Feed attractants in aquaculture - The chemical composition of aquacultured *Ulva lacinulata* (Chlorophyta)

LEKRAJ ETWARYSING

Thesis presented for the degree of Doctor of Philosophy in the Department of Biological Sciences

Supervisors:
Emeritus Professor John J Bolton (University of Cape Town)
Professor Denzil R Beukes (University of the Western Cape)
Dr Brett M Macey (Department of Forestry, Fisheries and the Environment)

February 2024

DECLARATION

I, Lekraj Etwarysing, declare that this thesis is my own unaided work, apart from the NMR analysis of samples that was carried out by the Department of Chemistry, University of the Western Cape and LC–MS analysis of samples performed by the Department of Chemistry, University of Cape Town. This thesis contains a culture experiment in Chapter 4, which was a joint research, both in the conception and execution, between Mr. Dhiren Vanmari of the Department of Biodiversity and Conservation Biology, University of the Western Cape and I, under the supervision of Dr. Mark D. Cyrus of Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University (then at the Department of Forestry, Fisheries and Environment), to grow the seaweed *Ulva* under different nutrient conditions. Mr. Vanmari looked at the effects of nutrients on protein contents for his MSc research while I looked at the effects of nutrients on the lipid and fatty acid contents of this seaweed for my PhD research. Mr. Vanmari and I, both participated in the setting up and optimization of the culture system (Chapter 4 Sections 4.3.3. and 4.3.4.), running the nutrient culture experiment (Chapter 4 Section 4.3.5.), recorded the same growth rates data (Chapter 4 Sections 4.3.4. and 4.4.1.) and collected the same seaweed grown from this culture experiment for our respective analyses. Description and findings of the abovementioned Sections of Chapter 4 presented in this thesis was written in my own words including graphics. Collection of environmental parameter data and water nutrient analysis were done by Mr. Vanmari. FAME analysis was carried out by the Department of Molecular and Cell Biology, University Cape Town and volatile compound analysis were carried out by the Central Analytical Facilities, Stellenbosch University. Neither the substance nor any part of the above thesis has been in the past, or is being, or is to be submitted for a degree at this University, or any other university, except for the information regarding the description and optimization of the culture system (Chapter 4 Sections 4.3.3. and 4.3.4.), nutrient enrichment experiment (Chapter 4 Section 4.3.5.) and growth rate data (Chapter 4 Sections 4.3.4 and 4.4.1.). Where the work of others has been used, it has been duly acknowledged in text and referenced. Where exact wordings have been used, the writing has been placed inside quotation marks and referenced. All experimental work presented in this thesis was carried out under the supervision of Emeritus Prof. John J. Bolton of the Department of Biological Sciences, University of Cape Town, Prof. Denzil R. Beukes of the School of Pharmacy, University of the Western Cape, Dr. Brett M. Macey of the Department of Forestry, Fisheries and Environment and Dr. Mark D. Cyrus of Centre for Sustainable Tropical Fisheries and Aquaculture, James Cook University. I hereby grant the University of Cape Town free license to reproduce this thesis in whole or in any part, for the purpose of research.

Signed: (10th February 2024)

This thesis is dedicated to:

Anita Etwarysing

Thank you for everything…

ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude and appreciation to my supervisors Emeritus Prof. John Bolton, Prof. Denzil Beukes and Dr. Brett Macey for their guidance, advice and support during the course of this research. John, thank you for sharing your lifelong knowledge and experience all things seaweed but most importantly for the patience that you have showed me throughout this journey. Denzil, thank you for the continuous support and motivation and for the countless hours that you have dedicated to my studies. Brett, thank you for the all the valuable insights that you have provided me from the beginning of this research, for all the time that you have spent reviewing my drafts and for always being there whenever I needed your help. A big thank you to Dr. Mark Cyrus for his key insights and guidance throughout this study and for providing me with the technical support and urchins for the trials.

I am very thankful to the National Research Foundation (NRF) for providing me with a bursary that allowed me to pursue this research. I would like to thank Prof. Edith Antunes for her valued assistance with the NMR analyses, Mr. Lucky Mokwena, Mr. William Arries and Mr. Arno Duvenhage for their assistance with the volatile and fatty acid analyses. I would also like to thank all the staffs at the Department of Forestry, Fisheries and the Environment Marine Research Aquarium for providing me with the space and logistics to run various experiments. A special thank you to Mr. Dhiren Vanmari for his assistance setting up the culture system and help throughout the culture experiment.

I would like to thank all my friends and colleagues from the Marine Biodiscovery Research group, Bas De Vos, Rifaat Aziz and Michael Bennett for their support.

To my family, thank you for the incredible support and encouragement. Thank you to my brother Abhi for his support and to always be there whenever I needed his help. I am forever grateful to my mother Anita for her unconditional love and persistent support throughout this whole journey and life in general. Lastly I would like to thank Anneke Groenewald for her love, support and patience, and for being there with me every step of the way.

ABSTRACT

For more than two decades, cultivation of the green seaweed *Ulva lacinulata* (Ulvales) has proven to be commercially viable and an integral part of the South African abalone aquaculture industry. This seaweed is not sold, but mainly used as feed for the abalone *Haliotis midae* (Lepetellida) and on some farms for the bioremediation of abalone effluent water to enable partial recirculation in integrated abalone-*Ulva* systems. The *Ulva* grown in farm effluent has higher protein content than material found in nature and has been found to be a suitable supplementary feed for secondary high commercial value macroalgivores, including the local sea urchin *Tripneustes gratilla* (Camarodonta). Under laboratory conditions, *T. gratilla* has demonstrated hierarchical feeding preference to this aquacultured *Ulva* over other seaweed species. Inclusion of this alga in the diet of this urchin has been shown to act as a feed attractant as well as act as a feed stimulant, but the mechanisms for this remain unknown. The aim of this research was to identify the fractions or compounds from aquacultured *Ulva* responsible for the attractant activity of this seaweed towards the sea urchin *T. gratilla* as well as to evaluate their effectiveness and minimum effective concentrations. This research also assessed the effect of nutrient variation on the lipid and fatty acid composition of aquacultured *Ulva* and evaluated the volatile organic compounds produced by this seaweed.

Crude *Ulva* extract, prepared from solvent extraction (dichloromethane - methanol), was fractionated to produce nine fractions (F1 → F9), with increasing polarity and solubility. The attractiveness and stimulatory effects of the *Ulva* fractions (extracts equivalent to 1 g fresh *Ulva*; FU) along with a control (no extract) were tested on *T. gratilla* in a chemosensory trial. The touch and grazing preferences of the urchin, placed in the center of a circular bioassay tank, were monitored over a period of 75 mins. A touch preference was assigned when the tube feet of *T. gratilla* touched the sample zone (Avicel® bioassay plate) containing an *Ulva* fraction, placed at the periphery of the tank, while a feed preference was allocated when the sample zone containing an extract was grazed. An urchin can show touch and grazing preference to one or multiple *Ulva* fractions in a bioassay. Bioassays were repeated using 120 individual sea urchins. Data collected showed clear evidence for the preferences of *T. gratilla* for the polar *Ulva* fractions F8 and F9. The predicted probability ranking for *Ulva* fractions to attract *T. gratilla* was F8 > F9 > F5 ≥ F6 > F4 > F7 > F3 > F2 ≥ F1, while the predicted probability ranking for *Ulva* fractions to stimulate *T. gratilla* to feed was F9 > F8 > F5 ≥ F6 > F4 ≥ F7 > F3 > F2 > F1. Glycerolipids, monogalactosyldiacylglycerol (MGDG) from F8 and a

mixture of compounds including diacylglyceryl-N,N,N-trimethyl-homoserine (DGTS), digalactosyldiacylglycerol (DGDG), dialkylglycerophosphate (PA), lysophospholipid (LPG) and sulfoquinovosylmonoacylglycerol (SQMG) from F9, were identified as the most probable feed stimulants for *T. gratilla*. The minimum effective concentrations (MECs) of the stimulatory *Ulva* fractions F8 and F9 were subsequently evaluated on *T. gratilla*. In total, five different concentrations for both F8 and F9 equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g fresh *Ulva* (FU) were tested on *T. gratilla* in chemosensory trials. The touch and grazing preferences of the urchins (n = 46 for each fraction) were recorded, and results showed that *T. gratilla* was able to perceive chemicals emitted by the treatment containing extracts equivalent to 0.05 g FU for both F8 and F9. However, the most effective treatments at attracting and stimulating *T. gratilla* to feed contained F8 equivalent to 2.0 g FU and F9 equivalent to 0.5 g FU. The effectiveness of F8 and F9 as attractants and feed stimulants were investigated further in a comparative study against fresh *Ulva* (FU), dry *Ulva* (DU) and crude *Ulva* extract (CE) on *T. gratilla* (n = 80). Data collected indicated that the treatment with FU was the most effective at both attracting and stimulating *T. gratilla* compared to the *Ulva* fractions. The predicted probability of occurrence for attracting *T. gratilla* was FU > F8 > CE ≥ F9 > DU, while the predicted probability for stimulating *T. gratilla* to graze was FU > DU > CE > F8 > F9.

To assess the effect of nutrient variation on the lipid and fatty acid composition of aquacultured *Ulva*, the seaweed was cultured for 10 days at six different nutrient concentrations: 0 (control), 25, 50, 75, 100 and 200% NE (NE: nutrient enrichment above ambient seawater concentration; 100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva*). Findings showed that nutrient variation did not significantly affect the specific growth rate (SGR) and lipid extracts of *Ulva* (solvent extraction; chloroform - methanol - phosphate buffer; 1:2:0.8). Lipid classes, obtained by the fractionation of the lipid extracts, were mainly characterized by glycolipids (GL) followed by neutral lipids (NL) and phospholipids (PL). Seaweed grown at low nutrient concentrations (0 and 25% NE; $p< 0.05$) had significantly lower neutral lipid (NL) contents than *Ulva* grown at higher nutrient concentrations, while glycolipid (GL) contents were significantly lower in *Ulva* cultured at a high nutrient concentration (200% NE; $p< 0.05$). Nutrient variation had no effect on the phospholipid (PL) contents of aquacultured *Ulva*. Analysis of the fatty acid methyl esters (FAMEs) of the crude *Ulva* lipid extracts, obtained from culture experiment, by gas chromatography coupled with mass spectrometry (GC-MS) revealed a total of 25 fatty acids (FA). The fatty acid profiled of aquacultured *Ulva* consisted primarily of saturated fatty acids (SFA) followed by

polyunsaturated fatty acids (PUFA) and monounsaturated fatty acids (MUFA). Nutrient variation had no significant effect on the fatty acid content except for the level of C18:0 FA (stearic acid), which was at its lowest value at the highest nutrient concentration (200% NE; $p < 0.05$). SFA content of *Ulva* consisted mainly of C16:0 FA (palmitic acid), PUFA of C16:4ω3 (hexadecatetraenoic acid) and MUFA of C18:1ω9 FA (oleic acid). The PUFA content of aquacultured *Ulva* was comprised mostly of omega-3 FAs (ω-3) compared to ω-6 FAs, with the ω-6:ω-3 ratios ranging between 0.08 to 0.12.

Aquacultured *U. lacinulata* produces a mixture of volatile organic compounds (VOCs) ranging from simple to complex volatile compounds that can contribute to its typical aroma. Tentative identification of VOCs from the solvent extract (dichloromethane - methanol; 2:1), essential oil and head space of aquacultured *Ulva* analysed by GC-MS, revealed a total of 63 compounds. VOCs from this alga were classed as aldehydes (18), hydrocarbons (15), ketones (10), esters (9), free fatty acids (6), alcohols (4) and aromatic compound (1). Based on the relative content (%) and information available from the literature, only the aldehydes, hydrocarbons, ketones, esters and alcohol are considered as the most important classes of compounds associated with the aroma of this seaweed. Findings also showed that aquacultured *Ulva* produces VOCs such as benzaldehyde and 6-methyl-5-hepten-2-one that have been shown to be flavour enhancers for high value species including sea urchin.

The research from this thesis clearly shows that local aquacultured *U. lacinulata* is an effective chemoattractant and feed stimulant for the sea urchin *T. gratilla*. Although the effects of *Ulva* in its fractionated form (*i.e. Ulva* fractions F8 and F9) towards *T. gratilla* was shown to be dose-dependent and less effective than fresh *Ulva*, findings from this research provide valuable information that may be used in the formulation or supplementation of compound diets to promote consumption, which could be beneficial for the aquaculture industry. This research also provided an improved understanding of the variability of lipid, lipid classes and the FA profile of aquacultured *Ulva*. Even though a high SFA content was observed, this seaweed has a beneficial PUFA profile with a low ω-6:ω-3 ratio that makes it suitable for human consumption and aquafeed production. Lastly, findings from this study showed that this seaweed produces a mixture of VOCs ranging from simple to complex compounds that may contribute to its typical aroma and the presence of compounds that may be used as flavour enhancers. With the growing interest looking at the commercial application of *Ulva* around the world, outcomes from this thesis may be of particular interest to the aquaculture, animal feed and human food industries.

TABLE OF CONTENTS

CHAPTER 1: General introduction

CHAPTER 2: Feed stimulants from the green alga *Ulva lacinulata* for the sea urchin *Tripneustes gratilla* in aquaculture

CHAPTER 3: Evaluation of the minimum effective concentrations and effectiveness of feed stimulatory *Ulva* fractions on the sea urchin, *Tripneustes gratilla*

CHAPTER 4: Effect of nutrient variation on the lipid and fatty acid composition of *Ulva lacinulata*: An NMR-based lipidomic approach

CHAPTER 5: Evaluation of volatile organic compounds from aquacultured *Ulva lacinulata*

CHAPTER 6: General discussion

References and Appendices

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

%	Percentage
%.d^{-1}	Percentage per day
±	Plus-minus
=	Equal to
≈	Approximately equal to
>	Greater than
≥	Greater than or equal to
®	Registered trademark symbol
°C	Degrees Celsius
°C.min^{-1}	Degrees Celsius per minute
.csv	Comma delimited
$[M - Gal]^+$	Mass minus galactose positive ion
$[M - H]^-$	Mass minus hydrogen negative ion
$[M + H]^+$	Mass plus hydrogen positive ion
$[M + NH_4]^+$	Mass plus ammonium positive ion
β	Beta
δ_C	Carbon chemical shift
δ_H	Proton chemical shift
Δρ	Probability of occurrence
µg	Microgram
µg.g^{-1}	Microgram per gram
µg.mL^{-1}	Microgram per millilitre
µL	Microlitre
µm	Micrometre
µM	Micromolar
µmol.L^{-1}	Micromole per litre
χ^2	Chi-square distribution
ω-1	Hydroxy fatty acid
ω-3	Omega-3 fatty acid
ω-6	Omega-6 fatty acid
ω-7	Omega-7 fatty acid
1D	One dimensional
^{1}H	Proton
2D	Two dimensional
^{13}C	Carbon - 13
AA	Arachidonic acid
Ace	Acetone
ACN	Acetonitrile
AgriSETA	Agricultural Sector Education Training Authority
ALA	α-linolenic acid
ANOVA	Analysis of variance
BF$_3$	Boron trifluoride
BP	Bromophenols
C	Carbon
ca.	About

CAF	Central Analytical Facilities
CAR	Carboxen
CD_3OD	Deuterated methanol-d4
$CDCl_3$	Deuterated chloroform-d
CE	Crude *Ulva* extract
CH_2Cl_2	Dichloromethane
$CHCl_3$	Chloroform
cm	Centimetre
COST	European Cooperation in Science and Technology
COSY	Correlated Spectroscopy
COVID-19	Coronavirus pandemic of 2019
D	Day
d	Doublet
Da	Dalton
DBE	Double bond equivalent
dd	Doublet of doublets
DEPT	Distortionless Enhancement by Polarization Transfer
df	Degrees of freedom
DFFE	Department of Forestry, Fisheries and Environment
DGDG	Digalactosyldiacylglycerol
DGTS	Diacylglyceryl-N,N,N-trimethyl-homoserine (also reported as DGTH)
DHA	Docosahexaenoic acid
DMS	Dimethylsulfide
DMSP	Dimethylsulfoniopropionate
DPA	Docosapentaenoic acid
dt	triplets
DU	Dry *Ulva*
DVB	Divinylbenzene
DW	Dry weight
E	East
e.g.	For example
EPA	Eicosapentaenoic acid
ESI	Electrospray ionization mode
et al.	et alia (and others)
EtOAc	Ethyl acetate
EtOH	Ethanol
eV	Electron volt
FA	Fatty acid
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization
FAO/WHO	Joint Food and Agriculture Organization and World Health Organization
FCR	Feed conversion ratio
FU	Fresh *Ulva*
F-value	Ratio of variance in ANOVA
FW	Fresh weight
g	Gram
$g.kg^{-1}$	Gram per kilogram
GC	Gas chromatography

