Microalgal species prospecting and characterisation for salinity tolerance, nutrient remediation and bio-product potential

Thesis submitted by

Nicolas von Alvensleben

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STATEMENT OF CONTRIBUTION BY OTHERS

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LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

Refereed publications

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2. Islam, M, A., Brown, R., Dowell, A., Eickhoff, W., Brookes, P., **von Alvensleben**, **N**., Heimann, K., 2014. Evaluation of a pilot-scale oil extraction from microalgae for biodiesel production. International Conference on Environment and Renewable Energy 2014, Volume 3, Pages 133-137.

3. Islam, M, A., Rahman, M, M., Heimann, K, Nabi, M,N., Ristovski, Z, D., Dowell, A., Thomas, G., Feng, B., **von Alvensleben**, **N**., Brown, R,J., 2015. Combustion analysis of microalgae methyl ester in a common rail direct injection diesel engine. Fuel, Volume 143, Pages 351-360.

4. **von Alvensleben**, **N**., Magnusson, M., Heimann, K., 2015. Salinity tolerance of four freshwater microalgal species and the effects of salinity and nutrient limitation on biochemical profiles. Journal of Applied Phycology. 1-16. doi:10.1007/s10811-015-0666-6

Contributed papers at national and international meetings

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2. Heimann, K., Huerlimann, R., Magnusson, M., **von Alvensleben, N.**, Hudson, S., Ellison, M. and de Nys, R. 2010. Lipid profiles of tropical microalgae, strain selection for biofuel production. 19th International Symposium on Plant Lipids, Cairns, Qld Australia, July 11-16. (Research contribution)

3. **von Alvensleben**, **N**. 2011. 'An overview of my research' presentation at the Advanced Manufacturing Cooperative Research Centre (AMCRC) conference, Melbourne, Australia. June 15. (Abstract, Oral presentation)

4. **von Alvensleben**, **N**. Magnusson, M. Heimann, K., 2012. *Picochlorum atomus* salinity tolerance and the effect on biochemical profiles and the use of salinity for contamination control of the freshwater cyanobacterium *Pseudanabaena limnetica.* Asia Pacific Conference of Algal Biotechnology (APCAB), Adelaide, Australia. July 9-12. (Abstract, Oral presentation)

5. **von Alvensleben**, **N**., Magnusson, M. and Heimann, K., 2014. Effects of salinity and nutrient limitation on growth and biochemical profiles of four freshwater microalgal species. 5th Congress of the International Society of Applied Phycology (ISAP). Sydney, Australia. June 22-27. (Abstract, Oral presentation)

ABSTRACT

Microalgae provide a multidisciplinary approach for waste-gas and –water remediation offering parallel production of bio-products including nutraceuticals, food, feed, fertiliser and fuel. The main challenges for microalgal biomass production in Australia are limited freshwater resources (most of which are slightly saline), high light intensities and high temperatures, the latter in particular in the dry tropics.

In line with the AMCRC-funded microalgae carbon dioxide emission abatement and bio-product development project at Australian coal-fired power plants to which this research was linked, establishing salinity tolerance of endemic microalgal species was a priority due to the varying salinities of available tailings-dam waters for cultivation at the different sites (freshwater to marine). Through complete biochemical profiling (total lipids, protein, carbohydrate and fatty acids and fatty acid profiles), this thesis provided much needed baseline information on the potential of endemic microalgae cultivation for bio-product potential of a carbon dioxide emissions abatement strategy. As tailing dam waters are nutrient-poor, fertilisation requirement was also investigated, which simultaneously also provided inferences for species selection with remediation potential of nutrient-rich waste-waters. The potential use of salinity for cyanobacterial contamination control in halotolerant microalgal species was also investigated. Microlagal carotenoid contents and profiles were investigated for high-value nutraceutical production potential. For this, nine microalgal species were screened for carotenoid responses under moderate high light, in nutrient-replete

and -deplete conditions and with added molybdenum and vanadium in concentrations found in Stanwell Corp. tailings-dam water.

Salinity tolerance (2 to 36 ppt) under nutrient-replete and –deplete conditions was established for *Picochlorum atomus*, *Desmodesmus armatus*, *Mesotaenium* sp., *Scenedesmus quadricauda* and *Tetraedron* sp. using growth rates. *Picochlorum atomus* was selected for its demonstrated growth performance under outdoor tropical conditions, while *Desmodesmus armatus*, *Mesotaenium* sp., *Scenedesmus quadricauda* and *Tetraedron* sp. were isolated from Stanwell Corp. tailings-dam water and were selected for their ability to tolerate the polluted waters at this site.

The euryhaline *Picochlorum atomus* was identified as suitable for nutrient remediation, as was *Scenedesmus quadricauda* up to 11 ppt. Lipid contents and fatty acid profiles of both species were suitable for biofuel production. *Mesotaenium* sp. (up to 8 ppt) was suitable for cultivation in oligotrophic tailings-dam waters at coal-fired power stations, leading to substantial potential savings on fertilisation costs for biofuel and bioethanol production. *Desmodesmus armatus* showed intermediate salinity tolerance and nutrient uptake and would be a suitable species for food and feed production due to high protein contents. These findings provide a basis for species selection based on site-specific salinity conditions and nutrient resource availability. Additional findings also indicate that high salinity (28-36 ppt) can be used to inhibit contamination by the freshwater cyanobacteria *Pseudanabaena limnetica*, a common problem in the tropics.

Transition metals have been shown to induce radical oxygen species production in microalgae, often resulting in the production of antioxidants and radical scavenging compounds such as carotenoids, which can be exploited for the production

of nutraceuticals and bioactive pharmaceuticals. Tailings-dam water at the Stanwell Corp. coal-fired power station contains significant amounts of these trace metals. Therefore, to enable pigment-product based species selection, a pilot-study (chapter 4) explored the effects of molybdenum and vanadium on carotenoid production in eight microalgal species (*Desmodesmus armatus*, *Desmodesmus maximus*, *Coelastrum proboscideum*, *Graessiella emersonii*, *Haematococcus* sp., *Scenedesmus quadricauda*, *Mesotaenium* sp., *Tetraedron* sp.), in nutrient-sufficient and -deplete conditions under increased irradiance (~400 µmol photons m^{-2} s^{-1}). The majority of species were isolated from Stanwell Corp. coal-fired power station and were chosen for their ability to grow in polluted tailings-dam waters, except *C. proboscideum* and *G. emersonii* which were isolated in North East tropical Queensland and were selected for their astaxanthin production potential. *Haematococcus* sp., which was obtained from CSIRO, was included as a positive control for astaxanthin production. Both vanadium and molybdenum induced small increases of astaxanthin, lutein, violaxanthin and β-carotene content in most species, in particular *Haematococcus* sp., which together with *D. armatus* also showed the highest pigment concentrations. *Coelastrum proboscideum* and *G. emersonii* both produced astaxanthin, making them interesting alternatives to *Haematococcus* sp. for commercial astaxanthin production. These species were selected for chapter 5 to investigate the interactive effects of molybdenum and high temperature under moderately high irradiance in a factorial design, which are likely on-site cultivation conditions during the Australasian summer.

Overall, the main driver of pigment concentration changes was high light in particular in *D. maximus*, whereas high temperature was the main stressor in *Haematococcus* sp. *Graesiella emersonii* produced the highest concentrations of

important xanthophyll cycle pigments (violaxanthin and zeaxanthin), with no effects of Mo making this species suitable for cultivation in tailings-dam waters. In contrast, high concentations of violaxanthin in *Desmodesmus armatus* could only be sustained without Mo, suggesting production in high-nutrient waters void of metal pollution should be considered. *Haematococcus* sp. had the highest concentrations of astaxanthin in response to high temperature stress which was enhanced slightly in the presence of Mo, making it suitable for tailings-dam water cultivation, but requiring a 2-step cultivation process for (i) biomass and (ii) astaxanthin production. This study identified *Coelastrum proboscideum* as an alternative astaxanthin producer. Although astaxanthin concentrations were lower, growth data suggest that a single-step cultivation approach could be feasible. Additionally, this study identified *Desmodesmus maximus*, *Desmodesmus* sp. and *G. emersonii* as potential species for commercial lutein production as an alternative to Marigold (*Tagetes* sp.) flowers due to higher lutein concentrations, offering the added advantage of independence of arable land and coupling production to remediation of tailings-dam water for *Desmodesmus* sp. and *G*. *emersonii*. In contrast, Mo treatment significantly lowered lutein concentrations in *D*. *maximus* making it a suitable species for coupled nutrient-rich water remediation and lutein production.

In summary, this thesis characterised endemic microalgal species for cultivation at industrial sites of varying salinity and nutrient availability, with further identification of low- to high-value bio-product potential.

LIST OF ABBREVIATIONS

μ	Specific growth rate
$\mu mol\ photons\ m^{-2}\ s^{-1}$	Photon flux density
$^{1}O_2$	Singlet oxygen
^{3}Chl	Triplet chlorophyll
3GP	Glyceraldehyde-3-phosphate
AA	Amino acids
Acetyl CoA	Acetyl Coenzyme A
AMCRC	Advanced Manufacturing Cooperative Research Center
AMD	Age-related macular degeneration
ANOVA	Analysis of variance
APX	Ascorbate peroxidase
ATP	Adenosine triphosphate
BBL	Barrel (crude oil)
BBM	Bold basal medium
BCH	β-carotene hydroxylase
BCK	β-carotene ketolase
BHT	Butylated hydroxytoluene
BOM	Australian Bureau of Meteorolgy
C. proboscideum	*Coelastrum proboscideum*
CAT	Catalase
Cd	Cadmium
Chl	Chlorophyll
CO_2	Carbon dioxide

Cr	Chromium
CSIRO	Commonwealth Scientific and Industrial Research Organization
CTI	Carotenoid isomerase
Cu^{2+}	Copper (II) complex
D. armatus	*Desmodesmus armatus*
D. maximus	*Desmodesmus maximus*
DHA	C22:6 (n-3), Docosahexaenoic acid
DHI	Danish Hydraulic Institute
DI water	De-ionized water
Div. day^{-1}	Divisions per day
DMPP	Dimethylallyl pyrophosphate
DW	Dry weight
EAA	Essential amino acids
EDTA	Ethylenediaminetetraacetic acid
EI-MS	Electron ionisation mass spectrometry
EPA	C20:5, (n-3), Eicosapentaenoic acid
EPA	Environmental Protection Agency
FA	Fatty acid
FAME	Fatty acid methyl ester
Fe^{2+}	Iron (II) complex
FID	Flame ionisation detector
FSW	Filtered seawater
G. emersonii	*Graesiella emersonii*
GC	Gas chromatography
GF	Glass fibre

GGPP	Geranyl geranyl pyrophosphate
GGR	Geranyl granyl reductase
GHP	Hydrophylic polypropylene
GPX	Glutathione peroxidase
GSH	Reduced glutathione
GSSG	Two glutathione molecules linked by disulphide bond
H_2O_2	Hydrogen peroxide
HEPA	High efficiency particulate arrestance
HL	High light
HPLC	High pressure liquid chromatography
ID	Inner diameter
IDI	Isopentenyl pyrophosphate isomerase
IPP	Isopentyl pyrophosphate
ITS2	Internal transcribed spacer
IUPAC	International Union of Pure and Applied Chemistry
LCY	Lycopene cyclase
LHC	Light harvesting pigment complex
LL	Low light
M	Molar
MDAsc	Monodehydroascorbate
MEP	Methyl erithritolphosphate
Mo	Molybdenum
MUFA	Monounsaturated fatty acids
MVA	Mevalonate
N	Nitrogen
N:P	Nitrate to phosphate ratio

N=3	Individual triplicate culture/sample replication
NaCl	Sodium chloride
NED	N-(1-naphthyl)-ethylenediamine
Nm	Nanometer
nMDS	Non-metric multidimensional scaling
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NO_x	Nitrous oxide
NPQ	Non-photochemical quenching
O_2	Oxygen
$O_2^{\bullet-}$	Superoxide anions
OD	Optical density
$OH^{\bullet}$	Hydroxyl radical
P. atomus	*Picochlorum atomus*
P. limnetica	*Pseudanabaena limnetica*
P	Phosphorus
PAG	Protein Calorie Advisory Group of the United Nations
PDA	Photo-diode array
PDS	Phytoene desaturase
PETC	Photosynthetic electron transport chain
P_{max}	Maximum irradiance saturation rate
PO_4^{3-}	Phosphate
PPT	Parts per thousand
PS	Photo-system
PSY	Phytoene synthase

PUFA	Polyunsaturated fatty acids
Re	Rhenium
ROS	Reactive oxygen species
S. table	Supplementary table
S. quadricauda	*Scenedesmus quadricauda*
SFA	Saturated fatty acids
SOD	Superoxide dismutase
Sp.	Species
TBAA	Tert-butyl acetoacetate
TFA	Total fatty acids
Ti	Titanium
U mg^{-1} DW	Units mg^{-1} DW
UPLC	Ultra pressure liquid chromatography
USD	United States dollars
UV	Ultra-violet
V	Vanadium
V:Z	Violaxanthin to zeaxanthin ratio
VDE	Violaxanthin de-epoxidase
Vs	Versus
W	Tungsten
ZE	Zeaxanthin epoxidase
λ	Wavelength
ω-3, -6	Omega-3, -6

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CHAPTER 1

General introduction

1.1 Project background

An ever increasing human population in combination with intensive industrial (e.g. mining, refining) and agricultural practices has led to a number of anthropogenically-induced global concerns. Specifically, increases in atmospheric carbon dioxide (CO_2) (globally ~35 billion tonnes of CO_2 per annum through the burning of fossil fuels, deforestation and intensive agriculture (BP, 2015)) are predicted to result in significant environmental problems affecting nations and their economies. Amongst them, and exacerbated by the growing population, are drought-induced freshwater shortages, water pollution/eutrophication through mining/agriculture, aquaculture and anthropogenic sewage, and natural resource depletion (e.g. fossil fuels) (Burke *et al.*, 2006; Chisti, 2008; Cordell *et al.*, 2009; Dismukes *et al.*, 2008).

Microalgae provide a multi-disciplinary solution to these issues, as large-scale cultivation can be used to remediate industrial waste-gases and waste-water pollutants, while also producing biomass which can be used as a feedstock for biodiesel, bioethanol, foods, feeds, fertilisers and bio-active pharmaceuticals. Like aquatic and terrestrial plants, microalgae fix CO_2 through photosynthesis (Dismukes *et al.*, 2008). Consequently, CO_2-rich industrial-waste gases can be supplemented to microalgal cultures for remediation in parallel to biomass production. Furthermore,

microalgae also utilize nitrogen and phosphate for growth which can be exploited for nutrient-rich water remediation from agriculture, aquaculture and human sewage facilities (Canter *et al.*, 2015; Pittman *et al.*, 2011), avoiding conflicts with freshwater use in agriculture. In contrast to terrestrial crops, microalgal cultivation can be carried out on waste-land rather than valuable arable land (Chisti, 2007). This represents distinct advantages for culture-site location flexibility, increases food, feed and nutraceutical production potential without competing with agricultural crop production for the fast growing human population.

The main challenges for microalgal biomass production in Australia are limited freshwater resources, high light intensities and high temperatures, the latter in particular in the dry tropics. In addition to using nutrient-rich waste water, freshwater scarcity can also be circumvented by using tailings-dam waters at industrial sites e.g. coal-fired power stations. However, this leads to further challenges with regards to species selection as water salinity at coal-fired power stations in Australia varies considerably from freshwater (2 ppt) to seawater (36 ppt). Even at low salinity culture sites, at scale, slightly saline ground-water needs to be used and salinity will fluctuate due to evaporation and replenishment with saline waste- or ground-water.

Additionally, using large volumes of waste-water often results in culture contamination by non-target organisms and represents one of the main hurdles to produce large quantities of target species biomass at low cost (Apel *et al.*, 2015; Wang *et al.*, 2013). The effects of contamination depend on the contaminant and may include grazing, resource competition, allelochemical inhibition or death of target species, toxin production and biomass biochemical composition modification. Common contaminants include zooplankton, bacteria, fungi, yeasts, protists and

viruses as well as non-target microalgae and cyanobacterial species (Becker, 1994; Borowitzka, 2005) (Table 1.1). A range of solutions are available to remove or prevent certain contaminants including manual separation (e.g. filtration or cytometry), chemical treatment (e.g. pesticides, biocides, antibiotics) and environmental/culture manipulation (e.g. salinity, pH) (Bartley *et al.*, 2014). Treatments, however, generally have limitations and considerable further research is required in this field to improve contamination control methods (Park *et al.*, 2011) (Table 1.1). Furthermore, research also needs to investigate the effects of contamination control methods on the biochemical composition of the resulting biomass as these may often interfere with the final product (Bartley *et al.*, 2014). Manual separation is only effective with different sized organisms and is often cost-prohibitive and ineffective for large-scale treatment. Culture techniques including selective biomass recirculation based on algal density to increase the population of easily harvestable algae, nutrient limitation and hydraulic retention time (culture dilution) have shown potential for algal species control (Park *et al.*, 2011). Benemann *et al.* (1977) demonstrated algal species control by selective recycling of harvested biomass and maintained *Spirulina* sp. dominance over the faster growing unicellular contaminant *Chlorella* sp. However, mechanisms of algal dominance are still not well understood and practical control methods for similar sized algae have not yet been defined in the literature (Park *et al.*, 2011). Chemical treatment including pesticides, biocides and antibiotics may be effective for a range of organisms, but has the disadvantages that they may interfere with the biochemical composition of target cells, leave residues in harvested biomass, represent an environmental concern with high concentration inputs into the environment and may also be subject to increasingly strict regulations (e.g. antibiotics) due to increasing

concerns of antibiotic-resistant bacteria (Bacellar Mendes *et al.*, 2013; Churro *et al.*, 2010). Environmental manipulations have proven effective for extremophile species such as *Dunaliella salina*, which tolerates high salinities up to 320 ppt (Chen *et al.*, 2009) with optimal cultivation salinities between 87 and 175 ppt (Farhat *et al.*, 2011), subsequently inhibiting the growth of most other non-halotolerant organisms (Bacellar Mendes *et al.*, 2013; Benamotz *et al.*, 1991). Similarly, *Arthrospira platensis* can be cultured in high pH conditions which also inhibit the development of many contaminant organisms (Apel *et al.*, 2015). However, most commercial microalgal species require culture conditions which are favourable to a range of organisms (Borowitzka, 2005).

Contamination by non-target microalgae and cyanobacteria represents a particularly complex problem as these are often of similar size and may have similar responses to treatments (Park *et al.*, 2011). Cyanobacteria are known to produce a wide range of secondary metabolites (e.g. harmane and norharmane (Volk, 2005; Volk *et al.*, 2006), nostocarboline (Blom *et al.*, 2006), glycosidase and peptidase inhibitors, microcystin and fischerellin toxins (Gross, 2003) with allelopathic activities including anti-algal, anti-fungal and anti-predation compounds (Gross, 2003; Legrand *et al.*, 2003). For example the cyanobacterium *Microcystis aeruginosa* and the dinoflagellelate *Alexandrium tamarense* have been shown to cause growth inhibition of microalgae and cyanobacteria (Singh *et al.*, 2001; Sukenik *et al.*, 2002).

Tailings-dam waters from coal-fired power stations or the mining industry are also generally polluted with heavy metals. Consequently, tailings-dam remediation-based microalgal biomass production for bio-product development requires strain selection for salinity tolerance, growth, nutrient requirements and removal potential,

Table 1.1. Common microlagal culture contaminants and management approaches

Contaminant	Management approaches	Reference
Zooplankton	- Pesticides: Trichlorphon, Decamethrin, Tralocythrin and Buprofezin	(Wang *et al.*, 2013)
	- Filtration	(Borowitzka, 2005)
Algae	- Negative allelopathy: *Peridinium aciculiferum* (Dinophyceae) negatively impacts *Synura petersenii* (Chrysophyceae), *Peridinium inconspicuum* (Dinophyceae), *Cyclotella* sp. (Bacillarophyceae), *Cryptomonas* sp. and *Rhodomonas lacustris* (Cryptophyceae) through lysis. The impact may be due to a single chemical or a combination of chemicals	(Rengefors *et al.*, 2007)
Bacteria	- Antibiotics	(Han *et al.*, 2015)
	- Phenolic compound 4, 4'- dihydroxybiphenyl found in *Nostoc insulare*	(Caicedo *et al.*, 2012; Volk *et al.*, 2006)
	- Selective spectrum biocides and anti-microbial compounds	(Bacellar Mendes *et al.*, 2013)
Cyanobacteria	- Phenolic compound 4, 4'- dihydroxybiphenyl and alkaloid nostocarboline found in *Nostoc* sp.	(Caicedo *et al.*, 2012; Volk *et al.*, 2006)
	- Negative allelopathy: *Peridinium gatunenese* (Dinophyceae) and the cyanobacteria *Microcystis aeruginosa* inhibit each other	(Vardi *et al.*, 2002)
	- UV Irradiation (*Microcystis aeruginosa* and *Anabaena variabilis*)	(Sakai *et al.*, 2007)
	- Selective spectrum biocides and anti-microbial compounds	(Bacellar Mendes *et al.*, 2013)
	- Salinity manipulations	(von Alvensleben *et al.*, 2013)
Protozoa	- Pulsed Electric Fields	(Rego *et al.*, 2015)
	- Quinine sulphate and ammonia bicarbonate	(Moreno-Garrido *et al.*, 2001)
	- Selective spectrum biocides and anti-microbial compounds	(Bacellar Mendes *et al.*, 2013)
	- Reduce pH to 3, briefly, to kill flagellates in microalgal cultures	(Becker, 1994)
Virus	- Selective spectrum biocides and anti-microbial compounds	(Bacellar Mendes *et al.*, 2013)
Fungi	- Phenolic compound 4, 4'- dihydroxybiphenyl found in *Nostoc insulare*	(Caicedo *et al.*, 2012; Volk *et al.*, 2006)

- heavy metal tolerance, suitable biochemical profiles and evaluation of the bio-product value to assess economic viability.

Additionally, microalgal biochemical profile plasticity (e.g. lipid, fatty acids, protein and carbohydrate contents) in response to variations in culture conditions affects bio-product potential. Therefore, specific effects of nutrient availability, salinity, high light, high temperature and transition metals (molybdenum (Mo) and vanadium (V), often present in high concentrations in tailings-dam waters at coal-fired power stations) on biochemical profiles and culture growth need to be evaluated. Furthermore, positive effects on culture growth and biochemical profiles can be exploited as a manipulation tool to improve the economics of specific bio-product production. Generally though, while stress conditions can improve bio-product outcomes, growth (biomass production) is typically impeded. Challenges of high light and temperature can be resolved through culture system design (e.g. shading, or water cooling); however this will generally add complexity and costs, potentially having a negative energy - and cost- balance for tailings-dam waste water remediation-based approaches.

To successfully carry out remediation projects and bio-product production, extensive physiological and physico-chemical profiling of microalgal culture species is required. Considerable research has been carried out globally for multiple microalgal species (James *et al.*, 1989; Pal *et al.*, 2011; Renaud *et al.*, 2002; von Alvensleben *et al.*, 2015). However, environmental conditions influence both morphological characteristics as well as biomass and biochemical productivities, e.g. lipid and carotenoid production (Pal *et al.*, 2011; Renaud *et al.*, 2002), which additionally also vary within species (James *et al.*, 1989; Sayegh *et al.*, 2011; Tanoi *et al.*, 2011; von Alvensleben *et al.*, 2015). As tailings-dam waters at Australian coal-fired power plants range in salinity (2-36 ppt) and Australian freshwater resources available for large-

scale microalgal cultivation are likely to be at least slightly saline (Hart *et al.*, 1991; Peck *et al.*, 2003), culture salinities will increase when used as make-up water for evaporative water loss.

1.2 Objectives and approach

The focus of this thesis was based on species selection for cultivation in varying salinities to provide biomass-based input into evaluating the bio-product potential of a CO_2-emissions abatement strategy using endemic microalgae. As tailings-dam waters are nutrient-poor, fertilisation requirement was also investigated, which simultaneously provided inferences for species selection with remediation potential of nutrient-rich waste-waters. Biochemical profiles were established for all cultivation conditions to evaluate the potential for the production of value-adding bio-products from these endemic species. The first part of the thesis investigated salinity tolerance of five tailings-dam water-derived microalgal species, and the effects of salinity and nutrient depletion on biochemical profiles (Chapters 1 and 2), as well as the potential use of salinity for cyanobacterial contamination control in halotolerant microalgal species (Chapter 2).

During the course of this industry-linked research, it became apparent that production of high-value bio-products is required to render the microalgae CO_2-abatement approach economically viable. Therefore, the second part focused on microlagal carotenoid production, for high-value nutraceutical production (Chapters 4 and 5). For this, nine microalgal species were screened for carotenoid responses under moderate high light, in nutrient-replete and -deplete conditions and with added Mo and V in concentrations found in Stanwell Corp. tailings-dam water.

Given the essential role bio-products from microalgae play in any waste-gas or –water remediation approach, the general introduction describes the various bio-products that can be derived from microalgae. To avoid repetition, introductions to the individual research chapters deal in depth with the relevant research background. The bio-product section of the introduction is structured from lowest to highest bio-product values and outlines current constraints to market where applicable.

1.3 Bio-products from microalgae

1.3.1 Biofuel

Commercial microalgae production is less than 60 years old (Borowitzka, 2013a). In 1942, Harder and von Witsch were the first to suggest that microalgal biomass has renewable fuel potential (Borowitzka, 2013a), however, following a significant period of low and stable oil prices, the need for alternative liquid fuels decreased and the research focus was redirected to the potential of microalgae as food and protein sources. Although, the urgency of fossil-fuel depletion has temporarily subsided, fossil fuels remain non-renewable and alternative energy sources will ultimately be required, in particular storable, transportable high-energy fuel such as biodiesel and bio-jet fuel which remain viable alternatives for hydrocarbon-fuel-dependent heavy transport systems (ships, planes, heavy-vehicles) which will be affected by fossil-fuel depletion (Stephens *et al.*, 2010a).

The feasibility of microalgal biofuel production and suitability as a liquid fuel has been demonstrated with considerable research for species selection and biochemical profiling (Lee *et al.*, 2015; Nwokoagbara *et al.*, 2015; von Alvensleben *et al.*, 2015), extraction methods including solvent extraction (Molina-Grima *et al.*,

2013b), direct transesterification (Ehimen *et al.*, 2010; Levine *et al.*, 2010) thermochemical liquefaction (Demirbas, 2010) and pyrolysis (Peng *et al.*, 2000), fuel characteristics (Islam *et al.*, 2015a; Islam *et al.*, 2013; Pourkhesalian *et al.*, 2014), applied test-engine studies (Islam *et al.*, 2015b) and particle emissions (Rahman *et al.*, 2015). However, the economic viability of microalgal biofuel production is an ongoing debate, as based on current crude oil prices (NASDAQ, 2015) USD 50 bbl^{-1} (i.e. USD 0.35 kg^{-1}), production costs for algae with 40% lipid (triglyceride) would need to be below USD 0.14 kg^{-1}, which is considerably lower than current production costs (7-70 USD kg^{-1}) and consequently not economically viable (Borowitzka, 2013b). Even at oil prices of USD 200 bbl^{-1} (i.e. USD 1.4 kg^{-1}), microalgae costs would need to be below USD 0.56 kg^{-1} to be economically viable (Stephens *et al.*, 2010a; Borowitzka, 2013c;) and therefore needs to be coupled with high value co-product production (Chisti, 2013; Enzing *et al.*, 2014; Lundquist *et al.*, 2010). Biofuel production experiments at the Stanwell Corp. coal-fired power station confirmed current approaches were not economically viable, mainly due to land requirements. Consequently, the industry partner, MBD Energy expanded their research focus to alternative and higher-value microalgal bio-products, such as animal feed (see section 1.1.2) and/or carotenoid pigments (see section 1.2) in order to attempt an economical approach to CO_2 flue gas emission abatement.

1.3.2 Food and feed

Aquaculture is currently an important microalgal feed market, utilizing ~20 % of global microalgae production (Muller-Feuga, 2000), mainly for larval rearing and as feed components or pigmentation additives for adult aquatic organisms e.g. prawns,

bivalves, salmonids (Benemann, 1992; Gagneux-Moreaux *et al.*, 2007; Spolaore *et al.*, 2006). Increasing interest in the use of microalgae as a novel crude protein source has prompted international organizations such as the Protein Calorie Advisory Group of the United Nations (PAG) and the International Union of Pure and Applied Chemistry (IUPAC) to publish recommendations and guidelines for the utilization of these unconventional protein sources (Becker, 1994). Recommendations include: detailed chemical composition, protein efficiency ratio and digestibility coefficients. These, however, are linked to a potentially highly variable biochemical profile induced by changes in cultivation conditions. Furthermore, the microalgal protein industry is still in its infancy due to technical and economic-viability constraints (Becker, 2007) but is forecast to improve with ongoing microalgal bio-product research (Enzing *et al.*, 2014). Amino acid composition is also an important consideration in particular when formulating animal feeds (Becker, 2007). A number of amino acids are often limiting in certain diets e.g. lysine, methionine and threonine in fish, shrimp, cattle and poultry feeds (D'Mello, 1993; Kung Jr *et al.*, 1996; Nunes *et al.*, 2014; Rawles *et al.*, 2013).

Modern diets are reported to have an unbalanced omega-6: omega-3 ratio (ω-3: ω-6) (Simopoulos, 2002), due to an excessive ω-6 intake through increased cereal grain-based product consumption; consequently there is also interest to produce ω-3 supplements from microalgae as a nutritional supplement. Currently the ω-3 supplement market is dominated by fish oil products; which is not sustainable in the long term due to over-fishing, eventually requiring replacement by alternative ω-3 sources (Dulvy *et al.*, 2003; Lenihan-Geels *et al.*, 2013; Worm *et al.*, 2006). Alternatives could include fish produced in aquaculture with formulated ω-3-rich algal diets which directly influence fatty acid composition, resulting in bio-accumualtion of ω-3's

(Benemann, 1992; Olsen *et al.*, 2004), or directly from microalgae which are the food chain-origin of long chain polyunsaturated ω-3's (Doughman *et al.*, 2007; Lenihan-Geels *et al.*, 2013). As maintenance of adequate fish oil ω-3 long chain polyunsaturated fatty acid profiles requires ω3-enriched feeds, the production of oily fish in aquaculture is at present still coupled to wild-stock-derived supplements (Turchini *et al.*, 2009). In this regard, the aquaculture industry has come under increasing scrutiny for environmental sustainability and research is ongoing to find economically viable alternatives for aquaculture feeds that will deliver a high-value product to market (Chauton *et al.*, 2015; Lenihan-Geels *et al.*, 2013; Turchini *et al.*, 2009).

Of particular interest for aquaculture and the human nutraceutical supplement market are the polyunsaturated fatty acids (PUFA) eicosapentaenoic acid (20:5 ω-3) (EPA) and docosahexaenoic acid (22:6 ω-3) (DHA), which are particularly important and beneficial in human diets for the prevention of cardiovascular disease as well as neurological and inflammatory conditions (Calder *et al.*, 2009). The heterotrophic protists *Schizochytrium* sp. and *Crypthecodinium* sp. have an established DHA market, predominantly for infant formula ω-3 enrichment (e.g. Martek Biosciences Corporation and Nutrinova) (Borowitzka, 2013b; Woodall *et al.*, 1997b). In contrast, microalgal production is restricted to certain marine classes (e.g. haptophytes, cryptophytes, diatoms) (Heimann *et al.*, 2015b). As freshwater chlorophytes, the focus of this research, do not produce appreciable amounts, the market potential will not be further considered here.

Substantial research has also been carried out using microalgae as terrestrial animal feeds (Lum *et al.*, 2013; Yaakob *et al.*, 2014). As the AMCRC-microalgae CO_2

emission abatement project at the Stanwell Corp. coal-fired power station was situated near a cattle feed-lot industry, the potential of microalgae as a value adding animal feed product was explored. However, endemic strain characteristics proved that the freshwater chlorophyte biomass did not contain the highly valued EPAs and DHAs. In addition, cultivation in the nutrient-poor metal-rich tailings-dam water cast doubt on the use of the biomass in high protein diets. Potential bioaccumulation of heavy metals in the microalgae, on the one hand, and the fertilisation requirements to increase protein content to commercially competitive levels with other feedstock on the other hand, as well as land availability at the power station led to severe reservations with regards to the economic viability of the project. This shifted the project focus to pigment-based high-value co-product generation, as other important components of animal feed formulation are pigments such as lutein in poultry feeds and astaxanthin in aquaculture feeds (Nelis *et al.*, 1991). However, due to lower costs, animal feeds generally contain synthetic versions of these pigments or extracts from low yield terrestrial plants (e.g. lutein from *Tagetes* sp.). Nevertheless, as increasing regulations become more restrictive for synthetic nutritional components and consumers demand proven health benefits, the market will likely shift to obtaining these pigments from micro-organisms including microalgae (discussed in detail in section 1.2.).

1.3.3 Bio-active pharmaceuticals/ nutraceuticals

Due to the vast diversity of algal species, their ability to produce a whole array of important biochemicals and their potential for mass production; current research is focussing on high-value chemical production (Gacheva *et al.*, 2014) (Table 1.2).

Important markets screening these natural products are: the cancer drug market (Gerwick *et al.*, 1994; Ördög *et al.*, 2004; Shanab *et al.*, 2012), the antibiotic market (Chu *et al.*, 2004; Robles Centeno *et al.*, 1999; Skulberg, 2000), the antiviral market (Huleihel *et al.*, 2001; Schaeffer *et al.*, 2000; Zainuddin *et al.*, 2002) and the cardiovascular market (Raposo *et al.*, 2015) (Table 1.2).

As outlined in table 1.2, carotenoids provide several therapeutic functions in humans, such as antioxidant effects including singlet oxygen quenching, prevention of age-related macular degeneration and cardiovascular disease, and immuno-modulatory, anti-tumor and anti-carcinogenesis activity (Fernandez-Sevilla *et al.*, 2010; Krinsky *et al.*, 2005; Maoka *et al.*, 2012; Valko *et al.*, 2006). This thesis focussed specifically on carotenoid contents in endemic microalgae, as the bio-product pathway to market is established, which was an important consideration in association with the CO_2 emission abatement from coal-fired power stations in the AMCRC-funded research project. Due to the complex functional responses, a thorough understanding of carotenogenesis and environmental conditions that generate reactive oxygen species is required for data interpretation of chapters 4 and 5, which are outlined below.

Table 1.2. Nutraceutical uses and properties for commercially-valuable microalgal pigments.

Carotenoid	Function and commercial uses	Bio-active effects/Nutraceutical properties	References
β-carotene	- Provitamin A activity - Antioxidant - Food colorant - Cosmetic preparations	Immune enhancement. Decreased incidence of cardiovascular events	(Chew *et al.*, 1999; Omenn *et al.*, 1996; Tanaka *et al.*, 2012)
Lutein	- Anti-photosensitzing agent - Anti-oxidant - Low-wavelength blue light filter - Cosmetic preparations	Decreased incidence of: - Age-related macular degeneration. - Cataract - Cardiovascular disease - Stroke - Certain cancers	(Michaud *et al.*, 2000; Seddon *et al.*, 1994; Sujak *et al.*, 1999; Tanaka *et al.*, 2012)
Astaxanthin	- Antioxidant - Anti-inflammatory - Food colorant	Decreased incidence of: - Certain cancers - Cataract - Diabetes - Inflammatory and neurodegenerative cardiovascular disease	(Chew *et al.*, 1999; Tanaka *et al.*, 2012; Yuan *et al.*, 2011)
Canthaxanthin	- Antioxidant	Immune enhancement Decreases the incidence of some cancers	(Chew *et al.*, 1999; Krinsky *et al.*, 2005; Tanaka *et al.*, 2012)
Zeaxanthin	- Anti-photosensitzing agent - Antioxidant	Decreased incidence of: - Age-related macular degeneration. - Cardiovascular disease - Certain cancers	(Seddon *et al.*, 1994; Sujak *et al.*, 1999)
Violaxanthin	- Antioxidant - Anti-inflammatory	Decreased incidence of: - Certain cancers	(Pasquet *et al.*, 2011; Soontornchaiboon *et al.*, 2012)

1.4 Microalgal carotenoids

Microalgae are ideal cell factories for the production of high value carotenoids as they combine the fast growth of unicellular organisms with an active isoprenoid metabolism and adequate storage capacity (León *et al.*, 2007). The global carotenoid market is estimated to be 1.2 billion USD in 2010, and has been projected to increase to 1.4 billion USD by 2018 (BCC-Research, 2011). Consequently, and in line with the project limitations encountered at the industrial production site at Stanwell Corp. coal-fired power station, as outlined above, the second part of this thesis focussed on microalgal carotenoid contents and potential avenues for improvement.

Current commercial carotenoid production is limited to a few algal species including *Haematococcus pluvialis* for astaxanthin and *Dunaliella salina* for β-carotene production; however other microalgal species could be viable alternatives for large-scale production (Table 1.3). Astaxanthin, β-carotene and lutein already have established markets in pharmaceutical, nutraceutical and aquaculture industries (Guedes *et al.*, 2011a), but are produced primarily *via* chemical synthesis, as the natural products, except for β-carotene, are not cost-competitive (Berman *et al.*, 2014). However a number of studies have shown, in particular for β-carotene and astaxanthin, that synthetic pigments do not provide the same health benefits as natural ones (Capelli *et al.*, 2013; Régnier *et al.*, 2015; Ribeiro *et al.*, 2011). Furthermore, the threshold of synthetic food additives legally permitted has been steadily decreasing due to their suspected role as promoters of carcinogenesis and claims of renal and liver toxicities leading to an increasing preference for natural pigments (Guedes *et al.*, 2011b).

Table 1.3. Microlagal species with commercial potential for pigment production.

Carotenoid	Microalgal species	References
Astaxanthin	*Haematococcus pluvialis** *Chlorella zofingensis* *Scenedesmus* sp.	(Del Campo *et al.*, 2004; Pirastru *et al.*, 2012)
β-carotene	*Dunaliella salina** *Nannochloropsis gaditana* *Scenedesmus almeriensis*	(Chen *et al.*, 2009; Garcia-Gonzalez *et al.*, 2005; Macias-Sanchez *et al.*, 2010; Macias-Sanchez *et al.*, 2005)
Canthaxanthin	*Chlorella zofingiensis* *Chlorococcum* sp. *Haematococcus pluvialis** *Nanochloropsis gaditana* *Nannochloropsis salina* *Scenedesmus* sp.	(Choubert *et al.*, 1993; Li *et al.*, 2006; Lubian *et al.*, 2000; Pirastru *et al.*, 2012; Rise *et al.*, 1994; Yuan *et al.*, 2002)
Lutein	*Chlorella protothecoides* *Chlorella zofingensis* *Scenedesmus almeriensis* *Muriellopsis* sp. *Picochlorum* sp.	(de la Vega *et al.*, 2011; Del Campo *et al.*, 2000; Del Campo *et al.*, 2001, 2004)
Violaxanthin	*Chlorella ellipsoidea* *Dunaliella tertiolecta*	(Pasquet *et al.*, 2011; Soontornchaiboon *et al.*, 2012)
Zeaxanthin	*Chlorella ellipsoidea* *Nannochloropsis oculata* *Microcystis aeruginosa* *Picochlorum* sp.	(Chen *et al.*, 2005; de la Vega *et al.*, 2011; Koo *et al.*, 2012; Liau *et al.*, 2011)

*Microalgal species cultivated commercially

1.4.1 Microalgal pigment functions

Microlagae pigments perform several functions including light harvesting, excess energy dissipation, reactive oxygen species (ROS) scavenging, triplet chlorophyll quenching but also contribute to structure stabilization and aid in the function of photosynthetic complexes (Demmig-Adams *et al.*, 2002). These pigments are categorized by their chemical structure which also determines their function. Many pigments have a number of isomers, resulting in different spectral properties which have implications for their ability to either transfer or remove excitation energy

(Falkowski *et al.*, 2007; Mulders *et al.*, 2014). The main pigment classes include chlorophylls, carotenoids and phycobiliproteins (the latter are present only in cyanobacteria, red algae and a small freshwater group of microalgae, the Glaucocystophyta, and will therefore not receive further consideration here). The carotenoids, a group of yellow to orange-red terpenoid pigments (Romero *et al.*, 2012), can be further divided into two groups: carotenes, which are oxygen-free 40 C-hydrocarbons and their oxygenated derivatives, the xanthophylls (Becker, 1994). The main photosynthetic pigments are the chlorophylls (Chl *a*, *b*, *c*) which harvest light and transduce it to chemical energy (Chen *et al.*, 2010). Certain carotenoids such as violaxanthin, neoxanthin and to a lesser extent lutein have light harvesting abilities and, like most carotenoids, also have a photo-protective role through filtering, quenching and/or scavenging mechanisms (Figure 1.1) (Falkowski *et al.*, 2007; Mulders *et al.*, 2014).

Pigments with filtering roles prevent the formation of over-excited Chl a by absorbing harmful radiation e.g. astaxanthin and β-carotene. Pigments with a quenching role prevent the formation of ROS by quenching (non-photochemical quenching) the energy of triplet or singlet excited Chl a (Frank et al., 1996; Krinsky, 1989; Krinsky et al., 2005; Pinto et al., 2003) (Figure 1.1). These include pigments involved in the xanthophyll cycle: violaxanthin, antheraxanthin and zeaxanthin, but also astaxanthin, β-carotene and lutein. Pigments with a scavenging role prevent cell damage by reacting with ROS e.g. astaxanthin, β-carotene, lutein and neoxanthin (Abdel Hameed, 2007; Guerin et al., 2003; Woodall et al., 1997a) (Figure 1.1, see section 1.2.3 for ROS scavenging mechanisms).

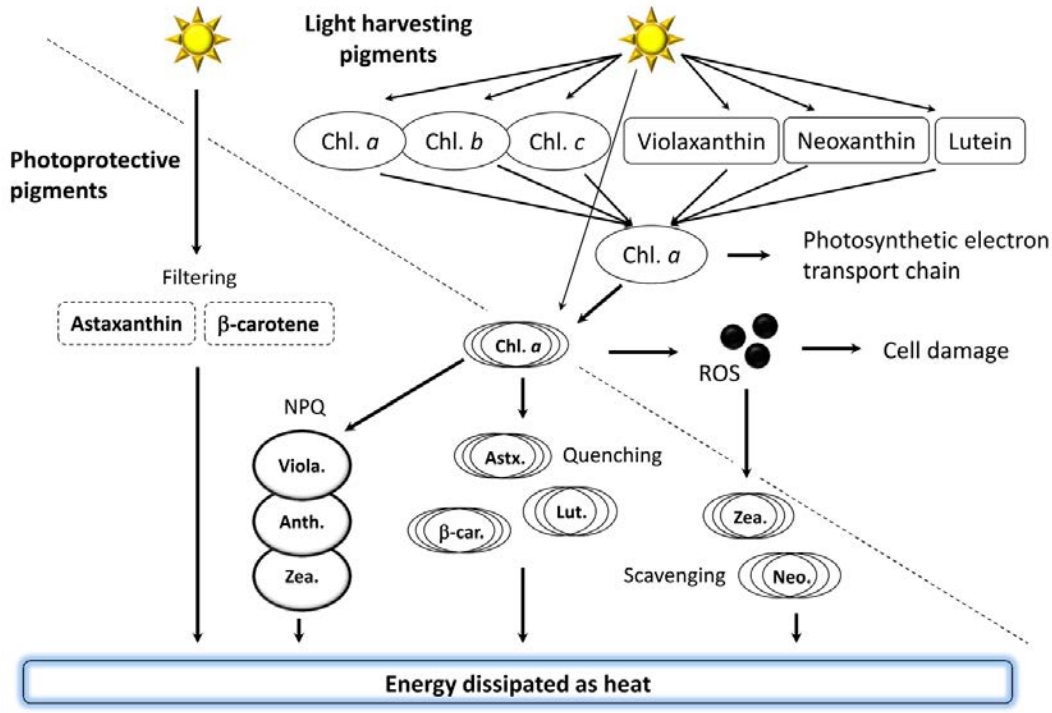

Figure 1.1. Schematic of pigment function adapted from Demmig-Adams *et al.* (1996); Falkowski *et al.* (2007); Mulders *et al.* (2014); Sukenik *et al.* (1992). Abbreviations: Anth: antheraxanthin, Astx: astaxanthin, β-car: β-carotene, Chl: chlorophyll, Lut: lutein, Neo: neoxanthin, Viola: violaxanthin, Zea: zeaxanthin.

1.4.2 Carotenoid synthesis

The first step in microalgal carotenoid synthesis is the production of the 5-carbon building-block, isopentenyl pyrophosphate (IPP) (Figure 1.2). The IPP is isomerized to its allylic isomer dimethylallyl pyrophosphate (DMPP) which is the initial, activated substrate for the formation of long chain polyisoprenoid compounds such as the C_{20} geranyl geranyl pyrophosphate (GGPP) (Cunningham *et al.*, 1998). Tail to tail condensation of two GGPP molecules forms the first colourless carotenoid, phytoene. Coloured carotenoids are synthesized by desaturation reactions of phytoene creating conjugated double bonds (Lee *et al.*, 2002). Phytoene desaturase catalyses the introduction of four double bonds forming the carotenoid lycopene. Lycopene serves

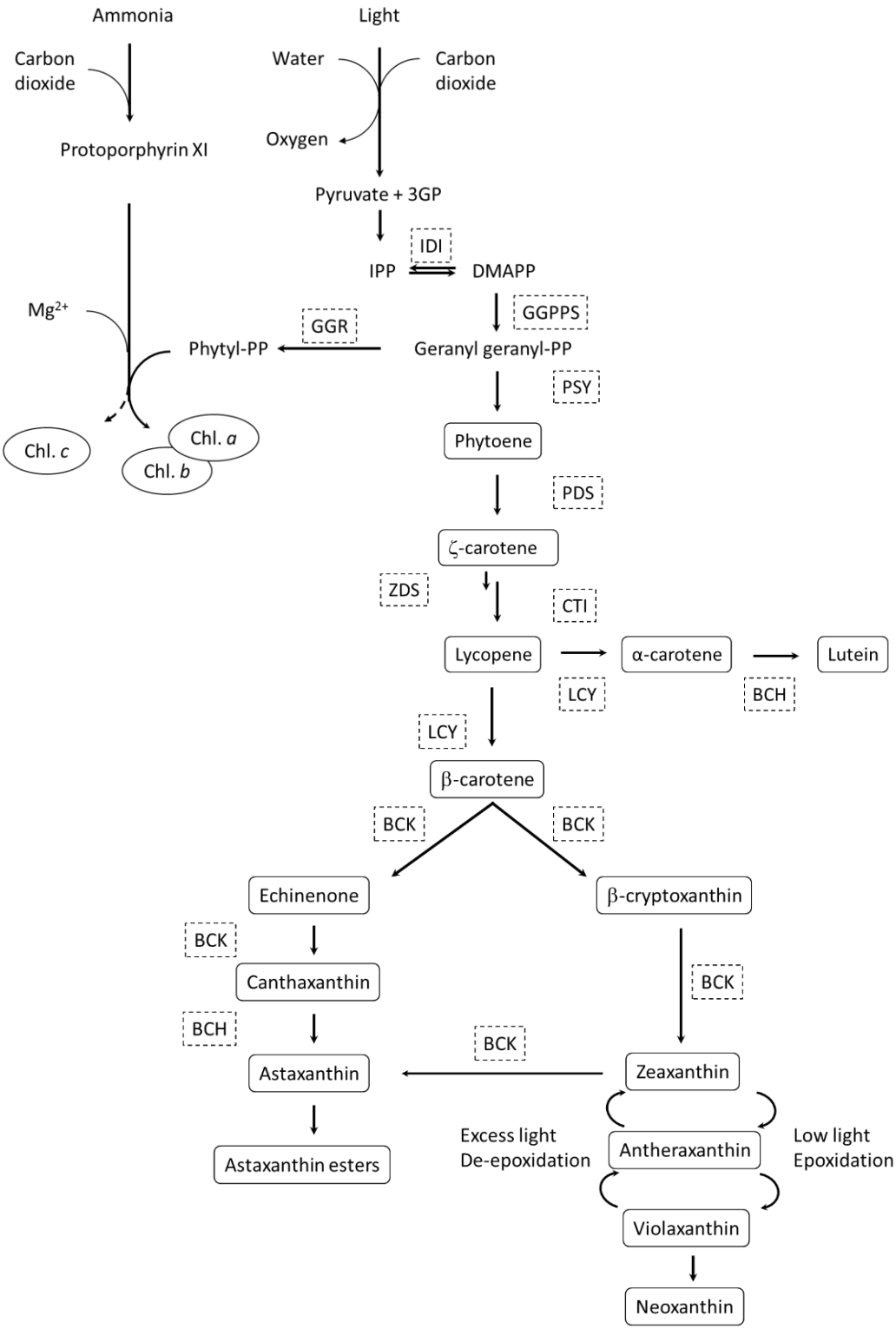

Figure 1.2. Schematic microalgal carotenoid synthesis pathway based on Cunningham *et al.* (1998); Demmig-Adams *et al.* (1996); Lohr *et al.* (2012); Lu *et al.* (2008); Mulders *et al.* (2014); Panaigua-Michel *et al.* (2012); Steinbrenner *et al.* (2001). Abbreviations: IDI: isopentyl pyrophosphate isomerase, GGPS: geranyl geranyl pyrophosphate synthase, GGR: geranyl geranyl reductase, PSY: phytoene synthase, PDS: phytoene desaturase, LCY: lycopene cyclase, BCH: β-carotene hydroxylase, CTI: carotenoid isomerase, BCK: β-carotene ketolase

-as a precursor for the formation of both α- and β- carotene. α- carotene can be converted to lutein, catalysed by β-carotene hydroxylase. β-carotene can either be the precursor for astaxanthin synthesis via two oxidation and two hydroxylation reactions, forming the intermediates echinenone and canthaxanthin, or converted to zeaxanthin by two hydroxylation steps (Mulders *et al.*, 2014). Zeaxanthin can be epoxidised in two steps to form antheraxanthin and violaxanthin (Demmig-Adams *et al.*, 1996; Panaigua-Michel *et al.*, 2012). A number of studies using for example *Chlorella zofingiensis* (Cordero *et al.*, 2011a; Wang *et al.*, 2008a) and *Haematococcus pluvialis* (Steinbrenner *et al.*, 2001) have shown that zeaxanthin can also be converted to astaxanthin, catalysed by β-carotene ketolase (Figure 1.2).

