.45-2.0) mg L^{-1}showed higher value compared to LIA4; 1.546 (1.3-1.8) mg L^{-1} but was not significantly difference. The NOEC values

recorded for both indicators ($rETR_{max}$ and α) in LIA4 and Es524 were 50 and 100 µg L^{-1}, respectively.

Table 3.4 indicates that DIU is the most toxic compound to *E. siliculosus*, followed by TBA and IPU. Generally, higher EC_{50} values were recorded in Es524 compared to LIA4, which clearly indicate higher tolerance of Es524 compared to LIA4 to the herbicides tested. Although exposure to TBA, has displayed higher EC_{50} values for the photosynthetic efficiency indicators in LIA4, but the differences were not statistically significant compared to Es524.

Table 3.4: Toxicity of diuron (DIU), terbuthylazine (TBA) and isoproturon (IPU) to LIA4 and Es524. EC_{50} values shown as means ± SD (n=4) together with the 95% C.I. in parentheses. Data sets for each indicator that are significantly different between the strains are represented by different letters (P< 0.05).

Herbicide	Indicator	*Ectocarpus siliculosus*					
		Es524			*LIA4*		
		7-d EC_{50} (µg L^{-1})	(µg L^{-1})		7-d EC_{50} (µg L^{-1})	(µg L^{-1})	
			NOEC	LOEC		NOEC	LOEC
DIU	RGR	24.65 ± 4.10 [a] (19.2-31.3)	10	50	9.94 ± 2.33 [b] (6.1-13.9)	5	10
	Fv/Fm	32.75 ± 2.96 [a] (27.1-38.2)	1	5	17.76 ± 2.70 [b] (13.2-21.8)	1	5
	ΦPSII	11.18 ± 1.07 [a] (10.3-14.2)	1	5	8.04 ± 0.74 [b] (5.1-8.8)	1	5
	qP	62.94 ± 9.44 [a] (52.2-73.1)	5	10	52.83 ± 6.68 [a] (45.3-62.4)	1	5
	qN	1.276 ± 0.36 [a] (0.8-1.7)	1	5	1.816 ± 0.34 [a] (1.3-2.3)	1	5
	$rETR_{max}$	24.15 ± 3.8 [a] (21.4-31.3)	10	50	16.93 ± 3.1 [b] (13.2-19.7)	5	10
	α	76.38 ± 10.4 [a] (65.4-88.3)	10	50	53.25 ± 9.7 [b] (41.2-62.6)	5	10
TBA	RGR	28.25 ± 5.62 [a] (23.4-36.1)	10	50	18.11 ± 4.71 [b] (12.1-21.7)	10	50
	Fv/Fm	80.06 ± 5.24 [a] (72.3-91.2)	5	10	88.13 ± 8.80 [a] (77.4-97.1)	5	10
	ΦPSII	62.32 ± 6.11 [a] (55.3-69.1)	5	10	68.17 ± 6.34 [a] (60.2-75.8)	5	10
	qP	83.37 ± 10.57 [a] (71.4-94.9)	5	10	90.16 ± 13.18 [a] (76.1-104.7)	10	50
	qN	5.063 ± 1.46 [a] (3.2-7.1)	1	5	6.345 ± 1.34 [a] (4.5-8.0)	1	5
	$rETR_{max}$	60.67 ± 9.3 [a] (47.2-72.8)	10	50	67.56 ± 10.2 [a] (55.1-78.7)	10	50
	α	95.32 ± 14.1 [a] (78.3-113.7)	10	50	89.89 ± 13.5 [a] (74.2-106.4)	10	50
IPU	RGR	315.2 ± 32.53 [a] (280.8-348.6)	50	100	257 ± 58.79 [a] (213.4-302.3)	50	100
	Fv/Fm	>10,000 [a]	100	500	3652 ± 378 [b] (3284.7-4021.9)	50	100
	ΦPSII	2666 ± 275 [a] (2404.2-2936.8)	< 10	50	2072 ± 198 [b] (1881.3-2268.8)	< 10	50
	qP	2704 ± 487 [a] (2238.7-3180.1)	50	100	1841 ± 270 [b] (1585.6-2112.4)	50	100
	qN	37.84 ± 9.50 [a] (28.1-47.3)	10	50	29.65 ± 6.39 [a] (22.9-37.1)	10	50
	$rETR_{max}$	1567 ± 219 [a] (1336.2-1804.9)	100	500	1411 ± 183 [a] (1214.7-1623.2)	50	100
	α	1715 ± 237 [a] (1451.7-2011.3)	100	500	1546 ± 204 [a] (1307.4-1825.6)	50	100

3.4 Discussion

3.4.1 *E. siliculosus* response to diuron (DIU)

Distinctive responses of *Ectocarpus siliculosus* (*E. siliculosus*) strains from two different areas to diuron (DIU) have been observed. Evaluations using different endpoints indicate that Es524 was more tolerant to DIU compared to LIA4 within the concentration range used in this study (1–500 µg L^{-1}). Additionally, a strong concentration dependent toxicity of DIU was observed for all endpoints tested.

For the relative growth rate (RGR), exposure to DIU at 50 µg L^{-1} and above caused a significant decrease of as much as 62-168% compared to the carrier control. This finding was in line to the report by Kumar *et al*., (2010), where the RGR of *Saccharina japonica* (seaweed) significantly reduced at 25 µg L^{-1} of DIU. Exposure to DIU probably damaged the capacity of *E. siliculosus* (LIA4 and Es524) strains to harvest light, and after 7 days this led to reduction in growth. A decrease in carbon assimilation and energy storage could be the factors that contribute to this condition. Additionally, the EC_{50}-RGR values recorded in Es524 and LIA4 to DIU exposure were 24.65 and 9.94 µg L^{-1} respectively. The significantly higher EC_{50} value for Es524 compared to LIA4 signifies higher tolerance of Es524 to DIU compared to LIA4. The values obtained are within the range of the EC_{50} (growth) values observed in two different green algae; *Chlamydomonas reinhardii*, EC_{50} =25 µg L^{-1}, Maule and Wright, 1984 and *Scenedesmus subspicatus*, EC_{50} =36 µg L^{-1}, Schäfer, *et al*. 1994). In fact, exposure of DIU to macrophytes such as *Lemna gibba* G3 and *Lemna minor* 1769 has also recorded EC_{50} values of 29 and 30 µg L^{-1} respectively (Okamura *et al*., 2003). Conversely, EC_{50} reported for *Hormosira banksii* (seaweed native to Australia and New Zealand) and lettuce, *Lactuca sativa* were 6.75 mg L^{-1} (Myers *et al*., 2006) and 9.5 mg L^{-1} (Okamura *et al*., 2003) respectively, which were much higher compared to *E. siliculosus*. This indicates far less sensitivity of the species (*Hormosira banksii* and

Lactuca sativa) to DIU. In addition, other studies have shown algae are more sensitive to the effects of DIU than fish, crustacean, and bacteria, where EC_{50} values in the range of 8-74 mg L^{-1} have been reported (Haynes *et al*., 2000; Fernandez-Alba *et al*., 2002; Okamura *et al*., 2002, 2003). This condition could be justified as a factor of the mode of action of DIU, which directly affects the photosystem II (PSII).

The effects of DIU on photosynthetic efficiency of *E. siliculosus* were evaluated via chlorophyll *a* fluorescence. It measures and quantifies alteration in PSII activity caused directly or indirectly by stress (Percival, 2005). Conrad *et al*., (1993) clearly stated the significance of measuring chlorophyll fluorescence yield to assess the toxic effect of DIU. In the present study, different chlorophyll fluorescence indicators; effective quantum yield of photosystem II (ΦPSII), maximum efficiency of photosystem II (Fv/Fm), photochemical quenching (qP), non-photochemical quenching (qN), maximum electron transport rate ($rETR_{max}$) and photosynthetic efficiency (α) have been used in order to evaluate the photosynthetic activity of *E. siliculosus*. For DIU effects on Fv/Fm and ΦPSII (**Figure 3.2** and **3.3** respectively), significant interactions between the strains (LIA4 and Es524) and the range of DIU treatments were observed. In Es524, significantly higher Fv/Fm and ΦPSII values were recorded at 10 and 5 μg L^{-1} of DIU respectively, indicating higher tolerance of Es524 compared to LIA4. In fact, the EC_{50} values obtained for both Fv/Fm and ΦPSII also indicated significantly higher values in Es524 compared to LIA4. The different tolerance recorded could be contributed by unique adaptation mechanisms possessed by each strain. The consistent decrease of Fv/Fm and ΦPSII indicators observed in both strains of *E. siliculosus* were in agreement with the reports on the sensitivity of photosystem II (PSII) in organisms exposed to pesticides reported earlier (Geoffroy *et al*., 2003, 2004; Olette *et al*., 2008; and Silkina *et al*., 2009). Moreover, a significant reduction (80%) of photosynthetic efficiency in *Elodea canadensis* (submersed macrophytes) was observed after exposure

to 5 µg L^{-1} of DIU (Knauert *et al*., 2010). In fact, Haynes *et al*., (2000b) also reported that DIU at 10 and 100 µg L^{-1} had reduced the ΦPSII in three different species of seagrass namely *Cymodocea serrulata*, *Halophila ovalis* and *Zostera capricorni* by 50-75% after a 5-day exposure period. Significant decrease of Fv/Fm and ΦPSII observed in *E. siliculosus* and other species at low concentrations (5 and 10 µg L^{-1}) probably indicate alteration in the structure of the photosynthetic apparatus. In addition, the detrimental effect of DIU on photosynthetic efficiency of *E. siliculosus* was better demonstrated by the ΦPSII rather than the Fv/Fm. This was proved by the lower EC_{50} values of ΦPSII recorded in both strains; LIA4 (8.04 µg L^{-1}) and Es524 (11.18 µg L^{-1}) compared to Fv/Fm which were 17.76 and 32.75 µg L^{-1} respectively. Previous study by Juneau *et al*., (2007) showed 100 mg L^{-1} of DIU (5 h exposure period) had caused a 55% decline in the effective quantum yield of photosystem II (ΦPSII) of seagrass *Halophila ovalis* but to a lesser extent to the maximum efficiency of photosystem II (Fv/Fm). Besides, lower EC_{50} values of ΦPSII and Fv/Fm; (EC_{50}-ΦPSII=1.8–2.9 µg L^{-1}, *Dunaliella tertiolecta, Phaeodactylum tricornutum, Thalassiosira pseudonana*, Sjollema *et al*., (2014); EC_{50}-Fv/Fm=10.9 µg L^{-1}, *Saccharina japonica*, Kumar *et al*., (2010); EC_{50}-Fv/Fm= 4.033, > 5 and > 5 µg L^{-1} for *Chara vulgaris*, *Myriophyllum spicatum* and *Apium nodiflorum*, respectively, Lambert *et al*., (2006)) reported from previous studies indicate better tolerance of *E. siliculosus* to DIU compared to aforementioned species. For the quenching response, maximum inhibitions of 85 and 89% photochemical quenching (qP) were observed in LIA4 and Es524 respectively, indicating that the redox state of the quinone pool in both strains was modified by the herbicide (Dosnon-Olette *et al*., 2010). Moreover, the binding of DIU to the D1 protein, has increased the fluorescence and non-photochemical quenching (qN, Figure 3.5), restricting the photochemical potential of the system (Negri *et al*., 2015). In fact, stimulation of qN by more than 100% may represent a disruption in the electron

transport necessary for energy dissipation by non-photochemical processes (transthylakoid pH gradient formation, antenna movements, photoinhibition) (Horton and Hague, 1988; Ruban and Horton, 1995 and Müller *et al*., 2001). As shown in the Figure 3.6 and 3.7, the patterns observed for the $rETR_{max}$ and α responses also indicated inhibitions of electron transport in both LIA4 and Es524 strains. The derived EC_{50} for $rETR_{max}$ and α showed significantly higher value in Es524 compared to LIA4 indicating higher tolerance of Es524 compared to LIA4. From the rETR-PAR curves, differences among treatments (DIU) and control could be detected, although high variations were observed. The results indicated that the method was able to produce reliable fluorescence measurements. These findings were in agreement to previous study by Figueredo *et al*., (2009) on three phytoplanktonic species (*Tetrallantos lagerheimii* Teiling, *Coelastrum sphaericum* Naegeli and *Pediastrum boryanum* (Turpin) Meneghini).

On the whole, the negative impacts of DIU as demonstrated through various indicators, might be driven by the ability of DIU to induce phytotoxicity by catalysing lethal photosensitized oxidation in the cell by two factors; inhibition of NADPH formation, which is necessary to maintain a functional carotenoid protective mechanism and also due to a high concentration of oxidized chlorophyll caused by an interruption of the electron flow (Stanger and Appleby, 1972). Additionally, Ralph (2000) mentioned that DIU inhibits the photoreduction side of PSII by strongly blocking the re-oxidation of the primary electron acceptor (Q_A), causing the magnitude of the minimum fluorescence emission to increase considerably, leading to a decrease in the variable fluorescence. Indeed, the reduction of the maximum quantum yield (Fv/Fm) observed in the present study, indicates a loss in photochemical energy conversion efficiency and /or damage at the level of photosystem II reaction centres. Moreover, the differences in DIU sensitivity between the species might indicate differential tolerance between them.

This could be due to the variations of their genetic profile, culture conditions, the range of concentrations used, uptake mechanisms, the binding strength of the chemical molecule to the target site, the unique metabolic pathways, or either of the compensation (defence) mechanisms of the species used (Knauert *et al*., 2010).

3.4.2 *E. siliculosus* response to terbuthylazine (TBA)

A stimulatory effect on algal growth observed at low pesticide concentrations (e.g. 1 µg L^{-1} TBA in present study) was also reported earlier and this was interpreted to be caused by physiological acclimation (El Dib *et al*., 1998; Franqueira *et al*., 1999; Rioboo *et al*., 2002). However, exposures to TBA at 50 µg L^{-1} and above caused significant reduction to the relative growth rates (RGR) in both strains (LIA4 and Es524). Although the EC_{50}-RGR recorded in Es524 was significantly higher compared to LIA4, no significant interaction between the strains and the range of TBA treatments was observed. The effect of TBA on *E. siliculosus* growth strongly depends on the herbicide concentrations. At 50 µg L^{-1}, as high as 86 and 70% reductions of RGR were observed in LIA4 and Es524 respectively. The effects of growth diminution were similarly observed for other triazines in different phytoplankton species by DeLorenzo *et al*. (2004) and Rioboo *et al*. (2002) albeit at a different level. These findings are also in line with reports by Fiori *et al*., (2013) and Singh and Wright (1999), who discovered significant growth reduction in *Gonyaulax spinifera* (a type of flagellate) and pea (*Pisum sativum*) respectively after being exposed to terbuthylazine (TBA). Moreover, Stepanova *et al*., (2012), also observed retardation in *Cyprinus carpio* (common carp) growth after being exposed to TBA at 520 and 820 µg L^{-1}. The variations in sensitivity to TBA by different organisms could be justified by different tolerances or could be equally due to the fact that TBA was affecting the photosystem II in photosynthetic

organisms compared to the non-photosynthetic organisms where the impact might become apparent at much higher concentration.

In addition, due to the specific effect of TBA on photosystem II (PSII), a drastic impact of the herbicide on photosynthetic efficiency of *E. siliculosus* was observed. The derived EC_{50} values for Fv/Fm and ΦPSII in Es524 were 80.06 and 62.32 µg L^{-1} respectively, which were not significantly different compared to LIA4 with 88.13 and 68.17 µg L^{-1}. Two-way analysis of variance (ANOVA) on both indicators also indicates similar response of LIA4 and Es524 to the range of TBA concentrations tested. Fv/Fm and ΦPSII were significantly inhibited at lower concentration (10 µg L^{-1}) compared to algal growth at 50 µg L^{-1}, attesting that photosynthetic efficiency is a more sensitive parameter than algal growth. These results were in line with those of Fiori and Pistocchi, (2014) and Macedo *et al*., (2008), who exposed *Skeletonema marinoi* to TBA and *Skeletonema costatum* to bentazon, respectively. This condition is probably driven by the fact that triazinic herbicides are known to be PSII inhibitors, acting biochemically by displacing the plastoquinone (Q_B) from its binding site in the D1 protein of photosystem II (PSII) (Hull, 1967). In the present study, both Fv/Fm and ΦPSII were significantly affected between 10 to 500 µg L^{-1} of TBA, with maximum inhibition of 76 and 80% recorded for each indicator respectively. However, our findings contradict those reported by Canero *et al*., (2011), which stated that TBA at 3 kg ha^{-1} did not caused any significant reduction either in Fv/Fm or ΦPSII parameters after being exposed for 24 hours and 15 days to *Olea europaea* (Olive). This condition could be justified by the fact that *E. siliculosus* is a type of macroalgae compared to the olive which is in the same group of advance terrestrial plants. This means that a much higher amount of TBA is probably required to cause significant change in their photosynthetic efficiency. Besides, the decrease of Fv/Fm values observed in the TBA treated cultures (Figure 3.9) are suggested due to the photoinhibition in the chlorophyll

of the brown seaweed. This photoinhibition is probably caused by the damage in photosynthetic components, as of chlorophyll molecules of PSII, and this effect can be either short-term and reversible (dynamic photoinhibition) or long-term and irreversible. For the effective quantum yield of PSII (ΦPSII), TBA at 50 µg L^{-1}, has caused 31 and 34% reduction in LIA4 and Es524 strains respectively. These values indicate that *E. siliculosus* was more tolerant to TBA compared to different species of flagellates such as *P.reticulatum*, *A. lusitanicum*, *S. trochoidea*, *H. hakashiwo*, *Lingulodinium polyedrum*, *Fibrocapsa japonica* and *Gonyaulax spinifera*, where the ΦPSII of these species were severely affected, with as much as an 80% reduction at 50 µg L^{-1} of TBA (Fiori *et al*., 2013). However, we also have found that *E. siliculosus* was more sensitive to TBA than *Prorocentrum minimum*.

As for the quenching responses of *E. siliculosus* to TBA exposure (Figure 3.11, qP and 3.12, qN), no significant interaction (P>0.05) was observed between the strains and the treatments ($S \times T$) in both parameters. However, significant decrease of qP values as compared to the carrier control were observed as early at 10 µg L^{-1} up to the highest concentrations tested (500 µg L^{-1}). As for the qN, significant stimulations were observed between 5 to 50 µg L^{-1}, with increases between 31 to 121% have been recorded. On the other hand, exposure to TBA at 100 and 500 µg L^{-1} have caused the qN to decrease significantly with maximum reduction of 49 and 54% compared to the carrier control in LIA4 and Es524 respectively. This condition could be related to the destruction of the PSII due to excessive blocking on the electron transport chain by the herbicide. Evaluations on the $rETR_{max}$ indicated that TBA at 50 µg L^{-1} and above had caused significant decrease in both strains. For α, significant reductions were observed at ≥ 50 and ≥ 100 µg L^{-1} of TBA in LIA4 and Es524 respectively. These results affirmed the effect of TBA on photosynthetic electron transport of *E. siliculosus*.

3.4.3 *E. siliculosus* response to isoproturon (IPU)

To date, no data have been published related to the effects of IPU against *E. siliculosus.* A previous study by Peres *et al*., (1996), indicated that a marked reduction in the diatom density was recorded after 34 days of exposure to 5 µg L^{-1} of IPU. Singh *et al*., (1997) also reported that IPU (0.25 – 16 kg a.i. ha^{-1}) caused significant reduction in the dry weight of different biotypes of *Phalaris minor*. On the other hand, Dewez *et al*., (2008) have observed that when *Scenedesmus obliquus* was exposed to low concentration of IPU (7 µg L^{-1}) the cells density (growth) had increased by 50%.

In the present study, no significant changes in the relative growth rate (RGR) of both strains (LIA4 and Es524) were observed at 10 and 50 µg L^{-1} of IPU. However, at 100 and 500 µg L^{-1}, the RGR of both strains were significantly reduced by IPU as much as 26 and 71% respectively in LIA4 and 25 and 62% in Es524. Moreover, at 50 µg L^{-1}, as much as 22 and 15% reductions were observed in LIA4 and Es524 strains respectively as compared to the carrier control. These values were almost two to three times less compared to the cell number of *Chlamydomonas reinhardtii* which was decreased by 44% of the control when exposed to the same level of IPU (50 µg L^{-1}) (Bi *et al*., 2012). These algistatic effects are in agreement with previous reports showing 50% decrease of growth when different algal species were exposed 96 h to 20 -25 µg L^{-1} of IPU (Anton *et al*., 1993; Kirby and Sheahan, 1994; Rioboo *et al*., 2002). From the studies reported, the decrease of algal growth in response to IPU was caused by photosynthesis deterioration. The derived EC_{50} (RGR) values for *E. siliculosus* strains; 257 and 315.2 µg L^{-1} for LIA4 and Es524 respectively were not significantly different between each other. However, the values were higher compared to *C. reinhardtii* (72 h EC_{50} RGR = 43.25 µg L^{-1}, Bi *et al*., 2012); *Botryococcus braunii* (7 d EC_{50} = 196 µg L^{-1} (0.95 µM) Lazar and Lazar., 2001) and *Lemna minor* (10 d EC_{50} = 33 µg L^{-1}, Kirby and Sheahan, 1994). The differences could be equally contributed either by the variations in

the length of exposure to IPU or the heterogeneity of the model species been used. In addition, the effects of IPU on macrophytes also have been reported by several researchers including Grollier *et al*., (1997) and Feurtet-Mazel *et al*., (1996). Grollier *et al*., (1997) observed that IPU at 60 µg L^{-1} caused a significant decrease in the stem length of *Elodea densa* of close to 43% compared with that of the control groups. Similarly, Feurtet-Mazel *et al*., (1996) whose working with the same species (*E. densa*) also observed that at 10 µg L^{-1}, IPU can caused significant negative effects on growth and a total growth inhibition was detected at concentrations higher than 200 µg L^{-1} IPU. The 'more tolerant' response of *E. siliculosus* may be caused by the lipophilic properties of IPU resulting in higher toxicity to macrophytes than to algae (Kirby and Sheahan, 1994). In addition, the effects of IPU on the growth of photosynthetic organisms could be directly linked to its herbicidal properties. IPU phytotoxicity is mainly due to the specific binding on the D1 protein within the thylakoid membranes (Arnaud *et al.*, 1994). The binding results in a breakdown of the photosynthetic synthesis of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADP), leading to an adverse impact on metabolism (Pietsch *et al*., 2006).

With respect to photosynthetic efficiency evaluation, Figures 3.16 and 3.17 show the effects of IPU on *E. siliculosus* under the dark (Fv/Fm) and light (ΦPSII) adapted state respectively. According to Dewez *et al*., (2008) photosynthetic fluorescence parameters were considered as reliable biomarkers for IPU toxicity. From the result obtained (Figure 3.16), IPU exposure at 500 µg L^{-1} indicates that Fv/Fm value of Es524 was significantly higher compared to LIA4. Moreover, the EC_{50}-Fv/Fm also showed significantly higher value in Es524 compared to LIA4 indicating higher tolerance compared to LIA4. At 10 and 50 µg L^{-1}, no significant effect on Fv/Fm was observed in both strains (Es524 and LIA4) after 7 days of exposure. This is in contrast to a report by Bi *et al*., (2012) who observed a reduction of 33.3% of Fv/Fm value in

green algae *(Chlamydomonas reinhardtii)* compared to the untreated group after been exposed to 50 µg L^{-1} of IPU. The non-significant reductions of 12 and 10% compared to the carrier control observed in LIA4 and Es524 respectively at 50 µg L^{-1} indicate that both strains of *E. siliculosus* were more tolerant to IPU compared to the green algae (*C. reinhardtii*). This condition may be justified by the fact that *C. reinhardtii* belongs to the group of microalgae instead of *E. siliculosus* which is in the group of macroalgae. Besides, the different sensitivity could also be explained by the distinct metabolism properties, uptake efficiency and morphological characteristics of different algal species (Bentley-Mowat and Reid, 1977; Blanck *et al*., 1984; Lawson and Mason, 1998; Juneau *et al*., 2001). The aforementioned factors that might contribute to the different response between the algae might be better interpreted through the ADME (Absorption, Distribution, Metabolism, and Excretion) concept (Doogue and Polasek, 2013). From previous studies on metal toxicity, we consider *E. siliculosus* to be a sufficiently sensitive organism for ecotoxicological studies. This hypothesis was tested in the current study. Furthermore, noticeable impacts of IPU on Fv/Fm values (significantly decreased) were detected at 100 µg L^{-1} in LIA4 and 500 µg L^{-1} in both strains. The significant decrease in photosynthetic efficiency in response to IPU exposure was in agreement with the observations by Pietsch *et al*., (2006) and Feurtet-Mazel *et al*., (1996), for *Ceratophyllum demersum* L. and *Elodea densa* respectively. The decrease in variable fluorescence observed, indicates that electron donation to PSII was inhibited and PSII functional reaction centres were abolished (Krause and Weis, 1991).

As for the ΦPSII response (Figure 3.17), significant decreases were observed at every concentration tested (10 – 500 µg L^{-1}) as compared to the carrier control. Reductions of ΦPSII as much as 18 – 28% and 14 - 25% were recorded for LIA4 and Es524 respectively. This result slightly contradicts the study by Knauert *et al*., (2010), in which photosynthetic efficiency of *Elodea Canadensis* and *Potamogeton lucens* was

not significantly affected when exposed to 14 µg L^{-1} of IPU. Again, the negative impact on photosynthetic efficiency was suggested due to inhibition of PSII electron transport by IPU interaction with the D1 protein affecting PSII primary photochemistry (Zer and Ohad, 1995). The changes of D1 protein function induce an alteration of Chl *a* fluorescence associated to PSII activity as what observed from the Fv/Fm and ΦPSII values. Moreover, the EC_{50} of both parameters (Fv/Fm and ΦPSII) were found to be > 10 mg L^{-1} and 2.666 mg L^{-1} respectively for Es524 and 3.652 and 2.072 mg L^{-1} for LIA4. These higher EC_{50} values recorded in both strains of *E. siliculosus* could indicate the signs of resistance mechanism that present in the brown seaweed. There is a resistance mechanism to the herbicide (IPU) contributed by the hydroxylation of the third carbon from the rest of the isopropyl from the IPU molecule with the aid of the P450 cytochrome (Reichart *et al*., 1982, quotation by Durst, 1991).

Further, the seaweed response to IPU exposure was also observed through quenching parameters (qP and qN). No significant interaction was observed between the strains and the treatments (*S* × *T*) in both parameters within the range of IPU concentrations tested. For qP, significant decrease as compared to the carrier control were observed at 100 and 500 µg L^{-1}, with maximum reduction of 37 and 24% was recorded in LIA4 and Es524 respectively. These values indicate qP was more affected by IPU than Fv/Fm. Previous study using *Scenedesmus obliquus* and *Scenedesmus quadricauda* also have reported 23% inhibition of qP at 20 µg L^{-1} of IPU. These results indicate that the redox state of the quinone pool was modified by the herbicide (Dosnon-Olette *et al*., 2010). As for the non-photochemical quenching (qN, Figure 3.19) significant changes as compared to the carrier control were observed at 50 till 500 µg L^{-1} of IPU. As much as 60 to 114% increments were recorded within the aforementioned concentrations. This finding was in agreement to the IPU effects on *Sc. obliquus* and *Sc. quadricauda*, where stimulation of qN up to 20% was reported

(Dosnon-Olette *et al*., 2010). Assessments of $rETR_{max}$ and α demonstrated less impact on electron transport compared to DIU and TBA. As for the *E. siliculosus* strains, significant decrease of $rETR_{max}$ and α were observed in LIA4 at 500 and ≥ 100µg L^{-1} of IPU respectively, while no significant change was observed in Es524.

3.5. Conclusion

The present study represents the first attempt to understand the effects of different herbicides on the model brown seaweed, *Ectocarpus siliculosus.* The different effects of these chemicals on the physiological health and growth of *E. siliculosus* demonstrated in this study highlight the need to examine the extent to which pollutants from terrestrial runoff and shipping activities can enhance the damaging effects at different levels of biological organizations. Moreover, besides elucidating the harm that these chemicals exerted towards *E. siliculosus*, one can surely ascertain that these weed killer and antifouling agents can also tremendously influence other marine communities as well.

A comparison of the NOEC relative growth rate (RGR) values to the measured environmental concentrations for terbuthylazine and isoproturon indicated that they were below the level that can caused adverse impacts to *E. siliculosus* growth. However, the much higher concentrations of diuron that can be encountered in the environmental could have a deleterious impact to *E. siliculosus*. Based on the EC_{50}s (Table 3.4), the order of toxicity of the three herbicides from high to low was: diuron > terbuthylazine > isoproturon in both strains of *E. siliculosus*. Our results clearly show a large difference in toxicity (up to two to three orders of magnitude) of the individual compounds, with DIU being the most toxic and IPU the least toxic compound. This toxicity ranking (DIU more toxic than IPU) is consistent with the study by Backhaues *et al*., (2004) and Knauert *et al*., (2008) reported previously. For TBA, it is a more potent chemical than

IPU since the seaweed cultures exposed to IPU showed an EC_{50} values higher than the TBA-treated cultures. *S*-Triazine herbicides (e.g. TBA), are considered one of the most efficient herbicides, at least when they are tested on freshwater algae (Abou-Waly *et al*., 1991), probably due to the presence of a methylthio group in position six of the triazine ring, which may tend to be linked with the relatively high inhibitory effect to algal cells (El-Dib *et al*., 1989). The differences in levels of toxicity can be associated with solubility of the herbicides in lipids; it is well known that lipid-soluble substances easily pass into cells through the cell wall (Tang *et al*., 1998) and, that the sorption of the herbicide to algal cells is a prerequisite for its action at the chloroplast membrane. IPU (log K_{ow} 2.25, Rioboo *et al*., 2002) can be considered a moderately lipophilic compound, but TBA (log K_{ow} 3.04, Gangolli S., 1999) could be rapidly taken up, as a passive uptake, from the medium by *E. siliculosus* cells due to affinity of this molecule to the algal cell (Reddy and Locke, 1996). This hypothesis will agree with the results obtained in the present work.

In addition, the results of the present study also revealed that chlorophyll fluorescent parameters (Fv/Fm, ΦPSII, qP, qN, $rETR_{max}$ and α) serve as important and sensitive indicators for assessing the impacts of DIU, TBA and IPU on *E. siliculosus*. The inhibition of photosynthetic electron transport by the herbicides was also observed in other studies of metal toxicity, which showed a concomitant decrease in photochemistry efficiency with an increase in non-photochemical quenching when the electron transfer was altered, in order to limit damage of the photosynthetic apparatus (Juneau *et al*., 2001, 2002, 2005; Mallick and Mohn, 2003). From the EC_{50} values obtained, qN is generally the most sensitive indicator, because it integrates all the toxic effects on the electron transport process, in contrast to qP, which is an indicator only of the active reaction centres of PSII or Fv/Fm which is only an indication of PSII charge separation (Juneau *et al*., 2001, 2002; Wang and Dei, 2006). Moreover, as shown in

Table 3.4, we discovered that the growth endpoint (RGR) was more sensitive than Fv/Fm and ΦPSII. This finding is similar to that of recent study by Park *et al*., (2017) who reported the root re-growth of three *Lemna* species (*L. minor, L. gibba and L. paucicostata*) to be more sensitive than the aforementioned endpoints in response to paraquat exposure. In fact, a previous report by Brown and Newman (2003) on Cu toxicity to the red seaweed *Gracilariopsis longissima*, affirmed that RGR was the most sensitive endpoints compared to photosynthetic rate (i.e. Fv/Fm), O_2 evolution and ion leakage. Therefore, the step to prioritize growth measurement endpoints for environmental risk assessment or to set the water quality standards by OECD and ISO international test guidelines cannot be ruled out (OECD, 1984; ISO 8692, 1989; Dahl *et al*., 2006). Overall, the differences in sensitivity of algae to individual herbicides has generally not been attributed to differences in the architecture of the herbicide binding site, as the amino acid sequence of the D1 protein in photoautotrophs is amongst the most highly conserved of photosynthetic proteins (Barber, 1992). Instead, differences in herbicide sensitivity between individual algal species have typically been attributed to unique defence mechanisms or differential cell permeability and herbicide uptake (Fahl *et al*. 1995).

Chapter 4

Biochemical response of *Ectocarpus siliculosus* strains with different pollution history to diuron, terbuthylazine and isoproturon

4.1 Introduction

Significant effects of diuron (DIU), terbuthylazine (TBA) and isoproturon (IPU) on growth and photosynthetic efficiency of LIA4 and Es524 have been discussed in the previous chapter. Inhibition of the electron transport in photosystem II (PSII) by the herbicides could be attributed to the displacement of the secondary plastoquinone acceptor by herbicides from the Qb site to D1 protein (Rensen, 1982). Due to this action, it is hypothesized that the herbicides might trigger **oxidative stress**, which consequently leads to the damage of lipids and other cellular components. Therefore, lipid peroxidation and hydrogen peroxide (H_2O_2) were measured as oxidative stress markers.

Besides, in response to this condition, a defensive mechanism called **antioxidative enzymes** will be stimulated to counteract the stress so that a stable homeostasis environment within the living cells can be maintained. Previous studies revealed that numerous pesticides can caused an increment of reactive oxygen species (ROS) in cells, consequently leading to the production of antioxidative defenses such as glutathione reductase or catalase (Mosleh *et al*., 2004; Dewez *et al*., 2005). In fact, different degree of resistance observed in LIA4 and Es524 could be attributed to the unique defence mechanisms possessed by each strain. Therefore, in the present study, evaluations of three different antioxidative enzymes; catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) could be useful to determine the factors that might responsible for the different sensitivity between the strains. Apart from the enzymatic scavenging mechanisms, the non-enzymatic responses (polyphenols) were also investigated in this study, since polyphenols are a well-known group of antioxidants that respond to abiotic stress (Dixon and Paiva, 1995; Ksouri *et al*., 2007; Zarzecka and Gugala, 2011; Lajayer *et al*., 2017).

4.2 Materials and methods

4.2.1 Algal material and culture conditions

Please refer to section **2.2**

4.2.2 Diuron, terbuthylazine and isoproturon treatments

Stock solutions of herbicides were prepared according to previous chapters (See **3.2.2**). Fresh biomass for each replicate was transferred to individual clear glass Wheaton jars containing 250 ml of Provasoli medium loaded with different concentrations of the herbicides. The pH, salinity and temperature of the test solutions were 7.4 ± 0.1, 33 ± 1.0 ppt and 15ºC respectively. The experiments were carried out for 7 days with a range of herbicides between 1 to 500 µg L^{-1} with four replicates for the H_2O_2 and lipid peroxidation assays, while three replicates for other biochemical studies per treatment. Once the exposure period finished, the biomass of both strains (LIA4 and Es524) were briefly rinsed with sterile seawater, promptly frozen in liquid nitrogen (-196 °C), kept in 15 mL tubes, and stored at -80 °C before analysis.

4.3 Evaluation of oxidative stress:

4.3.1 Hydrogen peroxide (H_2O_2) content

The H_2O_2 content was evaluated according to Sergiev *et al*., (1997) and Saez *et al*., (2015) with slight modification (smaller volume) in order to be suitable for plate reader analysis. Algal biomass (0.15 g) was extracted with 1.5 ml of 10% (W/V) trichloroacetic acid (TCA) in a centrifuge tube, and was kept in ice. The tubes were vortexed for 5 min after being added with glass beads (3 mm diameter). Later, the homogenate was centrifuged at $15{,}000^{X}$ g for 10 min (Sanyo Hawk 15/05). In each well, 50 µL of extract (supernatant) was added. This was followed by 150 µL of 50 mM

potassium buffer (pH 7.0) and 100 μL of 1 M potassium iodide (KI). The absorbance was measured at 390 nm (OPTImax Microplate Reader, Molecular Devices, CA, USA). H_2O_2 in 10% TCA solution was used as standard and expressed as nmol H_2O_2 g^{-1} wt tissue.

4.3.2 Level of lipid peroxidation (LPX)

The toxicity of reactive oxygen species (ROS) has often been monitored by measuring lipid peroxidation (LPX), where it gave an indication of damage to polyunsaturated fatty acids (Apel & Hirt, 2004). In present study, the thiobarbituric acid reactive substances (TBARS) assay was carried out according to Heath and Packer (1968) with modifications. The extraction of *E. siliculosus* tissues was performed as aforementioned. Later, 150 μL of the supernatant are mixed with 150 μL of 0.5% thiobarbituric acid solution (in 10% TCA). Then the mixture is heated at 95°C for 45 min and then followed by quick cooling on ice bath to stop the reaction. The absorbance of the mixture (300 μL) is read at 532nm and 600 nm using (OPTImax Microplate Reader, Molecular Devices, CA, USA). The latter is subtracted from the former to correct for non-specific turbidity. TBARS was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Wills 1969), and expressed as nmol g^{-1} wt tissue.

4.4 Antioxidative enzymes analysis:

According to Fayez *et al*., (2007), oxidative stress occurs when a plant is subjected to biotic or/and abiotic stresses, due to production of reactive oxygen species (ROS) beyond the homeostasis condition. It can occur mainly in chloroplasts, mitochondria and peroxisomes causing oxidative damage to lipids, proteins and DNA (Pinto *et al*., 2003; Møller *et al*., 2007; Anjum *et al*., 2015). Thus, to overcome this unfavourable condition, cells were equipped with ROS scavenging mechanisms (enzymatic and non-enzymatic) to detoxify increased amounts of ROS.

4.4.1 Preparation of protein extracts

The method by Ratkevicius *et al*., (2003) was adopted with minor modifications in order to prepare the protein extracts. (1.5 ± 0.2 g per replicate) of frozen *Ectocarpus* biomass (treated and control samples) was grounded in a mortar with liquid nitrogen (-196°C) to powder. Solution containing 0.1 M potassium phosphate buffer (pH 7) with 5mM 2-mercaptoethanol (extraction buffer) was added in a ratio of 1 g : 3 ml. The homogenate was filtered through Miracloth (Calbiochem) and centrifuged at 13,000 rpm for 10 min at 4°C (BioFuge 22R, Heraeus Sepatech GmBH, Germany). In order to precipitate the proteins, the supernatant was transferred to a new tube (50ml) and 0.5 gram per millilitre (ml) of ammonium sulphate were added; the mixture was vortexed at 400 rpm for 2 hours at 4°C. The mixture was then centrifuged at 13,000 rpm for 30 min at 4°C, and the pellet was re-suspended in 0.1 M potassium phosphate buffer (pH7), containing 2mM 2-mercaptoethanol and 10% glycerol. Protein extracts were adjusted to a final concentration of 1 mg ml^{-1} using Bradford method and bovine serum albumin (BSA) as standard (Bradford, 1976). Extracts were stored at -80°C for further enzymatic activity analysis.

4.4.2 Catalase (CAT)

Catalase (CAT) activity was measured spectrophotometrically by following the rate of decrease in absorbance at 240 nm (Jenway 7315, Bibby Scientific, UK) caused by the disappearance of H_2O_2 (Beers & Sizer, 1952). The method was adopted from Aebi (1984) with minor modifications. The absorption coefficient at 240 nm for H_20_2 was taken to be 43.6 M^{-1} cm^{-1} (Hildebrandt & Roots, 1975). The CAT activity was measured by adding 15µg (15µl) of protein extracts to 485 µL of 0.1 M potassium phosphate buffer (pH 7) containing 16 mM H_2O_2. The reaction was run at 20°C for 1 min, and only the initial linear rate was taken to determine the activity.

4.4.3 Ascorbate peroxidase (APX)

The method for APX activity evaluation was adopted from Nakano & Asada (1981) with minor modifications. The reaction was started by adding 16 mM H_2O_2 to a 500 µL mixture containing 15 µg (15µl) of protein extracts, 0.1 M potassium phosphate buffer (pH 7) and 0.5 mM ascorbate. APX activity was determined by monitoring the decreasing rate of ascorbate oxidation at 290 nm (Jenway 7315, Bibby Scientific, UK) for 0.5 min with an extinction coefficient of 2.8 mM cm^{-1} (Nakano and Asada (1981).

4.4.4 Glutathione reductase (GR)

GR activity was assayed using the method of Sen Gupta *et al*., (1993) with minor modifications. To 10 µg (10µl) of protein extracts, 290 µl of 0.1 M potassium phosphate buffer (pH 7) containing 0.5 mM oxidized glutathione (GSSG) and 0.15 mM NADPH (nicotinamide adenine dinucleotide phosphate) were added. The decrease in absorbance due to NADPH consumption was measured at 340 nm for 5 minutes and the activity was calculated using an extinction coefficient of 6.22 mM cm^{-1}.

4.5 Determination of total phenolic content

Phenolic contents in both strains of *E. siliculosus* were measured using the method by van Alstyne (1995) and Saez *et al*., (2015) with minor modifications. 100 mg of fresh biomass was added to a 15 ml tube containing 5 ml of 85% ethanol in distilled water. Glass beads (c. 10, 3 mm diameter) were added to aid the extraction. Tubes were placed on a mixer (Ika Labortechnik KS250) inside a cold room (4°C) and vortexed at 550 rpm for 24 h. Samples were centrifuged at 6000 g at 4°C for 10 min and the supernatant (12.5 µL) was added to 500 µL of 17% Folin-Ciocalteu reagent solution. After 5 min, the solution was alkalinized with 250 µL of 1 M Na_2CO_3 solution and the samples were heated for 30 min at 50 °C in a water bath. The absorbances of the samples were measured at 765 nm using concentrations of phloroglucinol between 0 and 0.17% as a standard.