GC–MS	Gas chromatography–mass spectrometry
GDP	Gross domestic product
GL	Glycolipid
GLM	Generalized linear model
GSI	Gonad somatic indices
H	Hydrogen
H_2O	Water
hex	Hexane
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High-performance liquid chromatography
hrs	Hours
HSQC	Hetereonuclear Single Quantum Coherence
HS–SPME	Headspace solid–phase microextraction
Hz	Hertz
I	Integral
IDC	Industrial Development Corporation
i.e.	That is
IMTA	Integrated Multi-Trophic Aquaculture
in vacuo	under vacuum
IQR	Inter-quantile range
ISO	International Standard Organization
ITS	Internal Transcribed Spacer
K	Potassium
$kg.m^{-2}$	Kilogram per square metre
kPa	Kilopascal
L	Litre
$L.day^{-1}$	Litre per day
LA	Linoleic acid
LCI	Lower confidence interval
LC–MS	Liquid chromatography–mass spectrometry
LiChrosolv®	Liquid chromatography solvent grade
LPG	Lysophospholipid
m	Metre
m	Multiplet
m/z	Mass to charge ratio
MAP	Mono-ammonium phosphate
MC	Moisture content
MEC	Minimum effective concentration
MeOH	Methanol
mg	Milligram
$mg.kg^{-1}$	Milligram per kilogram
$mg.mL^{-1}$	Milligram per millilitre
MGDG	Monogalactosyldiacylglycerol
MGTS	Monoacylglyceryl-4'-O-(N,N,N-trimethyl)-homoserine (also reported as MGTH)
MHz	Megahertz
m_i	Initial mass
min	Minute

MKP	Mono-potassium phosphate
mL	millilitre
mL.min^{-1}	Millilitre per minute
mm	Millimetre
m_{od}	Oven dry mass
MS	Mass spectra
ms	milli second
MUFA	Monounsaturated fatty acid
N	Nitrogen
n	number of sample
Na_2SO_4	Sodium sulfate
NaCl	Sodium chloride
NaN	Not a number
ND	Not detected
NE	Nutrient enrichment above ambient seawater concentration
NH_4^+	Ammonium
NL	Neutral lipid
NMR	Nuclear magnetic resonance spectroscopy
NO_2^-	Nitrite
NO_3^-	Nitrate
O	Oxygen
P	Phosphorus
PA	Dialkylglycerophosphate
PC	Principal component
PCA	Principal component analysis
PDMS	Polydimethylsiloxane
pers. comm.	Personal communication
Pg.	Page
PL	Phospholipid
ppm	Parts per million
PSI	Pound per square inch
PSU	Practical salinity unit
PTFE	Polytetrafluoroethylene
PUFA	Polyunsaturated fatty acid
p-value	Probability value (level of significance)
PVC	Polyvinyl chloride
*rbc*L	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RT	Retention time
S	South
SD	Standard deviation
SD–LLE	Steam distillation–liquid/liquid extraction
SE	Solvent extraction
sec	Second
SFA	Saturated fatty acid
SGR	Specific growth rate
Si	Silica
sn	Stereospecific numbering

sp.	Species
spp.	Several species
SPME	Solid phase microextraction
SQDG	Sulfoquinovosyldiacylglycerol
SQMG	Sulfoquinovosylmonoacylglycerol
t	Time
t	Triplet
TFA	Total fatty acid
TIC	Total ion chromatograms
TL	Total lipid
TLC	Thin layer chromatography
TOF-MS	Time-of-flight mass spectrometry
TAG	Triacylglycerols
tr	Trace
tufA	Elongation factor
UCI	Upper confidence interval
UFA	Unsaturated fatty acid
USD	United States Dollar
V	Volt
v/*v*	Volume per volume
v/v/v	Volume per volume per volume
VE.d^{-1}	Volume exchange rate
ver.	Version
VOC	Volatile organic compound
W	Watt
w/w	Weight by weight
W_o	Initial fresh biomass
W_t	Final fresh biomass
ZAR	South African rand
zg	Pulse program
z-value	Standardized score

CHAPTER 1

General introduction

1.1 Overview

Chemical cues play crucial roles in animal welfare and survival (Hay, 2009; Webster and Weissburg, 2009). In the aquatic environment, organisms can detect, discriminate and respond to a multitude of dissolved chemical signals that carry various messages (Derby and Sorensen, 2008; Webster and Weissburg, 2009). Detection of these external signals through chemical sensing affects various aspects of the organisms' behaviour, including the elicitation of feeding (*e.g.* Moore and Lepper, 1997, Coleman et al. 2007), deterrence of feeding (*e.g.* Hay et al. 1989; Toth and Pavia, 2000), predator avoidance and alarm responses (*e.g.* Jacobsen and Stabell, 2004; Smee and Weissburg 2006), conspecific recognition (*e.g.* Lecchini et al. 2017), mate identification and reproduction (*e.g.* Lonsdale et al. 1998; Hardege et al. 2011), selection of settlement surfaces by larvae (*e.g.* Swanson et al. 2004), continuity of symbiotic associations (*e.g.* Williamson et al. 2012), and migration (*e.g.* Boriss et al. 1999). However, our knowledge of which chemical compounds are involved and how they influence specific behaviour, such as feeding, is limited (Hay, 2009).

The production of the green seaweed *Ulva* (Ulvales) has proven to be commercially viable and is an integral part of the South African abalone aquaculture industry, with a continuous and increasing production since 2002 (*ca*. 2000 tonnes fresh weight; Bolton et al. 2016; Neveux et al. 2018; Rothman et al. 2020). This crop is not sold but is mainly used as feed (*i.e.* either fresh or dried - incorporated in formulated feed) for the abalone *Haliotis midae* (Lepetellida) and for bioremediation of farm effluent water to enable partial recirculation of water in abalone-*Ulva* integrated multi-trophic aquaculture (IMTA) production systems (Bolton et al. 2013, 2016; DFFE, 2016). Partial recirculation using *Ulva* can reduce farm's high pumping cost, alleviate pressure on natural kelps stock, has the potential to cut off the culture systems from the natural environment for short periods at a time (up to 3 days) during events of harmful algal bloom and warm up the water in abalone tanks, which can improve abalone growth (Bolton et al. 2009, 2013; Neveux et al. 2018; De Prisco, 2020). IMTA-farmed *Ulva* has a higher protein content than species found in nature and can have similar amino acid profiles to animal and other plant protein, thus making it an ideal ingredient for the partial replacement of fishmeal in formulated feeds (Robertson-Andersson et al. 2011; Shuuluka et al. 2013). Effluent grown *Ulva* has been found to be a suitable supplementary feed for secondary high commercial value macroalgivores such as abalone and sea urchin (Naidoo et al. 2006; Cyrus et al. 2013, 2015a, b). Laboratory-based research done by Cyrus et al. (2013) has demonstrated that a formulated diet containing 200 g.kg^{-1} dried aquacultured *Ulva* significantly increases the gonad somatic indices of the sea urchin *Tripneustes gratilla* (Camarodonta) and produces market quality gonads (roe) both in term of size and colouration, essential for this product. Additional testing of this formulated feed containing 200 g.kg^{-1} dried *Ulva* (25.7% protein) along with fresh *Ulva*, a formulated feed containing no *Ulva* and blank (control) on *T. gratilla* chemosensory response using a Y-shaped maze revealed that the animals were attracted to treatments with the feed containing dried *Ulva* and fresh *Ulva* compared to the feed containing no *Ulva* and the blank controls (Cyrus et al. 2015a). The researchers suggested that the urchins responded to the chemosensory properties of the seaweed found in the feed rather than its structural or morphological properties. Cyrus et al. (2015a) also reported that the urchins could not differentiate between the feed containing no *Ulva* and the blank and indicated that the dry formulated feed did not contain a chemical attractant. Incorporation of dried *Ulva* at 200 g.kg^{-1} acted as a feeding stimulant that significantly increased the consumption and protein intake of the prepared feeds (Cyrus et al. 2015a). Studies to date on the aquacultured *Ulva* in South Africa are mostly limited to data on the growth rate, nutrient uptake, bioremediation capacity, economic analysis, quantification of nutritional content as feed and its effect on the physiology of animals, while little research have been done on its chemistry with none focusing on the chemical compounds that may act as feed attractants or stimulants (Robertson-Anderson et al. 2008, 2011; Bolton et al. 2009; Shuuluka et al. 2013; Cyrus et al. 2013, 2015a, b; Madibana et al. 2017; Onomu et al. 2020).

The present thesis is structured into four experimental chapters, with each examining a different topic related to local aquacultured *Ulva*, including (1) the identification and characterization of the fractions or compounds from *Ulva* responsible for the attractant activity towards the cultured sea urchin *T. gratilla*; (2) investigation of the minimum effective concentrations (MECs) at which *Ulva* fractions initiate the feeding stimulatory response of *T. gratilla* and to evaluate the feed stimulatory effect of *Ulva* fractions in a direct comparison to crude *Ulva* extract, fresh and dry *Ulva* on *T. gratilla*; (3) an assessment of the effect of varying nutrient concentrations during the growth of aquacultured *Ulva* on the lipid and fatty acid composition of this alga; and (4) the evaluation of the volatile organic compounds produced by aquacultured *Ulva*. A review of the relevant literature on algal chemical ecology and chemical composition is included in Chapter 1 to provide insights on topics of importance including chemoattraction in marine organisms (*i.e.* feed attractant, stimulant and deterrent) and volatile compounds from seaweeds. Lastly, a review on the status and importance of *Ulva* in the South African aquaculture industry as well as the added benefits of using this seaweed as a feed-additive and/or supplementary feed is also included.

1.2 Status of global aquaculture and seaweed production

Despite the COVID-19 pandemic, the global aquaculture industry has been thriving and recorded a total production of 122.6 million tonnes live weight, estimated to be worth USD 281.5 billion in 2020 (FAO, 2022). This production value represented an increase of 6.7 million tonnes in live weight from the 115.9 million tonnes recorded in 2018 and equated to a growth of USD 18.5 billion from 2018 and USD 6.7 billion from 2019 (FAO, 2022). Of the 122.6 million tonnes live weight recorded in 2020, 87.5 million tonnes were from aquacultured animals estimated to be worth USD 264.8 billion, while the remaining 35.1 million tonnes were from algae valued at USD 16.5 billion (FAO, 2022). Almost all this algal production was seaweeds grown in marine aquaculture. Global aquaculture production in 2020 was dominated by countries from Asia (91.6%) followed by America (3.6%), Europe (2.7%), Africa (1.9%) and Oceania (0.2%; FAO, 2022).

In 2020, the total aquaculture production in Africa was estimated to be 2.35 million tonnes live weight, where 78.9% of the production came from inland aquaculture and 21.1% from marine and coastal aquaculture (FAO, 2022). Egypt dominated the production on the African continent in 2020 with an estimated production of 1.59 million tonnes live weight (67.6%), followed by Sub-Saharan African countries (excluding Nigeria) - 0.46 million tonnes live weight (19.6%), Nigeria - 0.26 million tonnes live weight (11.1%) and Northern African countries (excluding Egypt) - 0.04 million tonnes live weight (1.7%; FAO, 2022). Despite of the abundance of natural resources and an increasing need for food security, the contribution of African countries to the global aquaculture production in 2020 was still low, estimated at 1.9% of live weight (FAO, 2022; Hinrichsen et al. 2022).

The global algae (micro- and macroalgae) production by aquaculture in 2020 was estimated to be 35.1 million tonnes live weight (USD 16.5 billion; FAO, 2022). Global algae aquaculture was predominated by macroalgae (*i.e.* seaweeds), with countries from Asia, including China and Indonesia, being among the main producers (99.5% of total production; FAO, 2022). In 2019, farmed seaweeds accounted for an estimated 97.0% of the total 35.8 million tonnes wet weight of the total seaweed production (*i.e.* cultivated and wild-collected; Cai et al. 2021). An estimated 99.1% of farm seaweeds produced in 2019 came from Asia, where seven of the top seaweed producing countries by cultivation were located (Cai et al. 2021). Farmed seaweeds from the African continent in 2019 accounted for only 0.12 million tonnes wet weight of the global farmed seaweed production, an estimated 90.0% of this production coming from Tanzania (including Zanzibar) followed by Madagascar (7.5%), South Africa (1.8%) and the rest of Africa (0.6%; 3 countries; Cai et al. 2021; Msuya et al. 2022). Globally, the top six most cultivated seaweed species were *Saccharina japonica* (Laminariales; formally *Laminaria japonica*; commonly known as Kombu) [35.5%], *Eucheuma* spp. (Gigartinales) [23.2%], *Gracilaria* spp. (Gracilariales) [14.8%], *Undaria pinnatifida* (Laminariales; commonly known as Wakame) [8.0%], *Neoporphyra* and *Neopyropia* (formally *Porphyra* spp; Bangiales; commonly known as nori) [6.3%] and *Kappaphycus alvarezii* (Gigartinales) [4.6%] (FAO, 2022). Most algae produced by aquaculture are directly used for human consumption and as non-food applications in nutraceutical, pharmaceutical, cosmetic, feed, biofertilizer, textile and biofuel industries (McHugh, 2003; FAO, 2018).

1.3 Marine aquaculture in South Africa

In South Africa, the aquaculture industry produced a total production of 7085.65 tonnes in 2019, with mariculture (excluding seaweeds) contributing 5112.79 tonnes (72.2%) and freshwater aquaculture contributing 1972.86 tonnes (27.8%; DFFE, 2020). The South African aquaculture in 2019 was estimated to be worth ZAR 1217.3 million (USD 64.6 million), with mariculture contributing 87.0% of the total value estimated at ZAR 1061.8 million (USD 56.3 million; DFFE, 2020). Mariculture in South Africa was dominated by abalone production valued at ZAR 948.5 million (USD 50.3 million), representing 89.0% of the mariculture value and 78.0% of the total South African aquaculture value (DFFE, 2020). However, the industry compared to the rest of Africa and the World, remains a relatively small industry with the aquaculture industry contributing 0.2% of the country’s GDP (Adeleke et al. 2020; AgriSETA, 2020).

South Africa’s mariculture industry is the most developed in Africa, with a total of 35 farms culturing marine species in 2019 (DFFE, 2020; Adeleke et al. 2020). The main species that were commercially farmed include the abalone *Haliotis midae* (Lepetellida), oyster *Crassostrea gigas* (Ostreida), mussels *Mytilus galloprovincialis* and *Choromytilus meridionalis* (Mytilida), finfish *Argyrosomus japonicus* (Eupercaria incertae sedis), rock lobster *Panulirus homarus* (Decapoda) and seaweeds *Ulva* sp. (Ulvales) and *Gracilaria* sp. (Gracilariales; DFFE, 2020). The finfish *Oncorhynchus mykiss* (Salmoniformes) was also farmed at a pilot scale (DFFE, 2020). The finfish *Rhabdosargus globiceps* and *Pomadasys commersonnii* (Eupercaria incertae sedis), sea urchin *Tripneustes gratilla* (Camarodonta), scallop *Pecten sulcicostatus* (Pectinida) and clam *Venerupis corrugata* (as *V. corrugatus*; Venerida), were researched at the Department of Forestry, Fisheries and the Environment (DFFE) Aquaculture Research Facility in Cape Town, South Africa (DFFE, 2020).

The sea urchin *Tripneustes gratilla* Linnaeus, 1758 (Phylum: Echinodermata; Class: Echinoidea; Order: Camarodonta; Family: Toxopneustidae; Genus: *Tripneustes*) has been identified as a viable species for culture in South Africa (Cyrus et al. 2013). Over the last few years, extensive laboratory-based research has been carried out to develop technologies for the culture of this sea urchin (*e.g.* Scholtz et al. 2013; Cyrus et al. 2013, 2015a, b, 2020; Onomu et al. 2020; de Vos et al. 2023, 2024). A formulated diet developed by Cyrus et al. (2013) containing 200 g.kg^{-1} dried aquacultured *Ulva* (25.7% protein) was found to significantly increase the gonad somatic indices (GSI) of *T. gratilla*, compared to organisms fed solely with fresh *Ulva*, as well as produce market quality gonads (roe) in term of size and colouration. The inclusion of the dried aquacultured *Ulva* at 200 g.kg^{-1} was also shown to improve the palatability of dried formulated feeds and act as a feed stimulant that promotes feed consumption (Cyrus et al. 2015a). Cyrus et al. (2015b) demonstrated that *T. gratilla* fed on fresh *Ulva* can attain equivalent somatic growth rates to the formulated feed containing 200 g.kg^{-1} dried *Ulva* in a 20-week somatic growth phase trial. However, the researchers did not recommend the use of fresh *Ulva* only for the full life-cycle of *T. gratilla* since the gonad production was significantly lower than organism fed with the formulated feed containing 200 g.kg^{-1} dried *Ulva*. Cyrus et al. (2015b) subsequently suggested the use of fresh *Ulva* for the somatic growth phase and the formulated feed for the gonad enhancement phase to increase gonad growth prior to market, while retaining the marketable properties of the gonad, and increase product yield. A farm-based gonad enhancement study on *T. gratilla* was conducted more recently at Wild Coast Abalone farm in Eastern Cape Province of South Africa (Onomu et al. 2020). The effects of different fresh seaweeds (*U. lacinulata* [Bachoo et al. 2023], *Gracilaria gracilis* (Gracilariales) and 1:1 mixture of *U. lacinulata* and *G. gracilis*) and a formulated feed containing 200 g.kg^{-1} dried *Ulva* on the gonad growth (phase 1: 12 weeks) and gonad quality (phase 2: 6 weeks) were tested on wild-collected adult *T. gratilla* (50 – 85 mm test diameter). Findings from this study supported the findings from the laboratory-based research of Cyrus et al. (2015b) and revealed that the formulated feed containing 200 g.kg^{-1} dried *Ulva* (25.7% protein) could enhance gonad growth in *T. gratilla* and produce commercially acceptable gonads.