1.4.3 Up-regulation of carotenoid synthesis by reactive oxygen species

The photo-reduction of molecular oxygen in chloroplasts is unavoidable and leads to the production of ROS in all oxygenic photosynthetic organisms (Mallick, 2004). A number of different ROS occur transiently in microalgae as normal by-products of oxidative metabolism and additionally play an important role in cell signalling (Apel *et al.*, 2004), however high ROS concentrations can be extremely harmful as they can oxidize proteins, lipids and nucleic acids, often leading to alterations in cell structure and mutagenesis (Apel *et al.*, 2004). ROS species include: the photo-chemically generated singlet oxygen ($^1O_2$) as well as superoxide anions ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$) which are a consequence of high excitation inputs into photosynthesis (Figure 1.3).

Excessive light induces triplet chlorophyll (^{3}Chl) and singlet oxygen ($^1O_2$) formation in chloroplasts (Pinto *et al.*, 2003). Singlet $^1O_2$ is highly electrophilic and

capable of oxidizing many other molecules (Okamoto *et al.*, 2001). In addition, superoxide anions ($O_2^{\bullet-}$) can be generated by oxygen reduction in photosystem I (PSI) (Mehler reaction). The ($O_2^{\bullet-}$) diffuses into the stroma where it is dismutated into oxygen (O_2) and hydrogen peroxide (H_2O_2) (Takeda *et al.*, 1995). The reaction of H_2O_2 with reduced metal ions produces $OH^{\bullet}$ which is a strong oxidant that can react with and damage biomolecules (Demmig-Adams *et al.*, 1992; Noctor *et al.*, 1998; Pinto *et al.*, 2003; Takeda *et al.*, 1995) (Figure 1.3). Microalgae have developed a range of protective mechanisms to remove ROS before cellular damage occurs. These involve antioxidant enzymatic catalysts and low molecular weight compounds including phenolics, ascorbate, flavonoids, tocopherols and carotenoids (Figure 1.3).

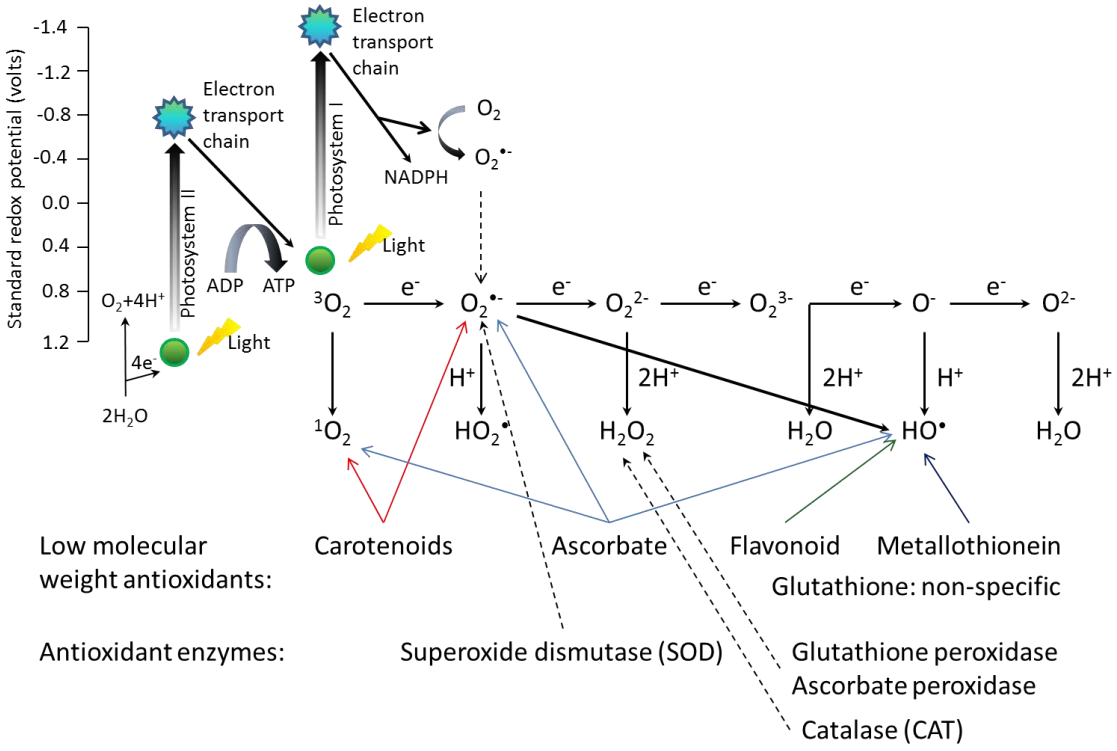

Figure 1.3. Schematic overview of ROS formation and microalgal ROS detoxification mechanisms.

Antioxidant enzymatic catalysts include the enzymes superoxide dismutase (SOD), which catalyse the dismutation of $O_2^{\bullet-}$ into O_2 and hydrogen peroxide H_2O_2, and catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX) (Figure 1.3), which reduce H_2O_2 to H_2O (Okamoto *et al.*, 2001; Pinto *et al.*, 2003).

Many microalgal species have the ability to modulate antioxidant levels, which is an important adaptive response to tolerating adverse conditions (Dat *et al.*, 1998; Pedrajas *et al.*, 1993; Thomas *et al.*, 1999). As microalgal carotenoid biosynthesis is one of the main microalgal responses to oxidative stress (Kobayashi *et al.*, 1993; Vaquero *et al.*, 2012), ROS-inducing environmental conditions, in particular temperature and irradiance, exposure to heavy metals, terpenes, ionones, amines, alkaloids and antibiotics (Bhosale, 2004) and nutrient availability (Lamers *et al.*, 2012; Mulders *et al.*, 2013) induce carotenogensis in microalgae. For example, previous studies have shown that high light-induced photo-oxidative stress and high temperatures increase β- carotene, astaxanthin and lutein contents of *Dunaliella salina*, *Haematococcus pluvialis* and *Muriellopsis* sp., respectively (Boussiba *et al.*, 1992; Del Campo *et al.*, 2000; Orset *et al.*, 1999) (Table 1.4).

Carotenoid responses to stressful environmental conditions can be potentially exploited for enhancing commercial production, particularly at cultivation sites in Australia, renowned for harsh environmental conditions (particularly light and temperature, Table 1.4) and heavy metal-polluted mining tailings-dam waters.

In an Australian commercial context, endemic microalgae strain response evaluations are important, as effects on carotenoid content are species-specific and outcomes are often dependent on exposure times (Margalith, 1999; Schoefs *et al.*, 2001). The responses of endemic freshwater chlorophytes to high irradiance, high

Table 1.4. Carotenoid induction studies using increased temperature, irradiance and nutrient limitation or exposure to iron to enhance carotenoid production.

Microalgal species	Carotenoid induction parameters	Target Carotenoid	References
Chlamydomonas acidophila	- High light - Temperature fluctuations	Lutein and β-carotene	(Garbayo *et al.*, 2008)
Dunaliella salina	- High light + nutrient limitation	β-carotene	(Benamotz *et al.*, 1983)
Dunaliella salina	- High light + salt stress + nutrient limitation	β-carotene	(Benamotz *et al.*, 1983)
Haematococcus pluvialis	- Increased light + ferrous salts + sodium acetate	Carotenoids	(Steinbrenner *et al.*, 2001)
Scenedesmus almeriensis	- Temperature + Irradiance	Lutein	(Sanchez *et al.*, 2008)

- temperature and Mo and V exposure were therefore systematically evaluated in chapters 4 (initial strain screening) and chapters 5 (factorial design of stress-responses of selected endemic species) of this thesis.

1.4.4 Carotenoids of commercial interest

1.4.4.1 Carotenes

Carotenes are primary carotenoids located in the thylakoid membranes with accessory pigment function in photosynthesis and radical scavenging in higher plants and algae (Lichtenthaler, 1999). Extensive studies have been carried out on the biological and health activities of carotenes in particular α- and β- carotene (Tanaka *et al.*, 2012). α- and β-carotene possess one and two retinyl groups, respectively, which are broken down by β-carotene monoxygenase to retinal, a form of vitamin A.

Consequently, β-carotene and to a lesser extent α-carotene are important vitamin A precursors (Guedes *et al.*, 2011a), with an increasing market demand as a pro-vitamin A and antioxidant in multivitamin preparations (Murthy *et al.*, 2005; Spolaore *et al.*, 2006). Additionally these carotenes have also shown to decrease the incidences of some cancers and degenerative diseases (Guedes *et al.*, 2011a; Tanaka *et al.*, 2012) influence immune responses (Hughes *et al.*, 1997) and intracellular communication (Sies *et al.*, 1997).

Currently, the β-carotene market is saturated, with 90 % of β-carotene produced synthetically (*www.ubic-consulting.com*) (UBIC-Consulting, 2012), and the remaining 10 % from *Dunaliella* sp. of which Australia is the largest producer (Del Campo *et al.*, 2007). However, synthetic food additives are being increasingly legally regulated (Guedes *et al.*, 2011a).

1.4.4.2 Astaxanthin

The keto-carotenoid astaxanthin (3, 3'- dihydroxy - β, β- carotene - 4, 4'- dione) is a xanthopyhll carotenoid produced by microalgae, fungi, yeasts and some plants (Cunningham *et al.*, 2011; Fassett *et al.*, 2011). Astaxanthin has two chiral centres, at the 3- and 3'-positions. Consequently, there are three stereoisomers: (3*S*, 3'*S*), (3*R*,3'*S*) and (3*R*,3'*R*) (Figure 1.4). Astaxanthin is produced by a number of microalgal species (Table 1.5), although none to the extent of *Haematococcus* sp., which is currently the predominant source of natural astaxanthin. In *Haematococcus pluvialis* the (3*S*, 3'*S*) stereoisomer is generally the predominant form which is often used in aquaculture and consequently the most commonly consumed by humans (Fassett *et al.*, 2011).In contrast, synthetic astaxanthin (disodium dissuccinate astaxanthin) contains a mixture

of two enantiomers (3*R*, 3'*R*) and (3*S*,3'*S*) and the inactive meso-form (3*R*, 3'*S*) (Vecchi *et al.*, 1979).

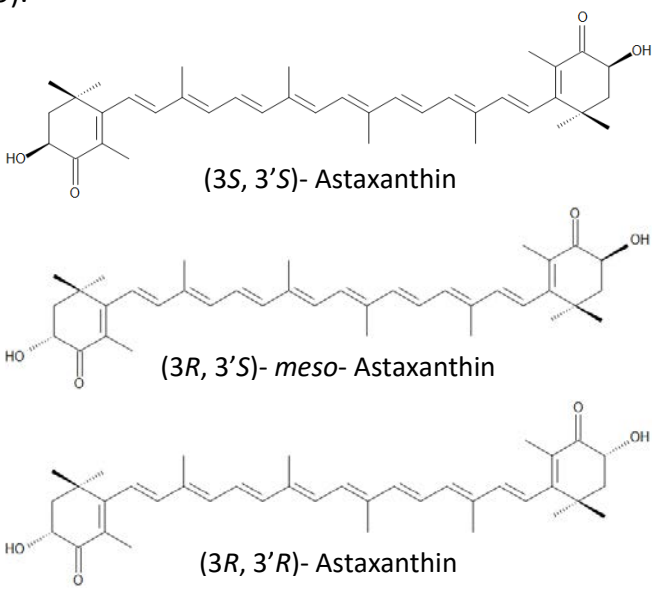

Figure 1.4. Chemical structures of astaxanthin isomers (Britton *et al.*, 2004).

Astaxanthin appears as a red pigment responsible for the pink to red colouration of flesh and shells in marine animals (Johnson *et al.*, 1991). Astaxanthin has widespread applications in nutraceutical, cosmetic, food and feed industries (Guerin *et al.*, 2003; Lorenz *et al.*, 2000). It is currently most frequently used in aquaculture as a pigment for salmon and rainbow trout cultivation representing the most cost-intensive feed additive (2500-3000 USD kg^{-1}) (Grewe *et al.*, 2007; Hussein *et al.*, 2006). There is also an increasing focus on its therapeutic properties as a neutraceutical, as astaxanthin cannot be synthesized by humans (Jyonouchi *et al.*, 1995a). Laboratory trials have shown astaxanthin to enhance the immune response (Jyonouchi *et al.*, 1995b), have anti-cancer properties (Chew *et al.*, 1999; Hix *et al.*, 2004), increase cardiovascular protection (Fassett *et al.*, 2011; Gross *et al.*, 2004), reduction of oxidative stress (Maoka *et al.*, 2012), and inflammation (Guerin *et al.*, 2003).

Table 1.5. Reported pigment contents in a number of microalgal species.

Microalgal species	Pigment [mg g^{-1} DW]	References
	β-carotene	
Chlorella citriforme	1.1	(Del Campo *et al.*, 2000)
Coccomyxa onubensis	1-3.5	(Vaquero *et al.*, 2012)
Dunaliella salina	37-90	(Garcia-Gonzalez *et al.*, 2005; Prieto *et al.*, 2011)
Picochlorum sp.	1-1.5	(de la Vega *et al.*, 2011)
	Astaxanthin	
Chlorella fusca	0.53	(Del Campo *et al.*, 2000)
Chlorella zofingiensis	1.7-6.8	(Del Campo *et al.*, 2000; Del Campo *et al.*, 2004; Liu *et al.*, 2012; Orosa *et al.*, 2001)
Chlorococcum sp.	1.7	(Zhang *et al.*, 2001)
Haematococcus pluvialis	21-98	(Boussiba *et al.*, 1999; Imamoglu *et al.*, 2009; Ip *et al.*, 2005b; Li *et al.*, 2011)
Neochloris wimmeri	19.2	(Orosa *et al.*, 2001)
Protosiphon botryoides	14.3	(Orosa *et al.*, 2001)
Scotiellopsis oocystiformis	10.9	(Orosa *et al.*, 2001)
	Lutein	
Chlamydomonas acidophila	4-5	(Garbayo *et al.*, 2008)
Chlorella protothecoides	4-5	(Del Campo *et al.*, 2007; Wei *et al.*, 2008)
Chlorococcum citriforme	7.2	(Del Campo *et al.*, 2000)
Coccomyxa sp.	7.2	(Garbayo *et al.*, 2012)
Coccomyxa onubensis	4-6	(Vaquero *et al.*, 2012)
Muriellopsis sp.	4-6	(Blanco *et al.*, 2007; Del Campo *et al.*, 2007)
Picochlorum sp.	3.5	(de la Vega *et al.*, 2011)
Scenedesmus almeriensis	4.5 - 5.5	(Sanchez *et al.*, 2008)
	Zeaxanthin	
Chlamydomonas reinhardtii	0.2-0.3	(Couso *et al.*, 2012)
Scenedesmus almeriensis	0.34	(Granado-Lorencio *et al.*, 2009)
Nannochloropsis gaditana	6	(Lubian *et al.*, 2000)
	Violaxanthin	
Chlamydomonas reinhardtii	0.7-1.4	(Couso *et al.*, 2012)
Chlorella citriforme	7.9	(Del Campo *et al.*, 2000)
Muriellopsis sp.	7.3	(Del Campo *et al.*, 2000)

Astaxanthin isomers occur esterified with FAs and unesterified. A current point of debate is whether the degree of astaxanthin esterification influences antioxidant capacity and assimilation (Fassett *et al.*, 2011; Lorenz *et al.*, 2000). Depending on environmental and physiological parameters (e.g. light intensity, temperature and nutrient availability) as well as species specificity (Margalith, 1999; Schoefs *et al.*, 2001), microalgae may produce astaxanthin with differing degrees of esterification (e.g. 99 % of astaxanthin from nitrate- and phosphate-starved *Haematococcus pluvialis* was esterified (Boussiba *et al.*, 1991).

It is becoming increasingly apparent that further research needs to be conducted to determine the importance of isomers and their nature of esterification for health benefits, cancer chemoprevention, inflammation and other uses, as apparently complex esterification and de-esterification reactions are involved for tissue- and organ-specific activity (Coral-Hinostroza *et al.*, 2002; Fassett *et al.*, 2011; Osterlie *et al.*, 1999; Showalter *et al.*, 2004), which has flow on considerations for a natural pigment-based industry.

1.4.4.3 Lutein and zeaxanthin

The xanthophyll carotenoid lutein ((3*R*, 3*R*', 6*R*') - β, ε- carotene- 3, 3'- diol) and its structural isomer zeaxanthin ((3*R*, 3*R*') - β, β- carotene- 3, 3'- diol) are antioxidants with numerous potential therapeutic properties and importance in human health, in particular visual health (e.g. age-related macular degeneration (AMD)). Lutein is the predominant component of the peripheral retina whereas zeaxanthin is the dominant component of the central macula (Granado *et al.*, 2003), both providing important high-energy blue light filtration and antioxidant protection from UV-induced reactive

oxygen species formation (ROS) as well as contributing to visual function (Granado *et al.*, 2003; Maci, 2010; Santocono *et al.*, 2006). Lutein and zeaxanthin have also been associated with the prevention of macular degeneration (Granado *et al.*, 2003; Snodderly, 1995), cataract (Arnal *et al.*, 2009; Moeller *et al.*, 2000), cancer (Nishino *et al.*, 2009; Tanaka *et al.*, 2012), cardiovascular hardening and coronary heart disease (Riccioni, 2009).

Similarly to astaxanthin, lutein and zeaxanthin must be acquired through diet (Granado *et al.*, 2003), mainly fruit and vegetables. The estimated daily uptake of ~1.5 mg day^{-1} (Johnson-Down *et al.*, 2002) is far from the recommended 6 mg day^{-1} daily requirements (Johnson *et al.*, 2010). Currently, the commercial source of natural lutein is marigold (*Tagetes* sp.) (Kumar *et al.*, 2010; Piccaglia *et al.*, 1998) which has a lutein content of ~0.03-0.1% DW (Bosma *et al.*, 2003; Sanchez *et al.*, 2008). In comparison, certain microalgal species have been shown to reach lutein contents between 0.3-0.7 % DW (Table 1.5), making these a promising alternative for commercial lutein production. In contrast, zeaxanthin contents are low in microalgae (e.g. 0.02-2.6 mg g^{-1} DW (Abd El-Baky *et al.*, 2009; Jin *et al.*, 2003)) and plant products (e.g. 3 and 5 μg g^{-1} in spinach and corn , respectively (Sajilata *et al.*, 2008)), which is driving research into metabolic engineering of bacteria (*E. coli*) for commercial natural zeaxanthin production (Li *et al.*, 2015).

1.4.5 Xanthophyll cycle pigments

Cellular concentrations of violaxanthin (5,6: 5,5'-Diepoxy-5,5'',6,6'-tetrahyrol-β–carotene-3,3'-diol) and zeaxanthin are tightly linked to light intensity as they represent the core molecules for protection of photosynthesis (Figure 1.5). Under high

irradiance, excess energy is dissipated by the step-wise de-epoxidation of two epoxy groups in violaxanthin which increases the conjugated double bonds from 9 in violaxanthin, to 10 in antheraxanthin and 11 in zeaxanthin (Figure 1.5). De-epoxidation occurs within minutes, whereas epoxidation can take minutes to days, depending on the degree of additional stressors (Demmig-Adams *et al.*, 1996).

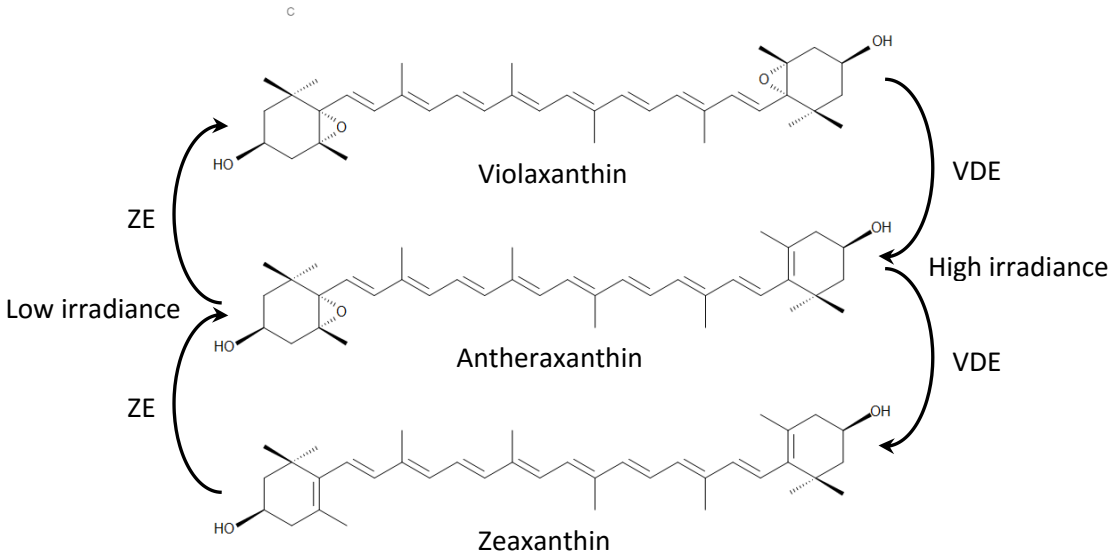

Figure 1.5. Irradiance regulation of epoxidation and de-epoxidation of xanthophyll cycle pigments (Britton *et al.*, 2004; Demmig-Adams *et al.*, 1996). VDE: violaxanthin de-epoxidase, ZE: zeaxanthin epoxidase.

In contrast to cancer research for astaxanthin, evaluation for violaxanthin in this field is limited, despite showing potential along with lutein and zeaxanthin for use as an anti-proliferative pigment for mammary and colon cancer cells (Pasquet *et al.*, 2011) and for its potent anti-inflammatory properties (Soontornchaiboon *et al.*, 2012). Similarly to zeaxanthin, an important limitation to violaxanthin commercialisation is its low yield in microalgae (~0.2-3 mg g^{-1} DW) (Cordero *et al.*, 2011a; Couso *et al.*, 2012; Del Campo *et al.*, 2000). A few species such as *Chlorella citriforme* and *Muriellopsis* sp.

have, however, been reported to contain high concentrations (~7-8 mg g^{-1} DW) (Del Campo *et al.*, 2000) (Table 1.5).

In summary, as carotenoids differ in their capacity to detoxify ROS (Miki, 1991; Shimidzu *et al.*, 1996; Woodall *et al.*, 1997b), violaxanthin has the same ROS quenching capacity as β-carotene and lutein, while astaxanthin has been reported to be significantly more effective (Beutner *et al.*, 2001; Miki, 1991). While violaxanthin and zeaxanthin concentrations are generally low, their epoxidation/de-epoxidation conversions can be used as an indicator for the adaptive potential of a species to stressful Australian cultivation conditions. Microalgae contain a number of carotenoids at sufficiently high concentrations for commercial interest, in particular astaxanthin, β-carotene and lutein (Table 1.5), which in theory can be increased by subjecting algae to ROS-inducing cultivation conditions (see section 1.2.3). In line with the requirement for high value bio-product production for economically sustainable CO_2 capture from flue gas emissions at the Stanwell Corp. coal-fired power plant in southeast Queensland, evaluation of such conditions formed the basis for research presented in chapters 4 and 5, with necessary fundamental background information presented in section 1.2.3.

CHAPTER 2

Salinity tolerance of *Picochlorum atomus* and the use of salinity for contamination control of the freshwater cyanobacterium *Pseudanabaena limnetica*[1]

2.1 Introduction

The depletion of fossil energy stores, climate change-associated increasing atmospheric levels of carbon dioxide and freshwater pollution have generated a renewed interest in industrial-scale microalgal biomass production (Stephens *et al.*, 2010). Industrial algal biomass production can utilize and sequester significant amounts of atmospheric or flue gas carbon dioxide (de Morais *et al.*, 2007) and remove pollutant nutrients such as nitrates, nitrites and phosphates from waste water ponds (Grönlund *et al.*, 2004).

To make industrial-scale microalgal cultivation successful, algal strain selection should focus on species with high production of target biochemicals and tolerance to a wide range of environmental conditions, such as salinity, temperature and nutrient or pollutant loads. Such algal 'super-species' should also show high biochemical productivity, which would considerably simplify production regarding standardisation of product quality across a range of production sites.

[1] Adapted from: von Alvensleben N, Stookey K, Magnusson M, Heimann K (2013) Salinity tolerance of *Picochlorum atomus* and the use of salinity for contamination control by the freshwater cyanobacterium *Pseudanabaena limnetica*. PLoS ONE 8 (5):e63569. doi:10.1371/journal.pone.0063569

Industry aims for microalgae cultivation at various power-stations in Australia for CO_2 and NO_x remediation from flue gas with parallel production of value-adding biochemicals. However, these sites differ in the water quality for cultivation. A cosmopolitan marine microalga, *Nannochloris atomus* Butcher (Chlorophyta, synonym for *Picochlorum atomus* (Butcher) Henley (Henley *et al.*, 2004)), has a suitable lipid and protein content for aquaculture (Becker, 2007; Chen *et al.*, 2012), high biomass production and a potentially broad tolerance to variations of salinity (Cho *et al.*, 2007; Witt *et al.*, 1981). However, the influence of salinity on growth patterns, nutrient requirements and biochemical profiles below 36 ppt, which are commonly encountered at potential production sites, have to date not been determined. Establishing species-specific growth parameters will identify optimal inoculation cell numbers and culture durations for achieving highest biomass productivity in the shortest possible timeframe**.** Understanding species-specific daily nutritional requirements will ensure minimal environmental impact (e.g. eutrophication through discharge of nutrient-rich harvest water effluent (Jarvie *et al.*, 2006)), whilst also minimising expenses associated with fertilisation.

Nitrate assimilation involves a two-step reduction reaction from nitrate to nitrite and nitrite to ammonium, ultimately resulting in the production of amino acids (Barea *et al.*, 1975). Nitrite reduction is rate-limiting and excessive nitrate provision results in an accumulation of cellular nitrite which is secreted, most likely due to its cytotoxicity at high concentrations (Becker, 1994). This has implications for the remediation of nitric oxide (NO) flue gas, which can be converted 1:1 to nitrite in water (Ignarro *et al.*, 1993) to be then used as a nitrogen source. Similarly, to reduce fertilisation costs, industry aims to remediate nutrient-polluted (waste) waters.

Optimal remediation requires correlation of inoculation cell numbers with nutrient loads.

Nitrogen and phosphorus availability also influences cellular protein, carbohydrate, and lipid content, as well as fatty acid profiles (Ahlgren *et al.*, 2003; Huerlimann *et al.*, 2010). Nitrogen limitation reduces the synthesis of chloroplastic proteins and chl *a*, but increases carotenoid content (Geider *et al.*, 1998) while the surplus of carbon metabolites are stored as storage lipids and - carbohydrates (Huerlimann *et al.*, 2010; Roessler, 1990). Higher lipid yields through nitrogen limitation have been obtained for several microalgal species (Huerlimann *et al.*, 2010; Li *et al.*, 2008; Sharma *et al.*, 2012) suggesting that target bio-product yields can be optimised through manipulation of culture nutrient status.

Microalgal culture contamination by rogue organisms is an ever-present risk in aquaculture industries (Meseck, 2007). Common contaminants include bacteria, viruses, fungi, other algae and zooplankton (e.g. ciliates, copepods, rotifers) (Borowitzka, 2005). Current procedures to minimise culture contamination include pH or salinity manipulations (Borowitzka, 2005; Meseck, 2007), the use of ammonium as a nitrogen source, or quinine treatment to reduce amoeba populations (Borowitzka, 2005; Lincoln *et al.*, 1983). Other remedies, such as the addition of antibiotics (Churro *et al.*, 2010) carry the risk of antibiotic resistance, placing restrictions on the use of the biomass and waste water disposal.

Culture contamination by non-target algae or cyanobacteria generally results in resource competition (Joint *et al.*, 2002) and/or the release of potentially toxic allelochemicals into the culture medium, inhibiting growth or killing the target species

(Hay, 2009). This often leads to lost productivity associated with disposal of contaminated cultures, sterilisation, re-inoculation and culture re-establishment. Adverse impacts on product quality can further negatively affect industry, even if productivity is unaffected.

Pseudanabaena limnetica (Lemmermann) Komàrek is a filamentous, non-heterocystous (Komarek, 2003) and non-toxic (Mischke, 2003) freshwater cyanobacterium (Willame *et al.*, 2006), with a certain degree of halotolerance (Acinas *et al.*, 2009) and is a frequent local nuisance contaminant in outdoor microalgal cultures during the tropical wet season. Consequently, methods must be developed to control levels of contamination, ideally not affecting the target species or influencing final products.

Given the potential importance of *P. atomus* in aquaculture, this chapter firstly aimed to determine the influence of salinity on growth, nutrient utilisation, biomass and lipid production and effects of nutrient limitation on biochemical profiles to determine end-product choice and industrial-scale cultivation protocols. Additionally, the effectiveness of salinity manipulations for contamination control of the freshwater cyanobacterial contaminant *P. limnetica* were investigated. It was shown that salinity had no effect on *P. atomus* growth and nutrient utilisation (except at 11 ppt for the latter) and had only a marginal effect on total lipid at 2 ppt and carbohydrate at 8 ppt, respectively, under nutrient-replete conditions. Nutrient status, however, significantly affected total lipid and fatty acid profiles, carbohydrate and protein contents. It was further shown that salinity can be used to control the establishment of *P. limnetica*.

2.2 Materials and Methods

2.2.1 Algal culture conditions

Batch cultures of *Picochlorum atomus* (culture accession # NQAIF 284) were maintained (24 °C, with a 12:12 h photoperiod and light intensity of 42 µmol photons m^{-2} s^{-1}) at the North Queensland Algal Identification/Culturing Facility (NQAIF) culture collection (James Cook University, Townsville, Australia), and were individually areated with 0.45 µm filtered air (Durapore; Millipore). Monoclonal cultures with low bacterial numbers (<1 mL^{-1}) were established in a total culture volume of 2 L in modified L1 culture medium (Andersen *et al.*, 2005), with 6 mg instead of 3 mg PO_4^{3-} L^{-1}. Cultures were re-fertilised with nitrate (~55 mg L^{-1}) and phosphate (6 mg L^{-1}) on day 5 after inoculation to generate sufficient biomass for biochemical analyses of nutrient-replete cultures.

The modified L1 culture medium was prepared at six different salinities: 2, 8, 11, 18, 28 and 36 ppt NaCl in filtered seawater (FSW) (pre-filtration Whatman GF/C, followed by 0.45 µm Durapore, Millipore). All materials were sterilised by autoclaving (Tomy, Quantum Scientific) and cultures were handled and inoculated aseptically in a laminar flow (AES Environmental Pty LTD fitted with HEPA filter). Replicate cultures (2 L, n = 3) of *P. atomus* were inoculated at a density of $4x10^9$ cells L^{-1} (~100 mg dry weight L^{-1}) for each salinity. Inoculation was carried out from 36 ppt mother-cultures with no acclimation to decreasing salinity. Cultures of *P. atomus* have been maintained at the above salinities for more than 200 generations showing the same growth and nutrient utilisation patterns.

2.2.2 Indirect methods for culture growth determination

Calibration curves were established from triplicate dilution series using *Picochlorum atomus* stock cultures to correlate cells L^{-1} (direct cell counts using a bright-line Neubauer-improved haemocytometer) and dry weights (DW) [g L^{-1}] (gravimetric analysis, modified from Rai *et al.* (1991)) with turbidity (% transmission [% TA at 750 nm, Spectramax Plus; Molecular Devices]). Turbidity and calibration curves were medium blanked for each salinity. Dry weight samples were corrected for salt content using salinity-specific blanks. Results were correlated to generate linear equations ($R^2 > 0.95$) used to determine cell numbers and respective dry weights of cultures of *P. atomus* from turbidity measurements.

2.2.3 Culture growth and nutrient analysis

Growth of *Picochlorum atomus* was determined daily using turbidity, from triplicate 250 µl samples per culture for 20 days and obtained data were transformed to cell numbers and dry weights as described above. Specific growth rates [µ] (eq. 2.1), were calculated from culture cell numbers (Levasseur *et al.*, 1993), as were the derived parameters divisions per day and generation time [days]. Biomass productivies were determined using equation 2.2 (modified from Su *et al.* (2011)). Where C_1 and C_2 = initial and final cell numbers [cells mL^{-1}], respectively, t_1 and t_2 = initial and final culture timepoints [days] per identified growth period, respectively, DW_1 and DW_2 = initial and final dry weight [g L^{-1}].

$$\mu = \frac{\ln(C_2 - C_1)}{(t_2 - t_1)} \qquad \text{eq. 2.1}$$

$$Volumetric\ biomass\ productivity[mg\ DW\ L^{-1}day^{-1}] = \frac{(DW_2 - DW_1)}{(t_2 - t_1)} \qquad \text{eq. 2.2}$$

Medium nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3-}) concentrations were determined every second day and on day 5, following nutrient addition, using the Systea EasyChem (Analytical Solutions Australia (ASA) auto-analyser following the manufacturer's EPA-approved and certified protocols (Systea User Manual, 2011).

2.2.4 Biochemical analyses

2.2.4.1 Total lipids, fatty acids, carbohydrate and protein

Biomass samples for biochemical analyses were harvested from 500 mL samples through centrifugation (20 min at 3000 g (Eppendorf 5810R), followed by 2 min at 16,000 g (Sigma 1-14, John Morris Scientific)) from all cultures when nitrate-replete during late logarithmic growth (day 11) and four days after nitrogen depletion during the initial stationary phase; i.e. days 18 and 22, for cultures at 11 and 2 ppt, respectively, and day 24 for cultures at 8, 18, 28 and 36 ppt. Cultures were classified as nutrient-replete and -deplete based on increasing and decreasing nitrite secretion patterns and the nutrient depletion was assured by harvesting four days after medium nutrient depletion (Malerba *et al.*, 2012). The biomass pellets were freeze-dried (VirTis benchtop 2K, VWR) and stored in air-tight vials under nitrogen at 4 °C until further analysis.

2.2.4.2 Total lipid determination

Total lipids were determined gravimetrically following a direct extraction and transesterification method adapted from Lewis *et al.* (2000) and modified following Rodriguez-Ruiz *et al.* (1998) and Cohen *et al.* (1988). Briefly, 2 mL freshly prepared methylation reagent (HPLC-grade methanol : acetyl chloride, 95:5 (v/v)) and 1 mL hexane were added to 30±0.1 mg lyophilized biomass. Following heating (100 ^{o}C, 60 min), 1 mL MilliQ purified water was added and the samples were centrifuged (1800 g for 5 min at 4 ^{o}C (Eppendorf 5810R, VWR) to achieve phase separation. The hexane layer was collected and the pellet was extracted twice more with 1 mL hexane, centrifuging as above between washes, to extract all lipids into the organic phase. The hexane extracts were combined (total of 3 mL) in a pre-weighed glass vial and evaporated to dryness under a gentle stream of nitrogen and weighed to determine total lipids.

2.2.4.3 Fatty acid extraction, transesterification and analysis

Fatty acids in lyophilised samples were simultaneously extracted and transesterified using a method adapted from Rodriguez-Ruiz *et al.* (1998) and Cohen *et al.* (1988), as described in Gosch *et al.* (2012). Briefly, 2 mL freshly prepared methylation reagent (methanol:acetylchloride, 95:5 (v/v)) and 300 μL internal standard (nonadecanoic acid, cat # 72332-1G-F, Sigma Aldrich, Australia), 0.2 mg L^{-1} in methanol) was added to approximately 30 mg (± 0.1 mg) dry biomass in Teflon-capped glass vials. Samples were heated at 100°C for 1h and allowed to cool, after which 1 mL hexane was added. To ensure complete partitioning of the formed fatty acid methyl esters (FAMEs) into the hexane layer, samples were heated again at 100°C for 1 min to

form a single methanol/hexane phase. One mL de-ionized water was then added to the cooled sample to facilitate phase separation. The hexane phase containing the FAMEs was collected and filtered through a 0.2 μm PTFE syringe filter prior to injection on the GC column. All solvents were HPLC grade. Butylated-hydroxy-toluene (BHT) (0.01 %) was added as an antioxidant during the extraction.

Fatty acid analysis was carried out on an Agilent 7890 GC (DB-23 capillary column with a 0.15 μm cyanopropyl stationary phase, 60 m x 0.25 mm ID (inner diameter)) equipped with flame ionisation detector (FID) and connected to an Agilent 5975C electron ionisation (EI) turbo mass spectrometer (Agilent technologies), for identification of fatty acid methyl esters (FAMEs) (split injection, 1/50). Injector, FID inlet and column temperatures were programmed following David et al. (David *et al.*, 2002). Fatty acid quantities were determined by comparison of peak areas of authentic standards (Sigma Aldrich) and were corrected for recovery of internal standard (C19:0) and total fatty acid content (mg g^{-1} DW) was determined as the sum of all FAMEs.

2.2.4.4 Fatty acid productivities

Total fatty acid productivities were determined using equation 2.3, where total FA_2 was determined in nutrient-deplete conditions, total FA_1 in nutrient-replete conditions, and t_1 and t_2 were harvest time points for FA determination.

$$FA\ producticvity\ [mg\ L^{-1} day^{-1}] = \frac{(Total\ FA_2 - Total\ FA_1)}{(t_2 - t_1)} \qquad \text{eq. 2.3}$$

2.2.4.5 Carbohydrate analysis

Total carbohydrate content was determined using the phenol-sulphuric acid assay (Dubois *et al.*, 1956). Prior to analysis, lyophilised algal samples were lysed in MilliQ-purified water with a Bullet Blender bead beater (ZrO_2 beads, 0.5 mm diameter) (Next Advance, Lomb Scientific) to enable collection of a homogenous sub-sample for extraction.

2.2.4.6 Ash and protein analysis

Ash-content (mg g^{-1} DW) was determined by combustion in air (500 °C, 24 h) (Yokogawa-UP 150, AS1044) while protein content was determined by difference (eq. 2.4) (Sims, 1978).

$$Protein\ (\%\ wt) = 100\% - (\%\ Ash + \%\ Total\ lipids + \%\ Carbohydrate) \quad \text{eq. 2.4}$$

2.2.5 Effect of salinity on contamination of Picochlorum atomus cultures with Pseudanabaena limnetica

To investigate if salinity could be used for contamination control, cultures of *Picochlorum atomus* were raised at 11, 18, 28 and 36 ppt (cultures at 2 and 8 ppt were not established as *P. limnetica* is a freshwater species) and seeded with *Pseudanabaena limnetica* colonies at a ratio of 1:100,000 cells mL^{-1} (*P. limnetica* : *P. atomus*). Cell counts (bright-line Neubauer improved haemocytometer) of both organisms commenced on day 8 after the first visible signs of *P. limnetica* dominance (culture colour change) in the lower salinity cultures (11 and 18 ppt).

2.2.6 Statistical analyses

All statistical analyses were carried out in Statistica 10 (StatSoft Pty Ltd.). Repeated measures analysis of variance (ANOVA) were used to determine the effects of salinity on growth rates, nitrite secretion, total nitrogen uptake and contaminant development through culture time. One-way ANOVAs were used to determine the effect of salinity on volumetric biomass productivities. For nutrient uptake analyses, data were divided into pre- and post- nutrient addition (days 0-4 and 5-10, respectively) and the slopes of each uptake period were analysed using one-way ANOVAs. Repeated-measures ANOVAs were used to determine the effects of salinity on nutrient uptake, over time. For total lipid, fatty acids, carbohydrate and protein content analyses, factorial ANOVAs were used to determine the effects of salinity, nutrient status and their interaction. Tukey post-hoc tests were used to determine significant differences assigned at $p< 0.05$. Homogeneity of variances and normality assumptions were verified using Cochran-Bartlett tests. Fatty acid and carbohydrate data required log transformation to fulfil normality assumptions.

2.3 Results

2.3.1 Effect of salinity on growth and nutrient uptake dynamics of Picochlorum atomus

Culture growth of *P. atomus* was divided into three phases (phase I; days 2-5, phase II; days 5-9 and phase III; days 9-18) (Figure 2.1) for which specific growth rates, divisions per day and generation times were calculated (Table 2.1). Within each growth phase, salinity had no significant effect ($F_{(5, 12)}= 0.99$, $p=0.46$) on growth rates,

while the effect of culture phase was significant ($F_{(2, 24)}$= 679.67, p<0.01) as growth rates decreased over culture time.

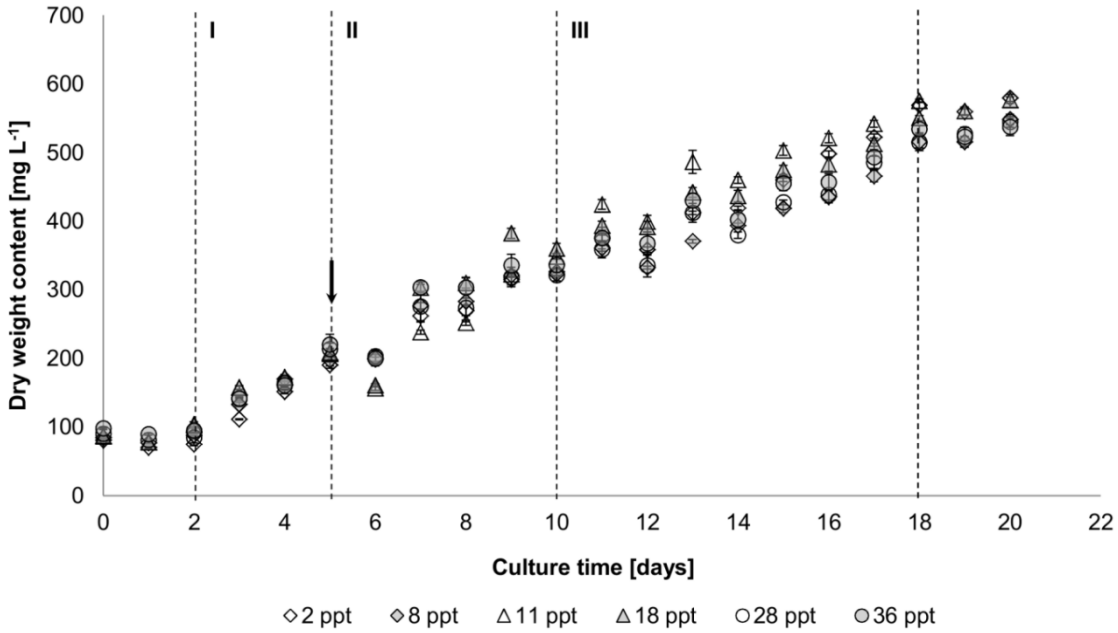

Figure 2.1. Mean biomass growth [mg DW L^{-1}] of *Picochlorum atomus* at 2, 8, 11, 18, 28 and 36 ppt determined using % transmittance at 750 nm. Arrow: indicates the addition of nutrients. Active growth was divided into 3 phases (I-III) based on log-transformed data for determination of specific growth rates [μ]. n=3. Standard error is shown. DW: dry weight.

Irrespective of salinity, specific growth rates [μ] were highest for the first two days following a one-day lag phase (μ=0.21-0.28), then decreased by ~50 % during phase II and a further ~50 % thereafter during phase III (Table 2.1). Nutrient addition on day 5 resulted in culture dilution (Figure 2.1).

Table 2.1. Effect of salinity on specific growth rates [μ], divisions per day [div. day^{-1}] and generation times [days] of *Picochlorum atomus.*

Culture time [days]	2 ppt growth rate [μ]	8 ppt growth rate [μ]	11 ppt growth rate [μ]	18 ppt growth rate [μ]	28 ppt growth rate [μ]	36 ppt growth rate [μ]
Days 2-5	0.28	0.25	0.21	0.27	0.28	0.26
Days 5-9	0.13	0.13	0.11	0.14	0.11	0.11
Days 9-18	0.06	0.05	0.06	0.04	0.05	0.05
Culture time [days]	**2ppt [Div. day^{-1}]**	**8 ppt [Div. day^{-1}]**	**11 ppt [Div. day^{-1}]**	**18 ppt [Div. day^{-1}]**	**28 ppt [Div. day^{-1}]**	**36 ppt [Div. day^{-1}]**
Days 2-5	0.4	0.35	0.3	0.39	0.4	0.37
Days 5-9	0.19	0.18	0.17	0.2	0.15	0.16
Days 9-18	0.09	0.07	0.09	0.06	0.07	0.07
Culture time [days]	**2 ppt gen. time [days]**	**8 ppt gen. time [days]**	**11 ppt gen. time [days]**	**18 ppt gen. time [days]**	**28 ppt gen. time [days]**	**36 ppt gen. time [days]**
Days 2-5	2.47	2.82	3.29	2.58	2.46	2.69
Days 5-9	5.39	5.51	6.04	4.94	6.6	6.35
Days 9-18	11.15	14.08	11.35	18.19	13.85	14.2

Biomass productivities during growth phase I were between 34-43 mg L^{-1} day^{-1} and 26-31 mg L^{-1} day^{-1} during phase II, with the exception of cultures at 18 ppt where biomass productivity remained similar at 36 mg L^{-1} day^{-1} (Table 2.2). Productivities, from the beginning of the logarithmic growth phase to the beginning of the stationary phase were approximately 27-30 mg L^{-1} day^{-1}.

Table 2.2. Effect of salinity on volumetric biomass productivities of *Picochlorum atomus* during growth phases I and II, and overall from days 2-18. n=3. Average ± standard error.

Culture time [days]	Total dry-weight productivity [mg DW L^{-1} day^{-1}]					
	2 ppt	**8 ppt**	**11 ppt**	**18 ppt**	**28 ppt**	**36 ppt**
Days 2-5	38 ± 1	37 ± 3	34 ± 1	35 ± 1	43 ± 2	42 ± 3
Days 5-9	31 ±1	31 ± 1	29 ± 1	36 ± 1	26 ±1	28 ± 1
Days 2-18	30 ± 0.5	27 ± 0.5	29 ± 0.5	28 ± 0.5	27 ± 0.5	27 ± 0.5

Except for cultures at 11 ppt, salinity had no effect on nitrate uptake of *P. atomus* for the first 4 days of the culture period with ~13-15 mg nitrate L^{-1} day^{-1} being assimilated. Following nutrient replenishment on day 5, a ~50% decrease in nitrate uptake was observed (Figure 2.2).

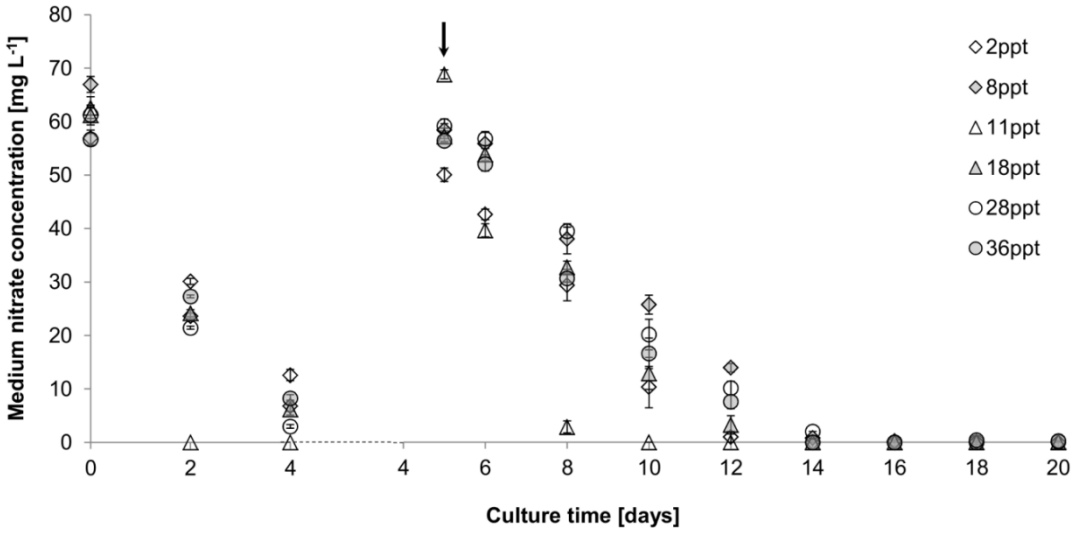

Figure 2.2. Effect of salinity on nitrate assimilation [mg L^{-1}] of *Picochlorum atomus*. n=3. Standard error is shown. Arrow: indicates measurements taken after nitrate and phosphate replenishment.