4.6 Evaluation of antioxidant activity

Non-enzymatic antioxidant activity of LIA4 and Es524 was measured according to the method by Kumar *et al*., (2010) with minor modifications. 1, 1-diphenyl-2-picryl-hydrazil (DPPH) was used to measure the free radical scavenging activity of the seaweed extracts. In the presence of antioxidants, the purple colour of the DPPH radical solution changes to a bright yellow. The intensity of this change can be monitored spectrophotometrically. Each replicate (0.2 g) was homogenized in 2 mL of absolute ethanol with a tissue homogenizer and the extract centrifuged at 10,000 rpm for 15 min at 4ºC. The supernatant (200 µL) was mixed with 0.2 mM DPPH ethanol solution (0.1 mL) and 0.1 M of acetate buffer (pH 5.5, 0.2 mL). After incubating in the dark for 30 min, the absorbance of the mixture was measured at 517 nm to determine DPPH scavenging activity (Abe *et al*., 1998). Activity is expressed as $(A_{control} - A_{treated})/A_{control} \times 100\%$, where $A_{control}$ and $A_{treated}$ are the absorbances at 517 nm of control and *Ectocarpus* treated sample.

4.7 Analysis of polyphenols

The method by Schmidt *et al*., (2012) was adopted for the polyphenols analysis with modifications. Initially, the tissues (control and treated, 0.5 g fresh mass, n=3) were pulverised with pestle and mortar by flash freezing the samples in liquid nitrogen (-196 °C). Later, polyphenols were extracted twice (24 h each) under continuous stirring (600 rpm) at 4 °C using ethanol 85% acidified with 1% HCL in 15 mL tubes. After each extraction, the samples were centrifuged at 4°C for 15 minutes at 6,000 rpm using a Harrier 18/80R refrigerated centrifuge (MSE, UK). The supernatants were pooled together from each replicate separately into clean cryotubes and filtered through a 0.22 µm nylon micro-membrane (Cronus filter) to remove particles before use for analysis. The extracts were loaded onto the HPLC vials and placed on an autosampler fitted to the high performance liquid chromatography (HPLC) system (Dionex UltiMate® 3000 HPLC, Germany) equipped with a C_{18} reverse-phase column (Thermo SCIENTIFIC, Aquasil C_{18}; 150 mm × 4.6 mm Ø column, 5 µm), protected by a 5 µm C_{18} reverse-phase guard column.

A gradient solvent system was employed, with solvent A being acetonitrile (ACN) and solvent B being 1% phosphoric acid (H_3PO_4) in Milli-Q water (99:1, v/v). The elution profile had the following proportions (v/v) of solvent B: 0.00–2.00 min, 90% ; 2.00–5.00 min, 88% ; 5.00–6.00 min, 85%; 6.00–10.00 min, 70%; 10.00–12.00 min, 65%; 12.00–15.00 min, 50%; 15.00–17.00 min, 65%; 17.00–20.00 min, 90%.

The column held at 25°C was flushed with a flow rate of 0.2 mL/min. Polyphenols were detected using a photo diode array UV detector at 254 nm. Identification of phenolic compounds was based on comparing retention times with the standards (Sigma-Aldrich, St. Louis, MO, USA). All the prepared samples were filtered through 0.22 µm membranes, and the mobile phase was degassed before injection on to HPLC.

Polyphenolic quantification was based on the measurement of the integrated peak area and the content was calculated using the analytical curves established from the HPLC output for standard solutions.

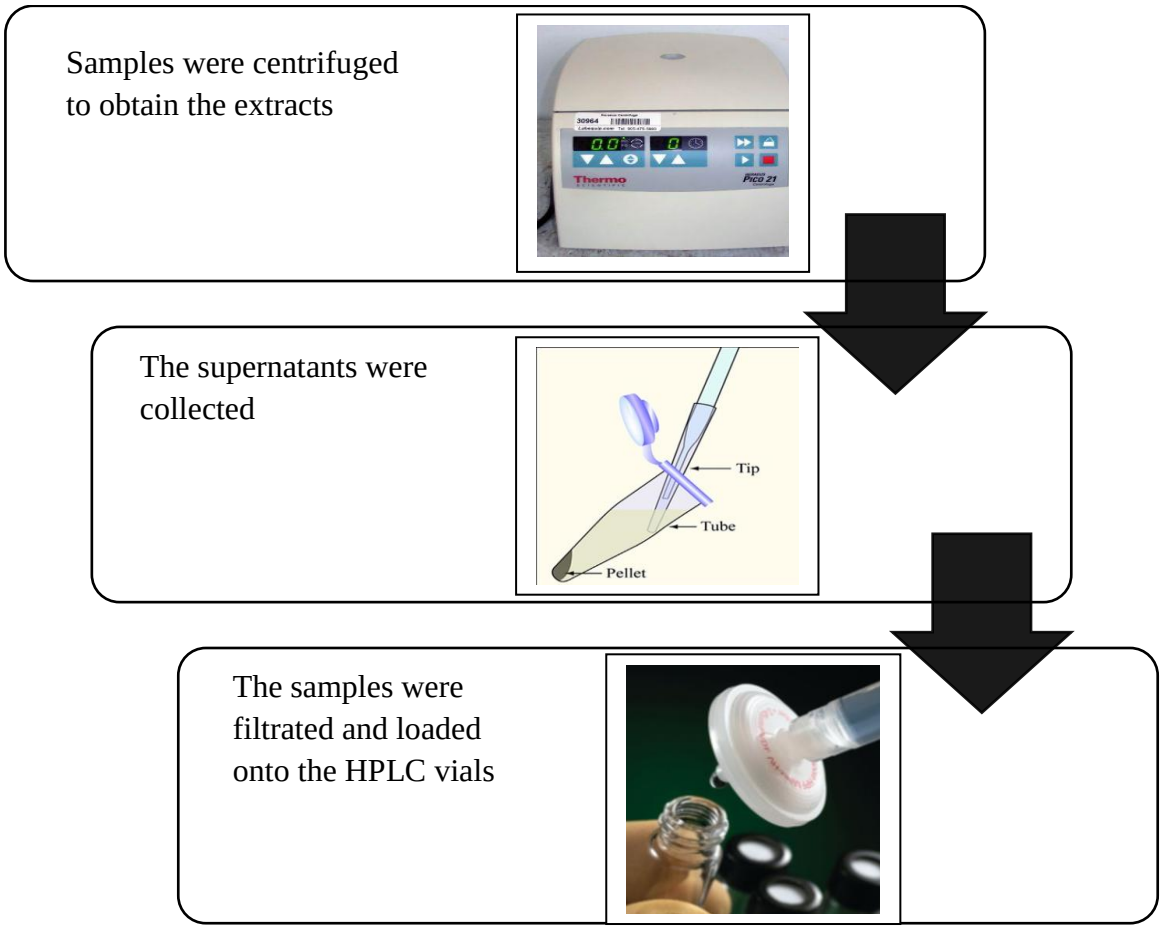

Figure 4.1: Sample preparations for HPLC analysis

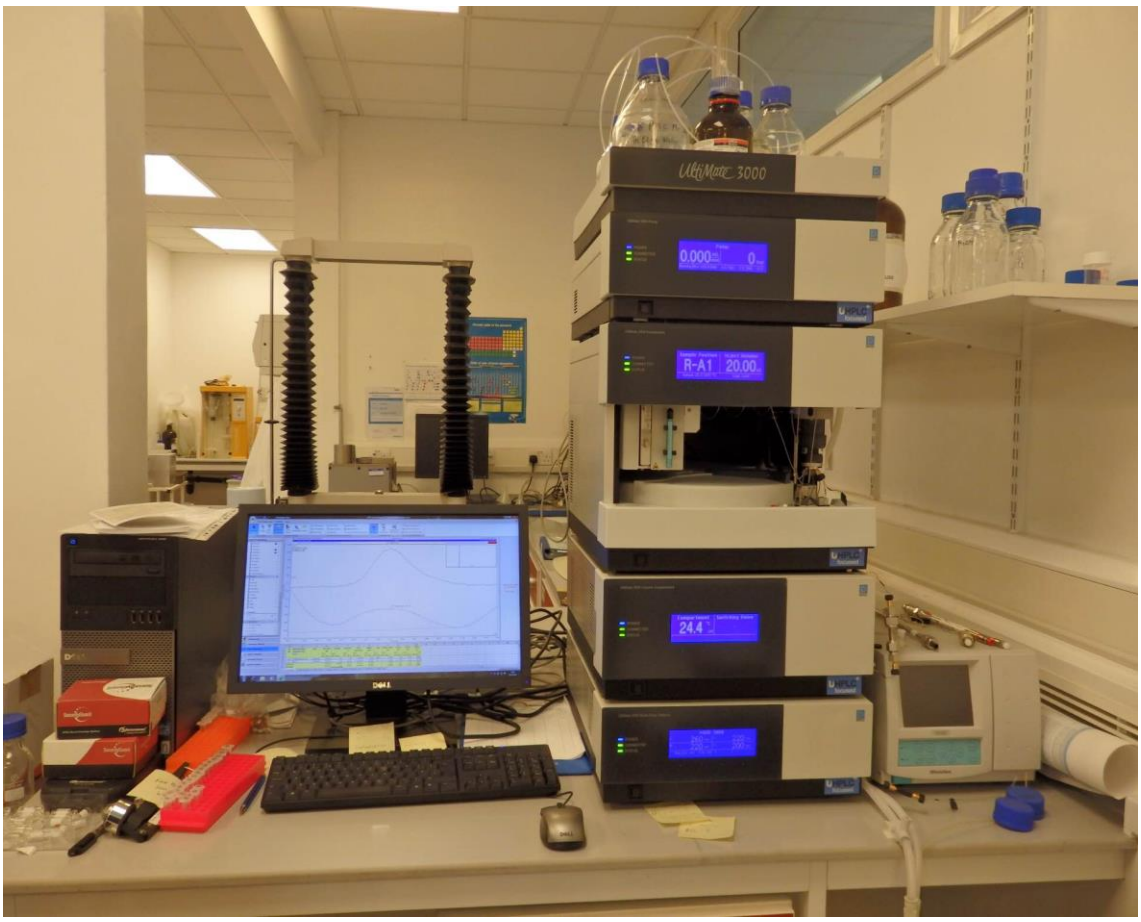

Figure 4.2 HPLC systems

4.8 Statistical analyses

Statistical analyses were carried out using the STATGRAPHICS Centurion (Version XVI, Statpoint Technologies, Inc., USA). The data were tested for homogeneity of variance (Bartlett test) and normality (Shapiro Wilk test) before subjected to one- or two- factor analyses of variance (ANOVA). Differences between individual means were determined by post hoc Tukey's test; significance was established at $P < 0.05$ for this procedure.

4.9 Results

4.9.1 The effects of DIU, TBA and IPU on H_2O_2 and lipid peroxidation levels in LIA4 and Es524

Exposure to diuron (DIU) (**Figure 4.3**) has induced the production of H_2O_2 in both strains of *E. siliculosus.* The mean H_2O_2 content of Es524 was not significantly affected by 1 -50 µg L^{-1} DIU, but at 100 and 500 µg L^{-1} the mean H_2O_2 have increased significantly by 119 and 179% respectively as compared to the carrier control. LIA4 was less tolerant to DIU with significant increments of H_2O_2 were recorded at lower concentrations of 10 and 50 µg L^{-1} with 89 and 105% respectively. The higher levels of H_2O_2 observed in LIA4 may signify higher sensitivity of the strain to DIU compared to Es524. Significant difference between the strains (S×T, $P<0.05$) was observed at 10 µg L^{-1}, with significantly higher level of H_2O_2 in LIA4 compared to Es524. At 100 and 500 µg L^{-1}, significant increases of H_2O_2 were observed in both strains probably due to excessive productions beyond the capacity of the strains to neutralize it.

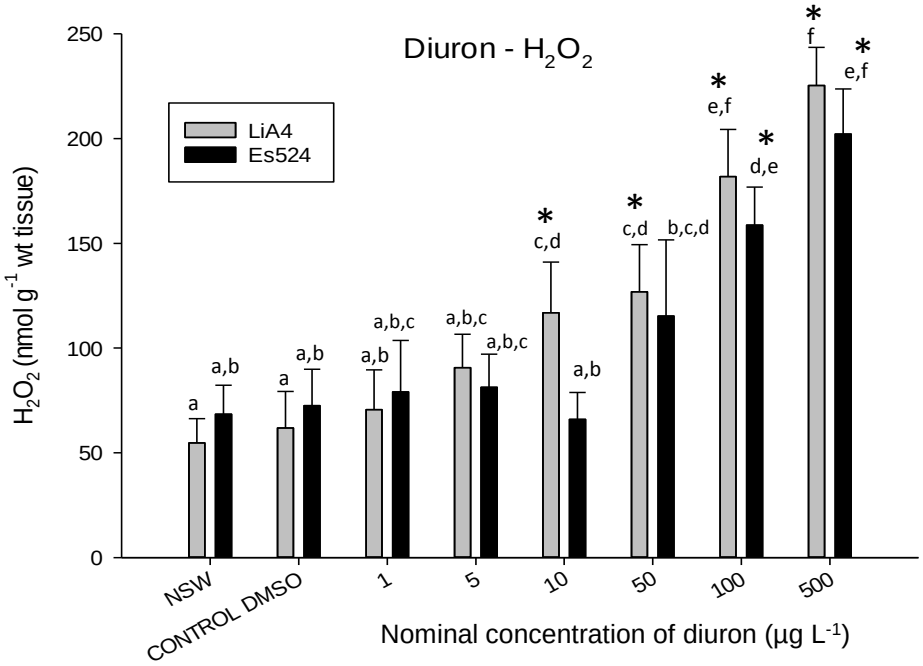

Figure 4.3: The effects of DIU on H_2O_2 levels in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P< 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

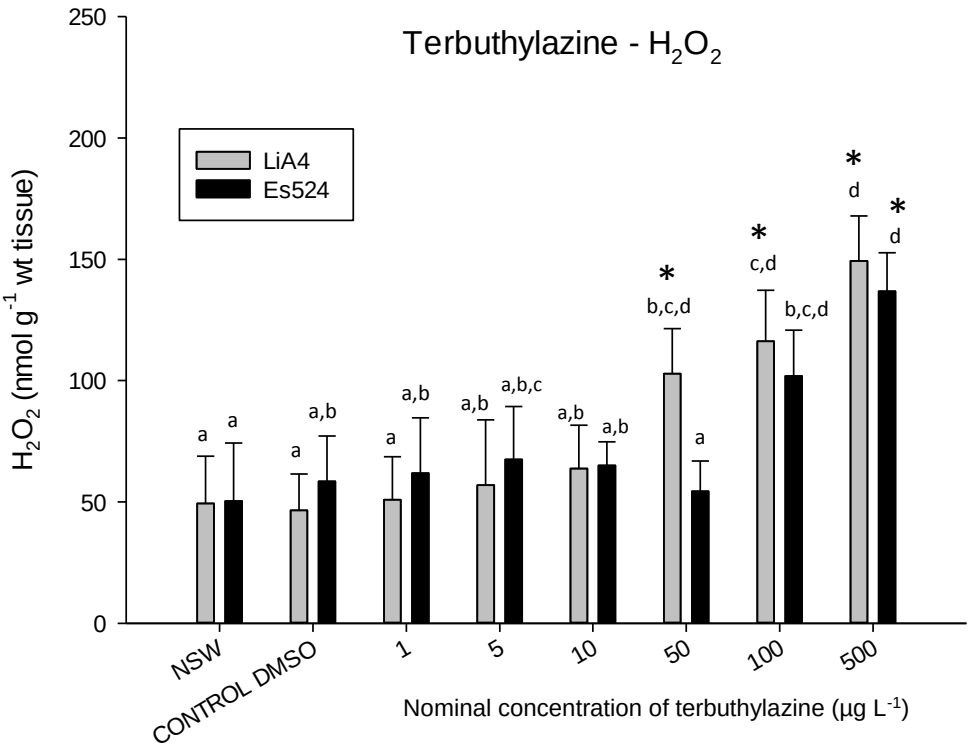

Figure 4.4: The effects of TBA on H_2O_2 levels in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

For TBA exposure (**Figure 4.4**) almost similar observations to DIU were recorded but at different levels of the herbicides treatments. H_2O_2 content of Es524 was not significantly changed up to 100 µg L^{-1} of TBA, but has increased significantly at 500 µg L^{-1} by 134% as compared to the carrier control. LIA4 was found to be more sensitive to TBA, with significant increments of H_2O_2 content as much as 121 and 150% were recorded at lower concentrations of 50 and 100 µg L^{-1} respectively. Significant difference between the strains (S×T, $P<0.05$) was observed at 50 µg L^{-1}, where higher H_2O_2 level was observed in LIA4 compared to Es524. At 500 µg L^{-1}, significant increases of H_2O_2 contents were observed in both strains, while no significant change was observed between 1-10 µg L^{-1} indicating the homeostasis conditions in both strains within that range.

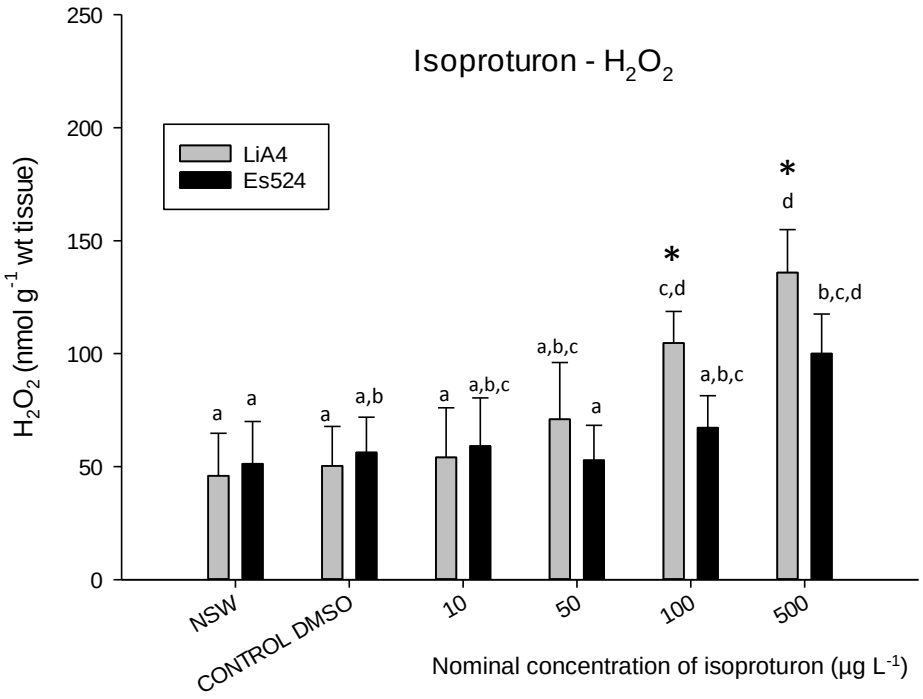

Figure 4.5: The effects of IPU on H_2O_2 levels in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

Figure 4.5 clearly indicates that H_2O_2 content in Es524 was not significantly affected within the IPU concentrations tested. LIA4 was more sensitive to IPU compared to Es524, with significant increments of H_2O_2 were observed at 100 and 500 µg L^{-1}, with 108 and 170% respectively. The non-significant change of H_2O_2 levels in Es524 strain could be due to the well balanced of homeostasis mechanisms within the strain.

Figure 4.6 shows the increase of TBARS levels in response to DIU exposure. In LIA4, significant increments were observed at 10 µg L^{-1} and above, in contrast to Es524 which occurs only at 100 and 500 µg L^{-1}. At 10 and 50 µg L^{-1} of DIU, the TBARS levels, compared to the carrier controls, increased significantly by 64–95% in LIA4, but only slight changes by 6–23% in Es524, which indicate higher tolerance of Es524 strain compared to LIA4. Significant difference between the strains (S×T, $P<0.05$) was recorded at 50 µg L^{-1}, with significantly higher level of TBARS in LIA4 compared to Es524, while exposure to 100 and 500 µg L^{-1} of DIU have shown significant increments of TBARS level in both strains probably due to the damage to the lipid membranes.

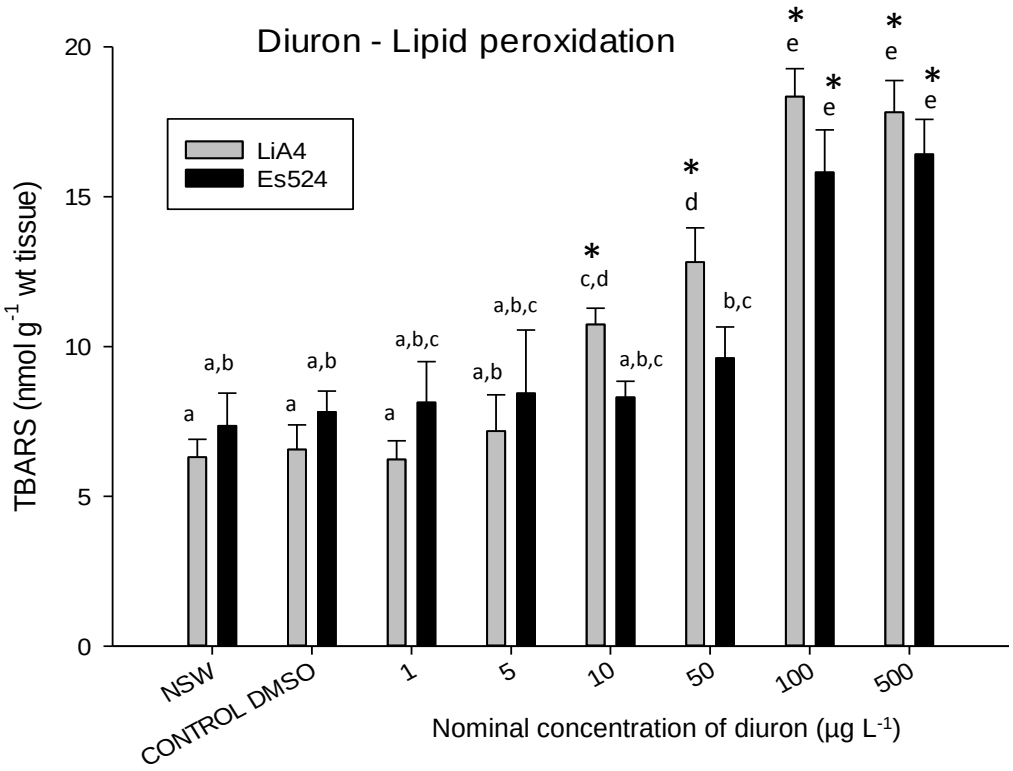

Figure 4.6: The effects of DIU on lipid peroxidation in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P< 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

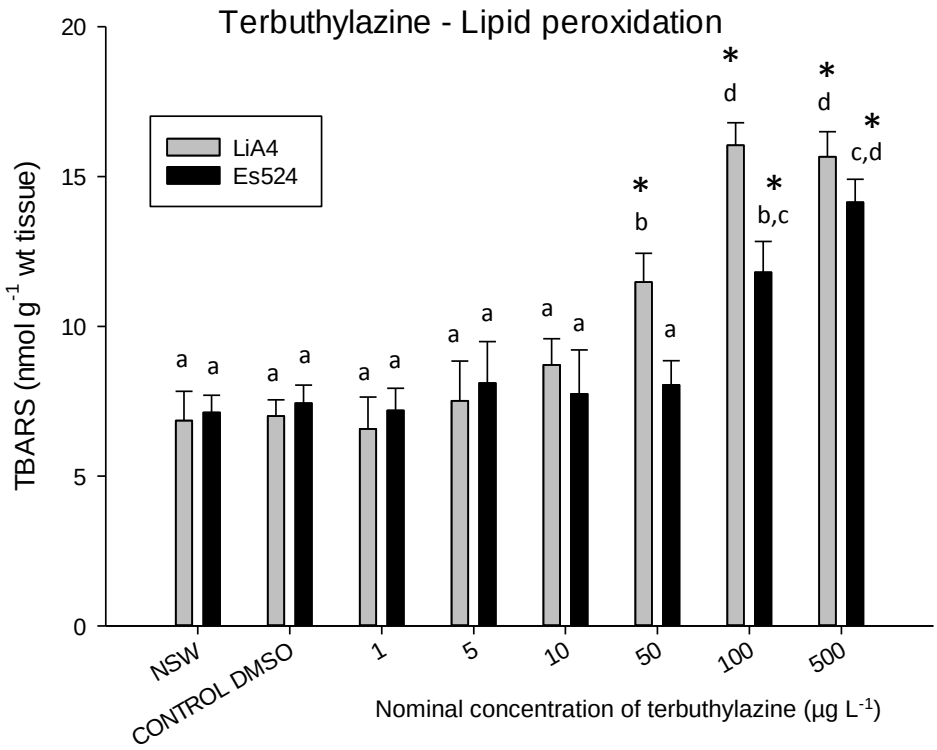

Figure 4.7: The effects of TBA on lipid peroxidation in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

Exposure to TBA for 7 d has produced different TBARS levels in LIA4 and Es524 (**Figure 4.7**). Significant increases of TBARS levels were observed in LIA4 at 50 µg L^{-1} and above, while in Es524 at 100 and 500 µg L^{-1}. At 50 and 100 µg L^{-1}, significant differences between the strains (S×T, $P<0.05$) were observed, with higher TBARS levels were recorded in LIA4 with 64 and 129% increments respectively, compared to Es524 (8.2 and 59%), which indicate more sensitive of LIA4 strain to TBA.

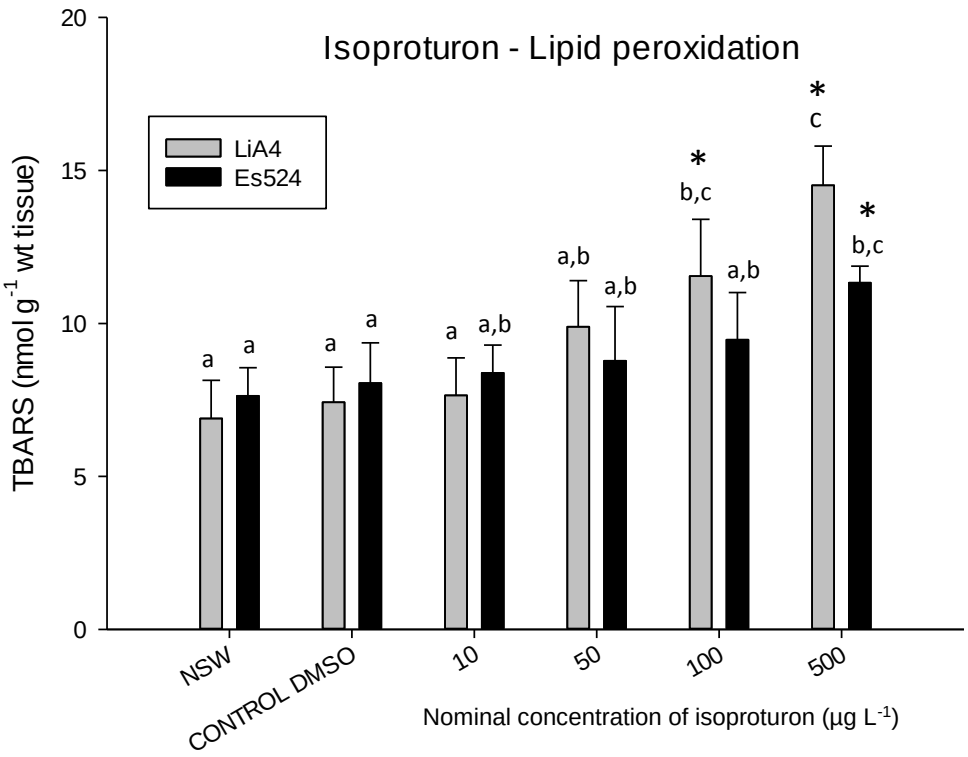

Figure 4.8: The effects of IPU on lipid peroxidation in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

For IPU (**Figure 4.8**), significant increases of TBARS levels were observed at 100 and 500 µg L^{-1} for LIA4 and at 500 µg L^{-1} for Es524. No significant change of TBARS level was observed up to 100 µg L^{-1} in Es524 which indicate better tolerant to IPU compared to LIA4. At the highest tested concentration (500 µg L^{-1}) of IPU, TBARS levels were significantly increased in both strains, although to lesser extent in Es524.

In relation to the production of reactive oxygen species (ROS), plants/algae were equipped with the antioxidative defense mechanisms, which will enable it to maintain the homeostasis conditions. Thus, evaluations on several antioxidative enzymes have been carried out in order to elucidate the mechanisms deployed by both strains to overcome the herbicides stresses.

4.9.2 Effects of DIU, TBA and IPU on different **antioxidant enzymes** in LIA4 and Es524

Exposure to DIU (1-500 µg L^{-1}) (**Figure 4.9**) has shown poor stimulation and to certain level suppression of the CAT activities in both strains of *E. siliculosus*. No significant difference (S×T, P>0.05) was observed between the strains, although Es524 seemed to possess higher level of CAT compared to LIA4. Significant change as compared to the carrier control was recorded only at the highest concentrations tested (500 µg L^{-1}) with 65 and 54% reduction in LIA4 and Es524 respectively, probably due to the damage of the enzyme protein.

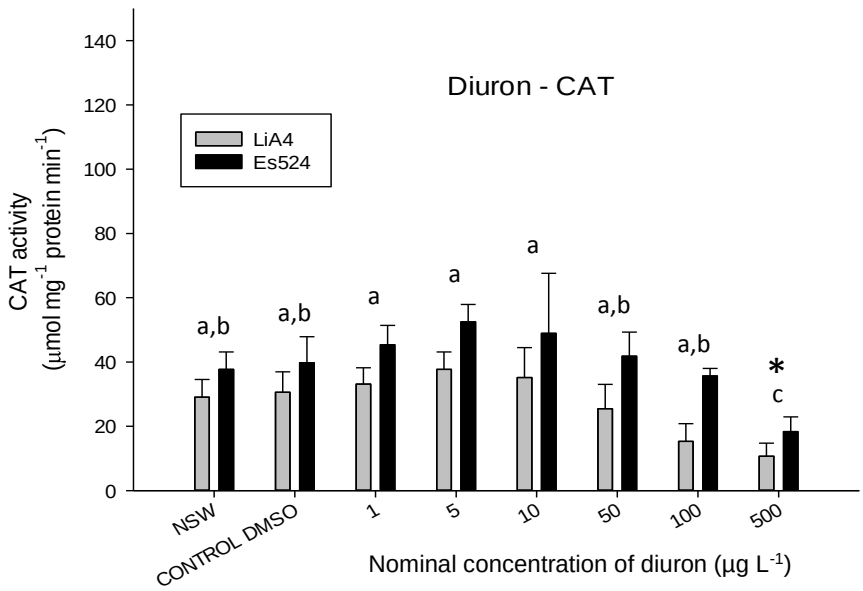

Figure 4.9: The effects of DIU on CAT activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

Figure 4.10 below has shown that CAT activity in both strains was slightly stimulated in response to TBA. Significant increase compared to carrier control was recorded only at 10 µg L^{-1} with 73 and 59% in LIA4 and Es524 respectively. At 5 and 50 µg L^{-1}, slight increments of CAT activities were observed in both strains, but it was not significantly different compared to the carrier control. No significant difference was observed between the strains (S×T, P>0.05) which indicates LIA4 and Es524 have shown similar response of CAT activities within the concentrations of TBA tested.

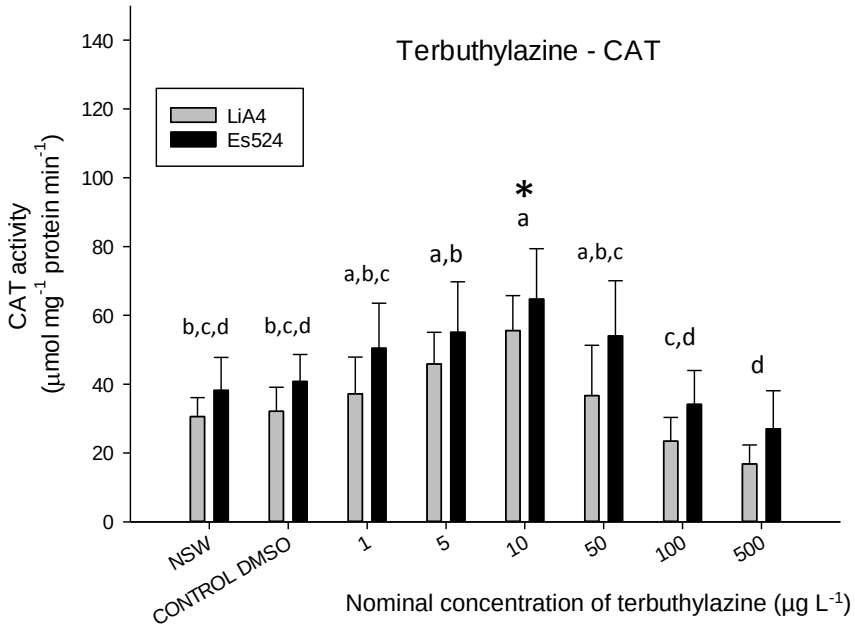

Figure 4.10: The effects of TBA on CAT activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

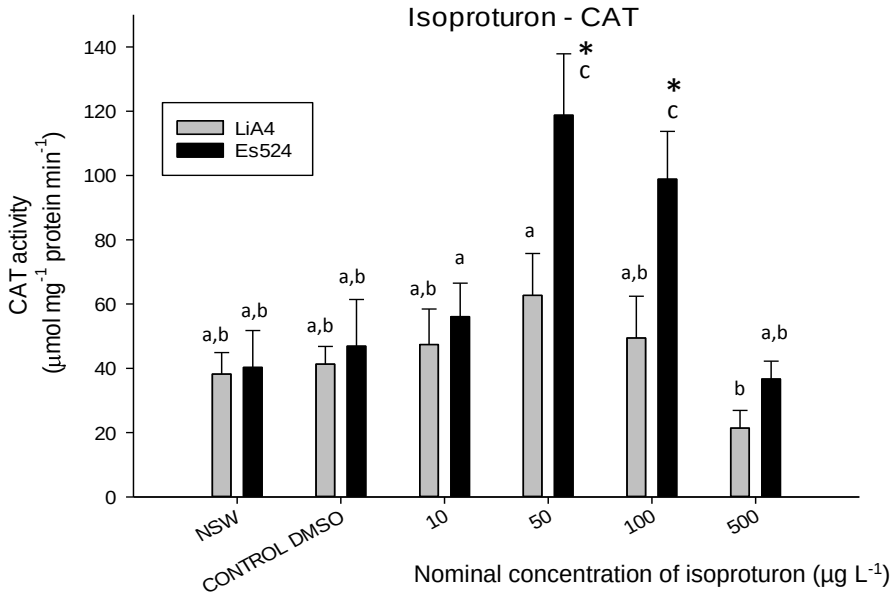

Figure 4.11: The effects of IPU on CAT activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

Exposure to IPU induced strong stimulation of CAT activity in the Es524 strain (**Figure 4.11**). On the other hand, only weak stimulation was demonstrated in LIA4. At 50 and 100 µg L^{-1}, CAT activities in Es524 were significantly higher (153 and 111% increase respectively) compared to LIA4 (52 and 19% increase respectively). The significantly higher levels of CAT stimulated in Es524 compared to LIA4 could be one of the factors that lead to its higher resistance to IPU. At 500 µg L^{-1}, CAT activity in both strains decreased, probably due to suppression of the enzyme activity.

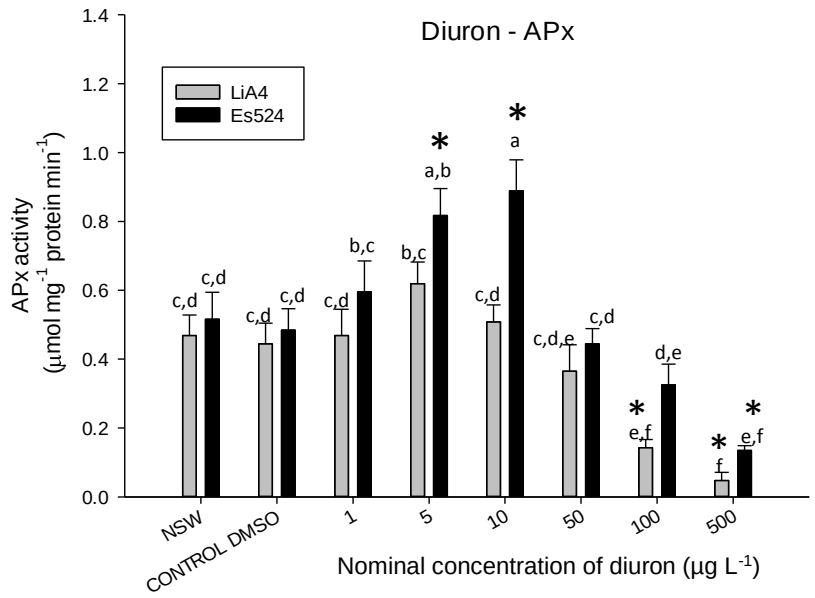

Figure 4.12: The effects of DIU on APX activity in two strains (Es524 and LiA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

Figure 4.12 shows that exposure to DIU has caused stimulation of APX activity up to 10 µg L^{-1}, before declined. Significant stimulations up to 69 and 83% were observed in Es524 at 5 and 10 µg L^{-1} respectively, while in LIA4, only slight inductions of 39 and 14% (not significant) were recorded at corresponding treatments. Significant difference (S×T, $P<0.05$) between the strains was observed at 10 µg L^{-1}, with higher APX activity was recorded in Es524 compared to LIA4. This might be one of the factors contributing to lower levels of H_2O_2 observed in Es524. At 50 µg L^{-1} and above, APX activities in both strains have reduced. Significant reductions as compared to the carrier control were observed at 100 and 500 µg L^{-1} in LIA4, and at 500 µg L^{-1} in Es524, with 68, 89 and 72%, respectively.

In contrast to CAT, strong induction of APX activity was observed in Es524 strain in response to TBA (**Figure 4.13**). Significant stimulations of APX activities were observed at 10 and 50 µg L^{-1} with 79 and 107%, respectively, in Es524 strain, while only 42 and 15% (not a significant increment) in LIA4 at corresponding treatments. Significant difference between the strains (S×T, $P<0.05$) was observed at 50 µg L^{-1}, where APX activity in Es524 was still showing increase while in LIA4 it started to decline. At 100 µg L^{-1} and above, APX activities in both strains of *E. siliculosus* have declined, probably due to suppression of the antioxidative enzyme.

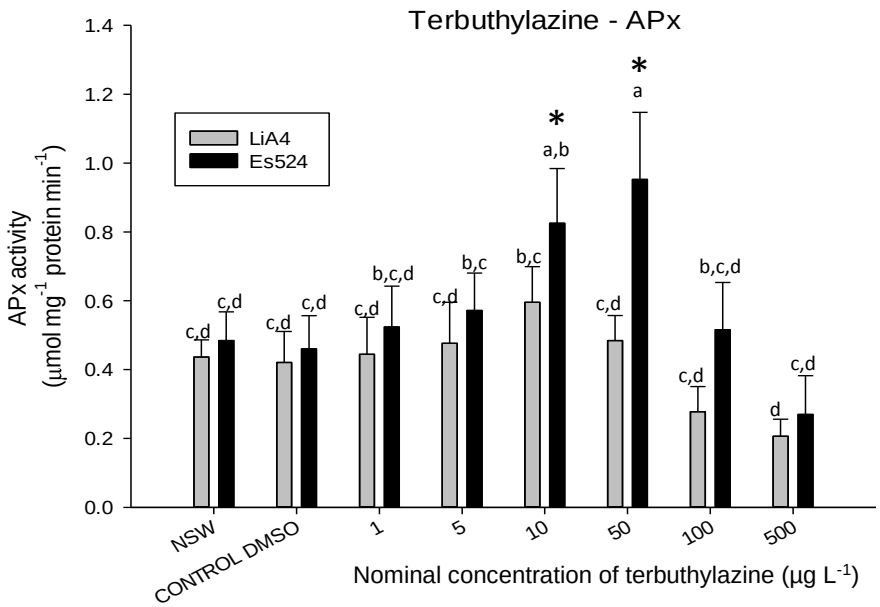

Figure 4.13: The effects of TBA on APX activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P< 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

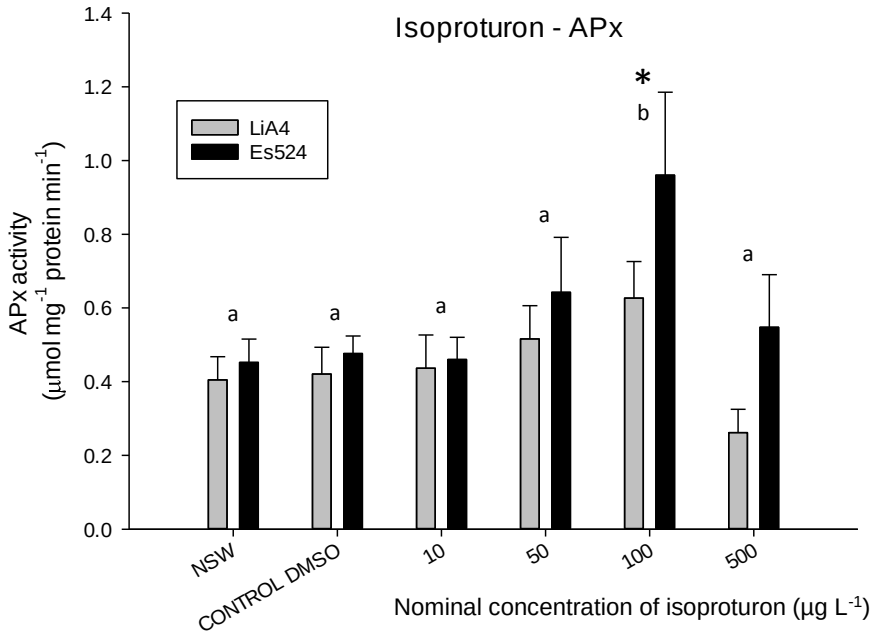

Figure 4.14: The effects of IPU on APX activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

For IPU treatments, APX activity was found to increase in both strains up to 100 µg L^{-1}, before declined (**Figure 4.14)**. Significant change of APX activity was recorded only at 100 µg L^{-1}, with 49 and 101% increase was recorded in LIA4 and Es524 respectively. No significant difference was observed between the strains (S×T, P>0.05), although higher levels of APX activity was recorded in Es524 compared to LIA4. Exposure to the highest IPU concentrations (500 µg L^{-1}), led to slight induction of APX activity in Es524 (15%), but diminution by 38% in LIA4, indicating higher tolerance of Es524 to IPU.