1.4 Integrated Multi-Trophic Aquaculture (IMTA) and *Ulva* aquaculture in South Africa

Integrated multi-trophic aquaculture (IMTA) is a farming technique that provides a more environmentally friendly, economically diverse and socially more acceptable way to produce aquatic organisms (Troell et al. 2009; Chopin et al. 2010). This method is aimed at increasing the utilization of resources by trophically linking cultured species to improve the use of nutrients, reduce environmental impacts and produce additional organisms with commercial value (Troell et al. 2009; Chopin et al. 2010, 2012). IMTA is accomplished by merging the production of fed aquaculture animals (*i.e.* fish, abalone or shrimp) with extractive organisms (*i.e.* seaweeds, shellfish or echinoderms), whereby the organic and inorganic waste products from the fed animals are used by the extractive species for growth (Chopin et al. 2012; Holdt and Edwards, 2014). Integration of extractive species such as seaweeds provides long-term sustainability and profitability to farms, whereby the wastes from fed aquaculture can be used as fertilizer to grow biomass, which may in turn be marketed or used as feed for secondary cultured macroalgivores (Neori et al. 2004; Chopin et al. 2010).

Ulva has been recognized as a model species for bioremediation applications and to sustainably produce high biomass in land-based IMTA systems due to their high growth rates as well their high nutrient uptake capacity (Robertson-Andersson et al. 2008; Lawton et al. 2013; Al-Hafedh et al. 2015; Shpigel et al. 2018a). In South Africa, *Ulva* is produced on abalone farms growing *Haliotis midae* (Lepetellida; Bolton et al. 2009). The annual production of this alga is reported to be around 2000 tonnes (wet or fresh weight) annually, where most of the *Ulva* is cultured in abalone wastewater (Bolton et al. 2016; Neveux et al. 2018). Recently, the production of *Ulva* on the abalone farms was estimated to be around 2300 tonnes per annum (*pers. comm.* Emeritus Professor John J. Bolton). Since 2002, *Ulva* has been cultivated at a commercial scale in large D-ended paddle-raceways (*ca.* 30 m long, 0.5-1 m deep; Bolton et al. 2009; Neveux et al. 2018; Rothman et al. 2020). This IMTA grown crop is not sold but is mainly used as abalone feed (*i.e.* either fresh or dried – incorporated in formulated feed) and for bioremediation of abalone wastewater (Bolton et al. 2013, 2016; DFFE, 2016). Currently, there are five abalone farms that are commercially cultivating *Ulva*, namely: Abagold Abalone Farm, Irvin & Johnson Cape Abalone, Diamond Coast Aquaculture Farm, Buffeljags Abalone Farm located in the Western Cape province and Wild Coast Abalone Farm Abalone located in the Eastern Cape province of South Africa. A few other farms are also growing *Ulva* in small quantities as a feed supplement (Bolton et al. 2013).

1.5 *Ulva* in aquafeed

Species from the genus *Ulva* have emerged as potential ingredients for aquafeeds due to their high protein contents as well as their high rate of biomass production in land-based and IMTA systems (Robertson-Andersson et al. 2011; Cyrus et al. 2013, 2015a, b; Silva et al. 2015; Mata et al. 2016; Shpigel et al. 2017, 2018a, b; Fleurence et al. 2018; Gadberry et al. 2018). *Ulva* biomass grown in aquaculture effluent can have higher protein content than materials collected from wild (up to 40% crude protein) and can have similar amino acid profiles to animal and other plant protein (Robertson-Andersson et al. 2011; Fleurence et al. 2012; Shuuluka et al. 2013; Angell et al. 2015, 2017; Bolton et al. 2016; Shpigel et al. 2018a, b). Member of this genus have also been reported to be an important source of soluble fibers, beneficial omega-3 fatty acids (ω-3), bioactive polar lipids, vitamins and trace elements, making them useful as functional feed ingredients in aquafeeds (Ortiz et al. 2006; García-Casal et al. 2007; Mata et al. 2016; Neto et al. 2018; Lopes et al. 2019; Moreira et al. 2020).

Inclusion of *Ulva* at low levels (up to 20%) in formulated feeds has been promising for several aquacultured species (*e.g.* Valente et al. 2006; Cyrus et al. 2013, 2015a, b; Silva et al. 2015; Shpigel et al. 2017; Madibana et al. 2017; Qiu et al. 2018; Yangthong and Ruensirikul, 2020). Dietary inclusion of *Ulva* at low levels (5 – 10%) into the practical feed of several marine and freshwater finfish including *Argyrosomus japonicus* (Eupercaria incertae sedis), *Dicentrarchus labrax* (Eupercaria incertae sedis), *Oreochromis niloticus* (Cichliformes) and *Oncorhynchus mykiss* (Salmoniformes), have been tested with no adverse effects, and can induce positive effects on growth, efficiency of the feed, nutrient utilization and overall body composition (Ergün et al. 2009; Güroy et al. 2013; Wassef et al. 2013; Silva et al. 2015; Valente et al. 2016; Madibana et al. 2017). Shpigel et al. (2018b) demonstrated that fresh IMTA-framed *Ulva* can be used as the only food source for somatic and gonadal growth in the sea urchin *Tripneustes gratilla elatensis* (Camarodonta) compared to the red seaweed *Gracilaria conferta* (Gracilariales) and the formulated diets containing 10 % dried *Ulva*. Supplementation of the diet of the juvenile abalone *Haliotis asinina* (Lepetellida) with protein-enriched *Ulva* can partially replace the use of fish and soybean meals in formulated feed (Santizo-Taan et al. 2020). Shpigel et al. (2017) demonstrated that the 100% substitution of fishmeal from the aquafeed of the juvenile finfish *Sparus aurata* (Eupercaria incertae sedis) with a feed containing 291 g.kg^{-1} of poultry meal and 146 g.kg^{-1} of IMTA-grown *Ulva* could produce similar specific growth rates, food conversion ratio and survival to fish fed with commercial aquafeed. Laramore et al. (2018) reported that replacement of the commercial feed of the shrimp *Penaeus vannamei* (as *Litopenaeus vannamei*; Decapoda) up to 25 % with fresh IMTA *U. lactuca* may be feasible without affecting production.

The use of *Ulva* as feed additive has also shown to enhance the colouration of cultured shrimp and sea urchin gonads, which is key to the product, mainly due to the β-carotene found in *Ulva* (*e.g.* Cruz-Suarez et al. 2010; Cyrus et al. 2013, 2015b; Shpigel et al. 2018b). Supplementation of the diet of juvenile finfish *Seriola lalandi* (as *Seriola dorsalis*; Carangiformes) with *Ulva* can produce muscle tissue with higher content of desirable omega-3 long-chain polyunsaturated fatty acids (ω-3 PUFAs; *e.g.* Legarda et al. 2021). Dietary supplementation with the *Ulva*-derived polysaccharide ulvan at 5 g.kg^{-1} has been shown to promote growth, enhance immune-related genes and increase immune responses against a pathogenic bacterium in shrimp (Klongklaew et al. 2021). Aquafeed supplemented with a water-soluble polysaccharide extract of *Ulva* at 10 mg.kg^{-1} has been found to improve growth and antioxidant activity of juvenile finfish, as well as increase immune responses of the host following a challenge with the pathogenic bacteria *Photobacterium damselae* (Vibrionales; Akbary and Aminikhoei, 2018). Brand (2023) demonstrated that the abalone *H. midae* fed a diet of fresh *U. lacinulata* grown in abalone effluent had a higher bacterial clearance efficiency, compared with abalone fed a formulated diet, following experimental challenge with a known dose of the bacterium *Listonella anguillara* (as *Vibrio anguillarum*; Vibrionales). Supplementation of *H. midae*'s diet with fresh and dried IMTA *U. lacinulata* has been shown to have a positive modulatory effect on the gut microbiome of the organisms (Macey et al. 2022). Inclusion of dried aquacultured *Ulva* at 200 g.kg^{-1} in aquafeed of the sea urchin *T. gratilla* increases the feed attractability and can act as a feed stimulant, therefore increasing acceptability of the feed, promoting feed consumption and protein digestibility (Cyrus et al. 2015a).

1.6 Taxonomy of aquacultured *Ulva* in South Africa

Ulva (Phylum: Chlorophyta, Class: Ulvophyceae, Order: Ulvales, Family: Ulvaceae) is a genus of green algae that was first described by Linnaeus in 1753 (Hayden et al. 2003). Species of this genus show a certain degree of phenotypic plasticity that is greatly controlled by seasons, environmental factors and age of the thallus, thus making them extremely difficult to identify by morphological features (Tanner, 1986; Malta et al. 1999; Loughnane et al. 2008; Hofmann et al. 2010). This genus also contains cryptic species that further complicates identification simply based on morphology (Hofmann et al. 2010; Kraft et al. 2010). Molecular sequencing has revolutionized the taxonomy of *Ulva* where molecular evidence has showed that specimens with tubular forms "*Enteromorpha*" and species with bladelike forms "*Ulva*", are molecularly polyphyletic and both genera were merged into a single genus, *Ulva* (Hayden et al. 2003).

For the last two decades, there has been an increasing body of research using molecular markers to identify and classify species from this genus (*e.g.* Hayden and Waaland 2004; Loughnane et al. 2008; Heesch et al. 2009; Kraft et al. 2010; Wolf et al. 2012; Kazi et al. 2016; Kang et al. 2019; Hughey et al. 2019; Bachoo et al. 2023). However, no real agreement has been reached on the species-level taxonomy of *Ulva* (Bolton 2020). The '*Ulva lactuca'* name that is commonly used in literature, particularly in aquaculture, is widely misannotated (O'Kelly et al. 2010; Bolton et al. 2016; Bolton, 2020; Fort et al. 2022). Recently, the Linnaean specimen of *U. lactuca* Linnaeus, on which the species is established, was shown to be *U. fasciata* Delile (Hughey et al. 2019; Bolton, 2020). Almost all the aquaculture literature reporting the culture of foliose species under the name *U. lactuca*, collected unattached in sheltered bays or estuaries, may in fact be an assortment of other *Ulva* species (Bolton et al. 2016; Bolton, 2020). With the increasing importance of *Ulva* in aquaculture, a logical and genetically based approach should be adopted to correctly identify species and strains from this genus (Bolton et al. 2016; Bolton, 2020). Proper identification will not only provide farmers with the proper information on the right conditions to grow specific species and strains, but will also enable farmers to select species and strains with desirable characteristics.

Recently, both morphoanatomical and molecular methods (using the biomarkers: Ribulose-1,5-bisphosphate carboxylase/oxygenase [*rbc*L], Internal Transcribed Spacer [ITS] and elongation factor [*tufA*]) were used to characterize *Ulva* cultured from five South African abalone farms (Bachoo et al. 2023). The research showed that the identification of farmed specimens based on morpho-anatomical features and literature descriptions suggested specimens collected from the abalone farms were composed of *U. rigida* and *U. lactuca* (Bachoo et al. 2023). Using molecular makers, *Ulva* specimens from the five main abalone farms cultivating *Ulva* were found to be from the same molecular species which was identified as *U. lacinulata* (Bachoo et al. 2023).

1.7 Chemoattraction

Aquatic organisms utilize dissolved chemical cues from other organisms to find and select food, mates and identify potential dangers (Kamio and Derby, 2017; Kamio et al. 2022). These specific chemicals are discovered regardless of the chemical complexity of the aquatic environments (Lee and Meyers, 1996). Feeding is usually achieved through a series of behavioural responses where each phase may be either a connected or separated event, governed by a set of physical and chemical conditions (Lindstedt, 1971).

‘Chemoattraction’ is attributed to the series of behavioural responses that an organism undergoes in the presence of chemical stimuli, which may eventually result in feeding (Lee and Meyers, 1996). A hierarchical feeding model was presented by Lindstedt (1971), describing the different behavioural responses that may occur in the presence of chemical stimuli. This model was adopted and further elaborated upon by Lee and Meyers (1996) to apply to crustaceans (Figure **1.1**). According to Lee and Meyers (1996), an organism’s response in the presence of chemical stimuli can be separated into five different stages: (1) “detection” [excitant]; (2) “orientation” [attractant, arrestant or repellent]; (3) “locomotion” [attractant or repellent]; (4) “initiation” [suppressant or incitant]; and (5) “continuation or termination” [stimulant or deterrent] of the feeding process. However, whatever the ecological role of the chemical stimuli, they have to be delivered at the right concentrations so that the recipients can recognize them and respond accordingly (Mollo et al. 2017). Compounds that stimulate the olfactory system (attractants, arrestants and repellents) are detectable from a distance at low amounts following a gradient of concentration through the water column, while compounds that influence taste and gustation (incitants, suppressants, stimulants and deterrents) are perceivable at extremely high concentrations depending on physicochemical characteristics of the molecules upon contact (Lee and Meyers, 1996; Mollo et al. 2017). From a distance a chemical compound may act as an attractant, but as a deterrent upon contact (Lindstedt, 1971; Lee and Meyers, 1996).

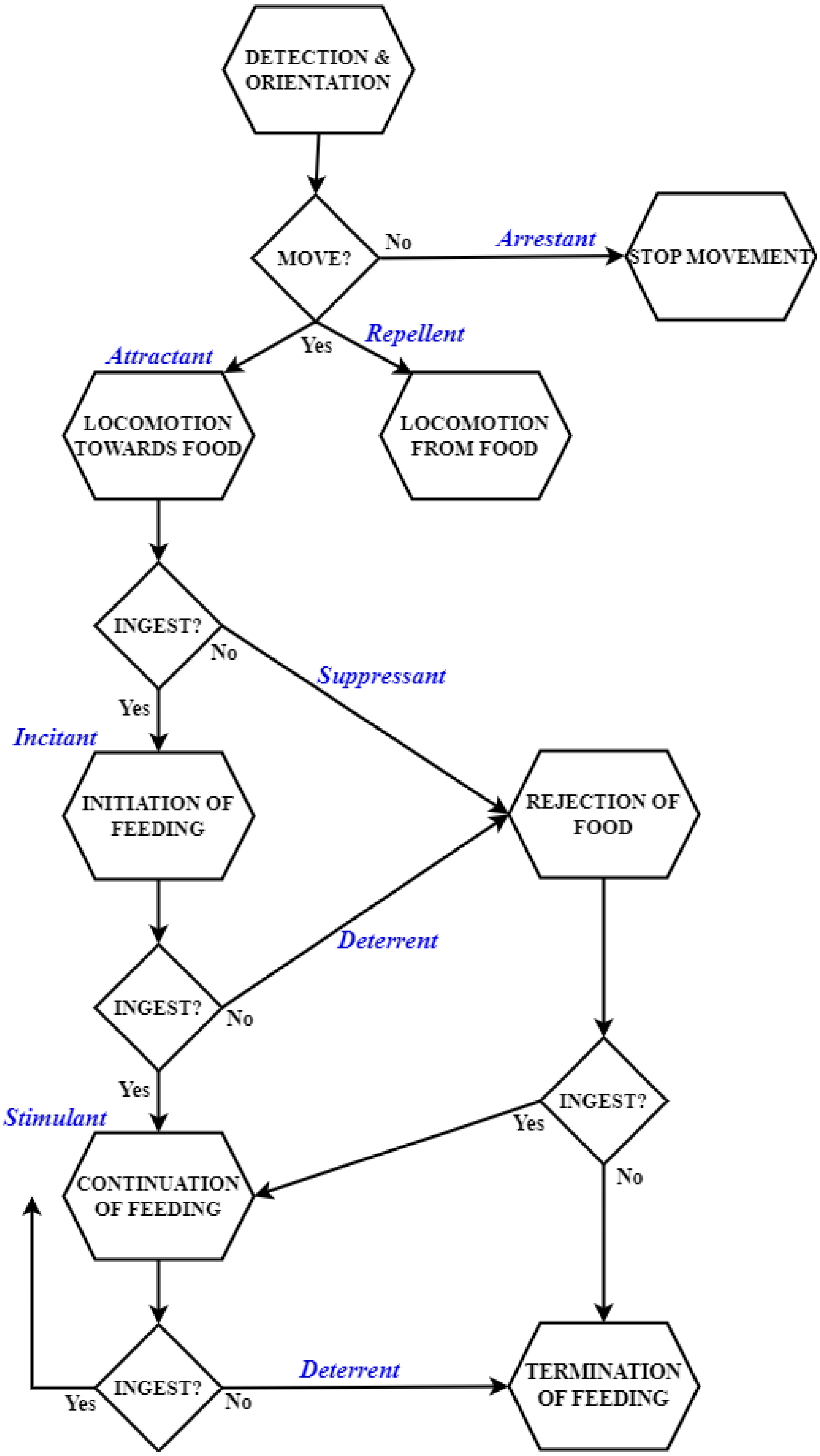


Figure 1.1: Classification of an organism's feeding behaviour in the presence of chemical stimuli. (Adapted from Lee and Meyers, 1996).