Cultures at 11 ppt took up nitrate significantly faster pre- ($F_{(5, 12)}$= 85.48, $p<0.01$) and post- ($F_{(5, 12)}$= 14.68, $p<0.01$) fertilisation, than cultures at the other salinities resulting in an uptake of 60 mg L^{-1} day^{-1} for the first two days and medium nitrate depletion. In contrast, a significant negative correlation between nitrite release and salinity ($F_{(1,4)}$= 35.03, $p<0.05$) was observed prior to re-fertilisation, except for cultures at 11 ppt which showed no nitrite release (Figure 2.3). Following fertilisation, all cultures released nitrite irrespective of salinity. Nitrite resorption started 4, 6, 10 and 12 days after fertilisation for cultures at 11 ppt, 2 ppt, 18 and 36 ppt, and 8 ppt, respectively, which correlated with medium nitrate depletion in most cultures (compare Figure 2.2 and Figure 2.3).

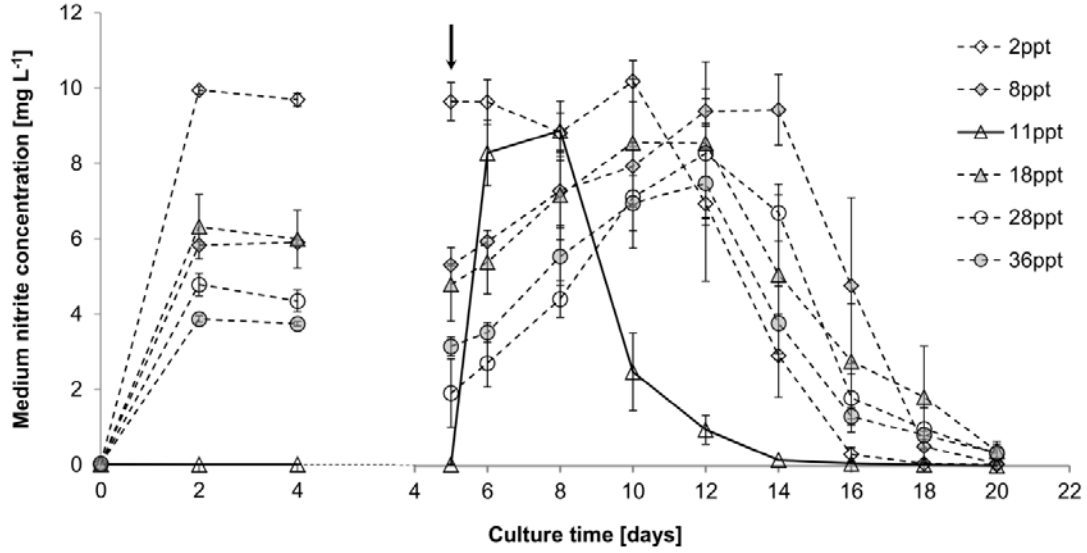

Figure 2.3. Effect of salinity on media nitrite dynamics [mg L^{-1}] of *Picochlorum atomus*. n=3. Standard error is shown. Arrow: indicates measurements taken after nitrate and phosphate replenishment.

Total daily nitrogen uptake (Figure 2.4) was similar between cultures at 2, 8, 18, 28 and 36 ppt but significantly higher at 11 ppt ($F_{(5, 12)} = 34.079$, $p<0.01$).

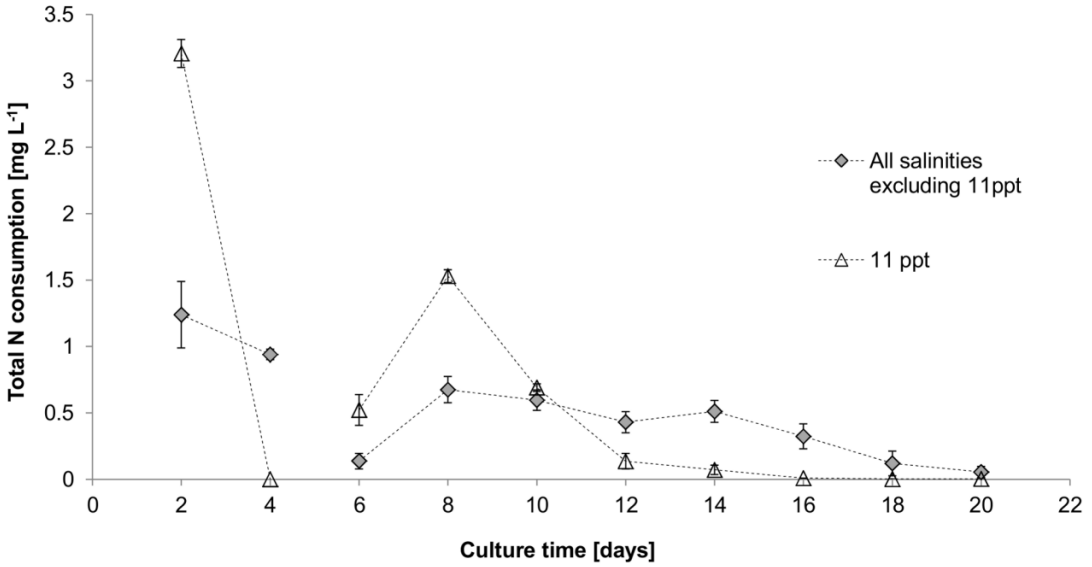

Figure 2.4. Mean total daily net N uptake [mg L^{-1}] of *Picochlorum atomus*. Average total nitrogen consumption is shown for salinities of 2, 8, 18, 28 and 36 ppt, while nitrogen consumption of cultures at 11 ppt is plotted individually to highlight the effect of 11 ppt. n=3. Standard error is shown.

Phosphate uptake followed a similar pattern to nitrate with a decrease in uptake rates following fertilisation. Initial phosphate uptake rates were 1.3-2.4 mg L^{-1} day^{-1} (Figure 2.5). Following phosphate addition, uptake rates decreased to 0.8-1 mg L^{-1} day^{-1}, except for cultures at 11 ppt. Initially, nitrate to phosphate uptake ratio was 6-9 :1 (N:P) and decreased to 4-7:1 (N:P) after nutrient addition.

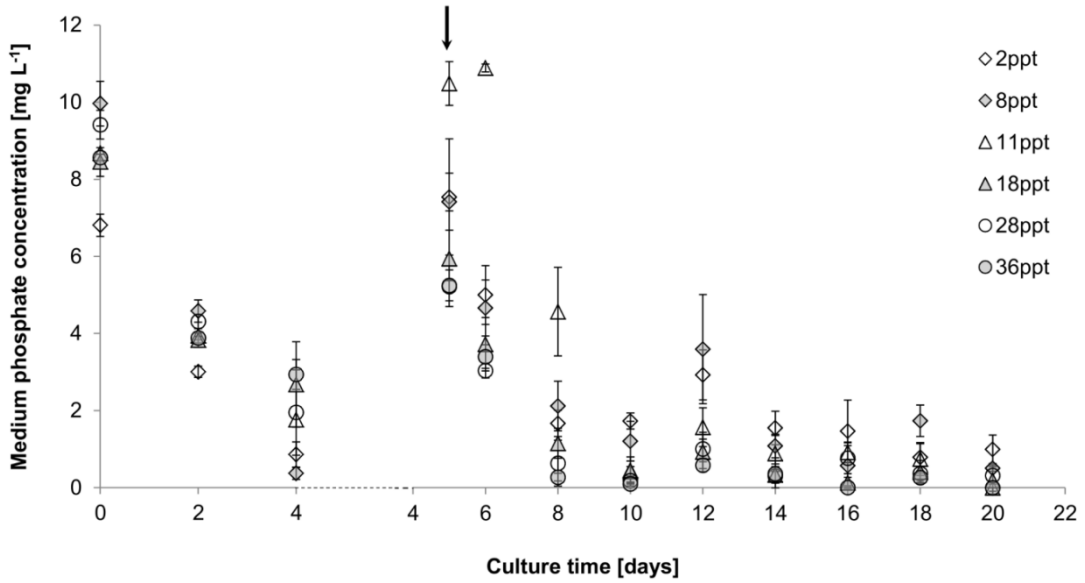

Figure 2.5. Effect of salinity on phosphate assimilation [mg L^{-1}] of *Picochlorum atomus*. n=3. Standard error is shown. Arrow: indicates measurements taken after nitrate and phosphate replenishment.

2.3.2 Effect of salinity and culture nutrient status on the biochemical profile of Picochlorum atomus

Post-hoc analyses identified marginally significant effects of salinity on total lipid content of *P. atomus* at 2 ppt compared to 28 and 36 ppt under nutrient-replete conditions (Figure 2.6A), whereas culture nutrient status had a large effect ($F_{(1, 24)}$ =229.63, p<0.01). Nutrient-starved cultures of *P. atomus* had significantly higher lipid content ($F_{(1, 24)}$ =229.63, p<0.01) than nutrient-replete cultures (Figure 2.6). After 4 days of nutrient starvation, biomass total lipid content increased by 3.5-11 % with the

lowest increase in cultures at 11 ppt and the highest increase in cultures at 28 and 36 ppt (Figure 2.6).

There was no significant effect of salinity on total fatty acid content, but there was a significant effect of culture nutrient status ($F_{(1, 1)} = 316.9$, $p<0.01$) where, as with lipid content, total fatty acid content in nutrient-deplete cultures was significantly higher than in replete biomass.

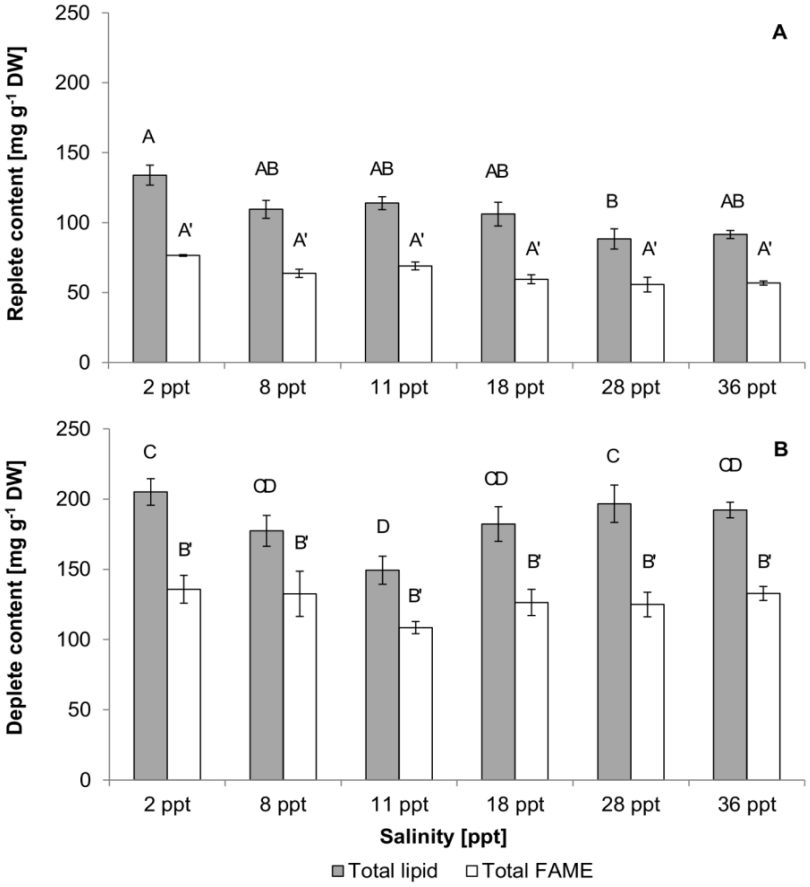

Figure 2.6. Effect of nutrient availability and salinity on total lipid and fatty acid content. Nutrient replete cultures (A) and nutrient deplete cultures (B). Grey bars: total lipid, white bars: total FAME. n =3. Standard error is shown. Different letters show statistical significance; A-D for lipids and A', B' for fatty acids.

Fatty acids represented 56-66 % of total lipids in nutrient-replete biomass and 66-74 % of total lipids in nutrient-deplete cultures, with cultures at 2 ppt showing the highest fatty acid content under both nutrient conditions (Figure 2.6). Lowest fatty

acid concentrations were recorded in nutrient-replete cultures at 28 ppt and 36 ppt (Figure 2.6A). Fatty acid productivities between nutrient-replete and -deplete conditions ranged from 4.7-6.2 mg L^{-1} day^{-1} with cultures at 11 ppt and 2 ppt showing the lowest and highest productivities, respectively (Table 2.3).

Table 2.3. Total FAME productivities [mg L^{-1} day^{-1}] of *Picochlorum atomus* from nutrient replete to deplete conditions. n=3. Average ± standard error.

Salinity	Total FAME productivity [mg L^{-1} day^{-1}]
2 ppt	6.2 ± 0.25
8 ppt	6.1 ± 0.13
11 ppt	4.7 ± 0.06
18 ppt	6.0 ± 0.09
28 ppt	5.9 ± 0.16
36 ppt	6.2 ± 0.13

While fatty acid content increased by up to 50 % following 4 days of nutrient starvation (Figure 2.6), nutrient status also had an influence on fatty acid profiles. A 9 and 11 % increase in saturated and mono-unsaturated fatty acids, respectively, and a corresponding decrease in polyunsaturated fatty acids was observed in nutrient-starved *P. atomus* cultures (Table 2.4). Specifically, C18:1 increased by ~ 13 % while C18:3 showed the largest decrease. The most abundant fatty acids were always C18:3 (n-3), C16:0, and C18:2 (n-6), equating to 54-68 % of the total fatty acids (Table 2.4). The observed ~50 % decrease in the proportion of omega-3 fatty acids and a small increase of omega-6 fatty acids led to a change in omega-6 to omega-3 ratios (ω6:ω3) from ~0.5:1 to ~1:1 under nutrient-limiting conditions.

Table 2.4. Effect of salinity and culture nutrient status (replete/deplete) on fatty acid profiles (proportion [%] of total FAME) of *Picochlorum atomus*. n=3.

	2 ppt		8 ppt		11 ppt		18 ppt		28 ppt		36 ppt	
	Replete	Deplete	Replete	Deplete	Replete	Deplete	Replete	Deplete	Replete	Deplete	Replete	Deplete
Saturated												
C12:0	0.24	0.16	0.48	0.20	0.84	0.45	0.38	0.19	0.50	0.24	0.40	0.21
C14:0	0.38	0.47	0.45	0.53	0.50	0.51	0.46	0.59	0.48	0.56	0.45	0.63
C16:0	14.95	22.50	15.00	22.22	15.68	20.86	15.38	21.69	15.27	21.81	15.82	21.54
C18:0	1.08	4.56	1.20	5.00	1.29	2.75	1.11	3.84	1.32	4.48	1.24	3.74
C20:0	2.21	1.85	2.22	1.94	2.44	1.95	2.29	1.96	2.86	1.94	2.88	2.00
Σ_{SFA}	18.86	29.55	19.35	29.89	20.76	26.51	19.62	28.27	20.42	29.03	20.79	28.11
Monounsaturated												
C16:1 (7)	1.03	1.26	0.93	1.05	1.44	1.26	0.99	1.07	1.09	0.93	0.98	0.85
C16:1 (9)	3.25	1.20	3.17	1.11	3.39	1.59	3.03	1.22	2.89	1.10	2.97	1.24
C18:1 (9)	1.82	14.76	1.99	17.01	4.50	11.63	1.68	14.21	2.07	19.13	1.95	17.96
C18:1 (x)	0.87	0.79	1.03	0.87	1.28	1.12	1.01	0.88	1.17	1.26	1.16	1.43
Σ_{MUFA}	6.99	18.01	7.12	20.04	10.61	15.60	6.71	17.39	7.22	22.43	7.06	21.48
Polyunsaturated												
C16:2 (7,10)	8.80	6.47	7.56	5.64	7.29	6.42	8.15	6.45	7.36	5.16	6.89	5.08
C16:2 (9, 12)	0.37	0.40	0.39	0.39	0.37	0.33	0.37	0.42	0.45	0.40	0.48	0.34
C16:3 (7,10,13)	12.35	6.15	12.81	6.18	9.76	7.47	12.08	6.57	11.69	5.22	11.70	5.82
C16:4 (4,7,10,13)	0.77	0.35	0.83	0.35	0.67	0.41	0.79	0.35	0.79	0.30	0.81	0.30
C18:2	14.26	18.93	12.36	17.20	15.39	17.84	13.23	19.15	13.30	19.31	13.18	19.31
C18:3 (6,9,12)	0.44	0.22	0.40	0.23	0.43	0.30	0.51	0.25	0.48	0.23	0.49	0.23
C18:3 (9,12,15)	34.62	18.48	36.09	18.59	31.90	23.29	35.27	19.60	34.80	16.29	35.19	17.83
Σ_{PUFA}	71.60	50.99	70.44	48.58	65.80	56.06	70.40	52.78	68.87	46.91	68.75	48.90
Sum of ω3	47.74	24.98	49.73	25.12	42.32	31.17	48.14	26.51	47.28	21.81	47.70	23.94
Sum of ω6	23.49	25.62	20.31	23.07	23.11	24.56	21.89	25.85	21.14	24.70	20.56	24.62
ω6:ω3 ratio	0.49	1.03	0.41	0.92	0.55	0.79	0.45	0.97	0.45	1.13	0.43	1.03

Carbohydrate contents were 120-250 mg g^{-1} DW in nutrient-replete cultures, with cultures at 2 ppt and 36 ppt containing the lowest and highest concentrations, respectively. Overall, cellular carbohydrate contents were not affected by salinity, but did increase two to three-fold across all salinities in nutrient-deplete cultures ($F_{(1, 24)}$ =86.98, p<0.01) (Figure 2.7).

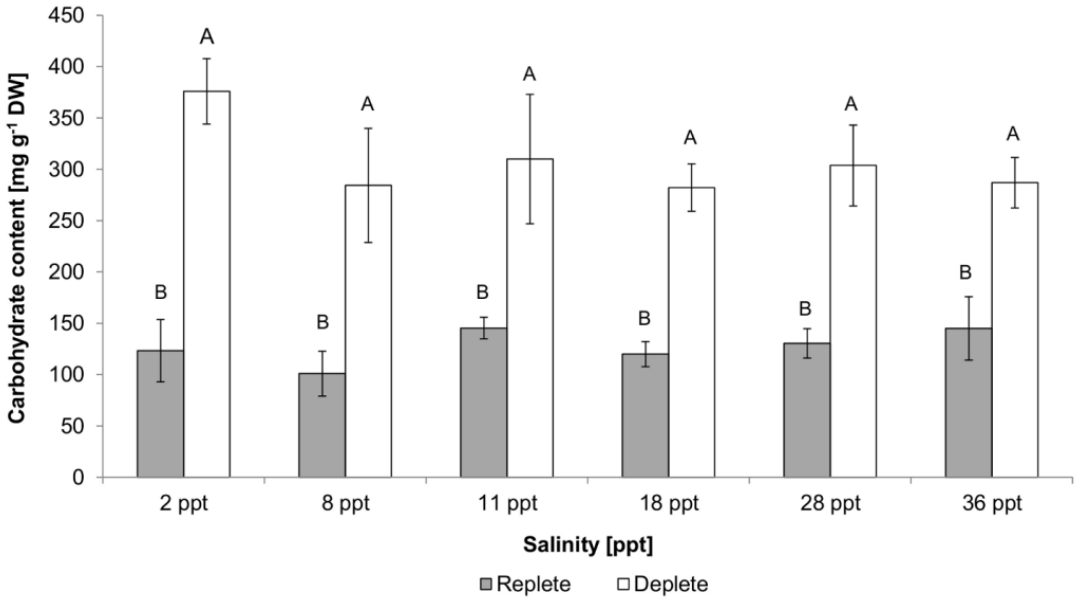

Figure 2.7. Effect of salinity and culture nutrient status (replete/deplete) on mean carbohydrate content [mg glucose g^{-1} DW] of *Picochlorum atomus*. n=3. Standard error is shown. Different letters show statistical significance.

Ash content increased with increasing salinity irrespective of nutrient status. Nutrient depletion led to a ~50 % decrease in ash content compared to replete cultures. Protein content was significantly higher ($F_{(5, 24)}$ =5.78, p < 0.01) in cultures at 8 ppt compared to cultures at 28 and 36 ppt in nutrient-replete conditions. Nutrient depletion induced a protein content decrease across all salinities with a significant decrease in cultures at 2 ppt (~40%) and 8 ppt (~30%) ($F_{(1, 24)}$ =34.34, p<0.01) (Figure 2.8). In both nutrient-replete and -deplete conditions, 8 ppt cultures had the highest protein content and cultures at 36 ppt the lowest.

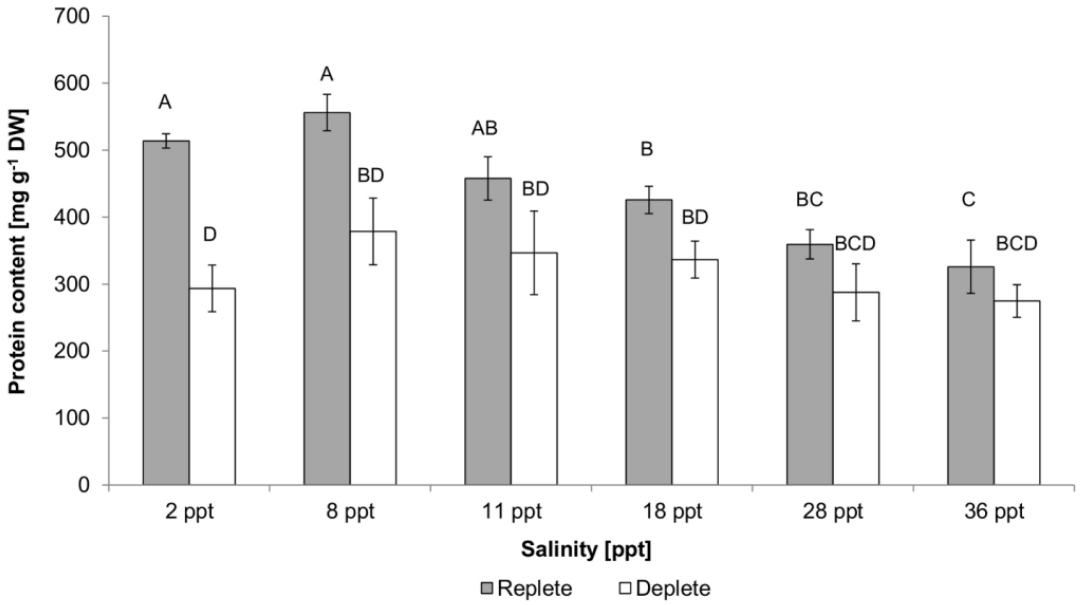

Figure 2.8. Effect of salinity and culture nutrient status (replete/deplete) on mean protein content [mg protein g^{-1} DW] of *Picochlorum atomus*. n=3. Standard error is shown. Different letters show statistical significance.

2.3.3 Effect of salinity on contamination of Picochlorum atomus cultures with Pseudanabaena limnetica

An increase in salinity significantly ($F_{(15, 40)}$ =5.7, p<0.01) slowed the establishment rate of *P. limnetica* (Figure 2.9), resulting in only 10 % of contaminant cells in culture at 36 ppt, compared to 60-70 % at 11 and 18 ppt on day 8. After 16 days, *P. limnetica* completely dominated cultures at 11 and 18 ppt (90-95 %), and reached ~70 % dominance at 28 ppt, whereas in cultures at 36 ppt, *P. atomus* was still dominant with 55 % (Figure 2.9). Specific growth rates [μ] for *P. limnetica* development from day 8 to 10 were ~0.25 in cultures at 11 and 18 ppt and ~0.6 in cultures at 28 and 36 ppt. Overall specific growth rates [μ] from days 8 to 16 were ~0.13 in cultures at 11 and 18 ppt and ~0.25 in cultures at 28 and 36 ppt. This shows that *P. limnetica* at 11 and 18 ppt were in late logarithmic growth around day 8 whereas at 28 and 36 ppt logarithmic growth was just commencing.

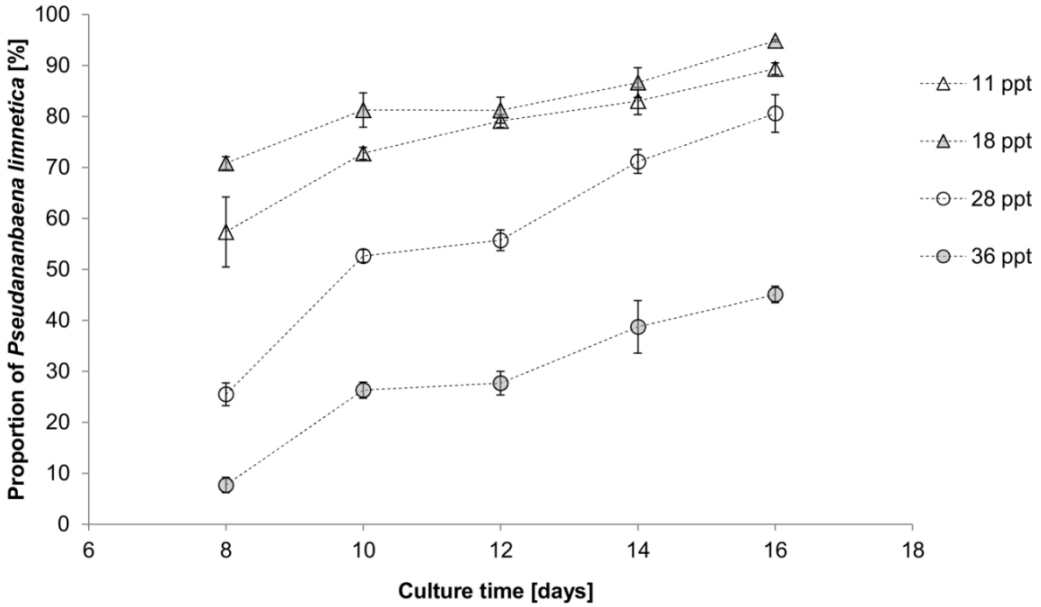

Figure 2.9. Effect of salinity (11, 18, 28 and 36 ppt) on the proportion [%] of *Pseudanabaena limnetica* in *Picochlorum atomus* cultures. n=3. Standard error is shown.

2.4 Discussion

2.4.1 Effect of salinity on growth and nutrient dynamics of Picochlorum atomus

Irrespective of salinity, *Picochlorum atomus* exhibited growth patterns typical of aerated batch cultures (Becker, 1994). The data established that *P. atomus* is a euryhaline microalga tolerating freshwater to marine salinities without adverse effects on growth and biomass productivities.

Initial specific growth rates [μ] were slightly lower than in previous reports, however maximum biomass [mg DW L^{-1}], maximum cell numbers [cells mL^{-1}] and initial volumetric productivities [mg DW L^{-1} day^{-1}] were comparable to previous reports using similar cultivation procedures for *Picochlorum* spp/*Nannochloris* spp (Table 2.5). Comparisons are however, difficult, as a summary of published biomass at harvest and biomass productivities for *Nannochloris* and *Picochlorum* spp shows great variability (Table 2.5). This variation is to be expected (Lim *et al.*, 2012) and is likely due to a

combination of effects, such as species-specific responses and cultivation/ environmental parameters, i.e. variable inoculation densities, differing light regimes, cultivation (batch *vs*. semi-continuous) and productivity calculations (direct *vs*. indirect) (de la Vega *et al.*, 2011; Negoro *et al.*, 1991; Roncarati *et al.*, 2004; Su *et al.*, 2011).

The decrease in growth rate during phases II and III (Figure 2.1, Table 2.1) is characteristic of batch cultures (Becker, 1994) and is generally the consequence of individual or combined effects of culture self-shading, nutrient limitation (MacIntyre *et al.*, 2005) and microalgal/bacterial exudate accumulation (Chiang *et al.*, 2004; Hay, 2009). Initially, these factors are unlikely to have a considerable effect on culture development, particularly considering the low inoculation densities, adequate nutrient provision and low bacteria cultures used in this study. However, over culture time, the accumulation of algal exudates followed by increased self-shading and bacterial growth-inhibiting exudates (negative allelopathic interactions) are likely to cause the observed decreasing growth rates. Nutrient limitation is unlikely to have affected growth as cultures were maintained nutrient-replete with high nitrite levels (Figure 3), indicating cellular nitrogen stores were filled throughout most of the cultivation period (Malerba *et al.*, 2012). Additionally, culture re-fertilisation on day 5 had no impact on culture growth; also implying cultures were not nutrient limited.

Table 2.5. Comparison of growth data in this chapter with growth data obtained for *Picochlorum* spp./*Nannochloris* spp. under similar cultivation conditions and the ranges reported for different cultivation approaches.

Species	**Specific growth rate** [μ]	**Cell numbers** [cells mL^{-1}]	**Maximum biomass** [mg DW L^{-1}]	**Volumetric productivities** [mg DW L^{-1} day^{-1}]	**References**
*Picochlorum atomus**	0.21-0.28	~$2.2x10^7$	~560	~26-43	This study
*Nannochloris atomus**	~0.32-0.38	~$3x10^8$	-	-	(Reitan *et al.*, 1994; Roncarati *et al.*, 2004; Sunda *et al.*, 2007)
Nannochloris spp./ *Picochlorum* spp.*	0.35-0.44	-	~330-410	~40	(Ben-Amotz *et al.*, 1985; Chen *et al.*, 2012; Witt *et al.*, 1981)
*Nannochloris maculata**	~0.36	~$1x10^8$	-	-	(Huertas *et al.*, 2000)
*Nannochloris bacillaris**	~0.41	~$1x10^7$	-	-	(Brown, 1982)
Nannochloris spp./ *Picochlorum* spp.	0.17-2.5	$3x10^6$-$3x10^8$	46-1800	7-320	(Ben-Amotz *et al.*, 1985; de la Vega *et al.*, 2011; Huertas *et al.*, 2000; Negoro *et al.*, 1991; Shifrin *et al.*, 1981b; Volkman *et al.*, 1989)

*: Comparable cultivation conditions

The observed growth patterns for *P. atomus* have direct implications for industrial cultivation, as optimal productivities are achieved in relatively dilute cultures for a brief period. Harvest effort and costs inversely correlate with culture cell densities. Consequently, future studies should investigate whether higher inoculation densities and/or semi-continuous culturing would improve biomass yield and overall productivity. In addition, the accumulation of microalgal/bacterial exudates and their effects on culture development require further investigation, as these may affect water treatment and recycling capacity on industrial-scales.

Nitrogen and phosphorus are essential macronutrients, where the first limiting nutrient reduces microalgal growth rates (MacIntyre *et al.*, 2005). Therefore, maximum biomass production requires adequate nutrient availability for each particular species in culture. However, excessive nutrient concentrations in harvest water pose environmental problems and unnecessary costs, unless harvest effluents can be efficiently recycled without compromising culture growth.

Initial nitrate uptake by *P. atomus* was similar at all salinities (except 11 ppt) and comparable to *Nannochloris maculata* (Huertas *et al.*, 2000). With the exception of cultures at 11 ppt, patterns of nitrite secretion until day 10 can be grouped into high (28 and 36 ppt), intermediate (18 and 8 ppt) and low (2 ppt) salinity patterns, where medium nitrite was highest in low salinity cultures. Medium nitrate depletion resulted in expected nitrite resorption as intracellular nitrogen stores became depleted (Malerba *et al.*, 2012). Nitrogen fluxes can provide insight into possible osmoregulatory mechanisms, often reflected in changes of biochemical profiles. The production of osmoregulatory solutes, such as proline in response to hyperosmotic stress has been reported for *Nannochloris* sp. (Brown, 1982), which would require

higher nitrogen provisions. However, despite the variable nitrite secretion, total nitrogen uptake patterns (except for 11 ppt) were not significantly different. This may indicate that higher nitrite secretion in the lower salinity cultures was potentially due to a slight swelling of cells, increasing cell surface area (Kirst, 1990), thereby increasing nitrate uptake. In contrast to nitrate (Dortch *et al.*, 1984), nitrite cannot be stored and is cytotoxic in higher concentrations (Becker, 1994). Reduction of nitrite to ammonium is limited by nitrite reductase activity (a reaction directly linked to photosynthesis and under circadian control (Rajasekhar *et al.*, 1987)). Thus, when nitrate reduction exceeds the reducing capacity of nitrite reductase, nitrite is secreted.

The significantly higher nitrogen requirements at 11 ppt are difficult to explain. Typically, higher nitrogen is required mainly for growth (Becker, 1994), which is not the case here (Figure 2.1) or hypersaline osmoregulation (Henley *et al.*, 2004), but no significant differences in protein contents were detected. Although this does not exclude the production of osmolytes such as glycine betaine or proline (Kirst, 1990), osmoregulatory responses would be expected to be higher at lower salinities, which should result in greater nitrogen requirements at lower salinities. As this was not observed, we hypothesise that 11 ppt may induce a transitional response where known hypo- or hyper-osmoregulatory responses are not induced.

At 11 ppt the biomass contained twice the amount of C18:1(9) and 2-3 % more C18:2 than at other salinities. Fatty acid changes in diacylglycerol (increases in phosphatidyl inositols and hydrolysis of phosphatidyl choline) and an increase in the fatty acid combinations of C16:0/C18:1 and C16:0/C18:2 was observed in *Dunaliella salina* as an immediate but transient response to hypo-saline osmotic shock (reducing salinity from 99 to 49 ppt) (Ha *et al.*, 1991). This indicates that salinity can affect

membrane composition. Hence, 11 ppt could induce changes in membrane lipids, perhaps increasing vacuolar storage capacity for nitrogen, which would explain the rapid uptake and the reduced nitrite secretion at 11 ppt.

Nitrate uptake of *P. atomus* was comparable or higher than reported for other species examined for wastewater treatment, including *Chlorella vulgaris* (Sydney *et al.*, 2011) and *Neochloris oleabundans* (Wang *et al.*, 2011a), suggesting that *P. atomus* could also be used in such applications. Nitrogen uptake potential also has important implications for industrial NO flue gas remediation. *Dunaliella tertiolecta* can remediate 21 mg day^{-1} of nitric oxide (NO) and showed a preference for NO uptake over NO_3^- (Nagase *et al.*, 2001). Future research should examine *P. atomus*'s nitrogen preferences and NO remediation potential from flue gas emitted by Australian coal-fired power stations.

As for nitrate uptake, initial phosphate uptake across all salinities was comparable to *Nannochloris maculata* (Huertas *et al.*, 2000) and uptake rates were comparable to *Chlorella stigmatophora*, showing potential for urban waste-water remediation (Arbib *et al.*, 2012). Remediation studies using *Neochloris oleabundans* have shown phosphate uptake to correlate with increasing medium phosphate availability (Wang *et al.*, 2011a). Consequently, further studies should investigate *P.atomus* phosphate uptake when exposed to higher concentrations.

The N:P ratio of *P. atomus* was similar to *Nannochloris atomus* (Reitan *et al.*, 1994). The N:P ratio decreased over culture time as nutrient availability per cell decreased and cell numbers increased. Downstream effects of the decreased N availability resulted in reduced total protein contents (Figure 2.8).

2.4.2 Effect of salinity and culture nutrient status on the biochemical profile of Picochlorum atomus

Culture salinity affected total lipid (at 2 ppt) and protein (at 8 ppt) contents of *Picochlorum atomus* under nutrient-replete conditions. However, nutrient availability was the main driver for significant differences in total lipid, carbohydrate, and protein contents, as well as fatty acid composition. Biochemical profile comparisons between studies are difficult, as species-specificity and environmental factors (nutrient availability, light intensity, photoperiod and cultivation stage) individually and combined affect the proximate chemical composition of microalgae (Ben-Amotz *et al.*, 1985; Piorreck *et al.*, 1984; Shifrin *et al.*, 1981a). Despite being a marine species, the highest total lipid content was observed when culturing *Picochlorum atomus* at 2 ppt, irrespective of nutrient status. Under nutrient-replete conditions, total lipid content of *P. atomus* was low, whereas nitrogen limitation increased total lipids to ~20%, corresponding to amounts reported for *Nannochloris atomus* and *Picochlorum* sp. (Ben-Amotz *et al.*, 1985; de la Vega *et al.*, 2011) and defining it as an oleaginous microorganism with the potential for oil-based biofuel production (Hu *et al.*, 2008). In contrast, a higher total lipid content was reported for *Nannochloris* sp. (~ 56 %) when CO_2 was added (Negoro *et al.*, 1991). Opportunistic biochemical profiling of very old cultures showed that *P. atomus* can also reach a total lipid content of ~60%. Consequently, studies should investigate high lipid yields in the context of remaining feasible and economically viable on a large-scale.

Total lipid content is not a good indicator for oil-based products, as this fraction contains all other lipid-soluble materials such as pigments. For oil-based products (e.g. biodiesel and bioplastics), the fatty acid content is more important (Gosch *et al.*, 2012;

Lim *et al.*, 2012). Nutrient-depletion increased fatty acid content by ~10%, suggesting that fertilisation adjustments can improve biomass suitability for such products. Fatty acid proportions of total lipids were comparable to (nutrient-replete) or higher (nutrient-deplete) than those reported for the same genus (de la Vega *et al.*, 2011). Fatty acid profiles were comparable to those described by Volkman et al. (Volkman *et al.*, 1989) but different to others for this genus (Ben-Amotz *et al.*, 1985; de la Vega *et al.*, 2011; Roncarati *et al.*, 2004) (which also differ between each other for many fatty acids). These outcomes highlight the importance to consider culture conditions (e.g. industry location) and species-specificity when considering industrial cultivation. Total fatty acid productivities by *P. atomus* were comparable to other species (e.g. *Nannochloropsis* sp.) (see Lim *et al.* (2012) for summary details).

Nutrient limitation considerably increased amounts of saturated (C16:0) and mono-unsaturated fatty acids (C18:1) but lowered amounts of polyunsaturated fatty acids (C18:3) consistent with responses reported for a wide variety of microalgal species (Reitan *et al.*, 1994). For nutritional/dietary purposes an ω6:ω3 ratio of approximately 1:1 has been shown to be beneficial for cardio-vascular health (Simopoulos, 2002), suggesting, that under the cultivation conditions reported here, *P. atomus* should be harvested when nutrient-deplete. In contrast, the suggested optimal fatty acid ratio for biofuel of 5:4:1 of C16:1, C18:1 and C14:0, respectively (Schenk *et al.*, 2008) was observed only under nutrient-replete conditions and low concentrations were observed. Identifying species with naturally occurring favourable fatty acid ratios for specific end-products could prove impossible under industrial conditions, therefore blending of fatty acids or oils from different microalgal species (Cha *et al.*, 2011) and/or fertilisation regimes must be considered to achieve the

specifications of a particular end-product. For example, for biofuel production, cultures of *P. atomus* will require nutrient starvation to increase lipid productivity and decrease the PUFA content.

Nutrient status also affected total carbohydrate and protein contents which increased and decreased, respectively, following nutrient limitation. Both carbohydrate and protein contents were similar under nutrient-replete conditions and slightly higher than reported for *Nannochloris atomus* under nutrient limitation (Ben-Amotz *et al.*, 1985). Similar patterns of protein decrease and concurrent carbohydrate increase as a result of nutrient depletion have been observed in a number of microalgal species e.g. *Chlorella vulgaris* and *Scendesmus obliquus* (Piorreck *et al.*, 1984), as N-limitation prevents the synthesis of proteins, channelling the photosynthetically acquired carbon into storage. Nutrient-replete *Picochlorum atomus* has been shown to be a promising replacement for *Nannochloropsis oculata* in aquaculture for grouper larval rearing (Chen *et al.*, 2012), which is rapidly expanding, and already one of the most valuable aquaculture species in Southeast Asia (Harikrishnan *et al.*, 2010).

2.4.3 Contaminant inhibition

In large-scale cultures, contamination by rogue organisms is a serious problem often resulting in significant economic losses (Meseck *et al.*, 2007). In tropical Australia, the freshwater cyanobacterium *Pseudanabaena limnetica* rapidly out-competes and dominates other microalgal species in culture. The observed broad salinity tolerance of *P. atomus*, with minimal effects on productivity or biochemical profiles, allows the use of salinity manipulations to inhibit or reduce culture

contamination by rogue organisms. Although increased culture salinity does not completely prevent the development of *P. limnetica*, it does delay its establishment and subsequent logarithmic growth at 28 and 36 ppt up to day 8. It is noteworthy however, that while establishment of *P. limnetica* at high salinities is considerably slower, once established, growth rates are high and culture take-over will occur. The extended time for establishment and logarithmic growth of *P. limnetica* provides an extended opportunity to harvest the biomass with low levels of contamination, which is an important aspect for end product quality control.

In conclusion, *Picochlorum atomus* has considerable advantages for large-scale cultivation as it can be cultivated at locations differing in water salinity ranging from 2 – 36 ppt, without adverse effects on biochemical profiles. High carbohydrate and protein content suggests use in aquaculture (Witt *et al.*, 1981) or as agricultural feed (e.g. for poultry) (Becker, 2007), when harvested under nutrient-replete conditions. In contrast, under nutrient-deplete conditions, fatty acid yields and the decrease in PUFA content is suitable for lipid-based biofuel production. Similarly, the improved ω6:ω3 ratio under these conditions, would allow cultivation of *P. atomus* as a health food supplement to improve cardiovascular health. In addition, salinity increase appears to be an effective tool for contamination delay, yielding biomass with guaranteed quality, which allows harvest and minimises economic losses due to culture re-establishment and end-product loss.

Based on these findings, chapter 3 investigated the salinity tolerance of four freshwater microalgal species, isolated from Stanwell Corp. tailings-dam water, and the effects of increasing salinity on biochemical profiles and applicability for

contamination control. As with *P. atomus*, nutrient requirements and effects of nutrient depletion on biochemical profile were also investigated.

CHAPTER 3

Salinity tolerance of four freshwater microalgal species and the effects of salinity and nutrient limitation on biochemical profiles[2]

3.1 Introduction

Cultivation of microalgae has the potential to provide critical ecosystem services through bioremediation of atmospheric industrial pollution (e.g. CO_2 and NO) (Brune *et al.*, 2009; Ho *et al.*, 2011) and nutrient-rich waters from agriculture, aquaculture or urban sewage (Chan *et al.*, 2014). In parallel, the resulting biomass can be used for production of commodities and high-value compounds such as protein or fatty acids (Mata *et al.*, 2010; Pulz *et al.*, 2004; Stephens *et al.*, 2010).

An important consideration for the feasibility of large-scale microalgae cultivation is water availability and salinity (Borowitzka *et al.*, 2013). Industrial sites, such as coal-fired power stations or sewage plants, may provide low salinity wastewaters, however in most cases groundwater is predominantly available, which, in many parts of Australia, is often saline (≤ 5ppt) (Hart *et al.*, 1991; Peck *et al.*, 2003). This is of particular concern in tropical areas, where high evaporation rates year-round may lead to problems of increased salinity. For example, in a 100,000L culture pond with a 5 ppt starting salinity and a ~5 % day^{-1} evaporation rate (South East Queensland, December) (BOM, 2006), salinity would increase to ~7.5 ppt in 10 days and ~10 ppt in 20 days despite daily replacement. Consequently, when screening

[2] Adapted from: von Alvensleben, N., Magnusson, M., Heimann, K., 2015. Salinity tolerance of four freshwater microalgal species and the effects of salinity and nutrient limitation on biochemical profiles. Journal of Applied Phycology: 1-16. doi:10.1007/s10811-015-0666-6

microalgae for biotechnological applications, it is important to determine species-specific halotolerance to identify species with broader salinity tolerance ranges providing greater flexibility in water requirements and applicability across different cultivation sites (Borowitzka *et al.*, 2013).

Many microalgae have the ability to tolerate fluctuations in salinity (Chapter 2) (Brown, 1982; Kirst, 1989; von Alvensleben *et al.*, 2013b) through the K^+/Na^+ pump as well as osmolyte production (e.g. glycine betaine, proline, sucrose, glycerol), which contributes to the osmotic potential for cell turgor and volume control and the latter protecting and restoring damaged proteins, nucleic acids and membrane lipids (Erdmann *et al.*, 2001). Despite this, salinity stress often leads to decreased biomass productivity due to the high energy-cost of osmoregulation (Oren, 1999) and is often associated with an over-production of reactive oxygen species (ROS) (Erdmann *et al.*, 2001; Mahajan *et al.*, 2005; Sudhir *et al.*, 2004). Importantly, this natural response to salinity stress can also be exploited in order to manipulate the biochemical composition of microalgae, as evidenced in e.g. increased fatty acid content with increasing salinity in the marine microalgae *Isochrysis* sp. and *Nannochloropsis oculata* (Renaud *et al.*, 1994) and in the freshwater microalga *Chlamydomonas mexicana* (Salama *et al.*, 2014).

Microalgal nutrient uptake (e.g. nitrogen and phosphate) varies widely between species (Aravantinou *et al.*, 2013; Dortch *et al.*, 1984) which has multiple implications for large-scale production depending on whether these nutrients need to be purchased or whether they are freely available in nutrient-rich wastewater, requiring remediation. If nutrient-rich wastewater is available, species-selection should identify species with high nutrient consumption and tolerance to eutrophic

conditions for timely wastewater remediation (Mata *et al.*, 2010). In contrast, if nutrient-rich wastewater is not available, nutrient provision will incur substantial costs, and species with lower nutrient consumption would be advantageous for biomass production. This is particularly important with the observed global peak of phosphate production, and fertilisers in general becoming increasingly expensive if effective recycling methods are not adopted (Cordell *et al.*, 2009; Dawson *et al.*, 2011). As microalgal growth is positively correlated with nutrient availability (MacIntyre *et al.*, 2005), nutrient provision at a cost will particularly affect economics of large-volume bio-products. Similarly to salinity, nutrient condition manipulations are commonly used to favourably alter the biochemical composition of microalgal biomass, for example to induce the rapid accumulation of triacylglycerols (TAG) (Gao *et al.*, 2013; Olofsson *et al.*, 2014; Rodolfi *et al.*, 2009) or pigments (Imamoglu *et al.*, 2009) in a number of algal species in commercial production. Whilst nutrient limitation leads to cessation of active biomass production, benefits are incurred if it leads to substantially higher accumulation of target compounds (Chapters 4 and 5) (e.g. β-carotene in *Dunaliella salina*, astaxanthin in *Haematococcus pluvialis* and lipids in *Nannochloropsis* spp (Richardson, 2011)) through diverting carbon usage for growth to carbon storage in biomolecules.

Considering the enormous diversity of algal species (Guiry, 2012) and the common stress-response to up-regulate the content of cellular components that are desirable in commercial production of algae, bioprospecting for new microalgal species amenable to cultivation and environmental tolerance trials connected to biochemical plasticity, remain important tasks. Research to-date for *Desmodesmus armatus*, *Mesotaenium* sp. and *Tetraedron* sp. is limited, in particular concerning their

potential biotechnological applications (Pulz *et al.*, 2004). *Scenedesmus* spp, however, have been extensively investigated with established potential for wastewater remediation and in biotechnological applications, e.g. pigment and biofuel production (Garcia-Moscoso *et al.*, 2013; Guedes *et al.*, 2011a; Martínez *et al.*, 2000; Muller *et al.*, 2005). Therefore this chapter investigated nutrient requirements and responses of four freshwater microalgal species (*Desmodesmus armatus*, *Mesotaenium* sp., *Tetraedron* sp. and *Scenedesmus quadricauda*) isolated from tailings-dam water of a Queensland power station to changes in salinity and flow-on effects on biochemical compositions. The second aim was to identify if nutrient limitation could be used to favourably alter the biochemical profiles and productivity of the same species, and if this effect was linked to the level of salinity stress.

3.2 Materials and Methods

3.2.1 Algal culture conditions

Freshwater microalgae were isolated from Tarong power station (Stanwell Corp.) and maintained at the North Queensland Algal Identification/Culturing Facility (NQAIF) culture collection (James Cook University, Townsville, Australia). Of the 13 successfully isolated species, *Desmodesmus armatus* (Trebouxiophyceae) (NQAIF301), *Mesotaenium* sp. (Conjugatophyceae) (NQAIF303), *Scenedesmus quadricauda* (Chlorophyceae) (NQAIF304), and *Tetraedron* sp. (Chlorophyceae) (NQAIF295), were chosen for the experiments as they were the dominant and most abundant species but also because of their cosmopolitan distribution which minimizes biosecurity concerns. The fact that they were isolated from tailings-dam waters from a coal fired power

plant points to their potential resilience in demanding environments and likely suitability for remediation purposes. Individually aerated (0.45 μm filtered air (Durapore; Millipore)) batch cultures were maintained at 24 °C, with a 12:12 h photoperiod and light intensity of 42 μmol photons m^{-2} s^{-1}. Monoclonal cultures with low bacterial numbers (<1 mL^{-1}) were established in a total culture volume of 2 L in modified L1 culture medium (Andersen *et al.*, 2005), with 5 mg L^{-1} instead of 3 mg L^{-1} phosphate. Preliminary studies identified high phosphate uptake rates by *Mesotaenium* sp. and *S. quadricauda*, consequently medium phosphate concentrations were increased to reduce phosphate replenishment requirements and avoid medium phosphate depletion. To account for differences in cell size, all species were inoculated at 100 mg dry weight L^{-1}. To maintain nutrient-replete biomass, cultures were re-fertilised with nitrate and phosphate when near depletion, until there was sufficient N-replete biomass for harvest for biochemical analyses. Modified L1 culture medium was prepared at four salinities: 2, 8, 11 and 18 parts-per-thousand (ppt) (0.03, 0.14, 0.19 and 0.31 M, respectively) NaCl in filtered seawater (FSW) (pre-filtration Whatman GF/C, followed by 0.45 μm Durapore, Millipore). All materials were sterilised by autoclaving (Tomy, Quantum Scientific) and cultures were handled and inoculated aseptically in a laminar flow (AES Environmental Pty LTD fitted with HEPA filter). Replicate cultures (2 L, n = 3) of all species were inoculated at a dry-weight (DW) of ~100 mg L^{-1} for each salinity. Inoculation was carried out from salinity-specific, 6-month-acclimated mother cultures.