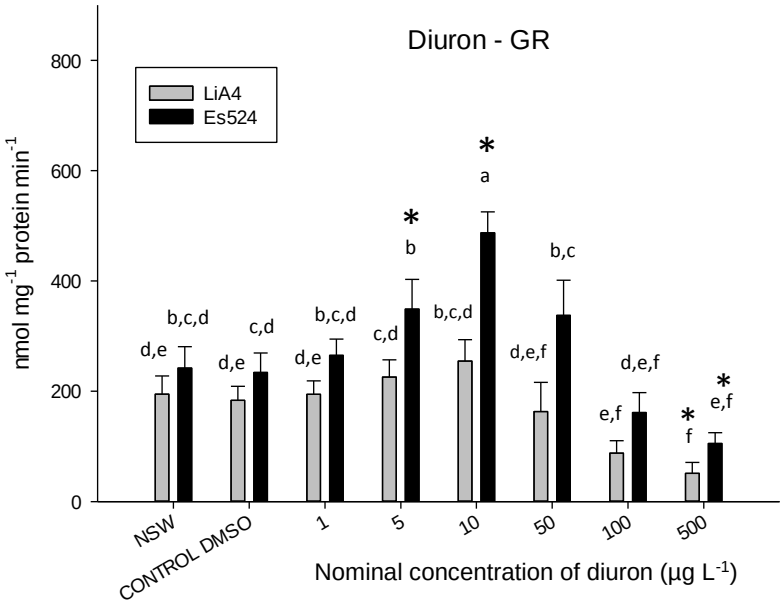

Figure 4.15: The effects of DIU on GR activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

A concentration-dependent increase of GR activity was observed in both strains up to 10 µg L^{-1} of DIU, before declining (**Figure 4.15**). Significant stimulations of GR activities have been recorded in Es524 at 5 and 10 µg L^{-1} with 49 and 108% increment respectively compared to the carrier control. On the other hand, only slight stimulations of 23 and 39% (not significant) were recorded in LIA4 at corresponding treatments, which may justify less tolerant of LIA4 to DIU compared to Es524. Significant differences between the strains (S×T, $P<0.05$) were observed at 5, 10 and 50 µg L^{-1}, with higher levels of GR activities displayed in Es524 compared to LIA4. At 100 µg L^{-1} and above, GR activity in both strains decreased, with significant reductions of as much as 72 and 55% observed at 500 µg L^{-1} in LIA4 and Es524, respectively.

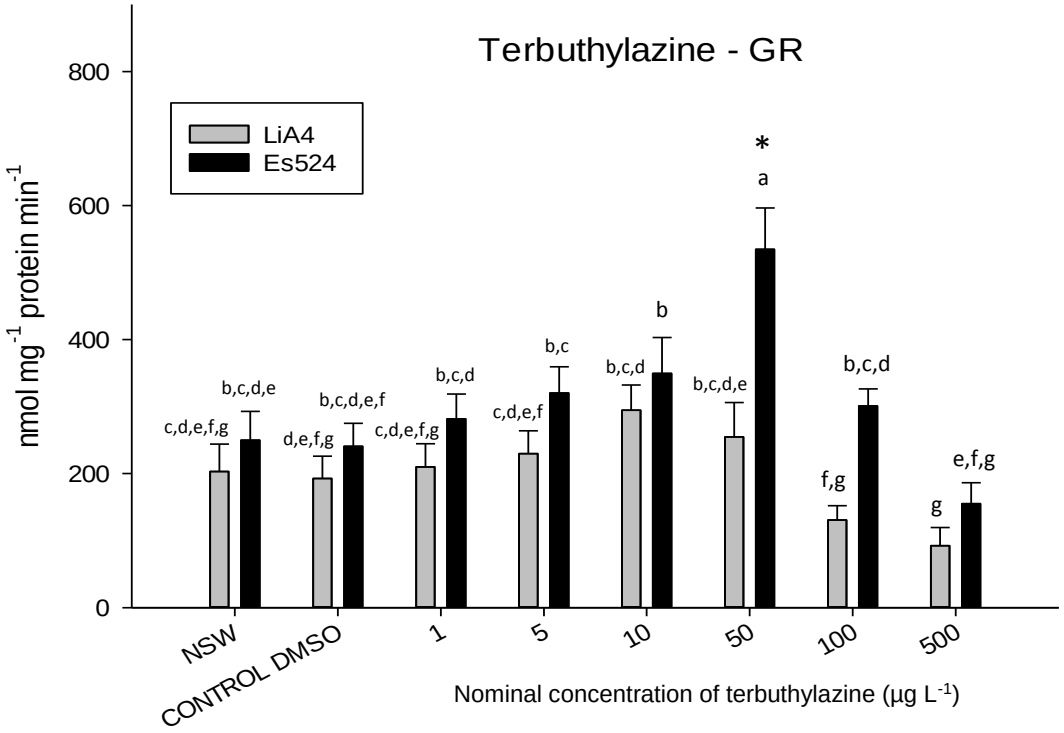

Figure 4.16: The effects of TBA on GR activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

For TBA exposure (**Figure 4.16**), significant stimulation of GR activity was observed only at 50 µg L^{-1} in Es524 strain, with 122% increment. On the other hand, no significant change was observed in LIA4, with maximum GR activity observed 10 µg L^{-1} with 53% increment. Significant differences between the strains (S×T, $P<0.05$) were observed at 50 and 100 µg L^{-1} with 122 and 25% increases in Es524 respectively, while only 32% induction and 32% diminution in LIA4 at corresponding treatments, which indicate higher levels of GR activities displayed in Es524 strain compared to LIA4. The decrease of the GR activity in both strains at the highest concentrations tested (500 µg L^{-1}) might indicate damage to the enzyme proteins.

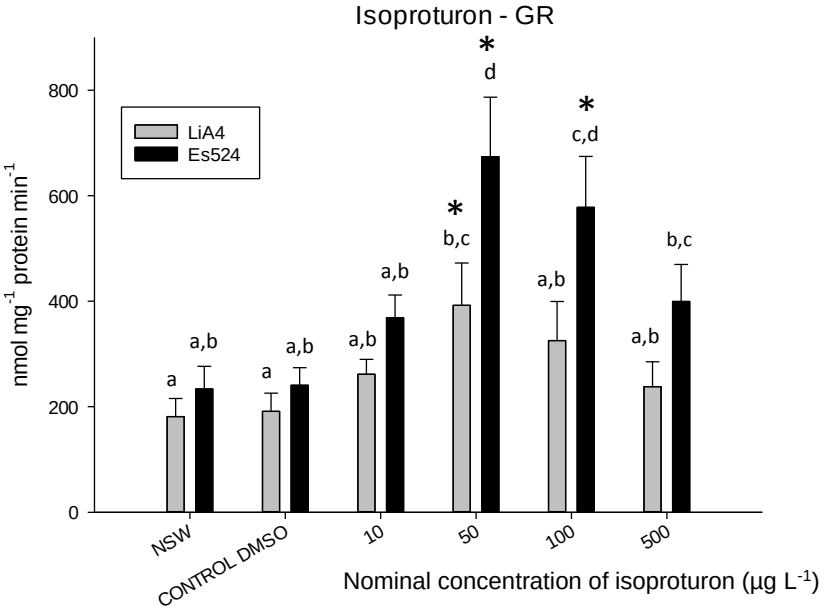

Figure 4.17: The effects of IPU on GR activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

Exposure to IPU between 10–500 µg L^{-1}, increased GR activity in both strains compared to the carrier control (**Figure 4.17**). However, significant increases were observed at 50 and 100 µg L^{-1} in the Es524 strain, and at 50 µg L^{-1} in LIA4. At 50 and 100 µg L^{-1}, significant differences between the strains (S×T, $P<0.05$) were observed, with 180 and 140% increases were recorded in Es524 respectively, while 105 and 70% in LIA4. At 500 µg L^{-1}, slight stimulations of GR activities were still exhibited in both strains, with 66 and 24% increment in Es524 and LIA4 respectively, but was not significantly different compared to the carrier control. The higher GR activity displayed in Es524 could contribute to its higher tolerance to IPU compared to LIA4.

4.9.3 Total phenolic content (TPC) and DPPH scavenging activity of LIA4 and Es524 in response to DIU, TBA and IPU exposure

Exposure to DIU has induced a concentration dependent increase of total phenolic content (TPC) in both strains up to 10 µg L^{-1}, before declined (**Figure 4.18**). Compared to the carrier control, total phenolic content has increased between 1 – 50 µg L^{-1} in Es524 and 1 -10 µg L^{-1} in LIA4. However, significant increases as much as 49 and 28.5% were observed only in Es524 at 10 and 50 µg L^{-1} respectively, which was significantly higher compared to LIA4, with a 31% increase and 16.4% decrease for corresponding treatments. This result indicates higher level of TPC in Es524 compared to LIA4 in response to DIU.

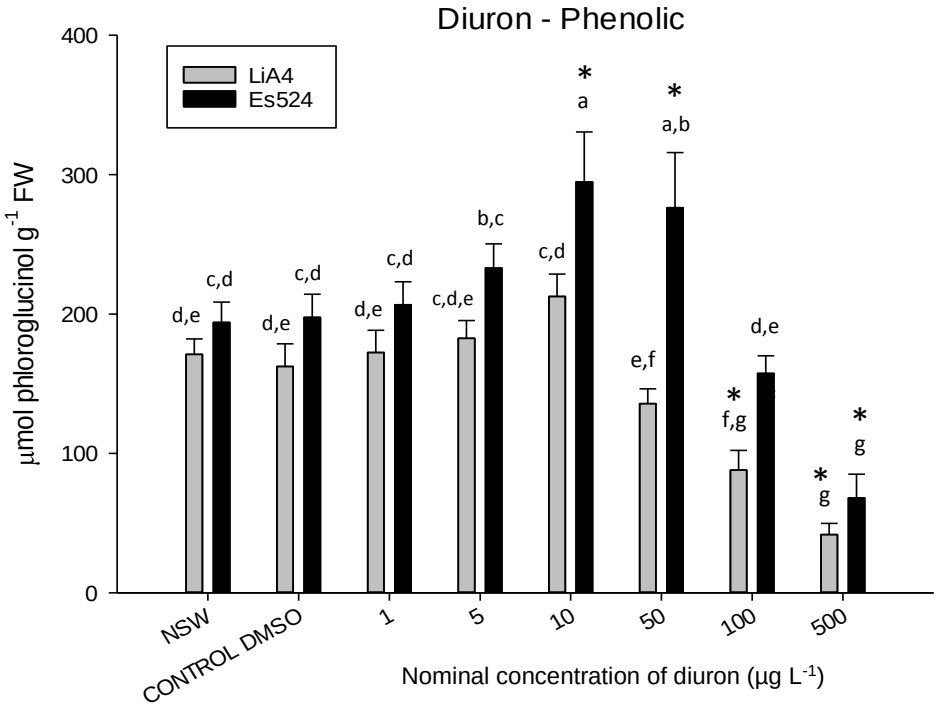

Figure 4.18: The effects of DIU on total phenolic content (TPC) in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$)**.** NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

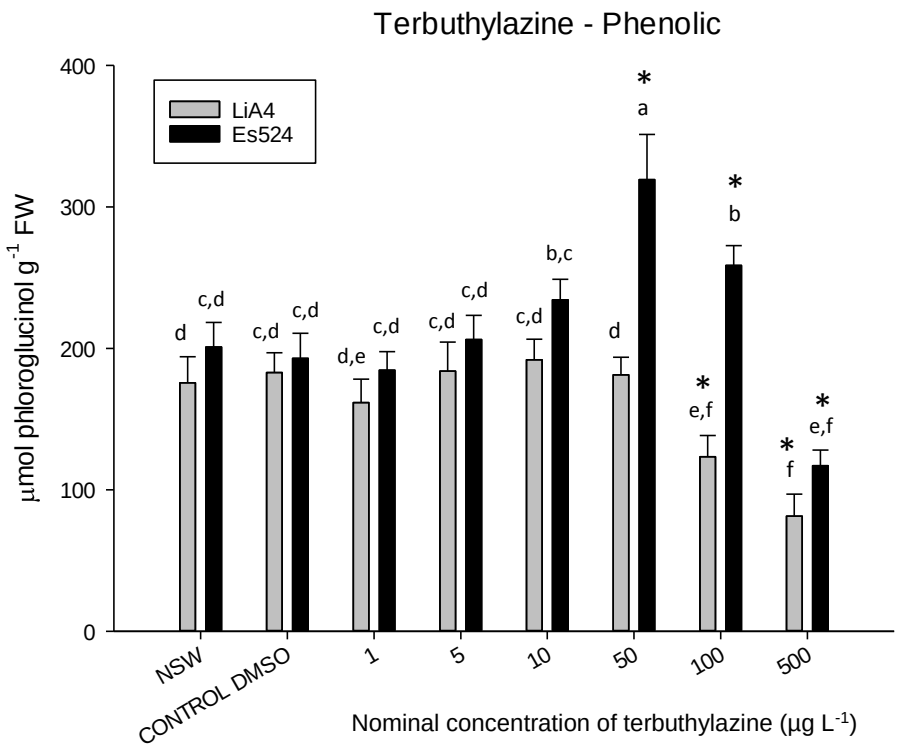

Figure 4.19: The effects of TBA on total phenolic content (TPC) in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

Figure 4.19 shows the distinct response of total phenolic content (TPC) between LIA4 and Es524 to TBA treatments. At 50 and 100 µg L^{-1}, the TPC were significantly increased in Es524 by 65.5 and 34% respectively, which was significantly higher compared to LIA4. On the other hand, no significant change was recorded in LIA4 up to 50 µg L^{-1} of TBA. The higher TPC observed in Es524 at 50 and 100 µg L^{-1} could be one of the factors that lead to higher resistance towards TBA compared to LIA4. Significant reductions of TPC in response to TBA were observed at 100 and 500 µg L^{-1} for LIA4 and at 500 µg L^{-1} for Es524.

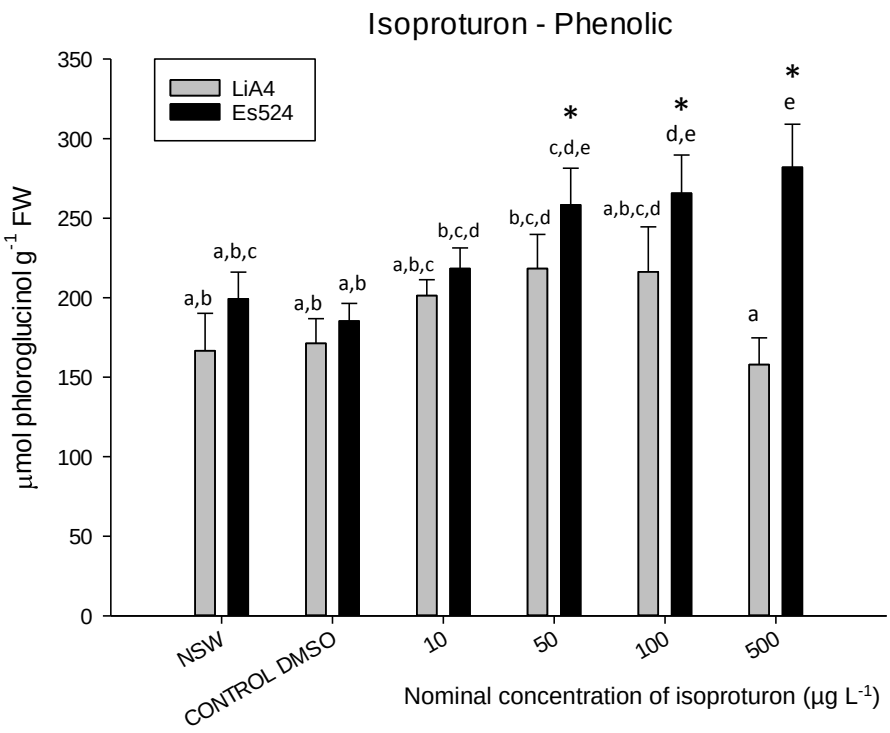

Figure 4.20: The effects of IPU on total phenolic content (TPC) in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

A concentration-dependant increase of TPC was observed in both strains of *E. siliculosus* in response to IPU exposure (**Figure 4.20**). No significant change was recorded in LIA4, in contrast to Es524 where significant increases of TPC were observed at 50 µg L^{-1} and above. At 500 µg L^{-1}, a significant difference between the strains (S×T, $P<0.05$) was observed, with a 52.2% increase recorded in Es524, and a 7.8% reduction in LIA4, which probably contributed to higher tolerance of Es524 to IPU.

Antioxidant capacity (AAC) of LIA4 and Es524 strains was measured as DPPH scavenging activity. Exposure to DIU has caused increase of DPPH scavenging activity in both strains up to 10 µg L^{-1}, before declined. Compared to the carrier control, significant increase of DPPH scavenging activity was observed at 10 µg L^{-1} in Es524. On the contrary, no significant change was observed in LIA4 up to 50 µg L^{-1} of DIU. At 10 and 50 µg L^{-1} of DIU, significant differences between the strains were recorded (S×T, $P<0.05$) with 90 and 42.6% increases in Es524 respectively, while 31.7% increase and 13% decrease in LIA4 at corresponding treatments. The higher DPPH scavenging activity observed in Es524 indicates better capacity of the strain to cope with adverse conditions that may lead to generation of reactive oxygen species.

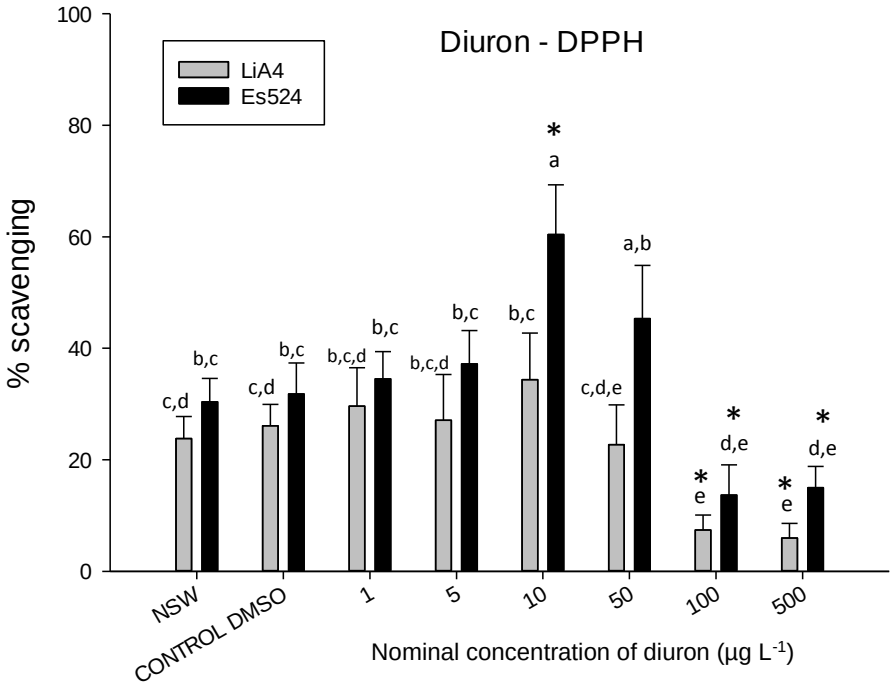

Figure 4.21: The effects of DIU on DPPH scavenging activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P< 0.05$)**.** NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

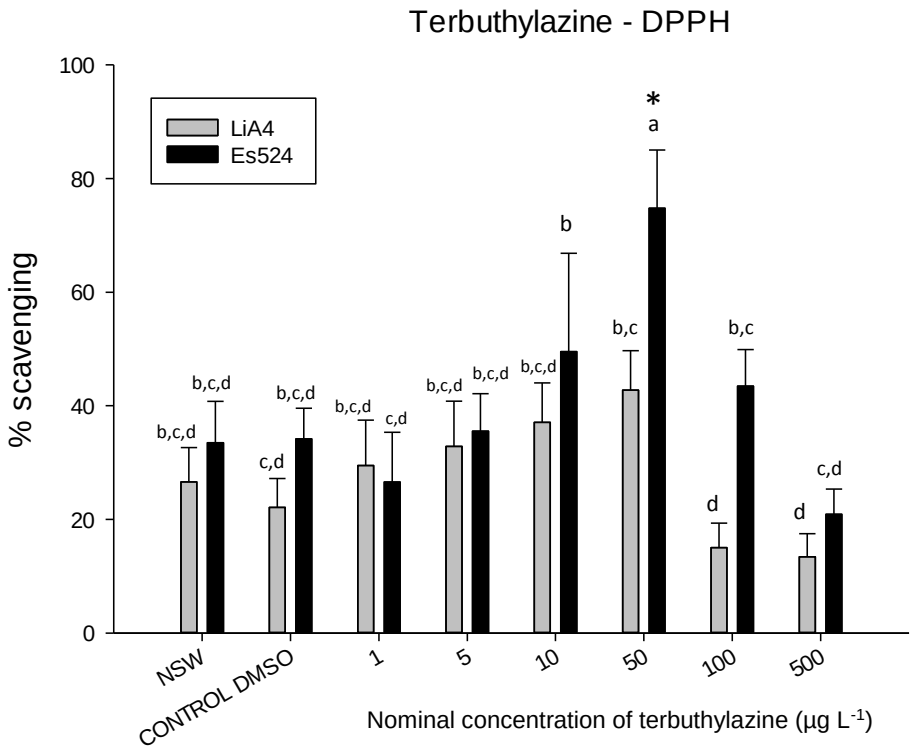

Figure 4.22: The effects of TBA on DPPH scavenging activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$)**.** NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

Figure 4.22 above shows a concentration dependent increase of DPPH scavenging activity in both strains of *E. siliculosus* up to 50 µg L^{-1} of TBA, before declined. Significant increase as compared to the carrier control was observed in Es524 at 50 µg L^{-1}, while no significant change was recorded in LIA4, although DPPH activities have increases by 48-93% between 5 to 50 µg L^{-1} of TBA. At 50 and 100 µg L^{-1}, DPPH scavenging activity in Es524 was significantly higher compared to LIA4 ($P<0.05$), with 119 and 27.2% increases recorded for Es524, respectively. On the other hand, 93% increase and 32% reduction were displayed in LIA4 at corresponding treatments.

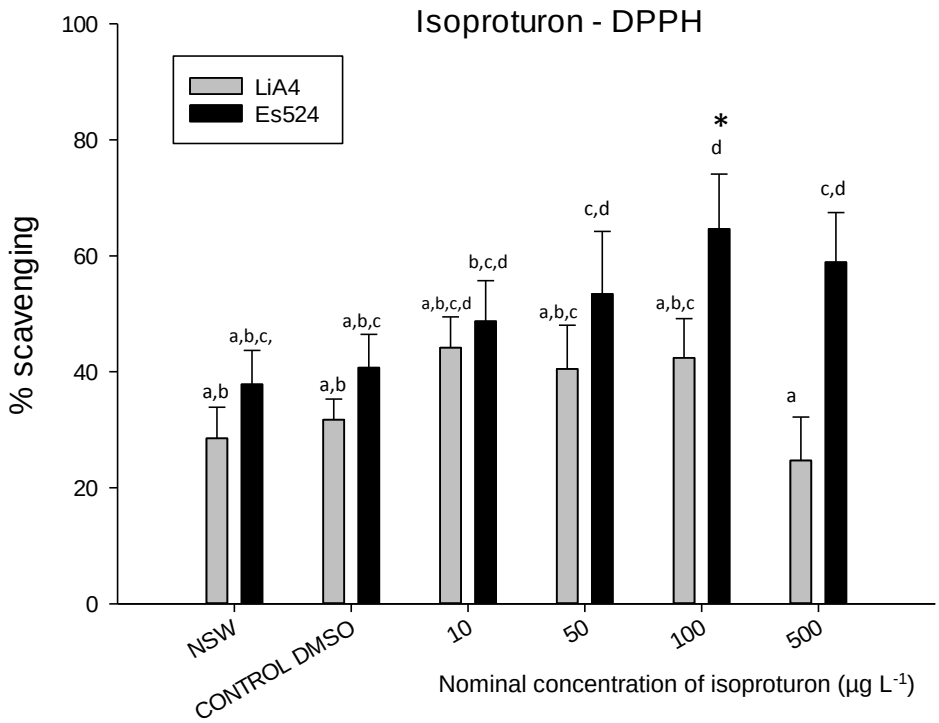

Figure 4.23: The effects of IPU on DPPH scavenging activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$). NSW= Natural seawater, DMSO concentration = 0.05 ml/L.

For IPU, 7 d of exposure on both strains of *E. siliculosus* caused an increase of DPPH scavenging activity (**Figure 4.23**). Compared to the carrier control, a significant increase of DPPH scavenging activity was observed at 100 µg L^{-1} in Es524, while no significant change was found in LIA4 within the IPU concentrations tested. At 100 and 500 µg L^{-1}, DPPH scavenging activity in LIA4 was significantly lower (33.6% increase, 22.2% decrease respectively) compared to Es524 (58.8%, 44.7% increased respectively), indicating the higher susceptibility of the LIA4 strain to IPU.

4.9.4 **Polyphenols** response in *E. siliculosus* strains to DIU, TBA and IPU exposure

High performance liquid chromatography (HPLC) analysis on both strains clearly indicated the presence of polyphenols such as caffeic acid, phloroglucinol and rutin in *E. siliculosus*. The identification of polyphenol compounds was based on the polyphenols mix standard that shown in **Figure 4.24**. **Table 4.1** indicates the standards mix been identified from the mobile phase used, while polyphenol compounds identified from *E. siliculosus* are shown in grey coloured box. There were other unidentified compounds found in the extracts by HPLC in addition to the standard compounds shown in Figure 4.22. Hence, the total of individual polyphenol compounds do not represent the total phenolic and total flavonoids in the extracts. Analysis of the polyphenols content, results presented in **Table 4.2 - 4.4** show various changes in *E. siliculosus* strains treated with the different herbicides. It seems likely that these non-enzymatic radical scavengers are an effective defense mechanisms, but are an inadequate solution to extreme herbicides toxicity.

Table 4.1 Detection of standard polyphenol compounds

Retention time (min)	Polyphenols
3.240	Hydroxybenzoic acid
6.727	Phloroglucinol
8.403	Chlorogenic acid
9.450	Esculetin
12.597	Catechin
13.000	Vanillic acid
14.053	Syringic acid
14.417	Caffeic acid
14.777	Rutin
16.147	Salicylic acid
16.907	Quercetin
17.47	Kaempferol

*Grey box = Polyphenols identified in *E. siliculosus*

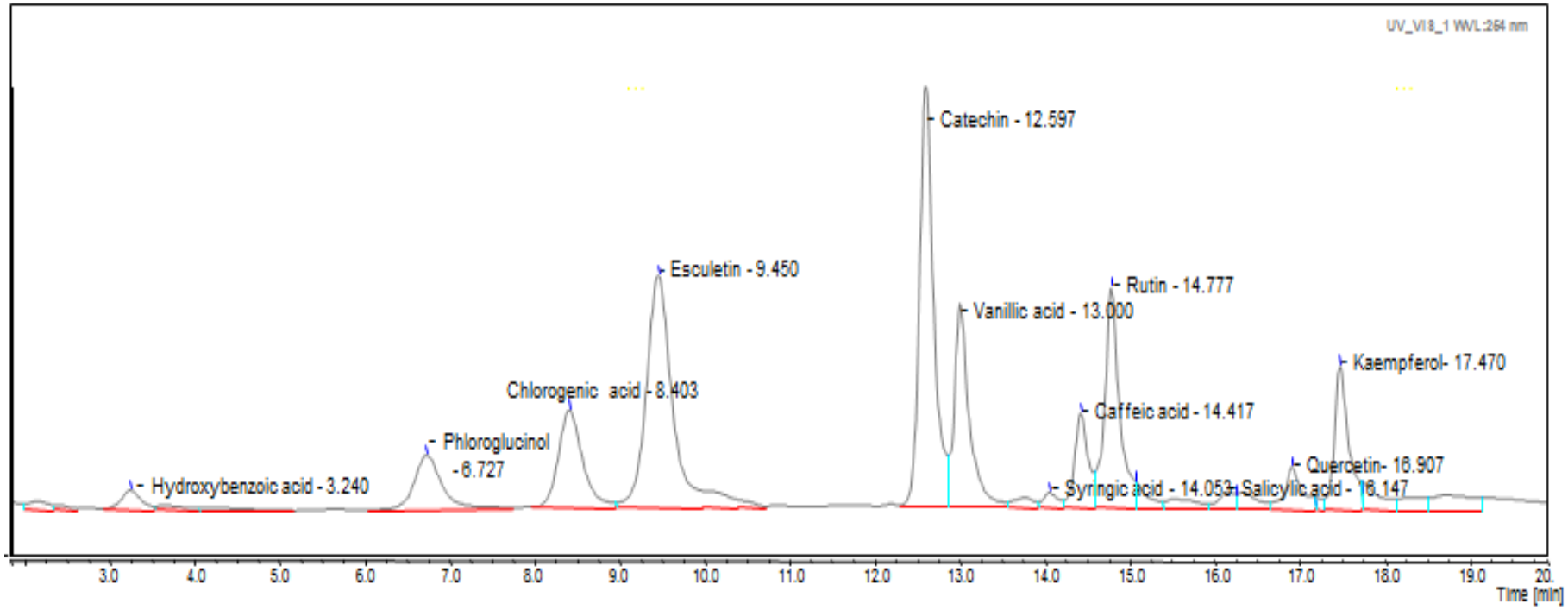

Figure 4.24 HPLC analysis of mixed polyphenol standards.

Table 4.2 shows exposure to DIU up to 10 µg L^{-1} has no significant effect on the amount of rutin in both strains. However, at 50 µg L^{-1}, significant increase of rutin was observed in Es524, in contrast to LIA4 which has decreased significantly as compared to the carrier control. At 100 µg L^{-1} of DIU, rutin was significantly decreased and detected in trace amount in Es524 and LIA4, respectively, while at 500 µg L^{-1} the compound was not detected in both strains.

For phloroglucinol (PG), significant increase was observed in Es524 at 10 and 50 µg L^{-1} of DIU which were significantly higher compared to LIA4 at corresponding treatments. Significant reduction of PG as compared to the carrier control was observed at ≥ 100 and ≥ 50 µg L^{-1} of DIU in Es524 and LIA4 respectively. At 500 µg L^{-1}, a trace amount of PG was recorded for Es524, while it was not detected in LIA4.

Caffeic acid (CA) was the least compound able to be quantified in both strains. Exposure to DIU up to 10 µg L^{-1} showed no significant difference between the strains. However, at 50 µg L^{-1}, significant difference of CA was observed in Es524 compared to LIA4 which was detected in trace amounts. At 100 and 500 µg L^{-1} of DIU, CA was not detected in both strains.

Diuron (µg L^{-1})	Polyphenols (µg g^{-1} FW)					
	Rutin		Phloroglucinol		Caffeic acid	
	Es524	LIA4	Es524	LIA4	Es524	LIA4
NSW	114.6 ± 13.7^{bcd}	98.2 ± 13^{bcd}	344.2 ± 22.7^{cd}	317.5 ± 28.1^{de}	18.4 ± 2.6^{ab}	14.6 ± 3.3^{ab}
DMSO	122.5 ± 14.4^{bc}	104.7 ± 9.4^{bcd}	350.8 ± 25.4^{cd}	326.3 ± 31.4^{cde}	16.8 ± 4.3^{ab}	13.8 ± 2.9^{ab}
1	108.3 ± 15.9^{bcd}	89.4 ± 10.6^{cd}	333.7 ± 30.4^{cde}	309.6 ± 24.2^{de}	17.9 ± 3.5^{ab}	16.1 ± 3.1^{ab}
5	119.5 ± 18.1^{bc}	98.6 ± 12.3^{bcd}	376.1 ± 28.7^{bc}	330.2 ± 36.5^{cde}	20.6 ± 4.8^{a}	15.5 ± 3.7^{ab}
10	133.8 ± 17.5^{ab}	115.5 ± 14.3^{bcd}	412 ± 36.9^{b}	347 ± 28.2^{cd}	22.2 ± 3.5^{a}	16.9 ± 2.8^{ab}
50	153.7 ± 11.6^{a}	48.4 ± 19.2^{e}	483 ± 43.4^{a}	195.9 ± 30.4^{f}	11.7 ± 3.9^{b}	++ ^{c}
100	79.6 ± 8.7^{de}	++ ^{f}	286.7 ± 32.6^{e}	127.6 ± 19.3^{g}	n.d ^{c}	n.d ^{c}
500	n.d ^{f}	n.d ^{f}	++ ^{h}	n.d ^{h}	n.d ^{c}	n.d ^{c}

n.d.= not detected, ++ = trace amount

Table 4.2: The effects of diuron (DIU) on polyphenolic compounds in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Results shown as means ± SD (n=3). Data sets which are significantly different from each other for each polyphenolic compounds are represented by different letters ($P < 0.05$).

For terbuthylazine (**Table 4.3**) exposure for 7 d indicate no significant increase on the amount of rutin in both strains. However, significant decreased of rutin were observed at ≥ 100 and 500 µg L^{-1} of TBA in LIA4 and Es524 respectively. At 50 and 100 µg L^{-1}, the amount of rutin was significantly higher in Es524 compared to LIA4.

For phloroglucinol (PG), no significant change was observed in both strains up to 10 µg L^{-1} of TBA. However, at 50 and 100 µg L^{-1} significant differences between the strains were recorded. PGs increased significantly in Es524, which were also significantly higher compared to LIA4 at corresponding treatments. Unlike rutin, PG was still detected at 500 µg L^{-1} of TBA, which significantly decreased in both strains as compared to the carrier control.

With respect to caffeic acid (CA), no significant increase of the compound was observed in both strains within the concentrations tested. However, at 10 and 50 µg L^{-1} of TBA, the amounts of CA in Es524 were significantly higher compared to LIA4. At 50 µg L^{-1}, only trace amount of CA was detected in LIA4, while in Es524 the amount of CA shows no significant difference as compared to the carrier control.

TBA (µg L^{-1})	Polyphenols (µg g^{-1} FW)					
	Rutin		Phloroglucinol		Caffeic acid	
	Es524	LIA4	Es524	LIA4	Es524	LIA4
NSW	131.4 ± 12.6^{ab}	105.6 ± 13.9^{abc}	362.4 ± 29.5^{bc}	325.8 ± 43.3^{cd}	20.7 ± 3.3^{ab}	15.1 ± 4.1^{b}
DMSO	122.1 ± 16.8^{abc}	107.3 ± 11.4^{abc}	355.3 ± 33.6^{bc}	319.7 ± 40.6^{cd}	19.5 ± 4.6^{ab}	14.2 ± 3.7^{b}
1	119.3 ± 19.2^{abc}	102.5 ± 16.3^{bc}	347.8 ± 42.3^{bc}	307.8 ± 45.6^{cd}	17.3 ± 4.1^{b}	15.5 ± 3.4^{b}
5	134.2 ± 21.6^{a}	108.2 ± 19.1^{abc}	331.6 ± 38.7^{cd}	315.1 ± 37.4^{cd}	18.6 ± 5.2^{ab}	14.9 ± 4.5^{b}
10	123.7 ± 24.8^{abc}	112.3 ± 17.4^{abc}	364.1 ± 48.5^{bc}	307.3 ± 42.8^{cd}	27.3 ± 5.1^{a}	17.5 ± 3.9^{b}
50	131 ± 30.5^{ab}	98.5 ± 18.7^{c}	461.5 ± 45.9^{a}	261.7 ± 31.2^{d}	14.2± 3.3^{b}	++ ^{c}
100	98.2 ± 17.7^{c}	36.4 ± 6.3^{d}	422.4 ± 52.1^{a}	168.9 ± 18^{e}	++ ^{c}	n.d ^{c}
500	++ ^{e}	n.d ^{e}	138.3 ± 22.7^{ef}	71.6 ± 15.3^{f}	n.d ^{c}	n.d ^{c}

n.d.= not detected, ++ = trace amount

Table 4.3: The effects of terbuthylazine (TBA) on polyphenolic compounds in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Results shown as means ± SD (n=3). Data sets which are significantly different from each other for each polyphenolic compounds are represented by different letters ($P < 0.05$).

In response to IPU exposure, both LIA4 and Es524 strains indicate distinct polyphenolic contents (**Table 4.4**). For rutin, no significant change was observed in both strains up to 50 µg L^{-1}. However, at 100 and 500 µg L^{-1}, the amounts of rutin were significantly higher in Es524 compared to LIA4.

Similarly for phloroglucinol (PG), significant increases in Es524, were recorded at 100 and 500 µg L^{-1} of IPU, which were significantly higher compared to LIA4. On the other hand, no significant change of PG content was observed in LIA4 as compared to the carrier control within the concentrations of IPU tested.

Exposure to IPU up to 50 µg L^{-1} indicated no significant change in the amount of caffeic acid (CA) in both strains. However, at 100 µg L^{-1}, caffeic acid was significantly higher in Es524 compared to LIA4, which may contribute to higher tolerance of the strain to oxidative stress. With the same treatment (100 µg L^{-1}), CA also has decreased significantly in LIA4 as compared to the carrier control. At the highest concentrations tested, only trace amount of CA was detected in Es524 while in LIA4 it was not detected.

IPU (µg L^{-1})	Polyphenols (µg g^{-1} FW)					
	Rutin		Phloroglucinol		Caffeic acid	
	Es524	LIA4	Es524	LIA4	Es524	LIA4
NSW	120.3 ± 15.4^{cde}	97.3 ± 14.6^{de}	357.6 ± 39.4^{bcd}	327.2 ± 31.7^{cd}	19.2 ± 4.1^{ab}	16.1 ± 3.2^{abc}
DMSO	131.5 ± 18.9^{bcd}	108.7 ± 17.1^{cde}	361.8 ± 45.8^{bc}	334.6 ± 40.2^{cd}	18.7 ± 3.6^{ab}	15.3 ± 3.5^{abc}
10	127.1 ± 21.3^{bcde}	102.5 ± 15.9^{de}	342.2 ± 50.3^{cd}	325.1 ± 32.8^{cd}	22.2 ± 3.7^{a}	16.5 ± 4.1^{abc}
50	151.6 ± 29.7^{abc}	111.6 ± 22.8^{cde}	395.3 ± 43.4^{abc}	342.7 ± 18^{cd}	14.3 ± 3.1^{bcd}	11.4 ± 2.8^{cd}
100	194.5 ± 22.6^{a}	136.9 ± 18.5^{bcd}	441.5 ± 40.9^{a}	365.8 ± 37.9^{bc}	17.4 ± 2.2^{abc}	8.1 ± 2.1^{d}
500	171.6 ± 17.8^{ab}	84.7 ± 12.5^{e}	426.1 ± 29.7^{a}	282.4 ± 30.5^{d}	++ ^{e}	n.d ^{e}

n.d.= not detected, ++ = trace amount

Table 4.4: The effects of isoproturon (IPU) on polyphenolic compounds in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Results shown as means ± SD (n=3). Data sets which are significantly different from each other for each polyphenolic compounds are represented by different letters ($P < 0.05$).