Electrophysiology and behavioural assays are the two most common methods that have been used to study chemoattraction in aquatic organisms (*e.g.* Derby and Ache, 1984; Hay et al. 1986; Voigt and Atema, 1992; Sola et al. 1993; Kidawa, 2005a; Fields et al. 2007). In some cases, a combination of both methods have been employed (*e.g.* Zimmer-Faust et al. 1988; Barlow, 1990; Kidawa, 2005b). Past studies using the electrophysiological method(s) has allowed researchers to study chemoreception in animals ranging from single olfactory neurons to whole organs (*e.g.* Laverack, 1963; Holland, 1978; Restrepo and Boyle, 1991). In contrast, behavioural assays have been employed to evaluate behavioural changes, such as increase of antennule flicking in lobsters (*e.g.* Devine and Atema, 1982), movements of spines, pedicellariae and tube feet in sea urchins (*e.g.* Solari et al. 2021; Addis et al. 2023), and chemically regulated odour search (chemotaxis) in fish (*e.g.* Baker et al. 2002), in the presence of various chemical stimuli. Studies on chemoattraction in marine organisms to date have mostly been focused on natural food components (*i.e.* extracts or whole dried components), singles or mixtures of artificial compounds (Klinger and Lawrence, 1984; Kidawa, 2005a; Erickson et al. 2006; Lyons and Scheibling, 2007; Luis and Gago, 2021; Solari et al. 2021; Addis et al. 2023).

1.7.1. Chemoattractants from seaweeds

Feed attractants are chemical compounds that make an organism to orient towards and move in the direction of the source (*i.e.* food; Lee and Meyers, 1996; Lawrence et al. 2013). These compounds belong to a group of low molecular weight and water/ethanol soluble compounds that are released from potential prey elements (Williams, 2007; Tantikitti, 2014). Chemical compounds such as amino acids and organic compounds including nucleotides, nucleosides, organic acids and quaternary ammonium bases derived from animal-based products, have been reported to elicit feeding behaviour responses in aquatic organisms (Hara, 1994a; Takaoka et al. 1995; Carr et al. 1996; Rolen et al. 2003; Velez et al. 2007; Sheybani et al. 2009; Lim et al. 2016).

Until now, specialized literature on chemical cues and compounds from seaweeds that marine herbivores use as a guide to search for food are still limited. Very few compounds of algal origin have been documented in the literature that may act as feed attractants for marine species (*e.g.* Van Alstyne et al. 2001; Rasher et al. 2015). *Ulva* is known to contain high amounts of sulfur-derived compound dimethylsulfoniopropionate (DMSP; up to 6977 $\mu g.g^{-1}$ fresh *Ulva*;

Smit et al. 2007). This compound has been reported to act as a feed attractant for the sea urchins *Strongylocentrotus purpuratus* and *S. droebachiensis* (Camarodonta; Van Alstyne et al. 2001). Past studies have documented distance attraction in marine organisms, such as sea urchins in laboratory-based trials, to the direction of seawater flowing over seaweeds (*e.g.* Vadas, 1977; Kupfermann and Carew, 1974; Garnick 1978; Mann et al. 1984; Scheibling and Hamm, 1991). Marine grazers, such as sea urchins have also been reported to exhibit hierarchical preferences when offered a choice of seaweeds, where some are strongly preferred over others (*e.g.* Prince and LeBlanc, 1992; Tuya et al. 2001; Dworjanyn et al. 2007; Angell et al. 2012; Seymour et al. 2013; Cyrus et al. 2015a; Yang et al. 2021; Addis et al. 2023). Several studies have shown the attractiveness of incorporating seaweed and seaweed derived extract(s) in formulated feeds of marine grazers (*e.g.* Dworjanyn et al. 2007; Cyrus et al. 2015a; Shpigel et al. 2017; Yu et al. 2023). Few analytical studies have however demonstrated the preferences of marine herbivores to certain types of seaweed extracts (*e.g.* Amsler et al. 1998; Schnitzler et al. 2001; Erickson et al. 2006; Bianco et al. 2010; Spiers et al. 2021). Erickson et al. (2006) reported that the sea urchin *Echinometra lucunter* (Camarodonta) showed preference to 1:1 ethanol:water extract (polar) over 1:1 ethyl acetate:methanol extract (non-polar) of the green seaweed *Ulva lactuca*. The researchers found that the consumption rates of the urchins were three times higher for artificial feed containing polar extract compared to control feed containing no extract and feed with non-polar extract. Preferences to certain types of seaweeds have occasionally been assigned to factors such as presence of an attractant and/or absence of a deterrent and the overall physical properties of the feed (Mann et al. 1984; Pelletreau and Muller-Parker, 2002; Chang et al. 2005; Wessels et al. 2006; Dworjanyn et al. 2007; Lyons and Scheibling, 2007).

1.7.2. Feed stimulants from seaweeds

Feeding stimulants are chemical compounds that encourage an organism to continue feeding once feeding process has been initiated (Linstedt, 1971; Lee and Meyers, 1996). Unlike feed attractants, these compounds are detected after direct contact between the animal’s chemoreceptors and the food source has occurred but, in some cases, they can also be waterborne (Lindstedt, 1971; Lee and Meyers, 1996). Several human food-related organic chemicals, including sugars, amino acids and fatty acids, have been perceived as feed stimulants for a number of marine organisms (Fuke et al. 1981; Klinger and Lawrence, 1984; Carr et al. 1996; Solari et al. 2021; Luis and Gago, 2021).

Lipids from seaweeds are mainly made-up of polar lipids including glycolipids, betaine lipids and phospholipids, and non-polar glycerolipids (neutral lipids), with fatty acids making up most of the total lipid content (up to 50%; Mišurcová et al. 2011; Gosch et al. 2012; Kumari et al. 2013; da Costa et al. 2017). Several polar lipids such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), 6-sulfoquinovosyldiacylglycerol (SQDG), phosphatidylcholine, 1-monoacylglyceryl-4'-O-(N,N,N-trimethyl)-homoserine (MGTS; reported as MGTH), and 1,2-diacylglyceryl-4'-O-(N, N, N-trimethyl)-homoserine (DGTS; reported as DGTH), have been identified from seaweeds that may act as feed stimulants towards a few marine herbivores (*e.g.* Sakata and Ina; 1985; Sakata et al. 1985, 1988, 1989). A few studies have also demonstrated the stimulatory effects of seaweed incorporation in the formulated feeds of several marine organisms (*e.g.* Dworjanyn et al. 2007; Xia et al. 2012; Cyrus et al. 2015a; Sánchez et al. 2016). Inclusion of seaweed in these formulated feeds have been reported to increase the palatability and acceptability of the artificials diets as well as enhance feed intake and in turn, increase protein and energy intake (Dworjanyn et al. 2007; Cyrus et al. 2015a).

1.7.3. Feed deterrents from seaweeds

Deterrents are classified as chemical compounds that inhibit the continuation or hasten termination of the feeding process (Linstedt, 1971). These chemicals are detected upon the contact of an organism’s chemoreceptors with the food source during gustation or tasting (Lee and Meyers, 1996). Unlike feed attractants and stimulants, feed deterrents from seaweeds have been well documented in the literature. In seaweeds, the lipophilic extracts (non-polar) have often been shown to be the major source of compounds associated with the chemical defences of the plants against grazers (*e.g.* Steinberg and van Altena, 1992; Cronin and Hay, 1996; Pelletreau and Muller-Parker, 2002; Barbosa et al. 2004; Ishii et al. 2004). However, in a few cases, polar extracts of seaweeds have also been reported to contain compounds that may have anti-grazing characteristic (*e.g.* Deal et al. 2003; Kubanek et al. 2004; Van Alstyne et al. 2006).

Secondary metabolites such as phenolics, terpenes, organic acids and organosulfur compounds have been documented from a number of seaweeds that can affect their palatability and act as deterrents (*e.g.* Steinberg and van Altena, 1992; Pelletreau and Muller-Parker, 2002; Van Alstyne and Houser, 2003; Barbosa et al. 2004; Bianco et al. 2010; Sudatti et al. 2018; Pereira et al. 2020). Dimethylsulfide (DMS) and acrylic acid produced from the cleavage of dimethylsulfoniopropionate (DMSP) in seaweed such as *Ulva* have been reported to function as potent feeding deterrents against the sea urchin *S. droebachiensis* and *S. purpuratus* (Van Alstyne et al. 2001; Van Alstyne and Houser 2003). Erickson et al. (2006) reported that the green macroalgae *U. lactuca* and *C. prolifera* were unpalatable to the sea urchin *E. lucunter* in feeding assays. The researchers reported that the polar extracts from *U. lactuca* and *C. prolifera* stimulated feeding in the urchins while the non-polar extract of *U. lactuca* deterred feeding. Several investigations have also shown that some compounds present in seaweeds can be deterrents to some grazers, but have no effects on, or at certain concentrations can stimulate the feeding response, in others (*e.g.* Duffy and Hay, 1994; Cronin and Hay, 1996; Hay et al. 1998; Barbosa et al. 2004). A natural food (*i.e.* seaweed) can contain feed stimulants as well as deterrents, thus making it unpalatable (Erickson et al 2006; Lawrence et al. 2013).

1.8 Volatile organic compounds (VOCs) from seaweeds

Marine organisms, such as seaweeds, produce an assortment of volatile organic compounds (VOCs) through several biological pathways (Fujimura et al. 1990; Akakabe et al. 2003; Garcia-Jimenez et al. 2013; Ferraces-Casais et al. 2013; de Alencar et al. 2017). These chemical compounds of high vapour pressure and low to moderate water solubility are released in the water column as metabolic wastes (de Alencar et al. 2017; Garicano Vilar et al. 2020). VOCs are known to act as infochemicals and play vital roles in shaping biotic interactions and regulating key ecological processes (Saha and Fink, 2022). VOCs produced by seaweed have been reported to be used as a defence system (*e.g.* Van Alstyne et al. 2001; Van Alstyne and Houser 2003; Wiesemeier et al. 2007; Lyons et al. 2007), pheromones (*e.g.* Pohnert and Boland, 2002) and feeding cues (*e.g.* Fink et al. 2006; Akakabe and Kajiwara, 2008). Chemical including sulphonated and halogenated compounds, terpenes, hydrocarbons, phenols, alcohols, aldehydes, ketones, fatty acids and esters, are distributed among the seaweeds (de Alencar et al. 2017; López-Pérez et al. 2017; Maruti et al. 2018; Keng et al. 2020). Several VOCs have strong odour or aroma and can be attributed to flavours of food (Ferraces-Casais et al. 2013; Urlass et al. 2023). However, the levels of VOCs may strongly be related to the physiological state of the seaweed species (Gressler et al. 2009; de Alencar et al. 2017). In *Ulva*, aldehydes have been reported to be the main group of VOCs (*e.g.* Fujimura et al. 1990; Kajiwara et al. 1992; Roussis et al. 2000; López-Pérez et al. 2017).

1.9 Aims of this study

In South Africa, the production of the green seaweed *Ulva* as an integral component of the abalone aquaculture industry has proven to be commercially viable. In addition to the benefits that it provides to the abalone industry, our knowledge of the chemistry and chemical compounds of this seaweed and how they may influence animal behaviour, such as feeding, is still lacking. Up to now, our understanding of how aquacultured *Ulva* affects the feeding of high value macroalgivores such as the sea urchin *T. gratilla* has only received cursory investigation (*e.g.* Cyrus et al. 2015a). Identification of compounds from this alga that may be incorporated in aquafeeds to promote consumption could be beneficial for the aquaculture industry.

The overall aims of this study are to identify the fractions or compounds from aquacultured *Ulva* responsible for the attractant activity towards the sea urchin *T. gratilla* (Chapter 2); to investigate the minimum effective concentrations (MECs) at which the *Ulva* fractions trigger the feed stimulatory response of *T. gratilla* as well as to evaluate the stimulatory effects *Ulva* fractions against crude *Ulva* extract, fresh and dry *Ulva* in a comparison study on *T. gratilla* (Chapter 3); to assess the effect of varying nutrient concentration during the growth of *Ulva* on the lipid and fatty acid composition of this seaweed (Chapter 4); and to evaluate the volatile organic compounds produced by aquacultured *Ulva* (Chapter 5).

Chapter 2 aimed to test the hypothesis of whether aquacultured *Ulva* possesses fractions or chemical compounds with attractant or feeding stimulant properties. Using a multidisciplinary approach of behavioural biology and analytical chemistry techniques, fractions prepared from the crude extract of aquacultured *Ulva* were evaluated on the sea urchin *T. gratilla* in trials for attractant activity, and compounds or chemical classes in stimulant fractions identified.

In Chapter 3, stimulatory fractions identified from Chapter 2 were evaluated on *T. gratilla* in trials to determine the minimum effective concentrations (MECs) required to trigger the urchins’ stimulatory response. Chapter 3 also investigated the effectiveness of the stimulatory *Ulva* fractions against crude extract, fresh and dried aquacultured *Ulva* on *T. gratilla* in a comparison test at equal concentration, to determine whether it would be advantageous to use *Ulva* fractions as a stimulant in artificial feeds over crude, fresh or dried aquacultured *Ulva*.

Chapter 4 aimed to investigate the effects of nutrient variation in the *Ulva* culture system on total lipid content, lipid classes and fatty acid profile of aquaculture *Ulva*. Using an experimental system, *Ulva* was grown at different nutrient concentrations mimicking the fertilization regime used on a commercial abalone farm. Through the application of Nuclear Magnetic Resonance spectroscopy (NMR), the analytical technique of gas chromatography coupled with mass spectrometry (GC-MS) and quantitative metabolomic approaches using multivariate statistics, the effects of nutrient variation on the total lipid, lipid classes and fatty acid profiles of this seaweed were evaluated. This chapter was aimed to provide insight on the variability of the lipid and fatty acid content of aquacultured *Ulva* under varying nutrient concentrations as well as to aid in selecting the appropriate fertilization regime to increase specific lipid classes.

Chapter 5 aimed to evaluate, identify and quantify the VOCs produced by aquacultured *Ulva* by analyzing its solvent extract, essential oil and headspace extract using GC-MS, and provide information about the main types and groups of volatile compounds associated with this seaweed that may potentially be linked with its chemosensory properties.

CHAPTER 2

Feed stimulants from the green alga *Ulva lacinulata* for the sea urchin *Tripneustes gratilla* in aquaculture.

2.1 Abstract

Like most echinoderms, sea urchins are slow moving organisms that rely on chemical cues to find potential food items. Under laboratory conditions, the local urchin *Tripneustes gratilla* has demonstrated hierarchical feeding preference to the sea lettuce, *Ulva lacinulata* over other seaweed species. Inclusion of this alga in the diet of this urchin has shown that it could act as a feed attractant. However, *T. gratilla*'s responses to this seaweed have so far only identified certain *Ulva* extracts that may attract this urchin in preliminary chemosensory trials, while the chemical compounds responsible for these responses remain unknown. The current study investigated the attractiveness and stimulatory effects of aquacultured *U. lacinulata* fractions on the feeding preferences of *T. gratilla* with the main aim of identifying the fractions or compounds responsible for the attractant activity. Crude *Ulva* extract, obtained from solvent extraction (dichloromethane - methanol), was fractionated to produce nine fractions (F1 → F9), with increasing polarity and solubility. The attractiveness and stimulatory effects of the fractions were tested on a total of 120 individual urchins in chemosensory trials. Outcomes from the trials indicated that 116 out of 120 urchins showed touch preferences to the nine fractions and the control with polar fractions F8 and F9 being the most effective at attracting *T. gratilla*. A total of 77 out of the 116 urchins showed grazing preferences for the different *Ulva* fractions with F8 and F9 stimulating the most urchins to feed. Predicted probability of occurrence ranking for *Ulva* fractions to attract an urchin was F8 > F9 > F5 ≥ F6 > F4 > F7 > F3 > F2 ≥ F1, while the predicted probability of occurrence for *Ulva* fractions to stimulate an urchin to feed in a single trial was F9 > F8 > F5 ≥ F6 > F4 ≥ F7 > F3 > F2 > F1. Further investigation of stimulatory fractions revealed the presence of compound lipids, with monogalactosyldiacylglycerol (MGDG) in F8 and a mixture of compounds including diacylglyceryl-N,N,N-trimethyl-homoserine (DGTS), digalactosyldiacylglycerol (DGDG), dialkylglycerophosphate (PA), lysophospholipid (LPG) and sulfoquinovosylmonoacylglycerol (SQMG) in F9, that can act as feed stimulants for the urchin. Results here provide clear evidence that certain *Ulva* fractions were effective at attracting and stimulating the urchin *T. gratilla* to feed. Glycerolipids and phospholipids identified in this study concur with other studies where similar compounds or groups of compounds have been identified that may act as feed stimulants for marine herbivores.