3.2.2 Culture growth

Culture growth was determined by measuring turbidity (OD_{750} nm)(Spectramax plus; Molecular Devices), correlated to cells L^{-1} and dry weights (DW) [g L^{-1}] following previously described methods (Section 2.2.2) (von Alvensleben *et al.*, 2013b). Turbidity calibration curves were medium-blanked for each salinity, while dry weight-samples were corrected for salt content using salinity-specific blanks. Results were correlated to generate linear equations ($R^2 > 0.95$) used to determine cell numbers and respective dry weights of each species at each salinity from turbidity measurements. Growth of all species was then determined every second day using turbidity for 20 days and obtained data were transformed to cell numbers and dry weights as described in von Alvensleben *et al.* (2013b). Volumetric biomass productivies (eq. 3.1) were determined using a method modified from Su *et al.* (2011).

$$\text{Volumetric Biomass Productivity [mg DW L}^{-1}\text{ day}^{-1}] = \frac{DW_2 - DW_1}{t_2 - t_1} \qquad \text{eq. 3.1}$$

Where DW_1 and DW_2=initial and final dry weight [g L^{-1}], respectively, t_1 and t_2=initial and final culture timepoints [days] per identified growth period, respectively.

3.2.3 Nutrient analyses

Medium nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3-}) concentrations were determined every second day. For this, 5 mL culture samples were centrifuged at 3000 g at 20 °C for 20 min.(Eppendorf 5810R, VWR), followed by 0.45 µm filtration (Durapore; Millipore Kilsyth) of the supernatant to remove any suspended material. From this, 1.25 mL was used for nitrate (NO_3^-), 1.25 mL for nitrite (NO_2^-) and 200 µL for

phosphate (PO_4^{3-}) determination. The phosphate assay required dilution with deionised water (DI) (Elix 5, Millipore) when PO_4^{3-} concentrations exceeded 2.5 mg L^{-1}, to ensure accuracy of the assay.

Nitrate and nitrite was determined spectrophotometrically (Spectramax Plus, Molecular Devices) in triplicate using a method adapted from Carvalho et al. (1998). The nitrite assay consisted of three reagents: Reagent A stock solution consisted of 13 g NH_4Cl and 1.7 g EDTA, pH 8.5 (adjusted with 28% NH_4Cl). This was diluted with DI water to 37 % of the original concentration to obtain reagent A working solution. The working solution of reagent B contained 5 g sulphanilamide dissolved in 300 mL of acidified DI water (5.29% HCl final concentration) before making the solution to 500 mL with DI water. The working solution of reagent C contained 500 mg of N-(1-naphthyl)-ethylenediamine (NED) (Sigma) 500 mL^{-1} DI water. All solutions were prepared in acid-washed (10% HCl) glassware. The nitrite assay was carried out in triplicate by adding 3.75 mL of working solution reagent A and 200 µL of working solution reagent B to 1.25 mL of centrifuged and filtered sample (see above). After 5 min, 200 µL of working solution reagent C was added. Absorbance of 250 µL sample was measured at 540 nm (Spectramax Plus, Molecular devices) in a 96-well plate, 20 min after the addition of working solution C.

Total nitrate + nitrite was measured in triplicate by adding 25 µL of 1 M HCl to 1.25 mL sample, followed by vortexing and immediate absorbance readings of 250 µL, at 220 nm in a 96-well plate format. Nitrite (λ = 540 nm) and nitrate + nitrite (λ = 220 nm) concentrations were calculated by comparison of absorbance's with serial-dilution linear regression calibration curves ($R^2 > 0.95$), generated previously from known nitrate and nitrite concentrations (0-32.21 µM and 0-43.38 µM sodium nitrate and

sodium nitrite, respectively). Nitrate concentrations were calculated by subtracting the nitrite concentration from the total nitrate + nitrite concentration.

Similarly, phosphate was determined spectrophotometrically (λ = 610 nm) (Spectramax Plus, Molecular Devices) in triplicate following standard methods adapted from Van Veldhoven and Mannaerts (1987) and R&D Systems, Malachite Green Phosphate Detection Kit (Cat. Number: DY996, www.RnDSystems.com). The assay consisted of two reagents: Reagent A contained 1.75 % (w/v) ammonium heptamolybdate x 4 H_2O in 6.3 N H_2SO_4 and reagent B contained 0.035 % (w/v) malachite green in DI water. For the assay, 40 µL of reagent A was added to 200 µL of filtered sample (see above) and, following incubation at room temperature for 10 min, 40 µL of reagent B was added. Samples were vortexed after addition of each reagent, then incubated at room temperature for an additional 20 min to allow complex formation between malachite green and phosphomolybdate formed in the first reaction. Absorbance of 70 µL was measured at 610 nm in a 96-well-plate. Phosphate concentrations were then calculated from serial-dilution linear regression calibration curves ($R^2 > 0.95$), generated previously from known phosphate concentrations (0-50 µM).

3.2.4 Biochemical analyses

Biomass samples for biochemical analyses were harvested from 500 mL samples by centrifugation as described in section 2.3.4 (von Alvensleben *et al.*, 2013b) when nitrate-replete during late-logarithmic growth (day 14 for *Desmodesmus armatus*, *Mesotaenium* sp. and *Tetraedron* sp. and day 12 for *S. quadricauda*) and four days after total nitrogen (N) depletion during the initial stationary phase (days 22-28 in

Desmodesmus armatus, days 20-24 in *Mesotaenium* sp., day 24 in *S. quadricauda* and days 24-30 in *Tetraedron* sp.). Cultures were classified as nutrient-replete and -deplete based on increasing and decreasing (NO_2^-) secretion patterns and nutrient depletion was assured by harvesting four days after medium nutrient depletion (Malerba *et al.*, 2012). Biomass pellets were freeze-dried (Virtis benchtop 2K, VWR) and stored in air-tight vials under nitrogen at 4 °C until further analysis.

3.2.4.1 Total lipid determination

Total lipids were determined gravimetrically following a direct extraction and transesterification method as described in detail in section 2.3.4.2 (von Alvensleben *et al.*, 2013a).

3.2.4.2 Fatty acid extraction, transesterification and analysis

Fatty acids were extracted from freeze-dried samples in a single-step extraction and transesterification procedure modified from Gosch *et al.* (2012) followed by GC-MS analysis as described in section 2.3.4.3 (von Alvensleben *et al.*, 2013a).

3.2.3.3 Amino acid analyses

Amino acid profiling of biomass from nutrient-replete and -deplete cultures at 2 and 11 ppt salinity was carried out by the Instrument Analysis Center of Shanghai Jiao Tong University using a Hitachi L8900 Amino Acid Analyzer (Tokyo, Japan) in accordance with the Chinese national standard protocol for amino acid determination (GB/T 5009.124-2003). Briefly, 10 mg samples were hydrolysed for 22 h at 110 ºC in a vacuum glass tube, using 6 M HCl. Samples were dried in a vacuum, and the residue

was diluted with 0.02 M HCl prior to analysis on the amino acid analyser, together with amino acid standards. Alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine were quantified. The sum of these amino acids is considered total protein throughout this chapter.

3.2.4.4 Carbohydrate determination

Total carbohydrate content was determined by difference, containing also dietary fibre and some organic acids, following the method by Sims (1978) using equation 3.2. For this, ash (dry inorganic) content (mg g^{-1} DW) was determined by combustion at 500 °C for 24 h in a muffle furnace (Yokogawa-UP 150, AS1044).

$$\text{Carbohydrate (\% wt)} = 100\ \% - (\%\ \text{Ash} + \%\ \text{Total lipids} + \%\ \text{Protein}) \quad \text{eq. 3.2}$$

3.2.5 Total lipid and FA productivities

Total fatty acid (TFA) productivities were determined using equation 3, where TFA_1 was determined in nutrient-replete conditions and TFA_2 in nutrient-deplete conditions, and t_1 and t_2 represent the -replete and -deplete harvest time points for FA determination, respectively.

$$\text{FA Productivity}\ [\text{mg L}^{-1}\ \text{day}^{-1}] = \frac{\text{TFA}_2 - \text{TFA}_1}{t_2 - t_1} \quad \text{eq. 3.3}$$

3.2.6 Statistical analyses

All statistical analyses were carried out in Statistica 12 (StatSoft Pty Ltd.). Repeated measures ANOVAs were used to determine the effects of salinity on culture growth and biomass productivity, and the effects of both salinity and nutrient status on biochemical profiles within- and between-species. One-way ANOVAs were used to clarify single variable effects generally within species across salinities. Tukey's post-hoc tests were used to determine significant differences assigned at $p< 0.05$. Homogeneity of variances and normality assumptions were verified using Levene's tests. Non-metric multidimensional scaling (nMDS, Primer 6) (Clarke, 1993) was used to evaluate patterns in fatty acid and amino acids quality (% of total FA or AA) relative to salinity and nutrient status. Increasing distances between points in the ordination plot represent decreasing similarity between those points. The Bray-Curtis similarity coefficient was used as a distance measure, derived from square root transformed data of individual fatty acid proportions (mean of replicates, n=3). Pearson product correlations for the relative proportions of the individual FAs or groups of FAs (>0.8) are shown in the vector loading plot. The length and direction of the vectors indicate the strength of the correlation and direction of change between the two axes, i.e. the relative differences in specific fatty acids between the demarcated groups.

3.3 Results

3.3.1 Effect of salinity on growth

Lower salinities (2, 8 and 11 ppt) had no effect on growth of *Desmodesmus armatus*, *Scenedesmus quadricauda* or *Tetraedron* sp., however, 18 ppt significantly

reduced or completely inhibited growth in all three species (Figure 3.1) (repeated-measures ANOVA: *D. armatus*; $F_{(1,3)}$=95.5, P<0.05, *S. quadricauda;* $F_{(1,3)}$=179.5, P<0.05 *Tetraedron* sp. $F_{(1,3)}$=44.3, P<0.05). In *Mesotaenium* sp., cultures at 2 and 8 ppt reached similar final biomass content (~1.1 g L^{-1}) but there was a significant decrease in biomass production with increasing salinity at 11 (0.8 g L^{-1}) and 18 ppt (0.2 g L^{-1}) (repeated-measures ANOVA, $F_{(1,3)}$=1549.4, P<0.05) (Figure 3.1).

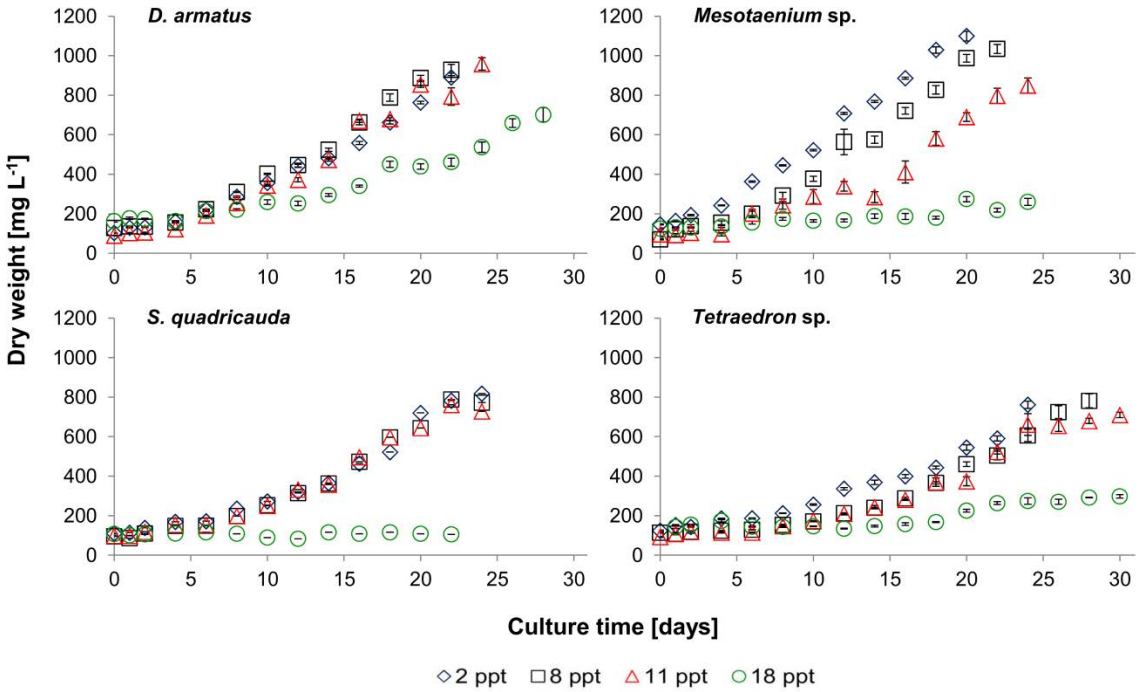

Figure 3.1. Mean biomass growth [mg DW L^{-1}] of *Desmodesmus armatus*, *Mesotaenium* sp., *Scenedesmus quadricauda* and *Tetraedron* sp. at 2, 8, 11, 18 ppt determined using % transmittance at 750 nm. n=3. Standard error is shown. DW: Dry weight.

At 2 and 8 ppt, highest final biomass density was achieved by *Mesotaenium* sp., > 1000 mg L^{-1}, whereas all other species reached between 650-950 mg L^{-1} (Figure 3.1). At 11 ppt, biomass density was highest for *D. armatus* and *Mesotaenium* sp. (~930 and 850 mg L^{-1}, respectively). With similar growth patterns from 2 to 11 ppt, *S. quadricauda* and *Tetraedron* sp. reached the lowest biomass density (730-770 and

710-760 mg L^{-1}, respectively) (Figure 3.1). At 18 ppt, *D. armatus* exhibited the highest growth reaching ~700 mg L^{-1} (Figure 3.1), whereas *Mesotaenium* sp. and *Tetraedron* sp. cultures showed minimal growth (260-300 mg L^{-1}) and *S. quadricauda* growth was completely inhibited, producing insufficient biomass for biochemical composition analysis at this salinity.

With the exception of *Mesotaenium* sp., salinity had no significant effect on biomass productivity [mg L^{-1} day^{-1}] from 2 ppt to 11 ppt for each species (repeated-measures ANOVA, $F_{(1, 2)}$=0.4, p=0.6) (Figure 3.2) but was significantly lower at 18 ppt in all species (repeated-measures ANOVA, $F_{(1, 3)}$=90.2, p<0.05) (Figure 3.2).

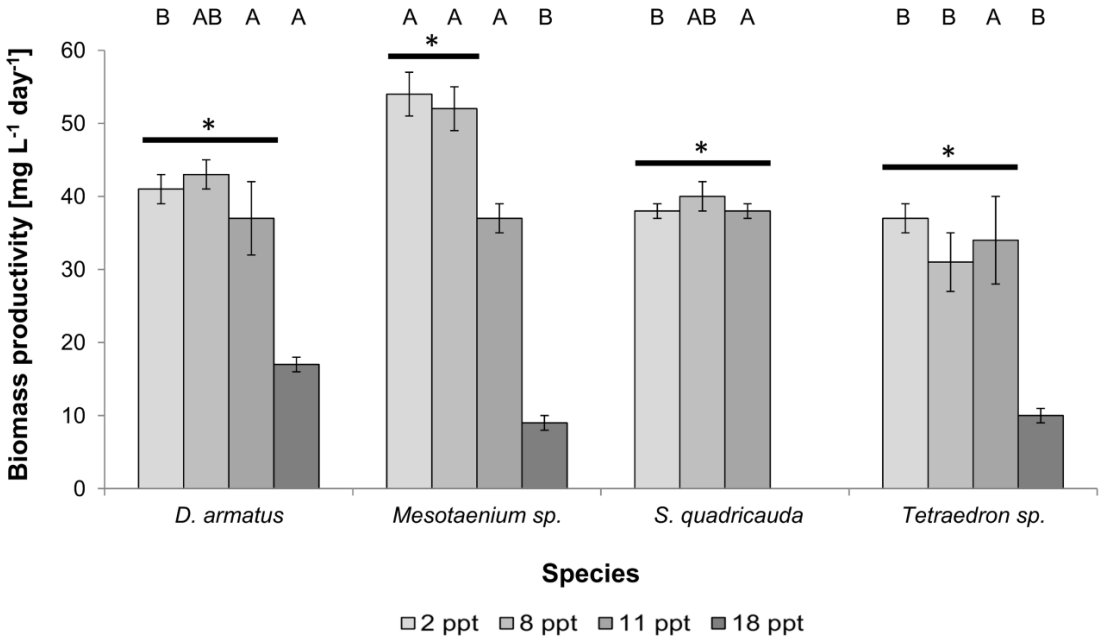

Figure 3.2. Biomass productivity [mg L^{-1} day^{-1}] of *Desmodesmus armatus, Mesotaenium* sp., *Scenedesmus quadricauda* and *Tetraedron* sp. n=3. Standard error is shown. Statistical relations within and between species are shown.

Mesotaenium sp. showed similar productivities at 2 and 8 ppt, but a significant decrease (one-way ANOVA, $F=_{(1, 3)}$=88.8, p<0.05) at both 11 and 18 ppt. Between

species, *Mesotaenium* sp. cultured at 2 and 8 ppt had the highest biomass productivity (52-54 mg L^{-1} day^{-1}) (repeated-measures ANOVA, $F_{(1, 9)} = 9$, $p<0.05$) (Figure 3.2). Productivities were similar (31-43 mg L^{-1} day^{-1}) in *D. armatus* and *S. quadricauda* cultures from 2-11 ppt, as well as *Mesotaenium* sp. at 11 ppt and *Tetraedron* sp. at 2 ppt. Overall, *Tetraedron* sp. had the lowest biomass productivity at salinities above 2 ppt (10-31 mg L^{-1} day^{-1}). At 18 ppt, *D. armatus* showed the highest productivity (17 mg L^{-1} day^{-1}) followed by *Tetraedron* sp. and *Mesotaenium* sp. with minimal productivity (9-10 mg L^{-1} day^{-1}) (Figure 3.2).

3.3.2 Nutrient dynamics

Within species, the highest total N uptake was always at 2 ppt (1.5 - 1.9 mg L^{-1} day^{-1}) (Table 3.1). Total N uptake was lower but not significantly different in cultures at 8 and 11 ppt in *D. armatus*, *Mesotaenium* sp. and *S. quadricauda* (1.4 - 1.5 mg L^{-1} day^{-1}) (repeated-measures ANOVA, $F=_{(1,2)}=2.7$, $p=0.1$) (Table 3.1). *Tetraedron* sp. showed a significantly lower intermediate uptake at 8 and 11 ppt (one-way ANOVA, $F=_{(1,3)}=27.3$, $p< 0.05$) (~1.1 mg L^{-1} day^{-1}). Between species, the highest total N uptake was observed in *S. quadricauda* and *Tetraedron* sp. cultures at 2 ppt (~1.9 mg L^{-1} day^{-1}), followed by *Mesotaenium* sp. (1.6 mg L^{-1} day^{-1}), then *D. armatus* (1.5 mg L^{-1} day^{-1}) (Table 3.1). Salinity had no significant effect on phosphate uptake in *D. armatus* (0.59-0.64 mg L^{-1} day^{-1}) (one-way ANOVA, $F_{(1,3)}=0.02$, $p=0.9$), *S. quadricauda* (1.5-2.1 mg L^{-1} day^{-1}) (one-way ANOVA, $F_{(1,2)}=1.7$, $p=0.2$) or *Tetraedron* sp. (0.36-0.57 mg L^{-1} day^{-1}) (one-way ANOVA $F_{(1,3)}=1.8$, $p = 0.2$).

Table 3.1. Total N (nitrate uptake corrected for nitrite secretion) and phosphate uptake rates [mg L^{-1} day^{-1}] of *Desmodesmus armatus*, *Mesotaenium* sp., *Scenedesmus quadricauda* and *Tetraedron* sp. n=3. Standard error is shown. Statistical relations within and between species are shown.

Species	Salinity [ppt]	Total N uptake rate [mg L^{-1} day^{-1}]	Statistical relation within species	Statistical relation between species	Phosphate uptake rate [mg L^{-1} day^{-1}]	Statistical relation within species	Statistical relation between species
D. armatus	2	1.50 ± 0.03	a	A	0.59 ± 0.3	a	A
	8	1.49 ± 0.05	a	A	0.62 ± 0.3	a	A
	11	1.39 ± 0.02	a	A	0.64 ± 0.3	a	A
	18	0.46 ± 0.03	b	A	0.59 ± 0.3	a	A
Mesotaenium sp.	2	1.64 ± 0.01	a	AB	2.19 ± 0.1	a	B
	8	1.38 ± 0.13	a	AB	0.75 ± 0.3	b	A
	11	1.31 ± 0.17	a	A	0.46 ± 0.1	b	AB
	18	0.75 ± 0.04	b	A	0.34 ± 0.1	b	AB
S. quadricauda	2	1.88 ± 0.22	a	AB	2.13 ± 0.6	a	B
	8	1.57 ± 0.20	a	AB	1.54 ± 0.5	a	B
	11	1.41 ± 0.08	a	A	1.50 ± 0.03	a	C
	18	-	-	-	-	-	-
Tetraedron sp.	2	1.85 ± 0.06	a	B	0.57 ± 0.1	a	A
	8	1.14 ± 0.03	b	B	0.40 ± 0.05	b	C
	11	1.17 ± 0.05	b	A	0.49 ± 0.1	ab	B
	18	0.40 ± 0.02	c	A	0.36 ± 0.2	b	B

In *Mesotaenium* sp., phosphate uptake was significantly higher at 2 ppt (~2.3 mg L^{-1} day^{-1}) (one-way ANOVA, $F_{(1,3)}$=49.1, P < 0.05), but not significantly different between cultures from 8 to 18 ppt (0.34-0.75 mg L^{-1} day^{-1}) (main-effects ANOVA, $F_{(1,2)}$=3.4, p=0.06) (Table 3.1). Between species, phosphate uptake was highest in *Mesotaenium* sp. at 2 ppt (2.2 mg L^{-1} day^{-1}) and *S. quadricauda* (1.5-2.1 mg L^{-1} day^{-1}) at all salinities (repeated-measures ANOVA, $F_{(1,3)}$=69.8, P < 0.05), requiring re-fertilisation every second day.

Correlating biomass productivity [mg L^{-1} day^{-1}] and total N and P uptake rates [mg L^{-1} day^{-1}] (Table 3.2) showed that, despite differences in uptake rates, these were closely correlated with biomass productivity resulting in similar N uptake per mg g^{-1} DW across all salinities within species.

Table 3.2. Nutrient consumption per unit biomass and protein.

Species	Salinity [ppt]	N uptake per mg biomass [mg N/mg DW L^{-1} day^{-1}]	PO_4^{3-} uptake per mg biomass [mg P/mg DW L^{-1} day^{-1}]	N uptake per mg protein [mg N/mg DW L^{-1} day^{-1}]
D. armatus	2	0.04	0.01	0.081
	8	0.03	0.01	-
	11	0.04	0.02	0.122
	18	0.03	0.03	-
Mesotaenium sp.	2	0.03	0.04	0.116
	8	0.03	0.01	-
	11	0.04	0.01	0.175
	18	0.08	0.04	-
S. quadricauda	2	0.05	0.06	0.128
	8	0.04	0.04	-
	11	0.04	0.04	0.141
	18	-	-	-
Tetraedron sp.	2	0.05	0.02	0.138
	8	0.04	0.01	-
	11	0.03	0.01	0.111
	18	0.04	0.04	-

The highest total N uptake per unit biomass was observed in *S. quadricauda* and *Tetraedron* sp. at 2 ppt (~0.05 mg N/mg DW L^{-1} day^{-1}) (Table 3.2). Phosphate uptake per unit biomass within species was generally lower at 2 ppt (except in *Mesotaenium* sp. and *S. quadricauda*) and increased at higher salinities. The highest phosphate uptake per unit biomass was observed in *S. quadricauda* at 2 ppt (~0.06 mg N/mg DW L^{-1} day^{-1}) (Table 3.2).

3.3.3 Biochemical composition

3.3.3.1 Total lipid and total fatty acid content

Total lipid content was 50 % higher than total fatty acid content, which is indicative of a large contribution of other lipid soluble compounds, particularly pigments, to the total lipid fraction (Lim *et al.*, 2012; von Alvensleben *et al.*, 2013b). Salinity had minor effects on total lipid content in nutrient-replete conditions, with *Mesotaenium* sp. showing the highest total lipid content (20-25 % of DW) and *Tetraedron* sp. the lowest (14-18 % of DW) (Figure 3.3). Nutrient depletion induced an increase of total lipid with increasing salinity in *S. quadricauda* (repeated-measures ANOVA, $F_{(1, 2)}$= 14.3, p= 0.03) and *Tetraedron* sp. (~5-10 % increase) (repeated-measures ANOVA, $F_{(1, 3)}$= 0.161, p=0.9) whereas nutrient depletion had no effect on total lipid content in *D. armatus* (repeated-measures ANOVA, $F_{(1,3)}$=1.1, p=0.4) and *Mesotaenium* sp. cultures ($F_{(1,3)}$=0.9, p=0.5) (Figure 3.3).

Salinity did, however, have a significant effect on TFA contents in all species in both nutrient-replete and -deplete conditions (species- and nutrient-specific one-way ANOVAs, P<0.05), except for *S. quadricauda* in -replete conditions (one-way ANOVAs, $F_{(1, 2)}$=2.2, p=0.2) (Figure 3.3). These significant effects were mainly driven by the small

variance between replicates. In nutrient-replete conditions, TFA content decreased with increasing salinity in *D. armatus* from 2 to 18 ppt (~2 % decrease) (one-way ANOVA, $F_{(1, 3)}$=71.6, p<0.05), *Mesotaenium* sp. (~1 % decrease) (one-way ANOVA, $F_{(1, 3)}$=204.9, p<0.05) and *S. quadricauda* (~0.6 % decrease) (one-way ANOVA, $F_{(1, 2)}$=2.2, p=0.2) from 2 to 11 ppt. In nutrient-replete *Tetraedron* sp. cultures, TFA content increased at 8 and 11 ppt (~6.5%). In all species, nutrient-replete cultures at 18 ppt contained the lowest TFA, except for *Mesotaenium* sp. which contained its highest TFA content (12-13% of DW) at this salinity.

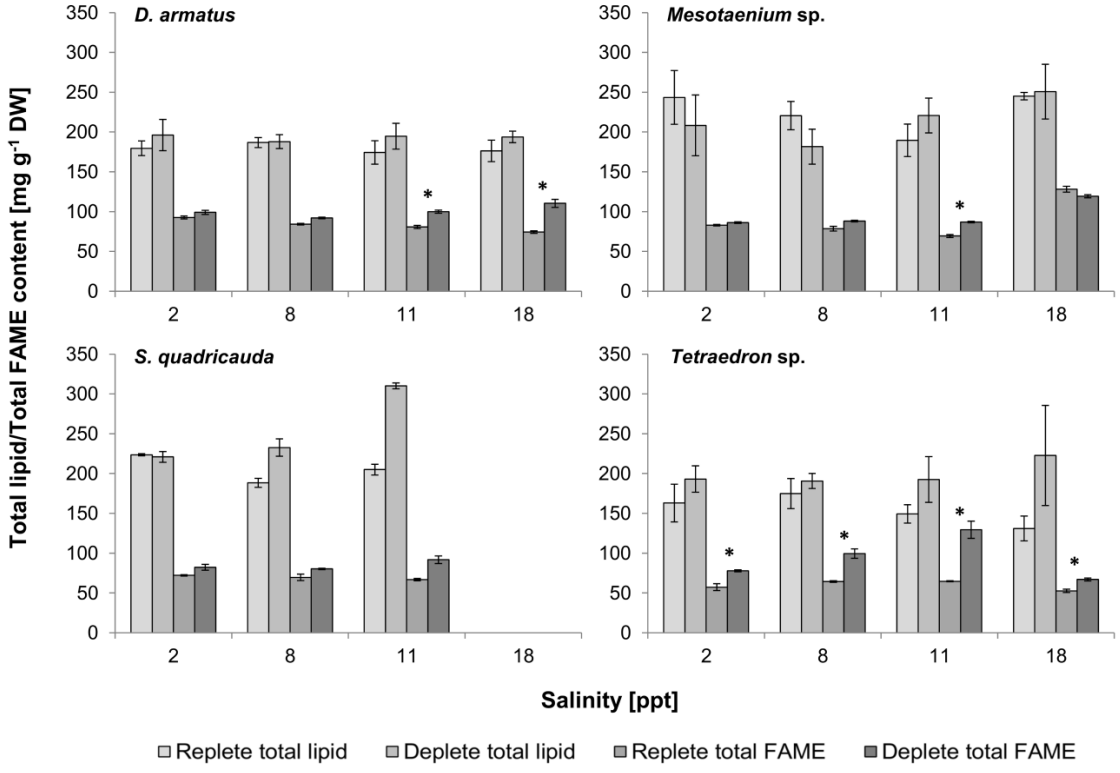

Figure 3.3. Total lipid and total FA contents [mg L^{-1}] of *Desmodesmus armatus*, *Mesotaenium* sp., *Scenedesmus quadricauda* and *Tetraedron* sp. in nutrient-replete and deplete conditions. n=3. Standard error is shown.

Nutrient depletion had a small but significant effect on TFA driving an increase (1-6 % increase) in TFA content in all species (repeated-measures ANOVAs, *D. armatus*:

$F_{(1, 1)}$=289, p<0.05, *Mesotaenium* sp.: $F_{(1, 1)}$=15, p<0.05, *S. quadricauda*: $F_{(1, 1)}$=58.8, p<0.05, *Tetraedron* sp.: $F_{(1, 3)}$=323.8, p<0.05) at all salinities, except in *Mesotaenium* sp. at 18 ppt where a decrease (1 %) was observed. Between species, TFA content was highest in *D. armatus* and *Mesotaenium* sp. (7-13 % of DW) and lowest in *S. quadricauda* and *Tetraedron* sp. (5-7 % of DW) (Figure 3.3).

3.3.3.2 Fatty acid profiles

The most abundant (> 5mg g^{-1} DW) fatty acids present in all species were C16:0, C18:1, C16:4, C18:2 and C18:3 (α-linolenic acid), except in *Mesotaenium* sp., which did not contain C16:4, but had high amounts of C16:2 (Supplementary table S3.2). Excluding cultures at 18 ppt, *Mesotaenium* sp. contained the highest C16:0 (15-35 mg g^{-1} DW) and C18:2 concentrations (10-35 mg g^{-1} DW), and *D. armatus* the highest C16:4 (9-15 mg g^{-1} DW) and C18:3 (α) (20-29 mg g^{-1} DW) concentrations. MDS analyses (Figure 3.4) showed distinct groupings of biomass samples based on their fatty acid profiles. In *D. armatus* (Figure 3.4A, stress 0.01), nutrient-replete cultures from 2 to 11 ppt and -deplete cultures at 2 ppt grouped together and were characterised by high proportions of omega-3 (ω-3) (52-56 %) and polyunsaturated fatty acids (PUFA) (~70 %, mainly C16:4 and C18:3) as indicated by the vector loadings to the top right of the ordination plot. Nutrient-replete cultures at 18 ppt had similar profiles to -deplete cultures at 8 and 11 ppt, and were characterised by higher proportions of omega-6 (ω-6) fatty acids (~18 %) (Pearson product correlation > 0.5, data not shown). Actual TFA concentrations (mg g^{-1} DW) for all species and treatments are listed in Supplementary tables S3.1-3.4. *Mesotaenium* sp. grouped by salinity rather than nutrient status (Figure 3.4B, stress 0). Cultures at 2 ppt were characterised

by a higher proportion of ω-3 (~30 %, mainly C18:3) whereas biomass from 8 and 11 ppt had a higher proportion of total PUFA (53-66 %, mainly C18:3) and ω-6 contents (43-53 %, mainly C20:3). The MDS of fatty acid profiles from *S. quadricauda* yielded groups strongly separated by culture nutrient status (Figure 3.4C, stress 0), with a higher proportion of ω-3 (~54 %, mainly C16:4 and C18:3) and PUFA (~67 %) in -replete cultures and an increase in proportion of ω-6 (3 % increase, mainly driven by an increase in C18:2) fatty acids in -deplete cultures.

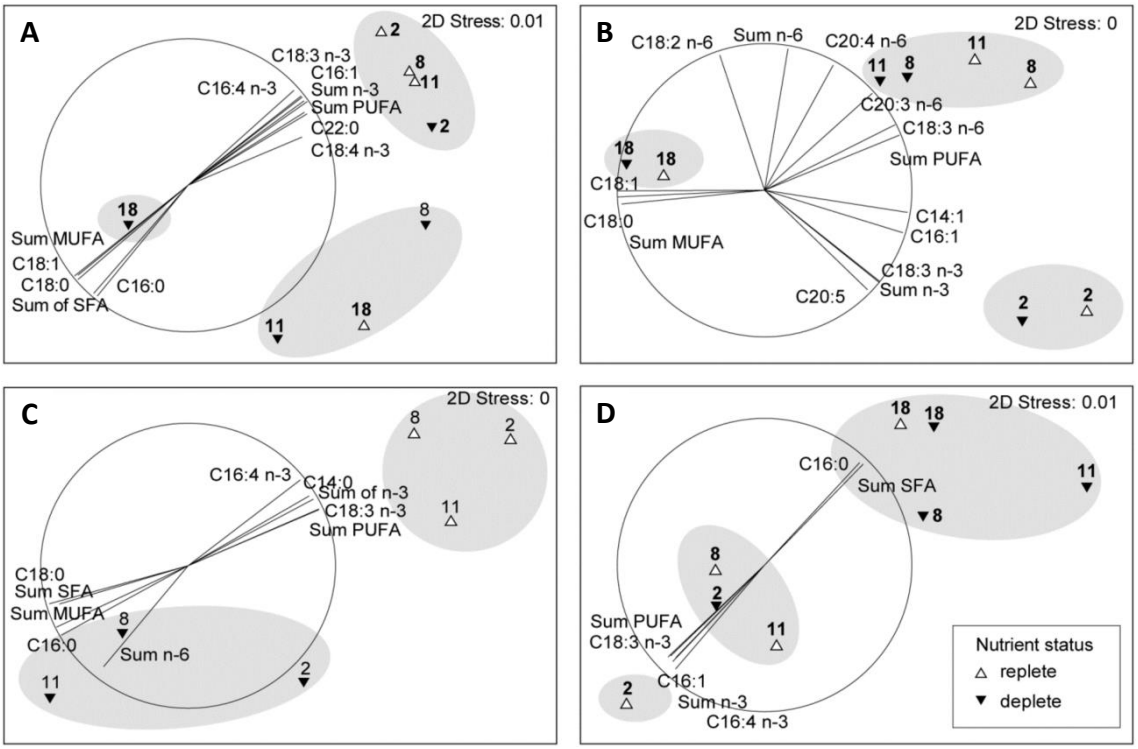

Figure 3.4. Fatty acid quality of *Desmodesmus armatus* (A), *Mesotaenium* sp. (B), *S. quadricauda* (C) and *Tetraedron* sp. (D) at different salinities and nutrient status. Multidimensional scaling (MDS) ordinations show clusters of samples based on the similarity of their fatty acid profiles (% of total FA). The vector loadings of the specific fatty acids relate to the clusters of samples in the ordination plots (correlations of R>0.8). The direction and size of each vector indicates the relative abundance of that variable in samples in the corresponding section of the ordination plot.

In *Tetraedron* sp., fatty acid profiles were affected by both nutrient status and salinity. Replete cultures at 2 ppt showed distinctly different profiles to all other

cultures (Figure 3.4D, stress 0.01) with the highest proportions of PUFA (57-64 %), lower proportions of saturated fatty acids (SFA) (~20 %) and monounsaturated fatty acids (MUFA) (~12 %) and the highest total ω-3 content (~52 %). Cultures at 8 and 11 ppt were most affected by nutrient depletion as indicated by the large distance between the points in the ordination plot, with an increase in proportion of MUFA contents (15-20 %, mainly C18:1) and decrease in PUFA contents (15-30 %, mainly C16:4 and C18:3). Cultures at 18 ppt were least affected by nutrient status with nutrient-replete and -deplete cultures grouping close together with similar FA proportions in both conditions.

3.3.3.3 Lipid and fatty acid productivities

Due to high biomass productivity (Figure 3.2), *Mesotaenium* sp. at 2 and 8 ppt showed the highest total lipid (11-13 mg L^{-1} day^{-1}) and total fatty acid productivities (4-4.5 mg L^{-1} day^{-1}) (Supplementary table S3.5). Nutrient-replete fatty acid productivities were highest from 2 to 11 ppt in all species. Fatty acid productivities were mainly driven by biomass productivities, with the exception of high productivities of C18:1 in *Mesotaenium* sp. at 18 ppt and C18:2 in *Tetraedon* sp. at 11 ppt which were driven by a high FA content rather than biomass productivity (Supplementary table S3.6). *Mesotaenium* sp. at all salinities had the highest C16:0, C16:2 and C18:2 productivities and *D. armatus* had the highest C16:4 productivity (Supplementary table S3.6). The highest C18:1 productivity was observed in *Tetraedron* sp. at 11 and *Mesotaenium* sp. at 18 ppt., while the highest C18:3 productivity occurred for *D. armatus* and *Mesotaenium* sp. at 2 ppt (Supplementary table S3.6).

3.3.3.4 Amino acid contents

Total protein content (sum of AA) and essential AA (EAA) content was highest in *D. armatus* (309-450 mg g^{-1} DW and 150-233 mg g^{-1} DW, respectively) and lowest in *Mesotaenium* sp. (179-260 mg g^{-1} DW, 92-135 mg g^{-1} DW, respectively) (Table 3.3). Except for *Mesotaenium* sp. and *Tetraedron* sp. at 2ppt and *S. quadricauda* at 11 ppt, nutrient depletion lead to a decrease in AA in all cultures.

With the exception of *Tetraedron* sp., AA contents at 11 ppt were always lower than at 2 ppt in both nutrient-replete and -deplete conditions. Although AA content decreased with nutrient depletion, proportions of total AA content (%) remained similar, maintaining a similar profile regardless of conditions. In all species, the predominant EAAs were histidine and leucine with the highest concentrations (34-64 mg g^{-1} DW and 21-39 mg g^{-1} DW, respectively), whereas methionine and arginine contents were lowest (5-9 mg g^{-1} DW and 5-10 mg g^{-1} DW, respectively). More specifically, the highest histidine contents (64 mg g^{-1} DW) were observed in *D. armatus* and *Tetraedron* sp. and the highest leucine contents (39 mg g^{-1} DW) in *D. armatus*. Lysine contents were highest in *D. armatus* and *S. quadricauda* (27 mg g^{-1} DW) (For detailed amino acid profiles, see Supplementary tables S3.7-3.10).

3.3.3.5 Carbohydrate contents

Carbohydrate content was highest in *Tetraedron* sp. (393-465 mg g^{-1} DW) and lowest in *S. quadricauda* (263-367 mg g^{-1} DW) (Table 3.3). At 2 ppt, nutrient depletion induced a carbohydrate content increase (20-50 mg g^{-1} DW) in all species. At 11 ppt, carbohydrate contents were higher than at 2 ppt in all species when nutrient-replete,

Table 3.3. Total amino acid, essential amino acid and carbohydrate contents [mg g^{-1} DW] of *Desmodesmus armatus*, *Mesotaenium* sp., *Scenedesmus quadricauda* and *Tetraedron* sp. at 2 and 11 ppt in nutrient-replete and deplete conditions.

Species	Total AA content [mg g^{-1} DW]				Total essential AA content [mg g^{-1} DW]				Carbohydrate content [mg g^{-1} DW]			
	2 ppt		11 ppt		2 ppt		11 ppt		2 ppt		11 ppt	
	Replete	Deplete	Replete	Deplete	Replete	Deplete	Replete	Deplete	Replete	Deplete	Replete	Deplete
D. armatus	451.2	402.4	309.3	297.3	233.7	204.8	160.6	152.7	305.2	355.6	405.5	403.3
Mesotaenium sp.	261.1	242.5	202.5	179.3	135.4	124.1	106.3	92.0	375.5	429.0	404.7	406.1
S. quadricauda	388.2	369.8	262.4	278.5	201.5	190.2	133.6	141.7	263.4	319.3	367.3	294.3
Tetraedron sp.	362.8	309.2	309.4	215.2	189.5	160.4	159.1	109.6	393.1	414.4	421.5	465.0

- however nutrient depletion only induced a further increase in *Tetraedron* sp. (~40 mg g^{-1} DW).

3.4 Discussion

This chapter identified species-specific effects of both salinity and nutrient status on growth and biochemical profiles of *Desmodesmus armatus*, *Mesotaenium* sp., *Scenedesmus quadricauda* and *Tetraedron* sp.

3.4.1 Growth

Despite being freshwater species, this study showed that *D. armatus*, *S. quadricauda* and *Tetraedron* sp. are relatively halotolerant, with similar growth up to 11 ppt, whereas *Mesotaenium* sp. has a lower salinity tolerance with optimal growth up to 8 ppt. Growth responses to salinity have implications for on-site cultivation of these four microalgal species. Although these species tolerated 18 ppt salinity to varying degrees, large-scale cultivation at this salinity will not be viable. Allowing for salinity increases due to evaporation, only *D. armatus* can potentially be cultured up to 11 ppt. *Scenedesmus quadricau*da and *Tetraedron* sp. should ideally be grown between 2 and 8 ppt, and *Mesotaenium* sp. at 2 to potentially 5 ppt, reducing the evaporation margin, after which growth will be compromised. For example, considering average tropical East Queensland evaporation rates (~5 % daily), these findings imply that *D. armatus*, *S. quadricauda* and *Tetraedron* sp. could be cultured in saline groundwater around 5 ppt with daily water replacements for up to ~24 days (~11 ppt) without adverse effects of salinity. In contrast, *Mesotaenium* sp. in these conditions would be affected by salinity after ~12 days (~8 ppt), consequently

requiring complete water replacement every 12 days, which has serious environmental implications depending on water availability at cultivation sites.

Biomass production of *D. armatus*, *Mesotaenium* sp. and *Tetraedron* sp. in this study are difficult to compare due to limited or absence of reports on growth patterns for these species. In general, biomass production was lower than in previous reports for *S. quadricauda* and other chlorophytes being examined for biotechnological potential (Dickinson *et al.*, 2013; Patil, 1991; Tiftickjian *et al.*, 1986; Zhou *et al.*, 2011). Lower biomass content was likely due to light limitation as the current study was performed under controlled laboratory conditions with an average light intensity of 40 μmol m^{-2} s^{-1} compared to 100-200 μmol m^{-2} s^{-1} (Dickinson *et al.*, 2013; Tiftickjian *et al.*, 1986) or natural daylight (Patil, 1991). This is of little concern as the aim was to identify suitably halotolerant freshwater species, and culture conditions were not optimised to maximize productivity. Furthermore, large-scale cultivation at high population densities is likely to reduce penetrating light intensities to similar levels.

The observed patterns of decreasing growth with increasing salinity are to be expected for freshwater microalgal species, as increasing culture salinity (mainly Na^+ and Cl^-) may lead to an over-production of reactive oxygen species which cause oxidative stress, enzyme inactivation and reduction of photosynthetic rates (Mahajan *et al.*, 2005; Sudhir *et al.*, 2004), but also cellular ionic imbalance and subsequent water loss (Erdmann *et al.*, 2001; Setter *et al.*, 1979). Acclimation to high salinities includes 3 processes: (i) restoration and maintenance of cell turgor and volume, (ii) changes in permeability of the cell membrane and regulated uptake (K^+) and expulsion (Na^+) of ions, and (iii) the accumulation of osmoprotectant compatible solutes and stress proteins (Brown, 1976; Erdmann *et al.*, 2001). Approximately 20 different

compatible solutes have been shown to occur in microalgae with variable degrees of osmoprotection and salinity compensation (Erdmann *et al.*, 2001). This has implications for growth, which will decrease, if ATP utilization is predominantly for osmotic regulation and/or nitrogen taken up is utilized for N- based osmoregulatory solute synthesis (e.g. proline, glycine betaine) (Erdmann *et al.*, 2001; Vanlerberghe *et al.*, 1987).

Nutrient dynamics, specifically nitrogen fluxes, can provide insight into possible osmoregulatory mechanisms, particularly when considered in combination with changes in biochemical profiles. In this study, the similarities in N uptake per unit biomass between salinities and the decrease of amino acid contents, specifically proline and glycine (Supplementary tables S3.7-3.10) with increasing salinity in all species indicate that these species are unlikely to produce N-containing osmolytes reported in microalgae (glycine betaine and/or proline). Although not specifically studied here, we hypothesise that osmoregulation in these species could be achieved *via* accumulation of carbohydrates which has been reported for other chlorophyte species e.g. *Chlamydomonas* sp., *Chlorella emersonii*, *Dunaliella* sp. and *Stichococcus bacilaris* (Benamotz *et al.*, 1983; Erdmann *et al.*, 2001). This is supported by increased carbohydrate contents with increasing salinity under nutrient-replete conditions.

For remediation/nutrient provision purposes, *S. quadricauda* and *Tetraedron* sp. at 2 ppt had the highest total N-uptake, and *Mesotaenium* sp. and *S. quadricauda* had the highest phosphate uptake. This has dual implications depending on cultivation site, where, if nutrients have to be added at a cost, strain selection should be towards low nutrient consumption species (e.g. *D. armatus*), and, if nutrient-rich wastewater is available, high nutrient uptake species (e.g. *Mesotaenium* sp. and *S. quadricauda*)

should be selected. The faster phosphate uptake in *Mesotaenium* sp. and *S. quadricauda* compared to the other species could be due to higher biomass production, specifically in *Mesotaenium* sp. or an indication of storage capacity by these species. Microalgae can store phosphate as polyphosphate for later use when external phosphate becomes limiting (Powell *et al.*, 2009). Excessive uptake and polyphosphate storage is either a consequence of nutrient starvation followed by re-exposure (Aitchison *et al.*, 1973), or 'luxury uptake', which does not require prior nutrient starvation (Eixler *et al.*, 2006). In this instance, 'luxury uptake' is most likely, as cultures were maintained nutrient-replete until intentional depletion.

Mesotaenium sp. at low salinities (2 to 8 ppt) had the highest biomass, lipid and FAME productivities using the least nutrients, making it the most suitable species for cultivation when fertilisation incurs a cost. For wastewater remediation of nitrate and phosphate, *S. quadricauda* is the most suitable species showing the highest removal rates of these nutrients. These findings correlate with previous reports that have identified *S. quadricauda* as an effective species for nutrient-rich wastewater remediation (Dickinson *et al.*, 2013; Martínez *et al.*, 2000; Shi *et al.*, 2007).

3.4.2 Biochemical profiles

Total lipid contents were at the lower end of the range reported for other green algae, but correspond to previous findings for a number of chlorophyte species (Griffiths *et al.*, 2009) (See comparisons in Table 3.4).

An increase in total lipid and FAs in microalgae following nutrient depletion has been shown in previous studies e.g. *Chlorella vulgaris* (Converti *et al.*, 2009), *Scenedesmus subspicatus* (Dean *et al.*, 2010) and is often used for large-scale

microalgal culture manipulations (Sharma *et al.*, 2012). However, this study shows that the degree of this effect varies between species and salinity conditions. In *D. armatus* and *Mesotaenium* sp. neither salinity nor nutrient depletion had any effects on total lipid content, whereas in *S. quadricauda* and *Tetraedron* sp. nutrient depletion only had significant effects at higher salinities from 8 ppt and 11 ppt, respectively. This shows that nutrient depletion and/or salinity stress are not universally effective lipid induction methods, supporting previous studies using *Chlorella sorokiniana* (Griffiths *et al.*, 2009), *Chlorella* sp., *Scenedesmus* sp. (Rodolfi *et al.*, 2009), *Tetraselmis* sp. and *Nannochloris* sp. (Reitan *et al.*, 1994).

N-limitation under continued photosynthetic carbon acquisition leads to diversion of carbon from growth to storage (Becker, 1994; Rodolfi *et al.*, 2009), as production of N-containing compounds such as proteins, nucleic acids and chlorophylls is inhibited, therefore resulting in reduced growth and biomass productivity. Although nutrient depletion for four days increased total fatty acid content and was statistically significant in all species, differences were small mainly driven by the small variance between replicate fatty acid samples. As such, larger nutrient depletion periods would need to be applied which cannot be recommended as a means to favourably alter the biochemical profiles and productivity of these species due to impacts on cultivation footprints required to sustain biomass yields.