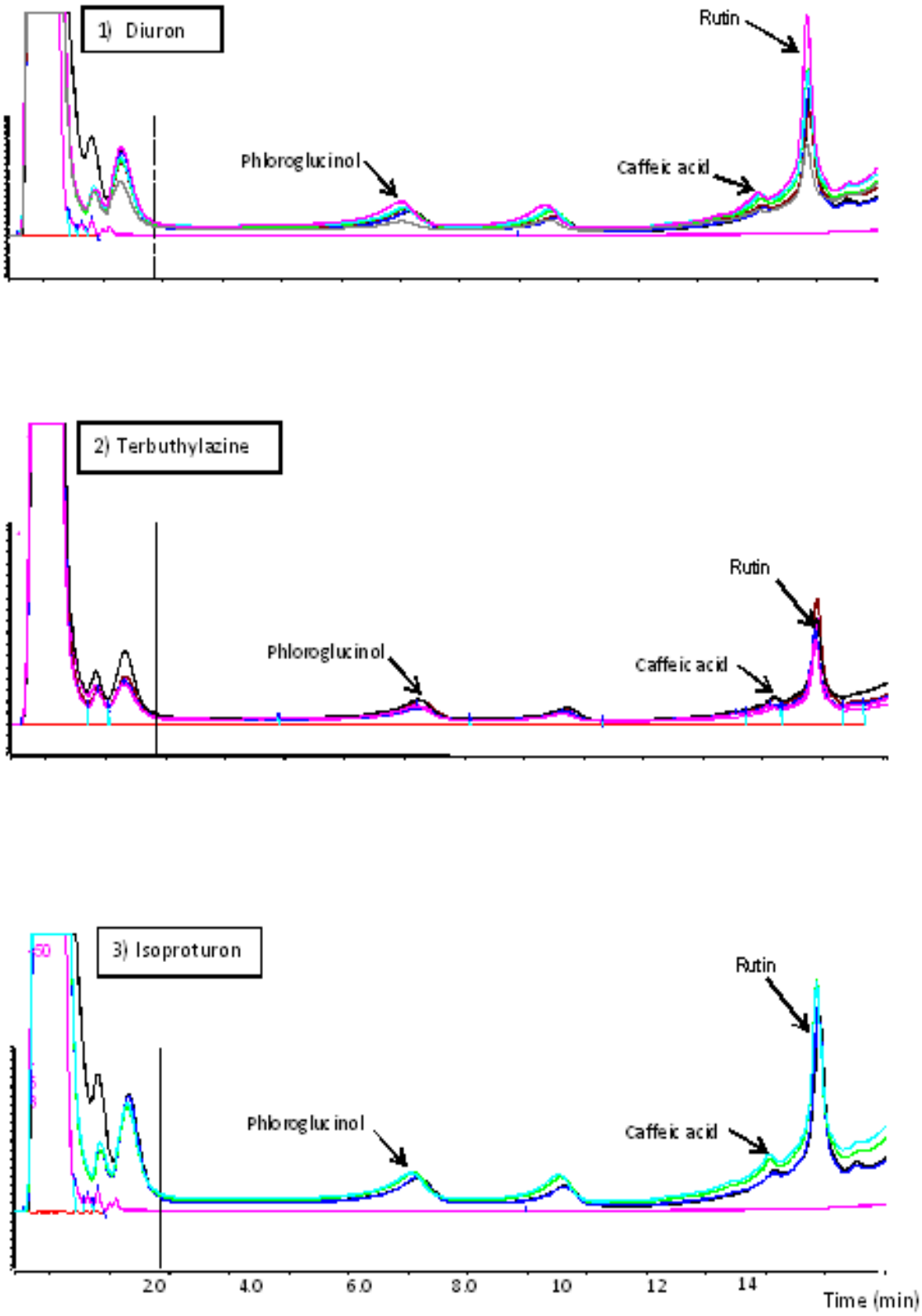

Figure 4.25: HPLC chromatogram at 254 nm of the *E. siliculosus* exposed to herbicides revealing several phenolic peaks

4.10 Discussion

4.10.1 Oxidative stress and antioxidative enzyme responses of *E. siliculosus* strains to **diuron (DIU)**

In the present study, exposure of diuron (DIU) on *E. siliculosus* strains (LIA4 and Es524) originated from different geographical locations; LIA4 (pristine site, Lon Liath, Scotland), Es524 (Cu-polluted site, Caleta Palito, Chile) demonstrate unique responses. For oxidative stress assessments, hydrogen peroxide (H_2O_2) content and lipid peroxidation (TBARS) served as important indicators. The relative increases of H_2O_2 contents in LIA4 and Es524 with increasing DIU concentrations reflect the stimulation of H_2O_2 generation in *E. siliculosus* by the PSII inhibitors. After 7 d of exposure to DIU, H_2O_2 contents were found to be significantly increased in LIA4 at ≥ 10 µg L^{-1} DIU, while in Es524 at ≥ 100 µg L^{-1}. In fact, at 10 µg L^{-1} of DIU, H_2O_2 content was found to be significantly lower in Es524 compared to LIA4 which indicate higher tolerance of the strain towards the herbicide/booster biocide. With respect to the mode of action of diuron (DIU), it is known to generate reactive oxygen species (ROS), which induce a shift of the balance between prooxidative and antioxidative reactions (Geoffroy *et al*., 2002). Thus, the significant increases of H_2O_2 levels observed as low at 10 and 100 µg L^{-1} of DIU in LIA4 and Es524 respectively might relate to this factor. According to Kleczowski, (1994), DIU inhibits the Hill reaction of the PSII by blocking electron flow between the primary electron acceptor Q and the D1 protein making up the electron chain. This blocking indirectly blocks the transfer of excitation energy from chlorophyll molecules to the PSII reaction centre (Trebst and Draber, 1986). As a result, the excited chlorophyll molecules (singlet chlorophyll) spontaneously form triplet chlorophyll through a non-radiative energy transformation of chlorophyll, where it (triplet chlorophyll) will reacts with molecular oxygen to form singlet oxygen ($^1O_2$), a type of

ROS (Fuerst and Norman, 1991). The transfer of energy to oxygen results in peroxidative destruction of pigments, proteins, and nucleic acid as well as disintegration of cellular membrane systems by lipid peroxidation (Watanabe *et al*., 2001). The increase in cellular ROS level is also linked to the reduction of fluorescence (reported in previous chapter), since it has been well documented that a photosynthetic efficiency decrease can enhance ROS production in microalgal cells, followed by oxidative damages and function abnormalities (Liu *et al*., 2012). Evaluation of the TBARS level indicates a similar response to H_2O_2, but significant differences between the strains were observed at 50 µg L^{-1} of DIU where the TBARS level was significantly higher in LIA4 compared to Es524. These findings of increased TBARS levels are parallel to the effect of heavy metal and pesticides on microalgal species reported previously (Choudhary *et al*., 2007; Kumar *et al*., 2008; Hong *et al*., 2009; Sabatini *et al*., 2009).

To overcome the hazardous impact of ROS, plants and algae have developed defence molecules and/or mechanisms such as antioxidative enzymes, α-tocopherol, carotenoids, ascorbate and glutathione, which located in various cell compartments (Geoffroy *et al*., 2002). The different oxidative responses exhibited by LIA4 and Es524 strains may be contributed to by this factor. Evaluation on catalase (CAT) clearly shows that DIU poorly stimulates the activity of the enzyme in both strains of *E. siliculosus*. Slight increases up to 23 and 32% (not significant) were observed in LIA4 and Es524 respectively, after 7 d of exposure to the range of DIU concentrations applied. Moreover, inhibition of CAT activity by DIU was observed at 50 µg L^{-1} and above. These findings of poor stimulation and inhibition of CAT activity by DIU are in line with studies performed by Geoffroy *et al*., (2002) and Teisseire and Vernet (2000) using *Scenedesmus obliquus* and *Lemna minor* respectively. Other pollutants, such as heavy metals (Cd, Zn, Cr, Al) or SO_2, were also reported to inhibit this enzyme (CAT) (Chaoui *et al*., 1997; Niewiedomska and Miszlaski, 1997; Willekens *et al*., 1997;

Boscolo *et al*., 2003). The suppression of CAT activity, observed from the DIU exposure, is likely due to the excess production of ROS caused by the herbicide/booster biocide, which lead to inactivation of the enzyme. In fact, a previous study by Viarengo (1985) reported that suppression of CAT might result from the binding of heavy metals onto a functional group of the enzyme.

For ascorbate peroxidase (APX) activity, different responses between LIA4 and Es524 strains were observed. Significant increases of as much as 69 and 83% were observed in Es524 at 5 and 10 µg L^{-1} of DIU respectively. These increments are suggested to keep the balance of cellular H_2O_2 components, in response to DIU exposure. In contrast, no significant increase of APX activity was observed in the LIA4 strain. The different responses observed between CAT and APX activities to DIU exposure, could be due to the factor that CAT has low affinity for H_2O_2, whereas APX has a high affinity for H_2O_2 and is able to detoxify low concentrations of H_2O_2 (Nakano and Asada, 1981; Amako *et al*., 1994). Other explanation to higher stimulation of APX activity compared to CAT in response to DIU exposure could be linked to the specific site of action of DIU towards the photosystem II. According to Link *et al*., (1997), APX which scavenges H_2O_2 using ascorbate as a donor of electrons is located mainly in chloroplasts. Our finding of stimulation of this enzyme was in agreement to previous reports in plants exposed to oxidative stress generating compounds, such as SO_2, O_3, herbicide (oxyfluorfen) or heavy metals (Knorzer *et al*., 1996; Ranieri *et al*., 1996; Chaoui *et al*., 1997). However, our finding in Es524 contradict to the reports by Teisseire and Vernet (2000) and Geoffroy *et al*., (2002) where lack of stimulation of APX activity to DIU exposure were observed in *L. minor* and *Sc. obliquus* respectively. The weak stimulation observed in the aforementioned organisms and in LIA4, might indicate either that DIU did not cause an overproduction of H_2O_2, or that constitutive

activity of the enzyme was high enough to cope with a possible excess of H_2O_2 generated by DIU (Teisseire and Vernet, 2000).

In addition, significant differences between LIA4 and Es524 strains were also observed in glutathione reductase (GR) activity. Our result indicates that GR was strongly stimulated in Es524, with a maximum increase of 108% recorded at 10 µg L^{-1} of DIU compared to the carrier control. On the other hand, only a slight induction (39%) was observed in LIA4 at corresponding treatment. Our findings in *E. siliculosus* strains were in agreement to the report by Geoffroy *et al*., (2002) where GR activity was induced by 46% in *Sc. obliquus* at 10 µg L^{-1} of DIU. Nevertheless, exposure to 100 µg L^{-1} of DIU has recorded a contrary result to the report by Teisseire and Vernet (2000) where slight increment of GR activity (117% of the control) was observed in *L. minor* while reduction as much as 31 and 52% were observed in Es524 and LIA4 respectively at similar concentration. Unlike CAT and APX, GR did not directly deactivate ROS, but it was indirectly involved in this process by maintaining glutathione in its predominantly reduced state (Foyer *et al*., 1997). The glutathione could quench ROS directly or indirectly by regenerating ascorbic acid, another antioxidant (Foyer *et al*., 1994). Overall, the increase of APX and GR activities in Es524 indicates that this strain possess an efficient ROS scavenging capacity compared to LIA4, to alleviate DIU toxicity by enhancing its defense mechanism. Thus, it is suggested that the non-significant change of H_2O_2 levels observed at 10 and 50 µg L^{-1} of DIU in Es524 may be linked to the increased in the activity of antioxidative defense systems.

4.10.2 Oxidative stress and antioxidative enzyme responses of *E. siliculosus* strains to **terbuthylazine (TBA)**

From our results, *E. siliculosus* strains (LIA4 and Es524) exposed to low concentrations of TBA (1-10 µg L^{-1}) showed no significant changes of H_2O_2 levels, with no visual signs of pigment loss and appeared healthy during the experimental period. However, at 50 µg L^{-1} of TBA, significantly higher level of H_2O_2 was observed in LIA4 compared to Es524. For LIA4, H_2O_2 levels have increased significantly at 50 µg L^{-1} and above while in Es524 it was only recorded at 500 µg L^{-1} indicating higher tolerance of Es524 to the herbicide. For lipid peroxidation, measured as TBARS levels, different response between the strains were observed at 50 and 100 µg L^{-1} of TBA with higher TBARS levels were recorded in LIA4 compared to Es524. Besides, as compared to the carrier control, significant increases of TBARS levels were observed at as low at 50 µg L^{-1} for LIA4, while in Es524 at 100 µg L^{-1}. The significant changes observed at lower concentrations of TBA indicate higher sensitivity of LIA4 strain compared to Es524. Our observation of triazine-induces the production of ROS, is in agreement with the reports by Sulmon *et al*., (2007), Erinle *et al*., (2016) and Esperanza *et al*., (2016) on *Arabidopsis thaliana* seedlings, *Pennisetum sp* and *Chlamydomonas reinhardtii* respectively. High production of ROS, as displayed from the results, could be due to severe structural damages to cells, caused by triazine intake into an algae/plant system.

In addition to the increase in the markers of oxidative stress, alterations in antioxidative enzyme activities were also observed. For CAT, both strains have response similarly to TBA with slight stimulation, which peaked at 10 µg L^{-1} (significantly different to carrier control) and then declined. The increase of CAT activity might indicate that TBA can cause production of ROS in cytosol. According to Chandlee *et al*., (1983) and Willekens *et al*., (1997), CAT activity is known to be suited

in the cytosol and peroxysomes representing importance sink for H_2O_2 induced by oxidative stress in photosynthetic organisms. Thus, the increase in CAT activity may signify that cellular tolerance to oxidative stress was initiated to cope with the overproduction of H_2O_2. However, at 100 µg L^{-1} of TBA and above, a decrease of CAT activities was observed in both strains, which contradict to the study by Mofeed and Mosleh (2013), where strong induction of CAT activity (210% increment) in *Scenedesmus obliquus* was recorded in response to another triazine (atrazine) at similar concentrations (100 µg L^{-1}). The diminution of CAT activities displayed in our results could be due to the herbicide inducing a deterioration effect on CAT protein when *E. siliculosus* strains were exposed to higher concentrations of TBA.

Compared to CAT, the induction of APX activity showed different responses between LIA4 and Es524. Higher APX activities were observed in Es524 strain compared to LIA4 in response to TBA. Unlike LIA4, APX activity in Es524 has shown a concentration-dependent increase up to 50 µg L^{-1}, followed by a decrease at higher concentrations. The peak of APX activity in Es524 was recorded at 50 µg L^{-1} with 107% increase compared to LIA4 with only 15%, which indicates that APX might responsible for the H_2O_2 detoxification in Es524. This finding of TBA-induces APX activity is concur to recent study by Erinle *et al*., (2016) where APX activity in *Pennisetum sp* had increased significantly upon exposure to triazine. Further, diminished APX activities observed at the highest concentrations of TBA (500 µg L^{-1}) might be due to the deficiency of ascorbate as substrate which led to low detoxification ability, together with high H_2O_2 accumulation (Dewez *et al*., 2005).

In addition, exposure of TBA on *E. siliculosus* strains (LIA4 and Es524) also has shown different glutathione reductase (GR) response between the strains. Compared to Es524, no significant changes of GR activity were observed in LIA4. In contrast,

peaked stimulation was observed in Es524 at 50 µg L^{-1} with 122% increase compared to carrier control while only 32% was observed in LIA4 at similar concentration. Significant increase of GR in Es524 strain indicated its role in detoxification of H_2O_2 which in line with previous study on *Scenedesmus obliquus*, where the activity of the enzyme has increased up to 111.3% in response to triazine exposure (Mofeed and Mosleh, 2013).

In conclusion, terbuthylazine (TBA) resulted in different response of H_2O_2 production in LIA4 and Es524. The increases of H_2O_2 suggest that oxidative stress was induced in both strains after exposure to the herbicide. To overcome this, different responses of antioxidant enzymes were recorded between the strains. With respect to CAT activity, both strains have showed similar responses to the range of TBA treatments. However, two other antioxidant enzymes; APX and GR have demonstrated different responses between the strains, which probably linked to the different tolerance observed between them. The intraspecific responses to TBA observed between LIA4 and Es524 was in line to previous findings by Behra *et al*., (1999) using twelve strains of *Scenedesmus subspicatus* exposed to triazine. In their study, variability between-strains were markedly different in response to triazine. According to them, the variations between the *S. subspicatus* strains can be explained by the significant role of genotype, versus environment interactions in determining intraspecific differences in adaptive physiological responses to the herbicide.

4.10.3 Oxidative stress and antioxidative enzyme responses of *E. siliculosus* strains to **isoproturon (IPU)**

Different response of H_2O_2 contents was observed between LIA4 and Es524 after exposure to isoproturon (IPU). Compared to Es524, a concentration-dependent increase of H_2O_2 levels were observed in LIA4, which significantly different compared to carrier control at 100 and 500 µg L^{-1} of IPU. On the other hand, no significant change was recorded in Es524 to the ranges of IPU concentrations tested which indicate better tolerance compared to LIA4. For the TBARS level, both strains have shown similar responses. Exposure to IPU up to 50 µg L^{-1}, displayed no significant change to the TBARS level, but once the IPU reached 100 µg L^{-1} and above, significant increases were observed. This finding contradict to the report by Bi *et al*., (2012) where maximum TBARS accumulation was observed at 50 µg L^{-1} of IPU, with the *Chlamydomonas reinhardtii* cells accumulated TBARS 3.43-folds higher than the control. The different results obtained, although at similar herbicide level, could be due to the fact that different species (micro / macroalgae) possess different tolerance levels. Moreover, due to disruption of electron transport in PSII attributed to the displacement of secondary plastoquinone acceptor by herbicides from the Qb site to D1 protein (Rensen, 1982), it was likely that IPU (a type of PSII inhibitor herbicide) triggered oxidative stress, which was evident by the increases of H_2O_2 and TBARS levels.

According to Yin *et al*., (2008), IPU-induced oxidative stress may be associated with antioxidant enzymes. In fact, hydrogen peroxide (H_2O_2) generated from the herbicide exposure is also highly toxic and must remain under tight control (Alscher *et al*., 2002; Choo *et al*., 2004). Therefore, in order to elucidate the factors that led to the different responses in H_2O_2 and TBARS levels between LIA4 and Es524, evaluations of different antioxidative enzymes (CAT, APX and GR) were carried out.

In contrast to DIU effect, our results clearly show that IPU strongly induced antioxidative enzyme activities of Es524 strain. However, the response of Es524 and LIA4 to IPU varies greatly with regards to the antioxidative enzyme activities measured. With respect to CAT activity, different responses were observed between the strains. CAT activity in Es524 was significantly induced at 50 and 100 µg L^{-1} of IPU with 153 and 111% increase respectively. On the other hand, only 52 and 19% stimulations (not significant) were observed in LIA4 at corresponding treatments. The high stimulation of CAT activity in Es524 could be the factor that leads to stable levels of H_2O_2 within the strain, which is in line to the study by Bi *et al*., (2012) on green algae exposed to IPU. In their finding, CAT activities in *C. reinhardtii* consistently increased with IPU at concentrations of 5 to 50 µg L^{-1}.

As for APX activity, both strains (LIA4 and Es524) were found to exhibit similar response to IPU, with significant stimulation observed at 100 µg L^{-1} and then declined. Even though both strains were found to respond similarly, the peaked induction of APX activity was hugely different between the strains with a 101% increase in Es524 and only 49% in LIA4. Exposure to the highest concentration (500 µg L^{-1}) indicates that positive induction of APX activity was still demonstrated in Es524, while in LIA4 it has reduced to below than the carrier control level. Our observations for APX activity contradict a report by Bi *et al*., (2012), which recorded similar patterns of APX activities to that of CAT in *C. reinhardtii* exposed to IPU.

Exposure to IPU also induced different response of glutathione reductase (GR) activities between LIA4 and Es524. Only moderate stimulations of GR activities (24-105%) were observed in LIA4, which peaked at 50 µg L^{-1} and then declined. On the other hand, strong induction of GR activities was displayed in Es524 with marked increment of 180 and 140% at 50 and 100 µg L^{-1} respectively. The huge difference in

GR activities demonstrated in both strains could be one of the factor that lead to high tolerance of Es524 against IPU compared to LIA4, due to the higher scavenging capacity of the ROS molecules.

It is clear from the above that IPU exposure demonstrated distinct homeostasis response of the defence mechanisms between LIA4 and Es524 strains. The antioxidative homeostasis in Es524 is markedly altered by IPU in gaining IPU tolerance. Significant stimulations of CAT, APX and GR activities help prevent oxidative damage in the strain (Es524) compared to LIA4 upon exposure to herbicide. These findings are in agreement with earlier reported results on antioxidative enzyme activities in *Triticum aestivum* exposed to IPU (0 – 20 mg/kg) (Yin *et al*., 2008), but contradict to the study by Nemat Alla *et al*., (2008), who have reported that IPU exposure on *Zea mays* had decreased the CAT and APX activities on day eighth of the treatment. The increases in activities of the aforementioned antioxidant enzymes are responsible for ROS scavenging in Es524 in response to IPU stress. This explains why H_2O_2 did not significantly increase in Es524 at 100 and 500 µg L^{-1} of IPU, unlike observed in LIA4.

4.10.4 Effects of DIU, TBA and IPU on non-enzymatic antioxidant activity

The occurrence of various antioxidants in seaweeds, including polysaccharides, dietary fibers, minerals, proteins, amino acids, vitamins, polyphenols and carotenoids have been reported previously (Burtin, 2003). Seaweeds are known to produce these antioxidants to counteract environmental stresses (Lesser, 2006). In fact, according to Steinberg *et al*., (1991) polyphenolics are the most abundant chemical defenses in temperate marine algae. Thus, besides the enzymatic antioxidative responses, investigations on non-enzymatic activities of both LIA4 and Es524 strains were also been carried out. In the present study, evaluations through the total phenolic content

(TPC) and DPPH scavenging activities revealed distinct antioxidant capacities between LIA4 and Es524 strains. From the results (**Figure 4.18 – 4.23**), exposure to the different herbicides have shown that greater DPPH scavenging activity and production of phenolic compounds were observed in Es524 compared to LIA4. Significantly higher DPPH scavenging activity and phenolic compounds were recorded in Es524 compared to LIA4 at different concentrations of the herbicides. These observations could explain the higher tolerance of Es524 strain towards the herbicides tested.

Further investigations using high performance liquid chromatography (HPLC) were carried out in order to determine the compounds that might contribute to the different adaptation responses between LIA4 and Es524 strains. To our knowledge, this is the first study to use HPLC protocol to quantify changes of phenolic compounds in brown seaweed *E. siliculosus* after exposure to various herbicides (DIU, TBA, IPU). Interestingly, both strains (LIA4 and Es524) investigated in this study also produced phenolic compounds without the herbicides exposure. The values were higher in the Es524 strain, which could relate to the environmental conditions from where it was isolated. The presence of three different phenolic compounds (phloroglucinol, rutin, caffeic acid) were proven in both strains of *E. siliculosus* (LIA4 and Es524) by using HPLC in control samples, as well as in 7 d herbicides exposed samples.

From the results obtained (**Table 4.2 - 4.4**), the levels of polyphenol compounds in Es524 were consistently higher than LIA4. Exposure to DIU, TBA and IPU indicated that the amount of phloroglucinol, rutin and caffeic acid were significantly higher in Es524 compared to LIA4 at different concentrations of the herbicides. We speculate that the higher levels of phenolic compounds observed in Es524 compared to LIA4 in response to the herbicides probably due to the higher number of physodes (a phlorotannin/phenolics containing structures frequently found in brown algae)

(Schoenwaelder, 2002; Schoenwaelder and Clayton, 1998). According to Van Alstyne and Paul (1990), brown algae typically possess high content of phenolic compounds and these substances can act as antioxidants by transferring hydrogen atoms to lipid peroxyl radicals (Foti *et al*., 1994). In fact, polyphenols have been found to involve in the reduction of H_2O_2 in vacuoles and apoplasts by acting as the donors of electrons to peroxidases (e.g. guaiacol peroxidase, pyrogallol peroxidase) (Yamasaki *et al*., 1997). Thus, they might be involved in the defence strategy for coping with H_2O_2 in the cells. It has been reported previously that the 'high adaptation' and acclimation potential of brown algae to abiotic stress was attributed to the ability of phenolic compounds such as phlorotannins (Pavia *et al*., 1997). Ruhland *et al*., (2007) also showed that increased concentrations of phenolic compounds are important to ameliorate damage caused by increased ROS. For example, Heo *et al*., (2009) and Schmidt *et al*., (2012) have observed that phlorotannins from *Ecklonia cava* and phenolic compounds of *Hypnea musciformis* respectively, were found to exert protective effects against photooxidative stress conditions induced by UV-B radiation. Therefore, the presence of such phenolics supports the hypothesis that their production might be crucial to overcome oxidative stress condition caused by herbicides. Moreover, Es524 which exhibits higher levels of phenolics compared to LIA4 after exposure to the herbicides, also indicates higher values of Fv/Fm (e.g. DIU (Fig. 3.2) and IPU (Fig. 3.16)), suggesting a protective role of the phenolics for photosynthetic apparatus. Similar observations of superior photosynthetic rate (rETR) and Fv/Fm values were found in cells of the chlorophyte *Zygnemopsis decussata* with high content of phenolics (Figueroa *et al*., 2009).

Overall, three types of polyphenol compounds—phloroglucinol, caffeic acid and rutin were detected in this study. Phloroglucinol appeared to be the highest compound, followed by rutin, while caffeic acid had the lowest concentration in the seaweed

extract. The more tolerance response of Es524 compared to LIA4 observed in the present study is in line to the report by Collen and Davison (1999 a,b) for intertidal brown and red macroalgae, where higher antioxidant levels are positively associated with their stress tolerance. These findings are consistent with the hypothesis that phenolic levels in Es524 which isolated from a Cu-polluted site are higher than LIA4 because these compounds are required for defense purposes. In fact, Steinberg *et al*., (1991) proposed that low levels of copper (Cu) might result in constrained phenolic production.

4.11 Conclusion

Overall, evaluations of various aspects of biochemical responses of *E. siliculosus* strains towards DIU, TBA and IPU have been carried out, comprised of the; i) levels of hydrogen peroxide and lipid peroxidation (TBARS), (ii) antioxidant enzymes activity (CAT, APX and GR) and (iii) non-enzymatic antioxidants activity (polyphenols). Thus, the results could be meaningful in evaluating the role of *E. siliculosus*'s antioxidative system in terms of herbicides tolerance and detoxification. It is clear that LIA4 and Es524 have displayed different biochemical responses to the herbicides tested. Elevated levels of H_2O_2 and lipid peroxidation indicate a state of oxidative stress possibly due to inhibition of electron transport in the photosystem II which leads to increased number of oxidised chlorophyll. Higher contents of H_2O_2 and lipid peroxidation demonstrated in LIA4 compared to Es524 in response to the herbicides exposure indicate that LIA4 experienced more severe oxidative stress conditions than Es524. Besides, stimulation of different antioxidative defence mechanisms, either enzymatic (CAT, APX, GR) or non-enzymatic (polyphenols), in both strains indicates that higher levels have been observed in Es524 compared to LIA4.

These results could explain the higher tolerance of Es524 to the herbicides compared to LIA4, as reported in previous chapter according to the EC_{50} values. Consequently, increased antioxidant defences in algae exposed to xenobiotic substances or metals is indicative of required protection against ROS production (Lee and Shin, 2003; Geoffroy *et al*., 2002). To sum up, the present study indicates that quantitative variation in the defense mechanisms between LIA4 and Es524 of *E. siliculosus* was found to have a pronounced effect on the susceptibility of the strains to the tested herbicides.

Chapter 5

The effects of herbicide singly and in combinations on physiological and biochemical responses of *Ectocarpus siliculosus*

5.1 Introduction

In the natural environment, organisms are rarely exposed to a single contaminant. Typically there is a mixture of numerous pesticides with varying constituents in varying concentrations and concentration ratios (Faust *et al*., 2003; Schuler and Rand, 2008). Due to different anthropogenic activities (e.g. agricultural practices, shipping activities), the receiving ecosystem is invariably contaminated with multiple chemicals. The toxic effect of multiple chemicals has been recognised as an important factor in ecotoxicology, because a chemical mixture can have a greater negative impact than the individual constituents of the mixture (Hernando *et al*., 2003).

Chapters 3 and 4 have shown that individual assessments on DIU, TBA and IPU have caused significant effects on physiology and biochemical in both strains of *E. siliculosus*. Therefore, it would be interesting to investigate the effects of the herbicides that might present in combination in the nature (e.g Bay of Vilaine area, Brittany, France, Caquet *et al*., 2013; Great Barrier Reef lagoon, Australia, Kroon *et al*., 2012). So far, toxic effects of diuron (DIU) have been investigated in combination with tebuconazole (Tlili *et al*., 2011), monolinuron and linuron (Gatidou *et al*., 2015), isoproturon and atrazine (Knauert *et al*., 2010), folpet and copper (Teisseire *et al*., 1999; Gatidou and Thomaidis, 2007), whereas the toxicity of TBA has been investigated in combination with chlorpyrifos (Perez *et al*., 2013).

To the best of our knowledge, no study has been reported in the literature which made reference to the effects of the combined application of diuron, terbuthylazine and isoproturon on marine macroalgae. Due to their successive use either in agriculture or antifouling purpose, residues of DIU, TBA and IPU may be found together in aquatic or nearshore marine ecosystems. Potent ecotoxicological risks might result from their association. Therefore, in the present study, the toxicity of DIU, TBA and IPU were

evaluated singly and in mixtures on two different strains of *E. siliculosus* with different pollution histories. Both physiological and biochemical activities have been evaluated in order to analyze the interactions between these herbicides.

5.2 Materials and methods

5.2.1. Biological material and test solutions

Both the LIA4 and Es524 strains of *E. siliculosus* used for the mixture studies were cultured and maintained according to the method described in section **2.2**. The pH, salinity and temperature of the test solutions either individual or mix of herbicides were 7.4 ± 0.1, 33 ± 1.0 ppt and 15ºC respectively.

5.2.2. Preparation of single herbicide solutions

A stock solution of individual herbicides; diuron (DIU), terbuthylazine (TBA) and isoproturon (IPU) were prepared according to the protocol described in section **2.4** using DMSO as the carrier solvent to reach final concentrations of 10 µg L^{-1} (DIU), 25 µg L^{-1} (TBA) and 300 µg L^{-1} (IPU) which caused ~40–60% of growth inhibition (RGR) in both strains of *E. siliculosus*. In chapter three, the inhibition by individual herbicides was determined at broad concentrations to ensure capturing possible inhibition effect ranges. For both exposures, either singly or in combinations, four replicates were used for the physiological and oxidative stress assessments while three replicates for the enzymatic/non-enzymatic analysis due to biomass constraint (difficulty to obtain large amounts of biomass from cultures).

5.2.3 Combination studies

To determine the possible interactions between DIU, TBA and IPU, combined media were prepared with binary mixtures of the herbicides, using [DIU (10 µg L^{-1}) + TBA (25 µg L^{-1})], [DIU (10 µg L^{-1}) + IPU (300 µg L^{-1})] and [TBA (25 µg L^{-1}) + IPU (300 µg

L^{-1})]. Possible interactions between the herbicides (diuron, terbuthylazine and isoproturon) were estimated using Abbot's formula (Gatidou and Thomaidis, 2007). In this widely used model, the expected effect of the mixture (C_{exp}), expressed as a percentage, can be predicted as follows:

$$C_{exp} = A + B - (A \times B / 100),$$

where A and B are the effect levels given by the single chemicals. The ratio of effect (RE) was then calculated as follows for each contaminant combination:

$$\text{RE} = \text{Observed effect} / C_{exp}$$

Interactive effects were evaluated by comparing RE to 1. A RE value > 1 indicates potential synergism between the two contaminants; RE equal to 1 indicated additivity; and RE < 1, an antagonism between the two chemicals. According to Green *et al*., (1997), the mixture that give more activity than expected is called synergism, less activity as antagonism, and expected according to a reference model is defined as additive. The RE of each replicate for each treatment was calculated and the mean and standard deviation were determined. Only if the mean value of RE was greater than one standard deviation from 1 (1 + S.D.), the interactive effect assumed to be significantly different from additivity (Chesworth *et al*., 2004).

5.2.4 Individual and mixture effects of herbicides on growth

The effects of the herbicides (DIU, TBA and IPU) singly and in mixtures on growth (RGR) of LIA4 and Es524 strains were carried out in accordance to the section **2.6.1** using different treatments as intended.

5.2.5 The effects of herbicides singly and in mixtures on photosynthetic efficiency of *E. siliculosus*

Different photosynthetic efficiency indicators of the treated samples were evaluated according to the methods mentioned in **2.6.2** previously.

5.2.6 The effects of herbicides singly and in mixtures on oxidative stress of *E. siliculosus*

Evaluation of oxidative stress was performed using hydrogen peroxide (H_2O_2) contents and lipid peroxidation levels (LPX) according to the protocols described in section **4.3.1** and **4.3.2** respectively.

5.2.7 The effects of herbicides singly and in mixtures on antioxidative enzyme activities of *E. siliculosus*

For the antioxidative enzyme responses, proteins from each sample were extracted according to the protocol described in section **2.7**. Different antioxidative enzymes assay; catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) were performed following the method stated in section **4.4.2, 4.4.3** and **4.4.4** respectively.

5.2.8 The effects of herbicides singly and in mixtures on non-enzymatic antioxidant activities of *E. siliculosus*

Evaluations of non-enzymatic activities of the *E. siliculosus* strains were carried out according to the methods mentioned in **4.7**.

5.2.9 Statistical analyses

STATGRAPHICS Centurion (Version XVI, Statpoint Technologies, Inc., USA) software was used for the statistical analyses. Prior subjected to one or two way analysis of variance (ANOVA) and post-hoc Tukey test at 95% confidence, the data were tested for normality (Shapiro Wilk test) and homogeneity of variance (Bartlett test).

5.3 Results

5.3.1 Individual and combined effects of herbicides on growth and photosynthetic efficiency of *E. siliculosus*

Exposure to individual and mixture of herbicides (diuron, terbuthylazine, isoproturon) for 7 days resulted in negative impacts on growth and photosynthetic efficiency of the brown seaweed.

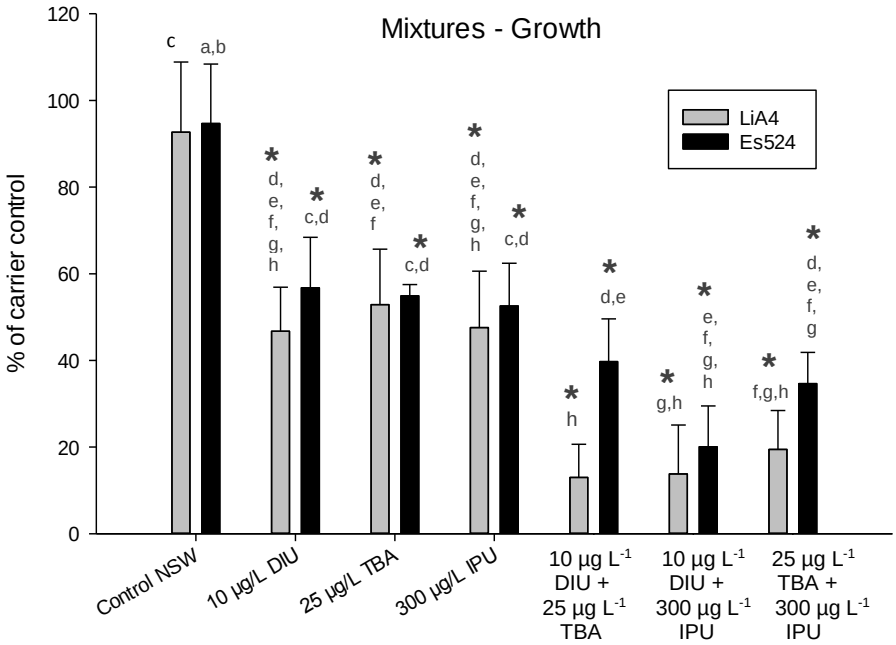

Figure 5.1: The effects of individual and binary combinations of herbicides on **RGR** in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean RGR values of the carrier control for LIA4 and Es524 were 5.07 and 7.38 % d^{-1} respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$)

For the relative growth rate response (RGR) (**Figure 5.1**), exposures to the herbicides singly and in mixtures have caused significant reductions in both LIA4 and Es524. Exposure to the herbicides singly showed no significant difference was observed between the strains. However, mixture of DIU + TBA indicated that RGR in Es524 was

significantly higher compared to LIA4 with antagonistic and synergistic interactions were recorded in each strain respectively, indicating better tolerance of Es524 strain towards herbicides. For the DIU + IPU and TBA + IPU mixtures, synergistic and additive interactions were observed for each combination respectively in both strains, with no significant difference was observed between the strains, although fewer impacts were observed for the Es524 strain compared to LIA4. Interactions between the binary mixtures for the growth response in both strains are shown in Table 5.1 below.

Table 5.1 Percentage of reductions of relative growth rate (RGR) in LIA4 and Es524 in response to the binary combination of the herbicides

Endpoints	**LIA4**		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
Growth (RGR)	87.01 ± 12.2 *75.31 ± 10.6* (1.155 ± 0.06)	86.21 ± 13.1 *77.78 ± 11.4* (1.108 ± 0.08)	80.54 ± 10.9 *74.86 ± 8.7* (1.076 ± 0.08)
	Es524		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
	60.30 ± 9.4 *68.82 ± 7.9* (0.876 ± 0.10)	79.94 ± 11.5 *70.16 ± 10.3* (1.139 ± 0.11)	65.36 ± 10.2 *71.14 ± 9.8* (0.919 ± 0.11)

*Note: Regular font indicates the percentage of reduction experimentally measured (mean ± SD); ***italic*** font indicates the percentage of reduction predicted with Abbot's model (mean ± SD); and the values in brackets indicate the ratio of reduction (mean ± SD)

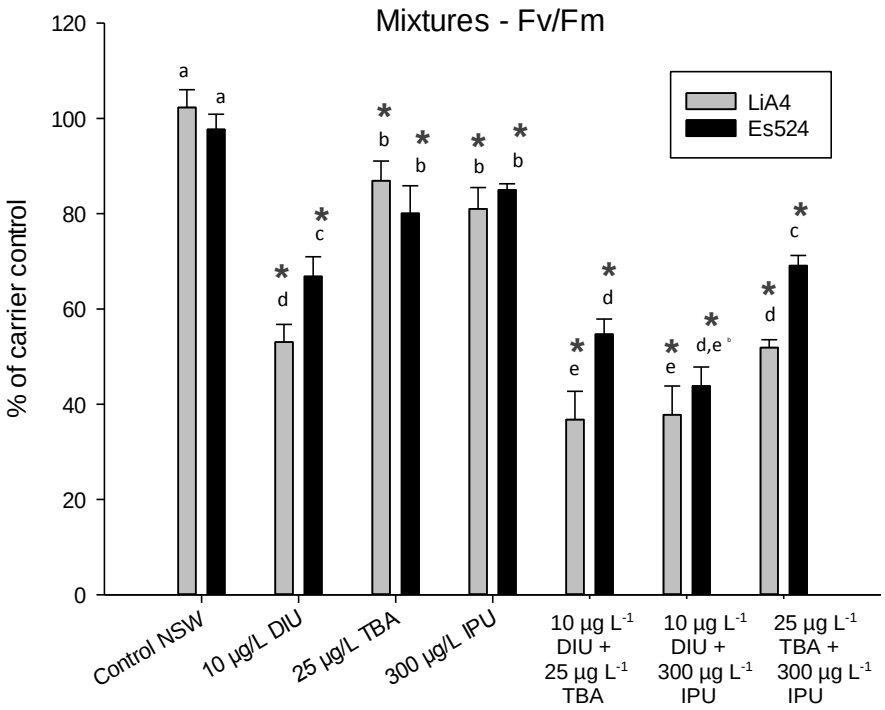

Figure 5.2: The effects of individual and binary combinations of herbicides on **Fv/Fm** in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean Fv/Fm values of the carrier control for LIA4 and Es524 were 0.708 and 0.685 respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$)

Significant decreases of maximal quantum yield (Fv/Fm) were observed for all treatment groups after exposure to the herbicides singly or in mixtures (**Figure 5.2**). In the results, Fv/Fm values in Es524 were significantly higher than LIA4 in response to the individual DIU, DIU + TBA and TBA + IPU treatments indicating higher tolerance of the strain compared to LIA4. DIU + TBA and TBA + IPU mixtures indicate antagonistic and additive interactions respectively in Es524, while synergistic interactions were observed in LIA4 for both combinations. For DIU + IPU mixture, synergistic interactions were observed in both strains. Table 5.2 shows the interactions of the binary mixtures for the Fv/Fm response.

Table 5.2 Percentage of inhibitions of Fv/Fm in LIA4 and Es524 in response to the binary combination of the herbicides

Endpoints	**LIA4**		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
Fv/Fm	63.22 ± 6.8 *51.29 ± 5.3* (1.233 ± 0.07)	62.21 ± 6.5 *54.59 ± 4.9* (1.140 ± 0.08)	48.13 ± 4.1 *29.56 ± 3.3* (1.628 ± 0.13)
	Es524		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
	45.29 ± 5.1 *52.78 ± 6.2* (0.858 ± 0.08)	56.18 ± 6.6 *49.88 ± 4.7* (1.126 ± 0.07)	30.88 ± 3.9 *31.94 ± 3.4* (0.967 ± 0.06)

*Note: Regular font indicates the percentage of inhibition experimentally measured (mean ± SD); ***italic*** font indicates the percentage of inhibition predicted with Abbot's model (mean ± SD); and the values in brackets indicate the ratio of inhibition (mean ± SD)

Similar to Fv/Fm, significant decreases of the effective quantum yield of PSII (Φ PSII) were observed for all treatment groups, with individual IPU showing the lowest decrease and DIU+IPU indicating the highest inhibition. No significant difference was observed between the strains except for the DIU + TBA and TBA + IPU mixtures, where significantly higher inhibitions were observed in LIA4 strain compared to Es524. Antagonistic interactions were recorded for both mixtures (DIU + TBA and TBA + IPU) in Es524 while LIA4 indicated synergistic interactions. Again, DIU + IPU mixture showed synergistic interactions in both strains. The interactions of the binary mixtures for the Φ PSII response are shown in Table 5.3.

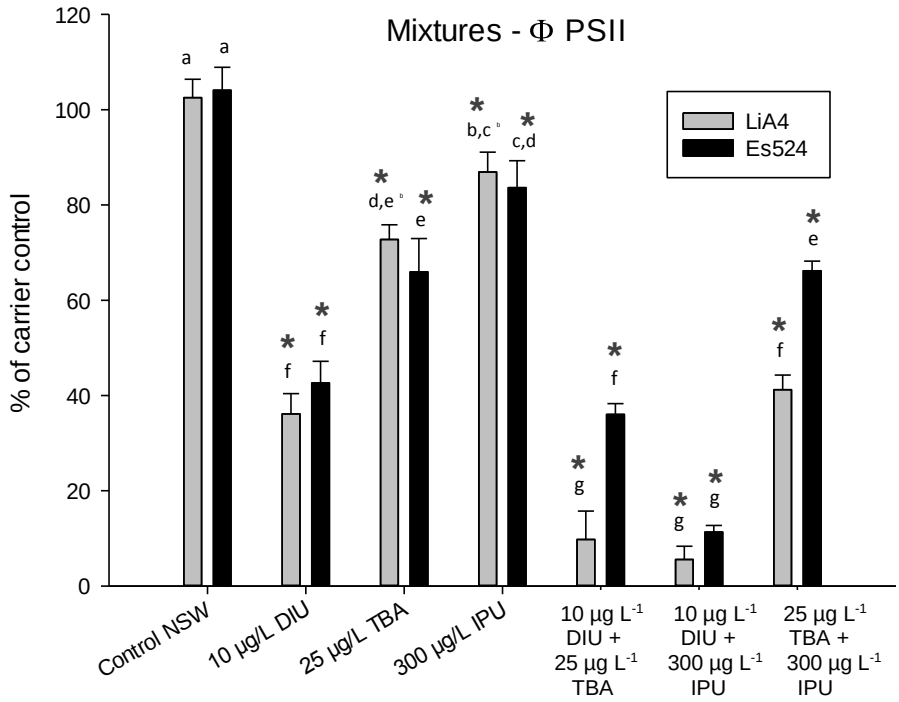

Figure 5.3: The effects of individual and binary combinations of herbicides on **ΦPSII** in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean ΦPSII values of the carrier control for LIA4 and Es524 were 0.672 and 0.653 respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$)

Table 5.3 Percentage of inhibitions of **ΦPSII** in LIA4 and Es524 in response to the binary combination of the herbicides

Endpoints	**LIA4**		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
ΦPSII	90.23 ± 8.3 *80.15 ± 7.4* (1.126 ± 0.08)	94.44 ± 7.6 *72.95 ± 7.1* (1.295 ± 0.11)	58.8 ± 5.9 *44.58 ± 4.2* (1.319 ± 0.10)
	Es524		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
	63.99 ± 4.7 *68.91 ± 4.4* (0.929 ± 0.06)	88.68 ± 4.5 *64.36 ± 4.9* (1.378 ± 0.13)	33.81 ± 3.8 *38.97 ± 4.4* (0.867 ± 0.09)

*Note: Regular font indicates the percentage of inhibition experimentally measured (mean ± SD); ***italic*** font indicates the percentage of inhibition predicted with Abbot's model (mean ± SD); and the values in brackets indicate the ratio of inhibition (mean ± SD)

Exposure to the herbicides singly and in mixtures caused a significant decrease of photochemical quenching (qP) in both strains (**Figure 5.4**). No significant difference was observed between the strains in response to the individual herbicide and DIU + IPU mixture. However, for the DIU + TBA and TBA + IPU mixtures, significantly higher inhibitions were observed in LIA4 strain compared to Es524 indicating lower tolerance of LIA4 strain to the mixtures. Additive and antagonistic interactions were recorded for the DIU + TBA and TBA + IPU respectively in Es524 strain, while synergistic interactions were observed for every binary mixtures tested in LIA4. Table 5.4 indicates the interactions of the binary mixtures for the qP response.