2.2 Introduction

Green seaweeds from the genus *Ulva* Linnaeus (Phylum: Chlorophyta; Class: Ulvophyceae; Order: Ulvales) occur in several habitats from exposed rocky shores to sheltered bays, estuaries and in some freshwater environments, either attached to solid substrate or free floating (Hayden and Waaland, 2004; Miladi et al. 2018). With more than 100 species currently taxonomically accepted, the cosmopolitan shallow marine distribution of this genus is attributed to its ability to withstand a wide range of climatic and ecological conditions thus making them ideal candidates for cultivation (Lotze and Worm, 2002; Sousa et al. 2007; Heesch et al. 2009; Bolton et al. 2016; Shpigel et al. 2017; Guiry and Guiry, 2023). *Ulva* (‘sea lettuce’) has an extensive history of being used as a human food product particularly as ‘aonori’ in Japan, and it has already been used as a food condiment in several countries such as China, South Korea, Philippines, Indonesia, British Isles, and Hawaii (Nisizawa et al. 1987; Kim et al. 2011). Currently, there is a European Cooperation in Science and Technology (COST) programme running called "SeaWheat" “(CA20106 – TOMORROW'S 'WHEAT OF THE SEA': ULVA, A MODEL FOR AN INNOVATIVE MARICULTURE)”, with the main aim of developing *Ulva*-based blue-biotech industries and using *Ulva* as a model species in European seaweed culture (COST, 2022; Buck and Shpigel, 2023).

A few *Ulva* species has been recognized as ideal candidates for bioremediation applications and to sustainably produce high biomass in land-based integrated multi-tropic aquaculture systems (IMTA) due to their high growth rates as well as high nutrient uptake capacity (Robertson-Andersson et al. 2008; Lawton et al. 2013; Al-Hafedh et al. 2015; Shpigel et al. 2018a). Effluent-grown *Ulva* has been demonstrated to have higher protein content than ones in nature (up to 40% crude protein) and can have similar amino acid profiles to animal and other plant protein (Robertson-Andersson et al. 2011; Fleurence et al. 2012; Shuuluka et al. 2013; Angell et al. 2015, 2017; Bolton et al. 2016; Shpigel et al. 2018a, b). Members from this genus have been reported to be a valuable source of soluble fibers, bioactive polar lipids, beneficial omega-3 fatty acids (ω-3), vitamins and trace elements (Ortiz et al. 2006; García-Casal et al. 2007; Mata et al. 2016; Neto et al. 2018; Lopes et al. 2019; Moreira et al. 2020). Aquacultured *Ulva* can be a suitable alternative for animal-based feed additives and as supplementary feed for high commercial value macroalgivores (Mulvaney et al. 2013; Cyrus et al. 2013, 2015a, b; Laramore et al. 2018; Elizondo-González et al. 2018; Shpigel et al. 2017, 2018b). Dietary inclusion of IMTA grown *Ulva* at low levels (5 - 10%) in the practical feed of fresh and marine finfish has been tested without any adverse effects on growth, feed utilization, nutrient retention, body composition, and organoleptic properties of the muscle tissue (Marinho et al. 2013; Silva et al. 2015; Valente et al. 2016; Madibana et al. 2017). In South Africa, several large-scale commercial abalone farms are practicing IMTA by growing the abalone *Haliotis midae* (Lepetellida) in land-based raceways interconnected to *Ulva* paddle raceways, where the *Ulva* is grown using abalone effluent, with an annual production of this seaweed estimated to be around 2000 tonnes fresh weight (Bolton et al. 2009, 2016; Neveux et al. 2018; Rothman et al. 2020).

Aquatic animals utilize water-borne chemical cues emitted by other organisms to find and select food, mates and to avoid predation (Kamio and Derby, 2017; Kamio et al. 2022). These infochemicals are recognized despite of the diversity of dissolved chemicals found in the aquatic ecosystems (Lee and Meyers, 1996). Like most echinoderms, sea urchins are slow-moving marine invertebrates that depend on chemical cues to initiate appropriate behavioral responses including escaping predators or damaged conspecifics, induction of settlement and metamorphosis, habitat selections and localization of potential food items and conspecific individuals (Mann et al. 1984; Burdett-Coutts and Metaxas, 2004; Swanson et al. 2004; Williamson et al. 2004; Morishita and Barreto, 2011; Kintzing and Butler, 2014; Pagès et al. 2021; Solari et al. 2021). Studies have shown that sea urchins can detect alarm cues from crushed individuals and nearby predators by moving away from the direction of the source (Mann et al. 1984; Parker and Shulman, 1986; Morishita and Barreto, 2011). In contrast, several researchers have also documented distance attraction in sea urchins to the direction of water flowing over seaweeds (Vadas, 1977; Garnick 1978; Mann et al. 1984; Scheibling and Hamm, 1991). Increases in spines and tube feet activities have been reported in sea urchins in the presence of chemical stimuli with occasional fully coordinated locomotion towards the source of the stimuli (Luis and Gago, 2021; Solari et al. 2021).

Olfaction and gustation are regarded as the main chemosensory systems for the detection and identification of chemical signals in aquatic organisms (Hara, 1994b; Derby and Sorensen, 2008). For food perceptions, compounds such as attractants, repellents and arrestants that stimulate the olfactory system are typically detected from a distance while chemicals such as incitants, suppressants, stimulants and deterrents influencing taste and gustation function upon direct contact with the chemoreceptors (Lee and Meyers 1996; Lindstedt 1971). However, whatever the ecological role of the chemical signals, they must be delivered at the right concentrations so that the recipients can recognize them and respond accordingly (Mollo et al. 2017). Compounds that stimulate the olfactory system of marine organisms are detectable at low amounts following a gradient of concentration through the water column, while compounds that influence taste or gustation are perceivable at extremely high concentrations depending on physicochemical characteristics of the molecules upon contact (Lee and Meyers 1996; Mollo et al. 2017).

Under laboratory conditions, several studies have successfully demonstrated sea urchin preferences for palatable seaweeds (Hay et al. 1986; Prince and LeBlanc, 1992; Dworjanyn et al. 2007; Stimson et al. 2007; Cyrus et al. 2015a; Yang et al. 2021). A few analytical studies have also reported the preferences of sea urchins to certain types of seaweed extracts (Bolser and Hay, 1996; Erickson et al. 2006). However, sea urchin responses to algal stimulation have so far only allowed the identification of a few compounds, namely the glycolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and phospholipid phosphatidylcholine, that can act as feed stimulants (Sakata et al. 1989). For local aquacultured *Ulva*, it has been reported that the inclusion of this seaweed in the diet of the sea urchin *Tripneustes gratilla* (Camarodonta) could act as a feed attractant (Cyrus et al. 2015a). It was demonstrated that the urchins were attracted to formulated feed containing 200 g.kg^{-1} dried *Ulva* compared to feed containing no *Ulva*. The researchers also observed that the urchins could not differentiate between a blank seawater treatment and formulated feed containing no *Ulva* and suggested that the urchins responded to the chemosensory properties of the alga in the feed rather than its structural or morphological properties.

Research studies on the aquacultured *Ulva* in South Africa are mostly limited to data on the growth rate, nutrient uptake, bioremediation capacity, economic analysis, quantification of nutritional content as feed and its effect on the physiology of animals, while little research has been done on its chemistry with none focusing on the chemical compounds that may act as feed attractants or stimulants (Robertson-Anderson et al. 2008, 2011; Bolton et al. 2009; Shuuluka et al. 2013; Cyrus et al. 2013, 2015a, b; Madibana et al. 2017; Onomu et al. 2020). Therefore, the aim of the current study was to identify the fractions or compounds in aquacultured *Ulva* responsible for the attractant activity towards urchins. The main objectives were: 1) evaluate the attractant activity of *Ulva* fractions on the sea urchin *T. gratilla*; and 2) identify the compounds or chemical classes in the stimulant fractions.

2.3 Material and Methods

2.3.1. Chemicals

All solvents used in this study were of chromatography grade (LiChrosolv®). Methanol (≥ 99.9%; MeOH), dichloromethane (≥ 99.8%; CH_2Cl_2), hexane (≥ 97.0%; hex), ethyl acetate (≥ 99.8%; EtOAc), acetonitrile (≥ 99.9 %; ACN), ammonium formate (≥99.0%), formic acid (97.5 – 98.5%), deuterated chloroform-d (≥ 99.8% atom; $CDCl_3$), methanol-d4 (≥ 99.8% atom; CD_3OD), silica gel 60 (0.040 – 0.063 mm) and Analtech TLC Uniplates™ were acquired from Merck® (Darmstadt, Germany).

2.3.2. Algal Material

Fresh *U. lacinulata* (Bachoo et al. 2023) was obtained from the Department of Forestry, Fisheries and Environment (DFFE) Aquaculture Research Facility in Cape Town, South Africa (33°55'13.7"S 18°22'52.1"E), where it is cultured as an experimental material (Figure **2.1**). This seaweed was initially obtained from Irvine and Johnson (I & J) Cape Abalone aquafarm, Gansbaai, South Africa (34°37'35.1"S 19°17'47.4"E) as material grown in abalone effluent in a commercial aquaculture system. The alga was thoroughly cleaned of sediments, epiphytes and visible surface contaminants, and washed several times with de-ionized water. *U. lacinulata* was then blotted-dried using paper towel to remove excess water before solvent extraction. The moisture content (%; MC) of the alga (blotted dry) was determined by drying 5.00 g (± 0.01 g; *n*=5) in an oven (Labotec EcoTherm, South Africa) set at 60 °C until a constant weight. Water content was determined gravimetrically using [Equation **2.1**; Kendel et al. (2015)].

$$\%\,MC = \left[\frac{m_{\mathrm{i}} - m_{\mathrm{od}}}{m_{\mathrm{i}}}\right] \times 100 \qquad \text{[Equation } \mathbf{2.1}\text{]}$$

where m_{i} = initial mass of wet *Ulva* and m_{od} = oven dry mass of *Ulva*.

Figure 2.1: (A) A 2500 L *Ulva* stocking tank at the Department of Forestry, Fisheries and the Environment (DFFE) Marine Research Aquarium in Sea Point (Cape Town, South Africa) [Photo taken by Bas De Vos]; **(B)** *Ulva lacinulata* thallus.

2.3.3. Sea urchin, Tripneustes gratilla

Sea urchins were obtained from the DFFE, where they are spawned and cultured for aquaculture research purposes. The broodstock *T. gratilla* were initially collected from shallow rock pools near Haga-Haga near East London, South Africa (32°45’4.23” S, 28°16’41.30” E; Cyrus et al. 2013). At the research facility, the organisms are held in plastic crates (60 × 40 × 20 cm; length × width × depth) suspended in large fibreglass tanks (282 × 182 × 50 cm; length × width × depth) supplied with heated seawater (24 – 25°C) at a salinity of 35 PSU under fluorescent light set at 12:12 hrs (light:dark) as previously described by Cyrus et al. (2013). The urchins were fed a mixed diet comprising of kelp *Ecklonia maxima* (Laminariales), fresh *Ulva* and a formulated feed consisting of 200 g.kg^{-1} dried *Ulva* (25.7% protein; Cyrus et al. 2013). For this experiment, 120 young adults ranging from 56 to 66 mm (59.8 ± 2.8 mm; mean ± SD; 8 months old) test diameter were used. Production size urchins were chosen to better understand the feeding behaviour for *Ulva* extracts, and therefore obtain useful data that could be used in feed production. The urchins were starved for period of 7 days prior to the start of the trials to increase their responsiveness to the stimuli (Figure **2.2**).

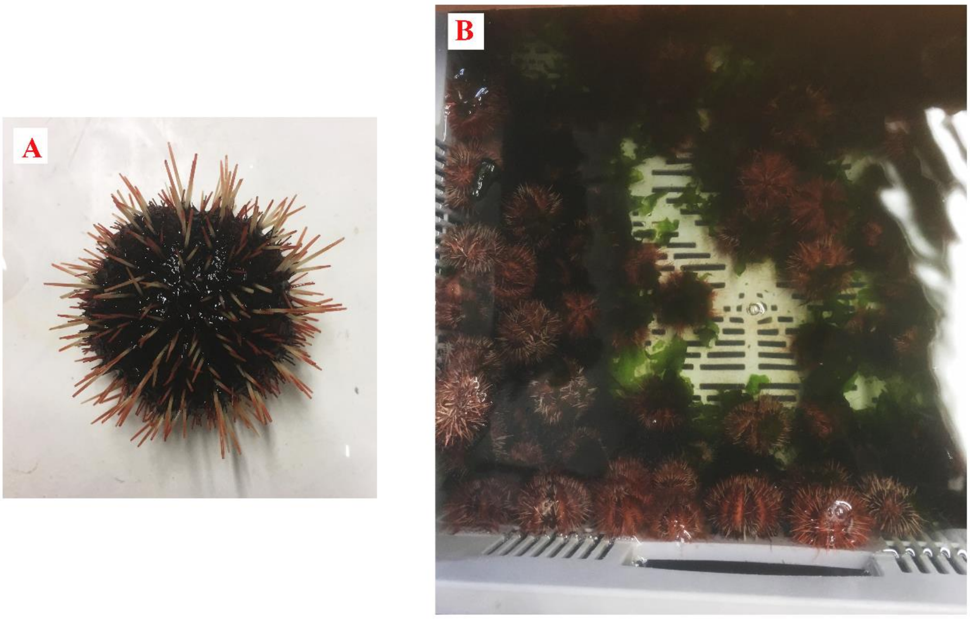


Figure 2.2: (A) Sea urchin *Tripneustes gratilla* (56 – 66 mm test diameter); **(B)** Plastic crate at the Department of Forestry, Fisheries and the Environment (DFFE) Marine Research Aquarium holding sea urchins in a recirculating aquaculture system.

2.3.4. Phytochemical extraction and fractionation

A crude extract and nine fractions (F1 – F9) were prepared from aquacultured *Ulva lacinulata* following a modified protocol used by Afolayan et al. (2008; Figure **2.3**). Algal material of 850 g (± 0.01 g wet weight) was roughly chopped into 2 – 3 cm pieces to increase the surface area. The seaweed was transferred to a conical flask where MeOH (1500 mL) was added, and maceration was allowed for 24 hrs at room temperature. The organic phase was collected through filtration, and the remaining algal biomass was re-extracted twice with CH_2Cl_2-MeOH (2:1; 1500 mL × 2) at room temperature for 24 hrs. The organic layers were combined, and sufficient de-ionised water was added until phase separation. The final organic (lower) phase was recovered and concentrated *in vacuo* (Büchi® Rotavapor R-210, Switzerland) to produce a crude extract (= **7.572 g**). Crude extract yield was estimated relative to dried *Ulva* (*w/w*) and expressed as percentage dry weight (%DW; see Table **2.1**; pg. 30).

The crude extract (1.0 ± 0.01 g) was further fractionated by silica gel step gradient column chromatography (10 g silica gel; column dimensions: 2.5 × 10 cm) using a combination of hex, EtOAc and MeOH (50 mL elution solvent per fraction) to produce nine fractions (F1 – F9) of increasing polarity (Figure **2.3**). The composition of the elution solvents were as follows: F1 (100% hex); F2 (9:1, hex:EtOAc); F3 (8:2, hex:EtOAc); F4 (7:3, hex:EtOAc); F5 (6:4, hex:EtOAc); F6 (4:6, hex:EtOAc); F7 (2:8, hex:EtOAc); F8 (100% EtOAc); F9 (1:1, MeOH:EtOAc).

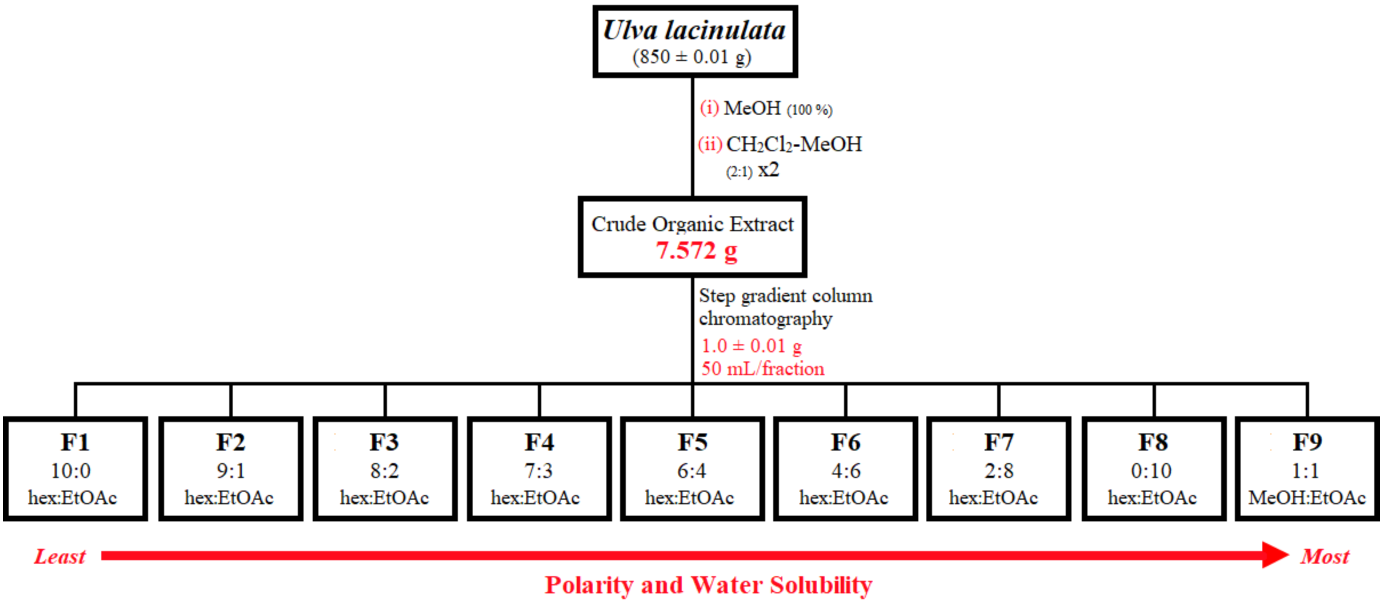


Figure 2.3: Schematic representation of crude *Ulva lacinulata* extract and fractions (F1 – F9) preparation. F1 (100% hexane); F2 (9:1, hexane:ethyl acetate); F3 (8:2, hexane:ethyl acetate); F4 (7:3, hexane:ethyl acetate); F5 (6:4, hexane:ethyl acetate); F6 (4:6, hexane:ethyl acetate); F7 (2:8, hexane:ethyl acetate); F8 (100% ethyl acetate); F9 (1:1, methanol:ethyl acetate).