While FA comparisons between studies show a degree of variability, generally due to differences in culture conditions, FA profiles here were similar to those of other chlorophytes, with C16:0, C16:2, C18:1, C18:2 and C18:3 being the predominant fatty acids (Dunstan *et al.*, 1992). More specifically, *D. armatus*, *Mesotaenium* sp. and

Table 3.4. Biomass productivity and biochemical content comparison between species isolated from the tailings-dam of a Queensland coal-fired power station (this study) and published data.

Species	Growth Productivity [mg L^{-1} day^{-1}]	Lipid content [% of DW]	FAME content [% of DW]	Total AA content [% of DW]	Carbohydrate content [% of DW]	References
D. armatus	43	17-20	7-11	16-45	30-40	This study
Mesotaenium sp.	54	18-25	7-12	9-26	38-43	This study
S. quadricauda	40	18-31	7-9	13-38	26-37	This study
Tetraedron sp.	37	13-22	5-12	11-36	39-47	This study
Chlorella sp.	170-230	18-19				(Rodolfi *et al.*, 2009)
Chlorella sp.		11-18		15-25	6-16	(Brown *et al.*, 1992)
Chlorella sp.		12-35		8-14	30-50	(Laurens *et al.*, 2014)
Chlorella pyrenoidosa		2			26	(Becker, 2007)
Chlorella vulgaris		14-22			12-17	(Becker, 2007)
Haematococcus pluvialis			50		40	(Recht *et al.*, 2012)
Isochrysis sp.		25-30	4-7	Protein: 36-38	10-12	(Renaud *et al.*, 1994)
Nannochloropsis sp.		15-55		10-21	10-20	(Laurens *et al.*, 2014)
Nannochloropsis sp.			15- 50		20	(Recht *et al.*, 2012)
Nannochloropsis sp.			13-35			(Pal *et al.*, 2011)
Nannochloropsis sp.		7-17		17-22	5-9	(Volkman *et al.*, 1993)
Nannochloropsis oculata		28-33	8-20	Protein: 48-50	6-8	(Renaud *et al.*, 1994)
Scendesmus sp.	190-260	18-21				(Rodolfi *et al.*, 2009)
Scendesmus sp.		10-30		9-15	30-45	(Laurens *et al.*, 2014)
S. quadricauda	22-25	20			20-25	(Pancha *et al.*, 2014)
S. obliquus	220,	12, 12-14			17, 10-17	(Becker, 2007; Ho *et al.*, 2012)
Tetraselmis sp.		10-17		31	12	(Brown, 1991)

- *Tetraedron* sp. had comparable FA profiles to other reports for these species (Lang *et al.*, 2011) and *S. quadricauda* FA profiles are comparable to those described for the same species by Ahlgren *et al.* (2003) (Compare with Supplementary tables S3.1-3.4).

Fatty acid profiles were affected differently between the four species: *Mesotaenium* sp. was affected by culture salinity, whereas in *D. armatus* and *S. quadricauda* nutrient availability had the greatest influence and *Tetraedron* sp. was affected by a combination of both. In all species, nutrient depletion induced an increase of SFA and MUFA driven mainly by an increase of C16:0 and C18:1, respectively, and a decrease of C18:3 (except *Tetraedron* sp. at 2 ppt). Similar changes have been reported for a number of green algae e.g. *Botryococcus braunii* and the eustigmatophyte *Nannochloropsis* sp. (Reitan *et al.*, 1994; Rodolfi *et al.*, 2009; Su *et al.*, 2011; Zhila *et al.*, 2005) and is most likely due to the accumulation of neutral lipids such as triacylglycerols, which in the Chlorophyceae, have been observed to contain mainly C16:0 and C18:1 FA (Becker, 1994).

Previous reports for the effects of increasing salinities on microalgal FA profiles have shown similar patterns to nutrient depletion with increases of C18:1 contents in *Botryococcus braunii* (Rao *et al.*, 2007; Zhila *et al.*, 2011), *Isochrysis* sp., *Dunaliella bardawil*, *D. salina* (Ben-Amotz *et al.*, 1985) and *D. abundans* (Xia *et al.*, 2014), increases of C16:0 in *Botryococcus braunii* (Rao *et al.*, 2007; Zhila *et al.*, 2011), *Nannochloropsis oculata* and *Nitzschia frustulum* (Renaud *et al.*, 1994). However, reports on the effects of sodium chloride on microalgal fatty acids are scarce and often contradictory (Zhila *et al.*, 2011). Furthermore, it is also unclear if fatty acid composition plays a role in microalgal osmoregulation (Renaud *et al.*, 1994). A primary role of fatty acids in algae are related to functions of cell membranes and metabolic

processes (Guschina *et al.*, 2006). The degree of membrane fatty acid unsaturation is also a significant parameter in algal adaptation to environmental conditions. Fatty acid changes in response to high salinities are required to maintain membrane fluidity and prevent destruction (Zhila *et al.*, 2011).

The distinct effect of salinity on *Mesotaenium* sp. FA profiles are difficult to explain and put into the context of current literature. The high concentrations of C16:0 and C18:1 at 18 ppt are potentially due to the inhibited growth at this salinity having similar effects to nutrient limitation (see above) which results in the accumulation of TAG containing C16:0 and C18:1 FA (Ben-Amotz *et al.*, 1985). Microalgal MUFAs and PUFAs have a promising biotechnological market for food, feed and material applications (Lligadas *et al.*, 2010; Pulz *et al.*, 2004). Examples include, oleic acid (C18:1, ω-9), linoleic (C18:2, ω-6), α-linolenic (C18:3, ω-3) and stearidonic acid (C18:4, ω-3). Oleic acid (C18:1) can be used to produce fatty acid-derived diols and polyols, from which polyurethanes can be synthesised through polyaddition reactions with organic isocyanates (Lligadas *et al.*, 2010). In this study, C18:1 content was significantly increased in all species by a combination of nutrient depletion and high salinities (11-18 ppt). The highest C18:1 content was observed for *Tetraedron* sp. at 11 ppt (54 mg g^{-1} DW) and *Mesotaenium* sp. at 18 ppt (35 mg g^{-1} DW). Both could be potential candidates for bioplastic manufacturing, however *Mesotaenium* sp. would require a 2-step cultivation process (Su *et al.*, 2011), with biomass production at 2 ppt followed by salinity stress (18 ppt).

For dietary applications linoleic acid (C18:2, ω-6) and α-linolenic acid (C18:3, ω-3) are essential nutrients for immune system function and tissue regeneration processes (de Jesus Raposo *et al.*, 2013). They are also important precursors for other

ω-6 and ω-3 FAs (Guil-Guerrero, 2007), with distinct cellular functions (Simopoulos, 2002) An imbalance in ω-6 and ω-3 FA ratios in current 'western diets' has been linked to a range of diseases such as cardiovascular disorders, diabetes, obesity, inflammatory processes, increased susceptibility to viral infections, certain types of cancer, autoimmune disorders, rheumatoid arthritis, asthma and depression (Guil-Guerrero, 2007; Simopoulos, 2002). Consequently, a ~1:1 ω-6:ω-3 uptake ratio has been recommended to ensure good health and normal development. This is an important consideration when identifying novel feed and FA sources. *Desmodesmus armatus*, *S. quadricauda* and *Tetraedron* sp. have low ω-6:ω-3 (generally <0.4:1) ratios and could therefore be beneficial as ω-3 nutritional supplements. *Mesotaenium* sp. on the other hand had a particularly high ω-6:ω-3 ratio at salinities above 8 ppt (3-10:1) driven by a high C18:2 content making it a possible candidate for pharmacological applications in the topical treatment of skin hyperplasias (Proksch *et al.*, 1993).

Stearidonic acid (C18:4) has also been shown to possess a number of health benefits and bioactive properties to prevent a range of conditions including certain cancers, arthritis and thrombosis (Guil-Guerrero (2007). Microalgae have previously been suggested as a potential source of C18:4 (Guil-Guerrero, 2007). In this study, C18:4 was present in *D. armatus* and *Tetraedron* sp. however only *D. armatus* at lower salinities contained notable amounts (7 mg g^{-1} DW) of this FA. Depending on the viability of targeting this FA for health purposes, *D. armatus* is therefore a suitable candidate for further research to improve C18:4 productivity yields. Lipid and fatty acid productivities were generally low in this study compared to the same species in other studies (Rodolfi *et al.*, 2009; Zhou *et al.*, 2011); which is likely due to the low growth rates, as actual total lipid and fatty acid contents were comparable to previous

studies (Ahlgren *et al.*, 2003; Dunstan *et al.*, 1992; Rodolfi *et al.*, 2009; Zhou *et al.*, 2011). Consequently, future research should focus on increasing biomass productivity.

Amino acid profiles in this study were similar to previous reports for *Chlorella* sp. and *Scenedesmus* sp. (Ahlgren *et al.*, 2003; Brown *et al.*, 1992), except for histidine concentrations which were considerably higher in species in this study (up to 6.5% of DW). The decrease of AA concentrations observed in all species in this study following nutrient depletion has been extensively documented and is most likely due to the diversion from protein production to carbohydrate or lipid production in the absence of N for protein synthesis (Flynn, 1990; Mata *et al.*, 2010; Rodolfi *et al.*, 2009). As mentioned earlier and as documented in other microalgal species (Brown *et al.*, 1978; Greenway *et al.*, 1979; Vanlerberghe *et al.*, 1987), the species in this study do not use AA-based osmoregulation to combat salinity stress, as indicated by salinity-induced decreases in AA content, particularly proline and glycine.

Feed protein quality is determined by amino acid digestion and absorption by animals and their respective amino acid requirements for metabolic processes. In general, the ideal protein source for an organism contains the same AA content and AA proportions as the organism itself (Brown *et al.*, 1992; De Silva *et al.*, 2012). Limitation of one or more specific amino acids restricts growth and results in the inability to utilize other essential amino acids (De Silva *et al.*, 2012) which becomes problematic when formulating feeds for farmed animals as certain essential AA are often limiting e.g. lysine, methionine and threonine in fish, shrimp, cattle, swine and poultry feeds (D'Mello, 1993; Kung Jr *et al.*, 1996; Nunes *et al.*, 2014; Rawles *et al.*, 2013). Currently, optimizing animal feed protein quality is carried out by supplementing feed with synthetic amino acids but can also be achieved by AA

blending from other sources with high concentrations of target amino acids e.g. plants, algae and insects (Boland *et al.*, 2013), or as a by-product of biotechnological processes such as biofuel production (Williams *et al.*, 2010). This study has shown that *D. armatus* had the highest AA contents and would be the most suitable species for amino acid production in particular lysine or as a feed supplement for species where lysine is often limiting e.g. giant clam (*Tridacna gigas*) aquaculture (Brown, 1991).

The carbohydrate content increase following N depletion is due to the diversion of carbon from protein synthesis to carbohydrate and lipid production (see above). This increase is consistent with previous studies showing a carbohydrate increase following N depletion in *Scenedesmus obliquus* (Ho *et al.*, 2012). Although this study did not specifically focus on carbohydrate production and composition in the four study species, microalgae are a potential source of sugars such as xylose, arabinose, mannose, galactose, glucose and the less common sugars rhamnose, fucose and uronic acids (Cheng *et al.*, 2011; Ho *et al.*, 2012; Krienitz *et al.*, 1999), with an interesting potential for commercialization (Draaisma *et al.*, 2013).

3.5 Conclusions

While all species cultured at salinities of 2-18 ppt, *Mesotaenium* sp. was the least salinity tolerant and *D. armatus* was the most halotolerant species of the dominant microalgae isolated from tailings-dam water of a Queensland coal-fired power station. Nitrogen uptake rates correlated with biomass irrespective of salinity, which together with decreased levels of proline and glycine at higher salinities suggest that salinity tolerance in these species is not achieved by glycine betaine or proline accumulation, as described for some other chlorophytes. Increased carbohydrate

contents suggest instead that carbohydrate-based osmoregulatory mechanisms could be involved in salinity acclimation. The total lipid content data of the examined species suggest that neither increased salinity nor nitrogen depletion should be viewed as universal mechanisms to increase total lipids or fatty acids, as *D*. *armatus* and *Mesotaenium* sp. did not respond significantly to either treatment and *S. quadricauda* and *Tetraedron* sp. were only significantly affected by higher salinities. This is further corroborated by the finding that the FA profile was predominantly influenced by salinity in *Mesotaenium* sp., by nutrient-status in *D*. *armatus* and *S*. *quadricauda* and by a combination of the two in *Tetraedron* sp. In general though, the isolated species responded to nutrient limitation with an increase in SFA and MUFA, particularly C16:0 and C18:1, which is well known from the literature. Generally, *D*. *armatus*, *S*. *quadricauda*, and *Tetraedron* sp. were characterised by low ω-6:ω-3 ratios making them potential candidates for ω-3 supplements. In contrast, *Mesotaenium* sp. was characterised by an ω-6:ω-3 ratio of 3-10:1, making it unsuitable for diet supplementation with ω-3 FAs, yet it could be a pharmacological candidate for the topical treatment of skin hyperplasias.

The overall species responses from this study can now be used to produce a species selection matrix to target species for scaled production based on their salinity tolerance and plasticity in biochemical composition (Table 3.5).

This study confirmed that *S*. *quadricauda* is an ideal candidate for environmental services, such as nitrogen and phosphate remediation, as it had the highest uptake rates. This study further identifies that the organism would be suitable across a salinity range of 2 < 11 ppt. *Desmodesmus armatus* and *Mesotaenium* sp. on the other hand stood out for biomass production under nutrient-poor conditions from

2<18 and 2<8 ppt, respectively. Such situations are typically encountered when producing carbon dioxide-supplemented biomass at coal-fired power stations in Australia where large amounts of nutrients and/or nutrient-rich water sources are generally unavailable.

Table 3.5. Decision matrix for species selection used in this study isolated from tailings-dam water of a Queensland power station for remediation, low nutrient-based cultivation, high fatty acid contents, bioplastic and nutritional potential based on salinity tolerance

Species	High N+P Remediation potential	Low N+P requirements Cost-effective	High FA	Bioplastics potential	Nutritional potential
D. armatus		2<18 ppt	2<8 ppt		2 ppt
***Mesotaenium* sp.**		2<8 ppt	2<8 ppt	>11<18 ppt	
S. quadricauda	2<11 ppt				
***Tetraedron* sp.**				8<11 ppt	

Both algae also had the highest FA content with a profile suitable for lipid-based biofuel production for on-site consumption. *Tetraedron* sp. and, *Mesotaenium* sp. in particular (5.4% of DW, 45% of TFA), excelled in accumulation of C18:1 at 8<11 and >11<18 ppt, respectively, a valuable precursor for bio-degradable plastic production. It needs to be recognised though that such production would require a two-step approach, where biomass accumulation would require cultivation at 2 ppt with subsequent salt stress used to shift the FA profile in favour of C18:1 accumulation, the feasibility of which still requires demonstration. *Desmodesmus armatus* also has demonstrated pharmaceutical potential through accumulation of Stearic acid when cultured at 2 ppt. While biomass yields and productivities are yet to be demonstrated on site, with regards to freshwater requirements, the results of this study suggest that *D*. *armatus*, *S*. *quadricauda* and *Tetraedron* sp. are sufficiently

salinity tolerant to only require freshwater make up water after 24 days cultivation based on East Queensland daily evaporation rates and salinity concentrations of available water sources, while *Mesotaenium* sp. could only be cultivated for 12 days under the same conditions. These results have major implications for cultivation-site and product range selection for these new isolates.

Following this research, it became evident that microalgal biomass production for biofuel and feed was not economically sustainable, requiring the simultaneous production of high value bio-products to offset expensive infrastructure and labour costs (Stephens *et al.*, 2010b). Consequently the following chapters (4 and 5) present research on high-value carotenoid production using current Stanwell Corp. tailings-dam water species and newly isolated strains, as pigment pathways to market are already established ensuring no delays to commercialisation.

CHAPTER 4

Carotenoid production in eight freshwater microalgal species

4.1 Introduction

Commercial-scale microalgal cultivation at coal-fired power stations (the origin of this research project) and agricultural or aquaculture facilities provides a multi-disciplinary solution to carbon sequestration and waste-water remediation while producing commercially valuable by-product potential from the algal biomass. However, establishing large-scale microalgal cultures is a costly venture due to requirements for specialized equipment and considerable manpower. As biofuels are not a high value commodity and economic viability can only be achieved in very specific circumstances which are highly sensitive to change (Lundquist *et al.*, 2010), microalgal industries need to establish parallel production of high-value end-products to ensure large-scale production of algal biomass is economically viable. There has therefore been a surge of interest in the discovery and production of valuable molecules from microalgae (Mayfield *et al.*, 2007; Rosenberg *et al.*, 2008). As mentioned in chapter 1, pigments such as astaxanthin, lutein and β-carotene already have established markets in pharmaceutical, nutraceutical and aquaculture industries, The global carotenoid market was estimated to be USD 1.2 billion in 2010 potentially increasing to USD 1.4 billion by 2018 (BCC-Research, 2011). This could lay a viable economic foundation for co-product development from microalgal biomass at remediation sites, but requires production enhancement of existing compounds of interest in microalgae suitable for the remediation purpose at hand. As such, effects of

factors known to influence pigment productivities and yields need to be explored for such strains to fully understand their potential economic potential in such applications.

4.1.1 Influences of light intensity on microalgal growth and carotenoid synthesis

Light intensity is a critical factor influencing microalgal growth (Cuaresma *et al.*, 2011; Masojidek *et al.*, 2008). Similarly to higher plants, the rate of photosynthesis in microalgae increases with increasing light intensity until reaching a maximum saturation rate (P_{max}) at a given light intensity (Melis, 2009). As discussed in chapter 1, light harvesting pigments transfer excitation energy to the photosynthetic electron transfer chain (PETC), *via* intermediate Chl *a*. When the energy transfer rate from light harvesting pigments to Chl *a* exceeds the electron transfer capacity of the PETC, the triplet Chl *a* can potentially pass its energy to ground state molecular oxygen instead. This creates reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), which can also be the result of adverse environmental conditions, such as salinity stress and large pH fluctuations, nutrient limitation, excessive high irradiance and temperature (Mulders *et al.*, 2014).

High light intensities typically result in photo-inhibition, which triggers carotenogenesis to combat photo-damage (Cuaresma *et al.*, 2011). Induction of high light-induced carotenogensis is well documented in studies examining pigment pathway enzyme activities, such as the rate-limiting phytoene synthase and β-carotene hydroxylase in *Haematococcus pluvialis* (Steinbrenner *et al.*, 2001) and phytoene desaturase in *Chlamydomonas reinhardtii* (Bohne *et al.*, 2002). High light stress has been identified as a key driver for inducing astaxanthin accumulation in

Haematococcus pluvialis (Masojidek *et al.*, 2003). Saturating light intensities are, however, species-dependent (Table 4.1). It must be considered though that culture cell densities exert a self-shading effect, artificially inflating light saturation tolerances (Cuaresma *et al.*, 2011).

Table 4.1. Saturating light intensities and maximum cultivation light intensities for several algal species.

Microalgal species	Saturating irradiance (P_{max}) [µmol photons m^{-2} s^{-1}]	Cultivation irradiance [µmol photons m^{-2} s^{-1}]	References
'Wild type' microalgae	400	-	(Melis, 2009)
Chlorella sorokiniana	900-1200	-	(Cuaresma *et al.*, 2009, 2011)
Nannochloris atomus	100	-	(Geider *et al.*, 1986)
Phaeodactylum tricornutum	220	-	(Geider *et al.*, 1985)
Selenastrum minutum	-	420	(Bouterfas *et al.*, 2002)
Nannochloropsis sp.	-	700	(Pal *et al.*, 2011)
Parietochloris incisa	-	400	(Solovchenko *et al.*, 2008)

4.1.2. Carotenogenesis responses to nutrient manipulation

Nutrient limitation, in particular nitrogen starvation, causes carotenoid synthesis in a number of microalgal species (Liaaen-jensen *et al.*, 1999). Similarly to temperature stress, nutrient stress has been shown to reduce photosynthetic electron transport rates (Demmig-Adams *et al.*, 1992) increasing the need for photo-protection by carotenoids. Nitrogen limitation re-directs carbon metabolites to storage as either lipids and/or carbohydrates (Huerlimann *et al.*, 2010; von Alvensleben *et al.*, 2013a). Carbon is also essential for the synthesis of geranyl-geranyl-PP (GGPP), a precursor molecule for both chlorophyll and carotenoids. Under nitrogen limitation, the

nitrogen-containing chlorophylls are not synthesised, making carbon available for carotenoid production (Geider *et al.*, 1998).

4.1.3 Mechanism of action of metal ions on carotenogenesis

In addition to temperature and irradiance, the exposure of microalgae to pollutant heavy metals triggers a number of ROS generating mechanisms (Conner *et al.*, 2003; Woodall *et al.*, 1997a; Zalups *et al.*, 2003) such as the disruption of the photosynthetic electron transport chain leading to superoxide anion ($O_2^{\bullet-}$) and subsequently hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$) formation (Pinto *et al.*, 2003) (Figure 4.1).

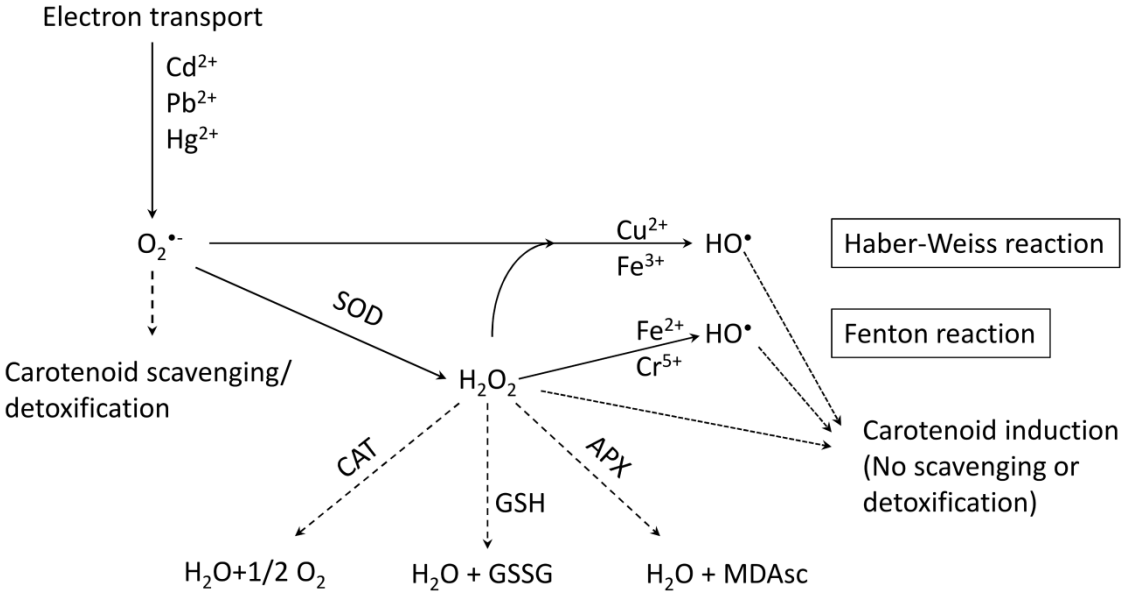

Figure 4.1. Heavy metal stress-induces cellular generation of ROS and hypothesized sites of carotenoid action adapted from Pinto *et al.* (2003). SOD: Superoxide dismutase, CAT: Catalase, GPX: Glutathione peroxidase, APX: Ascorbate peroxidase, GSSG: two molecules of glutathione linked by disulphide bond. MDAsc: Monodehydroascorbate.

Although carotenoids have only been shown to detoxify $^1O_2$, ^{3}Chl and $O_2^{\bullet-}$ (Boussiba, 2000; Pinto *et al.*, 2003) and are not directly involved in the degradation of

$HO^{\bullet}$ and H_2O_2, the induction of these harmful ROS species still induces carotenoid synthesis (Boussiba, 2000; Ip *et al.*, 2005a) producing radical scavengers to protect cells against oxidative damage (Fan *et al.*, 1998; Rise *et al.*, 1994; Shaish *et al.*, 1993).

Transition metals, such as Fe^{3+} and Cu^{2+}, and particularly those from groups 4-7 i.e. Ti, V, Cr, Mo, W and Re, have been shown to induce ROS formation due to their variable valences (Conte *et al.*, 2011), allowing them to undergo changes in oxidation state involving one electron (Mallick, 2004; Stohs *et al.*, 1995). This occurs either through the reaction of metal ions (e.g. Fe^{2+}) with H_2O_2 (Fenton 'like' reaction. eq. 1 and 2) (Kehrer, 2000) or through the decomposition of H_2O_2 (e.g. iron-catalysed Haber-Weiss reaction. eq. 3) (Haber *et al.*, 1934; Kehrer, 2000) both leading to $OH^{\bullet}$ production, inducing severe oxidative stress (Stohs *et al.*, 1995).

$$O_2^{\bullet-} + Fe^{3+} \longrightarrow Fe^{2+} + O_2 \qquad \text{(eq. 4.1)}$$

$$H_2O_2 + Fe^{2+} \longrightarrow Fe^{3+} + OH^- + OH^{\bullet} \qquad \text{(eq. 4.2)}$$

$$H_2O_2 + O_2^{\bullet-} \xrightarrow{Fe} O_2 + OH^- + OH^{\bullet} \qquad \text{(eq. 4.3)}$$

The effects of heavy metals on ROS metabolism in algae are strain-dependent (Stohs *et al.*, 1995) and vary between metals and concentrations (Okamoto *et al.*, 2001). In addition, chronic or acute metal treatments influence antioxidant responses (Okamoto *et al.*, 2001). For example, chronic exposure to metals generally resulted in high activities of the antioxidant enzymes SOD and APX, whereas only acute exposure induced carotenoid accumulation (Okamoto *et al.*, 2001; Pinto *et al.*, 2003). A summary of previous studies that investigated effects of metals on carotenoid production by microalgae is presented in Table 4.2.

Table 4.2. Previous studies on metal-induced ROS formation and carotenoid content enhancement in microalgae

Microalgal species	Carotenoid induction parameters	Target carotenoid enhancement	References
Chlorella protothecoides	- Fe + H_2O_2 - NaClO + H_2O_2	Lutein	(Wei *et al.*, 2008)
Coccomyxa onubensis	- Cu	Lutein	(Vaquero *et al.*, 2012)
Haematococcus pluvialis	- High light + Fe	Astaxanthin	(Kobayashi *et al.*, 1993)
Tetraselmis gracilis	- Cd	Carotenoids	(Okamoto *et al.*, 1996)

The tailings-dam water at Stanwell Corp. coal-fired power station contained a number of polluting metals (Table 4.3). Of these, molybdenum (Mo) and vanadium (V) are transition metals which could potentially induce ROS formation in microalgae.

Table 4.3. Elemental composition [mg L^{-1}] of Stanwell Corp. coal-fired power station tailings-dam water (Saunders *et al.*, 2012)

Element	Tailings-dam content [mg L^{-1}]	Element	Tailings-dam content [mg L^{-1}]
Aluminium	0.06	Mercury	<0.0001
Arsenic	0.0175	Molybdenum	0.8595
Boron	2.26	Nickel	0.016
Cadmium	0.0004	Phosphorous	<1
Calcium	197	Potassium	30
Chromium	<0.001	Selenium	0.06
Copper	0.004	Sodium	335.5
Iron	0.275	Strontium	1.365
Lead	<0.001	Vanadium	0.565
Magnesium	69.5	Zinc	0.231
Manganese	0.002		

Molybdenum, however, is also an essential trace element required for a number of biological functions, in particular as a cofactor in nitrogen fixation and reduction (Sakaguchi *et al.*, 1981), but concentrations in tailings-dam waters were ~9-fold higher than in defined trace metal solutions for microalgal cultivation (e.g. freshwater BBM or seawater f/2) (Andersen *et al.*, 2005). Higher than required concentrations of essential trace elements have nonetheless been shown to induce defence mechanisms in microalgae (Mallick, 2004). Because transition metals can induce carotenogenesis, metal pollution of industrial waste waters could potentially be exploited for enhancing carotenoid content in large-scale microalgal cultures, generating high value co-products from the microalgal biomass in addition to its intended deployment for carbon sequestration, metal remediation and other biomass-based co-products (e.g. animal feeds, biofuels). Table 4.4 summarizes molybdenum remediation potential by a number of green microalgae showing high concentration tolerances also for *Scenedesmus* spp (note: a number of *Scenedesmus* species have been transferred to the new genus *Desmodesmus* sp. based on ITS2 data (Palffy *et al.*, 2006)), which were particularly abundant in Stanwell Corp. coal-fired power station tailings-dam water.

Table 4.4. Molybdenum uptake by various green microalgae (Sakaguchi *et al.*, 1981)

Species	**Mo Absorbed (mg g^{-1} dry $weight^{-1}$)**
Chlorella regularis	13.2
Chlamydomonas angulosa	9.5
Chlamydomonas reinhardtii	21.2
Scenedesmus bijugatus	10
Scenedesmus chlorelloides	23.2
Scenedesmus obliquus	7.6

Environmental parameters such as irradiance, temperature and salinity have been shown to influence Mo uptake by *Chlorella regularis* (Sakaguchi *et al.*, 1981), which can therefore synergistically or antagonistically affect metal effects on carotenogenesis. This has not been investigated to date for either Mo or V, present at concentrations in Stanwell Corp. coal-fired power station tailings-dam water (~9-times higher than trace metal contents of defined media (Andersen *et al.*, 2005). Given the species-specific carotenoid synthesis in responses to various environmental stresses, it is important to determine carotenoid production patterns for each microalgal species to evaluate their commercial suitability and potential for high value carotenoid production. In the context of the carbon abatement project at the Stanwell Corp. coal-fired power station (see Chapter 1), this chapter served as a screening study to investigate the effects of light, culture nutrient status and transition tailings-dam metal (Mo or V) stress on carotenoid production, in eight chlorophyte microalgal species, with five isolates from Stanwell Corp. tailings-dam waters, two local tropical isolates, and one commercial astaxanthin producer, *Haematococcus* sp., as a reference organism. These data were used as a decision matrix for species selection for Chapter 5, where carotenoid production potential was examined in a multifactorial design testing the interactive effects of temperature and molybdenum stress under high light conditions.

4.2 Materials and Methods

4.2.1 Strain selection

Eight freshwater microalgal species were obtained from the North Queensland Algal Identification/Culturing Facility (NQAIF) culture collection (James Cook University, Townsville, Australia); *Desmodesmus armatus* (Chlorophyceae) (Culture accession NQAIF # 301)*, Mesotaenium* sp. (Conjugatophyceae) (NQAIF303), *Scenedesmus quadricauda* (Chlorophyceae) (NQAIF304), *Desmodesmus maximus* (Chlorophyceae) (NQAIF293) and *Tetraedron* sp. (Chlorophyceae) (NQAIF295), which were chosen for of their ability to grow in polluted tailings-dam water environments. Two newly isolated species from the Townsville region, *Graesiella emersonii* (Chlorophyceae) (NQAIF386) and *Coelastrum proboscideum* (Chlorophyceae) (NQAIF384)*,* were included as they showed potential for carotenoid production, and *Haematococcus* sp. (Chlorophyceae) (# CS-321, CSIRO culture collection) was included as a reference for astaxanthin production. Batch cultures of each species were maintained in Bold basal medium (BBM) (Andersen *et al.*, 2005) (24 °C, with a 12:12 h photoperiod and light intensity of 42 µmol photons m^{-2} s^{-1}) and were individually areated with 0.45 µm filtered air (Durapore; Millipore). All materials were sterilised by autoclaving (Tomy, Quantum Scientific) and cultures were handled and inoculated aseptically in a laminar flow (AES Environmental Pty LTD fitted with HEPA filter).

4.2.2 Treatments

Sub-cultures were established for each species in moderately nutrient-replete (~5 mg L^{-1} nitrate, ~2 mg L^{-1} phosphate) and nutrient-deplete conditions (4 days after

medium nitrate and phosphate depletion). Molybdenum (0.86 mg L^{-1}) and vanadium (0.57 mg L^{-1}) concentrations were based on tailings-dam water contents (Table 4.3) which were added separately to nutrient-replete and -deplete cultures (Figure 4.2). Control nutrient-replete cultures were maintained without heavy metal addition. All cultures were then exposed to light stress at 400-440 µmol photons m^{-2} s^{-1} for five days.

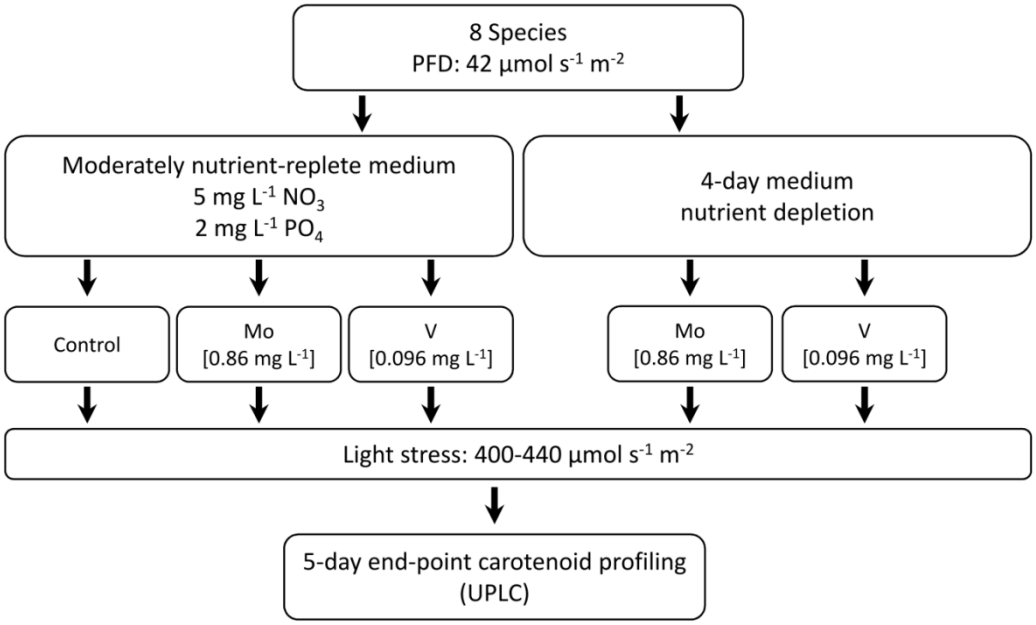

Figure 4.2. Experimental design

4.2.3 Carotenoid analyses

After three days, biomass was harvested by filtering 2 mL and 0.5 mL culture samples for dry weight (DW) and carotenoid determination, respectively, using 0.2 µm glass fibre filters (Whatman GF/F). Dry weights (DW) were determined gravimetrically using a method modified from Rai *et al.* (1991). Carotenoid extraction procedures were trialled prior to extractions to determine optimal methods and parameters including bead material and size, bead-beating duration and the number of extractions

required to ensure optimal and consistent carotenoid recovery from all species, in particular *Haematococcus* sp. Carotenoids were extracted by bead-beating (Biospec, OK, USA) using 0.7 mm zirconia beads (Biospec, OK, USA) in 1 mL ice-cold buffered methanol (98:2 methanol: 0.5M Tetrabutylammonium acetate (TBAA) solution, pH 6.5 with 0.01% butylated hydroxytoluene (BHT) added as an antioxidant). Spectrophotometric measurements of chlorophyll *a* content (652, 665 and 750 nm) were carried out to determine adequate dilutions for ultra-pressure liquid chromatography (UPLC) analysis. Pigment analyses were carried out using a Waters (Milford, MA, USA) Acquity UPLC system consisting of a binary solvent manager, sample manager, column heater and a photodiode array (PDA) detector. Immediately prior to injection, clarified extracts (0.2 µm hydrophilic polypropylene (GHP) Acrodisc® filters, Pall Corporation. Vic. Australia) were diluted 1:1 with 0.2 µm filtered 28 mM TBAA and injected on to a Waters HSS C18 column (2.1 x 100 mm; 1.8 µm, 100A) fitted with an in-line filter (2.1 mm, 0.2 mm). Separation followed a binary gradient of solvent A (45:35:20 [v/v/v], methanol:acetonitrile:aqueous pyridine solution [0.25 M pyridine pH adjusted to 5.9 with acetic acid]) to solvent B (60:40 [v/v] acetonitrile:acetone). Carotenoid chromatograms were monitored at 440 nm and identified by comparing retention time and photo-diode array (PDA) spectra with certified reference carotenoids (neoxanthin, violaxanthin, astaxanthin, zeaxanthin, lutein, chlorophyll *b* and *a*, and β-carotene) sourced from DHI (Danish Hydraulic Institute, Denmark) with quantification *via* external calibration curves. Throughout carotenoid extraction and analyses, samples were maintained on ice and light exposure was avoided. Only free astaxanthin content was identified and quantified, as

no astaxanthin esters were detected. Free astaxanthin is referred to as astaxanthin throughout this chapter.

4.2.4 De-epoxidation state

As antheraxanthin was not quantified in this study, the approximate de-epoxidation state was calculated to quantify the de-epoxidized proportion of the total xanthophyll cycle pigment pool. Calculations were modified from those described in Couso *et al.* (2012) (eq. 4.4), to exclude antheraxanthin concentrations (eq. 4.5).

$$De - epoxidation\ state = ([Anth.] + [Zea.])/([Anth.] + [Zea.] + [Viola.]) \quad \text{(eq. 4.4)}$$

$$Approximate\ de - epoxidation\ state = [Zea.]/([Zea] + [Viola.]) \quad \text{(eq. 4.5)}$$

4.3 Results

Effects of nutrient status and metal addition were species-and carotenoid-specific. In most species, carotenoid contents were highest in nutrient-replete cultures with added Mo or V when compared to nutrient-replete control cultures (Figures 4.3 and 4.4). Most distinct effects were observed in *Haematococcus* sp with total carotenoids and xanthophylls 14-17 % higher than respective control cultures.

Overall, *Haematococcus* sp. contained the highest carotenoid concentrations with up to 4.5 mg g^{-1} DW total carotenoids, followed by *D. armatus* and *S. quadricauda* reaching 3 mg g^{-1} DW and 2 mg g^{-1} DW, respectively. In contrast, *G*. *emersonii* showed higher total carotenoid and xanthophylls under nutrient-deplete conditions with added V (Figure 4.3).

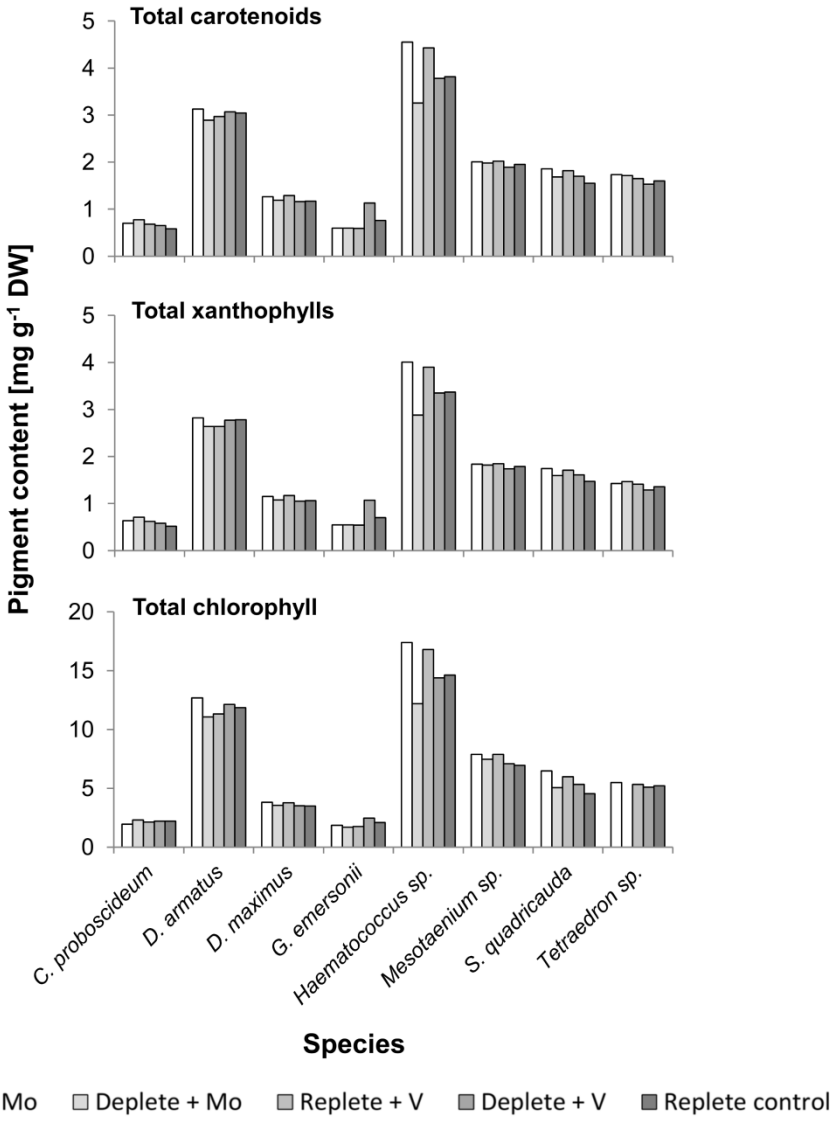

Figure 4.3. Effect of nutrient-status and Mo and V addition on total carotenoid, chlorophyll and xanthophyll contents [mg g^{-1} DW] in eight freshwater chlorophyte microalgae. Note the different axis scales.

As expected, total chlorophyll contents [mg g^{-1} DW] were higher than carotenoid contents, but followed similar patterns to total carotenoids with slightly higher concentrations in nutrient-replete metal-treated cultures in *D. maximus*, *Haematococcus* sp., *Mesotaenium* sp. and *S. quadricauda*. Chlorophyll contents were generally not greatly affected by treatments, except for nutrient-deplete

molybdenum-stressed *Haematococcus* sp., where contents were noticeably lower but higher in metal-treated nutrient-replete cultures compared to controls.

Except for *Haematococcus* sp. and violaxanthin contents, treatment responses were small (Figure 4.4).

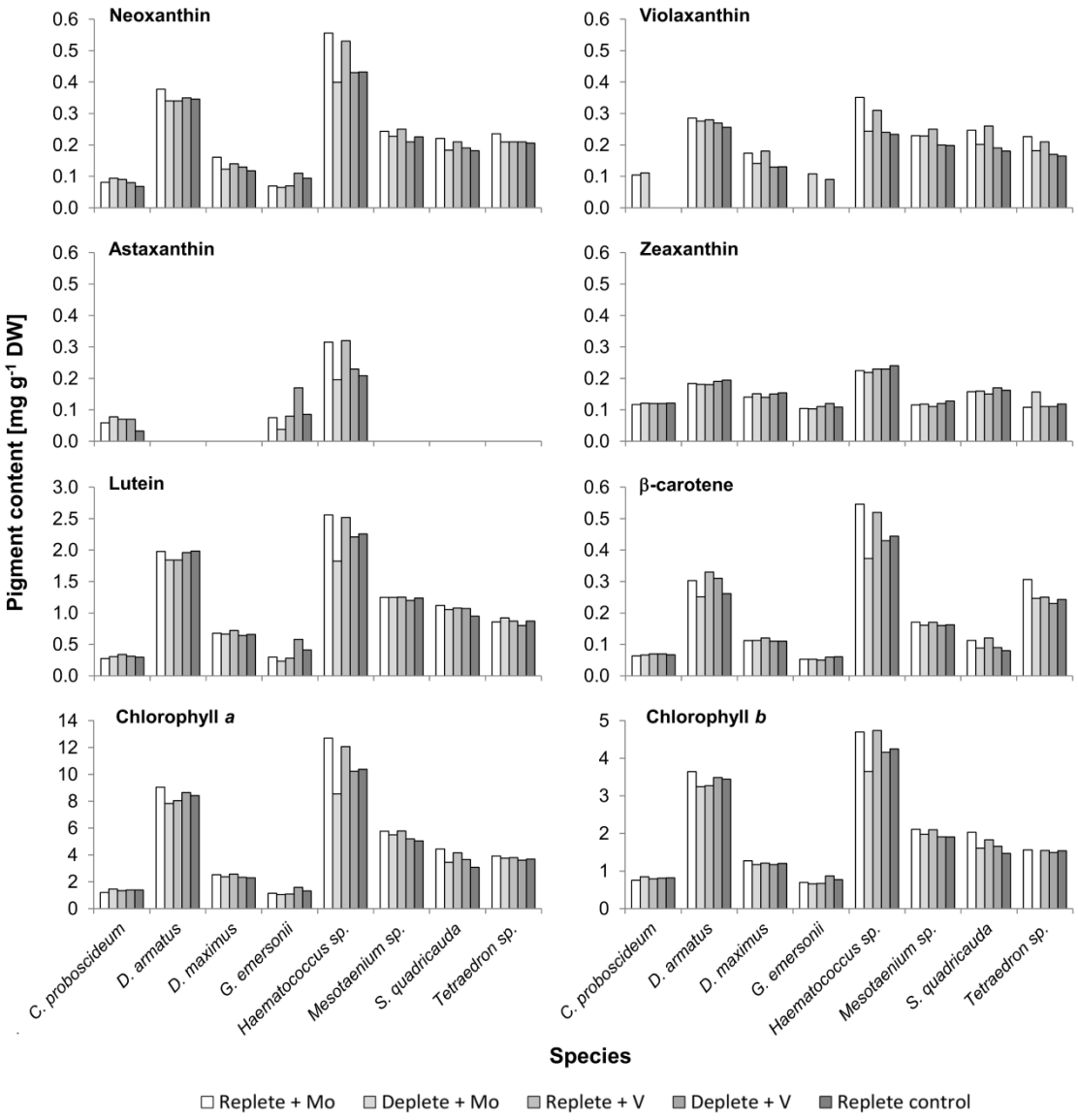

Figure 4.4. Effect of nutrient-status and Mo and V addition pigment content profiles [mg g^{-1} DW] in eight freshwater chlorophyte microalgae. Note the different axis scales.

Of interest was that astaxanthin was detected only in *Haematococcus* sp. (as expected) and *C*. *proboscideum* and *G*. *emersonii* (Figure 4.4). In *C. proboscideum*, all metal treated cultures contained 0.06-0.07 mg g^{-1} DW, representing 45-50 % more astaxanthin than control cultures, whereas *Haematococcus* sp. nutrient-replete metal-treated cultures contained 0.3 mg g^{-1} DW astaxanthin, representing ~34% more than control cultures (Figure 4.4). The highest lutein contents were found in *Haemataococcus* sp. (2.5 mg g^{-1} DW), *D. armatus* (2 mg g^{-1} DW) and *S. quadricauda* (1 mg g^{-1} DW), with nutrient-replete, metal-treated cultures containing 10-15 % more lutein, than control cultures in *S. quadricauda* and *Haematococcus* sp. (Figure 4.4). Similarly, the highest β-carotene contents were in *Haematococcus* sp. (0.5 mg g^{-1} DW), *D*. *armatus* (0.3 mg g^{-1} DW) and *Tetraedron* sp. (0.3 mg g^{-1} DW) (Figure 4.4). With regards to nutrient status and metal treatments, *Haematococcus* sp. showed 9-34 % higher concentrations of all carotenoids (except zeaxanthin) in nutrient-replete cultures with added metals when compared to controls (Figure 4.4). Violaxanthin contents also increased by 20-30% compared to controls under nutrient-replete conditions with added metals in all species, except in *D. armatus*, while effects could not be evaluated for *C. proboscideum* and *G. emersonii*, as no violaxanthin was detected under certain conditions (Figure 4.4). Zeaxanthin content was least affected by metal addition and nutrient status with treatment cultures containing 6-9 % lower concentrations than control cultures and similar contents within species and across species, with *G. emersonii* showing the lowest concentrations (~0.1 mg g^{-1} DW) and *Haematococcus* sp. the highest (~0.2 mg g^{-1} DW) (Figure 4.4).

Xanthophyll cycle pigment content ratios were used as a measure for evaluating the effectiveness or degree of irradiation stress. Ratios were lowest in *D.*

armatus and *Mesotaenium* sp. (30-40 % of the de-epoxidized xanthophyll pool) and highest in *C. proboscideum* and *G. emersonii* (>50 %), suggesting lower high-light tolerance of the latter two species (Table 4.5).

Table 4.5. Approximate de-epoxidation state (Z:Z+V) (excluding antheraxanthin).