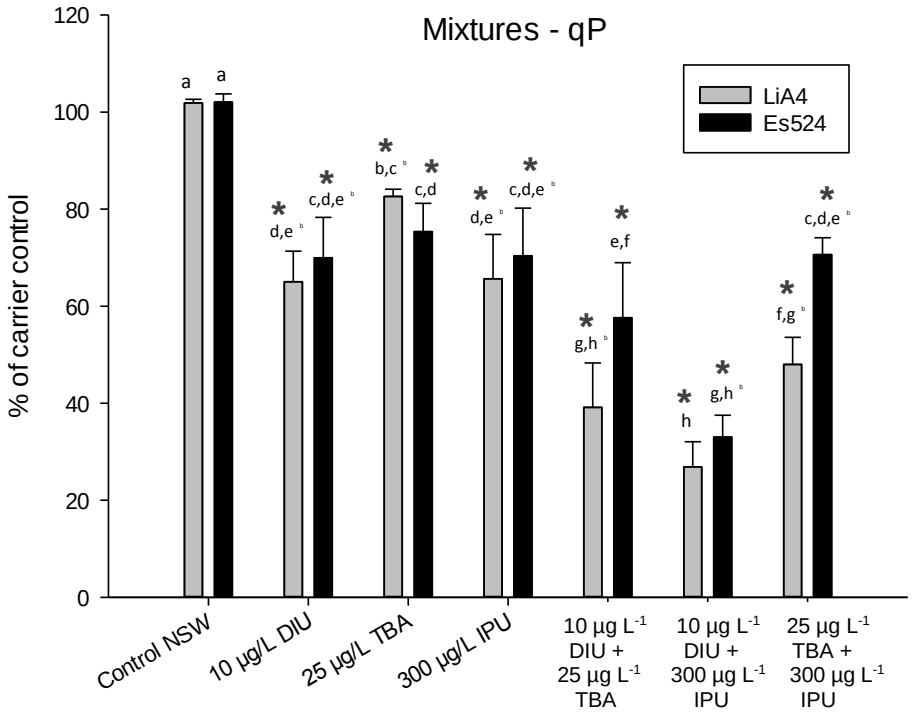

Figure 5.4: The effects of individual and binary combinations of herbicides on **qP** in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean qP values of the carrier control for LIA4 and Es524 were 0.647 and 0.623 respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$)

Table 5.4 Percentage of inhibitions of **qP** in LIA4 and Es524 in response to the binary combination of the herbicides

Endpoints	**LIA4**		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
qP	60.87 ± 8.4 *50.80 ± 7.1* (1.198 ± 0.08)	73.14 ± 6.1 *54.03 ± 5.8* (1.354 ± 0.15)	52.02 ± 6.4 *46.57 ± 5.5* (1.117 ± 0.07)
	Es524		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
	42.38 ± 8.1 *42.33 ± 6.7* (1.001 ± 0.08)	66.98 ± 5.8 *54.22 ± 4.9* (1.235 ± 0.09)	29.37 ± 4.4 *46.13 ± 5.2* (0.637 ± 0.2)

*Note: Regular font indicates the percentage of inhibition experimentally measured (mean ± SD); ***italic*** font indicates the percentage of inhibition predicted with Abbot's model (mean ± SD); and the values in brackets indicate the ratio of inhibition (mean ± SD)

An increase of non-photochemical quenching (qN) response was observed in both strains after 7 d of exposure to the herbicides singly and in mixtures. Single treatments indicate significant increases in LIA4 and Es524, but no significant difference was observed between the strains. As for the mixtures, DIU + TBA and TBA + IPU showed significant increases of qN in Es524 but not in LIA4 as compared to the carrier control. Antagonistic and additive interactions were recorded for each combination respectively in Es524 while in LIA4 both mixtures showed antagonistic interactions. DIU + IPU mixture indicate no significant change in either strain with antagonistic interactions recorded in both LIA4 and Es524.

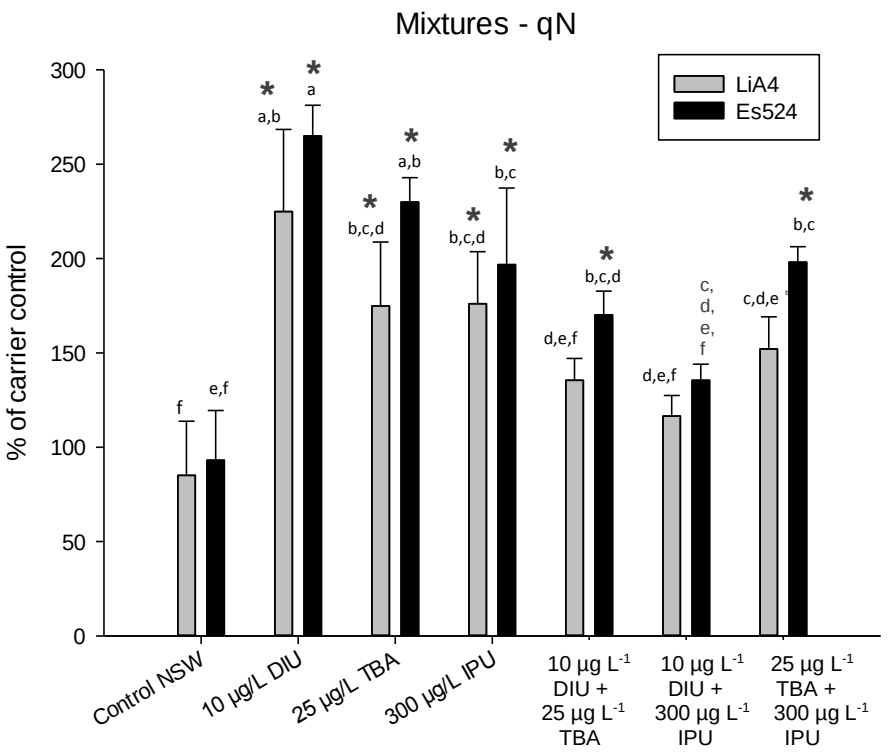

Figure 5.5: The effects of individual and binary combinations of herbicides on **qN** in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean qN values of the carrier control for LIA4 and Es524 were 0.058 and 0.052 respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$)

Table 5.5 Percentage of increase of **qN** in LIA4 and Es524 in response to the binary combination of the herbicides

Endpoints	**LIA4**		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
qN	35.50 ± 7.4 *106.25 ± 16.1* (0.334 ± 0.26)	16.45 ± 3.8 *105.97 ± 18.2* (0.155 ± 0.33)	52.12 ± 8.9 *94 ± 14.7* (0.554 ± 0.22)
	Es524		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
	70.24 ± 9.2 *80.56 ± 6.7* (0.872 ± 0.10)	35.52 ± 5.1 *102.08 ± 12.4* (0.348 ± 0.24)	98.08 ± 11.7 *100.96 ± 15.1* (0.972 ± 0.18)

*Note: Regular font indicates the percentage of increase experimentally measured (mean ± SD); ***italic*** font indicates the percentage of increase predicted with Abbot's model (mean ± SD); and the values in brackets indicate the ratio of increase (mean ± SD)

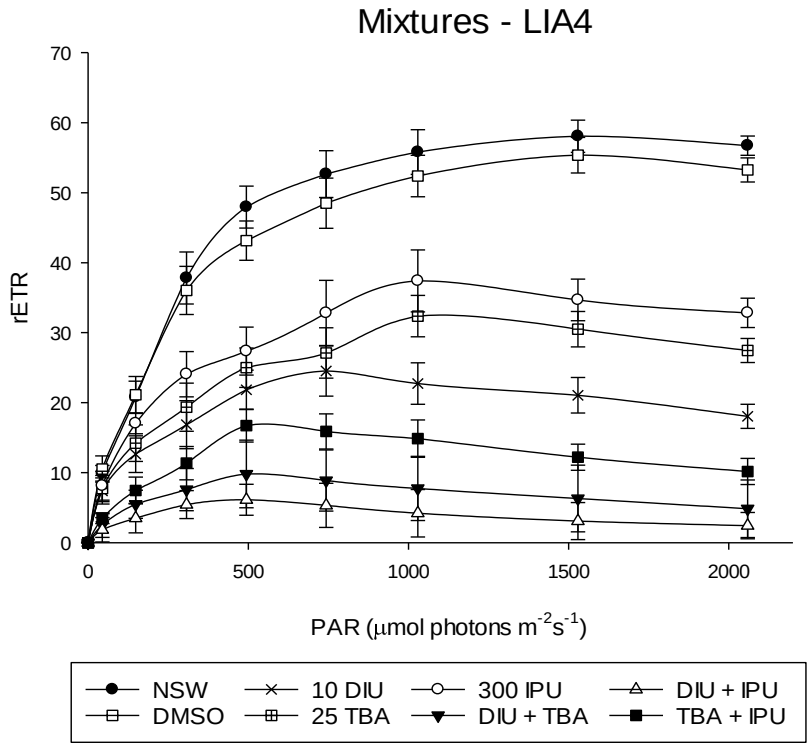

Figure 5.6 Relative electron transport rate (rETR) of LIA4 exposed to mixtures of herbicide for a period of 7 days. Means ± SD, n=4.

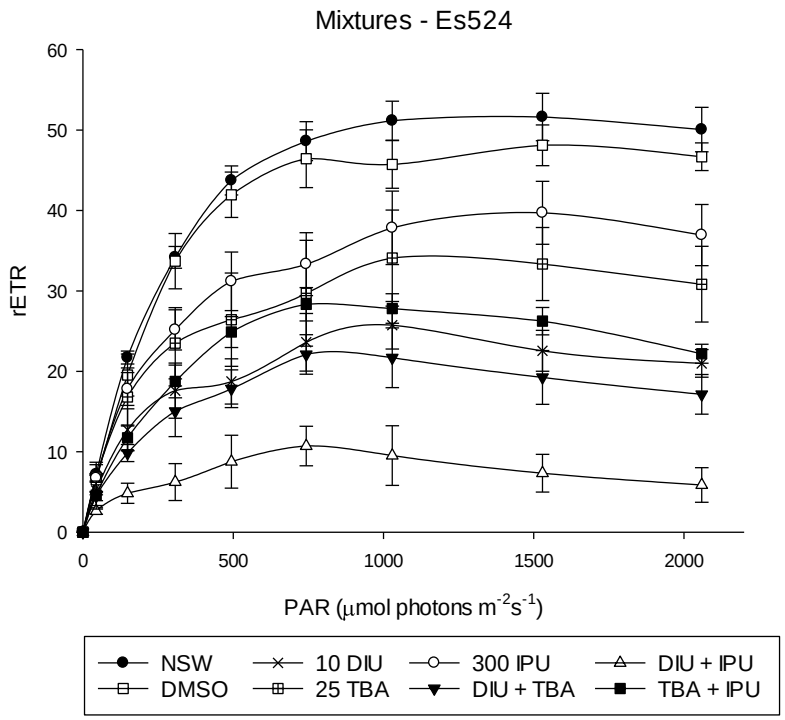

Figure 5.7 Relative electron transport rate (rETR) of Es524 exposed to mixtures of herbicide for a period of 7 days. Means ± SD, n=4.

Inhibition of electron transport and decrease in photosynthetic efficiency (α) were observed in both strains of *E. siliculosus*, in response to the herbicides singly and in mixtures. For the $rETR_{max}$, exposures to the herbicides singly have caused significant decreases as compared to the carrier control in both strains, except for the TBA and IPU singly in Es524. The effects of the combinations were more pronounced, with significant inhibitions were observed in both strains. Although no significant difference was recorded between the strains for the $rETR_{max}$ responses, exposures to the DIU + TBA and TBA + IPU mixtures indicated different interactions between the strains with LIA4 exhibited additive and synergistic interactions respectively, while Es524 showed antagonistic and additive interactions for the corresponding mixtures. For DIU + IPU mixture, synergistic interactions were observed in both strains. As for α, significant decreases as compared to the carrier control were observed in the LIA4 strain but not in Es524 when exposed to the herbicides singly. However, no significant difference was found between the strains when exposed to the herbicides singly or in mixtures. On the other hand, exposures to the different binary mixtures indicating significant decreases were recorded in both strains. For DIU + TBA mixture, synergistic and additive interactions were observed in LIA4 and Es524 respectively, while TBA + IPU mixture, additive interactions were recorded in both strains. Synergistic interactions were observed for the DIU + IPU mixture in both strains.

Variable	Treatments	*Ectocarpus siliculosus*	
		LIA4	**Es524**
$rETR_{max}$	NSW	57.60 ± 8.13 [a]	51.5 ± 7.59 [a,b]
	DMSO	53.90 ± 8.17 [a,b]	47.76 ± 7.33 [a,b,c]
	10 µg L^{-1} Diuron	21.56 ± 5.41 [d,e,f,g]	22.94 ± 5.95 [d,e,f,g]
	25 µg L^{-1} Terbuthylazine	29.64 ± 5.65 [d,e,f]	32.03 ± 6.96 [c,d,e]
	300 µg L^{-1} Isoproturon	34.50 ± 6.22 [c,d,e]	37.76 ± 7.26 [b,c,d]
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	7.54 ± 3.07 [g,h]	19.98 ± 10.20 [e,f,g,h]
	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	4.414 ± 2.98 [h]	8.365 ± 5.99 [g,h]
	25 µg L^{-1} TBA + 300 µg L^{-1} IPU	13.85 ± 4.09 [f,g,h]	26.48 ± 4.41 [d,e,f]
α	NSW	0.221 ± 0.023 [a,b]	0.207 ± 0.017 [a,b,c]
	DMSO	0.234 ± 0.024 [a]	0.195 ± 0.023 [a,b,c,d]
	10 µg L^{-1} Diuron	0.133 ± 0.032 [e,f,g]	0.143 ± 0.028 [d,e,f]
	25 µg L^{-1} Terbuthylazine	0.154 ± 0.031 [c,d,e]	0.156 ± 0.028 [c,d,e]
	300 µg L^{-1} Isoproturon	0.159 ± 0.019 [c,d,e]	0.164 ± 0.025 [b,c,d,e]
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	0.066 ± 0.023 [h,i]	0.118 ± 0.022 [e,f,g,h]
	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	0.046 ± 0.012 [i]	0.077 ± 0.010 [g,h,i]
	25 µg L^{-1} TBA + 300 µg L^{-1} IPU	0.092 ± 0.015 [f,g,h,i]	0.133 ± 0.030 [e,f,g]

Table 5.6 Photosynthetic efficiency of *E. siliculosus* exposed to different concentrations of herbicides singly and in mixtures over a period of 7 days. Data shown as means ± SD (*n*=4). Different letters represent significant differences between the strains (LIA4 and Es524) for each variable at $P < 0.05$.

Table 5.7 Percentage of inhibitions of **rETRmax and α** in LIA4 and Es524 in response to the binary combination of the herbicides

Endpoints	**LIA4**		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
$rETR_{max}$	86.01 ± 12.6 *77.99 ± 10.3* (1.103 ± 0.12)	91.81 ± 10.5 *74.39 ± 9.1* (1.234 ± 0.09)	74.30 ± 11.2 *64.82 ± 12.4* (1.147 ± 0.13)
α	71.79 ± 10.5 *62.59 ± 8.4* (1.147 ± 0.10)	80.34 ± 8.9 *61.38 ± 7.3* (1.309 ± 0.12)	60.68 ± 9.4 *55.28 ± 7.8* (1.098 ± 0.14)
	Es524		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
$rETR_{max}$	58.19 ± 8.3 *67.84 ± 8.6* (0.858 ± 0.11)	82.49 ± 9.2 *62.08 ± 7.3* (1.329 ± 0.15)	44.6 ± 6.5 *47.07 ± 8.8* (0.948 ± 0.14)
α	39.48 ± 5.4 *41.33 ± 5.1* (0.955 ± 0.12)	60.51 ± 6.3 *38.42 ± 4.4* (1.575 ± 0.16)	31.79 ± 5.2 *32.82 ± 4.5* (0.968 ± 0.13)

*Note: Regular font indicates the percentage of inhibition experimentally measured (mean ± SD); ***italic*** font indicates the percentage of inhibition predicted with Abbot's model (mean ± SD); and the values in brackets indicate the ratio of inhibition (mean ± SD)

5.3.2. Effects of DIU, TBA and IPU singly and in mixtures on oxidative stress of *E. siliculosus* strains

Figure 5.8 shows that exposure to the herbicides singly and in mixtures triggered the production of H_2O_2 in both strains. No significant difference was observed between LIA4 and Es524 strains in response to the herbicides exposure singly. However, unlike TBA (25 µg L^{-1}), DIU (10 µg L^{-1}) and IPU (300 µg L^{-1}) exposure singly have caused significant increases of H_2O_2 contents in LIA4 as compared to the control. Mixtures of DIU + TBA and TBA + IPU showed H_2O_2 contents in LIA4 was significantly higher compared to Es524, with synergistic interactions were recorded for both mixtures in LIA4. For Es524, the aforementioned mixtures indicated synergistic and antagonistic interactions respectively (Table 5.6). Significant increases of H_2O_2 content were observed in both strains in response to DIU + IPU mixture with additive and synergistic interactions in LIA4 and Es524, respectively.

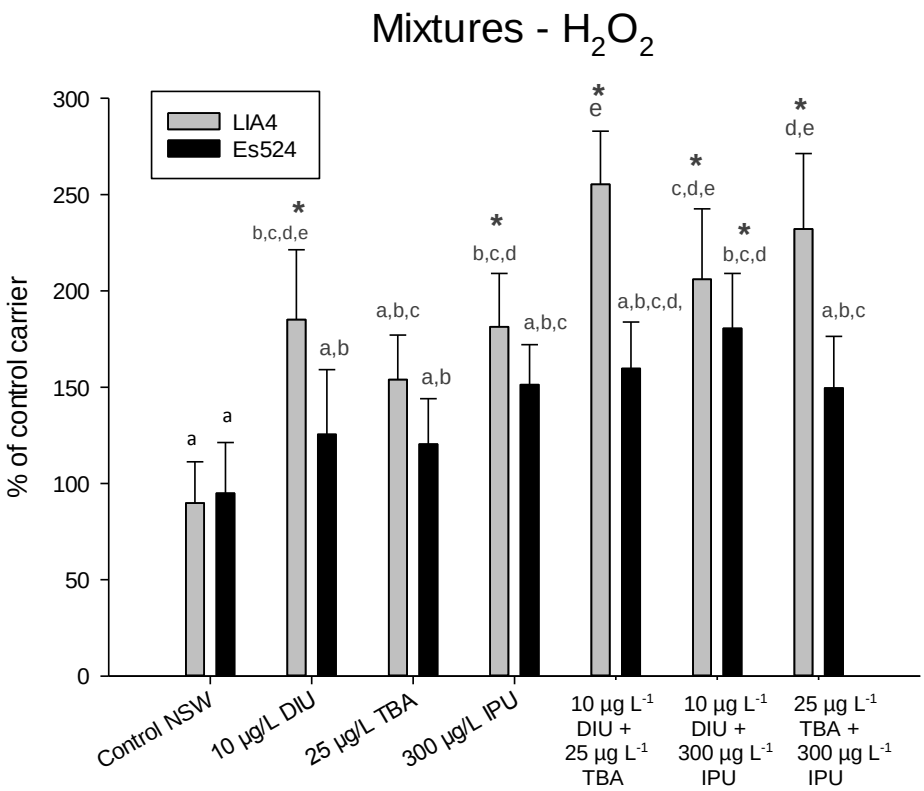

Figure 5.8: The effects of individual and binary combinations of herbicides on **H_2O_2** in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean H_2O_2 values of the carrier control for LIA4 and Es524 were 54.22 and 63.85 nmol g^{-1} wt tissue respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$)

Table 5.8 Percentage of increases of hydrogen peroxide (H_2O_2) in LIA4 and Es524 in response to the binary combination of the herbicides

Endpoints	**LIA4**		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
H_2O_2	155.35 ± 16.8 *93.15 ± 10.9* (1.668 ± 0.24)	106.05 ± 18.8 *97.23 ± 11.4* (1.091 ± 0.16)	132.09 ± 22.2 *91.43 ± 10.5* (1.445 ± 0.31)
	Es524		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
	59.72 ± 9.1 *40.65 ± 6.4* (1.469 ± 0.25)	80.56 ± 12.7 *63.77 ± 9.8* (1.263 ± 0.20)	49.54 ± 8.9 *61.29 ± 9.2* (0.808 ± 0.17)

*Note: Regular font indicates the percentage of increase experimentally measured (mean ± SD); ***italic*** font indicates the percentage of increase predicted with Abbot's model (mean ± SD); and the values in brackets indicate the ratio of increase (mean ± SD)

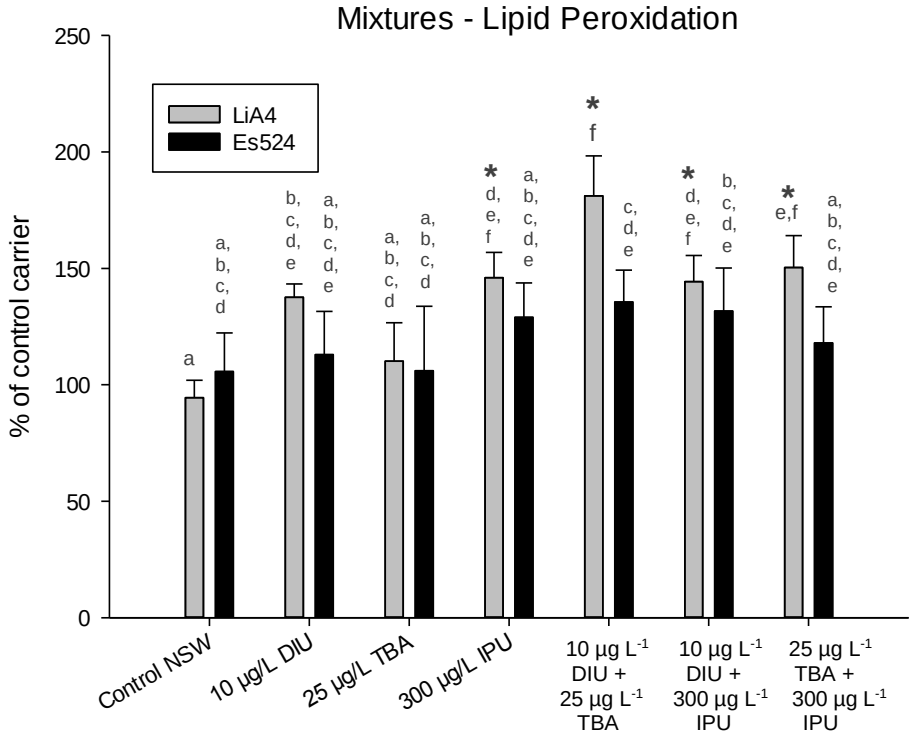

Figure 5.9: The effects of individual and binary combinations of herbicides on **lipid peroxidation** in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=4). Mean TBARS values of the carrier control for LIA4 and Es524 were 6.81 and 7.63 nmol g^{-1} wt tissue respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$)

Evaluation of lipid peroxidation (**Figure 5.9**) indicated no significant change in the Es524 strain in response to the herbicides singly or in mixtures. In contrast, significant increases of lipid peroxidation levels were observed in LIA4 strain against the DIU and IPU exposure singly and all the binary combinations tested. Significant difference between LIA4 and Es524 was observed only for the DIU + TBA mixture, with the lipid peroxidation level significantly lower in Es524 compared to LIA4. For DIU + TBA mixture, both strains showed synergistic interactions for the lipid peroxidation response,

while DIU + IPU mixture, antagonistic interactions were observed in both strains. Additive and antagonistic interactions were recorded in LIA4 and Es524 respectively in response to the TBA + IPU mixture.

Table 5.9 Percentage of increases of lipid peroxidation in LIA4 and Es524 in response to the binary combination of the herbicides

Endpoints	**LIA4**		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
Lipid peroxidation	81.18 ± 7.7 *43.94 ± 4.6* (1.847 ± 0.26)	44.36 ± 3.4 *66.35 ± 5.9* (0.669 ± 0.24)	50.42 ± 4.6 *51.48 ± 5.4* (0.979 ± 0.19)
	Es524		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
	35.59 ± 3.6 *18.27 ± 2.3* (1.947 ± 0.32)	31.7 ± 4.5 *38.27 ± 4.2* (0.828 ± 0.15)	18.05 ± 2.3 *33.33 ± 4.7* (0.542 ± 0.23)

*Note: Regular font indicates the percentage of increase experimentally measured (mean ± SD); ***italic*** font indicates the percentage of increase predicted with Abbot's model (mean ± SD); and the values in brackets indicate the ratio of increase (mean ± SD)

5.3.3 Effects of DIU, TBA and IPU singly and in mixtures on antioxidative defense mechanisms (enzymatic and non-enzymatic) of *E. siliculosus* strains

Figure 5.10 shows CAT activity in both strains was induced in response to the herbicides exposure. Single exposure to TBA (25 µg L^{-1}) significantly increases CAT activity in both strains, while for DIU (10 µg L^{-1}) no significant change was observed. Exposure to IPU singly (300 µg L^{-1}) and mixtures of DIU + TBA and TBA + IPU showed CAT activity in Es524 was significantly higher compared to LIA4. Antagonistic and synergistic interactions were observed in LIA4 and Es524 strain respectively for CAT response to both mixtures (DIU + TBA and TBA + IPU). Exposure to DIU + IPU mixture, on the other hand, showed no significant changes of CAT activity with antagonistic interactions were observed in both strains.

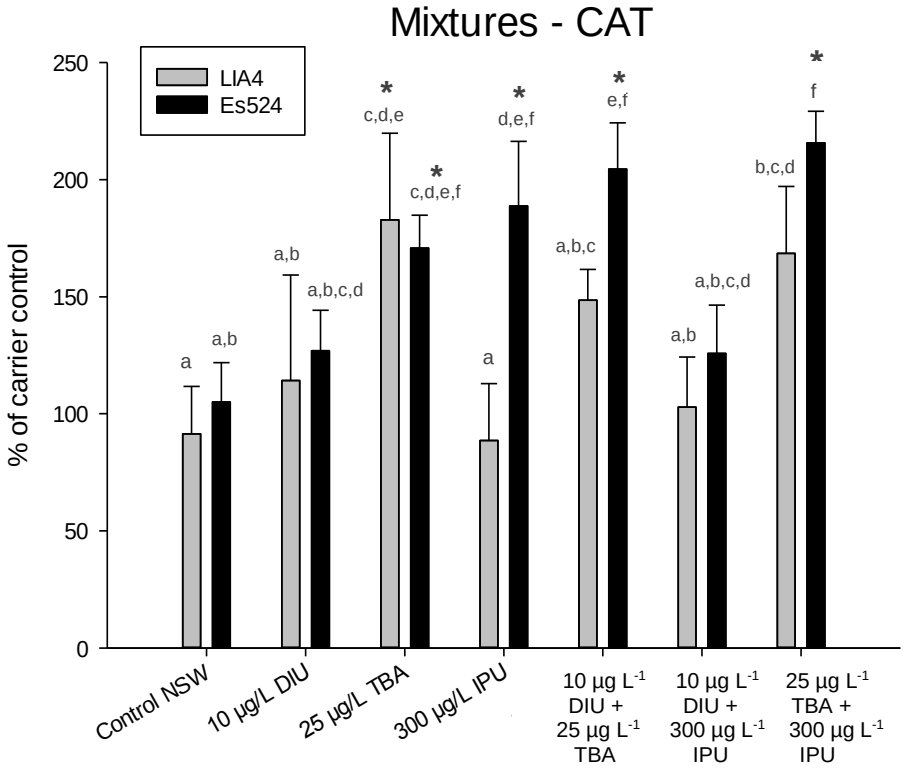

Figure 5.10: The effects of individual and binary combinations of herbicides on **catalase** activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Mean CAT activity of the carrier control for LIA4 and Es524 were 35.74

and 43.12 µmol mg^{-1} protein min^{-1} respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$)

Table 5.10 Percentage of changes of catalase (CAT) activity in LIA4 and Es524 in response to the binary combination of the herbicides

Endpoints	**LIA4**		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
Catalase (CAT)	48.57 ± 4.3 *85.31 ± 9.2* (0.569 ± 0.28)	2.86 ± 0.59 *4.49 ± 0.82* (0.636 ± 0.17)	68.57 ± 11.6 *80.9 ± 8.1* (0.848 ± 0.14)
	Es524		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
	104.49 ± 10.3 *78.66 ± 8.9* (1.328 ± 0.18)	25.84 ± 4.3 *91.79 ± 10.1* (0.282 ± 0.35)	115.73 ± 7.2 *96.72 ± 9.3* (1.197 ± 0.17)

*Note: Regular font indicates the percentage of change experimentally measured (mean ± SD); ***italic*** font indicates the percentage of change predicted with Abbot's model (mean ± SD); and the values in brackets indicate the ratio of change (mean ± SD)

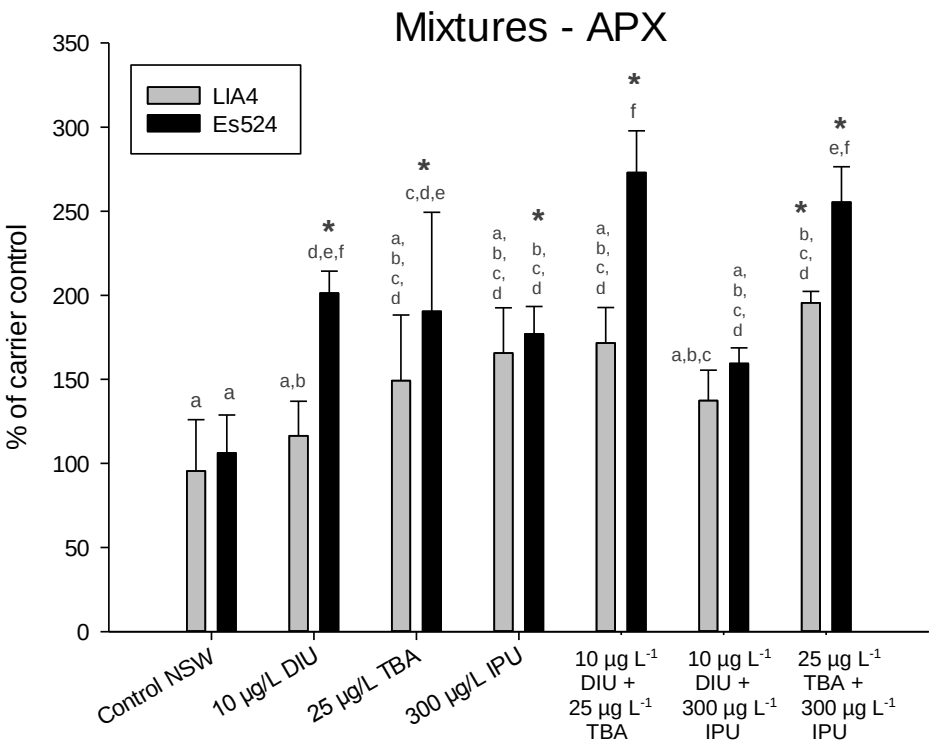

Figure 5.11: The effects of individual and binary combinations of herbicides on **ascorbate peroxidase** activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Mean APX activity of the carrier control for LIA4 and Es524 were 0.46 and 0.52 µmol mg^{-1} protein min^{-1} respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$)

Exposures to the herbicides singly and in mixtures have stimulated APX activity in both LIA4 and Es524 strains (**Figure 5.11**). However, in contrast to LIA4, exposure to herbicides singly caused significant increases of APX activity in Es524 strain, but not in LIA4 as compared to the control. At 10 µg L^{-1} of DIU and binary mixtures of DIU + TBA and TBA + IPU, APX activity in Es524 was significantly higher compared to LIA4. Synergistic and additive interactions were observed for the aforementioned

mixtures in LIA4 respectively, while for Es524, synergistic interactions were recorded for both combinations. As for DIU + IPU mixture, antagonistic interactions were observed in both strains with no significant change of APX activity recorded.

Table 5.11 Percentage of changes of ascorbate peroxidase (APX) activity in LIA4 and Es524 in response to the binary combination of the herbicides

Endpoints	**LIA4**		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
Ascorbate peroxidase (APX)	71.64 ± 8.8 *57.59 ± 6.7* (1.244 ± 0.19)	37.31 ± 4.9 *71.31 ± 5.8* (0.523 ± 0.17)	95.52 ± 3.4 *82.58 ± 4.2* (1.157 ± 0.16)
	Es524		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
	172.97 ± 15.8 *100.13 ± 10.4* (1.728 ± 0.29)	59.46 ± 3.5 *100.31 ± 7.1* (0.593 ± 0.16)	155.41 ± 12.8 *97.83 ± 13.3* (1.589 ± 0.24)

*Note: Regular font indicates the percentage of change experimentally measured (mean ± SD); ***italic*** font indicates the percentage of change predicted with Abbot's model (mean ± SD); and the values in brackets indicate the ratio of change (mean ± SD)

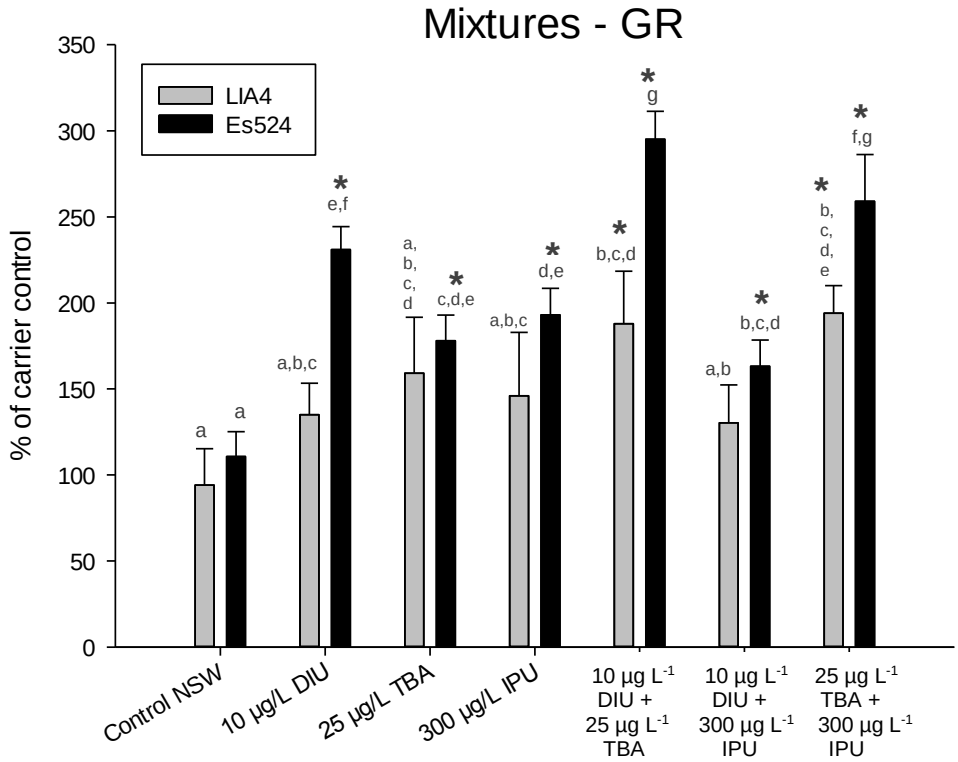

Figure 5.12: The effects of individual and binary combinations of herbicides on **glutathione reductase** activity in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Data shown as means ± SD (*n*=3). Mean GR activity of the carrier control for LIA4 and Es524 were 204 and 243 nmol mg^{-1} protein min^{-1} respectively. Values with an asterisk are significantly different at $P < 0.05$ compared to carrier control. Data sets which are significantly different from each other are represented by different letters ($P < 0.05$)

Poor inductions of GR activity in LIA4 compared to Es524 were observed in response to the herbicides singly and in mixtures (**Figure 5.12**). Exposure to the herbicides singly caused significant increases of GR activity in Es524 but not in LIA4, with significant differences between the strains observed at 10 µg L^{-1} DIU and 300 µg L^{-1} IPU. Mixtures of DIU + TBA and TBA + IPU showed GR activity in Es524 was significantly higher

compared to LIA4 with synergistic interactions were recorded for both combinations in both strains. Instead, the DIU + IPU mixture indicated antagonistic interactions in both strains, but a significant increase was observed only in Es524 strain.

Table 5.12 Percentage of changes of glutathione reductase (GR) activity in LIA4 and Es524 in response to the binary combination of the herbicides

Endpoints	**LIA4**		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
Glutahione reductase (GR)	87.96 ± 14.3 *73.49 ± 9.2* (1.197 ± 0.24)	30.23 ± 5.1 *64.91 ± 7.9* (0.466 ± 0.21)	94.03 ± 7.8 *77.96 ± 8.5* (1.206 ± 0.22)
	Es524		
	10 µg L^{-1} DIU + 25 µg L^{-1} TBA	10 µg L^{-1} DIU + 300 µg L^{-1} IPU	25 µg L^{-1} TBA + 300 µg L^{-1} IPU
	195.12 ± 10.7 *106.81 ± 8.3* (1.827 ± 0.28)	63.24 ± 5.8 *102.16 ± 7.7* (0.619 ± 0.20)	159.06 ± 16.7 *98.47 ± 9.2* (1.615 ± 0.31)

*Note: Regular font indicates the percentage of change experimentally measured (mean ± SD); ***italic*** font indicates the percentage of change predicted with Abbot's model (mean ± SD); and the values in brackets indicate the ratio of change (mean ± SD)

In **Table 5.13,** evaluations on non-enzymatic antioxidant response showed exposure to the herbicides singly and in mixtures have induced distinct tolerance level between LIA4 and Es524 to certain treatments. DIU (10 µg L^{-1}) and TBA (25 µg L^{-1}) singly showed no significant change of the polyphenolic compound in both strains (LIA4 and Es524) as compared to the carrier control. However, exposure to IPU (300 µg L^{-1}) singly has induced significant increases of rutin in both strains, while for phloroglucinol (PG) a significantly higher amount was observed in Es524 compared to LIA4. As for caffeic acid (CA), a similar treatment (300 µg L^{-1} IPU) indicated no significant change in Es524, while in LIA4 it was not detected.

For the mixtures, no significant change of rutin and caffeic acid were observed in response to DIU + TBA mixture with antagonistic interactions were recorded in both LIA4 and Es524 strains. On the other hand, significant increase of PG was observed in both strains, which was significantly higher in Es524 compared to LIA4. Additive and synergistic interactions were recorded in LIA4 and Es524 respectively for the PG response towards DIU + TBA mixture. For the DIU + IPU mixture, significant decreases of PG and CA were observed in both strains, with additive and antagonistic interactions were recorded in LIA4 and Es524 respectively for PG, while for CA, antagonistic interactions were recorded in both strains. No significant change was observed for rutin, with antagonistic interactions were observed in both strains. Exposure to TBA + IPU mixture indicated significant increases of PG in both strains, while for rutin only in Es524. Additive and antagonistic interactions were observed for the PG and rutin respectively in LIA4, while synergistic and antagonistic interactions were noticed in Es524. No significant change of CA was observed in both strains in response to the TBA + IPU mixture, with antagonistic and additive interactions were recorded in LIA4 and Es524 respectively.