2.3.5. Bioassay technique

The bioassay technique in this study was based on a modified version of the 'Avicel® plate method' first used by Sakata et al. (1989). Bioassay plates made of glass coated microcrystalline Avicel® (5 × 5 cm; length × width; 0.25 µm Avicel® thickness; Figure **2.4A**) were used to test *Ulva* fractions in a circular plastic tank of 80 cm diameter. The plates were placed 13 cm apart from one another and 20 cm from the centre on the bottom of each tank (Figure **2.4B**). The positions of the plates were marked on the bottom of the tank to facilitate plate placements. A sample zone (3 cm diameter) was drawn using a pencil on each bioassay plate (Figure **2.4A**).

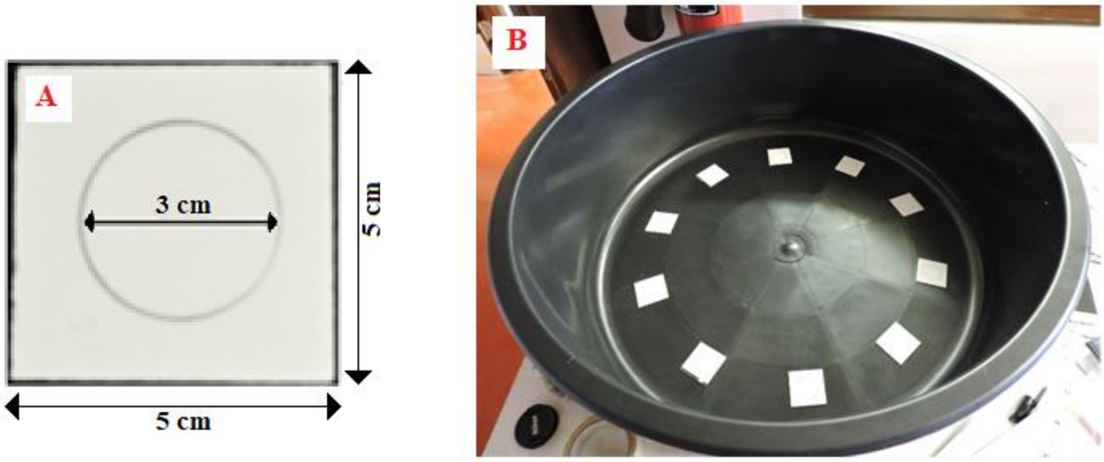


Figure 2.4: **(A)** A 5 × 5 cm (length × width) Avicel® bioassay plate with 3 cm diameter sample zone; (**B)** Bioassay plates placements in a 80 cm diameter plastic tank; plates positioned 13 cm apart from each other and 20 cm from the centre.

Concentrated *Ulva* extracts (F1 – F9) were suspended in MeOH:CH_2Cl_2 (1:1; *v*/*v*) and extracts equivalent to 1 g fresh *Ulva* were applied onto the sample zones on the Avicel® plates. The solvents were allowed to dry for 10 mins, and the bioassay plates were placed in the tank (as described above) containing 20 L (9.4 cm deep) of temperature-controlled seawater (24 – 25°C) at salinity of 35 PSU (Figure **2.5A**). For each of the trials, nine *Ulva* fractions along with a control plate, and a plate containing no extract, were tested.

Figure 2.5: (A) Example of a trial with Avicel® bioassay plates containing *Ulva lacinulata* fractions before the addition of a urchin; **(B)** Example of the beginning of a trial where an urchin was placed at the centre of the tank surrounded by the Avicel® bioassay plates containing the *U. lacinulata* fractions.

Chemosensory trials were performed by placing a starved urchin at the centre of the tank surrounded by bioassay plates and its movement monitored for 75 mins (Figure **2.5B**). A touch preference was established when the sea urchins' tube feet touched the sample zone of a bioassay plate, and a feed preference was allocated when the sample zone of a bioassay plate was grazed with the urchin leaving its characteristic traces looking like ‘star marks’ (Figure **2.6**). An urchin may show a touch preference to one or multiple *Ulva* fractions in a single trial. Likewise, an urchin may graze on one or multiple *Ulva* fractions in a single trial. Therefore, all the touch and graze preferences of an individual urchin in a single trial were recorded over the 75 min period. The trials were repeated using 120 individual sea urchins. Between each trial, the tank was emptied and washed, the water and bioassay plates were replaced. Moreover, the sequence in which the bioassay plates were offered was randomized for every trial without repetition to avoid directional placement of specific bioassay plates.

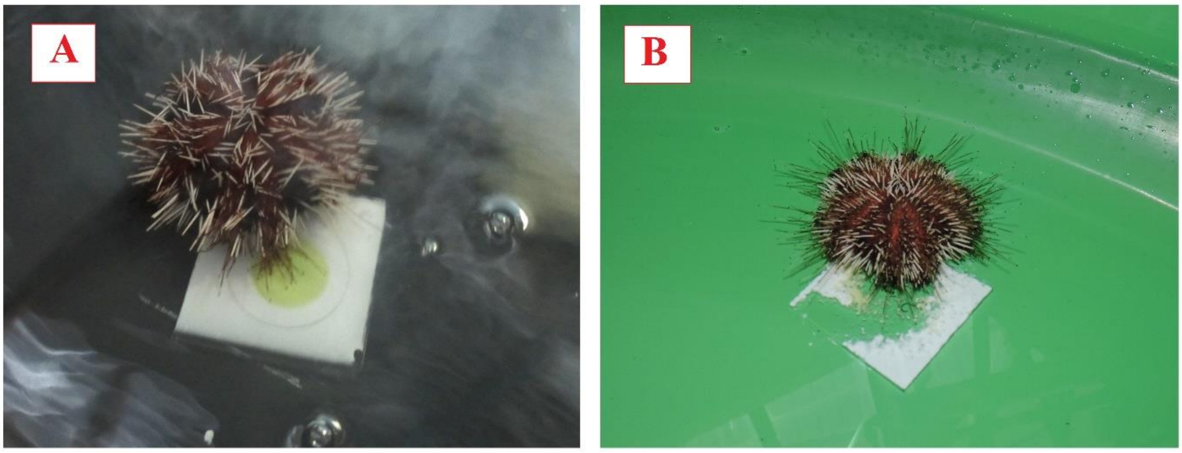


Figure 2.6: (A) Example of an urchin’s tube feet touching the sample zone containing *Ulva lacinulata* fraction (touch preference); **(B)** Example of an Avicel® bioassay plate containing *U. lacinulata* fraction being grazed by an urchin (grazing preference).

2.3.6. Nuclear magnetic resonance (NMR) spectroscopy

NMR (1D and 2D) analyses were performed at the Department of Chemistry, University of the Western Cape, Bellville, South Africa using a Bruker 400 MHz Avance III HD equipped with a 5 mm PABBO BB probe. NMR experiments were carried out using standard pulse sequences. TopSpin® (ver. 3.6.2, Bruker TopSpin® Inc) software was used for NMR data acquisition. Data were further processed using MestReNova (ver. 6.0.2, Mestrelab Research). All spectra were baseline (Whittaker Smoother) and manual phase corrected and referenced according to residual undeuterated solvent ($CDCl_3$ – δ_H 7.26 and δ_C 77.0 or CD_3OD – δ_H 3.350 and δ_C 49.3).

2.3.7. High-performance liquid chromatography (HPLC)

Semi-preparative HPLC was performed at the School of Pharmacy, University of the Western Cape, Bellville, South Africa, using Agilent Technologies 1260 Infinity LC System equipped with UV100 and refractive index detectors. *Ulva* fractions (*i.e.* F8 and F9; ≈ 100 mg) were purified on a reverse phase Phenomenex Luna® 10 µm Prep C18(2) 100 A LC column (250 × 10 mm) using ACN:H_2O (55:45)% as mobile phase at a flow rate of 3 mL.min^{-1}.

2.3.8. Liquid chromatography-mass spectrometry (LC-MS) and compound identification

LC-MS was performed at the Department of Chemistry, University of Cape Town, Rondebosch, South Africa using a SCIEX X500 QTOF system (AB Sciex Pte. Ltd., Singapore) equipped with an InfinityLad Poroshell 120 EC-C18 analytical LC column (4.6 × 150 mm, 4 µm particle size). *Ulva* fractions equivalent to a concentration of 100 µg.mL^{-1} were prepared using methanol and a volume of 10 µL was injected into the LC-MS. The mobile phase consisted of A (water containing 0.05% formic acid and 10 mM ammonium formate as additive) and B (acetonitrile). Analyses were performed using a linear gradient solvent system optimized as follows: 0.00 – 1.00 min, 2% B; 1.00 – 25.00 min, 2 – 98% B; 25.00 – 27.00 min, 98% B; 27.00 – 27.10 min, 98 – 2% B; 27.10 – 30.00 min, 2% B. The flow rate was 0.7 mL.min^{-1}, while the ion source temperature and ion spray voltage were set to 450 ºC and 5500 V, respectively. *Ulva* fractions were analysed in both positive and negative mode electrospray ionization mode (ESI). High resolution data were obtained using an information dependent acquisition method consisting of a TOF-MS survey (100 – 2000 Da for 150 ms) and 7 dependent MS/MS scans (50 – 1500 Da for 100 ms). Declustering potential was set to 80 V and MS/MS fragmentation was attained using a collision energy of 35 V with a collision energy spread of 15 V. All data was acquired and processed using SCIEX OS software (ver. 1.6.1).

The data were further evaluated in MZmine 2 (ver. 2.51; Pluskal et al. 2010) using the following parameters: (1) Mass detection: MS_1 (retention time, RT: 0.01 – 29.99 min; noise level: 1000) and MS_2 (RT: 0.01 – 29.99 min; noise level: 50); (2) Chromatogram builder: MS_1 (RT: 0.01 – 29.99 min; minimum time span: 0.01 min; minimum peak height: 10,000 counts; m/z tolerance: 0.01 m/z or 10 ppm); (3) Chromatogram deconvolution: local minimum search algorithm (chromatographic threshold: 0.01%; search minimum in RT range: 0.20 min; minimum relative height: 0.01%; minimum absolute height: 1000 counts; minimum ratio of peak top/edge: 2; peak duration: 0.01 – 3.00 min), m/z center calculation (median), m/z range for MS_2 scan pairing (0.05 Da) and RT range for MS_2 scan pairing (0.20 min); (4) Isotopic peaks grouper: m/z tolerance (0.02 m/z or 10.0 ppm), RT tolerance (0.2 min), maximum charge (2); (5) Join aligner: m/z tolerance (0.01 m/z or 10 ppm), RT tolerance (0.2 min), weight for m/z and RT (50); (6) Filtering: peak list rows filter (minimum peak in a row: 1; minimum peaks in an isotope pattern: 1; keep only peaks with MS_2 scan.

2.3.9. Statistical analyses

Statistical analyses were executed using R (ver. 4.0.2, R Development Core Team 2020) and Microsoft® Excel® 365. Non-parametric chi-squared tests were conducted to test for significant differences in the touch preference and grazing data. Furthermore, binomial logistic regression was performed on both the touch and grazing preference data to assess the outcome of the predictor variable on whether *Ulva* fractions had any effect on the urchins (whether urchins responded or not). Binomial logistic regression uses a generalized linear model (GLM) approach to estimate the maximum probability of model parameters (Ruff et al. 2020). Additionally, Wald’s test was performed to assess the significance of *Ulva* fractions in the model, since it is fitting for models without overdispersion (Bolker et al. 2009). Outcomes from the logistic model were expressed as probabilities, lower (LCI) and upper (UCI) 95% confidence intervals, Wald *z*-values and *p*-values of an urchin to be attracted and/or to feed on a particular treatment. Significance was accepted at $p < 0.05$.

2.4 Results

2.4.1. Crude extract content and **Ulva** *fraction distributions*

The crude extract yield, seaweed water content and fractionation contents (F1 – F9) are shown in Table **2.1**. The crude extract from *U. lacinulata* used in this study was 6.58% of dry weight (DW), where the moisture content of the alga was determined to be 86.46 ± 0.30%. Fractionation of the crude extract produced nine fractions (F1 – F9), with increasing polarity and water solubility where F1 is the least polar and least water soluble, while F9 is the most polar and most water soluble. Polar fraction F9 consisted of 27.70 ± 2.28% (highest) relative to the crude *Ulva* extract, while fraction F8 only comprised of 1.72 ± 0.24% (lowest) relative to the crude extract. The content recovery from the Si-column was 92.35 ± 1.29%.

Table 2.1: Crude extract content (%), moisture content (%) and *Ulva lacinulata* fraction (%) distributions. Values for [b] are mean of replicates (*n*=5) ± standard deviation [SD]; Values for [c] and [d] are mean of replicates (*n*=3) ± SD.

	Percentage (%)
Crude extract yield [a]	6.58
Water content fresh seaweed [b]	86.46 ± 0.30
Fractionation of crude extract (1.0 ± 0.01 g) [c]	
F1	12.50 ± 0.70
F2	9.03 ± 0.77
F3	16.72 ± 2.08
F4	9.90 ± 0.75
F5	7.40 ± 0.19
F6	5.25 ± 0.86
F7	2.13 ± 0.29
F8	1.72 ± 0.24
F9	27.70 ± 2.28
Recovery from Si-column [d]	92.35 ± 1.29

[a] = % *w/w* relative to dried *Ulva*; [b] = % *w/w* relative to fresh (blotted dry) *Ulva*; [c] = % relative to crude *Ulva* extract.

2.4.2. Touch preferences

The touch preferences of the 120 urchins for each of the *Ulva* fractions (F1 – F9) are shown in Figure **2.7**. In total, 116 out of 120 urchins showed preferences to the *Ulva* fractions and control (FC). Data from the touch preference showed that the distribution of urchins among the *Ulva* fractions were significantly different (χ^2= 85.062, *df*= 9, *p*< 0.001).

Figure 2.7: Touch preferences of *Tripneustes gratilla* for the various *Ulva lacinulata* fractions (F1 – F9) and control (FC).

Most touch preferences were attributed to polar fractions (F8= 51 and F9= 48 observations), followed by semi-polar fractions (F5= 29, F6= 29, F4=25 and F7= 20 observations) and non-polar fractions (F3= 17, F2= 16 and F1= 16 observations). The total number of observations of the urchins' preferences to one or more *Ulva* fraction (F1 – F9) and the control (FC) in single trials are shown in Table **2.2**.

Table 2.2: Total number of observations of the urchins' preferences to one or more of the *Ulva lacinulata* fractions (F1 – F9) and control (FC) per single trial.

	Number of observations per single trial									
Fraction	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***
FC	0	2	1	1	0	0	1	2	0	1
F1	2	4	3	0	0	1	3	2	0	1
F2	2	4	4	1	1	0	1	2	0	1
F3	0	3	3	1	4	1	2	2	0	1
F4	6	6	3	3	2	1	2	1	0	1
F5	8	4	6	1	4	1	2	2	0	1
F6	6	6	5	2	5	1	2	1	0	1
F7	2	4	7	0	2	1	2	1	0	1
F8	12	18	8	3	4	0	3	2	0	1
F9	14	11	11	4	3	0	3	1	0	1
Total no. urchins	**52**	**31**	**17**	**4**	**5**	**1**	**3**	**2**	**0**	**1**

2.4.3. Grazing preferences

The grazing preferences of the 120 urchins for *Ulva* fractions (F1 – F9) are shown in Figure **2.8.** In total, 77 out of the 116 urchins that responded positively to the chemical stimuli in the touch preference trials actively grazed on different *Ulva* fractions. Data from the grazing preference showed that the distribution of urchins among the *Ulva* fractions were significantly different (χ^2= 48.412, *df*= 9, *p*< 0.001).