Species	Control	Replete + Mo	Deplete + Mo	Replete + V	Deplete + V
C. proboscideum	-	0.53	0.52	-	-
D. armatus	0.43	0.39	0.40	0.39	0.41
D. maximus	0.54	0.45	0.52	0.44	0.54
G. emersonii	-	-	0.49	-	0.57
Haematococcus sp.	0.51	0.39	0.47	0.43	0.49
Mesotaenium sp.	0.39	0.34	0.34	0.31	0.38
S. quadricauda	0.47	0.39	0.44	0.37	0.47
Tetraedron sp.	0.42	0.32	0.46	0.34	0.39

4.4 Discussion

4.4.1 Treatment effects

Tolerance to high irradiance levels is a pre-requisite for large-scale microalgal production in Australia and is species-specific. At the same time, light stress, particularly in response to high light, has been identified as an important driver for carotenogenesis in diverse microalgal species (Lubian *et al.*, 1998; Orosa *et al.*, 2000; Steinbrenner *et al.*, 2003), providing a manipulation tool for high value co-product development to provide economic incentive for carbon dioxide abatement and waste water remediation. Zeaxanthin is synthesised as the initial xanthophyll cycle pigment and is epoxidized to violaxanthin in low light conditions. Under stressful light conditions, violaxanthin is de-epoxidized to zeaxanthin as part of non-photochemical quenching (NPQ) to dissipate energy from singlet excited state chlorophylls (Demmig-

Adams *et al.*, 1996). Subsequently, the ratios between these pigments can be used to evaluate the effectiveness of light stress on microalgae. Xanthophyll cycle pigment patterns in this study correlated with previous findings that saturating light intensities are species-specific (Table 4.5). The high violaxanthin to zeaxanthin ratio (~1.5:1, V:Z) and low de-epoxidation state, generally between 30-40 %, observed in *D. armatus, Mesotaenium* sp. and *Tetraedron* sp suggest the irradiance of 400-440 µmol photons m^{-2} s^{-1} was not stressful. In contrast, <1:1, V:Z and high de-epoxidation state in *G. emersonii*, *C. proboscideum* and to a lesser degree *D. maximus, Haematococcus* sp. (although unlikely, see below) and *S. quadricauda* suggest that provided light intensities were more stressful to these isolates (but see chapter 5.4.2 for outcomes when accounting for antheraxanthin). Violaxanthin is also a precursor for neoxanthin synthesis (Mulders *et al.*, 2015) and cellular ratios of these pigments can be used as an additional indicator of light stress. Neoxanthin concentrations were similar to violaxanthin concentrations in most species but higher in *D. armatus* further indicating this species was the least light stressed. With the exception of *Haematococcus* sp., neoxanthin content was typically lower in species with a <1:1, V:Z, inferring light induced de-epoxidation of violaxanthin to zeaxanthin, resulting in decreased violaxanthin availability as the precursor for neoxanthin synthesis. Similar patterns have been shown in *Chlamydomonas reinhardtii* where neoxanthin concentrations decreased with decreasing violaxanthin concentrations (Couso *et al.*, 2012). The high neoxanthin content found in *Haematococcus* sp. could be indicative that irradiances used were either not stressful or high concentrations of other carotenoids (in particular astaxanthin) provide sufficient photoprotective activity, reducing

requirements for xanthophylls involved in NPQ responses, thereby allowing for violaxanthin to neoxanthin conversion.

Slight increases in carotenoid pigment content were generally observed for metal-treated nutrient-replete cultures compared to nutrient-replete controls (except for *G. emersonii* and *C. proboscideum*). This indicates both of these metals induced ROS formation, triggering carotenoid-based (e.g. astaxanthin, β-carotene, lutein and neoxanthin) radical scavenging responses (Mulders *et al.*, 2014). *Haematococcus* sp. showed the strongest responses to metal addition under moderately nutrient-replete conditions, suggesting an ability to cope with metal-induced ROS stress. The absence of astaxanthin esters in *Haematococcus* sp. further indicates that cells were in the intial "brown cell" stages (intermediate encystment stage of *Haematococcus* sp.) inferring experimental conditions, including light intensities, were unlikely stressful resulting in a slow encystment rate (transformation from "green cell" to "red cell" stage, *via* the intermediate "brown cell" stage) (Margalith, 1999; Solovchenko, 2015). Furthermore, Borowitzka *et al.* (1991a) demonstrated that nitrogen limitation induced the formation of red-palmelloid cells in *H. pluvialis* (corresponding to microscopy observations of *Haematococcus* sp. in this study, data not shown). Generally lower pigment contents, in particular β-carotene and lutein have been well documented for nutrient starved cultures of *Haematoccoccus* sp. (Boussiba, 2000; Del Campo *et al.*, 2004), which was generally accompanied by an increase in astaxanthin. In this study, no noticeable increase with nutrient limitation was observed, which could indicate that the nutrient stress applied was too moderate. This conclusion is supported by the demonstrated positive correlation between nutrient starvation, chlorophyll break down and cessation of astaxanthin synthesis in *Haemotococcus pluvialis* at chlorophyll

threshold concentrations of 20 pg cell^{-1} (Boussiba *et al.*, 1999). As chlorophyll contents were moderately reduced only in Mo-treated nutrient-limited *Haematococcus* sp., it is therefore not surprising that astaxanthin content was not severely reduced compared to nutrient-replete controls. In the other species, lower pigment contents were generally also observed in nutrient-deplete cultures with added metals, which are in accord with findings for *Chlorella vulgaris*, *Phaeodactylum tricornutum* and *Tetraselmis suecica,* where total carotenoid contents decreased significantly in cultures subject to eight-day nutrient limitation (Goiris *et al.*, 2015). This infers that moderate nutrient contents are required for maintaining cell functionality for optimal carotenoid synthesis.

Lower chlorophyll concentrations in nutrient-deplete cultures were to be expected as chlorophyll synthesis requires nitrogen (Senge *et al.*, 2006) and has been described in a number of microalgal species (Bar *et al.*, 1995; Hagen *et al.*, 2001; Solovchenko *et al.*, 2013). However, the lower concentrations in nutrient-replete control cultures compared to slightly higher concentrations in metal-treated -replete cultures were unexpected, as chlorophylls are not involved in radical scavenging and contents have generally been reported to decrease in the presence of metals (Mallick, 2004; Pokora *et al.*, 2014; Sadiq *et al.*, 2011). Metal tolerance thresholds vary considerably between microalgal species and metal type (Zhou *et al.*, 2012); inferring metal concentrations in this study were unlikely detrimental to the photosynthetic apparatus of the species selected for this screening study.

Specifically with regards to astaxanthin as a high value co-product, only free astaxanthin was identified and quantified in *Haematococcus* sp. (0.2-0.3 mg g^{-1} DW), irrespective of treatment, making comparisons with published results difficult, as

these typically report astaxanthin contents for commercial production which generally contain a large proportion of astaxanthin esters (Boussiba *et al.*, 1999). Results were however comparable to those reported by Torzillo *et al.* (2003) for *H. pluvialis* in its intial stages, shifting from green- to red-cells. This low astaxanthin content and the absence of esters are indicative of insufficient light stress or induction period for optimal concentrations. This conclusion is supported by the low abundance (data not shown) of cysts, which contain higher total astaxanthin concentrations compared to vegetative cells (Boussiba, 2000), typically at the expense of lutein and chlorophyll content (Del Campo *et al.*, 2004; Margalith, 1999). The high proportion of lutein (56-59 % of total carotenoids) and chlorophyll (~80% of total pigments) corroborate that nutrient and metal stress was insufficient to induce encystment and optimal total astaxanthin accumulation in *Haematococcus* sp.

The purpose of this screening study was to identify species with the potential for pigment production as a co-product in remediation applications of CO_2 and metal-rich waste waters at coal-fired power stations. Both Mo and V addition to moderately nutrient-replete cultures of *Haematococcus* sp. induced higher astaxanthin content making *Haematococcus* sp. a potential candidate particularly when these stresses are applied in conjunction with well-established astaxanthin induction methods. Other astaxanthin producers were the local tropical isolates *C. proboscideum* and *G. emersonii*. Although contents were lower than for *Haematococcus* sp. and nutrient and metal stress effects were less pronounced, warranting an inclusion of these species for further detailed analyses of pigment responses (Chapter 5).

Lutein contents of ~0.2 % of DW in *D. armatus* and *Haematococcus* sp. are of particular interest for commercial applications, as the current pure source of

commercial lutein is marigold (*Tagetes* sp.) which has a lutein content of ~0.03-0.1 % (Bosma *et al.*, 2003; Fernandez-Sevilla *et al.*, 2010; Lin *et al.*, 2015), making these species potentially suitable alternatives for commercial lutein production. These species were therefore also selected (Chapter 5).

In summary, this study screened eight freshwater green algal species of which five were selected for further carotenoid induction experiments. *Haematococcus* sp. and *S. quadricauda* were chosen based on high total carotenoid content, distinctive positive pigment responses to metal treatments and serving as commercial and research benchmarks, respectively. In addition to *Haematoccoccus* sp., *D. armatus* was selected for lutein production potential, general high carotenoid content and its origin from tailings-dam water of the Stanwell Corp. coal-fired power station, while *C. proboscideum* and *G. emersonii* were selected due astaxanthin production. Although astaxanthin content was significantly lower than observed for *Haematococcus* sp., cultivation of *Haematococcus* sp. for the primary purpose of remediation at coal-fired power stations could prove difficult, as growth rates are typically low, particularly when high astaxanthin content is the aim (Ip *et al.*, 2004), it is sensitive to environmental stresses (Lee *et al.*, 1999; Margalith, 1999), and prone to contamination (Gutman *et al.*, 2011). Consequently, *C. proboscideum* and *G. emersonii* were selected as growth trials have shown these species to be resilient and have high growth rates which may compensate for lower carotenoid contents and provide a simpler commercial alternative for astaxanthin production under conditions experienced at coal-fired power stations.

CHAPTER 5

Interactive effects of temperature and molybdenum on microalgal carotenoid synthesis

5.1 Introduction

Commercial production for high volume low value products such as biodiesel and/or for the purpose of remediation requires the development of high-value products to offset production costs. Microalgal carotenoids for the food and feed industry are one particular high value product with already established pathways to market. Furthermore, microalgae are ideal cell factories for the production of high value carotenoids as they combine the fast and easy growth of unicellular organisms with an active isoprenoid metabolism, ensuring sufficient precursors for the carotenogenic pathway and an adequate storage capacity (León *et al.*, 2007). As described in section 1.2, the global carotenoid market was estimated to be 1.2 billion USD in 2010, and with a projected increase to US$ 1.4 billion USD by 2018 (BCC-Research, 2011).

Carotenoids can only be synthesized *de novo* in microorganisms and plants, consequently humans and animals obtain these compounds solely through diet (Delgado-Vargas *et al.*, 2000). In humans, carotenoids provide several therapeutic functions such as antioxidant effects including singlet oxygen quenching, prevention of age related macular degeneration, cardiovascular disease, and immuno-modulatory, anti-tumor and anti-carcinogenesis activity (Fernandez-Sevilla *et al.*, 2010; Krinsky *et*

al., 2005; Maoka *et al.*, 2012; Valko *et al.*, 2006). Carotenoids are also extensively used in the animal feed industry for example astaxanthin in salmonid feeds and lutein in poultry feeds (Delgado-Vargas *et al.*, 2000; Yaakob *et al.*, 2014) (detailed in section 1.2.4).

Currently, the predominant sources of natural microalgal β-carotene and astaxanthin are *Dunliella salina* and *Haematococcus pluvialis*, respectively (Del Campo *et al.*, 2007), whereas for commercial production lutein is extracted from marigold flowers (*Tagetes* sp.) (Kumar *et al.*, 2010; Piccaglia *et al.*, 1998). Most carotenoids can be produced synthetically (Delgado-Vargas *et al.*, 2000) and at lower costs than their natural counterparts (Grewe *et al.*, 2007; Guerin *et al.*, 2003), however the threshold of synthetic food additives legally permitted has been steadily decreasing due to their suspected role as promoters of carcinogenesis and claims of renal and liver toxicities leading to an increasing preference for natural pigments (Guedes *et al.*, 2011a). Consequently there is a renewed commercial interest to identify natural carotenoid sources from plants and microorganisms.

Although current commercial carotenoid production is limited to a few algal species including *Haematococcus pluvialis* and *Dunaliella salina*, these species require very specific culture conditions for successful production. *Haematococcus* sp., in particular, requires costly cultivation infrastructure and has low biomass productivities, is sensitive to environmental fluctuations and is particularly prone to contamination (Margalith, 1999). Furthermore, astaxanthin extraction from *Haematococcus* sp. is increasingly difficult as encystment proceeds due to the formation of a rigid algaenan cell wall (Choi *et al.*, 2015; Cuellar-Bermudez *et al.*, 2015). Consequently, research is ongoing to identify and characterise alternative,

simpler and more cost-effective microalgal species for carotenoid production with a number of potential alternative species summarized in Table 1.3. Similarly, the current commercial lutein source (*Tagetes* sp.) generally contains low lutein concentrations (~0.03 % DW) (Sanchez *et al.*, 2008) and requires large areas of agricultural land for production. In comparison, certain microalgal species not only have higher lutein contents (0.3-0.7 % DW) (Table 1.5) but also do not require arable land and can be further coupled with waste-water remediation projects, making these promising alternatives for commercial lutein production.

Microalgal carotenoid production is generally tightly linked to culture growth and photosynthetic rates, where decreased growth rates due to sub-optimal growth conditions including excess light, nutrient depletion and exposure to transition metals (described in detail in sections 1.2.3 and 4.1.3) result in culture stress and subsequent antioxidant enzyme production and carotenogenesis (Demmig-Adams *et al.*, 1992). Furthermore, high temperatures, in particular in addition to high irradiance, will also generally lead to enhanced formation of reactive oxygen species (ROS) in microalgal cells.

5.1.1 Influences of temperature on microalgal growth and carotenoid synthesis

Temperature regulates the concentration of enzymes involved in carotenoid biosynthesis, which ultimately dictates carotenoid concentrations in microorganisms (Hayman *et al.*, 1974). Studies on *Dunaliella* sp. have shown that temperature influences production of individual carotenoids differently, for example α-carotene increased at lower (17°C) temperatures, while β-carotene increased at higher temperatures (34°C) (Orset *et al.*, 1999), inferring temperature manipulations can be

used to influence/manipulate carotenoid profiles. Certain microalgal strains tolerate a broad temperature range between 15-35°C (e.g. *Chlorella* and *Spirulina*) whereas others have a considerably narrower temperature tolerance requiring rigorous monitoring and regulation e.g. *Haematococcus* sp. (25-27°C) (Masojidek *et al.*, 2008). Numerous temperature studies show clear distinctions in temperature tolerances and influences on carotenoid profiles between different algal species but also within species, emphasizing the importance of temperature-induced growth responses, which can further be used to determine species-specific optimal temperature ranges (James *et al.*, 1989). Stressful temperature ranges can then potentially be used to induce carotenogenesis or further exacerbate carotenoid production in combination with other environmental factors such as high irradiance, as for example, lutein content in *Scenedesmus almeriensis* (Sanchez *et al.*, 2008).

The aims of this study were to determine the effects of high light intensity, temperature and molybdenum addition on pigment production in six freshwater microalgal species. Species selection was based on outcomes of the pilot study (Chapter 4) (two of which were isolated from the tailings-dam of Stanwell Corp coal-fired power station in SE Queensland, three were regional isolates and *Haematococcus* sp. served as a well-studied high astaxanthin-producing bench mark) to investigate the effects of high temperature and light and molybdenum (Mo) stress in a fully factorial design.

5.2 Materials and methods

5.2.1 Strain selection

Six freshwater microalgal species selected from previous experiments (Chapter 4) were obtained from the North Queensland Algal Identification/Culturing Facility (NQAIF) culture collection (James Cook University, Townsville, Australia). Species included: *Desmodesmus armatus* (culture accession NQAIF301)*, Desmodesmus maximus* (NQAIF293)[3], chosen for their ability to grow in polluted tailings-dam water environments, the tropical regional isolates *Graesiella emersonii* (NQAIF386), *Coelastrum proboscideum* (NQAIF384) and *Desmodesmus* sp. (NQAIF385), showing potential for pigment production, and *Haematococcus* sp. (CSIRO, CS-321) as a reference for astaxanthin production yields and productivities. Cultures were maintained in Bold basal medium (BBM) (Andersen *et al.*, 2005) batch cultures individually aerated with 0.45 μm filtered air (Durapore; Millipore) at 24 °C, with a 12:12 h photoperiod and light intensity of 42 μmol photons m^{-2} s^{-1}. All materials were sterilised by autoclaving (Tomy, Quantum Scientific) and cultures were handled and inoculated aseptically in a laminar flow (AES Environmental Pty LTD fitted with HEPA filter).

5.2.2 Treatments

Triplicate sub-cultures were established for each species when moderately nutrient-replete (5 mg L^{-1} NO_3, 2 mg L^{-1} PO_4) (Figure 5.1). Molybdenum (0.86 mg L^{-1}, based on Stanwell Corp. average tailings-dam water concentrations) was added to

[3] *Desmodesmus maximus* was chosen to replace *Scenedesmus quadricauda* (chapter 4), as the *Scenedesmus* culture was lost during the transfer of NQAIF laboratories.

treatment cultures, while control cultures were maintained without heavy metal addition (Figure 5.1). All cultures were moved to a high light environment (600 µmol photons m^{-2} s^{-1}) and subjected to two different cultivation temperatures (24°C and 30°C) for 10 days (Figure 5.1).

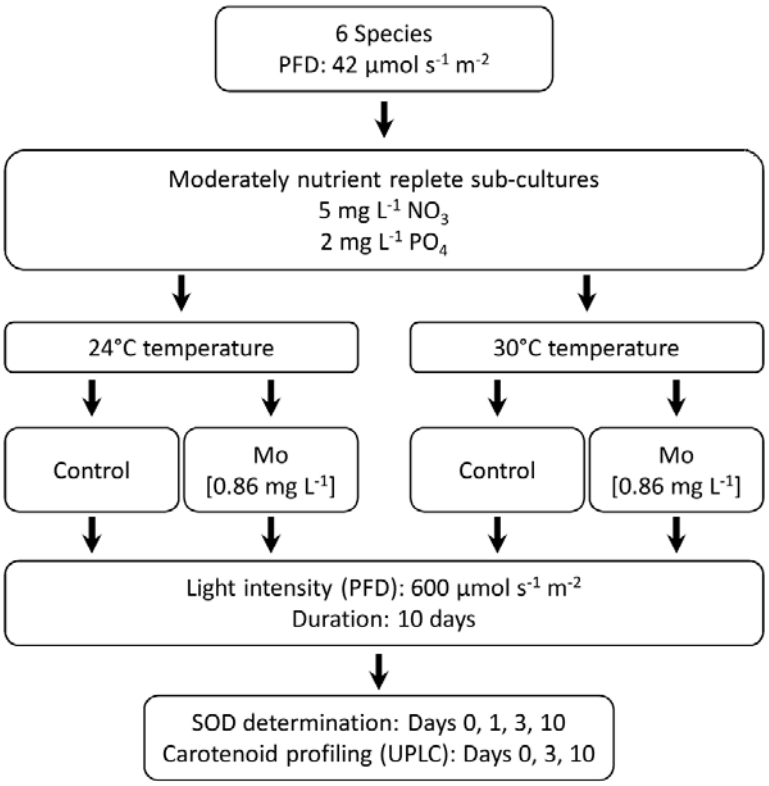

Figure 5.1. Experimental design

Cultures were sampled for superoxide dismutase (SOD) activity at inoculation and days 1, 3 and 10 and pigment profiles were determined at inoculation and on days 3 and 10 (Figure 5.1).

5.2.3 SOD determination

Superoxide dismutase activity was determined using a colorimetric SOD determination kit (Cat. No. 19160, Sigma-Aldrich, NSW. Australia). This kit utilizes

Dojindo's water soluble tetrazolium salt (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl-2H-tetrazolium, monosodium salt) which produces a water soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with $O_2^{\bullet-}$ is linearly related to xanthine oxidase activity and is inhibited by SOD, therefore the IC_{50} (50% inhibition activity of SOD or SOD-like materials) can be determined using a colorimetric method. Enzyme extraction from cultures was carried out following the method by Dewez *et al.* (2005). Cells were harvested from 2 mL culture by centrifugation at 2000g for 10 min. The pellet was then resuspended in 500 µL 0.1 M sodium phosphate buffer, pH 7. Samples were homogenized using a bead beater (Biospec, OK, USA) using 500 µL 212-300 µm glass beads (Biospec, OK, USA) for 10 min., followed by centrifugation at 2300 g for 20 min. at 4°C. 20 µL of supernatant was used for SOD determination following kit procedures.

5.2.4 Pigment analyses

Pigment extractions and analyses were carried out following procedures as described in chapter 4 (Section 4.2.3). Astaxanthin esters were identified by comparing retention time and PDA spectra in addition to literature comparisons (Britton *et al.*, 2004; Holtin *et al.*, 2009; Mulders *et al.*, 2015). As molar extinction coefficients are similar between astaxanthin esters and their non-esterified counterparts (Mulders *et al.*, 2015), ester concentrations were quantified as astaxanthin equivalents, using calibration curves from certified astaxanthin reference standards (DHI) (Danish Hydraulic Institute, Denmark).

5.2.5 De-epoxidation state

De-epoxidation state was calculated to quantify the de-epoxidized proportion of the total xanthophyll cycle pigment pool. Calculations were as described in (Couso *et al.*, 2012) using equation 5.1, where *Anth*: Antheraxanthin, *Zea*: Zeaxanthin and *Viola*: Violaxanthin.

$$De - epoxidation\ state = [Anth. + Zea.]/[Anth. + Zea. + Viola.] \quad \text{e.q. 5.1}$$

5.2.6 Statistical analyses

All statistical analyses were carried out in Statistica 12 (StatSoft Pty Ltd.). Factorial ANOVAs were used to determine the interactive effects of treatment and temperature on SOD concentrations and carotenoid contents on individual sampling days. One-way ANOVAs were used to clarify single variable effects including individual pigment content and proportion comparisons between species, at individual treatments. Fisher LSD post-hoc tests were used to determine significant differences assigned at $p< 0.05$. Partial eta-squared was used to determine effect sizes where significant interactions of temperature and treatment were identified (Richardson, 2011). Homogeneity of variances and normality assumptions were verified using Cochran-C and Levene's tests, respectively. Data not fulfilling assumptions of homogeneity of variance and normality were log transformed. As this research aimed to investigate effect of high light, temperature and Mo stress for enhancement of pigment products, only considerable treatment impacts are highlighted in the figures of this chapter. An additional summary of detailed statistical analyses can be made available upon request.

5.3 Results

5.3.1 Time response of SOD activity to high light, temperature and molybdenum stress in six freshwater chlorophytes

SOD production responses showed two general patterns (Figure 5.2): either an activity spike on day 1 (1.2-2.5, 0.3-1.2 and 0.2-0.8 units (U) mg^{-1} DW for *D. armatus*, *C. proboscideum* and *Haematococcus* sp., respectively), followed by decline to levels similar to those at inoculation, or increasing SOD activity on day 3 followed by a slow decline reaching similar levels on day 10 as those on day 1 (0.6-1 and 0.7-0.9 U mg^{-1} DW in *D. maximus* and *G. emersonii*, respectively). *Desmodesmus* sp. and *Haematococcus* sp. showed the lowest SOD activity (0.1 and 0.8 U mg^{-1} DW, respectively) with *Desmodesmus* sp. showing no response of SOD activity with treatment. In general SOD responses to high light (compare 24 °C control to inoculum levels on day 0) were modest not exceeding responses to molybdenum and/or temperature treatments. Molybdenum-treatment and temperature had no significant effects on SOD activity for *Desmodesmus* sp., *G. emersonii* and *Haematococcus* sp. In contrast, significant interactive effects of both parameters were identified in *D. armatus* on days 1 and 10 (Factorial ANOVA, $F_{(1, 1)}=27.85$, $p<0.05$ and $F_{(1, 1)}=10.34$, $p=0.01$, respectively), and significant effects of Mo only, on day 3 (Factorial ANOVA, $F_{(1, 1)}=19.71$, $p<0.05$).

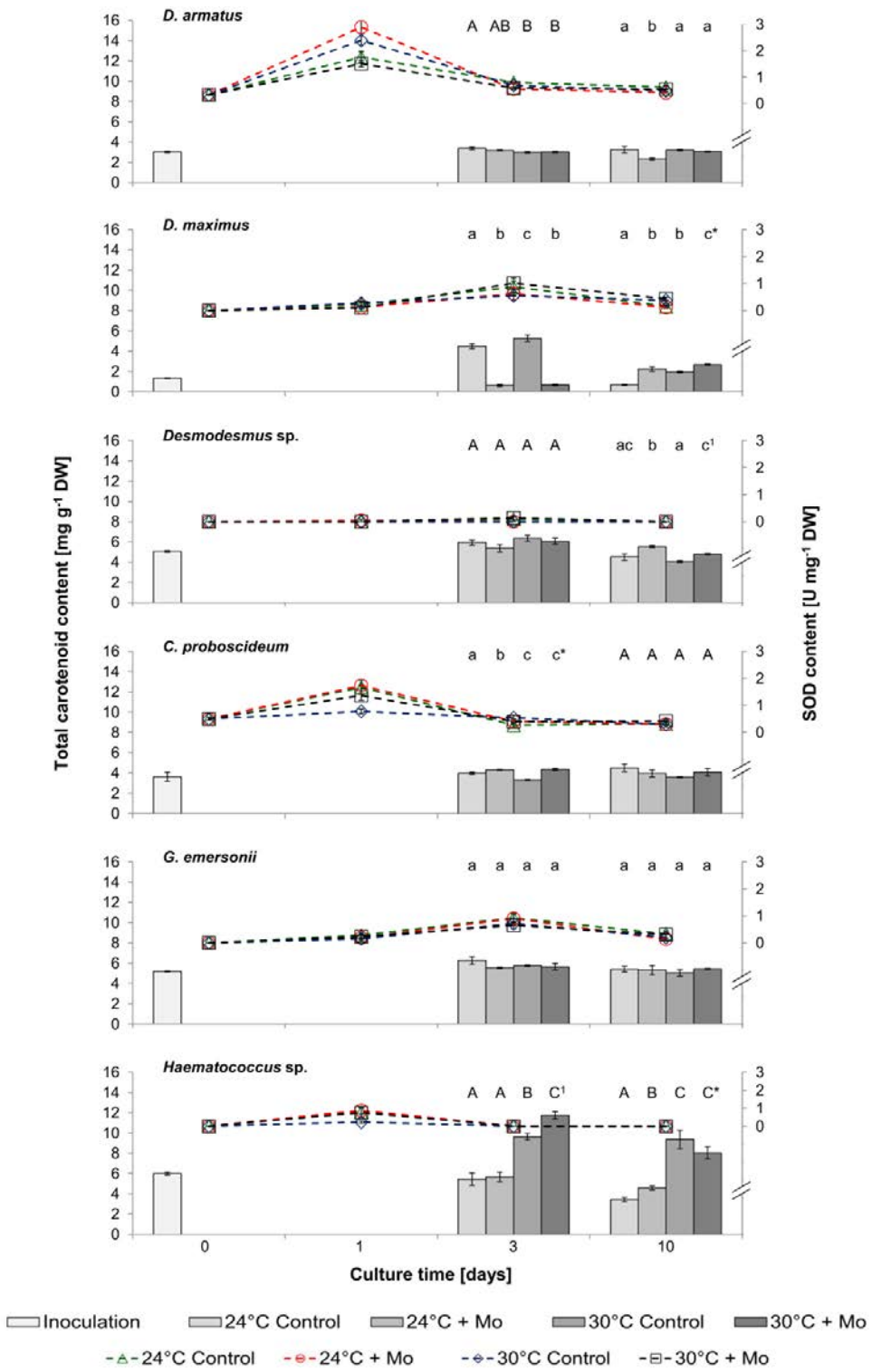

Figure 5.2. Ten-day time course of the effect of high light, temperature and molybdenum (Mo) treatments on total carotenoid contents [mg g^{-1} DW] and SOD activities [U mg^{-1} DW] in *D. armatus*, *D. maximus*, *Desmodesmus* sp., *C. proboscideum*, *G. emersonii* and *Haematococcus* sp. n=3. Standard error is shown. Letters used reflect the main effect driver determined by partial-eta squared value. Capital letters: statistical interactions driven by temperature. Lower case letters: statistical interactions driven by treatment. *: Interactive effects of temperature and treatment. [1]: Individual effects of both temperature and treatment.

A significant effect of temperature was also observed in *C. proboscideum* on day 1 (Factorial ANOVA, $F_{(1, 1)}$=15.48, p<0.05). Effects of metal treatment, however, did not show any consistent patterns with temperature within species or between species. For example, SOD activity was highest in *D. armatus* in 24 °C + Mo treatment and 30°C control cultures on day 3. In contrast, SOD activity was lowest for 30 °C controls in *C. proboscideum* on day 1 compared to those at 24 °C and the Mo-treatments at both temperatures, while in *D. maximus*, temperature induced a 2.5-4 times higher SOD activity at 30°C compared to 24 °C on day 10.

5.3.2 Time response of total carotenoid content to high light, temperature and molybdenum stress in six freshwater chlorophytes

Total carotenoid content varied with species and only in some instances with treatment, with the highest contents observed in *Haematococcus* sp. (3.4-11.8 mg g^{-1} DW), followed by *G*. *emersonii* (5-6.2 mg g^{-1} DW), *Desmodesmus* sp. (4-6.2 mg g^{-1} DW) and *C*. *proboscideum* (3.3-4.5 mg g^{-1} DW), being lowest in *D*. *armatus* (2.3-3.3 mg g^{-1} DW) (Figure 5.2). Except for *D*. *maximus* and *Haematococcus* sp., irrespective of whether or not an SOD response was observed on day 1 or day 3, total carotenoid content did not differ greatly from those of the inoculi in response to high light exposure, Mo-treatment, high temperature and combined high temperature-Mo treatments and appeared to be influenced by treatment time in some instances and were species-specific (Figure 5.2). For example, despite a marked SOD activity response on day 1 following exposure to high light, carotenoid contents decreased only marginally in *D*. *armatus*, while no treatment effect was observed in *C*. *proboscideum* compared to controls at 24°C (Figure 5.2). While a slight negative effect

of Mo treatment was visible on day 10 in *D. armatus*, which was more pronounced for 24°C cultures, that also showed the highest SOD activity, *C. proboscideum* showed no significant carotenoid responses with treatment time (Figure 5.2). Coinciding with no SOD response or very small SOD activity increases on day 3, no statistically significant effect of treatment on carotenoid content was evident for *Desmodesmus* sp. and *G. emersonii* after 3 days of cultivation. In contrast to *G. emersonii*, however, cultivation time led to slight reductions with temperature (more pronounced at 30 °C) (20-36 %), but declines were negligible or lower in Mo-treatments (4.8-5.5 *vs.* 4-4.5 mg g^{-1} DW for temperature controls) (Figure 5.2). Following a small increase of SOD activity on day 1 (except for 30 °C controls), carotenoid content was significantly increased by temperature on day 3 in *Haematococcus* sp., which was further enhanced by Mo-treatment (from 5.5 to 9.6-11.8 mg g^{-1}, respectively, decreasing by 20-37 % with incubation time, except for 30 °C controls (Figure 5.2). In contrast, significant carotenoid content increases above 24 °C inoculi contents were observed in *D. maximus* on day 3 at both temperatures (4.5-5.2 mg g^{-1} DW), coinciding with a minor peak in SOD activity, while Mo-treatments had lower than inoculi contents (0.6-0.7 mg g^{-1} DW), increasing with incubation time for the Mo treatments (72-75 %) but decreasing significantly by (63 and 85 %) for the temperature controls (Figure 5.2).

5.3.3 Time response of pigment profiles to high light, temperature and molybdenum stress in six freshwater chlorophytes

In general, no large effects of treatments on pigment proportions of the total carotenoid content were observed for *D. armatus*, *Desmodesmus* sp., *C. proboscideum*

and *G. emersonii*, while large changes were observed in *D. maximus* and *Haematococcus* sp. (Figure 5.3).

For *Haematococcus* sp., astaxanthin content increased dramatically with treatment and treatment time becoming the dominant pigment. In *D. maximus*, lutein content increased noticeably with treatment, specifically high light and temperature, with enhanced proportions being observed with cultivation time, while the inverse was recorded for the proportion of neoxanthin (Figure 5.3).

Detailed pigment content changes are described below comparing between species responses to treatments. Pigment profiles were species-specific and affected differently by high light, temperature, Mo treatment and culture time. In general, neoxanthin, β-carotene and total chlorophyll contents (Figures 5.4-5.9) followed the same responses to treatments as described for the individual species for total carotenoids in section 5.2. Content ranges for the treatments were highest for total chlorophyll ranging from (19-33 mg g^{-1} DW) in *Desmodesmus* sp., also containing the highest β-carotene (0.5-0.8 mg g^{-1} DW) and neoxanthin (0.6-1.2 mg g^{-1} DW) contents (Figure 5.6), followed by *D. maximus*, *G*. *emersonii* and *C*. *proboscideum* with total chlorophyll, β-carotene and neoxanthin contents of (10-22, 14-20 and 9-13 mg g^{-1} DW total chlorophyll (Figure 5.5), 0.02-0.6, 0.3-0.5 and ~0.3 mg g^{-1} DW β-carotene (Figure 5.8) and 0.1-1, 0.5-0.8 and 0.3-0.5 mg g^{-1} DW neoxanthin (Figure 5.7), respectively).

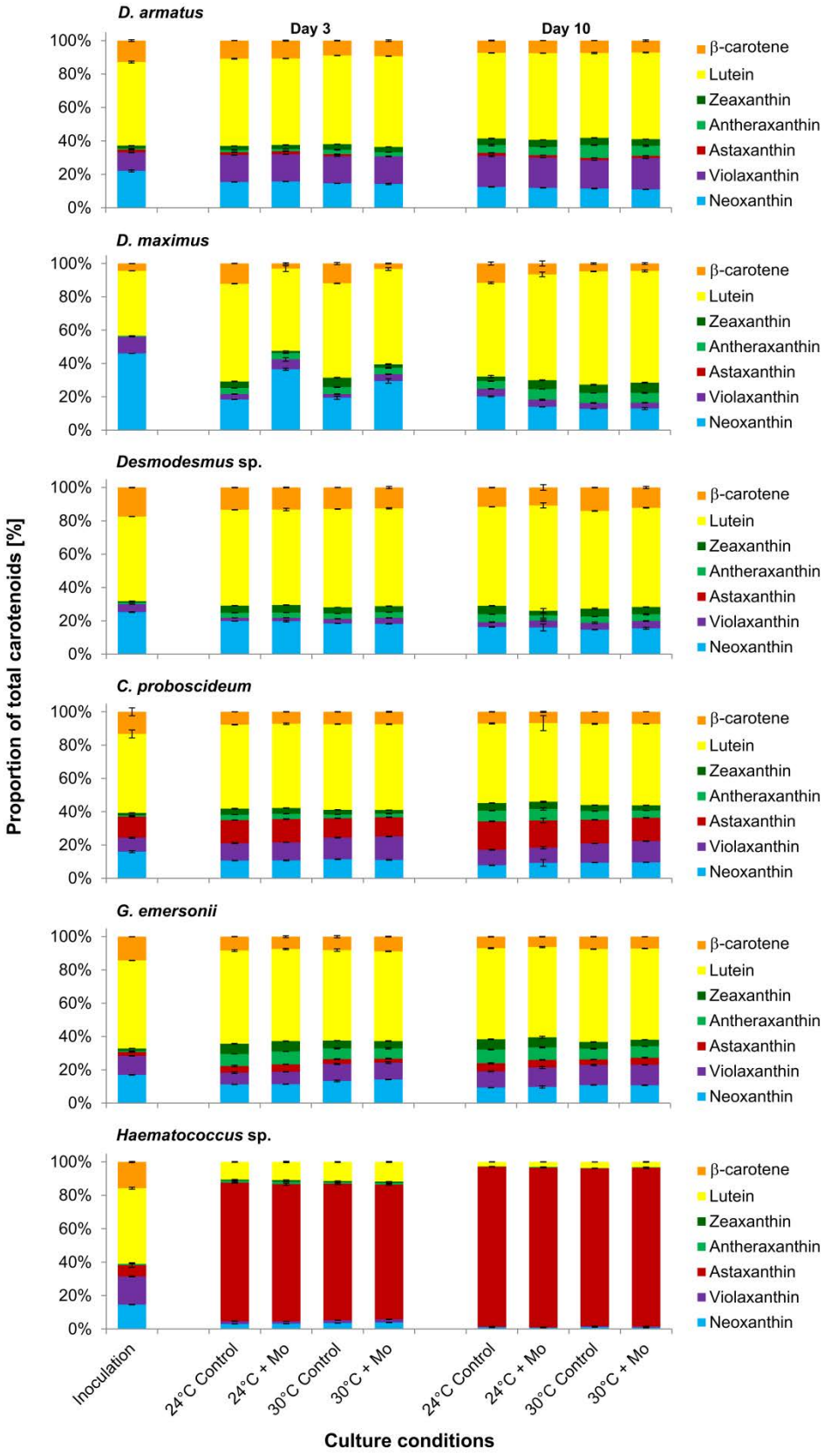

Figure 5.3. Time responses of individual pigment proportions [%] of total carotenoids in *D. armatus*, *D. maximus*, *Desmodesmus* sp., *C. proboscideum*, *G. emersonii*, and *Haematococcus* sp.to high light, temperature and molybdenum treatment. n=3. Standard error is shown.

In comparison, total chlorophyll, β-carotene and neoxanthin contents were only slightly lower for *D*. *armatus* (8-14, 0.2-0.4 and 0.3-0.5 mg g^{-1} DW, respectively) (Figure 5.4), while treatments resulted in 85-99 %, 100% and 47-97 % decreased contents of total chlorophyll, β-carotene and neoxanthin, respectively, in *Haematococcus* sp., with residual contents of 0.3-5 and 0.03-0.5 mg g^{-1} DW chlorophyll and neoxanthin, respectively remaining (Figure 5.9).

For the other pigments, antheraxanthin content varied between 0.1-0.5 mg g^{-1} DW, with *G*. *emersonii* having the highest and *Haematococcus* sp. the lowest (Figures 5.4 – 5.9), while violaxanthin content was high in *Haematococcus* sp. at inoculation (~1 mg g^{-1} DW) (Figure 5.9), followed by *D*. *armatus* (~0.6 mg g^{-1} DW, Figure 5.4), *C*. *proboscideum* (~0.6 mg g^{-1} DW, Figure 5.7) and *G*. *emersonii* (~0.7 mg g^{-1} DW, Figure 5.8). *G*. *emersonii* also reached the highest zeaxanthin content (~0.4 mg g^{-1} DW, Figure 5.8), followed by *D*. *maximus* (~0.3 mg g^{-1} DW, Figure 5.5), *Desmodesmus* sp. (~0.3 mg g^{-1} DW, Figure 5.6) and *C*. *proboscideum* (~0.2 mg g^{-1} DW, Figure 5.7). The highest lutein content was observed in *Desmodesmus* sp. (~3.8 mg g^{-1} DW, Figure 5.6), *G*. *emersonii* (~3.5 mg g^{-1} DW, Figure 5.8) and *D*. *maximus* (~3 mg g^{-1} DW, Figure 5.5), with an exceptionally strong 3-day-responses to high light and temperature observed in *D*. *maximus* (Figure 5.5). Compared to inoculum content, treatment responses for these pigments were small for *D*. *armatus* (except for antheraxanthin, Figure 5.4) and small for violaxanthin for *C*. *proboscideum* (Figure 5.7) and *G*. *emersonii* (Figure 5.8). Except for *D*. *maximus*, where levels increased (Figure 5.5), high light had a negative effect on total chlorophyll, β-carotene and neoxanthin contents (Figures 5.4 – 5.9), while it generally positively affected antheraxanthin, zeaxanthin and lutein contents, except for the latter in *Haematococcus* sp. (Figure 5.9).

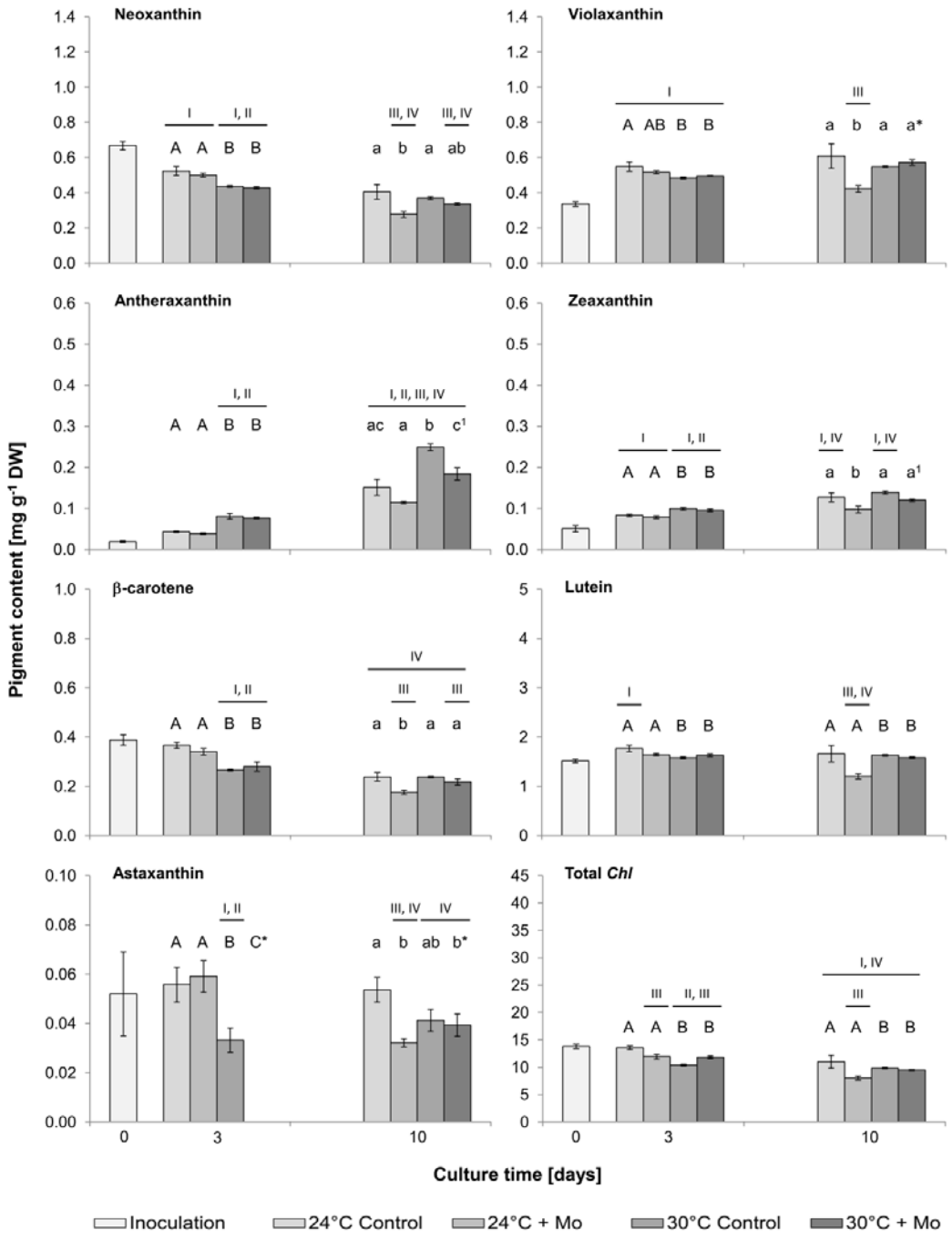

Figure 5.4. Time response of pigment contents [mg g^{-1} DW] to high light, temperature and molybdenum stress in *D. armatus*. n=3. Standard error is shown. Axes are standardized where possible; however in certain cases different scales are required to visualise responses. Roman numerals describe significant effects between all treatments over time. I: effect of light, II: effect of temperature, III: effect of molybdenum, IV: effect of time. Lettering describes interactive effects between temperature and molybdenum on pigment contents on days 3 and 10. Capital letters: statistical interactions driven by temperature; lower case letters: statistical interactions driven by molybdenum treatment. *: interactive effects of temperature and molybdenum treatment. [1]: Individual effects of both temperature and molybdenum treatment.

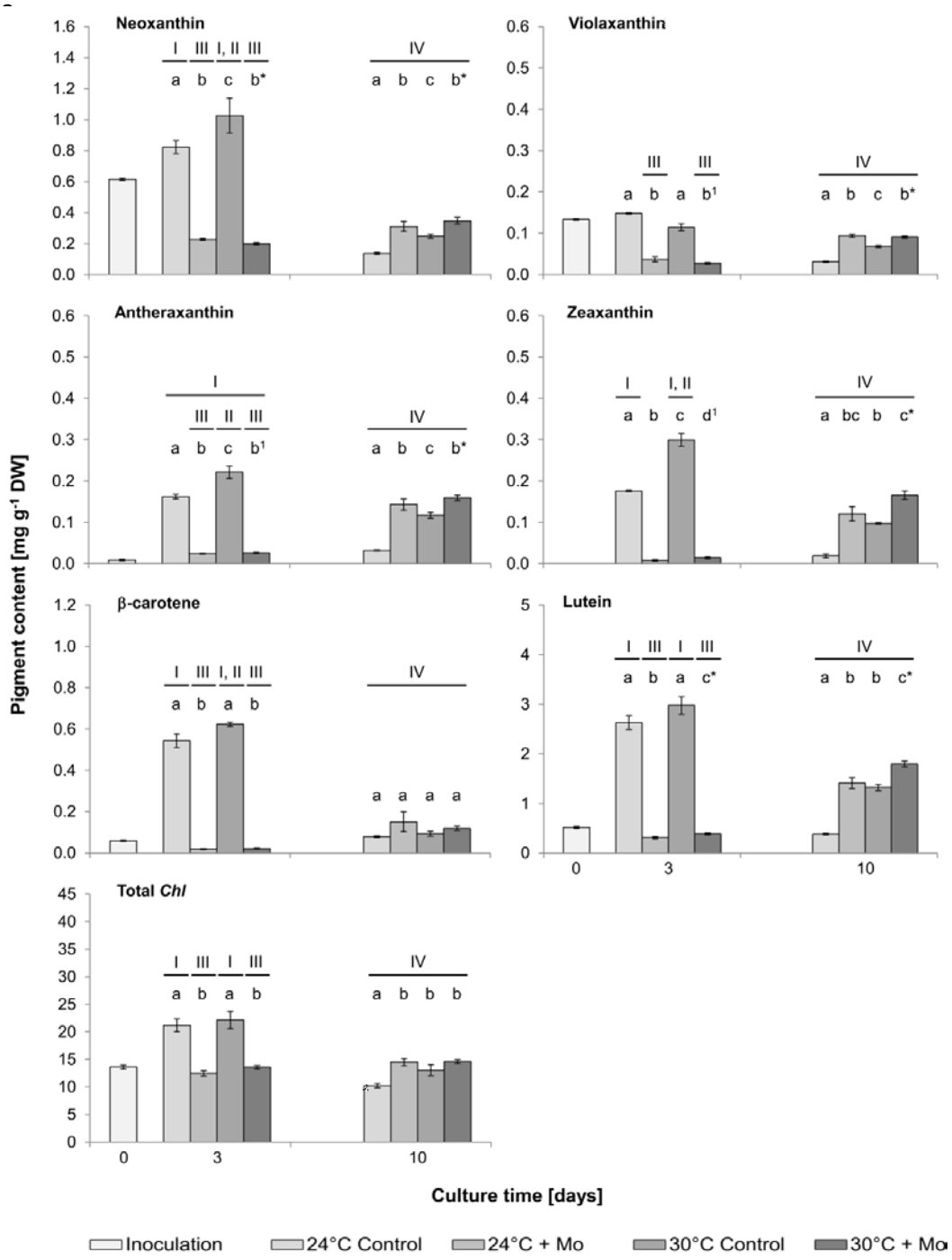

Figure 5.5. Time response of pigment contents [mg g^{-1} DW] to high light, temperature and molybdenum stress in *D. maximus*. n=3. Standard error is shown. Axes are standardized where possible; however in certain cases different scales are required to visualise responses. Roman numerals describe significant effects between all treatments over time. I: effect of light, II: effect of temperature, III: effect of molybdenum, IV: effect of time. Lettering describes interactive effects between temperature and molybdenum on pigment contents on days 3 and 10. Capital letters: statistical interactions driven by temperature; lower case letters: statistical interactions driven by molybdenum treatment. *: interactive effects of temperature and molybdenum treatment. [1]: Individual effects of both temperature and molybdenum treatment.