Single / Mixture	Polyphenols (µg g^{-1} FW)					
	Rutin		Phloroglucinol		Caffeic acid	
	Es524	LIA4	Es524	LIA4	Es524	LIA4
NSW	123.9 ± 13.8^{bcd}	101.4 ± 13.5^{def}	351.3 ± 22.6^{efgh}	314.7 ± 46.8^{ghi}	23.5 ± 4.2^{ab}	17.4 ± 3.8^{abcd}
DMSO	115.2 ± 15.4^{cde}	97.8 ± 13.1^{def}	369.2 ± 16.7^{efg}	327.5 ± 39.5^{fgh}	19.1 ± 3.1^{abc}	15.5 ± 3.3^{abcde}
10 µg L^{-1} DIU	126.4 ± 18.4^{bcd}	109.1 ± 15.4^{cde}	398.6 ± 23.7^{cdef}	350.2 ± 41.3^{efgh}	23.9 ± 3.4^{a}	18.1 ± 3.6^{abcd}
25 µg L^{-1} TBA	140.2 ± 19.2^{abcd}	122.6 ± 16.7^{cd}	409.5 ± 41.4^{bcde}	372.8 ± 50.2^{efg}	22.2 ± 3.2^{ab}	16.4 ± 2.9^{abcd}
300 µg L^{-1} IPU	186.2 ± 21.6^{a}	150.4 ± 20.5^{abc}	471.3 ± 52.7^{abc}	394.2 ± 47.4^{def}	11.3 ± 3.8^{cde}	n.d ^{f}
10 µg L^{-1} DIU + 25 µg L^{-1} TBA	129.5 ± 24.8^{bcd}	113.6 ± 15.2^{cde}	480.2 ± 40.5^{a,b}	405.7 ± 42.6^{cde}	14.9 ± 3.7^{bcde}	10.8 ± 2.4^{cde}
10 µg L^{-1} DIU + 300 µg L^{-1} IPU	73.7 ± 13.6^{ef}	62.9 ± 10.4^{f}	285.4 ± 36.7^{h,i}	251.3 ± 34.9^{i}	7.5 ± 2.1^{ef}	n.d ^{f}
25 µg L^{-1} TBA + 300 µg L^{-1} IPU	169.1 ± 15.6^{ab}	131.7 ± 19.3^{bcd}	533.8 ± 42.7^{a}	449.6 ± 53.1^{bcd}	15.8 ± 3.8^{abcde}	9.7 ± 3.4^{de}

n.d.= not detected, ++ = trace amount

Table 5.13: The effects of DIU, TBA and IPU singly and in mixtures on polyphenolic compounds in two strains (Es524 and LIA4) of *Ectocarpus siliculosus* after 7 days of exposure. Results shown as means ± SD (n=3). Data sets which are significantly different from each other for each polyphenolic compounds are represented by different letters (P< 0.05).

5.5 Discussion

Applications of herbicides in mixture are common nowadays. Due to the wider effects that can be expected from the mixtures, herbicides can be deliberately or unintentionally released into the environment in multiple combinations. In fact, in the environment, organisms are rarely exposed to only one xenobiotic at a time, which make evaluation of xenobiotics in mixtures is essential especially in the field of ecotoxicology. Evaluations of mixture effects in terms of additivity, synergisms or antagonisms, depends crucially on definitions of what the expected effect of a mixture should be (Payne *et al*., 2000). If the observed effects are stronger than expected, there is said to be synergism; likewise, if they are weaker, there is antagonism. When expectations are met, the combination effect can be called additive (Berenbaum, 1989). In the present study, the effects of DIU, TBA and IPU were assessed on two different strains of *E. siliculosus* with different pollution histories in order to determine the interactions of the herbicides on different endpoints when exposed in binary combinations.

Exposure of LIA4 and Es524 strains to 10 µg L^{-1} DIU, 25 µg L^{-1} TBA and 300 µg L^{-1} IPU indicated that the herbicides were toxic on both strains. These values represent 40 - 60% effects of the individual herbicides on the growth rate of *E. siliculosus* strains. The inhibition values obtained from our studies were within the range of previously reported on microalgae and aquatic plants realized with DIU, IPU or other triazines (Hatgers *et al*., 1998, *Selenastrum capricornutum*, EC_{50} DIU = 15 µg L^{-1}; Teisseire *et al*., 1999, *L. minor*, IC_{50} DIU = 25 µg L^{-1}; Vallotton *et al*., 2009, *Scenedesmus vacuolatus*, EC_{50} Isoproturon = 164 µg L^{-1}; Leboulanger *et al*., 2001, *Chlorella vulgaris*, EC_{50} Atrazine = 42 µg L^{-1}). No significant difference on the relative growth rate (RGR) was observed between the strains after been exposed to the herbicides singly. However, exposure to the TBA + IPU and DIU

+ TBA mixtures showed that growth inhibitions were higher in LIA4 strain compared to Es524, with latter mixture indicated significant difference between the strains. Additive and antagonistic interactions were observed for the mixtures in Es524 respectively, while in LIA4 additive and synergistic interactions were respectively recorded. For DIU + IPU mixture, synergistic interactions were observed in both stains. The lesser effect (antagonistic interaction) observed for the DIU + TBA mixture on the growth of Es524 strain showed that the effect of the mixture was significantly lower compared to LIA4. Reduction of growth as much as 87% was observed in LIA4, while 60.3% was seen in Es524 as compared to the carrier control. The antagonistic interactions of DIU with different kind of herbicides and even metals have been reported by several researchers. For instance, Ohki *et al*., (1997) reported a decrease of phytotoxicity of chlorophtalim in the presence of DIU, while Nicolaus *et al*., (1989) observed a similar antagonistic interaction between DIU and oxyfluorfen. In fact, this protective effect of the phenylurea was also found in a binary mixture with copper (Teisseire *et al*., 1999). We speculate that the different interactions observed between LIA4 and Es524 to the DIU + TBA mixture are probably due to the different homeostasis mechanisms possessed by each strain to ameliorate the effect of the mixture.

Evaluations on different photosynthetic efficiency indicators revealed that exposures to the herbicides singly and in mixtures have caused detrimental effects on both strains of *E. siliculosus*. For the maximal quantum yield (**Fv/Fm**), significant decrease was observed in both strains after exposure to the herbicides singly, with DIU showing the highest inhibition compared to TBA and IPU. Exposure to the DIU singly and mixtures of DIU + TBA and TBA + IPU indicated significantly higher inhibitions were recorded in LIA4 compared to Es524, signifying higher tolerance of the strain originated from polluted

environment. Synergistic interactions were observed for all the binary mixtures in LIA4 strain and DIU + IPU mixture in Es524. In contrast, antagonistic and additive effects were recorded for the DIU + TBA and TBA + IPU mixtures respectively in Es524.

The effects of the herbicides on effective quantum yield of photosystem II (Φ PSII) showed very similar responses to the Fv/Fm, except for the individual DIU treatment (10 µg L^{-1}) where no significant difference was observed between the strains. Synergistic interactions were recorded for all the mixtures in LIA4 strain, while in Es524, only DIU + IPU mixture exhibits the same interaction. DIU + TBA and TBA + IPU mixtures on the other hand showed antagonistic interactions in Es524 strain.

As for the photochemical quenching (qP), significant decreases were observed in both strains in response to the herbicides singly and in mixtures. Significant differences between LIA4 and Es524 were recorded for the DIU + TBA and TBA + IPU mixtures, where stronger inhibitions were observed in LIA4 compared to Es524. Unlike qP, non-photochemical (qN) quenching response in both strains indicated no significant difference between the strains after exposure to the herbicides singly and in mixtures. For LIA4 strain, all the binary combinations showed synergistic interactions for the photochemical quenching response, while antagonistic interactions were observed for the non-photochemical quenching. Photochemical quenching (qP) response in Es524 on the other hand showed additive, synergistic and antagonistic interactions for the DIU + TBA, DIU + IPU and TBA + IPU mixtures respectively, while the non-photochemical quenching exhibited antagonistic, antagonistic and additive effects for the corresponding mixtures respectively.

In addition, inhibitions of electron transport measured through the $rETR_{max}$ and α indicate significant decreases of $rETR_{max}$ in both strains in response to the herbicides singly

and in mixtures. Although no significant difference was observed between the strains, different interactions were exhibited in each strain for the DIU + TBA (additive,LIA4; antagonis,Es524) and TBA + IPU (synergy,LIA4; additive,Es524) mixtures. As for α, exposure to the herbicides singly or in mixture showed no significant difference between the strains. However, exposures to the herbicides singly have significant effect on LIA4, but not in Es524, probably indicating higher tolerance of Es524. On the other hand, significant decreases of α were observed in both strains in response to the mixtures, with DIU + TBA and TBA + IPU mixtures exhibiting synergistic and additive interactions respectively in LIA4 while additive interactions were observed for both in Es524.

Collectively, evaluations on various photosynthetic efficiency indicators showed higher impacts of DIU + TBA and TBA + IPU mixtures on LIA4 strain compared to Es524. The distinct tolerance displayed by LIA4 and Es524 could be contributed by the different adaptations mechanisms employed by the seaweed. Therefore, further investigations on biochemical activities of the seaweeds were carried out in order to elucidate the intrinsic metabolism that lead to the difference tolerance between the strains. Previously, there have been studies on the effect of herbicides singly and in mixtures, however, most of it were restricted to parameters such as growth and photosynthesis activity with only few researchers have investigate the biochemical responses in such a case (Geoffroy *et al*., 2002; Mofeed and Mosleh, 2013). Therefore, in the present study, the different responses/interactions observed between LIA4 and Es524 strains to DIU + TBA and TBA + IPU mixtures might linked to their unique adaptation mechanisms which make biochemical evaluations in response to the binary mixtures are desirable.

Unlike Es524, further investigations on oxidative stress revealed that exposure to DIU (10 µg L^{-1}) and IPU (300µg L^{-1}) singly have induced significant increases of H_2O_2

content in LIA4 as compared to the carrier control. In contrast, no significant change was observed in both strains in response to TBA at 25 µg L^{-1}. Exposure to the herbicides in mixtures showed significant difference between the strains were observed for the DIU + TBA and TBA + IPU mixtures, with H_2O_2 content in LIA4 was found to be significantly higher compared to Es524. Synergistic and antagonistic interactions were recorded for the aforementioned mixtures respectively in Es524 while in LIA4 both mixtures have showed synergistic interactions. DIU + IPU mixture on the other hand indicated significant increase of H_2O_2 but no significant difference was recorded between the strains, with additive and synergistic interactions were observed in LIA4 and Es524 respectively. Moreover, lipid peroxidation levels indicated similar responses in both strains when exposed to the herbicides singly, with a significant increase observed only in LIA4 at 300 µg L^{-1} of IPU. For the mixtures, significant increase were observed in LIA4 but not in Es524, with significant difference between the strains was recorded against the DIU + TBA mixture. Synergistic and antagonistic interactions were recorded for the DIU + TBA and DIU + IPU mixtures respectively in both strains, while TBA + IPU showed additive and antagonistic interactions in LIA4 and Es524, respectively.

The effects on antioxidative enzyme activities showed distinct responses between LIA4 and Es524 for certain treatments that lead to different tolerance between the strains. Our results indicated that DIU + TBA and TBA + IPU mixtures act synergistically on the antioxidative enzyme activities in Es524 strain by promoting stimulation of the radical scavengers. CAT, APX and GR activities were observed to be significantly higher compared to LIA4 strain which linked to the higher tolerance of Es524 strain to the herbicide mixtures. On the other hand, in LIA4, DIU + TBA and TBA + IPU mixtures showed antagonistic interactions for CAT response which may indicate suppression of the

enzyme activity. CAT known to play an important role in detoxification of H_2O_2, therefore poor stimulation of the enzyme in response to the DIU + TBA and TBA + IPU mixtures have reduced the ability of LIA4 to cope with ROS. Significant increases of lipid peroxidation observed in LIA4 in response to the mixtures may due to the peroxidation of membranes and pigments due to an overproduction of ROS (Geoffroy *et al*., 2002). Besides, similar mixtures (DIU + TBA and TBA + IPU) act synergistically and additively in LIA4 on APX activity respectively, while GR activity indicated additive interactions for both mixtures. For DIU + IPU mixture, both strains indicate antagonistic interactions for all the antioxidative enzyme activities, probably due to the damage to the enzymes protein. The higher tolerance of Es524 against DIU + TBA and TBA + IPU mixtures compared to LIA4 may due to the efficient and higher antioxidative enzymes stimulation in Es524 strain. The additive interactions recorded for the aforementioned mixtures on GR and APX activities may indicate inhibition or lesser production of the antioxidative enzymes in LIA4 strain.

With respect to non-enzymatic antioxidant responses, analysis of polyphenols indicates that exposures to DIU and TBA singly have no significant effects on polyphenol content in both strains. However, IPU singly has caused significant increase of rutin in both strains and phloroglucinol level was significantly higher in Es524 compared to LIA4. The distinct responses between LIA4 and Es524 towards the DIU + TBA and TBA + IPU mixtures mentioned earlier were also observed on the polyphenols level. For DIU + TBA mixture, although no significant change of rutin or caffeic acid was observed in both strains, the phloroglucinol level has increase significantly which was significantly higher in Es524 compared to LIA4. As for TBA + IPU mixture, significant increases of phloroglucinol and rutin were observed in Es524 while for LIA4 only phloroglucinol level

was observed to increase significantly in response to the mixture. It is suggested that the different tolerance between the strains could also be contributed by the different content of polyphenols stimulated by the mixtures. On the other hand, the highest impacts observed for the DIU + IPU mixture could be linked to the significant decreases of phloroglucinol and caffeic acid in both LIA4 and Es524.

Overall, our results contradict those reported by other authors for similarly acting compounds. For instance, Arrhenius *et al*., (2004) investigated the joint effects of DIU, linuron and monolinuron in a mixture with nine other phenylureas on the photosynthetic activity of marine microalgal communities and reported that the mixture toxicity is well predicted using the concentration addition concept. Pesce *et al*., (2010) also found that DIU in combination with its metabolite DCPMU, which also acts in the same way as DIU and other PSII herbicides, indicated additive effects on natural phototrophic biofilm communities. However, all three types of interactions (synergistic, additive, antagonistic) observed through different endpoints in the present study were in line with a recent study by Gatidou *et al*., (2015) where various interactions were recorded between phenylureas (DIU, linuron, monolinuron) in binary combinations on *Vibrio fischeri*. In fact, the different interaction observed between LIA4 and Es524, is also in agreement to previous report by Liu *et al*., (2013) in two different species; *Chlorella pyrenoidosa* and *Vibrio qinghaiensis* exposed to different combinations of pesticides (simetryn, bromacil, hexazinone, dodine, propoxur, metalaxyl). In their findings, they have concluded that the interaction between similar compounds is closely related to the test organism, where the interaction conclusion obtained in an indicator organism cannot be extrapolated into the other organism. Nevertheless, they did not elucidate the reason why some binary pesticide mixtures produce antagonism to *C. pyrenoidosa* but synergism to *V. qinghaiensis*.

From our studies, it is clear that the different interactions observed for the DIU + TBA and/or TBA + IPU mixtures on growth and various photosynthetic efficiency responses (Fv/Fm, ΦPSII, qP, $rETR_{max}$) between the strains could be explained by the higher inductions of antioxidative defense mechanisms either enzymatic (CAT, APX , GR) or non-enzymatic (antioxidant compounds) in Es524. In fact, the stimulations have hardened Es524 strain against oxidative stress generated by the herbicides. Moreover, biochemical responses were also proved to be sensitive to the herbicides in the present study since DIU and TBA as low as 10 and 25 µg L^{-1} respectively were enough to significantly stimulate the activity of APX and GR enzymes in Es524. Previous studies on aquatic animals (Di Giulio *et al*., 1989; Doyotte *et al*., 1997) and plants (Subhadra *et al*., 1991; Teisseire *et al*., 1998) also showed high sensitivity of biochemical responses for ecotoxicological studies.

5.6 Conclusion

Our results show that the higher tolerance of Es524 to DIU + TBA and TBA + IPU mixtures compared to LIA4 was attributable to a strengthening of antioxidative defenses in response to the combinations. These effects probably due to the mixtures have triggered the production of ROS, later increase the stimulation of antioxidative defense mechanisms. Among the herbicide mixtures, DIU + IPU was highly toxic compared to DIU + TBA and TBA + IPU. The main hazard generated by the mixture (DIU + IPU) was its potential to act synergistically in both strains, thus reinforcing the ecotoxicological risk of both herbicides in the environment. This synergistic effect might even be more pronounced when more pollutants interact. Indeed, for the DIU + IPU mixture, antagonistic interactions were observed in both strains on the antioxidant enzyme activities. CAT, APX and GR activities

were poorly stimulated or probably suppressed in both strains in response to the mixture. Due to their importance in the metabolism of H_2O_2, the impaired of their activity reduced the ability of *E. siliculosus* strains to cope with ROS. Variations in interaction (synergism, antagonism and additivity) observed from the herbicide mixtures in *E. siliculosus* suggest that complex interactions between these types of contaminants may exist in the natural environment. Since the herbicides rarely occur alone in the environment, but rather in combination, a better understanding of these interactions will be of major importance when assessing the real-world potential effects of these contaminants in the natural environment.

Chapter 6

General Discussion and Conclusions

6.1 Risks posed to *E. siliculosus* from herbicide environmental concentrations

In the environment, measured concentrations of diuron (DIU) range from 3.05 µg L^{-1}, detected in the Seto Inland Sea, (Okamura *et al*., 2003); 42 µg L^{-1} in lagoon water in Italy (Gennaro *et al*., 1995); 0.768 µg L^{-1} in the marina UK, (Boxall *et al*., 2000); 6.742 µg L^{-1} estuaries UK, (Thomas *et al*., 2001), 2 µg L^{-1} in Mediterranean coast, Spain, (Martinez *et al*., 2000), and 1.13 µg L^{-1} in Marinas Netherlands, (Lamoree *et al*., 2002). Besides, high concentrations of DIU have also been recorded in French (10 µg L^{-1}) (Blanchoud *et al*., 2004) and North American (30 µg L^{-1}) (Field, 2003) surface waters. In the present study, EC_{50} values for RGR (9.9-25 µg L^{-1}), Fv/Fm (17.7-33 µg L^{-1}), ΦPSII (8.0-11.1 µg L^{-1}), qN (1.2-1.8 µg L^{-1}) and rETRmax (16.9-24.1 µg L^{-1}) were all less than 40 µg L^{-1} (the highest reported in the environment was 42 µg L^{-1}, Table 6.1). The respective values for NOEC and LOEC of RGR were 5 µg L^{-1} and 10 µg L^{-1} for LIA4, while 10 µg L^{-1} and 50 µg L^{-1} for the Es524. From the results, some of the concentrations in the environment were higher than the measured effects data, and therefore might exert a detrimental effect on the seaweed.

As for terbuthylazine (TBA), qN and RGR were the most sensitive indicators with a range of EC_{50} values of 5.06-6.3 µg L^{-1} and 18.1-28.2 µg L^{-1}, respectively. The NOEC and LOEC values recorded for qN were 1 and 5 µg L^{-1} respectively, while for RGR; 10 and 50 µg L^{-1} were derived respectively. Although TBA is a replacement for the formerly widely used atrazine, reported concentrations in the environment remain limited. A maximum TBA concentration of 694.32 ng L^{-1} and 234.5 ng L^{-1} was reported by Carafa *et al*., (2007) in the Sacca di Goro Lagoon and Northern Adriatic Sea, Italy respectively.

Table 6.1 The effects of diuron, terbuthylazine and isoproturon in relation to the reported environmental concentrations

Herbicides	End-points	7d EC_{50} (µg L^{-1}) (95% C.I)		NOEC (µg L^{-1})		LOEC (µg L^{-1})		Reported environmental concentrations
		LIA4	Es524	LIA4	Es524	LIA4	Es524	
Diuron	RGR	9.94 (6-14)	24.65 (19-31)	5	10	10	50	3.05 µg L^{-1} (Japan), 42 µg L^{-1} (Italy), 0.768 -6.742 µg L^{-1} (UK), 2.0 µg L^{-1} (Spain), 1.130 µg L^{-1} (Netherlands), 10 µg L^{-1} (France), 30 µg L^{-1} (North America)
	Fv/Fm	17.76 (13-22)	32.75 (27-38)	1	1	5	5	
	ΦPSII	8.04 (5-9)	11.18 (10-14)	1	1	5	5	
	qN	1.82 (1.3-2.3)	1.28 (0.8-1.7)	1	1	5	5	
	$rETR_{max}$	16.93 (13-20)	24.15 (21-31)	5	10	10	50	
Terbuthy-lazine	RGR	18.11 (12-22)	28.25 (23-36)	10	10	50	50	0.1 - 2.8 µg L^{-1} (Czech Rep.), 0.23-0.69 µg L^{-1} (Italy), Up to 1.27 µg L^{-1}(Spain), 13- 261 ng L^{-1} (France)
	ΦPSII	68.17 (60-76)	62.32 (55-69)	5	5	10	10	
	qN	6.35 (4.5-8.0)	5.063 (3.2-7.1)	1	1	5	5	
Isoprotu-ron	RGR	257 (213-302)	315.2 (281-349)	50	50	100	100	0.05 - 23.18 µg L^{-1}(Germany) Up to 2.6 µg L^{-1} (France) Up to 17 µg L^{-1} (UK) 500 µg L^{-1}(UK)
	qN	29.65 (22.9-37.1)	37.84 (28.1-47.3)	10	10	50	50	

*Certain endpoints were not mentioned due to much higher values compared to reported environmental concentrations

According to the Czech State Phytosanitary Administration, about 107 tonnes of TBA was applied in the Czech Republic in 2010 (Stepanova *et al*., 2012). Previous studies have found TBA to be present in 50% of water samples taken from Czech rivers during the period 2005 to 2009. The highest environmental concentrations reached 0.1 µg L^{-1} in 2005 to 2.8 µg L^{-1} in 2006 (Kodes *et al*., 2010). In addition, various concentrations ranging from less than detection limits up to 1.27 µg L^{-1} have been reported in surface and groundwater in northern Spain (Hildebrandt *et al*., 2008). A three-year survey of the Scheldt Estuary, northern France, by Noppe *et al*., (2007) recorded concentration of between 13 and 261 ng L^{-1}. With reference to the data obtained, the NOEC and LOEC values of RGR (10 and 50 µg L^{-1} respectively) were higher than the levels been reported in the environment, thus indicate low-risk impact posed by TBA to the seaweed. However, long-term risk assessment and multi combination effects of the herbicide with other pollutant still present a threat to the environment, thus should be monitored accordingly.

Of the three herbicides tested, isoproturon (IPU) had the highest EC_{50}, NOEC and LOEC values, indicating that it was the least toxic (Table 6.1). The EC_{50} values for RGR and qN ranged from 257-315 µg L^{-1} and 29.6-37.8 µg L^{-1}, respectively. The respective values for NOEC and LOEC were 10 and 50 µg L^{-1} for qN and 50 and 100 µg L^{-1} for RGR. To place these values in an environmental context, reported concentrations of IPU in the environment have ranged from 0.05 and 23.18 µg L^{-1}, with a median at 0.21 µg L^{-1} in the Zwester Ohm Catchment, Germany (Muller *et al*., (2002). Likewise, in the River Vannetin, France, concentrations of up to 2.6 µg L^{-1} have been recorded (Irace–Guigand *et al*., 2004). Kirby and Sheahan (1994) have reported short-lived values of up to 17 µg L^{-1} in agricultural run-off waters and a maximum concentration of 500 µg L^{-1} was recorded by Johnson *et al*., (1996) for drain water from a crop (cereals/oilseed) field after rainfall event.

Compared to the EC_{50}, NOEC and LOEC of RGR data, the reported environmental concentrations were lower than the laboratory effects data (except to Johnson *et al*., 1996, (500 µg L^{-1}) which was measured in a designated environment at the Oxford University Farm, Wytham), thus indicates minimal risk of IPU in order to cause adverse impact on the seaweed.

Collectively, although some of the data reported in the present study fall within the environmental realistic concentrations, without further investigations these data do not warrant any changes in environmental quality standards for these herbicides. This was not a stated aim of this study. To inform legislators about water quality standards further research would be required on a wider range of species together with chronic toxicity assessments for more precise risk estimations of the tested herbicides and to ensure any legislation related to the use of the herbicides are properly justified.

6.2 Intra-specific responses of *E. siliculosus* strains to herbicides (DIU, TBA and IPU)

Diverse interactions involving numerous environmental factors have led plants to evolve specific mechanisms, allowing them to adapt and survive stressful events. Both biotic and abiotic stresses impose challenges to plants survival and developments, thus, the timely perception of the stress in order to respond in a rapid and efficient manner is vital to minimize the biological damage caused by the stress (Rejeb *et al*., 2014). In the near-shore ecosystem, seaweed is constantly exposed to various stresses that require efficient adaptation mechanism in order to survive. Previous studies by Pawlik-Skowronska *et al*., 2007; Gonzalez *et al*., 2010, 2012a and Brown *et al*., 2012 in response to metal stress by seaweeds have elucidated the mechanisms by which they detoxify and tolerate metals. For instance, green seaweed, *Ulva compressa* induced an oxidative stress condition after

chronic exposure to copper (Cu), stimulate the activation of the antioxidant enzyme ascorbate peroxidase and synthesis of ascorbate, whereas in brown seaweed *Scytosiphon lomentaria* protection from oxidative stress occurs *via* increased activities of primarily catalase, glutathione peroxidase, ascorbate peroxidase and production of ascorbate (Ratkevicius *et al*., 2003; Contreras *et al*., 2005, 2009; Gonzalez *et al*., 2012b; Mellado *et al*., 2012). In fact, continuous exposure to metal stress may lead to the directional selection of traits that aid survivability of individuals in polluted environments (Saez *et al*., 2015), evidence for which has been obtained for the brown seaweeds *Fucus serratus* (Nielsen *et al*., 2003) and *Ectocarpus siliculosus* (Russell and Morris, 1970).

The present study provides insight into the physiological responses and antioxidative defense mechanism of two different *E. siliculosus* strains (LIA4 and Es524) after exposure to different herbicides. Using growth and chlorophyll fluorescence as indicators, we observed that Es524 strain which originated from a polluted environment in Chile (Caleta Palito) was more tolerance to the herbicides tested compared to LIA4. Since Es524 strain indicated higher tolerance against the PSII inhibitors, biochemical evaluations were carried out as well. The underlying mechanisms of protection and defense against oxidative stress were studied in detail.

The use of growth and photosynthetic efficiency parameters are common in plant ecophysiology and stress research. For instance, the maximum quantum yield of PSII, expressed as the ratio of variable to maximum chlorophyll fluorescence (Fv/Fm), have been used widely in xenobiotics assessments (Bi *et al*., 2012; Macinnis-Ng and Ralph, 2003). The pulse amplitude modulated (PAM) fluorometry method applied in the present study offers the advantage that the algae is not stressed or damaged by the technique itself (Bilger *et al*., 1995) plus it allows fast assessment of photosynthetic activity. As oxidative stress

directly intervenes in the photosynthetic process (Foyer and Shigeoka, 2011), we found that this method can also be used to determine the relative antioxidative properties of *E. siliculosus*. From our results, exposures to the different herbicides/booster biocide have caused changes in the amount of H_2O_2 contents in both strains of *E. siliculosus*, which are highly related to the variable fluorescence obtained. Therefore, PAM fluorometry is a suitable technique to rapidly screen for the antioxidative potential in comparative studies in macroalgae, which is in line to previous study by Dummermuth *et al*., (2003).

Moreover, *in vivo* chlorophyll a fluorescence as a means to determine effects on photosynthetic efficiency of seaweeds turned out to be a sensitive and thus valuable biomarker to monitor PSII herbicide contamination in the estuarine and coastal environments. However, plant growth remains the more relevant ecological endpoint when assessing the ecotoxicological risk of herbicides for the estuarine and coastal environment since mainly reduction in growth will have a serious impact on the ecological functioning of the near-shore ecosystems. In fact, the OECD and ISO international test methods have prioritize growth measurement for regulatory decision making (OECD, 1984; ISO 8692, 1989; Dahl *et al*., 2006).

Biochemical evaluations on both strains, indicated that Es524 possess higher antioxidative potential compared to LIA4. The higher antioxidative properties of Es524 can be explained by enzyme and non-enzymatic mechanisms. In chapter 4, exposure to diuron (DIU) lead to poor stimulation of catalase (CAT) activity in both strains of *E. siliculosus*. However, stronger activation of ascorbate peroxidase (APX) and glutathione reductase (GR) enzymes were observed in Es524 strain compared to LIA4. These observations indicate APX and GR probably had more important role in H_2O_2 detoxification upon exposure to DIU than CAT in Es524 strain. As for terbuthylazine (TBA) exposure, both

strains have showed similar response of CAT activity to the range of TBA treatments. However, evaluations on APX and GR activities indicated different response between the strains, with significantly higher stimulation of the aforementioned enzymes were observed in Es524 strain compared to LIA4. Isoproturon (IPU) exposure on both strains on the other hand, demonstrated stronger inductions of CAT and GR activities in Es524 compared to LIA4. As for APX activity, although both strains were found to respond similarly, the peaked stimulation of APX activity was hugely different between the strains with 101% increase in Es524 and only 49% in LIA4. Higher antioxidative enzymes activity possessed by Es524 may explain the high capability of resistance of the strain against the herbicides tested. Previous studies have reported the activities of antioxidant enzymes such as APX, catalases and SODs are up regulated in response to several abiotic stresses such as drought (Smirnoff and Colombe, 1988), low temperatures (Schöner and Krause, 1990), high light intensities (Cakmak and Marschner, 1992), ozone, SO_2, UV-B (Willekens *et al*., 1994) and salinity (Lopez *et al*., 1996), which is also true for Es524 in the present study, upon exposure to the herbicides. Besides the increases, there are also studies showing decrease of antioxidative enzymes under stress. For instances, the decreased of GR activities in the diatoms (*Entemoneis kufferathii*) and *Chaetoceros sp* with increasing temperature and light intensities was observed by Schriek (2000). In another study by Aguilera *et al*., (2002b) showed decreased SOD activities in *Palmaria palmata* and decreased GR activities in *Monostroma aff. arcticum* under UV radiation. With respect to pesticide, exposure of DIU (100 µg L^{-1}) on *Lemna minor* has resulted decreases on antioxidative enzyme activities (CAT, P-POD, G-POD) (Teisseire and Vernet, 2000) probably due to enzyme damage. In fact, Dewez *et al*., (2005) also have reported diminished of CAT and APX activities at 48 h of fludioxonil exposure on *Scenedesmus obliquus*, probably due to the deterioration of

cellular system functions by the fungicide. These findings are in agreement to our study for LIA4 strain treated with ≥ 50 μg L^{-1} DIU, ≥ 100 μg L^{-1} TBA and ≥ 500 μg L^{-1} IPU. The decrease of antioxidative defense mechanisms can be interpreted as a classical stress response in which the intensity of the stress is too high and the stage of exhaustion is reached (Lichtenthaler, 1996). The significant decrease of relative growth rate observed at similar concentrations after 7 d of exposure to the herbicides, strengthen this hypothesis. Our study clearly shows that increased antioxidative enzyme activities have been found to be a good indicator of toxicity as reported previously by (Ferrari *et al*., 1999) in three terrestrial plants, oats, Chinese cabbage and lettuce, exposed to different concentrations of the herbicide sodium trichloroacetate. In fact, the increases in the antioxidant enzyme activities were found to be more sensitive than effects on biomass and germination rate (Radetski *et al*., 2000). Therefore, the use of these enzyme activities may allow the detection of early impact in algae, although further improvement is required.

As a model of brown seaweed, further investigations of extracts from *E. siliculosus* evince the presence of phenolic compounds with antioxidative properties. Total phenolics and DPPH scavenging assays indicate distinct antioxidative abilities, with Es524 exhibits higher antioxidative potential compared to LIA4 in response to the herbicides. In addition, several polyphenols such as caffeic acid, phloroglucinol and rutin have been identified by high performance liquid chromatography (HPLC) in both strains. Exposure to the herbicides (DIU, TBA and IPU) showed significantly higher amounts of polyphenols in Es524 strain compared to LIA4 at various concentrations. In fact, the correlation of the polyphenols with high antioxidative activity at different concentrations of the herbicides tested, indicated that Es524 contain higher amount of phloroglucinol, rutin and caffeic acid

as low molecular weight antioxidants. Since there is an unidentified peak observed from the HPLC analysis, other low molecular weight antioxidants may also be present in this alga.

In addition, since pesticides never occur alone in the environment, but rather in combination, studies were conducted in which *E. siliculosus* strains (LIA4 and Es524) were exposed to three different binary mixtures. Our results showed that the toxicity effect of DIU + TBA and TBA + IPU mixtures to LIA4 and Es524 strains are different. The mixtures exhibited higher toxicities to LIA4 strain than the one isolated from polluted site, Es524. All three types of interactions (antagonism, additivity and synergism) were recorded and the results varied according to the endpoints. Similar results were reported by Fernandez-Alba *et al*., (2002), in which diuron and Irgarol 1051 had synergistic effects on the cell growth of *Selenastrum capricornutum*. On the other hand, when exposed to the seagrass *Zostera marina*, the two chemicals (DIU and Irgarol 1051) acted both antagonistically and additively to Fv/Fm (Chesworth *et al*., 2004). Therefore, it is obvious that despite the similarity of specific interactions with the molecular target sites, the toxicological behavior of DIU (phenylurea) and Irgarol 1051 (*S*-triazines) can be quite different. Thus, the prediction of the combined action of such chemicals with similar modes of action remains unapparent, whether it would increase, decrease, or have no effect, for any particular toxic effects under consideration (Koutsaftis and Aoyama, 2006). In the present study, the presence of terbuthylazine (TBA) together with the phenylureas DIU/IPU potentiated the stimulations of antioxidative enzymes (CAT, APX, GR) in both LIA4 and Es524 strains of *E. siliculosus*, although at different levels. Synergistic interactions were observed when Es524 were exposed to TBA mixtures containing either DIU or IPU. Possibly the presence of TBA enhanced the inductions of the radical scavengers, therefore

reducing toxicity by stimulations of different antioxidative defense mechanisms (enzymatic and non-enzymatic).

In general, Es524 strain which originated from a Cu-polluted site in Chile has to cope with more adverse environmental conditions than LIA4, which was isolated from a pristine site in Scotland. This was in line with a recent study by Pise *et al*., (2013) on marine red seaweed *Porphyra vietnamensis*, inhabiting the central west coast of India, that indicates higher levels of lipid peroxidation, H_2O_2, CAT and GST in samples collected from a high pollution area (Dona Paula) than in samples from a less-polluted areas (Malvan and Kunkeshwar). This condition was attributed to higher levels of metals such as cadmium, lead and mercury in Dona Paula compared to Malvan and Kunkeshwar. In another study, Maharana *et al*., (2010) reported that the oxidative stress and antioxidant defence systems (lipid peroxidation, H_2O_2, CAT and GST) in a marine brown alga *Padina tetrastromatica* were higher in samples from relatively polluted localities (Colaba and Karwar) compared to less polluted ones (Anjuna). For this reason, it is obvious that species inhabiting harsh environments exhibit higher antioxidant enzyme activities and antioxidant concentrations as species inhabiting deeper waters. This relation between antioxidant capabilities and environment (e.g depth distribution) has been suggested by Aguilera *et al*., (2002,a,b) and accounts for the high resistibility to oxidative H_2O_2 stress of the *Chaetomorpha* species and *Fucus distichus* living in the eulittoral and upper sublittoral. The increase of activities of reactive oxygen scavenging (ROS) enzymes with increased environmental stress in the higher intertidal for *Mastocarpus stellatus* was also reported by Collen and Davison (1999b). Davison and Pearson (1996) also explained high tolerance to various stresses of species from the uppermost sublittoral zone. A recent study using the same strains of *E. siliculosus* by Saez *et al*., (2015) also observed higher antioxidant

responses in Es524 strain compared to LIA4 after been treated with copper (Cu), which probably relate to the prevailing environmental conditions from where they were isolated. Besides, dissolved organic carbon (DOC) also has been reported to influence the tolerance of certain species to metal stress through altering metal speciation and reduce the bioavailability to algae (De Palma *et al*., 2011; Rainbow *et al*., 2011), however, this is not necessarily applicable to organic pollutants since speciation is relevant for metals but not organics.

Overall, this study extends the brown seaweed ecotoxicological data to diuron, terbuthylazine and isoproturon. The varied responses observed between LIA4 and Es524 strains indicated different strategies against the PSII inhibitors. Effective and higher antioxidant responses exhibited by Es524 strain isolated from polluted habitat, lead to higher tolerance against the herbicides tested compared to LIA4 which originated from a pristine site. The important element in reactive oxygen metabolism that determine the balance between production and protection within cells will undoubtedly contribute to the distinctive tolerance between the strains.

6.3 Future works

The data obtained in the present study have shown the detrimental impacts of DIU, TBA and IPU on *E. siliculosus*. However, it is also important to identify potential effects of the degradation products that originate from it (e.g. dichloroalanine (DCA), desetil-terbuthylazine (D-TBA)) so as to assess the impact of the herbicides as a whole. Indeed, herbicides could be converted into another compound which could be more toxic than their parent molecules leading to a magnified pollution impact. Thus, toxicity studies must focus

not only on the parent compound of a polluting agent, but also on the degradation products in order to define the real environmental impact of a pollutant.

The present study also describes the effects of DIU, TBA and IPU observed in the laboratory under controlled environment, which might lead to low external validity. Therefore, the impacts of the herbicides through application of mesocosm study should also be performed in order to integrate all the factors that could influence their toxicity. With reference to Rimet and Bouchez (2011) study, diatoms have been shown to be affected in lotic mesocosms at environmentally relevant concentrations of a pesticide mixture with diuron (1.5 µg L^{-1} for chronic pollution and 20 µg L^{-1} for acute pollution lasting few hours). Thus, the ability of mesocosms to provide high ecological relevance to the experiment indicates that environmental concentrations of the pollutant might affect diatom life forms and ecological guilds (Rimet and Bouchez, 2011).

In addition, more studies regarding for long-term exposure to herbicides and the effects of TBA and IPU on estuarine and marine organisms are required. In addition, environmental factors should also be taken into account. The interaction of herbicide effects with other environmental factors (e.g salinity, temperature) and the possible compound effects of different pollutants should also be investigated in order to evaluate the real-world impact of these herbicides on the environment and before establishing water quality guidelines.