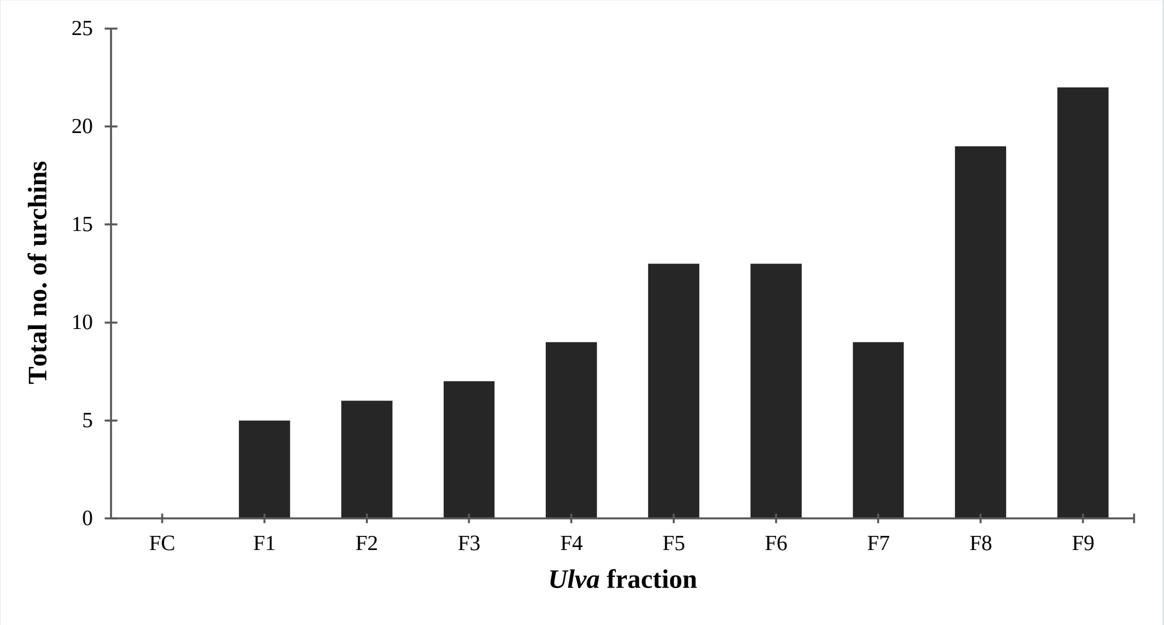


Figure 2.8: Grazing preferences of *Tripneustes gratilla* for the various *Ulva lacinulata* fractions (F1 – F9) and control (FC).

Most grazing preferences were attributed to polar fractions (F9= 22 and F8= 19 urchins), followed by semi-polar fractions (F5= 13, F6= 13, F7= 9 and F4= 9 urchins) and non-polar fractions (F3= 7, F2= 6 and F1= 1 urchins). The grazing preferences of the urchins to one or more of the *Ulva* fractions (F1 – F9) and the control (FC) in single trials are shown in Table **2.3**.

Table 2.3: Total number of *Ulva lacinulata* fractions (F1 – F9) and control (FC) grazed by the urchins in single trial.

	Number of *Ulva* fraction grazed in a single trial									
Fraction	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***	***10***
FC	0	0	0	0	0	0	0	0	0	0
F1	5	0	0	0	0	0	0	0	0	0
F2	3	1	1	1	0	0	0	0	0	0
F3	1	1	3	2	0	0	0	0	0	0
F4	7	0	0	1	1	0	0	0	0	0
F5	9	2	1	0	1	0	0	0	0	0
F6	8	1	1	2	1	0	0	0	0	0
F7	2	3	2	2	0	0	0	0	0	0
F8	11	5	2	0	1	0	0	0	0	0
F9	16	3	2	0	1	0	0	0	0	0
Total no. urchins	**62**	**8**	**4**	**2**	**1**	**0**	**0**	**0**	**0**	**0**

2.4.4. Binomial logistic regression - probability of occurrence

2.4.4.1. Touch preferences

The probability of occurrence for the touch preferences of *T. gratilla* for *U. lacinulata* fractions are shown in Table **2.4**. Outcomes showed that polar fractions F8 ($\Delta\rho$= 0.425, *z*-value= 4.816, *p*< 0.001) and F9 ($\Delta\rho$= 0.400, *z*-value= 4.486, *p*< 0.001) have the highest predicted probabilities of attracting an urchin in a single trial. Moreover, semi-polar fractions F5 and F6 have equal predicted probabilities ($\Delta\rho$= 0.242, *z*-value= 2.124, *p*< 0.05) of attracting an urchin in a single experiment, while non-polar fraction F1 has the lowest predicted probability ($\Delta\rho$= 0.133, *z*-value= -6.970, *p*< 0.001) of attracting an urchin in a single trial.

Table 2.4: Predicted probabilities (Δρ) of *Tripneustes gratilla* being attracted to an *Ulva lacinulata* fraction (F1 – F9) and control (FC) in a single trial with lower (LCI) and upper (UCI) 95% confidence interval, Wald *z*-values and *p*-values showing significant differences.

Fraction	Probability (Δρ)	LCI	UCI	z–value	*p*– value
FC	0.067	0.034	0.128	-1.690	0.091
F1	0.133	0.083	0.207	-6.970	< 0.001
F2	0.133	0.083	0.207	0.000	1.000
F3	0.142	0.090	0.216	0.187	0.851
F4	0.208	0.145	0.290	1.533	0.125
F5	0.242	0.173	0.326	2.124	< 0.05
F6	0.242	0.173	0.326	2.124	< 0.05
F7	0.167	0.110	0.244	0.722	0.470
F8	0.425	0.340	0.515	4.816	< 0.001
F9	0.400	0.316	0.490	4.486	< 0.001

2.4.4.2. Grazing preferences

The probability of occurrence for the grazing preferences of *T. gratilla* for *U. lacinulata* fractions are shown in Table **2.5**. Results obtained from the binomial model showed that polar fraction F9 has the highest predicted probability (Δρ= 0.183, *z*-value= 3.193, *p*< 0.01) of stimulating an urchin to graze on this extract in a single trial followed by F8, which exhibited the second highest predicted probability (Δρ= 0.158, *z*-value= 2.813 *p*< 0.01) of stimulating an urchin to feed on this fraction in a single trial. In contrast, non-polar fraction F1 exhibited the least predicted probability (Δρ= 0.042, *z*-value= -6.864, *p*< 0.001) of stimulating an urchin to graze on this fraction in a single trial.

Table 2.5: Predicted probabilities (Δρ) of *Tripneustes gratilla* grazing on *Ulva lacinulata* fractions (F1 – F9) and control (FC) in a single trial with lower (LCI) and upper (UCI) 95% confidence interval, Wald *z*-values and *p*-values showing significant differences.

Fraction	Probability (Δρ)	LCI	UCI	z-value	p-value
FC	0.000	0.000	NaN	-0.026	0.979
F1	0.042	0.017	0.096	-6.864	< 0.001
F2	0.050	0.023	0.107	0.308	0.758
F3	0.058	0.028	0.117	0.590	0.555
F4	0.075	0.039	0.138	1.087	0.277
F5	0.108	0.064	0.178	1.892	0.058
F6	0.108	0.064	0.178	1.892	0.058
F7	0.075	0.039	0.138	1.087	0.277
F8	0.158	0.103	0.235	2.813	< 0.01
F9	0.183	0.124	0.263	3.193	< 0.01

2.4.5. Identification of compounds from feeding stimulatory fractions

Based on the data collected from the touch and grazing preferences of *T. gratilla*, stimulatory fractions F8 and F9 were further elucidated using HPLC, NMR and LC-MS for compound identification.

2.4.5.1. Fraction F8

The HPLC chromatogram as well as the NMR spectra (Figure **2.9**) of fraction F8 revealed the presence of compound **2.1**, as the main constituent (see Supplementary Information **2** for HPLC; Figure **S2.2**). The structure of the compound was elucidated using 1D and 2D NMR spectroscopy. Overall, the NMR data were characteristic of a glycolipid. More specifically, the ^{1}H and ^{13}C NMR spectra (Figure **2.9**) of this compound showed signals characteristic of a terminal methyl group (CH_3) of a omega-3 (ω-3) fatty acid (*t*; δ_H 0.97, δ_C 14.3), methylene groups (CH_2) alpha to a carbonyl carbon between δ_H 2.30 – 2.45 (*t*; δ_C 33.9 – 34.25), bis-allylic methylene (CH_2) groups of a polyunsaturated fatty acid δ_H 2.73 (*m*; δ_C 25.6), and methylene groups (CH_2)n of saturated fatty acid chains between δ_H 1.20 – 1.68 (*m*; δ_C 20.0 – 30.0), that indicated the presence of fatty acids. Signals between δ_H 5.36 – 5.43 (*m*; δ_C 126.7 – 128.8), corresponded to the protons at double bonds, while the region between δ_H 3.5 and 4.5 is characteristic of a sugar moiety.

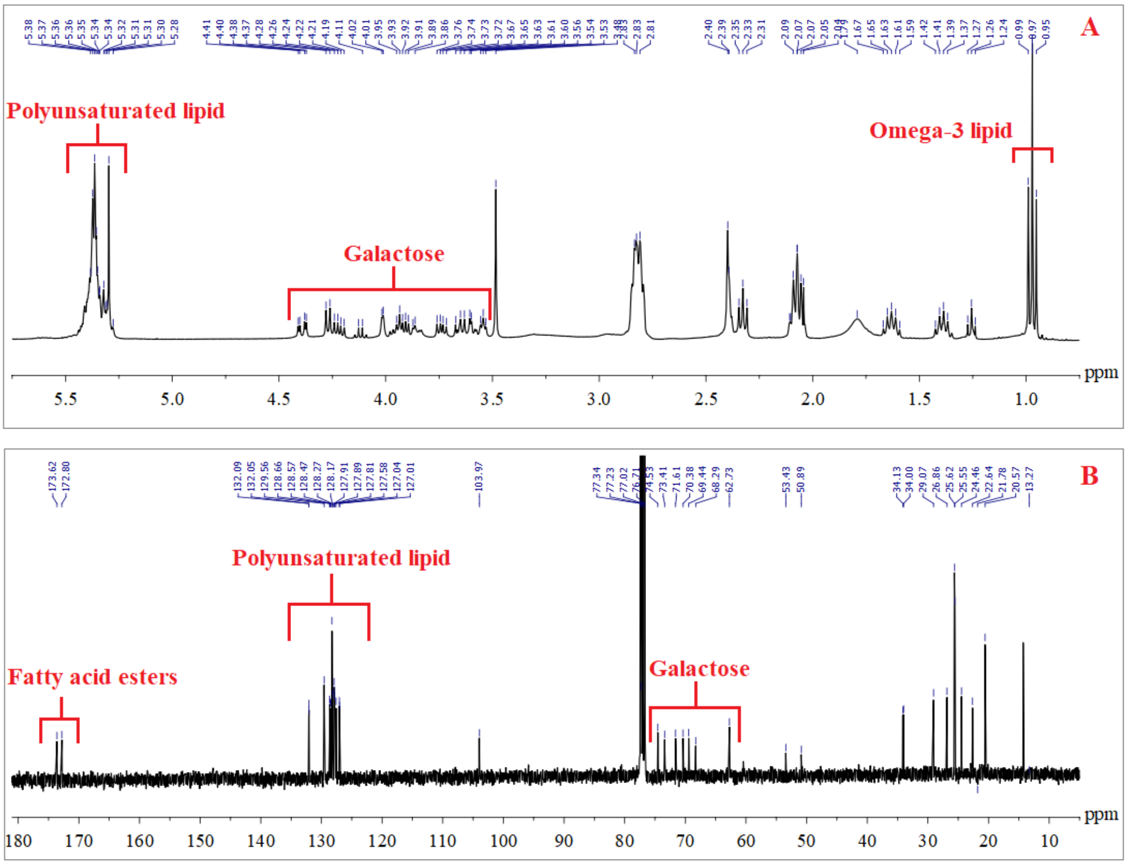


Figure 2.9: Nuclear magnetic resonance (NMR) spectra of compound **2.1** from *Ulva lacinulata* fraction F8 **(A)** proton NMR [^{1}H; $CDCl_3$, 400 MHz] and **(B)** carbon NMR (^{13}C; $CDCl_3$, 100 MHz).

Evaluation of the ^{1}H-, ^{13}C- and ^{1}H – ^{1}H correlation spectroscopy (COSY) NMR spectra of compound **2.1** indicated the presence of a glycerol backbone and hexose moiety (Table **2.6**; see Supplementary Information **2** for COSY; Figure **S2.3**). The carbon and proton signal assignments were made by the analysis of the heteronuclear single quantum correlation (HSQC) and ^{1}H – ^{1}H COSY spectra together with heteronuclear multiple bond correlation spectrum (HMBC; Figure **2.10**; see Supplementary Information **2** for HSQC; Figure **S2.4**). Determination of the number of attached protons was performed by analyzing 2D and distortionless enhancement by polarization transfer (DEPT-135) spectroscopic data (see Supplementary Information **2** for DEPT-135; Figure **S2.5**).

HMBC correlations between the methylene proton signals located at δ_H 4.39 (*dd*; CH_2 – 1a) and δ_H 4.24 (*m*; CH_2 – 1b) and the methine signals at δ_H 5.32 (*m*; CH-2, δ_C 70.4) of the glycerol moiety to the carbonyl carbons at C-1′ (δ_C 172.8) and C-1″ (δ_C 173.6), respectively, confirmed the attachment of the fatty acid side chains to carbon atoms at C-1 and C-2 of the glycerol moiety. The anomeric proton H-1‴ of the hexose at δ_H 4.27 (*d*) showed HMBC cross-peaks with C-3 (δ_C 68.3) of the glycerol moiety and C-2‴ (δ_C 71.6), thus establishing its connection to C-3. Carbon signals at δ_C 104.0 (C-1‴), 71.6 (C-2‴), 73.4 (C-3‴), 69.4 (C-4‴), 74.5 (C-5‴) and 60.4 (C-6‴) indicated the presence of a terminal β-galactopyranose unit that was linked to C-3 of the glycerol moiety. Integration of the peak areas of the olefinic methine (δ_H 5.37), allylic (δ_H 2.02), bis-allylic (δ_H 2.82) and saturated methylene (δ_H 1.25 – 1.6) signals provided an estimate of the number of double bonds and chain length of the fatty acids. Proton chemical shifts of the hexose moiety together with their coupling constants indicated that compound **2.1** had the structure of the glyceroglycolipid 1-(6Z,9Z,12Z,15Z-octadecatetraenoyl)-2-(4Z,7Z,10Z,13Z-hexadecatetraenoyl)-3-*O*-*β*-D-galactosyl-*sn*-glycerol (Figure **2.11**).

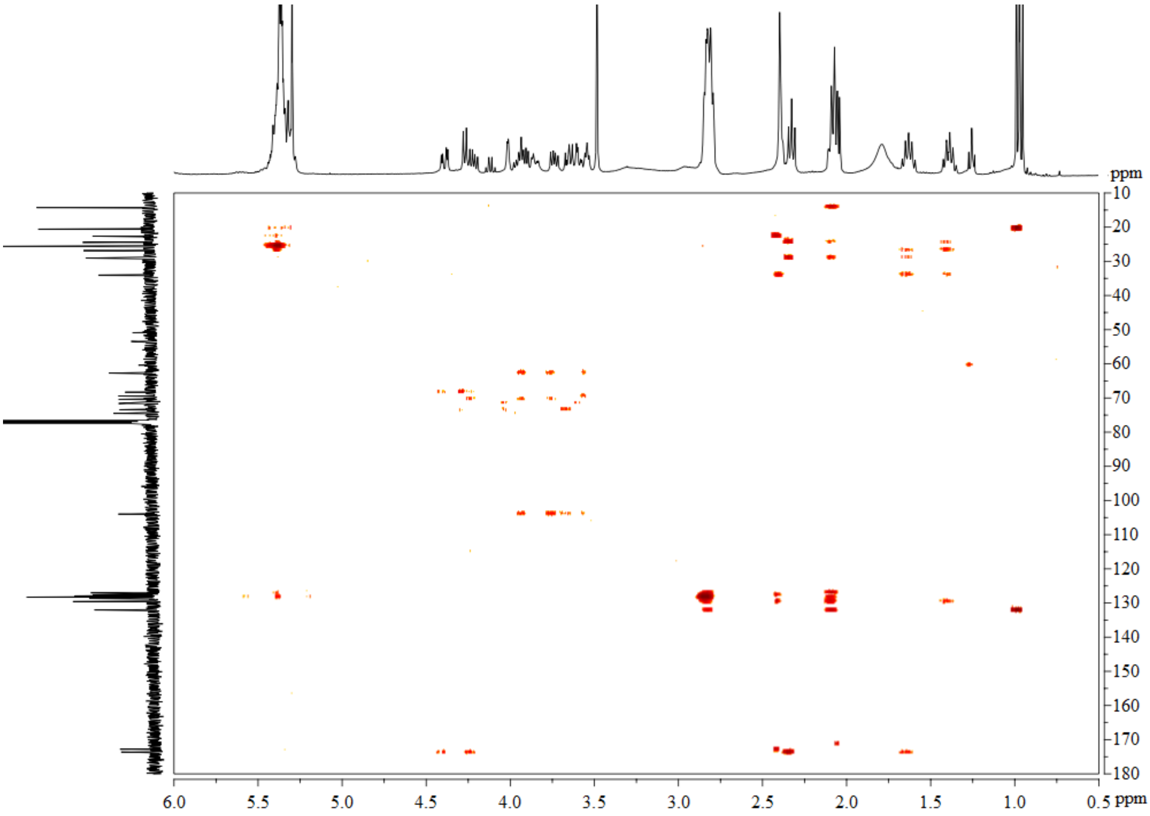
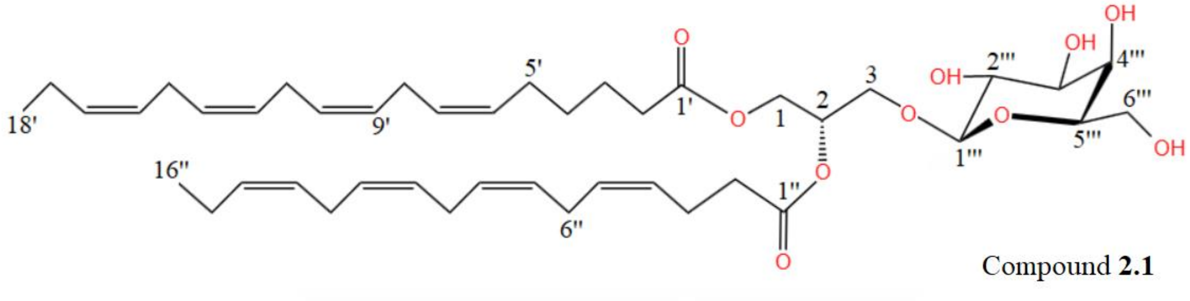


Figure 2.10: Heteronuclear multiple bond correlation spectrum (HMBC; $CDCl_3$, 400 MHz) of compound **2.1** from *Ulva lacinulata* fraction F8.