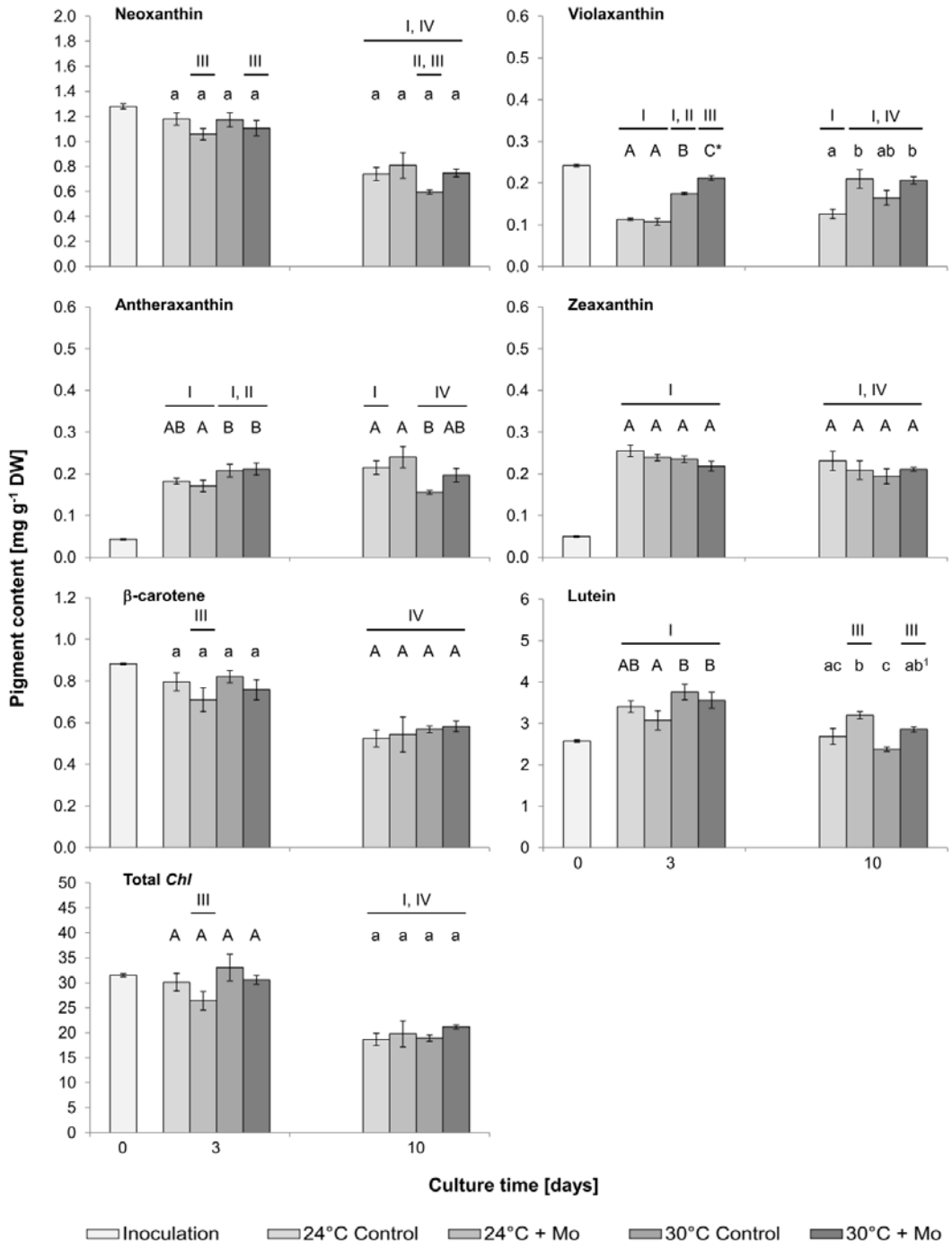

Figure 5.6. Time response of pigment contents [mg g^{-1} DW] to high light, temperature and molybdenum stress in *Desmodesmus* sp. n=3. Standard error is shown. Axes are standardized where possible; however in certain cases different scales are required to visualise responses. Roman numerals describe significant effects between all treatments over time. I: effect of light, II: effect of temperature, III: effect of molybdenum, IV: effect of time. Lettering describes interactive effects between temperature and molybdenum on pigment contents on days 3 and 10. Capital letters: statistical interactions driven by temperature; lower case letters: statistical interactions driven by molybdenum treatment. *: interactive effects of temperature and molybdenum treatment. [1]: Individual effects of both temperature and molybdenum treatment.

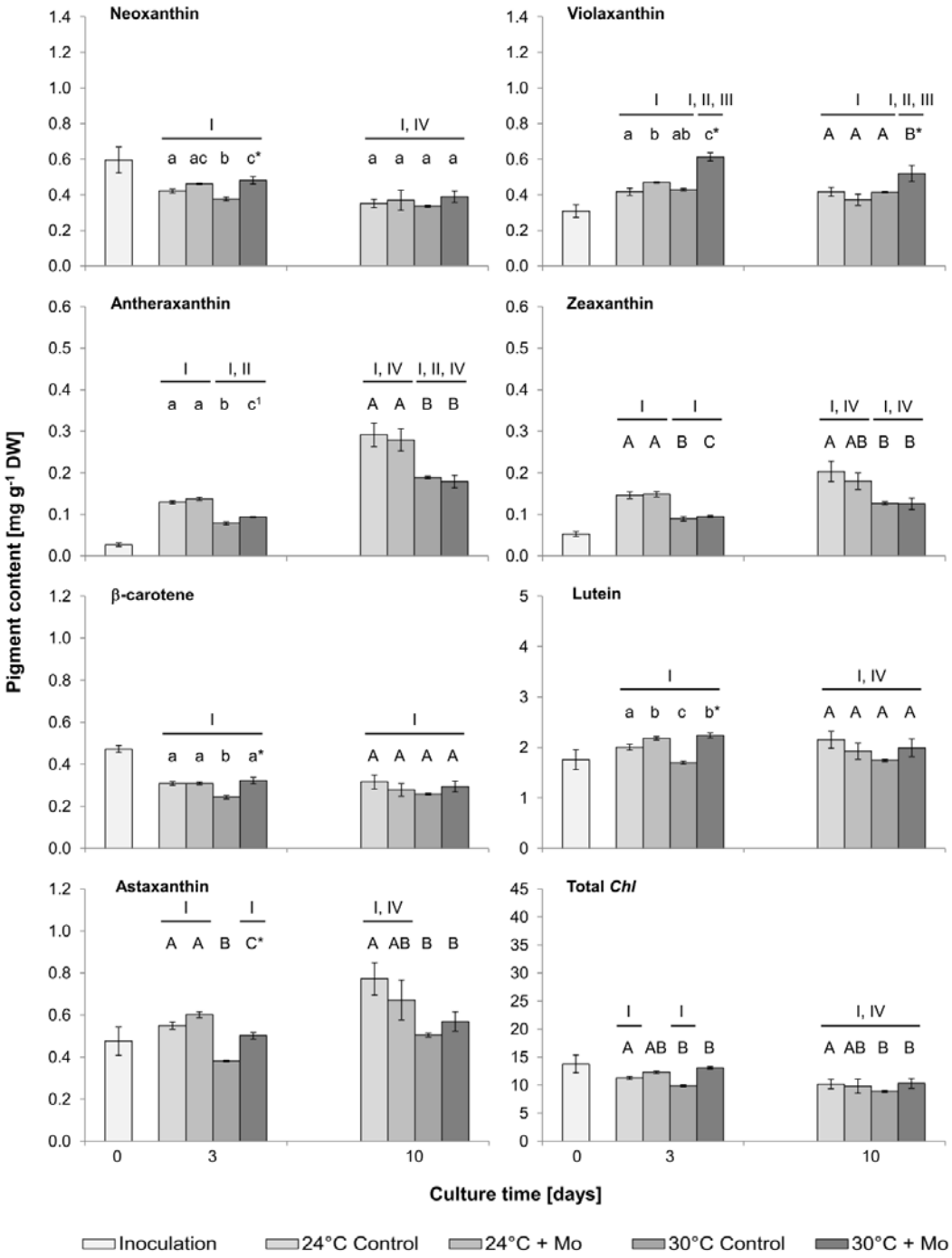

Figure 5.7. Time response of pigment contents [mg g^{-1} DW] to high light, temperature and molybdenum stress in *C. proboscideum*. n=3. Standard error is shown. Axes are standardized where possible; however in certain cases different scales are required to visualise responses. Roman numerals describe significant effects between all treatments over time. I: effect of light, II: effect of temperature, III: effect of molybdenum, IV: effect of time. Lettering describes interactive effects between temperature and molybdenum on pigment contents on days 3 and 10. Capital letters: statistical interactions driven by temperature; lower case letters: statistical interactions driven by molybdenum treatment. *: interactive effects of temperature and molybdenum treatment. [1]: Individual effects of both temperature and molybdenum treatment.

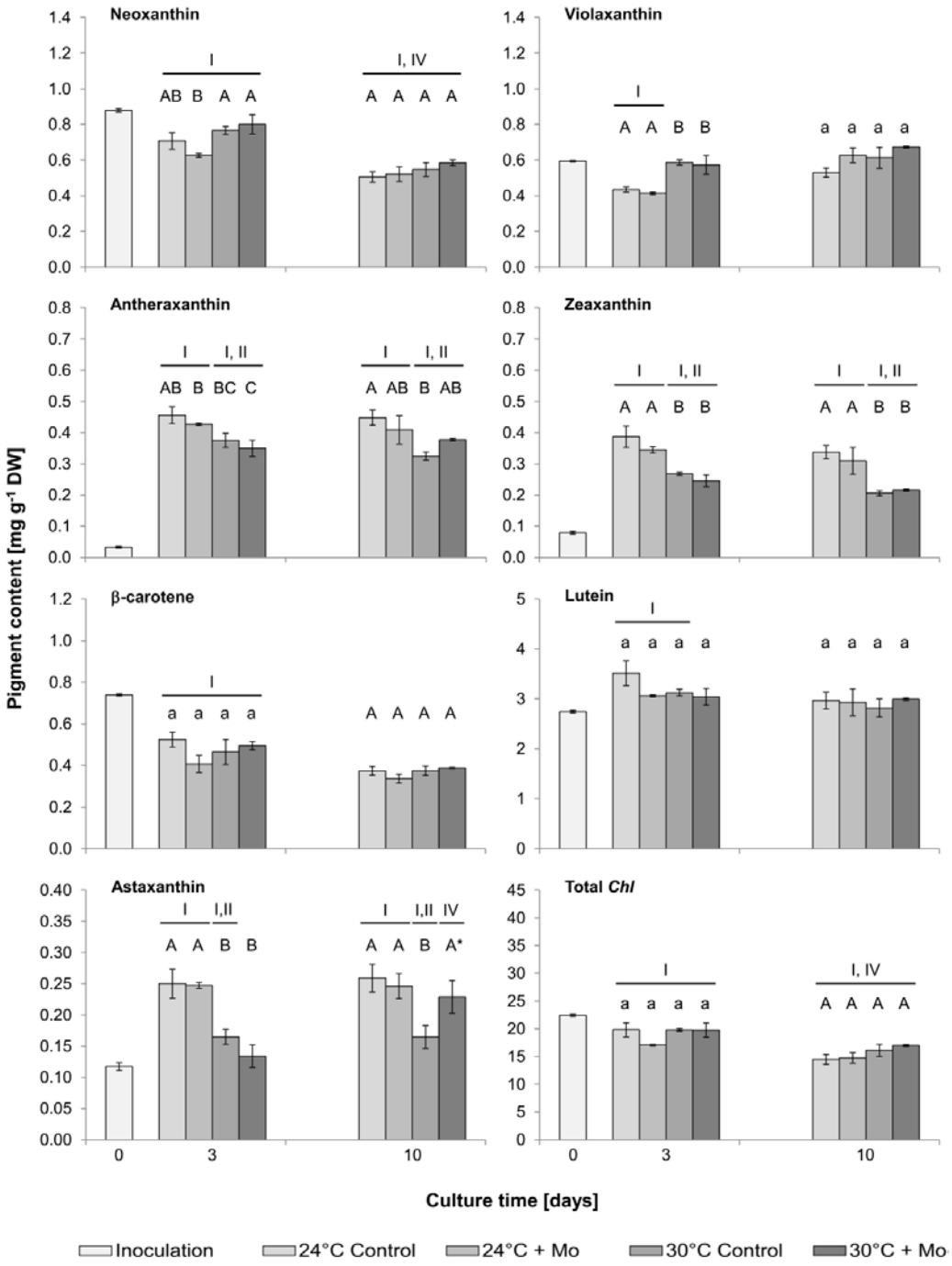

Figure 5.8. Time response of pigment contents [mg g^{-1} DW] to high light, temperature and molybdenum stress in *G. emersonii*. n=3. Standard error is shown. Axes are standardized where possible; however in certain cases different scales are required to visualise responses. Roman numerals describe significant effects between all treatments over time. I: effect of light, II: effect of temperature, III: effect of molybdenum, IV: effect of time. Lettering describes interactive effects between temperature and molybdenum on pigment contents on days 3 and 10. Capital letters: statistical interactions driven by temperature; lower case letters: statistical interactions driven by molybdenum treatment. *: interactive effects of temperature and molybdenum treatment. [1]: Individual effects of both temperature and molybdenum treatment.

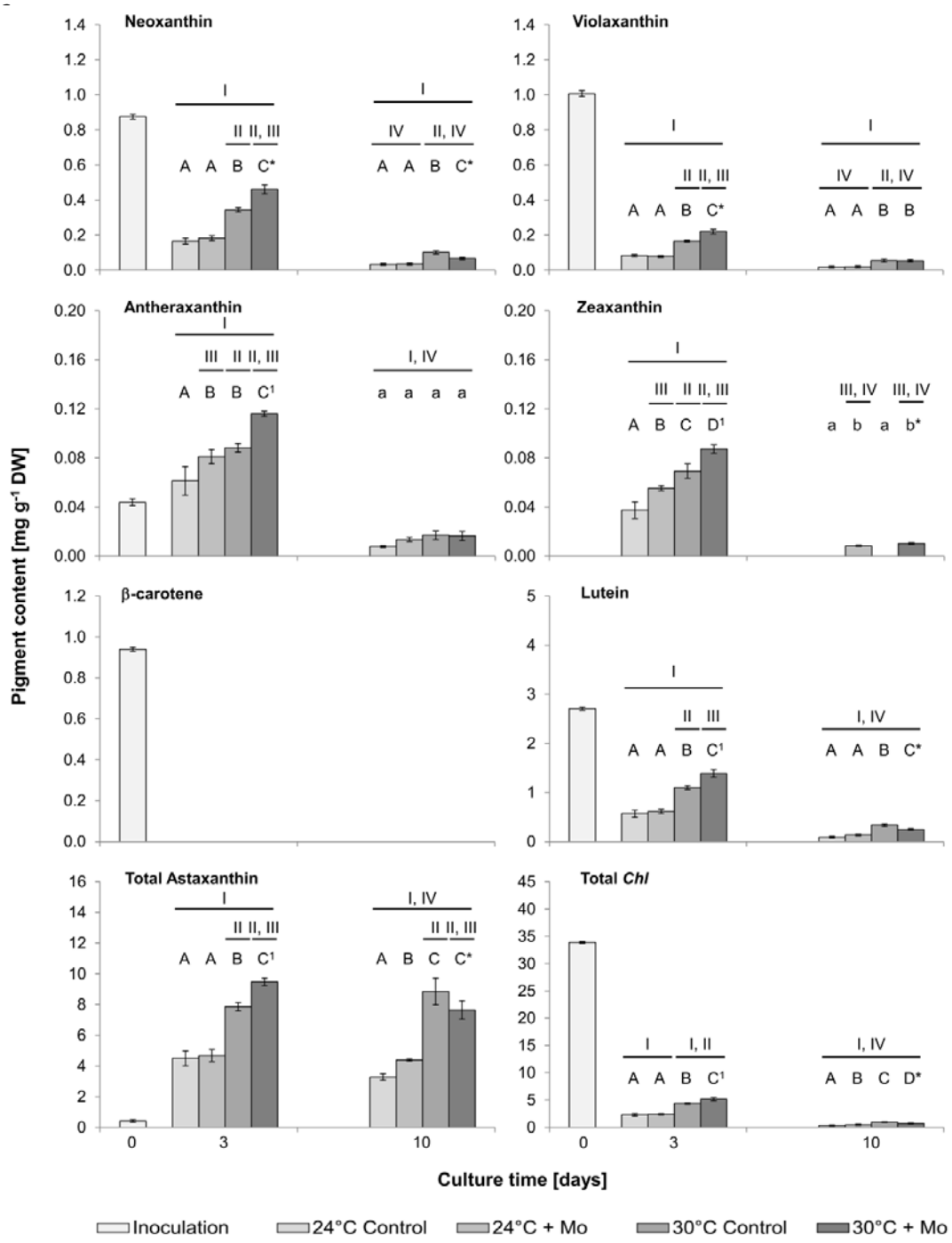

Figure 5.9. Time response of pigment contents [mg g^{-1} DW] to high light, temperature and molybdenum stress in *Haematococcus sp.* n=3. Standard error is shown. Axes are standardized where possible; however in certain cases different scales are required to visualise responses. Roman numerals describe significant effects between all treatments over time. I: effect of light, II: effect of temperature, III: effect of molybdenum, IV: effect of time. Lettering describes interactive effects between temperature and molybdenum on pigment contents on days 3 and 10. Capital letters: statistical interactions driven by temperature; lower case letters: statistical interactions driven by molybdenum treatment. *: interactive effects of temperature and molybdenum treatment. [1]: Individual effects of both temperature and molybdenum treatment.

In contrast, effects on violaxanthin contents were mixed, with positive responses only observed for *D*. *armatus* (Figure 5.5) and *C*. *proboscideum* (Figure 5.8).

Of the six species investigated, effects of high light alone and in conjunction with incubation time were detrimental to most pigments in *Haematococcus* sp., out-weighing the effects of temperature and molybdenum treatments, except for astaxanthin content, which will be described separately together with the other two astaxanthin producers, *D*. *armatus* and *G*. *emersonii*. Initially, large positive effects of high light were observed for antheraxanthin and zeaxanthin in *Haematococcus* sp., with contents additionally increasing in response to elevated temperature and molybdenum treatment on day 3, but decreasing sharply with prolonged exposure to high light (Figure 5.9). In contrast, while high light induced large positive responses of these pigments in the astaxanthin-producer *G*. *emersonii*, high temperature and molybdenum treatment had negative effects which remained largely unchanged with incubation time (Figure 5.8). While initial responses of these pigments to high light were modest in the other astaxanthin-producer *D*. *armatus*, incubation time and high temperature led to improved contents, which were less pronounced in the presence of molybdenum (Figure 5.4).

In contrast to *Haematococcus* sp., the effects of high light did not out-weigh effects of temperature and molybdenum treatments in the non-astaxanthin producing *D*. *maximus* (Figure 5.5). For the first three days, strong positive effects of high light were generally enhanced by high temperature (except for violaxanthin), while molybdenum had a negative effect, reducing all pigment concentrations to, or below inoculum levels (Figure 5.5). Prolonged treatment with high light (day 10) also reduced pigment levels to or below inoculum levels, but high temperature and molybdenum

treatments had positive effects, with responses to the latter stressor being larger than to elevated temperature alone (Figure 5.5). In contrast, while high light responses were similar for *Desmodesmus* sp., high temperature, treatment time and, molybdenum treatment in particular, had less of an effect (except for violaxanthin) (Figure 5.6). Violaxanthin content decreased with exposure to high light and, while molybdenum treatment initially had no effect at 24 °C compared to the combined effect of molybdenum and high temperature treatment, which elicited a stronger positive response than high temperature alone (day 3), prolonged exposure at 24 °C (day 10) improved violaxanthin contents to those of the combined treatment (Figure 5.6).

Of the astaxanthin producers, *Haematococcus* sp. showed the strongest total astaxanthin response and highest levels (7.7-9.5 mg g^{-1} DW) under high light, temperature and temperature + Mo treatments, with increased levels being sustained compared to the inoculum, but increasing slightly for 30 °C controls, whilst reducing for 24 °C controls and 30 °C + Mo treatment with culture time (Figure 5.9). Significantly lower levels of total astaxanthin of 0.4-0.8, 0.03-0.06 and 0.1-0.3 mg g^{-1} DW were achieved by *C. proboscideum* (Figure 5.7), *D. armatus* (Figure 5.4) and *G. emersonii* (Figure 5.8), respectively. In contrast to *Haemotcoccus* sp., positive responses to high light were modest in *C. proboscideum* and slightly enhanced by Mo-treatment at 24 °C, while high temperature had a negative effect, with a slight positive response observed by Mo-treatment at 30 °C (Figure 5.7). Compared to *C. proboscideum*, total astaxanthin content was affected similarly by high light and molybdenum treatment in *G. emersonii*, as was the sustained or enhanced response (30 °C + Mo) with cultivation time, but responses to temperature and temperature + Mo treatments were inversed

(Figure 5.8). In contrast, total astaxanthin content improved only marginally in response to high light and molybdenum treatment in *D. armatus*, while temperature and molybdenum treatments had a negative effect particularly over culture time (Figure 5.4).

In contrast to *Haematococcus* sp. and *C. proboscideum*, no astaxanthin esters were detected in *D. armatus* and *G. emersonii*, consequently astaxanthin concentrations reported for these species is free astaxanthin. While in *Haematococcu*s sp. free astaxanthin was present at similar concentrations to astaxanthin esters, ~75 % was free astaxanthin in *C. probscideum* at the time of inoculation (Table. 5.1).

Table 5.1. Time effect of high light, temperature and molybdenum stress on free astaxanthin and astaxanthin ester content [mg g^{-1} DW] in *Haematococcus* sp. and *C. proboscideum*.

		***Haematococcus* sp.**		***C. proboscideum***	
		Astaxanthin		**Astaxanthin**	
		Free	**Esters**	**Free**	**Esters**
Inoculation	24°C, LL	0.3 ± 0.01	0.2 ± 0.01	0.3 ± 0.05	0.2 ± 0.02
Day 3	24°C, HL Control	0.7 ± 0.08	3.8 ± 0.4	0.3 ± 0.01	0.2 ± 0.01
	24°C, HL + Mo	0.8 ± 0.06	3.8 ± 0.3	0.3 ± 0.01	0.3 ± 0.01
	30°C, HL Control	1.3 ± 0.1	6.5 ± 0.2	0.2 ± 0.01	0.2 ± 0.01
	30°C, HL + Mo	1.8 ± 0.1	7.7 ± 0.1	0.2 ± 0.01	0.3 ± 0.01
Day 10	24°C, HL Control	0.3 ± 0.02	3 ± 0.2	0.5 ± 0.05	0.3 ± 0.03
	24°C, HL + Mo	0.4 ± 0.01	4 ± 0.03	0.4 ± 0.06	0.3 ± 0.04
	30°C, HL Control	0.8 ± 0.05	8.1 ± 0.8	0.3 ± 0.01	0.2 ± 0.02
	30°C, HL + Mo	0.6 ± 0.09	7 ± 0.5	0.3 ± 0.03	0.3 ± 0.03

LL: low light, HL: high light

High light and high temperature greatly induced astaxanthin ester accumulation in *Haematococcus* sp., while molybdenum treatment had a marginal or no effect (Table 5.1). In contrast, no large or consistent changes were identifiable with treatment in *C. proboscideum* (Table 5.1).

5.3.4 Time effect of high light, temperature and molybdenum stress on the de-epoxidation state in six freshwater chlorophytes

De-epoxidation state was calculated to quantify the de-epoxidized proportion of the total xanthophyll cycle pigment pool as an indication of the degree of light stress. All species showed the lowest de-epoxidation state at inoculation when cultivated at 24°C and low light (Table 5.2) prior to exposure to increased light conditions.

By day 3, this ratio increased in all species with the lowest increase (11-37 %) in *D. armatus* and the highest increase in *Desmodesmus* sp., followed by *G. emersonii*, *Haematococcus* sp. and *D. maximus* (~60-90 %). Ratios increased a further 30-40 % with culture time in *D. armatus* and *C. proboscideum*, but decreased 10-50 % in *G. emersonii*, *Haematococcus* sp. and *D. maximu*s control cultures, suggesting acclimation or protective responses (e.g. astaxanthin in *Haematococcus* sp.) to high light and temperature. *Desmodesmus* sp. showed the highest effects of light stress with 70-80 % of the xanthophyll pool de-epoxidized irrespective of treatment, whereas *D. armatus* showed the least effect of light stress with 20-40 % de-epoxidation. *Desmodesmus maximus* showed distinct effects of molybdenum treatment with lower de-epoxidation in molybdenum- treated cultures on day 3.

Table 5.2. Time effect of high light, temperature and molybdenum stress on the de-epoxidation state (Anth.+ Zea. : Anth. +Zea.+Viola.) of six freshwater chlorophytes.

		D. armatus	*D. maximus*	*Desmodesmus* sp.	*C. proboscideum*	*G. emersonii*	*Haematococcus* sp.
Inoculation	24°C, LL	0.17	0.06	0.28	0.21	0.16	0.04
Day 3	24°C, HL Control	0.19	0.70	0.79	0.40	0.66	0.54
	24°C, HL + Mo	0.19	0.46	0.79	0.38	0.65	0.63
	30°C, HL Control	0.27	0.82	0.72	0.28	0.52	0.49
	30°C, HL + Mo	0.26	0.59	0.67	0.24	0.51	0.48
Day 10	24°C, HL Control	0.31	0.62	0.78	0.54	0.60	0.32
	24°C, HL + Mo	0.34	0.74	0.68	0.55	0.53	0.56
	30°C, HL Control	0.41	0.76	0.68	0.43	0.46	0.24
	30°C, HL + Mo	0.35	0.78	0.66	0.37	0.47	0.34

LL: low light; HL: high light

5.4 Discussion

The outcomes of this research identified mixed time responses of SOD, pigment contents and de-epoxidation states to high light, temperature and molybdenum stress in six freshwater chlorophytes. High light and temperature, as well as metal stress are factors known to induce reactive oxygen species (ROS) responses, affect the de-epoxidation state of pigments and affect pigment profiles in general (Benamotz *et al.*, 1983; Couso *et al.*, 2012; Sanchez *et al.*, 2008; Steinbrenner *et al.*, 2001). Two of these chlorophytes (*D*. *armatus*, *D*. *maximus*) were isolated from the tailings-dam of the Stanwell Corp. operated coal-fired power station in southeast Queensland, three were isolated from tropical north Australia (*Desmodesmus* sp., *C*. *proboscideum* and *G*. *emersonii*) and *Haematococcus* sp. was included as a reference response organism and for benchmarking astaxanthin contents. These species and the conditions were chosen to evaluate pigment product potential in tailings-dam water at the Stanwell Corp. coal-fired power station.

5.4.1 Effect of high light, temperature and molybdenum on ROS responses

Two basic mechanisms have been identified to protect photosynthetic species against reactive oxygen stress, namely reduction of ROS and detoxification (Jahns *et al.*, 2012). ROS formation can be triggered either at the acceptor site of the photosystems leading to the generation of the superoxide radical (O_2^-) or via energy transfer from triplet chlorophyll ($^3Chl^\bullet$) yielding singlet oxygen ($^1O_2$). Superoxide radicals are converted to hydrogen peroxide (H_2O_2), which can be detoxified by SOD, whereas carotenoids primarily serve the function to detoxify $^3Chl^\bullet$ and $^1O_2$.

Xanthophyll cycle responses are discussed in detail in section 5.4.2, but are integrated here to infer information on ROS species and defense strategies adopted by the six freshwater chlorophytes to the different stressors.

A number of SOD studies on *Desmodesmus* sp. and *Scendesmus* sp. showed that SOD responses (quantities, duration and production patterns) were species- and strain-specific (Pokora *et al.*, 2014; Pokora *et al.*, 2003; Pokora *et al.*, 2013), which was also observed in the present study. Rapid SOD production was generally induced in microalgae exposed to ROS (e.g. *Haematococcus* exposed to the superoxide anion generator methyl viologen) (Liu *et al.*, 2010), but similarly in ageing algal cultures (e.g. 28 day *Scenedesmus* sp. cultures) (Pokora *et al.*, 2003). Direct comparisons between studies are difficult due to the variety of units used (U mg^{-1} protein, U mg^{-1} Chl, U mg^{-1} DW or U $cell^{-1}$) in addition to differences in stressor selection and experimental parameters. In general, the largest SOD responses were observed on day 1, while responses on day 3 were lower. *Desmodesmus* sp. was the only species showing no SOD response and minor fluctuations in total carotenoids making it the most tolerant species to the stressors applied. The high light-induced large increase in the de-epoxidation state (Table 5.2) suggests effective management of high light-induced ROS ($^3Chl^{\bullet}$ and $^1O_2$) *via* non-photochemical quenching (NPQ) (Figure 5.6) (see section 5.4.2). There were few indications in this study to show that SOD activity was linked to or influenced by carotenoid production, as species with significant day 1-SOD responses (*D*. *armatus*, *C*. *proboscideum*) to temperature and metal treatment showed no large effects on total carotenoid content, suggesting that detoxification of O_2^--derived H_2O_2 was required early. This is supported by xanthophyll cycle pigment content (Figures 5.4 and 5.7, respectively) and moderate increases in the de-epoxidation state (Table

5.2), suggesting an acclimation with time to the stressful environmental conditions imposed. While no quantitatively comparable studies exist for *C. proboscideum* and *D. armatus* SOD production, SOD activity patterns of *D. armatus* were similar to those reported for *Scenedesmus obliquus*, with activity increases for the first 1-2 days (Pokora *et al.*, 2003).

Three day exposure to high light in combination with Mo and elevated temperature elicited a measurable SOD response in *G*. *emersonii* and *D*. *maximus*, suggesting some detoxification requirement of ROS *via* SOD. In contrast to *G*. *emersonii*, the total carotenoid content response to high light exposure was far more extensive for *D*. *maximus*. Nonetheless, the xanthophyll cycle pigment profiles (Figures 5.5 and 5.8), and large increases in de-epoxidation states (Table 5.2) suggesting that high light stress induced $^{3}Chl^{\bullet}$- and $^{1}O_2$-ROS, which is more efficiently dealt with via NPQ (Jahns *et al.*, 2012). In contrast to *G*. *emersonii*, *D*. *maximus* showed a large increase in total lutein content (Figure 5.5) in response to 3-day high light exposure, with levels reducing for day 10, suggestive of an acclimation to continuous stress (see section 5.4.3). The strong lutein response could imply that high light induces ^{3}Chl and $^{1}Chl^{\bullet}$, which have been shown to be effectively managed by lutein in higher plants (Jahns *et al.*, 2012).

In *Haematococcus* sp., a moderate increase in SOD activity was observed on day 1 elicited by high temperature and Mo treatment, rather than high light. This increase in SOD activity has been described previously as a short term strategy to survive oxidative stress prior to carotenoid synthesis which is a long-term strategy in *H. pluvialis* (Wang *et al.*, 2011b). Which also corresponds to reports that SOD activity decreases during the transition of green flagellated cells to “brown cells” as

encystment proceeds (Liu *et al.*, 2010). Superoxide dismutase activity in *Haematococcus* sp. in this study was slightly lower (~0.75 U mg^{-1} DW) than the only comparable data for *H. pluvialis* (~1 U mg^{-1} DW), which was grown outdoors under sunlight (Chaumont *et al.*, 1995). Superoxide dismutase and astaxanthin production responses have been reported to work separately and differently in different *Haemtococcus* sp. cell types with antioxidant enzymes produced only in the absence or at low concentrations of astaxanthin, as this pigment was shown to provide more effective protection from oxidative stress (Liu *et al.*, 2010). Consequently, the low SOD activity in *Haematococcus* sp. in this study is likely due to the presence of sufficient astaxanthin to protect the cells from oxidative stress. Nonetheless, the impact of high light and temperature on xanthophyll cycle pigments (Figure 5.9) (see section 5.4.2) and the de-epoxidation state (Table 5.2) suggest strong ROS induction by the chosen cultivation conditions, which, while beneficial in a two-stage commercial astaxanthin production, highlights that the species is not tolerant to growth conditions experienced in the Australasian summer at Tarong (see section 6.3).

Taken together, in species with large and early SOD responses, NPQ–lowering of ROS was moderate and not reflected in the total carotenoid content; which is perhaps indicative of acclimation to the environmental conditions applied. Based on this, it might be possible to use SOD activity monitoring as a measure to evaluate acclimation capability of species. In contrast, for species showing no or delayed SOD responses, noticeable increases in total carotenoid content could be used as an indicator of stressor-induced ROS. This would then have direct implications for species selection for bioremediation and cultivation strategies commercial production for the pigment market.

5.4.2 Effect of high light, temperature and Mo treatment on xanthophyll cycle pigments

Inoculation pigment concentrations provided baseline pigment profiles in non-stressful culture conditions (24 °C; 42 µmol photons m^{-2} s^{-1}) and allow determining the single and combined effects of high light (600 µmol photons m^{-2} s^{-1}), temperature (30°C) and molybdenum treatments over time. The protective function of the xanthophyll cycle pigments in chloroplasts through non-photochemical quenching has been extensively described and is still subject to considerable studies (Demmig-Adams *et al.*, 1996; Goss *et al.*, 2010; Jahns *et al.*, 2012; Ladygin, 2000). Xanthophyll cycle kinetics, capacity and de-epoxidation state can be used as a marker for the photo-adaptive state/capacity of microalgal cells (Goss *et al.*, 2010). The decrease of violaxanthin and the corresponding increase of antheraxanthin and zeaxanthin in *Desmodemsus* sp., *Haematococcus* sp. and *D. maximus* are driven by increased irradiance and are due to the de-epoxidation of violaxanthin to zeaxanthin *via* antheraxanthin by violaxanthin de-epoxidase (Goss *et al.*, 2010; Jahns *et al.*, 2012). These patterns are common in microalgae exposed to high/saturating light intensities and have been observed in *Chlamydomonas reinhardtii*, *Nannochloropsis gaditana* and *Dunaliella salina* (Couso *et al.*, 2012; Gentile *et al.*, 2001; Jin *et al.*, 2003). Zeaxanthin has been reported to have a rigidifying effect on the membrane lipid matrix, which counterbalances the disorganization of the photosynthetic membranes induced by high irradiance and heat, and protects from peroxidative damage (Havaux, 1998), whereas violaxanthin is a better light harvesting pigment than zeaxanthin (Havaux *et al.*, 1999). Consequently, the xanthophyll cycle provides an effective mechanism for

cells to rapidly convert efficient low-light light harvesting pigments to effective photo-protectors under high light.

Haematococcus sp. initially exhibited similar xanthophyll cycle pigment responses to other species to deal with stress conditions on day 3, however by day 10 stressors applied here induced the typical and well-described life cycle progression to encystment encompassed by the over-production of astaxanthin (Borowitzka *et al.*, 1991b; Boussiba, 2000; Han *et al.*, 2012; Imamoglu *et al.*, 2009) (see section 5.4.3 for additional detail).

Steady or increasing violaxanthin contents observed for *D. armatus*, *C. proboscideum* and *G. emersonii* suggest that the chosen light intensities were less stressful than for the other species. However, antheraxanthin and zeaxanthin concentrations also increased in these species indicating violaxanthin de-epoxidation, in particular in *G. emersonii* where 50-60% of the xanthophyll pigment pool was de-epoxidized by day 3. These results could suggest an acclimation in these species to the light conditions *via* active violaxanthin synthesis (Lohr *et al.*, 1999) and subsequent de-epoxidation, or the ability to synthesize antheraxanthin and zeaxanthin as described for *Nannochloropsis gaditana* when exposed to high light (Gentile *et al.*, 2001).

Neoxanthin is the product of violaxanthin oxidation (Lichtenthaler, 2012), consequently neoxanthin synthesis will generally decrease if violaxanthin availability decreases as it is de-epoxidised to zeaxanthin in high irradiance conditions. This was observed in all species in response to high light and temperature, except for *D. maximus*. Patterns of neoxanthin decrease under high light conditions similar to those in this study have also been observed in *Chlamydomonas reinhardtii* (Couso *et al.*, 2012). The decline in neoxanthin concentrations and the maintenance of the

violaxanthin pool in *D. armatus*, *C. proboscideum* and *G. emersonii* in this study suggest re-conversion of neoxanthin to zeaxanthin, which has been demonstrated in higher plants and algae (Ladygin, 2000). In higher plants and algae the assembly of the major light harvesting pigment-protein complexes (LHC) (associated with photosystems I and II) requires the carotenoids lutein and neoxanthin in addition to Chls *a* and *b*, although the precise mechanism/contribution by the individual pigments is still a matter of debate (Croce *et al.*, 1999). In this study, both neoxanthin and, in particular, lutein contents increased in response to high light and temperature in *D*. *maximus*, which could be indicative of requirement of increased protection of the LHCs. Additionally, the observed xanthophyll pigment responses of *D*. *maximus* to high light and temperature suggest an active synthesis of violaxanthin, as an increase in neoxanthin concentrations was observed, while concentrations of its prercursor violaxanthin remained relatively constant (Figure 5.5), which has been described for green algae and higher plants (Ladygin, 2000). Furthermore, the strong increase in zeaxanthin concentrations could imply that zeaxanthin might have been partially synthesized *de novo*, as also described for *N*. *gaditana* (Gentile *et al.*, 2001).

Interestingly, and in contrast to the other species responses, Mo treatment led to a significant decrease in neoxanthin and violaxanthin content compared to controls in *D*. *maximus* without eliciting a strong SOD response, suggesting that metal treatment did not induce detoxification of ROS via this pathway. Similar pigment responses following metal treatments have been reported for *Scenedesmus quadricauda* exposed to cadmium (Çelekli *et al.*, 2013) as well as *S. incrassatulus* and *S. quadricauda* exposed to copper (Kováčik *et al.*, 2010; Perales-Vela *et al.*, 2007). It has been shown that metal stress led to the accumulation of proline and/or reduced

glutathione (GSH) (a thiol peptide, free radical scavenger, found in plants and eukaryotic algae) in *Scenedesmus* sp., *S. armatus*, *S. quadricauda* and *S. acutus* (Çelekli *et al.*, 2013; El-Enany *et al.*, 2001; Gorbi *et al.*, 2006; Tripathi *et al.*, 2006), however this remains to be confirmed. It could be possible that *D*. *maximus* utilizes a similar metal tolerance strategy, as it was isolated from high Mo concentrations-containing tailings-dam water (Stanwell Corp. Tarong coal-fired power station), which could explain that no effect on growth was observed (Figure S5.1).

Taken together, the data suggest that the xanthophyll pigment pool and derived de-epoxidation states could be used to make inferences on the light tolerance of the investigated species, as three distinct groupings could be distinguished: (i) high light tolerant species with large xanthophyll pigment pools and slow de-epoxidation rates (*D. armatus* and *C. proboscideum*), (ii) low light adapted species with small xanthophyll pigment pools and fast de-epoxidation rates (*Desmodesmus* sp., *D. maximus*) and (iii) intermediate pigment pools and de-epoxidation rates (*G. emersonii*, *Haematococcus* sp.), as suggested by Dimier *et al.* (2009).

5.4.3 Time response of pigments

In addition to changes in xanthophyll cycle pigments, exposure to increased irradiance, temperature in this study caused noticeable decreases of β-carotene and total chlorophyll concentrations in *Desmodesmus* sp. and *Haematococcus* sp., while total chlorophyll content in the other species remained similar throughout the 10-day time course. These decreases correspond to previous patterns in *Desmodesmus* sp. (Solovchenko *et al.*, 2013; Xie *et al.*, 2013), *Dunaliella salina* (Saha *et al.*, 2013), *Haematococcus pluvialis* (Kobayashi *et al.*, 1997; Torzillo *et al.*, 2003), *Scenedesmus*

armatus (Tukaj *et al.*, 2003), *Chlamydomonas reinhardtii* (Couso *et al.*, 2012) and *Nannochloropsis gaditana* (Gentile *et al.*, 2001). This shows that the chosen cultivation conditions were more stressful for *Desmodesmus* sp. and for *Haematococcus* sp. in particular.

Carotenoid profiles and their relative proportions may provide insight whether these pigments participate in active protection against stress or whether alternative protective mechanisms (e.g. NPQ via xanthophyll cycle pigments (see sections 5.4.2 and 1.2.5) or antioxidant enzymes (see sections 5.4.1 and 1.2.3)) are used. This is best exemplified by astaxanthin-proportions of the pigment pool of *D*. *armatus*, *C*. *proboscideum*, *G*. *emersonii* and *Haemotcoccus* sp. in response to stress. Astaxanthin proportions were more or less maintained for three of the four species, but increased dramatically in *Haematococcus* sp. at the expense of production of all other pigments with cultivation time (Figure 5.3). *Haematococcus* sp. in this study behaved similarly to most reports on this genus (Boussiba *et al.*, 1999; Del Campo *et al.*, 2004; Torzillo *et al.*, 2003) with highest concentrations on day 3 (8-9.5 mg g^{-1} DW), representing ~80% of total carotenoids, which were similar to reports by Orosa *et al.* (2001) for *Haematococcus* sp. after 3 days under similar conditions. Astaxanthin was primarily stored as esters, but free astaxanthin proportions (8-18%) were higher in this study than in previous reports (e.g. 0.6 % of total astaxanthin) following encystment (Boussiba *et al.*, 1999; Torzillo *et al.*, 2003). Astaxanthin synthesis generally occurs at the expense of β-carotene, which is the precursor to astaxanthin synthesis (Lemoine *et al.*, 2010; Lohr *et al.*, 2012) and is accompanied by chlorophyll breakdown (Torzillo *et al.*, 2003), yet small amounts of chlorophyll are necessary for astaxanthin synthesis as astaxanthin production and accumulation ceases when chlorophyll contents drop

below 20 pg cell (Boussiba *et al.*, 1999). Consequently, the observed time-dependent decrease in other accessory pigments in *Haematococcus* sp. was to be expected. Although astaxanthin synthesis does not conflict with the lutein biosynthesis pathway, in agreement with our results, high lutein content in *Haematococcus* sp. are typically only found in green flagellated cells (Margalith, 1999; Montsant *et al.*, 2001), and decline strongly with exposure to stress, suggesting that astaxanthin is used as the sole protectant against photo-oxidative damage in encysted stages. This conclusion is supported by the fact that astaxanthin has been shown to have the highest ROS scavenging and protective activity of synthesized carotenoids (Lemoine *et al.*, 2010; Lorenz *et al.*, 2000) which, when combined with encystment within a hard cell wall (Montsant *et al.*, 2001), can protect the cell from adverse conditions for substantial periods of time.

In addition to the commercial exploitation of over production of astaxanthin in *Haematococcus* sp., environmentally induced overproduction of β-carotene (Mulders *et al.*, 2015) is commercially exploited in *Dunaliella* sp. (Cifuentes *et al.*, 1992; Cowan *et al.*, 1992; Fu *et al.*, 2014) where accumulation of up to 14% of cell dry weight can be achieved (Milledge, 2011). In the microalgae studied here, β-carotene was not one of the main photo-protective pigments, as its concentration generally decreased in all species (except for *D. maximus*) after exposure to experimental conditions and over culture time. β-carotene decreases in microalgae following exposure to high light have been reported for *Desmodesmus* sp., *C. reinhardtii* and *Nannochloropsis* sp. (Couso *et al.*, 2012; Lubian *et al.*, 2000; Xie *et al.*, 2014). This is to be expected, as β-carotene is the precursor for the xanthophyll cycle pigments including astaxanthin (Han *et al.*, 2013), exposure to stressful conditions generally results in the production of

protective pigments at the expense of β-carotene (Bar *et al.*, 1995; Rise *et al.*, 1994; Schoefs *et al.*, 2001). This is most obvious in *Haematococcus* sp., where β-carotene was no longer detectable once astaxanthin production was initiated and corresponds to previous research by Schoefs *et al.* (2001). β-carotene is essential in the assembly of pigment-proteins and photosystem II (PSII) (Senger *et al.*, 1993; Telfer *et al.*, 1994; Trebst *et al.*, 1997) and is present in reaction centers as an accessory light harvesting pigment but also protects PSII from photo-oxidative damage (de las Rivas *et al.*, 1993). Consequently the observed decrease of chlorophyll contents in response to high light could have resulted in a concomitant decrease of β-carotene. β-carotene can also be hydroxylated to zeaxanthin (up to 30%) in *Chlamydomonas reinhardtii* under high light (Depka *et al.*, 1998). This may explain the observed zeaxanthin increase in *D. armatus*, *C. proboscideum* and *G. emersonii*, as violaxanthin concentrations remained similar over culture time in these species (see section 5.4.2).

Desmodesmus maximus was the only species in this study showing β-carotene concentration increases in response to high light and slightly further increased by high temperature compared to inoculation. Similar findings have been described for number of *Scenedesmus* spp, including *Scenedesmus almeriensis* (Bishop, 1996; Sánchez *et al.*, 2008). This may suggest that *D. maximus* was subject to more $^{1}O_{2}^{\bullet}$ which is specifically deactivated by β-carotene in PSII reaction centers (Jahns *et al.*, 2012), compared to the other species, which alleviated ROS stress using xanthophyll cycle NPQ (see section 5.4.2).

Lutein is the third pigment of commercial interest, after astaxanthin and β-carotene (BCC-Research, 2011). Lutein and zeaxanthin are particularly important under high light stress for the efficient transition of light harvesting complexes of PSII

from a conformation that favours light harvesting to one more efficient at thermal energy dissipation of excess excitation energy (Niyogi *et al.*, 1997; Pogson *et al.*, 1998). In this study, generally species with high lutein content under non-stressful conditions (except for *Haematococcus* sp. see above) did not respond with large and sustained changes in lutein content to stressful conditions. Similar patterns have been reported in *Chlamydomonas reinhardtii* exposed to high light (Niyogi *et al.*, 1997) and *Coccomyxa onubensis* exposed to Fe^{2+} (Garbayo *et al.*, 2012). In contrast, *D. maximus* contained low lutein concentrations under non-stressful conditions and showed a six-fold increase in lutein content when exposed to high light, corresponding to observations in *Scenedesmus* sp. (Yen *et al.*, 2011), *Scenedesmus obliquus* (Ho *et al.*, 2014) and *Scenedesmus almeriensis* (Sánchez *et al.*, 2008). Higher temperatures caused a further slight lutein increase, while Mo treatment had no large initial effects (Figure 5.5), suggesting energy-dissipation from ^{3}Chl and ^{1}Chl$^{\bullet}$ via lutein (see section 5.4.1).

Taken together these results show that *D. maximus* is the only species of those investigated here using photo-protection *via* β-carotene and lutein, which could show significant potential in commercial applications for pigment production. However, the pigment profile data also imply that cultivation in tailings-dam water would be counter-productive due to the inhibitory effects of Mo (see section 6.3). In contrast, the species with generally high lutein content and no noticeable responses to the environmental conditions including Mo treatment could perhaps be further investigated for their potential in a tailings-dam metal-remediation application providing opportunity for lutein co-product development (see section 6.4). The viability of astaxanthin co-product development using *Haematococcus* sp. requires

further investigation, as the pigment response data suggest that extensive protection of the green flagellated cell cycle stage against high irradiance and temperature would be required (see section 6.3).

5.5 Conclusions

High light, temperature and Mo treatment induced moderate time- and species-specific increases in SOD activity, with *Desmodemus* sp. being the only microalga investigated showing no SOD response. Generally SOD activity responses were larger in day-1 (*D*. *armatus*, *C*. *proboscideum*, and *Haematococcus* sp.) responding microalgae than in day-3 responding species (*D*. *maximus* and *G*. *emersonii*), indicating that conditions induced H_2O_2 generation from the superoxide anion, requiring detoxification via SOD. Generally, except for *Haematococcus* sp., day-1 SOD response species showed lower de-epoxidation states and little to no responses in total carotenoids, while those responding on day 3 showed noticeable changes in total carotenoids to the stressors, except for *G*. *emersonii*, suggesting that early SOD responses are indicative of acclimation potential and changes in total carotenoids could be a measure for stress-susceptibility. These findings have direct implications for species selection for bioremediation and cultivation strategies commercial production for the pigment market.

Xanthophyll pigment pool and derived de-epoxidation states data could be useful to determine light tolerance of microalgal species. This study revealed three distinct groupings: (i) high light tolerance/adapted species (large xanthophyll pigment pools and slow de-epoxidation rates: *D. armatus* and *C. proboscideum*), (ii) low light-adapted species (small xanthophyll pigment pools and fast de-epoxidation rates:

Desmodesmus sp., *D. maximus*) and (iii) intermediate pigment pools and de-epoxidation rates: *G. emersonii*, *Haematococcus* sp.

Overall, high light stress was the major driver of pigment concentration changes for *D*. *maximus*, while high temperature was the main stressor for *Haematococcus* sp. Molybdenum treatment in addition to high light stress generally had little effect, except for in *Haematococcus* sp. at 30°C further improving astaxanthin contents, while it completely counteracted the high light effect in *D. maximus*. These findings have major implications for cultivation approaches in southeast and tropical Australia; particularly with regards to cultivation in Mo-rich tailing dam waters (see section 6.3).

Of the species investigated, *G. emersonii*, *Desmodesmus sp.* and *Haematococcus sp.* showed pigment product development potential (see section 6.3). *Graesiella emersonii* produced the highest xanthophyll cycle pigments, antheraxanthin and zeaxanthin, sustained over the cultivation period of 10-days in response to high light. These data indicate that this species detoxifies high light-induced ROS via NPQ, and, while it appears to show no acclimation to high light, sustained growth (Figure S5.1) indicates effective lowering of ROS. Low light and low temperature conditions were favourable for β-carotene contents highest in *Desmodesmus* sp. and *Haematococcus* sp., with pigment responses to increasing temperature allowing for the development of cultivation strategies for the more lucrative lutein and astaxanthin markets, respectively in Mo-rich water resources.

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Table 5.3: Species containing highest pigment concentrations [mg g^{-1} DW] at respective time-points and conditions over the experimental period.