References

Abad, M.J., Bedoya, L.M. and Bermejo, P., 2008. Natural marine anti-inflammatory products. *Mini reviews in medicinal chemistry*, 8, pp.740-754

Abe, N., Murata, T., Hirota, A., 1998. Novel 1,1-diphenyl-2-picryhydrazylradical scavengers, bisorbicillin and demethyltrichodimerol, from a fungus. *Biosci. Biotechnol. Biochem.* 62, pp.661–662

Abou-Waly, H., Abou-Setta, M.M., Nigg, H.N. and Mallory, L.L., 1991. Growth response of freshwater algae, *Anabaena flos-aquae* and *Selenastrum capricornutum* to atrazine and hexazinone herbicides. *Bulletin of environmental contamination and toxicology*, 46, pp.223-229

Aebi, H., 1984. [13] Catalase *in vitro*. *Methods in enzymology*, 105, pp.121-126

Aguilera, J., Bischof, K., Karsten, U., Hanelt, D. and Wiencke, C., 2002a. Seasonal variation in ecophysiological patterns in macroalgae from an Arctic fjord. II. Pigment accumulation and biochemical defence systems against high light stress. *Marine Biology*, 140, pp.1087-1095

Aguilera, J., Dummermuth, A., Karsten, U., Schriek, R. and Wiencke, C., 2002b. Enzymatic defences against photooxidative stress induced by ultraviolet radiation in Arctic marine macroalgae. *Polar Biology*, 25, pp.432-441

Ahel, M., Evans, K.M., Fileman, T.W. and Mantoura, R.F.C., 1992. Determination of atrazine and simazine in estuarine samples by high-resolution gas chromatography and nitrogen selective detection. *Analytica chimica acta*, 268, pp.195-204

Alscher, R.G., Erturk, N. and Heath, L.S., 2002. Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *Journal of Experimental Botany*, 53, pp.1331-1341

Amako, K., Chen, G.X. and Asada, K., 1994. Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. *Plant and Cell Physiology*, 35, pp.497-504

Anderson, B.S. and Hunt, J.W., 1988. Bioassay methods for evaluating the toxicity of heavy metals, biocides and sewage effluent using microscopic stages of giant kelp *Macrocystis pyrifera* (Agardh): a preliminary report. *Marine Environmental Research*, 26, pp.113-134

Anjum, N.A., Sofo, A., Scopa, A., Roychoudhury, A., Gill, S.S., Iqbal, M., Lukatkin, A.S., Pereira, E., Duarte, A.C. and Ahmad, I., 2015. Lipids and proteins—major targets of oxidative modifications in abiotic stressed plants. *Environmental Science and Pollution Research*, 22, pp.4099-4121

Anton, F.A., Ariz, M. and Alia, M., 1993. Ecotoxic effects of four herbicides (glyphosate, alachlor, chlortoluron and isoproturon) on the algae *Chlorella pyrenoidosa* Chick. *Science of the Total Environment*, 134, pp.845-851

Apel, K. and Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annual Review of Plant Biology*, 55, pp.373-399

APVMA (Australian Pesticides and Veterinary Medicine Authority), 2012. Diuron Chemical Review. (see <https://apvma.gov.au/node/12511> (accessed 16.09.13)

Arias-Estévez, M., López-Periago, E., Martínez-Carballo, E., Simal-Gándara, J., Mejuto, J.C. and García-Río, L., 2008. The mobility and degradation of pesticides in soils and the pollution of groundwater resources. *Agriculture, Ecosystems & Environment*, 123, pp.247-260

Arnaud, L., Taillandier, G., Kaouadji, M., Ravanel, P. and Tissut, M., 1994. Photosynthesis inhibition by phenylureas: A QSAR approach. *Ecotoxicology and Environmental Safety*, 28, pp.121-133

Arrhenius, Å., Grönvall, F., Scholze, M., Backhaus, T. and Blanck, H., 2004. Predictability of the mixture toxicity of 12 similarly acting congeneric inhibitors of photosystem II in marine periphyton and epipsammon communities. *Aquatic Toxicology*, 68, pp.351-367

Arun, A., Peters, N.T., Scornet, D., Peters, A.F., Mark Cock, J. and Coelho, S.M., 2013. Non-cell autonomous regulation of life cycle transitions in the model brown alga *Ectocarpus*. *New Phytologist*, 197, pp.503-510

Athukorala, Y., Kim, K.N. and Jeon, Y.J., 2006. Antiproliferative and antioxidant properties of an enzymatic hydrolysate from brown alga, *Ecklonia cava*. *Food and Chemical Toxicology*, 44, pp.1065-1074

Backhaus, T., Faust, M., Scholze, M., Gramatica, P., Vighi, M. and Grimme, L.H., 2004. Joint algal toxicity of phenylurea herbicides is equally predictable by concentration addition and independent action. *Environmental Toxicology and Chemistry*, 23, pp.258-264

Baldauf, S.L., 2003. The deep roots of eukaryotes. *Science*, 300, pp.1703-1706

Barber, J., 1992. Photosynthetic reaction centres: a common link. *Trends in Biochemical Sciences*, 12, pp.321-326

Battaglin, W.A., Furlong, E.T., Burkhardt, M.R. and Peter, C.J., 2000. Occurrence of sulfonylurea, sulfonamide, imidazolinone, and other herbicides in rivers, reservoirs and ground water in the Midwestern United States, 1998. *Science of the Total Environment*, 248, pp.123-133

Beers, R.F. and Sizer, I.W., 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry*, 195, pp.133-140

Behra, R., Genoni, G.P. and Joseph, A.L., 1999. Effect of atrazine on growth, photosynthesis, and between-strain variability in *Scenedesmus subspicatus* (Chlorophyceae). *Archives of Environmental Contamination and Toxicology*, 37, pp.36-41

Bentley-Mowat, J.A. and Reid, S.M., 1977. Survival of marine phytoplankton in high concentrations of heavy metals, and uptake of copper. *Journal of Experimental Marine Biology and Ecology*, 26, pp.249-264

Berenbaum, M.C., 1989. What is synergy?. *Pharmacological Reviews*, 41, pp.93-141

Berger, B. and Heitefuss, R., 1991. Use of isoproturon, alone and in combination with other compounds, on winter wheat and winter barley. *Weed Research*, 31, pp.9-18

Bi, Y.F., Miao, S.S., Lu, Y.C., Qiu, C.B., Zhou, Y. and Yang, H., 2012. Phytotoxicity, bioaccumulation and degradation of isoproturon in green algae. *Journal of Hazardous Materials*, 243, pp.242-249

Bilger, W., Schreiber, U. and Bock, M., 1995. Determination of the quantum efficiency of photosystem II and of non-photochemical quenching of chlorophyll fluorescence in the field. *Oecologia*, 102, pp.425-432

Blanchoud, H., Farrugia, F. and Mouchel, J.M., 2004. Pesticide uses and transfers in urbanised catchments. *Chemosphere*, 55, pp.905-913

Blanchoud, H., Moreau-Guigon, E., Farrugia, F., Chevreuil, M. and Mouchel, J.M., 2007. Contribution by urban and agricultural pesticide uses to water contamination at the scale of the Marne watershed. *Science of the Total Environment*, 375, pp.168-179

Blanck, H., Wallin, G. and Wängberg, S.Å., 1984. Species-dependent variation in algal sensitivity to chemical compounds. *Ecotoxicology and Environmental Safety*, 8, pp.339-351

Boland, W., Pohnert, G. and Maier, I., 1995. Pericyclic reactions in nature: spontaneous Cope rearrangement inactivates algae pheromones. *Angewandte Chemie International Edition*, 34, pp.1602-1604

Bolhar-Nordenkampf, H.R., Long, S.P., Baker, N.R., Oquist, G., Schreiber, U.L.E.G. and Lechner, E.G., 1989. Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: a review of current instrumentation. *Functional Ecology*, pp.497-514

Boscolo, P.R., Menossi, M. and Jorge, R.A., 2003. Aluminum-induced oxidative stress in maize. *Phytochemistry*, 62, pp.181-189

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72, pp.248-254

Brown, M.T. and Newman, J.E., 2003. Physiological responses of *Gracilariopsis longissima* (SG Gmelin) Steentoft, LM Irvine and Farnham (Rhodophyceae) to sub-lethal copper concentrations. *Aquatic Toxicology*, 64, pp.201-213

Brown, M.T., Newman, J.E. and Han, T., 2012. Inter-population comparisons of copper resistance and accumulation in the red seaweed, *Gracilariopsis longissima*. *Ecotoxicology*, 21, pp.591-600

Bulleri, F., Benedetti-Cecchi, L., Acunto, S., Cinelli, F. and Hawkins, S.J., 2002. The influence of canopy algae on vertical patterns of distribution of low-shore assemblages on rocky coasts in the northwest Mediterranean. *Journal of Experimental Marine Biology and Ecology*, 267, pp.89-106

Burridge, T.R., Karistianos, M. and Bidwell, J., 1999. The use of aquatic macrophyte ecotoxicological assays in monitoring coastal effluent discharges in Southern Australia. *Marine Pollution Bulletin*, 39, pp.89-96

Burtin, P., 2003. Nutritional value of seaweeds. *Electronic Journal of Environmental, Agricultural and Food Chemistry*, 2, pp.498-503

Butler, L.G., Price, M.L. and Brotherton, J.E., 1982. Vanillin assay for proanthocyanidins (condensed tannins): modification of the solvent for estimation of the degree of polymerization. *Journal of Agricultural and Food Chemistry*, 30, pp.1087-1089

Cakmak, I. and Marschner, H., 1992. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase in bean leaves. *Plant Physiology*, 98, pp.1222-1227

Callow, M.E. and Willingham, G.L., 1996. Degradation of antifouling biocides. *Biofouling*, 10, pp.239-249

Canero, A.I., Cox, L., Redondo-Gómez, S., Mateos-Naranjo, E., Hermosín, M.C. and Cornejo, J., 2011. Effect of the herbicides terbuthylazine and glyphosate on photosystem II photochemistry of young olive (*Olea europaea*) plants. *Journal of Agricultural and Food Chemistry*, 59, pp.5528-5534

Caquet, T., Roucaute, M., Mazzella, N., Delmas, F., Madigou, C., Farcy, E., Burgeot, T., Allenou, J.P. and Gabellec, R., 2013. Risk assessment of herbicides and booster biocides along estuarine continuums in the Bay of Vilaine area (Brittany, France). *Environmental Science and Pollution Research*, 20, pp.651-666

Carafa, R., Wollgast, J., Canuti, E., Ligthart, J., Dueri, S., Hanke, G., Eisenreich, S.J., Viaroli, P. and Zaldívar, J.M., 2007. Seasonal variations of selected herbicides and related metabolites in water, sediment, seaweed and clams in the Sacca di Goro coastal lagoon (Northern Adriatic). *Chemosphere*, 69, pp.1625-1637

Carbery, K., Owen, R., Frickers, T., Otero, E. and Readman, J., 2006. Contamination of Caribbean coastal waters by the antifouling herbicide Irgarol 1051. *Marine Pollution Bulletin*, 52, pp.635-644

Chalifour, A. and Juneau, P., 2011. Temperature-dependent sensitivity of growth and photosynthesis of *Scenedesmus obliquus*, *Navicula pelliculosa* and two strains of *Microcystis aeruginosa* to the herbicide atrazine. *Aquatic Toxicology*, 103, pp.9-17

Chandlee, J.M., Tsaftaris, A.S. and Scandalios, J.G., 1983. Purification and partial characterization of three genetically defined catalases of maize. *Plant Science Letters*, 29, pp.117-131

Chaoui, A., Mazhoudi, S., Ghorbal, M.H. and El Ferjani, E., 1997. Cadmium and zinc induction of lipid peroxidation and effects on antioxidant enzyme activities in bean (*Phaseolus vulgaris* L.). *Plant Science*, 127, pp.139-147

Charrier, B., Coelho, S.M., Le Bail, A., Tonon, T., Michel, G., Potin, P., Kloareg, B., Boyen, C., Peters, A.F. and Cock, J.M., 2008. Development and physiology of the brown alga *Ectocarpus siliculosus*: two centuries of research. *New Phytologist*, 177, pp.319-332

Chesworth, J.C., Donkin, M.E. and Brown, M.T., 2004. The interactive effects of the antifouling herbicides Irgarol 1051 and Diuron on the seagrass *Zostera marina* (L.). *Aquatic Toxicology*, 66, pp.293-305

Choo, K.S., Snoeijs, P. and Pedersén, M., 2004. Oxidative stress tolerance in the filamentous green algae *Cladophora glomerata* and *Enteromorpha ahlneriana*. *Journal of Experimental Marine Biology and Ecology*, 298, pp.111-123

Choudhary, M., Jetley, U.K., Khan, M.A., Zutshi, S. and Fatma, T., 2007. Effect of heavy metal stress on proline, malondialdehyde, and superoxide dismutase activity in the cyanobacterium *Spirulina platensis*-S5. *Ecotoxicology and Environmental Safety*, 66, pp.204-209

Chynoweth, D.P., 2002. Review of biomethane from marine biomass. *University of Florida*, pp.1-207

Cock, J.M., Sterck, L., Rouzé, P., Scornet, D., Allen, A.E., Amoutzias, G., Anthouard, V., Artiguenave, F., Aury, J.M., Badger, J.H. and Beszteri, B., 2010. The *Ectocarpus* genome and the independent evolution of multicellularity in brown algae. *Nature*, 465, pp.617-621

Coelho, S.M., Taylor, A.R., Ryan, K.P., Sousa-Pinto, I., Brown, M.T. and Brownlee, C., 2002. Spatiotemporal patterning of reactive oxygen production and Ca2+ wave propagation in *Fucus* rhizoid cells. *The Plant Cell*, 14, pp.2369-2381

Coelho, S. M., Scornet, D., Rousvoal, S., Peters, N. T., Dartevelle, L., Peters, A. F., & Cock, J. M., 2012a. How to cultivate *Ectocarpus*. *Cold Spring Harbor Protocols*, pp.258-261

Coelho, S. M., Scornet, D., Rousvoal, S., Peters, N. T., Dartevelle, L., Peters, A. F., & Cock, J. M., 2012b. *Ectocarpus*: a model organism for the brown algae. *Cold Spring Harbor Protocols*, pp.pdb-emo065821

Collén, J. and Davison, I.R., 1999a. Reactive oxygen metabolism in intertidal *Fucus* spp.(Phaeophyceae). *Journal of Phycology*, 35, pp.62-69

Collén, J. and Davison, I.R., 1999b. Stress tolerance and reactive oxygen metabolism in the intertidal red seaweeds *Mastocarpus stellatus* and *Chondrus crispus*. *Plant, Cell & Environment*, 22, pp.1143-1151

Conrad, R., Büchel, C., Wilhelm, C., Arsalane, W., Berkaloff, C. and Duval, J.C., 1993. Changes in yield of *in-vivo* fluorescence of chlorophyll *a* as a tool for selective herbicide monitoring. *Journal of Applied Phycology*, 5, pp.505-516

Contreras, L., Moenne, A. and Correa, J.A., 2005. Antioxidant responses in *Scytosiphon lomentaria* (phaeophyceae) inhabiting copper-enriched coastal environments. *Journal of Phycology*, 41, pp.1184–1195

Contreras, L., Mella, D., Moenne, A. and Correa, J.A., 2009. Differential responses to copper-induced oxidative stress in the marine macroalgae *Lessonia nigrescens* and *Scytosiphon lomentaria* (Phaeophyceae). *Aquatic Toxicology*, 94, pp.94-102

Croteau, R., Kutchan, T.M. and Lewis, N.G., 2000. Natural products (secondary metabolites). *Biochemistry and Molecular Biology of Plants*, 24, pp.1250-1319

Dahl, B. and Blanck, H., 1996. Toxic effects of the antifouling agent Irgarol 1051 on periphyton communities in coastal water microcosms. *Marine Pollution Bulletin*, 32, pp.342-350

Dahl, U., Gorokhova, E. and Breitholtz, M., 2006. Application of growth-related sublethal endpoints in ecotoxicological assessments using a harpacticoid copepod. *Aquatic Toxicology*, 77, pp.433-438

Dalton, R.L. and Boutin, C., 2010. Comparison of the effects of glyphosate and atrazine herbicides on nontarget plants grown singly and in microcosms. *Environmental Toxicology and Chemistry*, 29, pp.2304-2315

Davis, T.A., Volesky, B. and Mucci, A., 2003. A review of the biochemistry of heavy metal biosorption by brown algae. *Water Research*, 37, pp.4311-4330

Davison, I.R. and Pearson, G.A., 1996. Stress tolerance in intertidal seaweeds. *Journal of Phycology*, 32, pp.197-211

De Franco, P.O., Rousvoal, S., Tonon, T. and Boyen, C., 2009. Whole genome survey of the glutathione transferase family in the brown algal model *Ectocarpus siliculosus*. *Marine Genomics*, 1, pp.135-148

Delaroque, N., Müller, D.G., Bothe, G., Pohl, T., Knippers, R. and Boland, W., 2001. The complete DNA sequence of the *Ectocarpus siliculosus* virus EsV-1 genome. *Virology*, 287, pp.112-132

Delgado-Moreno, L. and Peña, A., 2009. Compost and vermicompost of olive cake to bioremediate triazines-contaminated soil. *Science of The Total Environment*, 407, pp.1489-1495

DeLorenzo, M.E., Leatherbury, M., Weiner, J.A., Lewitus, A.J. and Fulton, M.H., 2004. Physiological factors contributing to the species-specific sensitivity of four estuarine microalgal species exposed to the herbicide atrazine. *Aquatic Ecosystem Health & Management*, 7, pp.137-146

Deng, X., Gao, K. and Sun, J., 2012. Physiological and biochemical responses of *Synechococcus sp*. PCC7942 to Irgarol 1051 and diuron. *Aquatic Toxicology*, 122, pp.113-119

DePalma, S.G., Arnold, W.R., McGeer, J.C., Dixon, D.G. and Smith, D.S., 2011. Effects of dissolved organic matter and reduced sulphur on copper bioavailability in coastal marine environments. *Ecotoxicology and Environmental Safety*, 74, pp.230-237

Dewez, D., Marchand, M., Eullaffroy, P. and Popovic, R., 2002. Evaluation of the effects of diuron and its derivatives on *Lemna gibba* using a fluorescence toxicity index. *Environmental Toxicology*, 17, pp.493-501

Dewez, D., Geoffroy, L., Vernet, G. and Popovic, R., 2005. Determination of photosynthetic and enzymatic biomarkers sensitivity used to evaluate toxic effects of copper and fludioxonil in alga *Scenedesmus obliquus*. *Aquatic Toxicology*, 74, pp.150-159

Dewez, D., Didur, O., Vincent-Héroux, J. and Popovic, R., 2008. Validation of photosynthetic-fluorescence parameters as biomarkers for isoproturon toxic effect on alga *Scenedesmus obliquus*. *Environmental Pollution*, 151, pp.93-100

Dezfuli, B.S., Simoni, E., Giari, L. and Manera, M., 2006. Effects of experimental terbuthylazine exposure on the cells of *Dicentrarchus labrax* (L.). *Chemosphere*, 64, pp.1684-1694

Di Giulio, R.T., Washburn, P.C., Wenning, R.J., Winston, G.W. and Jewell, C.S., 1989. Biochemical responses in aquatic animals: a review of determinants of oxidative stress. *Environmental Toxicology and Chemistry*, 8, pp.1103-1123

Dillwyn, L.W., 1809. In British Confervae or Colored Figures and Descriptions of the British Plants Referred by Botanists to the Genus Conferva pp. 1–87; plates 69 and 100–109; W. Phillips, London, UK

Dittami, S.M., Scornet, D., Petit, J.L., Ségurens, B., Da Silva, C., Corre, E., Dondrup, M., Glatting, K.H., König, R., Sterck, L. and Rouzé, P., 2009. Global expression analysis of the brown alga *Ectocarpus siliculosus* (Phaeophyceae) reveals large-scale reprogramming of the transcriptome in response to abiotic stress. *Genome Biology*, 10, p.R66

Dixon, R.A. and Paiva, N.L., 1995. Stress-induced phenylpropanoid metabolism. *The Plant Cell*, 7, p.1085

Dobšíková, R., Blahová, J., Modrá, H., Škorič, M. and Svobodová, Z., 2012. The effect of acute exposure to herbicide Gardoprim Plus Gold 500 SC on haematological and

biochemical indicators and histopathological changes in common carp (*Cyprinus carpio* L.). *Acta Veterinaria Brno*, 80, pp.359-363

Doogue, M. P. and Polasek, T. M., 2013. The ABCD of clinical pharmacokinetics. *Therapeutic Advances in Drug Safety*, 4, pp. 5–7. http://doi.org/10.1177/2042098612469335

Doppler, T., Camenzuli, L., Hirzel, G., Krauss, M., Lück, A. and Stamm, C., 2012. Spatial variability of herbicide mobilisation and transport at catchment scale: insights from a field experiment. *Hydrology and Earth System Sciences*, 16, pp.1947-1967

Dorigo, U. and Leboulanger, C., 2001. A pulse-amplitude modulated fluorescence-based method for assessing the effects of photosystem II herbicides on freshwater periphyton. *Journal of Applied Phycology*, 13, pp.509-515

Dosnon-Olette, R., Trotel-Aziz, P., Couderchet, M. and Eullaffroy, P., 2010. Fungicides and herbicide removal in *Scenedesmus* cell suspensions. *Chemosphere*, 79, pp.117-123

Doyotte, A., Cossu, C., Jacquin, M.C., Babut, M. and Vasseur, P., 1997. Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. *Aquatic Toxicology*, 39, pp.93-110

Draisma, S.G., Peters, A.F. and Fletcher, R.L., 2003. Evolution and taxonomy in the *Phaeophyceae*: effects of the molecular age on brown algal systematics. *Out of the Past*, pp.87-102

Dröge, W., 2002. Free radicals in the physiological control of cell function. *Physiological Reviews*, 82, pp.47-95

Dubber, D. and Harder, T., 2008. Extracts of *Ceramium rubrum*, *Mastocarpus stellatus* and *Laminaria digitata* inhibit growth of marine and fish pathogenic bacteria at ecologically realistic concentrations. *Aquaculture*, 274, pp.196-200

Duke, N.C., Bell, A.M., Pederson, D.K., Roelfsema, C.M. and Nash, S.B., 2004. Herbicides implicated as the cause of severe mangrove dieback in the Mackay region,

NE Australia: consequences for marine plant habitats of the GBR World Heritage Area. *Marine Pollution Bulletin*, 51, pp.308-324

Dummermuth, A.L., Karsten, U., Fisch, K.M., König, G.M. and Wiencke, C., 2003. Responses of marine macroalgae to hydrogen-peroxide stress. *Journal of Experimental Marine Biology and Ecology*, 289, pp.103-121

Durst, F., 1991. Métabolisation des herbicides. In : Les herbicides - mode d'action et principes d'utilisation. Institut National de la Recherche Agronomique (INRA), Paris : 193-236

EC, 2000., Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for the Community action in the field of water policy. Official Journal of the European Communities L 327, 1–72

Edwards, P. and D. F. Kapraun., 1973. Benthic marine algal ecology in the Port Aransas, Texas area. *Contributions in Marine Science*, 17, pp.15-52

EFSA, European Food Safety Authority., 2011. Conclusion on the peer review of the pesticide risk assessment of the active substance terbuthylazine. *EFSA Journal*, 9, pp.1-133

Eklund, B.T. and Kautsky, L., 2003. Review on toxicity testing with marine macroalgae and the need for method standardization—exemplified with copper and phenol. *Marine Pollution Bulletin*, 46, pp.171-181

El-Dib, M.A., Shehata, S.A. and Waly, H.F.A., 1989. Response of freshwater alga *Scenedesmus* to triazine herbicides. *Water, Air, and Soil Pollution*, 48, pp.307-316

Elstner, E., 1991. Mechanisms of oxygen activation in different compartments of plant cells. *Active Oxygen/Oxidative Stress and Plant Metabolism*, 6, pp.13-26

Environment Agency., 1998. Environmental problems from antifouling agents: surveys of manufacturers, chandlers (suppliers) and treatment sites. *Environment Agency Technical Report*, No. P2F(97)03.

Erinle, K.O., Jiang, Z., Ma, B., Li, J., Chen, Y., Ur-Rehman, K., Shahla, A. and Zhang, Y., 2016. Exogenous calcium induces tolerance to atrazine stress in *Pennisetum* seedlings and promotes photosynthetic activity, antioxidant enzymes and psbA gene transcripts. *Ecotoxicology and Environmental Safety*, 132, pp.403-412

Eser, S., 2001. Ecotoxicological investigations of periphyton communities using HPLC analysis. PhD thesis, Technical University of Munich, p.239

Esperanza, M., Seoane, M., Rioboo, C., Herrero, C. and Cid, Á., 2016. Early alterations on photosynthesis-related parameters in *Chlamydomonas reinhardtii* cells exposed to atrazine: A multiple approach study. *Science of the Total Environment*, 554, pp.237-245

Fahl, G.M., Kreft, L., Altenburger, R., Faust, M., Boedeker, W. and Grimme, L.H., 1995. pH-dependent sorption, bioconcentration and algal toxicity of sulfonylurea herbicides. *Aquatic Toxicology*, 31, pp.175-187

Faust, M., Altenburger, R., Backhaus, T., Blanck, H., Boedeker, W., Gramatica, P., Hamer, V., Scholze, M., Vighi, M. and Grimme, L.H., 2003. Joint algal toxicity of 16 dissimilarly acting chemicals is predictable by the concept of independent action. *Aquatic Toxicology*, 63, pp.43-63

Fayez, K.A. and Abd-Elfattah, Z., 2007. Alteration in growth and physiological activities in *Chlorella vulgaris* under the effect of photosynthetic inhibitor diuron. *International J. of Agricul. and Biol*, 1560, pp.09-4

Federal Environmental Protection Agency., 2000. Estimation of the pesticide input in surface waters of Germany. Federal Environmental Protection Agency Report 3/2000, Berlin, Germany

Fent, K., 1996. Ecotoxicology of organotin compounds. *Critical Reviews in Toxicology*, 26, pp.3-117

Fernández-Alba, A.R., Hernando, M.D., Piedra, L. and Chisti, Y., 2002. Toxicity evaluation of single and mixed antifouling biocides measured with acute toxicity bioassays. *Analytica Chimica Acta*, 456, pp.303-312

Fernando, I.S., Kim, M., Son, K.T., Jeong, Y. and Jeon, Y.J., 2016. Antioxidant activity of marine algal polyphenolic compounds: a mechanistic approach. *Journal of Medicinal Food*, 19, pp.615-628

Ferrari, B., Radetski, C.M., Veber, A.M. and Ferard, J.F., 1999. Ecotoxicological assessment of solid wastes: A combined liquid-and solid-phase testing approach using a battery of bioassays and biomarkers. *Environmental Toxicology and Chemistry*, 18, pp.1195-1202

Field, J.A., Reed, R.L., Sawyer, T.E., Griffith, S.M. and Wigington, P.J., 2003. Diuron occurrence and distribution in soil and surface and ground water associated with grass seed production. *Journal of Environmental Quality*, 32, pp.171-179

Fuerst, E.P. and Norman, M.A., 1991. Interactions of herbicides with photosynthetic electron transport. *Weed Sci*, 39, pp.458-464

Feurtet-Mazel, A., Grollier, T., Grouselle, M., Ribeyre, F. and Boudou, A., 1996. Experimental study of bioaccumulation and effects of the herbicide isoproturon on freshwater rooted macrophytes (*Elodea densa* and *Ludwigia natans*). *Chemosphere*, 32, pp.1499-1512

Figueredo, C.C., Giani, A. and Lemos Filho, J.P., 2009. Photosynthetic capacity of three phytoplanktonic species measured by a pulse amplitude fluorometric method. *Brazilian Journal of Plant Physiology*, 21, pp.167-174

Figueroa, F.L., Korbee, N., Carrillo, P., Medina-Sánchez, J.M., Mayte, M.A.T.A., Bonomi, J. and Sanchez-Castillo, P.M., 2009. The effects of UV radiation on photosynthesis estimated as chlorophyll fluorescence in *Zygnemopsis decussata* (Chlorophyta) growing in a high mountain lake (Sierra Nevada, Southern Spain). *Journal of Limnology*, 68, pp.206-216

Fiori, E., Mazzotti, M., Guerrini, F. and Pistocchi, R., 2013. Combined effects of the herbicide terbuthylazine and temperature on different flagellates from the Northern Adriatic Sea. *Aquatic Toxicology*, 128, pp.79-90

Fiori, E. and Pistocchi, R., 2014. *Skeletonema marinoi* (Bacillariophyceae) sensitivity to herbicides and effects of temperature increase on cellular responses to terbuthylazine exposure. *Aquatic Toxicology*, 147, pp.112-120

Foti, M., Ingold, K.U. and Lusztyk, J., 1994. The surprisingly high reactivity of phenoxyl radicals. *Journal of the American Chemical Society*, 116, pp.9440-9447

Foyer, C.H., Lopez-Delgado, H., Dat, J.F. and Scott, I.M., 1997. Hydrogen peroxide-and glutathione-associated mechanisms of acclimatory stress tolerance and signalling. *Physiologia Plantarum*, 100, pp.241-254

Foyer, C.H., Lelandais, M. and Kunert, K.J., 1994. Photooxidative stress in plants. *Physiologia Plantarum*, 92, pp.696-717

Foyer, C.H. and Shigeoka, S., 2011. Understanding oxidative stress and antioxidant functions to enhance photosynthesis. *Plant Physiology*, 155, pp.93-100

Franqueira, D., Cid, A., Torres, E., Orosa, M. and Herrero, C., 1999. A comparison of the relative sensitivity of structural and functional cellular responses in the alga *Chlamydomonas eugametos* exposed to the herbicide paraquat. *Archives of Environmental Contamination and Toxicology*, 36, pp.264-269

Fuerst, E.P. and Norman, M.A., 1991. Interactions of herbicides with photosynthetic electron transport. *Weed Sci*, 39, pp.458-464

Gachon, C.M., Strittmatter, M., Müller, D.G., Kleinteich, J. and Küpper, F.C., 2009. Detection of differential host susceptibility to the marine oomycete pathogen *Eurychasma dicksonii* by real-time PCR: not all algae are equal. *Applied and Environmental Microbiology*, 75, pp.322-328

Galassi, S., Guzzella, L., Mingazzini, M., Vigano, L., Capri, S. and Sora, S., 1992. Toxicological and chemical characterization of organic micropollutants in River Po waters (Italy). *Water Research*, 26, pp.19-27

Gamble, D.S., Khan, S.U. and Tee, O.S., 1983. Atrazine hydrolysis. Part I: Proton catalysis at 25° C. *Pest Management Science*, 14, pp.537-545

Gangolli, S., 1999. The Dictionary of Substances and Their Effects*: C* (Vol. 2). Royal Society of chemistry.

Gatidou, G. and Thomaidis, N.S., 2007. Evaluation of single and joint toxic effects of two antifouling biocides, their main metabolites and copper using phytoplankton bioassays. *Aquatic Toxicology*, 85, pp.184-191

Gatidou, G., Stasinakis, A.S. and Iatrou, E.I., 2015. Assessing single and joint toxicity of three phenylurea herbicides using *Lemna minor* and *Vibrio fischeri* bioassays. *Chemosphere*, 119, pp.S69-S74

Geoffroy, L., Teisseire, H., Couderchet, M. and Vernet, G., 2002. Effect of oxyfluorfen and diuron alone and in mixture on antioxidative enzymes of *Scenedesmus obliquus*. *Pesticide Biochemistry and Physiology*, 72, pp.178-185

Geoffroy, L., Dewez, D., Vernet, G. and Popovic, R., 2003. Oxyfluorfen toxic effect on *S. obliquus* evaluated by different photosynthetic and enzymatic biomarkers. *Archives of Environmental Contamination and Toxicology*, 45, pp.445-452

Geoffroy, L., Frankart, C. and Eullaffroy, P., 2004. Comparison of different physiological parameter responses in *Lemna minor* and *Scenedesmus obliquus* exposed to herbicide flumioxazin. *Environmental Pollution*, 131, pp.233-241

Giacomazzi, S. and Cochet, N., 2004. Environmental impact of diuron transformation: a review. *Chemosphere*, 56, pp.1021-1032

Gonzalez, A., Vera, J., Castro, J., Dennett, G., Mellado, M., Morales, B., Correa, J.A. and Moenne, A., 2010. Co-occurring increases of calcium and organellar reactive oxygen species determine differential activation of antioxidant and defense enzymes

in *Ulva compressa* (Chlorophyta) exposed to copper excess. *Plant, Cell & Environment*, 33, pp.1627-1640

González, A., de los Ángeles Cabrera, M., Henríquez, M.J., Contreras, R.A., Morales, B. and Moenne, A., 2012a. Cross talk among calcium, hydrogen peroxide, and nitric oxide and activation of gene expression involving calmodulins and calcium-dependent protein kinases in *Ulva compressa* exposed to copper excess. *Plant Physiology*, 158, pp.1451-1462

González, A., de los Ángeles Cabrera, M., Mellado, M., Cabello, S., Márquez, S., Morales, B. and Moenne, A., 2012b. Copper-induced intracellular calcium release requires extracellular calcium entry and activation of L-type voltage-dependent calcium channels in *Ulva compressa*. *Plant Signaling & Behavior*, 7, pp.728-732

Graham, L. and Wilcox, L., 1999. Algae. Prentice-Hall, Upper Saddle River, NJ.

Gramatica, P. and Di Guardo, A., 2002. Screening of pesticides for environmental partitioning tendency. *Chemosphere*, 47, pp.947-956

Green, J.M., Jensen, J.E. and Streibig, J.C., 1997. Defining and characterizing synergism and antagonism for xenobiotic mixtures. *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants. Dordrecht, The Netherlands: Kluwer Academic*, pp.263-274

Grenni, P., Rodríguez-Cruz, M.S., Herrero-Hernández, E., Marín-Benito, J.M., Sánchez-Martín, M.J. and Caracciolo, A.B., 2012. Effects of wood amendments on the degradation of terbuthylazine and on soil microbial community activity in a clay loam soil. *Water, Air, & Soil Pollution*, 223, pp.5401-5412

Grollier, T., Feurtet-Mazel, A., Boudou, A. and Ribeyre, F., 1997. Role of temperature on isoproturon bioaccumulation and effects on two freshwater rooted macrophytes: *Elodea densa* and *Ludwigia natans*. *Ecotoxicology and Environmental Safety*, 36, pp.205-212

Grouselle, M., Grollier, T., Feurtetmazel, A., Ribeyre, F. and Boudou, A., 1995. Herbicide isoproturon-specific binding in the freshwater macrophyte *Elodea densa*-a single-cell fluorescence study. *Ecotoxicology and Environmental Safety*, 32, pp.254-259

Guardiola, F.A., Cuesta, A., Meseguer, J. and Esteban, M.A., 2012. Risks of using antifouling biocides in aquaculture. *International Journal of Molecular Sciences*, 13, pp.1541-1560

Hall, A., 1980. Heavy metal co-tolerance in a copper-tolerant population of the marine fouling alga, *Ectocarpus siliculosus* (dillw.) lyngbye. *New Phytologist*, 85, pp.73-78

Hall, D.O. and Rao, K.K., 1995. Photosynthesis. Cambridge University Press, Cambridge

Han, T., Kang, S.H., Park, J.S., Lee, H.K. and Brown, M.T., 2008. Physiological responses of *Ulva pertusa* and *U. armoricana* to copper exposure. *Aquatic Toxicology*, 86, pp.176-184

Hartgers, E.M., Aalderink, G.R., Van den Brink, P.J., Gylstra, R., Wiegman, J.W.F. and Brock, T.C., 1998. Ecotoxicological threshold levels of a mixture of herbicides (atrazine, diuron and metolachlor) in freshwater microcosms. *Aquatic Ecology*, 32, pp.135-152

Haynes, D. and Johnson, J.E., 2000a. Organochlorine, heavy metal and polyaromatic hydrocarbon pollutant concentrations in the Great Barrier Reef (Australia) environment: a review. *Marine Pollution Bulletin*, 41, pp.267-278

Haynes, D., Ralph, P., Prange, J. and Dennison, B., 2000b. The impact of the herbicide diuron on photosynthesis in three species of tropical seagrass. *Marine Pollution Bulletin*, 41, pp.288-293

Heath, R.L. and Packer, L., 1968. Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics*, 125, pp.189-198

Hela, D.G., Lambropoulou, D.A., Konstantinou, I.K. and Albanis, T.A., 2005. Environmental monitoring and ecological risk assessment for pesticide contamination

and effects in Lake Pamvotis, northwestern Greece. *Environmental Toxicology and Chemistry*, 24, pp.1548-1556

Heo, S.J., Ko, S.C., Cha, S.H., Kang, D.H., Park, H.S., Choi, Y.U., Kim, D., Jung, W.K. and Jeon, Y.J., 2009. Effect of phlorotannins isolated from *Ecklonia cava* on melanogenesis and their protective effect against photo-oxidative stress induced by UV-B radiation. *Toxicology In Vitro*, 23, pp.1123-1130

Hernando, M.D., Ejerhoon, M., Fernandez-Alba, A.R. and Chisti, Y., 2003. Combined toxicity effects of MTBE and pesticides measured with *Vibrio fischeri* and *Daphnia magna* bioassays. *Water Research*, 37, pp.4091-4098

Hildebrandt, A.G. and Roots, I., 1975. Reduced nicotinamide adenine dinucleotide phosphate (NADPH)-dependent formation and breakdown of hydrogen peroxide during mixed function oxidation reactions in liver microsomes. *Archives of Biochemistry and Biophysics*, 171, pp.385-397

Hildebrandt, A., Guillamón, M., Lacorte, S., Tauler, R. and Barceló, D., 2008. Impact of pesticides used in agriculture and vineyards to surface and groundwater quality (North Spain). *Water Research*, 42, pp.3315-3326

Holtkamp, A.D., Kelly, S., Ulber, R. and Lang, S., 2009. Fucoidans and fucoidanases—focus on techniques for molecular structure elucidation and modification of marine polysaccharides. *Applied Microbiology and Biotechnology*, 82, pp.1-11

Hong, Y., Hu, H.Y., Xie, X., Sakoda, A., Sagehashi, M. and Li, F.M., 2009. Gramine-induced growth inhibition, oxidative damage and antioxidant responses in freshwater cyanobacterium *Microcystis aeruginosa*. *Aquatic Toxicology*, 91, pp.262-269

Horton, P. and Hague, A., 1988. Studies on the induction of chlorophyll fluorescence in isolated barley protoplasts. IV. Resolution of non-photochemical quenching. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, 932, pp.107-115

Hull, H.M., 1967. Herbicide handbook of the Weed Society of America. W.F. Humphrey Press, Geneva, New York, Humphrey, p.281

Hunt, R., 1982. Plant growth curves. Edward Arnold, London. ISBN 0-7131-2844-5

INRA, 2004. Agritox, Base de données sur les substances actives phytopharmaceutiques. (see <http://www.inra.fr/agritox/> (accessed 4.11.13)

Irace-Guigand, S., Aaron, J.J., Scribe, P. and Barcelo, D., 2004. A comparison of the environmental impact of pesticide multiresidues and their occurrence in river waters surveyed by liquid chromatography coupled in tandem with UV diode array detection and mass spectrometry. *Chemosphere*, 55, pp.973-981

ISO 8692., 1989. Water quality—Fresh water algal growth inhibition test with *Scenedesmus subspicatus* or *Selenastrum capricornutum* International Organization for Standardization, Geneva

Jacobson, A.H. and Willingham, G.L., 2000. Sea-nine antifoulant: an environmentally acceptable alternative to organotin antifoulants. *Science of the Total Environment*, 258, pp.103-110

Johnson, A.C., Haria, A.H., Bhardwaj, C.L., Williams, R.J. and Walker, A., 1996. Preferential flow pathways and their capacity to transport isoproturon in a structured clay soil. *Pest Management Science*, 48, pp.225-237

Jones, R., 2005. The ecotoxicological effects of Photosystem II herbicides on corals. *Marine Pollution Bulletin*, 51, pp.495-506

Juneau, P., Dewez, D., Matsui, S., Kim, S.G. and Popovic, R., 2001. Evaluation of different algal species sensitivity to mercury and metolachlor by PAM-fluorometry. *Chemosphere*, 45, pp.589-598

Juneau, P., El Berdey, A. and Popovic, R., 2002. PAM fluorometry in the determination of the sensitivity of *Chlorella vulgaris*, *Selenastrum capricornutum*, and *Chlamydomonas reinhardtii* to copper. *Archives of Environmental Contamination and Toxicology*, 42, pp.155-164

Juneau, P., Green, B.R. and Harrison, P.J., 2005. Simulation of Pulse-Amplitude-Modulated (PAM) fluorescence: Limitations of some PAM-parameters in studying environmental stress effects. *Photosynthetica*, 43, pp.75-83

Juneau, P., Qiu, B. and Deblois, C.P., 2007. Use of chlorophyll fluorescence as a tool for determination of herbicide toxic effect. *Toxicological and Environ Chemistry*, 89, pp.609-625

Katsaros, C., Karyophyllis, D. and Galatis, B., 2006. Cytoskeleton and morphogenesis in brown algae. *Annals of Botany*, 97, pp.679-693

Kawai, H., Hanyuda, T., Draisma, S.G. and Müller, D.G., 2007. Molecular phylogeny of *Discosporangium mesarthrocarpum* (Phaeophyceae) with a reinstatement of the order Discosporangiales. *Journal of Phycology*, 43, pp.186-194

Kirby, M.F. and Sheahan, D.A., 1994. Effects of atrazine, isoproturon, and mecoprop on the macrophyte *Lemna minor* and the alga *Scenedesmus subspicatus*. *Bulletin of Environmental Contamination and Toxicology*, 53, pp.120-126

Klarzynski, O., Plesse, B., Joubert, J.M., Yvin, J.C., Kopp, M., Kloareg, B. and Fritig, B., 2000. Linear β-1, 3 glucans are elicitors of defense responses in tobacco. *Plant Physiology*, 124, pp.1027-1038

Kleczkowski, L.A., 1994. Inhibitors of photosynthetic enzymes/carriers and metabolism. *Annual Review of Plant Biology*, 45, pp.339-367

Kloareg, B. and Quatrano, R.S., 1988. Structure of the cell walls of marine algae and ecophysiological functions of the matrix polysaccharides. *Oceanography and Marine Biology: An Annual Review*, 26, pp.259-315

Knauert, S., Escher, B., Singer, H., Hollender, J. and Knauer, K., 2008. Mixture toxicity of three photosystem II inhibitors (atrazine, isoproturon, and diuron) toward photosynthesis of freshwater phytoplankton studied in outdoor mesocosms. *Environmental Science & Technology*, 42, pp.6424-6430

Knauert, S., Singer, H., Hollender, J. and Knauer, K., 2010. Phytotoxicity of atrazine, isoproturon, and diuron to submersed macrophytes in outdoor mesocosms. *Environmental Pollution*, 158, pp.167-174

Knörzer, O.C., Burner, J. and Boger, P., 1996. Alterations in the antioxidative system of suspension-cultured soybean cells (*Glycine max*) induced by oxidative stress. *Physiologia Plantarum*, 97, pp.388-396

Kobayashi, N. and Okamura, H., 2002. Effects of new antifouling compounds on the development of sea urchin. *Marine Pollution Bulletin*, 44, pp.748-751

Kodes, V., Vejvodova, J. and Kollerova, M., 2010. "Screening pesticidu v hydrosfere CR. *Hydrologicke´ dny* (2010)," in Voda v menicim se prostredı, pp. 193–198, Hradec Kralove, Czech Republic

Konstantinou, I.K. and Albanis, T.A., 2004. Worldwide occurrence and effects of antifouling paint booster biocides in the aquatic environment: a review. *Environment International*, 30, pp.235-248

Koutsaftis, A. and Aoyama, I., 2006. The interactive effects of binary mixtures of three antifouling biocides and three heavy metals against the marine algae *Chaetoceros gracilis*. *Environmental Toxicology*, 21, pp.432-439

Kraan, S., 2013. Mass-cultivation of carbohydrate rich macroalgae, a possible solution for sustainable biofuel production. *Mitigation and Adaptation Strategies for Global Change*, 18, pp.27-46

Krause, G.H. and Weis, E., 1984. Chlorophyll fluorescence as a tool in plant physiology. *Photosynthesis Research*, 5, pp.139-157

Krause, G.H. and Weis, E., 1991. Chlorophyll fluorescence and photosynthesis: the basics. *Annual Review of Plant Biology*, 42, pp.313-349

Kroon, F.J., Kuhnert, P.M., Henderson, B.L., Wilkinson, S.N., Kinsey-Henderson, A., Abbott, B., Brodie, J.E. and Turner, R.D., 2012. River loads of suspended solids,

nitrogen, phosphorus and herbicides delivered to the Great Barrier Reef lagoon. *Marine Pollution Bulletin*, 65, pp.167-181

Ksouri, R., Megdiche, W., Debez, A., Falleh, H., Grignon, C. and Abdelly, C., 2007. Salinity effects on polyphenol content and antioxidant activities in leaves of the halophyte *Cakile maritima*. *Plant Physiology and Biochemistry*, 45, pp.244-249

Kumar, S., Habib, K. and Fatma, T., 2008. Endosulfan induced biochemical changes in nitrogen-fixing cyanobacteria. *Science of The Total Environment*, 403, pp.130-138

Kumar, K.S., Choo, K.S., Yea, S.S., Seo, Y. and Han, T., 2010. Effects of the phenylurea herbicide diuron on the physiology of *Saccharina japonica* aresch. *Toxicology and Environmental Health Sciences*, 2, pp.188-199

Kumar, K.S. and Han, T., 2010. Physiological response of *Lemna* species to herbicides and its probable use in toxicity testing. *Toxicology and Environmental Health Sciences*, 2, pp.39-49

Kumar, S., Bhanjana, G., Sharma, A., Sidhu, M.C. and Dilbaghi, N., 2014. Synthesis, characterization and on field evaluation of pesticide loaded sodium alginate nanoparticles. *Carbohydrate Polymers*, 101, pp.1061-1067

Lajayer, B.A., Ghorbanpour, M. and Nikabadi, S., 2017. Heavy metals in contaminated environment: Destiny of secondary metabolite biosynthesis, oxidative status and phytoextraction in medicinal plants. *Ecotoxicology and Environmental Safety*, 145, pp.377-390

Lam, C. and Harder, T., 2007. Marine macroalgae affect abundance and community richness of bacterioplankton in close proximity. *Journal of Phycology*, 43, pp.874-881

Lambert, S.J., Thomas, K.V. and Davy, A.J., 2006. Assessment of the risk posed by the antifouling booster biocides Irgarol 1051 and diuron to freshwater macrophytes. *Chemosphere*, 63, pp.734-743

Lamoree, M.H., Swart, C.P., Van der Horst, A. and Van Hattum, B., 2002. Determination of diuron and the antifouling paint biocide Irgarol 1051 in Dutch marinas and coastal waters. *Journal of Chromatography A*, 970, pp.183-190

Lamoureux, G.L., Simoneaux, B. and Larson, J., 1998. The metabolism of atrazine and related 2-chloro-4 6-bis(alkylamino)-s-triazines in plants (Chapter 6). In: Bal-lantine, L.G., McFarland, J.E., Hackett, D.S. (Eds.), Triazine Herbicides: RiskAssessment, vol. 683. American Chemical Society, Symp. Series, Washington,D.C, pp. 60–81

Langlois, J., Sassi, J.F., Jard, G., Steyer, J.P., Delgenes, J.P. and Hélias, A., 2012. Life cycle assessment of biomethane from offshore-cultivated seaweed. *Biofuels, Bioproducts and Biorefining*, 6, pp.387-404

Laviale, M., Morin, S. and Créach, A., 2011. Short term recovery of periphyton photosynthesis after pulse exposition to the photosystem II inhibitors atrazine and isoproturon. *Chemosphere*, 84, pp.731-734

Lawson, N.M. and Mason, R.P., 1998. Accumulation of mercury in estuarine food chains. *Biogeochemistry*, 40, pp.235-247

Lazãr, D. A. and Lazãr, C., 2001. The influence of the isoproturon herbicide on growth, gaseous exchanges and assimilatory pigment contents in *Botryococcus braunii* kuetz. *Romanian Agricultural Research*, 15, pp.49-53

Le Bail, A., Charrier, B., Cock, M.J., de Franco, P.O., Dittami, S.M., Rousvoal, S. and Tonon, T., 2008. Normalisation genes for expression analyses in the brown alga model *Ectocarpus siliculosus*. *BMC Molecular Biology*, 9, p.75

Leboulanger, C., Rimet, F., de Lacotte, M.H. and Bérard, A., 2001. Effects of atrazine and nicosulfuron on freshwater microalgae. *Environment International*, 26, pp.131-135

Lee, R. K. S., 1980. A catalogue of the marine algae of the Canadian Arctic. Ottawa: National Museums of Canada, Publications in Botany, No. 9, p. 82

Lee, M.Y. and Shin, H.W., 2003. Cadmium-induced changes in antioxidant enzymes from the marine alga *Nannochloropsis oculata*. *Journal of Applied Phycology*, 15, pp.13-19

Lesser, M.P., 2006. Oxidative stress in marine environments: biochemistry and physiological ecology. *Annual Review Physiology*, 68, pp.253-278

Lewis, S.E., Brodie, J.E., Bainbridge, Z.T., Rohde, K.W., Davis, A.M., Masters, B.L., Maughan, M., Devlin, M.J., Mueller, J.F. and Schaffelke, B., 2009. Herbicides: a new threat to the Great Barrier Reef. *Environmental Pollution*, 157, pp.2470-2484

Lichtenthaler, H.K., 1996. Vegetation stress: an introduction to the stress concept in plants. *Journal of Plant Physiology*, 148, pp.4-14

Lin, Y.J., Karuppiah, M., Shaw, A. and Gupta, G., 1999. Effect of simulated sunlight on atrazine and metolachlor toxicity of surface waters. *Ecotoxicology and Environmental Safety*, 43, pp.35-37

Linders, J. B. H., Jansma, J. W., Mensink, B. J. W. and Otermann, K., 1994. Pesticides: Benefaction or Pandora's Box? A synopsis of the environmental aspects of 243 pesticides. R.I.V.M., Bilthoven, The Netherlands

Link, G., Tiller, K. and Baginsky, S., 1997. Glutathione, a regulator of chloroplast transcription. *Regulation of Enzymatic Systems Detoxifying Xenobiotics in Plants. Kluwer Academic Publishers, Dordrecht, The Netherlands*, pp.125-137

Liu, W., Ming, Y., Huang, Z. and Li, P., 2012. Impacts of florfenicol on marine diatom *Skeletonema costatum* through photosynthesis inhibition and oxidative damages. *Plant Physiology and Biochemistry*, 60, pp.165-170

Liu, S.S., Wang, C.L., Zhang, J., Zhu, X.W. and Li, W.Y., 2013. Combined toxicity of pesticide mixtures on green algae and photobacteria. *Ecotoxicology and Environmental Safety*, 95, pp.98-103

Loos, R., Gawlik, B.M., Locoro, G., Rimaviciute, E., Contini, S. and Bidoglio, G., 2009. EU-wide survey of polar organic persistent pollutants in European river waters. *Environmental Pollution*, 157, pp.561-568

Lopez, F., Vansuyt, G., Casse-Delbart, F. and Fourcroy, P., 1996. Ascorbate peroxidase activity, not the mRNA level, is enhanced in salt-stressed *Raphanus sativus* plants. *Physiologia Plantarum*, 97, pp.13-20

Luna-Acosta, A., Renault, T., Thomas-Guyon, H., Faury, N., Saulnier, D., Budzinski, H., Le Menach, K., Pardon, P., Fruitier-Arnaudin, I. and Bustamante, P., 2012. Detection of early effects of a single herbicide (diuron) and a mix of herbicides and pharmaceuticals (diuron, isoproturon, ibuprofen) on immunological parameters of Pacific oyster (*Crassostrea gigas*) spat. *Chemosphere*, 87, pp.1335-1340

Macedo, R.S., Lombardi, A.T., Omachi, C.Y. and Rörig, L.R., 2008. Effects of the herbicide bentazon on growth and photosystem II maximum quantum yield of the marine diatom *Skeletonema costatum*. *Toxicology in Vitro*, 22, pp.716-722

Macinnis-Ng, C.M. and Ralph, P.J., 2003. Short-term response and recovery of *Zostera capricorni* photosynthesis after herbicide exposure. *Aquatic Botany*, 76, pp.1-15

Maharana, D., Jena, K., Pise, N.M. and Jagtap, T.G., 2010. Assessment of oxidative stress indices in a marine macro brown alga *Padina tetrastromatica* (Hauck) from comparable polluted coastal regions of the Arabian Sea, west coast of India. *Journal of Environmental Sciences*, 22, pp.1413-1417

Maier, I., Parodi, E., Westermeier, R. and Müller, D.G., 2000. *Maullinia ectocarpii* gen. et sp. nov.(Plasmodiophorea), an intracellular parasite in *Ectocarpus siliculosus* (Ectocarpales, Phaeophyceae) and other filamentous brown algae. *Protist*, 151, pp.225-238

Mallick, N. and Mohn, F.H., 2003. Use of chlorophyll fluorescence in metal-stress research: a case study with the green microalga *Scenedesmus*. *Ecotoxicology and Environmental Safety*, 55, pp.64-69

Manzo, S., Buono, S. and Cremisini, C., 2008. Predictability of copper, irgarol, and diuron combined effects on sea urchin *Paracentrotus lividus*. *Archives of Environmental Contamination and Toxicology*, 54, pp.57-68

Marchini, S., Passerini, L., Cesareo, D. and Tosato, M.L., 1988. Herbicidal triazines: Acute toxicity on *Daphnia*, fish, and plants and analysis of its relationships with structural factors. *Ecotoxicology and Environmental Safety*, 16, pp.148-157

Maule, A. and Wright, S.J.L., 1984. Herbicide effects on the population growth of some green algae and cyanobacteria. *Journal of Applied Microbiology*, 57, pp.369-379

Maxwell, K. and Johnson, G.N., 2000. Chlorophyll fluorescence—a practical guide. *Journal of Experimental Botany*, 51, pp.659-668

McClanahan, T.R., Cokos, B.A. and Sala, E., 2002. Algal growth and species composition under experimental control of herbivory, phosphorus and coral abundance in Glovers Reef, Belize. *Marine Pollution Bulletin*, 44, pp.441-451

Mellado, M., Contreras, R.A., González, A., Dennett, G. and Moenne, A., 2012. Copper-induced synthesis of ascorbate, glutathione and phytochelatins in the marine alga *Ulva compressa* (Chlorophyta). *Plant Physiology and Biochemistry*, 51, pp.102-108

Misheer, N., Kindness, A. and Jonnalagadda, S.B., 2006. Seaweeds along KwaZulu-Natal coast of South Africa—3: elemental uptake by *Ulva lactuca* (sea lettuce). *Journal of Environmental Science and Health Part A*, 41, pp.1249-1259

Mitchell, C., Brodie, J. and White, I., 2005. Sediments, nutrients and pesticide residues in event flow conditions in streams of the Mackay Whitsunday Region, Australia. *Marine Pollution Bulletin*, 51, pp.23-36

Mladinic, M., Zeljezic, D., Shaposhnikov, S.A. and Collins, A.R., 2012. The use of FISH-comet to detect c-Myc and TP 53 damage in extended-term lymphocyte cultures treated with terbuthylazine and carbofuran. *Toxicology Letters*, 211, pp.62-69

Mofeed, J. and Mosleh, Y.Y., 2013. Toxic responses and antioxidative enzymes activity of *Scenedesmus obliquus* exposed to fenhexamid and atrazine, alone and in mixture. *Ecotoxicology and Environmental Safety*, 95, pp.234-240

Møller, I.M., Jensen, P.E. and Hansson, A., 2007. Oxidative modifications to cellular components in plants. *Annu. Rev. Plant Biol*, 58, pp.459-481

Morris, O.P. and Russell, G., 1974. Inter-specific differences in responses to copper by natural populations of *Ectocarpus*. *British Phycological Journal*, 9, pp.269-272

Mosleh, Y.Y., Paris-Palacios, S., Arnoult, F., Couderchet, M. and Vernet, G., 2004. Metallothionein induction in the aquatic oligochaete *Tubifex tubifex* exposed to the herbicide isoproturon. *Environmental Toxicology*, 19, pp.88–93

Müller, D.G., 1972. Studies on reproduction in *Ectocarpus siliculosus*. *Bulletin de la Société Botanique de France*, 119(sup1), pp.87-97

Müller, D.G., Kapp, M. and Knippers, R., 1998. Viruses in marine brown algae. *Advances in Virus Research*, 50, pp.49-67

Müller, P., Li, X.P. and Niyogi, K.K., 2001. Non-photochemical quenching. A response to excess light energy. *Plant Physiology*, 125, pp.1558-1566

Müller, K., Bach, M., Hartmann, H., Spiteller, M. and Frede, H.G., 2002. Point-and nonpoint-source pesticide contamination in the Zwester Ohm catchment, Germany. *Journal of Environmental Quality*, 31, pp.309-318

Murchie, E.H. and Lawson, T., 2013. Chlorophyll fluorescence analysis: a guide to good practice and understanding some new applications. *Journal of Experimental Botany*, 64, pp.3983-3998

Myers, J.H., Gunthorpe, L., Allinson, G. and Duda, S., 2006. Effects of antifouling biocides to the germination and growth of the marine macroalga, *Hormosira banksii* (Turner) Desicaine. *Marine Pollution Bulletin*, 52, pp.1048-1055

Nakano, Y. and Asada, K., 1981. Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant and Cell Physiology*, 22, pp.867-880

Navarro, S., Vela, N., Giménez, M.J. and Navarro, G., 2004. Persistence of four s-triazine herbicides in river, sea and groundwater samples exposed to sunlight and darkness under laboratory conditions. *Science of the Total Environment*, 329, pp.87-97

Negri, A.P., Flores, F., Mercurio, P., Mueller, J.F. and Collier, C.J., 2015. Lethal and sub-lethal chronic effects of the herbicide diuron on seagrass. *Aquatic Toxicology*, 165, pp.73-83

Nemat Alla, M.M., Hassan, N.M. and El-Bastawisy, Z.M., 2008. Changes in antioxidants and kinetics of glutathione-S-transferase of maize in response to isoproturon treatment. *Plant Biosystems*, 142, pp.5-16

Neushul, P., 1989. Seaweed for war: California's World War I kelp industry. *Technology and Culture*, 30, pp.561-583

Nicolaus, B., Sandmann, G., Watanabe, H., Wakabayashi, K. and Böger, P., 1989. Herbicide-induced peroxidation: influence of light and diuron on protoporphyrin IX formation. *Pesticide Biochemistry and Physiology*, 35, pp.192-201

Nielsen, H.D., Brownlee, C., Coelho, S.M. and Brown, M.T., 2003. Inter-population differences in inherited copper tolerance involve photosynthetic adaptation and exclusion mechanisms in *Fucus serratus*. *New Phytologist*, 160, pp.157-165

Niewiadomska, E. and Miszalski, Z., 1997. Determination of some oxidative stress parameters in variegated leaves of *Chlorophytum comosum* (Thunb) Bak. *Acta Physiologiae Plantarum*, 19, pp.33-39

Noppe, H., Ghekiere, A., Verslycke, T., De Wulf, E., Verheyden, K., Monteyne, E., Polfliet, K., Van Caeter, P., Janssen, C.R. and De Brabander, H.F., 2007. Distribution and ecotoxicity of chlorotriazines in the Scheldt Estuary (B-Nl). *Environmental Pollution*, 147, pp.668-676

Noyrod, P., Chailapakul, O., Wonsawat, W. and Chuanuwatanakul, S., 2014. The simultaneous determination of isoproturon and carbendazim pesticides by single drop analysis using a graphene-based electrochemical sensor. *Journal of Electroanalytical Chemistry*, 719, pp.54-59

OECD., 1984. Algal growth inhibition test. OECD guidelines for testing chemicals, No. 201, Organization for Economic Cooperation and Development, Geneva, Switzerland

Ohki, A., Ohki, S., Koizumi, K., Böger, P., Sato, Y., Kohno, H. and Wakabayashi, K., 1997. Phytotoxicity caused by peroxidizing herbicides is alleviated by 2-substituted 4, 6-bis (ethylamino)-1, 3, 5-triazines. *Journal of Pesticide Science*, 22, pp.309-313

Okamura, H., Watanabe, T., Aoyama, I. and Hasobe, M., 2002. Toxicity evaluation of new antifouling compounds using suspension-cultured fish cells. *Chemosphere*, 46, pp.945-951

Okamura, H., Nishida, T., Ono, Y. and Shim, W.J., 2003. Phytotoxic effects of antifouling compounds on nontarget plant species. *Bulletin of Environmental Contamination and Toxicology*, 71, pp.881-886

Olaniran, A.O., Balgobind, A. and Pillay, B., 2013. Bioavailability of heavy metals in soil: impact on microbial biodegradation of organic compounds and possible improvement strategies. *International Journal of Molecular Sciences*, 14, pp.10197-10228

Olette, R., Couderchet, M., Biagianti, S. and Eullaffroy, P., 2008. Toxicity and removal of pesticides by selected aquatic plants. *Chemosphere*, 70, pp.1414-1421

Paris-Palacios, S., Mosleh, Y.Y., Almohamad, M., Delahaut, L., Conrad, A., Arnoult, F. and Biagianti-Risbourg, S., 2010. Toxic effects and bioaccumulation of the herbicide isoproturon in *Tubifex tubifex* (Oligocheate, Tubificidae): a study of significance of autotomy and its utility as a biomarker. *Aquatic Toxicology*, 98, pp.8-14

Park, J.S., Brown, M.T. and Han, T., 2012. Phenol toxicity to the aquatic macrophyte *Lemna paucicostata*. *Aquatic Toxicology*, 106, pp.182-188

Park, J., Brown, M.T., Depuydt, S., Kim, J.K., Won, D.S. and Han, T., 2017. Comparing the acute sensitivity of growth and photosynthetic endpoints in three *Lemna* species exposed to four herbicides. *Environmental Pollution*, 220, pp.818-827

Parks, R., Donnier-Marechal, M., Frickers, P.E., Turner, A. and Readman, J.W., 2010. Antifouling biocides in discarded marine paint particles. *Marine Pollution Bulletin*, 60, pp.1226-1230

Pavia, H., Cervin, G., Lindgren, A. and Åberg, P., 1997. Effects of UV-B radiation and simulated herbivory on phlorotannins in the brown alga *Ascophyllum nodosum*. *Marine Ecology Progress Series*, pp.139-146

Pawlik-Skowrońska, B., Pirszel, J. and Brown, M.T., 2007. Concentrations of phytochelatins and glutathione found in natural assemblages of seaweeds depend on species and metal concentrations of the habitat. *Aquatic Toxicology*, 83, pp.190-199

Payne, J., Rajapakse, N., Wilkins, M. and Kortenkamp, A., 2000. Prediction and assessment of the effects of mixtures of four xenoestrogens. *Environmental Health Perspectives*, 108, p.983

Peakall, D.B. and Walker, C.H., 1994. The role of biomarkers in environmental assessment (3). Vertebrates. *Ecotoxicology*, 3, pp.173-179

Percival, G.C., 2005. The use of chlorophyll fluorescence to identify chemical and environmental stress in leaf tissue of three oak (*Quercus*) species. *Journal of Arboriculture*, 31, p.215

Pérès, F., Florin, D., Grollier, T., Feurtet-Mazel, A., Coste, M., Ribeyre, F., Ricard, M. and Boudou, A., 1996. Effects of the phenylurea herbicide isoproturon on periphytic diatom communities in freshwater indoor microcosms. *Environmental Pollution*, 94, pp.141-152

Pérez, J., Domingues, I., Monteiro, M., Soares, A.M. and Loureiro, S., 2013. Synergistic effects caused by atrazine and terbuthylazine on chlorpyrifos toxicity to early-life stages of the zebrafish *Danio rerio*. *Environmental Science and Pollution Research*,

Pesce, S., Lissalde, S., Lavieille, D., Margoum, C., Mazzella, N., Roubeix, V. and Montuelle, B., 2010. Evaluation of single and joint toxic effects of diuron and its

main metabolites on natural phototrophic biofilms using a pollution-induced community tolerance (PICT) approach. *Aquatic Toxicology*, 99, pp.492-499

Peters, A.F., Scornet, D., Müller, D.G., Kloareg, B. and Cock, J.M., 2004. Inheritance of organelles in artificial hybrids of the isogamous multicellular chromist alga *Ectocarpus siliculosus* (Phaeophyceae). *European Journal of Phycology*, 39, pp.235-242

Phang, S.M., 2010. Potential products from tropical algae and seaweeds, especially with reference to Malaysia. *Malaysian Journal of Science*, 29, pp.160-166

Pietsch, C., Krause, E., Kent-Burnison, B., Steinberg, C.E. and Pflugmacher, S., 2006. Effects and metabolism of the phenylurea herbicide isoproturon in the submerged macrophyte *Ceratophyllum demersum* L. *Journal of Applied Botany and Food Quality*, 80, pp.25-30

Pinto, E., Sigaud-kutner, T., Leitao, M.A., Okamoto, O.K., Morse, D. and Colepicolo, P., 2003. Heavy metal–induced oxidative stress in algae. *Journal of Phycology*, 39, pp.1008-1018

Pise, N.M., Gaikwad, D.K. and Jagtap, T.G., 2013. Oxidative stress and antioxidant indices of the marine red alga *Porphyra vietnamensis*. *Acta Botanica Croatica*, 72, pp.197-209

Potin, P., Bouarab, K., Salaün, J.P., Pohnert, G. and Kloareg, B., 2002. Biotic interactions of marine algae. *Current Opinion in Plant Biology*, 5, pp.308-317

Provasoli, L. and Carlucci, A.F., 1974. Vitamins and growth regulators. In 'Algal Physiology and Biochemistry.' (Ed. WDP Stewart) pp. 741-778. (Blackwell: Oxford)

Radetski, C.M., Cotelle, S. and Férard, J.F., 2000. Classical and biochemical endpoints in the evaluation of phytotoxic effects caused by the herbicide trichloroacetate. *Environmental and Experimental Botany*, 44, pp.221-229

Rainbow, P.S., Kriefman, S., Smith, B.D. and Luoma, S.N., 2011. Have the bioavailabilities of trace metals to a suite of biomonitors changed over three decades in SW England estuaries historically affected by mining?. *Science of The Total Environment*, 409, pp.1589-1602

Ralph, P.J., 2000. Herbicide toxicity of *Halophila ovalis* assessed by chlorophyll a fluorescence. *Aquatic Botany*, 66, pp.141-152

Rana, B.C. and Kumar, J.N., 1995. Observations on the effect of the herbicide isoproturon on aquatic targets and associated organisms. *Bulletin of Environmental Contamination and Toxicology*, 55, pp.104-110

Ranieri, A., D'urso, G., Nali, C., Lorenzini, G. and Soldatini, G.F., 1996. Ozone stimulates apoplastic antioxidant systems in pumpkin leaves. *Physiologia Plantarum*, 97, pp.381-387

Rascher, U., Liebig, M. and Lüttge, U., 2000. Evaluation of instant light-response curves of chlorophyll fluorescence parameters obtained with a portable chlorophyll fluorometer on site in the field. *Plant, Cell & Environment*, 23, pp.1397-1405

Ratkevicius, N., Correa, J.A. and Moenne, A., 2003. Copper accumulation, synthesis of ascorbate and activation of ascorbate peroxidase in *Enteromorpha compressa* (L.) Grev.(Chlorophyta) from heavy metal-enriched environments in northern Chile. *Plant, Cell & Environment*, 26, pp.1599-1608

Reddy, K.N. and Locke, M.A., 1996. Molecular properties as descriptors of octanol-water partition coefficients of herbicides. *Water, Air, & Soil Pollution*, 86, pp.389-405

Rejeb, I.B., Pastor, V. and Mauch-Mani, B., 2014. Plant responses to simultaneous biotic and abiotic stress: molecular mechanisms. *Plants*, 3, pp.458-475

Rensen, J.J., 1982. Molecular mechanisms of herbicide action near photosystem II. *Physiologia Plantarum*, 54, pp.515-521

Revitt, D.M., Ellis, J.B. and Llewellyn, N.R., 2002. Seasonal removal of herbicides in urban runoff. *Urban Water*, 4, pp.13-19

Rimet, F. and Bouchez, A., 2011. Use of diatom life-forms and ecological guilds to assess pesticide contamination in rivers: lotic mesocosm approaches. *Ecological Indicators*, 11, pp.489-499

Rioboo, C., González, O., Herrero, C. and Cid, A., 2002. Physiological response of freshwater microalga (*Chlorella vulgaris*) to triazine and phenylurea herbicides. *Aquatic Toxicology*, 59, pp.225-235

Ritter, A., Goulitquer, S., Salaün, J.P., Tonon, T., Correa, J.A. and Potin, P., 2008. Copper stress induces biosynthesis of octadecanoid and eicosanoid oxygenated derivatives in the brown algal kelp *Laminaria digitata*. *New Phytologist*, 180, pp.809-821

Rivard, L., 2003. Environmental fate of metolachlor. *Chemistry*, 51218, pp.45-2

Roberts, T. R., Hutson, D.H., Lee, P.W., Nicholls, P.H. and Plimmer, J. R., 1998. Metabolic pathways of agrochemicals, in Part 1: Herbicides and Plant Growth Regulators, The Royal Society of Chemistry, Cambridge, UK, 1st edition, 1998

Ruban, A.V. and Horton, P., 1995. Regulation of non-photochemical quenching of chlorophyll fluorescence in plants. *Functional Plant Biology*, 22, pp.221-230

Ruhland, C.T., Fogal, M.J., Buyarski, C.R. and Krna, M.A., 2007. Solar ultraviolet-B radiation increases phenolic content and ferric reducing antioxidant power in *Avena sativa*. *Molecules*, 12, pp.1220-1232

Russell, G. and Morris, O.P., 1970. Copper tolerance in the marine fouling alga *Ectocarpus siliculosus*. *Nature*, 228, pp.288-289

Russell, G. and Morris, O.P., 1972. Ship-fouling as an evolutionary process. In *Proceedings of the Third International Congress on Marine Corrosion and Fouling. Northwestern University Press, Evanston, Illinois* (pp. 719-730)

Russell, G. and Bolton, J.J., 1975. Euryhaline ecotypes of *Ectocarpus siliculosus* (Dillw.) Lyngb. *Estuarine and Coastal Marine Science*, 3, pp.91-94

Rutherford, A.W. and Krieger-Liszkay, A., 2001. Herbicide-induced oxidative stress in photosystem II. *Trends in Biochemical Sciences*, 26, pp.648-653

Sabatini, S.E., Juarez, A.B., Eppis, M.R., Bianchi, L., Luquet, C.M. and de Molina, M.D.C.R., 2009. Oxidative stress and antioxidant defenses in two green microalgae exposed to copper. *Ecotoxicology and Environmental Safety*, 72, pp.1200-1206

Sáez, C.A., Pérez-Matus, A., Lobos, M.G., Oliva, D., Vásquez, J.A. and Bravo, M., 2012. Environmental assessment in a shallow subtidal rocky habitat: Approach coupling chemical and ecological tools. *Chemistry and Ecology*, 28, pp.1-15

Sáez, C.A., Roncarati, F., Moenne, A., Moody, A.J. and Brown, M.T., 2015. Copper-induced intra-specific oxidative damage and antioxidant responses in strains of the brown alga *Ectocarpus siliculosus* with different pollution histories. *Aquatic Toxicology*, 159, pp.81-89

Saladin, G. and Clément, C., 2005. Physiological side effects of pesticides on non-target plants. *Agriculture and Soil Pollution: New Research*, pp.53-86

Sandrin, T.R. and Maier, R.M., 2003. Impact of metals on the biodegradation of organic pollutants. *Environmental Health Perspectives*, 111, p.1093

Sbrilli, G., Bimbi, B., Cioni, F., Pagliai, L., Luchi, F. and Lanciotti, E., 2005. Surface and ground waters characterization in Tuscany (Italy) by using algal bioassay and pesticide determinations: comparative evaluation of the results and hazard assessment of the pesticides impact on primary productivity. *Chemosphere*, 58, pp.571-578

Schafer, H., Hettler, H., Fritsche, U., Pitzen, G., Roderer, G. and Wenzel, A., 1994. Biotests using unicellular algae and ciliates for predicting long-term effects of toxicants. *Ecotoxicology and Environmental Safety*, 27, pp.64-81

Schiel, D.R. and Foster, M.S., 2006. The population biology of large brown seaweeds: ecological consequences of multiphase life histories in dynamic coastal environments. *Annual Review of Ecology, Evolution and Systematics*, 37, pp.343-372

Schiel, D.R., 2006. Rivets or bolts? When single species count in the function of temperate rocky reef communities. *Journal of Experimental Marine Biology and Ecology*, 338, pp.233-252

Schiel, D.R. and Lilley, S.A., 2007. Gradients of disturbance to an algal canopy and the modification of an intertidal community. *Marine Ecology Progress Series*, 339, pp.1-11

Schmidt, É.C., Pereira, B., dos Santos, R.W., Gouveia, C., Costa, G.B., Faria, G.S., Scherner, F., Horta, P.A., de Paula Martins, R., Latini, A. and Ramlov, F., 2012. Responses of the macroalgae *Hypnea musciformis* after in vitro exposure to UV-B. *Aquatic Botany*, 100, pp.8-17

Schmitt-Jansen, M. and Altenburger, R., 2005. Toxic effects of isoproturon on periphyton communities–a microcosm study. *Estuarine, Coastal and Shelf Science*, 62, pp.539-545

Schoenwaelder, M.E., 2002. The occurrence and cellular significance of physodes in brown algae. *Phycologia*, 41, pp.125-139

Schoenwaelder, M.E. and Clayton, M.N., 1998. Secretion of phenolic substances into the zygote wall and cell plate in embryos of *Hormosira* and *Acrocarpia* (Fucales, Phaeophyceae). *Journal of Phycology*, 34, pp.969-980

Schöner, S. and Krause, G.H., 1990. Protective systems against active oxygen species in spinach: response to cold acclimation in excess light. *Planta*, 180, pp.383-389

Schreiber, U., Schliwa, U. and Bilger, W., 1986. Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. *Photosynthesis Research*, 10, pp.51-62

Schriek, R., 2000. Effects of light and temperature on the enzymatic antioxidative defense systems in the Antarctic ice diatom *Entomoneis kufferathii Manguin*. *Reports on Polar Research*, 349, pp.1-130

Schuler, L.J. and Rand, G.M., 2008. Aquatic risk assessment of herbicides in freshwater ecosystems of south Florida. *Archives of Environmental Contamination and Toxicology*, 54, pp.571-583

Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., Von Gunten, U. and Wehrli, B., 2006. The challenge of micropollutants in aquatic systems. *Science*, 313, pp.1072-1077

Sen-Gupta, A., Webb, R.P., Holaday, A.S. and Allen, R.D., 1993. Overexpression of superoxide dismutase protects plants from oxidative stress (induction of ascorbate peroxidase in superoxide dismutase-overexpressing plants). *Plant Physiology*, 103, pp.1067-1073

Sergiev, I., Alexieva, V. and Karanov, E., 1997. Effect of spermine, atrazine and combination between them on some endogenous protective systems and stress markers in plants. *Compt Rend Acad Bulg Sci*, 51, pp.121-124

Shaw, M. and Müller, J.F., 2005. Preliminary evaluation of the occurrence of herbicides and PAHs in the wet tropics region of the Great Barrier Reef, Australia, using passive samplers. *Marine Pollution Bulletin*, 51, pp.876-881

Sheikh, M.A., Oomori, T., Fujimura, H., Higuchi, T., Imo, T., Akamatsu, A., Miyagi, T., Yokota, T. and Yasumura, S., 2012. Distribution and potential effects of novel antifouling herbicide diuron on coral reefs. In *Herbicides-Environmental Impact Studies and Management Approaches*. InTech

SIABAVE., 2002. Synthèse des etudes menées sur le basin versant du champ captant de Couraux (Marne) Rapport BRGM, FREDONCA, SIABAVE, Reims, France, pp. 67–92

Silkina, A., Bazes, A., Vouvé, F., Le Tilly, V., Douzenel, P., Mouget, J.L. and Bourgougnon, N., 2009. Antifouling activity of macroalgal extracts on *Fragilaria pinnata* (Bacillariophyceae): a comparison with diuron. *Aquatic Toxicology*, 94, pp.245-254

Singh, S., Kirkwood, R.C. and Marshall, G., 1997. Effects of isoproturon on photosynthesis in susceptible and resistant biotypes of *Phalaris minor* and wheat. *Weed Research*, 37, pp.315-324

Singh, G. and Wright, D., 1999. Effects of herbicides on nodulation, symbiotic nitrogen fixation, growth and yield of pea (*Pisum sativum*). *The Journal of Agricultural Science*, 133, pp.21-30

Sjollema, S.B., MartínezGarcía, G., van der Geest, H.G., Kraak, M.H., Booij, P., Vethaak, A.D. and Admiraal, W., 2014. Hazard and risk of herbicides for marine microalgae. *Environmental Pollution*, 187, pp.106-111

Smirnoff, N. and Colombé, S.V., 1988. Drought influences the activity of enzymes of the chloroplast hydrogen peroxide scavenging system. *Journal of Experimental Botany*, 39, pp.1097-1108

Spliid, N.H. and Køppen, B., 1998. Occurrence of pesticides in Danish shallow ground water. *Chemosphere*, 37, pp.1307-1316

Stachowski-Haberkorn, S., Jérôme, M., Rouxel, J., Khelifi, C., Rincé, M. and Burgeot, T., 2013. Multigenerational exposure of the microalga *Tetraselmis suecica* to diuron leads to spontaneous long-term strain adaptation. *Aquatic Toxicology*, 140, pp.380-388

Stanger, C.E. and Appleby, A.P., 1972. A proposed mechanism for diuron-induced phytotoxicity. *Weed Science*, 20, pp.357-363

Starr, R.C. and Zeikus, J.A., 1993. The culture collection of algae at the University of Texas at Austin. *Journal of Phycology*, 29, pp.90-95

Steinberg, P.D., Edyvane, K., De Nys, R., Birdsey, R. and Van Altena, I.A., 1991. Lack of avoidance of phenolic-rich brown algae by tropical herbivorous fishes. *Marine Biology*, 109, pp.335-343

Štěpánová, S., Plhalová, L., Doleželová, P., Prokeš, M., Maršálek, P., Škorič, M. and Svobodová, Z., 2012. The effects of subchronic exposure to terbuthylazine on early developmental stages of common carp. *The Scientific World Journal*, 2012. Article ID 615920

Stern, J.L., Hagerman, A.E., Steinberg, P.D., Winter, F.C. and Estes, J.A., 1996. A new assay for quantifying brown algal phlorotannins and comparisons to previous methods. *Journal of Chemical Ecology*, 22, pp.1273-1293

Subhadra, A.V., Nanda, A.K., Behera, P.K. and Panda, B.B., 1991. Acceleration of catalase and peroxidase activities in *Lemna minor* L. and *Allium cepa* L. in response to low levels of aquatic mercury. *Environmental Pollution*, 69, pp.169-179

Sulmon, C., Gouesbet, G., Binet, F., Martin-Laurent, F., El Amrani, A. and Couée, I., 2007. Sucrose amendment enhances phytoaccumulation of the herbicide atrazine in *Arabidopsis thaliana*. *Environmental Pollution*, 145, pp.507-515

Tang, J., Hoagland, K.D. and Siegfried, B.D., 1998. Uptake and bioconcentration of atrazine by selected freshwater algae. *Environmental Toxicology and Chemistry*, 17, pp.1085-1090

Teisseire, H., Couderchet, M. and Vernet, G., 1998. Toxic responses and catalase activity of *Lemna minor* L. exposed to folpet, copper, and their combination. *Ecotoxicology and Environmental Safety*, 40, pp.194-200

Teisseire, H., Couderchet, M. and Vernet, G., 1999. Phytotoxicity of diuron alone and in combination with copper or folpet on duckweed (*Lemna minor*). *Environmental Pollution*, 106, pp.39-45

Teisseire, H. and Vernet, G., 2000. Is the “Diuron effect” due to a herbicide strengthening of antioxidative defenses of *Lemna minor* ?. *Pesticide Biochemistry and Physiology*, 66, pp.153-160

Teisseyre, A. and Mozrzymas, J.W., 2006. The inhibitory effect of copper ions on lymphocyte KV1. 3 potassium channels. *Journal of Physiology and Pharmacology*, 57, pp.301-314

Tejada, M., Del-Toro, M., Osta, P., Parrado, J. and Gómez, I., 2013. Sorption of terbuthylazine in organic matter amended soils: Effects on *Eisenia Fetida* and *Lumbricus Terrestris*. In *Herbicides - Advances in Research*, Dr. Andrew Price (Ed.), ISBN: 978-953-51-1122-1, InTech, DOI: 10.5772/54948

Terlizzi, A., Fraschetti, S., Gianguzza, P., Faimali, M. and Boero, F., 2001. Environmental impact of antifouling technologies: state of the art and perspectives. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 11, pp.311-317

Thomas, K.V., Fileman, T.W., Readman, J.W., Waldock, M.J., (2001a) Monitoring of antifouling paint booster biocides and tributyltin in U.K. marinas, estuaries and coastal waters. A report for the Department of the Environment, Transport and the Regions and the Environment Agency, EPG/1/5/90 and P2-118, p.45

Thomas, K.V., Fileman, T.W., Readman, J.W. and Waldock, M.J., 2001b. Antifouling paint booster biocides in the UK coastal environment and potential risks of biological effects. *Marine Pollution Bulletin*, 42, pp.677-688

Thomas, K.V., McHugh, M., Hilton, M. and Waldock, M., 2003. Increased persistence of antifouling paint biocides when associated with paint particles. *Environmental Pollution*, 123, pp.153-161

Thomas, N.V. and Kim, S.K., 2011. Potential pharmacological applications of polyphenolic derivatives from marine brown algae. *Environmental Toxicology and Pharmacology*, 32, pp.325-335

Thomas, D.N. and Kirst, G.O., 1991a. Salt tolerance of *Ectocarpus siliculosus* (Dillw.) Lyngb.: comparison of gametophytes, sporophytes and isolates of different geographic origin. *Plant Biology*, 104, pp.26-36

Thomas, D.N. and Kirst, G.O., 1991b. Differences in osmoacclimation between sporophytes and gametophytes of the brown alga *Ectocarpus siliculosus*. *Physiologia Plantarum*, 83, pp.281-289

Tlili, A., Montuelle, B., Bérard, A. and Bouchez, A., 2011. Impact of chronic and acute pesticide exposures on periphyton communities. *Science of the Total Environment*, 409, pp.2102-2113

Tomás-Barberán, F.A., Ferreres, F. and Gil, M.I., 2000. Antioxidant phenolic metabolites from fruit and vegetables and changes during postharvest storage and processing. *Studies in Natural Products Chemistry*, 23, pp.739-795

Trebst, A. and Draber, W., 1986. Inhibitors of photosystem II and the topology of the herbicide and Q B binding polypeptide in the thylakoid membrane. *Photosynthesis Research*, 10, pp.381-392

UNEP (United Nations Environment Programme), 2013. Land-based pollution. (see <http://web.unep.org/regionalseas/what-we-do/land-based-pollution> (accessed 12.10.13)

Van Alstyne, K.L., 1995. Comparison of three methods for quantifying brown algal polyphenolic compounds. *Journal of Chemical Ecology*, 21, pp.45-58

Van Alstyne, K.L. and Paul, V.J., 1990. The biogeography of polyphenolic compounds in marine macroalgae: temperate brown algal defenses deter feeding by tropical herbivorous fishes. *Oecologia*, 84, pp.158-163

Van Hal, J.W., Huijgen, W.J.J. and López-Contreras, A.M., 2014. Opportunities and challenges for seaweed in the biobased economy. *Trends in Biotechnology*, 32, pp.231-233

Vallotton, N., Eggen, R.I.L. and Chèvre, N., 2009. Effect of sequential isoproturon pulse exposure on *Scenedesmus vacuolatus*. *Archives of Environmental Contamination and Toxicology*, 56, pp.442-449

Velisek, J., Stara, A., Koutnik, D. and Machova, J., 2014. Effect of terbuthylazine-2-hydroxy at environmental concentrations on early life stages of common carp (*Cyprinus carpio* L.). *BioMed Research International*, 2014

Viarengo, A., 1985. Biochemical effects of trace metals. *Marine Pollution Bulletin*, 16, pp.153-158

Wal, H., Sperber, B.L., Houweling-Tan, B., Bakker, R.R., Brandenburg, W. and López-Contreras, A.M., 2012. Production of acetone, butanol, and ethanol from biomass of the green seaweed *Ulva lactuca*. *Bioresource Technology*, 128, pp.431-437

Wang, W.X. and Dei, R.C., 2006. Metal stoichiometry in predicting Cd and Cu toxicity to a freshwater green alga *Chlamydomonas reinhardtii*. *Environmental Pollution*, 142, pp.303-312

Wang, T., Jónsdóttir, R., Kristinsson, H.G., Hreggvidsson, G.O., Jónsson, J.Ó., Thorkelsson, G. and Ólafsdóttir, G., 2010. Enzyme-enhanced extraction of antioxidant ingredients from red algae *Palmaria palmata*. *LWT-Food Science and Technology*, 43, pp.1387-1393

Wargacki, A.J., Leonard, E., Win, M.N., Regitsky, D.D., Santos, C.N.S., Kim, P.B., Cooper, S.R., Raisner, R.M., Herman, A., Sivitz, A.B. and Lakshmanaswamy, A., 2012. An engineered microbial platform for direct biofuel production from brown macroalgae. *Science*, 335, pp.308-313

Watanabe, K., Ohori, Y., Sato, Y., Böger, P. and Wakabayashi, K., 2001. Changes in fatty acid composition of neutral lipid in mung bean cotyledons by oxyfluorfen-induced peroxidation. *Pesticide Biochemistry and Physiology*, 69, pp.166-173

Willekens, H., Chamnongpol, S., Davey, M., Schraudner, M., Langebartels, C., Van Montagu, M., Inzé, D. and Van Camp, W., 1997. Catalase is a sink for H_2O_2 and is indispensable for stress defence in C 3 plants. *The EMBO Journal*, 16, pp.4806-4816

Willekens, H., Van Camp, W., Van Montagu, M., Inze, D., Langebartels, C. and Sandermann Jr, H., 1994. Ozone, sulfur dioxide, and ultraviolet B have similar effects on mRNA accumulation of antioxidant genes in *Nicotiana plumbaginifolia* L. *Plant Physiology*, 106, pp.1007-1014

Wills, E.D., 1969. Lipid peroxide formation in microsomes. General considerations. *Biochemical Journal*, 113, pp.315-324

Wilson, S., 2002. Nutritional value of detritus and algae in blenny territories on the Great Barrier Reef. *Journal of Experimental Marine Biology and Ecology*, 271, pp.155-169

Wilson, K.A., Able, K.W. and Heck Jr, K.L., 1990. Predation rates on juvenile blue crabs in estuarine nursery habitats: evidence for the importance of macroalgae (*Ulva lactuca*). *Marine Ecology Progress Series*, 58, pp.243-251

Yamasaki, H., Sakihama, Y. and Ikehara, N., 1997. Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H_2O_2. *Plant Physiology*, 115, pp.1405-1412

Yebra, D.M., Kiil, S. and Dam-Johansen, K., 2004. Antifouling technology—past, present and future steps towards efficient and environmentally friendly antifouling coatings. *Progress in Organic Coatings*, 50, pp.75-104

Yin, X.L., Jiang, L., Song, N.H. and Yang, H., 2008. Toxic reactivity of wheat (*Triticum aestivum*) plants to herbicide isoproturon. *Journal of Agricultural and Food Chemistry*, 56, pp.4825-4831

Zarzecka, K. and Gugała, M., 2011. The effect of herbicides and soil tillage systems on the content of polyphenols in potato tubers. *Polish Journal of Environmental Studies*, 20.

Zer, H. and Ohad, I., 1995. Photoinactivation of photosystem II induces changes in the photochemical reaction center II abolishing the regulatory role of the QB site in the Dl protein degradation. *The FEBS Journal*, 231, pp.448-453

Zhang, W., Jiang, F. and Ou, J., 2011. Global pesticide consumption and pollution: with China as a focus. *Proceedings of the International Academy of Ecology and Environmental Sciences*, 1, pp.125-144

Zsolnay, A., 1994. The lack of effect of the dissolved organic material in soil on the water solubility of the herbicide, terbuthylazine. *Science of the Total Environment*, 152, pp.101-104