Pigments	Inoculation	Day 3				Day 10			
		Control		Mo-treatment		Control		Mo-treatment	
	24°C, LL	24°C, HL	30°C, HL	24°C, HL	30°C, HL	24°C, HL	30°C, HL	24°C, HL	30°C, HL
Neoxanthin	*Desmodesmus* sp. [1.3 ± 0.02]	*Desmodesmus* sp. [1.2 ± 0.05]							
Violaxanthin	*Haematococcus* sp. [1 ± 0.02]	*D. armatus* [0.6± 0.03]		*D. armatus* [0.6± 0.01]	*G. emersonii* [0.6 ± 0.05]	*D. armatus* [0.6± 0.03]	*G. emersonii* [0.6 ± 0.04]	*G. emersonii* [0.6 ± 0.06]	*G. emersonii* [0.7 ± 0.06]
Antheraxanthin		*G. emersonii* [0.5 ± 0.03]		*G. emersonii* [0.5 ± 0.03]		*G. emersonii* [0.5 ± 0.03]		*G. emersonii* [0.5 ± 0.03]	
Zeaxanthin		*G. emersonii* [0.4 ± 0.03]		*G. emersonii* [0.4 ± 0.03]					
β-carotene	*Desmodesmus* sp. *Haematococcus* sp. [0.9 ± 0.01]								
Lutein			*Desmodesmus* sp. [3.8 ± 0.2]		*Desmodesmus* sp. [3.8 ± 0.2]				
Astaxanthin					*Haematococcus* sp. [9.5 ± 0.2]				

LL: low light, HL: high light

Three of the six investigated chlorophytes (*Desmodesmus* sp., *G*. *emersonii*, and *D*. *maximus*) showed promise for commercial production of lutein with contents higher than 3 mg g^{-1} DW, exceeding contents in *Tagetes* sp., currently used for commercial lutein production (see section 6.3) but cultivation times and conditions will need to be optimized for sustained production.

CHAPTER 6

Synthesis and conclusions

6.1 Commercial context

Microalgal biotechnology is a growing field with ongoing interest in bio-product production for food, feed and health purposes as well as sustainable production of biodiesel, bioethanol, bioplastics and fertilisers coupled to waste-water, -gas and -metal remediation (Becker, 1994; Hochman *et al.*, 2015). Total production volumes and market size of food, feed and nutraceutical supplements from microalgae are still small in comparison to alternative sources (Enzing *et al.*, 2014) such as agricultural crops, mircrobial sources or synthetic manufacturing (Demain, 2007); however microalgal production has increased five-fold since the beginning of the century and is projected to keep growing with ongoing research into strain selection and improvements to cultivation, harvesting and biomass processing technologies (Enzing *et al.*, 2014). Currently, low volumes and high production costs of microalgae encourage exclusively the production of high-value products (Borowitzka, 2013b; Lundquist *et al.*, 2010). One of the main research focuses is lowering production costs of commercial microalgae cultivation, required to increase the economic viability of lower value products such as biofuels (Borowitzka, 2013c; Enzing *et al.*, 2014).

A study by the European commission identified a number of key aspects required to accomplish microalgal product commercialisation (Enzing *et al.*, 2014):

1. The production of astaxanthin, β-carotene, DHA, EPA for food and feed applications solely from algae requires significant science and technology.

2. The challenges in production costs are driven by production upscaling, biomass production, extraction, harvesting, species selection and contamination, in addition to the lack of capital investments.

Improvements to large-scale microalgae cultivation require a multi-disciplinary approach due to the low density cultures of the single-celled organisms, i.e. often not exceeding 0.1% total solids of the cultivation broth (Berner *et al.*, 2014), providing engineering challenges due to the species-specific nature of low cost approaches for biomass harvesting (dewatering) (Difusa *et al.*, 2015; Molina-Grima *et al.*, 2013a). Contamination is another hurdle particularly in freshwater remediation scenarios (Bacellar-Mendes *et al.*, 2013; Wang *et al.*, 2013). Furthermore, differences in environmental conditions between different cultivation sites and seasonal fluctuations within sites impact on growth and biochemical composition, requiring the identification and characterization of suitable endemic species for cultivation at the different sites (Heimann *et al.*, 2015a).

6.2 Research context and outcomes

As part of the AMCRC carbon-capture project from Australian coal-fired power stations, whose water resources range from freshwater to marine, this research investigated salinity tolerance of endemic microalgal species, isolated from tailings-dams from the Stanwell Corp. power-station in SE Queensland. In addition, water resources in inland Australia are often slightly saline (Hart *et al.*, 1991; Peck *et al.*, 2003) with culture salinity levels subject to rise due to the saline nature of the make-

up water to mitigate evaporative water loss in large-scale inland cultivation (von Alvensleben *et al.*, 2015). This would also demand selection of endemic microalgal species with a certain degree of halotolerance. Therefore this research characterised growth, effect of nutrient limitation and biochemical profile variabilities to identify potential species for bio-product production, using nutrient utilisation and limitation responses to infer suitability for cultivation in oligotrophic tailings-dam waters and/or suitability for bioremediation of nutrient-rich waste waters.

Microalgae species salinity tolerances ranged from euryhaline (*Picochlorum atomus*) to 8 ppt being the lowest for active growth of *Mesotaenuium* sp. (Table 6.1). The next most halotolerant species were *Desmodesmus armatus* and *Tetraedron* sp. (up to 18 ppt), while *Mesotaenium* sp. although least halotolerant showed the highest productivities. While, salinity had little effect on biochemical profiles, nutrient limitation caused lipid and fatty acid (FA) increases in addition to changes in fatty acid profiles in *P. atomus* and to a lesser extent *S. quadricauda* and *Tetraedron* sp. indicating that nutrient manipulation is a valid, but species-specific, tool for lipid-based bioproduct enhancement (von Alvensleben *et al.*, 2015; von Alvensleben *et al.*, 2013a). Nutrient limitation decreased PUFA contents with a concurrent increase of MUFA, making *P. atomus* a suitable candidate for fatty acid-based biofuels (Schenk *et al.*, 2008) and bio-plastics from C18:1 (Lligadas *et al.*, 2010). Nutrient depletion also resulted in an ω6:ω3 fatty acid ratio close to 1:1 which has potential as a health food supplement to improve cardiovascular health (Simopoulos, 2002). In contrast, nutrient-replete conditions led to high carbohydrate and protein contents, making it suitable for agriculture or aquaculture feeds. *Tetraedron* sp. and, *Mesotaenium* sp. in particular (5.4% of DW, 45% of TFA), excelled in accumulation of C18:1 at 8<11 and

>11<18 ppt, respectively, a valuable precursor for bio-degradable plastic production (Lligadas *et al.*, 2010). *Desmodesmus armatus* also has demonstrated pharmaceutical potential through accumulation of Stearic acid when cultured at 2 ppt.

Nutrient uptake patterns provided an insight in the suitability of species for cultivation in oligotrophic (tailings-dam waters), i.e. requiring low fertilisation, or for remediation of nutrient-rich waste water, i.e. high fertilisation requirements (Table 6.1). *Scenedesmus quadricauda* could be an ideal candidate for environmental services, such as nitrogen and phosphate remediation, as it had the highest uptake rates and low salinity tolerance (2-11 ppt). *Desmodesmus armatus* and *Mesotaenium* sp., on the other hand, stood-out for biomass production under nutrient-poor conditions from 2-18 and 2-8 ppt, respectively.

In addition, salinity experiments identified high salinity as a tool for culture contamination control in euryhaline tolerant microalgal cultures. It was shown that salinities of ≥28 ppt slowed the establishment of the common tropical freshwater cyanobacterial contaminant *P. limnetica* in cultures of *P. atomus*. Contamination by non-target organisms is a significant problem, particularly in large-scale open freshwater cultures, contributing to the high costs of microalgal biomass production (Borowitzka, 1999; Meseck *et al.*, 2007; Zhou *et al.*, 2015).

The overall species responses from these studies can be used to produce a species selection matrix to target species for scaled production based on their salinity tolerance and plasticity in biochemical composition (Table 6.1)

Table 6.1. Species selection matrix for salinity tolerance, nutrient requirements, biomass productivity and bio-product potential.

Species	Salinity tolerance [ppt]	Nutrient uptake[1]	Biomass productivity	Bio-product
Desmodesmus armatus	2-18	++	●●●	Violaxanthin Protein
Mesotaenium sp.	2-8	+	●●●	Lipid/Biofuel Carbohydrate/Bioethanol feedstock
Picochlorum atomus	2-36	+++	•	Lipid/Biofuel Picoplankton: Larval aquaculture feed
Scenedesmus quadricauda	2-11	+++	••	Biofuel
Tetraedron sp.	2-11	+	••	Bioplastics (C18:2)

[1] +: Preferable for cultivation requiring fertilisation (e.g. tailings-dam water), requiring low fertiliser inputs. +++: Suitable for nutrient-rich wastewater remediation.

Within the carbon capture project from Australian coal-fired power stations, it became evident that microalgal biomass production for biofuel and feed was not economically sustainable, requiring the simultaneous production of high value bio-products to offset expensive infrastructure and labour costs (Heimann pers. com). Therefore, the second part of this thesis characterised pigment profiles of eight tailings-dam water and tropical microalgal species isolates to identify those with suitable carotenoid profiles for commercial pigment production (Chapter 4 and 5). This research priority was based on the fact that pathways to market are established for low-volume, high value carotenoids (Enzing *et al.*, 2014; Spolaore *et al.*, 2006). It also embraced that presently used microalgae, e.g. *Haematococcus* spp or *Dunaliella salina* for the commercial production of astaxanthin and β-carotene, respectively, would be unlikely candidates for successful low-cost cultivation at a coal-fired power station in SE Queensland. Moreover, it tested the effects of known ROS and therefore

carotenogenesis inducers (high light and temperatures) and heavy metals (Mo and V), prevalent in tailings-dam waters at coal-fired power stations, on carotenoid content and profiles. A pilot study for species selection was required to identify likely species and bio-product outcomes for cultivation at coal-fired power stations during the Australasian summer. Selected promising microalgae species were then subjected to a factorial design experiment to investigate interactive effects of high light, high temperature and Mo stress for enhancing pigment product potential.

The research identified species with potential for astaxanthin, lutein, violaxanthin and zeaxanthin production (Table 6.2). In addition, carotenoid concentrations were highest in all species in nutrient-sufficient cultures, a response that was shown to be species- and cultivation condition-specific (Varela *et al.*, 2015). Addition of Mo and V allowed identification of species suitability for carotenoid production in tailings-dam water. While effects were minor for five of the six species, exposure of *D*. *maximus* to Mo concentrations present in tailings-dam water had negative effects on carotenoid contents and profiles, eliminating this species for cultivation in these water resources for pigment production. High light was identified as the principal driver for carotenoid accumulation, except for *D*. *armatus* and *Haematococcus* sp. where high temperatures were the principal drivers producing lower and higher pigment contents, respectively. Light intensities from 400-600 µmol m^{-2} s^{-1} were least stressful for *D*. *armatus* which initially showed a considerable SOD response followed by acclimation. *Graesiella emersonii* and *Coelastrum proboscideum* also showed acclimation potential to irradiances used through NPQ. Furthermore the data suggest that these species may produce violaxanthin, zeaxanthin or antheraxanthin *de novo*. This has implications for using these species in high light

environments and for identifying new sources of commercially valuable zeaxanthin and violaxanthin (see 6.4).

Encystment of *Haematococcus* sp. in response to moderately high temperatures (30 °C) accompanied by high contents of esterified astaxanthin implies that cultivation at freshwater-based coal-fired power stations could be possible. The results confirmed that a two-step production process is required, with temperate conditions required for biomass production and carotenogenesis inducing conditions for astaxanthin accumulation (Boussiba, 2000; Guedes *et al.*, 2011a). *Coelastrum proboscideum* was identified as a potential alternative to *Haematococcus* sp. for astaxanthin production.

Table 6.2. Summary of species suitability and cultivation conditions for carotenoid product development.

Species	Mo tolerance	Bioproduct and conditions
Desmodesmus armatus	Yes	Violaxanthin (HL, 3-10 days) Protein (N-replete, 2 ppt)
Desmodesmus maximus	No	Lutein (HL, 3 days)
Desmodesmus sp.	Yes	Lutein (HL, 3 days)
Coelastrum proboscideum	Yes	Astaxanthin (HL, 24°C, 3-10 days)
Graesiella emersonii	Yes	Violaxanthin Zeaxanthin (HL, 24°C, 3 days) Lutein (HL, 3-10 days)
Haematococcus sp.	During encystment	Astaxanthin (HL, 30°C, 3-10 days)

Days: stress exposure duration; HL: high light, N-replete: nutrient-replete

Although astaxanthin concentrations in *C. proboscideum* were lower than *Haematococcus* sp., potentially simpler cultivation requirements (which needs to be confirmed for on-site across season outdoor production) could be economically attractive for production at sites which are unsuitable for *Haematococcus* sp.

Three commercially attractive microalgal lutein producers were identified. The tropical isolate, *Desmodesmus* sp. had the highest lutein concentrations, followed by *G*. *emersonii* and *D*. *maximus*, with a ten-fold higher lutein content than Marigold flowers (Fernandez-Sevilla *et al.*, 2010).

6.3 Commercial implications for pigment co-product development

6.3.1 Applicability to Stanwell Corp. power-station

This research has identified the cultivation and composition characteristics for a number of microalgal species for bio-product production at industrial sites, using Stanwell Corp. environmental and water quality conditions as a reference. Predominantly suitable temperatures (19-30°C) (BOM, 2015_a) and high irradiance (12-24 MJ m^{-2}, equivalent to 2,160-4,320 µmol m^{-2} at midday) (BOM, 2015_b) cause fluctuations in tailings-dam water salinity, which when coupled with heavy metal pollutants result in a generally high-stress environment for microalgal cultivation. Species characterization has highlighted aspects for consideration for species selection at this site: Conditions of high irradiance and high temperature during the Australasian summer are unlikely to be suitable for the sensitive *Haematococcus* sp. The two-step process would require substantial infrastructure to provide shading and water cooling for the actively growing green flagellate state and timed exposure to stressors (e.g.

high temperature, light) for commercial astaxanthin production. This adds significant infrastructure costs, compromising profit margins, unless a carbon-offset and/or bioremediation of metal incentive could be included in economic forecasts. *Desmodesmus maximus* would be unsuitable for carotenoid production using tailings-dam water due to the inhibitory effect of Mo on carotenoid concentrations. As *D. maximus* has particularly high lutein production in cultures without Mo, it would be preferable to cultivate this species in nutrient-rich wastewater for remediation, coupled with lutein production. *Mesotaenium* sp. would be most suitable for culturing using tailings-dam water due to its low nutrient requirements and high biomass production; however its sensitivity to salinity may result in reduced productivity if culture salinity is not maintained below 8 ppt. The high nutrient requirements by *P. atomus* and *S. quadricauda* and to a lesser extend *D. armatus* suggest these species should be preferably cultured for bio-product production in parallel to nutrient-rich wastewater remediation from agriculture, aquaculture or sewage, as these would incur large fertilisation costs in oligotrophic environments such as tailings-dam water, unless costs could be offset with high-value products such as lutein from *D. armatus* or carbon/metal remediation offsets.

6.3.2 Species selection for commercial carotenoid production

Lutein contents of 0.3-0.4 % of DW in *Desmodesmus* sp., *G. emersonii* and *D. maximus* are of particular interest for commercial applications, as the current natural source of commercial lutein is Marigold (*Tagetes* sp.) which has a lutein content of ~0.03-0.1 % (Bosma *et al.*, 2003; Fernandez-Sevilla *et al.*, 2010; Lin *et al.*, 2015), making these species a potential alternative for commercial lutein production.

Furthermore, microalgae have the added advantages of higher growth rates per unit area than *Tagetes* sp., year-round productivity, no need for arable land and additional production of other value-adding products (Lin *et al.*, 2015).

Further research improving carotenoid induction methods and timeframes may also improve lutein content in these species as previous research has shown microalgae such as *Scenedesmus almeriensis*, *Chlorococcum citriforme* and *Coccomyxa onubensis*, *Desmodesmus* sp. to reach lutein contents up to 0.5-0.7 % of DW (Del Campo *et al.*, 2000; Garbayo *et al.*, 2012; Xie *et al.*, 2014) (Table 6.3). Studies have also shown *Tagetes* sp. to contain mainly lutein esters, whereas microalgae generally contain un-esterified lutein (Lin *et al.*, 2014). Nutraceutical, food or feed benefits of esters *vs.* free lutein are, however, not clearly established (Lin *et al.*, 2015).

The astaxanthin market is forecast to expand in particular with increasing regulation on the use of the synthetic pigments, and will likely further increase as therapeutic uses for this pigment are discovered and established in the current pharmacopeia. Furthermore, recent ongoing research suggests that astaxanthin from natural sources, in particular astaxanthin esters, have stronger antioxidant activity for therapeutic uses, than their synthetic counterpart (Capelli *et al.*, 2013; Régnier *et al.*, 2015).

Astaxanthin synthesis in microalgae has been extensively studied, in particular in *Haematococcus* sp. (Boussiba, 2000; Lemoine *et al.*, 2010; Margalith, 1999; Solovchenko, 2015). A number of species such as *Nannochloropsis* sp. (Lubian *et al.*, 2000), *Chlorella* sp. (Del Campo *et al.*, 2000; Pelah *et al.*, 2004) and *Scenedesmus* sp. (Cordero *et al.*, 2011b; Orosa *et al.*, 2001) have been reported to synthesize and accumulate astaxanthin, however none to the extent of *Haematococcus* sp. which

overproduces and accumulates up to 30-40 mg g^{-1} DW (Boussiba *et al.*, 1999; Cai *et al.*, 2009; Imamoglu *et al.*, 2009) (Table 6.3). However, *Haematococcus* sp. has a number of disadvantages including low productivities, sensitive to environmental fluctuations, a predisposition to contamination and complex pigment extraction requirements (Choi *et al.*, 2015; Cuellar-Bermudez *et al.*, 2015; Margalith, 1999). Furthermore, as mentioned previously, conditions of high irradiance and high temperature found at Stanwell Corp. power station are unlikely to be suitable for *Haematococcus* sp. biomass production without a two-step prcess and shading during the Australasian summer. Consequently, research is ongoing to identify more effective species for astaxanthin production for example *Chlorella zofingiensis* which is reported to accumulate up to 7 mg g^{-1} DW astaxanthin (Del Campo *et al.*, 2004) (Table 6.3)

Apart from *Haematococcus* sp., this study identified traces of astaxanthin in *D. armatus* and *G. emersonii*, however these did not increase under stress conditions and are unlikely to have any commercial applicability. Similar trace concentrations have been identified in *Scenedesmus vacuolatus* and *Spirulina platensis* (Abd El-Baky *et al.*, 2009; Orosa *et al.*, 2001) (Table 6.3). In contrast, *Coelastrum proboscideum* showed a more substantial astaxanthin accumulation (up to 0.8 mg g^{-1} DW), slightly higher than previous reports for this species (~0.6 mg g^{-1} DW) (Del Campo *et al.*, 2000) and similar to *Chlorella zofingiensis* (1 mg g^{-1} DW) (Table 6.3), proposed as a potential commercial astaxanthin producing species due to its high productivity (Wang *et al.*, 2008b). These are still twelve times lower than *Haematococcus* sp. astaxanthin contents in this study (up to 9.5 mg g^{-1} DW) which were in themselves low- to mid-range concentrations compared to reported astaxanthin concentrations for *Haematococcus* sp. (Table 6.3).

Table 6.3. Pigment comparisons between studies. For this study (chapter 5), highest pigment contents [mg g^{-1} DW] of commercially valuable pigments were included for each species.

Species	Neoxanthin	Violaxanthin	Zeaxanthin	β-carotene	Lutein	Astaxanthin	Reference
Desmodesmus armatus	0.7 ± 0.02	0.6 ± 0.07	0.1 ± 0.002	0.4 ± 0.02	1.8 ± 0.1	0.1 ± 0.006	Chapter 5
Desmodesmus maximus	1 ± 0.1	0.1 ± 0.001	0.3 ± 0.02	0.6 ± 0.01	3 ± 0.2	-	Chapter 5
Desmodesmus sp.	1.3 ± 0.02	0.2 ± 0.003	0.3 ± 0.01	0.9 ± 0.003	3.8 ± 0.2	-	Chapter 5
C. proboscideum	0.6 ± 0.07	0.6 ± 0.04	0.2 ± 0.02	0.5 ± 0.06	2.2 ± 0.1	0.8 ± 0.04	Chapter 5
Graesiella emersonii	0.9 ± 0.01	0.7 ± 0.06	0.4 ± 0.03	0.7 ± 0.01	3.5 ± 0.2	0.3 ± 0.02	Chapter 5
Haematococcus sp.	0.9 ± 0.01	1 ± 0.02	0.1 ± 0.003	0.9 ± 0.01	2.7 ± 0.03	9.5 ± 0.2	Chapter 5
Chlamydomonas reinhardtii	1.2	1.4	0.3	2	2.5	-	(Couso *et al.*, 2012)*
Chlorella citriforme	-	1.6	-	1.2	7.4	0.3	(Del Campo *et al.*, 2000)
Chlorella fusca	-	0.6	-	0.8	4.7	0.5	(Del Campo *et al.*, 2000)
Chlorella sorokiniana	-	0.1	0.1	0.2	3	-	(Cordero *et al.*, 2011b)*
Coccomyxa onubensis	-	-	0.4	1.1	4	-	(Vaquero *et al.*, 2012)*
C. proboscideum	-	0.7	-	0.7	3.4	0.6	(Del Campo *et al.*, 2000)
Desmodesmus sp.					2.6-3.9		(Xie *et al.*, 2013)
Dunaliella salina		0.6		1.2	4.5	-	(Ahmed *et al.*, 2014)
Haematococcus sp.	0.3-0.7	0.3-0.7		0.25-1	0.5-4.5	23-44	(Orosa *et al.*, 2001; Torzillo *et al.*, 2003)
Muriellopsis sp.	-	1.4	-	1.1	5.6	0.2	(Del Campo *et al.*, 2000)
Nannochloropsis sp.	-	1.1	-	0.5	-	0.3	(Ahmed *et al.*, 2014)
Picochlorum sp.	1.5	1	0.4-1.8	0.9	3.5	-	(de la Vega *et al.*, 2011)
Scenedesmus armatus	-	0.3	-	0.5	2.8	-	(Cordero *et al.*, 2011b)*
Scenedesmus almeriensis	-	-	0.3	0.1	2.9-5.5	-	(Sanchez *et al.*, 2008)* (Granado-Lorencio *et al.*, 2009)

*similar culture conditions to this study

The aim of this study was, however, not carotenoid production optimization, but rather responses to stressful environmental conditions. In this regard, *C. proboscideum* is an interesting species for further astaxanthin production optimization, as it responded to stress induction, has fast growth rates (data not shown) and is often dominant in tropical NE Queensland freshwater bodies, suggesting it is a competitive species. Furthermore, unlike *Haematococcus* sp. where free astaxanthin proportions of total astaxanthin decreased as astaxanthin accumulation proceeded, the proportion of free astaxanthin in *C. proboscideum* remained around 50 % of total astaxanthin. Free astaxanthin has been shown to be preferable for food and feed applications (Choubert *et al.*, 1993; Goswami *et al.*, 2010; Sommer *et al.*, 1990; Storebakken *et al.*, 1987), however, this is currently under debate (Fassett *et al.*, 2011), as more recent studies report no differences in activity or assimilability between the free and esterified forms (Lorenz *et al.*, 2000).

6.4 Future directions

Although recent research has shown that inferences based on laboratory-scale experimental set ups have limited applicability in regard to biomass productivities and bio-product potential in up-scaled systems, they provide preliminary characterization and a guide for effective future research and development to achieve economical and environmentally friendly bio-products from microalgae (Borowitzka, 2013c). The salinity tolerances established for the microalgae characterised here are applicable, as cultures have been acclimated to increasing salinities. It is however acknowledged, that biomass productivities along with bio-product productivities will require year-round cultivation under relevant outdoor conditions to extrapolate the real potential

of the species investigated, which could not be achieved within this PhD due to time and research infrastructure constraints (replication of sufficiently large outdoor systems). Of the outdoor conditions likely to influence projected bio-product potential, high light and temperatures are the most likely variables to influence biomass and bio-product outcomes, as nutrient status can be controlled via fertilisation and limitation. This implies that suggested species choice for cultivation at sites with either oligotrophic or nutrient-rich water resources will be an asset in establishing demonstration-scale projects.

Although outdoor light intensities in Queensland can be much higher than those applied here, these are more likely to affect freshly inoculated low density cultures (depending on the cultivation system), as culture growth will ultimately lead to self-shading thereby attenuating the light effect (MacIntyre *et al.*, 2005). High or low temperatures, on the other hand, can have significant impact on biomass – and bio-product productivities (Bhosale, 2004; Gacheva *et al.*, 2014; Ras *et al.*, 2013; Wei *et al.*, 2015), which could not be pursued in this study due to research infrastructure limitations at the start to the mid-term of this project. Therefore, results and conclusions presented here will need to be validated, either through fully replicated outdoor-year round cultivation or in factorial design indoor experiments at sufficiently large scale. Irrespective of these constraints, this research has provided new species growth and biochemical profile data that can inform species selection for follow up research and commercial-scale validation, constraining associated costs. Additionally, the potential bio-product outcomes allow for selecting a cultivation system aligned in expense and complexity to the likely bio-product revenue generated, further constraining costs of outdoor fully replicated year-round validation studies, which will

allow additionally investigating optimal inoculation, harvesting and biomass processing regimes for maximal biomass and bio-product productivities. Furthermore, large-scale cultivation will be required to evaluate cost-effective harvesting (dewatering) and bio-product extraction methods. Thus in essence this research laid the foundation for species selection based on the nature of the water resource available, enabling follow up demonstration-scale validation for the economic assessment of bio-product viability: bio-product value relative to establishment and maintenance costs. This will determine required product productivities and the potential requirement for multi-product production.

In the light of the above, resource limitations did not allow for carrying out experiments in actual tailings-dam water, primarily due to the high shipment - and elemental analytical costs and the deterioration of the elemental signature upon storage (Newman, 1996), which is, however, an important aspect when considering bio-product development for feed applications, due to the bioaccumulation potential of heavy metals. Nonetheless, for pigment product development, it was established that the most prevalent tailings-dam water heavy metals, Mo and V, had no detrimental effects on *D*. *armatus*, *Desmodesmus* sp., *G*. *emersonii*, *C*. *proboscideum*. Therefore, future on-site research can, for example, capitalise with regards to species selection on the here established astaxanthin potential of *C*. *proboscideum* and/ or the lutein potential and apparent high light and temperature hardiness of *Desmodesmus* sp. to assess bioaccumulation of these metals and to also determine the effect of the complex tailings-dam water heavy metal mixtures. Similarly, with the onset of the violaxanthin and zeaxanthin markets (Bhosale *et al.*, 2005; Nishino *et al.*, 2009; Pasquet *et al.*, 2011; Soontornchaiboon *et al.*, 2012), further research should identify

species with higher concentrations of these pigments but potentially also those with the ability for continuous *de novo* synthesis of these pigments as suggested in this research for *D. armatus, G. emersonii* and *C. proboscideum.*

While salinity increases proved beneficial for slowing the growth of the tropical freshwater cyanobacterium *Pseudanabaena limnetica* in the cultivation of the euryhaline *Picochlorum atomus*, there is undeniably a great need to determine effective contamination control, particularly for the cultivation of freshwater species. As shown here, salinity increases that were effective had to be quite large, which is likely not feasible on a sufficiently large scale also due to environmental concerns for freshwater sites. Such research has to focus on the scalability of the contamination control mechanism (be it chemically or system design) and cost-effectiveness, which again will be informed by the bio-product selected. The latter, in turn, can be pre-selected from the established profiles presented in this research, which can facilitate and narrow down selection of approaches for contamination control.

6.5 Conclusions

To ensure economic viability of bioremediation approaches and high volume, low value bio-product development using microalgae, large-scale microlagal production needs to initially target established markets, with pigments offering an established pathway to markets. Production sites should preferentially be located near water sources requiring remediation to benefit from the remediation potential of microalgae. Water quality availability at a given production site, in turn though, can limit bio-product potential of the microalgal biomass (e.g. metal accumulation potential from mine tailings-water could impede feed or food supplement

applications). While this research could not investigate all facets (e.g. large-scale replicated outdoor designs, metal bioaccumulation potential, year round outdoor biomass – and bio-product productivities) due to resource limitation, it laid the much needed foundation for species selection based on determined salinity tolerances and biochemical profiles in regard to nutrient requirements and bio-product potential specifically for coal-fired power stations in SE Australia.

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APPENDIX

Supplementary tables

Table S3.1. Effect of salinity and culture nutrient status (replete/deplete) on *Desmodesmus armatus* fatty acid profiles (FA content [mg g^{-1} DW]).

FAME	2 ppt		8 ppt		11 ppt		18 ppt	
	Rep.	Dep.	Rep.	Dep.	Rep.	Dep.	Rep.	Dep.
Saturated								
C14:0	0.35	0.32	0.32	0.36	0.36	0.32	0.34	0.44
C16:0	14.89	17.41	15.00	18.45	14.02	22.49	16.34	26.85
C18:0	0.35	0.34	0.32	0.52	0.44	0.66	2.97	3.09
Σ_{SFA}	15.24	17.75	15.31	18.97	14.46	23.15	19.30	29.94
Monounsaturated								
C16:1 (7) n-9	1.18	1.05	0.97	1.28	1.11	1.47	1.46	2.12
C16:1 (9)	3.33	3.26	2.73	2.47	2.60	2.04	1.81	1.57
C18:1 (cis, 9) n-9	4.95	7.86	4.94	9.69	5.50	18.97	10.68	29.13
Σ_{MUFA}	9.46	12.17	8.64	13.44	9.22	22.48	13.95	32.81
Polyunsaturated								
C16:2 (7,10) n-6	1.12	1.44	1.10	1.45	1.00	1.32	0.87	1.27
C16:4 n-3	15.20	13.04	12.35	10.92	11.91	9.03	7.16	6.76
C18:2 (trans, trans 9,12) n-6	2.28	2.26	2.22	1.84	1.90	2.14	2.11	2.80
C18:2 (cis 9,12) n-6	7.19	11.05	7.14	10.71	7.12	11.52	7.89	13.00
C18:3 n-6 γ-linolenic	2.01	1.93	2.01	1.87	1.92	1.59	1.68	1.57
C18:3 n-3, α-linolenic	29.61	28.58	26.63	23.42	24.98	19.86	17.04	16.27
C18:4 n-3	5.92	6.20	4.67	5.09	4.26	4.08	3.14	3.09
Σ_{PUFA}	63.32	64.50	56.12	55.30	53.09	49.54	39.89	44.77
Sum of ω3	50.73	47.82	43.65	39.43	41.16	32.97	27.33	26.13
Sum of ω6	10.31	14.42	10.25	14.03	10.04	14.44	10.44	15.84
ω6:ω3 ratio	0.20	0.30	0.23	0.36	0.24	0.44	0.38	0.61

Rep.: replete, Dep.: deplete.

Table S3.2. Effect of salinity and culture nutrient status (replete/deplete) on *Mesotaenium* sp. fatty acid profiles (FA content [mg g^{-1} DW]).

FAME	2 ppt		8 ppt		11 ppt		18 ppt	
	Rep.	**Dep.**	**Rep.**	**Dep.**	**Rep.**	**Dep.**	**Rep.**	**Dep.**
Saturated								
C14:0	0.25	0.26	0.24	0.29	0.25	0.29	0.42	0.45
C16:0	16.28	20.27	15.36	23.39	15.14	23.02	35.80	35.13
C18:0	0.35	0.34	0.32	0.52	0.44	0.66	2.97	3.09
Σ_{SFA}	16.63	20.61	15.68	23.90	15.59	23.68	38.77	38.22
Monounsaturated								
C16:1 (9)	3.31	2.60	2.38	1.67	1.72	1.47	0.90	0.87
C18:1 (9)	1.97	4.07	2.57	9.12	4.13	11.28	35.56	33.81
Σ_{MUFA}	5.29	6.67	4.95	10.79	5.84	12.75	36.46	34.68
Polyunsaturated								
C16:2 (7,10) n-6	7.40	6.51	12.01	10.93	9.36	8.96	6.69	5.05
C18:2 (cis 9, 12) n-6	10.57	11.77	22.81	23.88	22.39	23.84	34.57	30.44
C18:3 (6,9,12) n-6	1.39	1.31	1.75	1.61	1.35	1.44	1.09	0.95
C18:3 (9,12,15) n-3	23.71	21.75	10.86	7.87	6.86	7.52	3.65	3.65
C20:4 (all cis, 5,8,11,14) n-6	1.73	1.86	3.02	2.92	2.91	2.97	2.90	2.11
C20:5 (EPA) (all cis, 5, 8, 11, 14, 17) n-3	1.69	2.03	0.71	0.75	0.42	0.58	0.33	0.28
Σ_{PUFA}	46.50	45.24	51.18	47.97	43.29	45.31	49.23	42.48
Sum of ω3	25.41	23.78	11.57	8.63	7.29	8.10	3.99	3.94
Sum of ω6	19.36	19.59	36.58	36.43	33.09	34.24	42.34	36.43
ω6:ω3 ratio	0.76	0.82	3.16	4.22	4.54	4.23	10.63	9.26

Rep.: replete, Dep.: deplete.

Table S3.3. Effect of salinity and culture nutrient status (replete/deplete) on *Scenedesmus quadricauda* fatty acid profiles (FA content [mg g^{-1} DW]).

FAME	2 ppt		8 ppt		11 ppt	
	Rep.	Dep.	Rep.	Dep.	Rep.	Dep.
Saturated						
C14:0	0.30	0.32	0.29	0.31	0.28	0.31
C16:0	12.49	17.43	12.77	19.18	12.88	22.23
C18:0	0.30	0.45	0.33	0.71	0.34	0.94
C22:0	3.14	0.45	1.16	0.92	0.78	0.85
Σ_{SFA}	15.93	18.34	14.26	20.81	14.00	24.02
Monounsaturated						
C18:1 (9) n-9	5.06	9.86	5.19	13.77	5.46	17.37
Σ_{MUFA}	5.06	9.86	5.19	13.77	5.46	17.37
Polyunsaturated						
C16:2 (7,10) n-6	1.09	1.26	0.95	1.18	0.82	1.41
C16:4 (4, 7, 10, 13) n-3	11.73	10.35	12.03	8.76	10.86	9.84
C18:1 (cis 9) n- 9	5.06	9.86	5.19	13.77	5.46	17.37
C18:2 (cis 9,12) n-6	6.91	10.41	6.24	10.23	5.82	11.18
C18:3 (6,9,12) n-6	1.32	1.35	1.21	1.20	1.49	1.36
C18:3 (9,12,15) n-3	26.17	26.61	26.17	20.94	25.00	22.82
Σ_{PUFA}	52.28	59.85	51.78	56.08	49.45	63.98
Sum of ω3	37.90	36.96	38.20	29.71	35.86	32.66
Sum of ω6	9.31	13.03	8.40	12.61	8.13	13.95
ω6:ω3 ratio	0.25	0.35	0.22	0.42	0.23	0.43

Rep.: replete, Dep.: deplete.

Table S3.4. Effect of salinity and culture nutrient status (replete/deplete) on *Tetraedron* sp. fatty acid profiles (FA content [mg g^{-1} DW]).

FAME	2 ppt		8 ppt		11 ppt		18 ppt	
	Rep.	Dep.	Rep.	Dep.	Rep.	Dep.	Rep.	Dep.
Saturated								
C14:0	0.23	0.26	0.27	0.30	0.26	0.28	0.23	0.26
C16:0	10.64	14.87	14.12	21.61	11.37	25.25	13.15	14.45
C18:0	0.42	0.67	0.87	1.44	0.63	1.80	1.14	1.53
Σ_{SFA}	11.06	15.54	14.99	23.04	12.00	27.04	14.29	15.98
Monounsaturated								
C16:1 (7)	0.63	0.71	0.62	0.66	0.53	0.63	0.44	0.53
C16:1 (9)	1.72	2.00	1.80	1.58	1.66	1.29	0.93	0.92
C18:1 (9)	4.32	9.99	7.79	29.07	10.51	54.28	14.35	21.78
Σ_{MUFA}	6.67	12.70	10.21	31.31	12.70	56.20	15.72	23.23
Polyunsaturated								
C16:2 (7,10) n-6	0.79	1.40	1.16	1.37	1.34	1.15	0.47	0.53
C16:4 n-3	8.40	9.27	6.93	5.93	6.11	5.01	2.38	2.86
C18:2 (trans, trans 9,12) n-6	2.31	2.55	2.42	2.94	2.49	3.28	3.22	3.79
C18:2 (cis, 9, 12) n-6	3.13	6.09	4.71	7.80	6.46	8.45	2.59	3.24
C18:3 (6,9,12) n-6	0.35	0.17	0.00	0.16	0.16	0.16	0.16	0.18
C18:3 (9,12,15) n-3	18.07	21.33	17.56	18.46	17.57	19.71	1.27	12.11
C18:4 n-3	3.75	0.17	2.69	0.16	2.48	0.16	0.16	0.18
Σ_{PUFA}	36.80	40.98	35.47	36.82	36.61	37.92	10.25	22.88
Sum of ω3	30.22	30.77	27.18	24.55	26.16	24.88	3.81	15.15
Sum of ω6	3.92	7.49	5.87	9.17	7.80	9.60	3.06	3.77
ω6:ω3 ratio	0.13	0.24	0.22	0.37	0.30	0.39	0.80	0.25

Rep.: replete, Dep.: deplete.

Table S3.5. Total lipid and total FAME productivities [mg L^{-1} day^{-1}] of *Desmodesmus armatus*, *Mesotaenium* sp., *Scenedesmus quadricauda* and *Tetraedron* sp. at 2, 8, 11 and 18 ppt salinity. Productivities were derived from biomass productivities during the exponential growth phase.

Species	Salinity [ppt]	Total lipid productivity [mg L^{-1} day^{-1}]	Total FAME productivity [mg L^{-1} day^{-1}]
D. armatus	2	7.36 ± 0.4	3.80 ± 0.06
	8	8.03 ± 0.3	3.62 ± 0.004
	11	6.45 ± 0.5	2.99 ± 0.05
	18	3.00 ± 0.2	1.26 ± 0.02
Mesotaenium sp.	2	13.14 ± 1.8	4.48 ± 0.05
	8	11.47 ± 0.9	4.09 ± 0.2
	11	7.01 ± 0.7	2.57 ± 0.1
	18	2.21 ± 0.04	1.15 ± 0.03
S. quadricauda	2	8.49 ± 0.05	2.74 ± 0.02
	8	7.54 ± 0.2	2.78 ± 0.2
	11	7.79 ± 0.2	2.54 ± 0.05
	18	-	-
Tetraedron sp.	2	6.03 ± 0.9	2.12 ± 0.1
	8	5.42 ± 0.6	2.00 ± 0.02
	11	5.07 ± 0.4	2.20 ± 0.02
	18	1.31 ± 0.2	0.53 ± 0.02

Table S3.6. Individual FAME productivities [mg L^{-1} day^{-1}] of *Desmodesmus armatus*, *Mesotaenium* sp., *Scenedesmus quadricauda* and *Tetraedron* sp. at 2, 8, 11 and 18 ppt salinity. Productivities were derived from biomass productivities during the exponential growth phase.

FAME	Salinity [ppt]	FAME productivity [mg L^{-1} day^{-1}]			
		D. armatus	***Mesotaenium* sp.**	***S. quadricauda***	***Tetraedron sp.***
C16:0	2	0.61	0.88	0.47	0.39
	8	0.65	0.8	0.51	0.44
	11	0.52	0.56	0.49	0.39
	18	0.28	0.32	-	0.13
C16:2 n-6	2	-	0.4	-	-
	8	-	0.62	-	-
	11	-	0.35	-	-
	18	-	0.06	-	-
C16:4 n-3	2	0.62	-	0.45	0.31
	8	0.53	-	0.48	0.22
	11	0.44	-	0.41	0.21
	18	0.12	-	-	0.02
C18:1 n-9	2	0.2	0.11	0.19	0.16
(cis)	8	0.21	0.13	0.21	0.24
	11	0.2	0.15	0.21	0.36
	18	0.18	0.32	-	0.14
C18:2 n-6	2	0.3	0.57	0.26	0.12
(cis)	8	0.31	1.19	0.25	0.15
	11	0.26	0.83	0.22	0.22
	18	0.13	0.31	-	0.03
C18:3 n-3	2	1.21	1.28	0.99	0.67
(α-linolenic)	8	1.15	0.56	1.05	0.54
	11	0.92	0.25	0.95	0.6
	18	0.29	0.03	-	0.01

Table S3.7. Amino acid profiles [mg g^{-1} DW] of *Desmodesmus armatus* at 2 and 11 ppt in nutrient-replete and deplete conditions.

Amino Acid	**2 ppt**		**11 ppt**	
	Replete	**Deplete**	**Replete**	**Deplete**
Aspartic Acid	43.3	38.6	30.8	28.8
Threonine*	21.5	19.6	15.2	14.5
Serine	18.6	17.6	13.5	13.0
Glutamic Acid	48.9	44.8	33.8	32.4
Glycine	24.5	22.1	17.1	16.2
Alanine	33.1	30.3	22.4	21.9
Cysteine	2.0	1.8	1.3	1.1
Valine*	24.5	21.8	16.3	15.3
Methionine*	9.7	9.0	5.9	6.4
Isoleucine*	18.5	15.9	12.5	11.3
Leucine*	39.0	34.9	26.8	25.1
Tyrosine	19.1	18.6	12.2	14.1
Phenylalanine*	26.0	22.3	17.6	15.8
Lysine*	31.0	27.9	21.3	20.1
Histidine*	63.6	53.4	45.1	44.2
Arginine	11.8	9.7	7.5	7.6
Proline	16.2	14.1	10.1	9.5
Σ_{AA}	451.1	402.4	309.3	297.3
$\Sigma_{Essential\ AA}$	233.7	204.8	160.6	152.7

*:Essential AA.

Table S3.8. Amino acid profiles [mg g^{-1} DW] of *Mesotaenium* sp. at 2 and 11 ppt in nutrient-replete and deplete conditions.

Amino Acid	2 ppt		11 ppt	
	Replete	Deplete	Replete	Deplete
Aspartic Acid	24.8	22.6	19.0	17.4
Threonine*	13.0	11.7	10.0	8.9
Serine	11.7	10.4	8.9	7.9
Glutamic Acid	28.3	27.3	22.1	20.6
Glycine	14.2	12.7	11.0	9.7
Alanine	19.0	17.7	14.3	13.0
Cysteine	0.7	0.8	0.4	0.5
Valine*	14.4	13.0	11.1	9.8
Methionine*	4.4	4.7	2.5	3.0
Isoleucine*	10.8	9.9	8.4	7.4
Leucine*	24.3	21.3	19.1	16.2
Tyrosine	11.2	9.9	8.7	7.5
Phenylalanine*	17.2	14.8	13.7	11.3
Lysine*	16.0	14.7	12.2	11.5
Histidine*	35.3	34.0	29.3	23.8
Arginine	6.3	5.6	4.8	4.0
Proline	9.5	11.5	6.8	6.6
Σ_{AA}	261.1	242.5	202.5	179.3
$\Sigma_{Essential\ AA}$	135.4	124.1	106.3	92.0

*:Essential AA.

Table S3.9. Amino acid profiles [mg g^{-1} DW] of *Scenedesmus quadricauda* at 2 and 11 ppt in nutrient-replete and deplete conditions.

Amino Acid	**2 ppt**		**11 ppt**	
	Replete	**Deplete**	**Replete**	**Deplete**
Aspartic Acid	37.7	35.5	27.2	27.8
Threonine*	18.1	17.8	13.0	13.7
Serine	15.9	16.1	11.5	12.5
Glutamic Acid	42.6	41.0	29.7	30.9
Glycine	21.0	20.1	14.8	15.5
Alanine	28.1	27.4	19.1	20.3
Cysteine	1.8	1.8	1.1	1.2
Valine*	20.5	19.6	13.7	14.4
Methionine*	7.8	8.1	5.2	5.9
Isoleucine*	15.6	14.4	10.5	11.0
Leucine*	33.2	31.8	22.5	23.9
Tyrosine	16.1	15.9	10.3	12.6
Phenylalanine*	21.6	20.2	14.7	15.3
Lysine*	27.0	25.6	18.3	19.4
Histidine*	57.9	52.6	35.7	38.0
Arginine	10.1	8.8	6.6	7.1
Proline	13.3	13.1	8.5	9.1
Σ_{AA}	388.2	369.8	262.4	278.5
$\Sigma_{Essential\ AA}$	201.5	190.2	133.6	141.7

*:Essential AA.

Table S3.10 Amino acid profiles [mg g^{-1} DW] of *Tetraedron* sp. at 2 and 11 ppt in nutrient-replete and deplete conditions.

Amino Acid	2 ppt		11 ppt	
	Replete	Deplete	Replete	Deplete
Aspartic Acid	30.6	25.7	26.9	18.8
Threonine*	17.4	13.3	15.2	10.0
Serine	16.3	12.3	14.3	8.9
Glutamic Acid	36.2	30.9	32.5	24.4
Glycine	20.6	16.9	18.1	12.4
Alanine	31.2	24.4	26.4	18.4
Cysteine	2.6	1.6	2.0	1.1
Valine*	19.2	15.6	16.9	11.6
Methionine*	6.9	6.0	6.1	4.3
Isoleucine*	13.6	11.1	12.3	8.3
Leucine*	27.7	23.7	25.6	17.6
Tyrosine	14.1	11.7	12.8	8.4
Phenylalanine*	17.9	15.3	16.3	11.0
Lysine*	22.2	17.1	19.7	12.5
Histidine*	64.7	58.2	47.1	34.3
Arginine	7.5	6.6	6.4	4.5
Proline	14.2	18.7	10.9	8.7
Σ_{AA}	362.8	309.2	309.4	215.2
$\Sigma_{Essential\ AA}$	189.5	160.4	159.1	109.6

*:Essential AA.

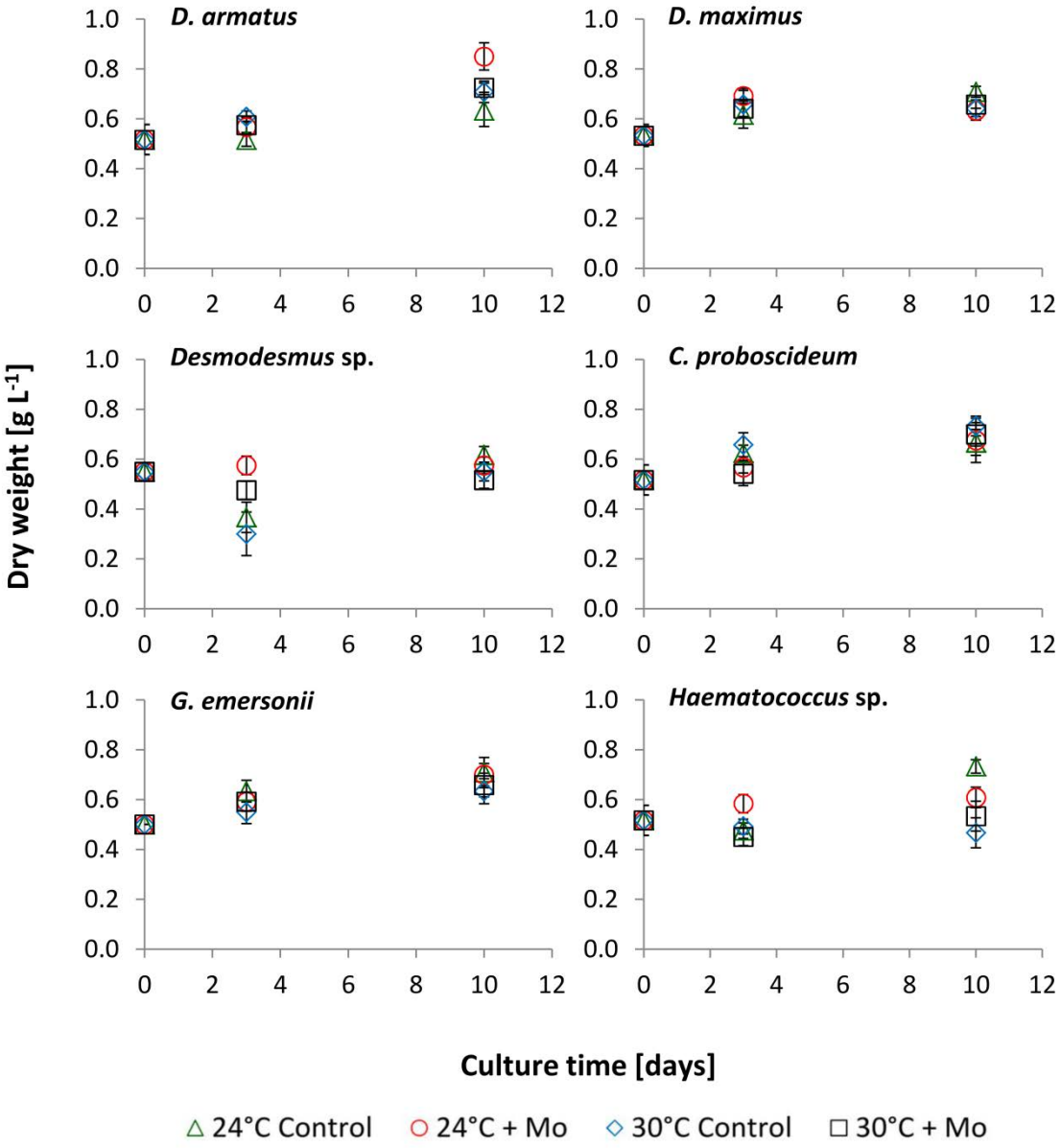

Figure S5.1. Culture dry-weights [g L^{-1}] of *D. armatus*, *D. maximus*, *Desmodesmus* sp., *C. proboscideum*, *G. emersonii* and *Haematococcus* sp.