Table 2.6: Nuclear magnetic resonance (NMR) spectroscopic data of compound **2.1** from *Ulva lacinulata* fraction F8 ($CDCl_3$; 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR). 'δ' is represented in parts per million (ppm).

No.	**δ_C**	**δ_H, mult, *J* (Hz)**	**COSY**	**HMBC**
1	62.7	4.39, *dd*, 12.0, 3.4 4.24, *m*	3, 2	2, 1′
2	70.4	5.32, *m*	1, 3	
3	68.3	3.94, *m* 3.74, *dd*, 11.2, 6.4	2, 1	2, 1, 1‴
1′	172.8			
2′	34.0	2.36, *m*	3′	1′, 3′, 4′
3′	129.6	5.39, *m*	2′	
4′	127.0	5.36, *m*	5′	
6′ - 7′	127.6/128.7	5.37, *m*	5′/8′	
9′ - 10′	127.6/128.7	5.37, *m*	8′/11′	
12′ - 13′	127.6/128.7	5.37, *m*	11′/14′	
15′	128.3	5.39, *m*	14′	
16′	132.1	5.39, *m*	17′	
17′	20.6	2.07, *m*	16′	15′, 16′, 18′
18′	14.3	0.97, *t*, 7.5	17′	16′, 17′
5′	25.6	2.82, *m*	4′, 6′	3′, 4′, 6′, 7′
8′	25.6	2.82, *m*	7′, 9′	6′, 7′, 9′, 10′
11′	25.6	2.82, *m*	10′, 12′	9′, 10′, 12′, 13′
14′	25.6	2.82, *m*	13′, 15′	12′, 13′, 15′, 16′
1″	173.6			
2″	34.1	2.36, *m*	3″	1″, 3″, 4″
3″	24.5	1.63, *dt*, 15.3, 7.5	2″	1″, 2″, 4″, 5″
4″ - 5″	127.6/128.7	5.37, *m*	3″/6″	
7″ - 8″	127.6/128.7	5.37, *m*	6″/9″	
10″ - 11″	127.6/128.7	5.37, *m*	9″/12″	
13″ - 14″	127.6/128.7	5.37, *m*	12″/15″	
15″	33.8	2.38, *dd*, 9.5, 4.6	14″	13″, 14″, 16″
16″	22.6	2.39, *m*		14″, 15″
6″	25.6	2.82, *m*	5″, 7″	4″, 5″, 7″, 8″
9″	25.6	2.82, *m*	8″, 10″	7″, 8″, 10″, 11″
12″	25.6	2.82, *m*	11″, 13″	10″, 11″, 13″, 14″
1‴	104.0	4.27, *d*, 7.4	2‴	3, 2‴
2‴	71.6	3.64, *m*	1‴	3‴
3‴	73.4	3.59, *dd*, 9.5, 3.1	4‴	2‴
4‴	69.4	4.01, *d*, 2.3	3‴, 5‴	2‴
5‴	74.5	3.54, *t*, 4.9	4‴, 6‴	1‴, 4‴, 6‴
6‴	60.4	4.12, *dd*, 14.3, 7.1	5‴	4‴, 5‴



Compound **2.1**

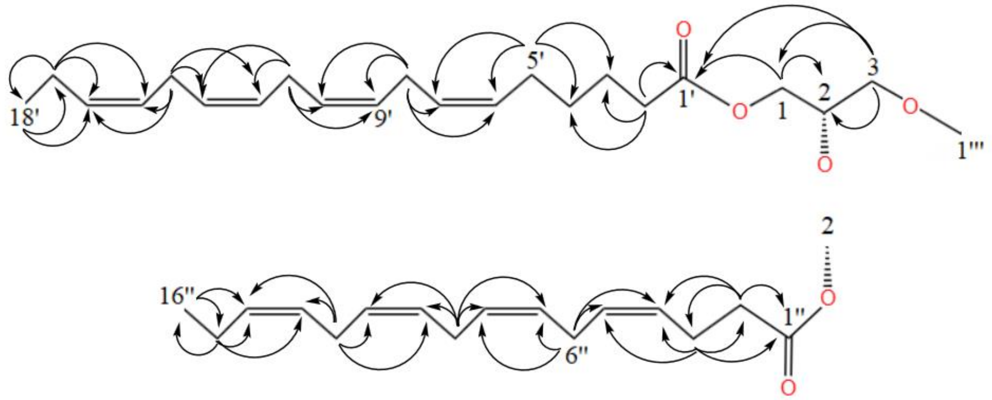


Figure 2.11: Chemical structure of compound **2.1** from *Ulva lacinulata* fraction F8. Arrows represent the heteronuclear multiple bond correlation (HMBC) in the fatty acid chains.

Liquid chromatography-mass spectrometry (LC-MS) of compound **2.1** showed a strong molecular ion peak at m/z 760.4960 in the positive LC-MS spectrum as $[M + NH_4]^+$ ion (see Supplementary Information **2** for Figure **S2.6**). The mass spectrum was characterized by its characteristic fragment ion at m/z 581.4 $[M - Gal]^+$ and by fragment ions for fatty acid tails at m/z 305.2 and m/z 333.2 (see Supplementary Information **2** for Figure **S2.7**). The LC-MS data therefore confirmed compound **2.1** as MGDG (34:8).

2.4.5.2. Fraction F9

Unlike F8, purification of stimulatory fraction F9 using reverse phase HPLC revealed the presence of a mixture of compounds as its constituents (see Supplementary Information **2** for HPLC; Figure **S2.8**). Attempts were made to separate individual compounds from F9. However, due to the overlapping of the HPLC signals, three major subfractions (A, B and C) were collected. Figure **2.12** depicts the ^{1}H NMR spectra of fraction F9 and subfractions of F9 (A, B and C) collected from HPLC. Although different, the spectra showed proton signals at varying intensities between regions of the CH_3 terminals (δ_H 0.82 – 1.12), CH_2 (δ_H 2.30 – 2.50) and $(CH_2)n$ (δ_H 1.32 – 1.58) groups, and regions between δ_H 5.30 – 5.72 corresponding to double bonds. The spectra also showed that the proton signals were not clearly separated, indicating the presence of several compounds sharing similar proton signals. Therefore, elucidation of the structures of the compounds from A, B and C using NMR spectroscopy was inconclusive.

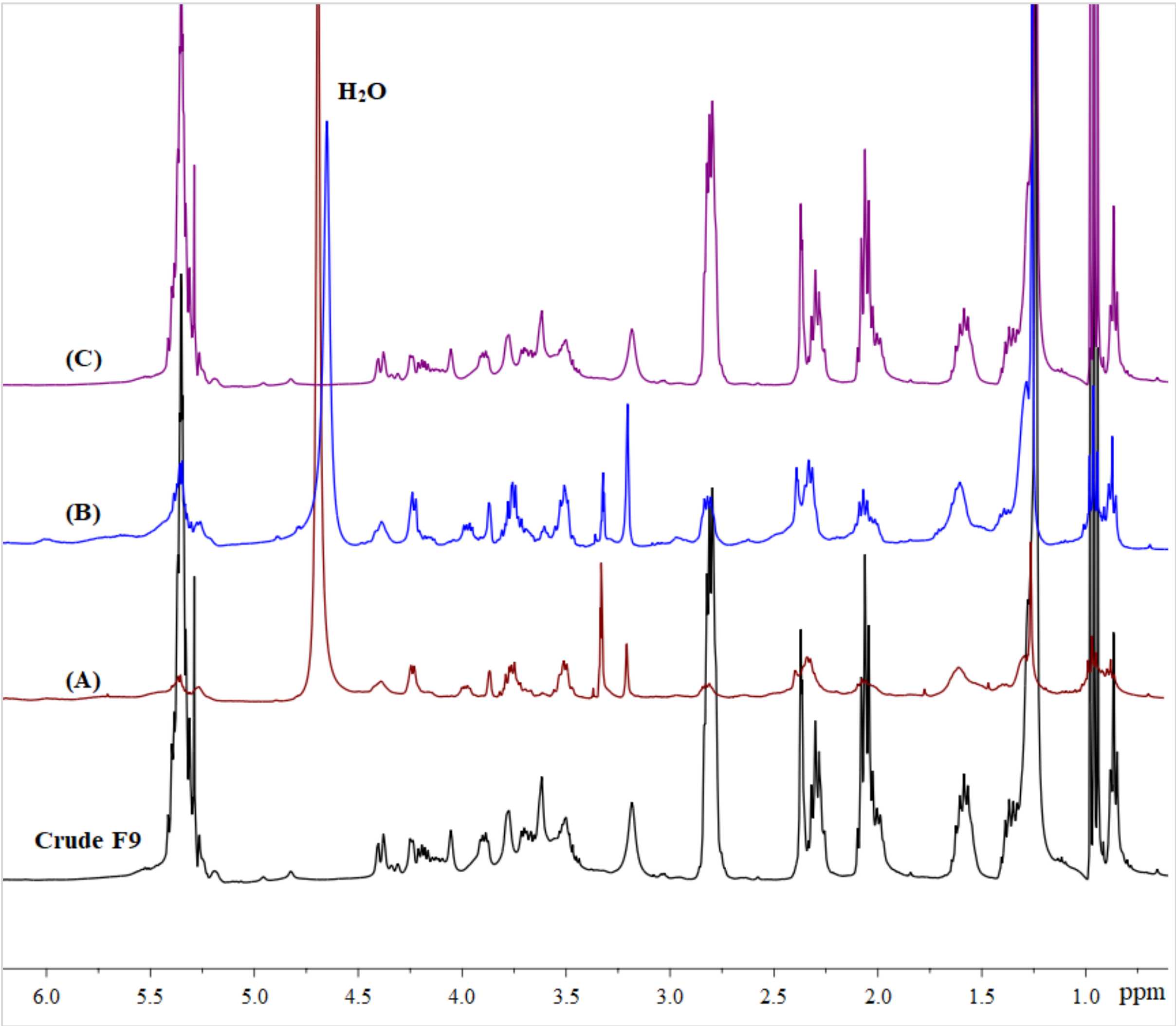


Figure 2.12: Proton nuclear magnetic resonance [^{1}H NMR; CD_3OD, 400 MHz] spectra of *Ulva lacinulata* F9 and F9 subfractions (**A**, **B** and **C**) collected from high-performance liquid chromatography (HPLC).

Preliminary evaluation of the compounds from fraction F9 by LC-MS using MZmine onboard library and comparison of mass spectra to the literature, allowed the tentative identification of glycolipids, betaine lipid, and phospholipids. Two compounds namely diacylglyceryl-N,N,N-trimethyl-homoserine (DGTS) and digalactosyldiacylglycerol (DGDG), were identified in the positive ESI mode. DGTS was identified as $[M + H]^+$ ions with a detected m/z 680.5105, while DGDG was identified as $[M + NH_4]^+$ ions with detected m/z 888.4590 (see Supplementary Information **2** for MS; Figures **S2.11** and **S2.12**). Moreover, three compounds, namely dialkylglycerophosphate (PA), sulfoquinovosylmonoacylglycerol (SQMG) and lysophospholipid (LPG), were identified in the negative ESI mode. All three compounds were identified as $[M-H]^-$ ions, where PA had a detected m/z 723.4934 while SQMG and LPG had detected m/z 555.2773 and m/z 481.2511, respectively (see Supplementary Information **2** for MS; Figures **S2.14**, **S2.15** and **S2.16**).

2.5 Discussion

Chemotactic responses of marine herbivores, such as sea urchins, to food-related compounds have been extensively documented (Klinger and Lawrence, 1984; Solari et al. 2021; Luis and Gago, 2021; Casal-Porras et al. 2021). In the presence of food, feeding in urchins is typically achieved, and may exclusively be based on factors such as the presence/absence of feed attractants/deterrents, nutritive value and physical properties of the food (Mann et al. 1984; Pelletreau and Muller-Parker, 2002; Chang et al. 2005; Wessels et al. 2006; Lyons and Scheibling, 2007; Dworjanyn et al. 2007; Cyrus et al. 2015a). Based on prior investigations on food perception and capture in local *Tripneustes gratilla* (*e.g.* Cyrus et al. 2015a), in the current study, the chemosensory properties of the different *Ulva* fractions were expected to have an effect on the feeding behaviour in this species.

Results presented in the current study suggested that *T. gratilla* is capable of distance food perception as it was evident that the animals elicited a much stronger response to certain *Ulva* fractions than others. Indeed, the chemical preferences of the urchins were found to be the most pronounced for polar fractions (F8 and F9) compared to non-polar (F1 – F3) and semi non-polar (F4 – F7) fractions, thus giving clear evidence that chemical characteristics of the fractions influenced the preferences of the urchins. Even though a very small amount of polar fraction F8 resulted from the fractionation of the crude extract compared to F9, this fraction was the most effective at attracting the urchins (Table **2.1** and Figure **2.6**). The possibility of induced chemotaxis in *T. gratilla* from the 7-day starvation period is undetermined, since it was not addressed in the present study.

Chemotactic responses of *T. gratilla* to the different fractions showed a clear pattern, revealing that the solubility and polarity of the fractions may have played a role in either the attraction or inhibition of the animals. Depending on the chemical nature of the compounds, a polar solvent (*i.e.* MeOH) would extract polar and water-soluble metabolites, while non-polar solvents (*i.e.* hex) would extract the opposite. Combinations of hex and EtOAc (semi-polar) at various ratios with increasing polarities would ensure the extraction of a wide range of compounds with different polarities and solubilities. This concept of solvent extraction "like dissolve like," means that a solvent will only dissolve compounds with the same polarity as the solvent used (Shipeng et al. 2015). Therefore, compounds from polar fractions (*i.e.* F8 and F9) were likely detected by the urchins from a distance due to their water solubility, while semi non-polar (*i.e.* F4 – F7) and non-polar fraction (*i.e.* F1 – F3) compounds were more likely to be detected upon contact (*i.e.* interaction between sea urchins' tube feet and sample zone on the bioassay plate containing *Ulva* fraction).

Numerous investigations had implicated non-polar extracts (lipophilic) of seaweeds as the major source of secondary metabolites associated with their chemical defence against grazers (*e.g.* Steinberg and van Altena, 1992; Cronin and Hay, 1996; Pelletreau and Muller-Parker, 2002; Barbosa et al. 2004; Ishii et al. 2004). Polar extracts in contrast, have been associated with lipid compounds that can stimulate feeding in marine herbivores (*e.g.* Sakata and Ina; 1985; Sakata et al. 1985). However, in some cases, compounds found in the polar extracts have been found to deter grazers (*e.g.* Deal et al. 2003; Kubanek et al. 2004; Van Alstyne et al. 2006). In the present study, it can be suggested that the strong response of *Tripneustes gratilla* for polar fractions (F8 and F9) is elicited by the presence of water-soluble compounds that act as feed attractants and trigger the olfactory ability of the urchins from a distance (≈ 14.4 – 15.4 cm). The low to moderate responses from the non-polar (F1 – F3) and semi non-polar fractions (F4 – F7) also indicate that the compounds present in these fractions stimulated the olfactory responses of the urchins to some extent. However, the data collected from the touch and grazing preferences of *T. gratilla* suggested that after the initiation of feeding (tasting), the compounds present in these fractions (F1 – F7) could have acted as incitants/deterrents. Research has shown that some compounds present in seaweeds can be deterrents to some grazers but have no effects on, or at certain concentrations can stimulate a feeding response, in others (Duffy and Hay, 1994; Cronin and Hay, 1996; Hay et al. 1998).

In the current study, MGDG was identified as the main compound from fraction F8 that appears to act as the feed stimulant for *Tripneustes gratilla*. In addition, a mixture of compounds including DGTS, DGDG, PA, SQMG and LPG were identified by LC-MS from fraction F9 that may also act as feed stimulants for the urchins. Similar group of compounds were reported by Sakata et al. (1989), where glycerolipids including sulfoquinovosyldiacylglycerol (SQDG), phosphatidylcholine, DGDG and MGDG from the brown seaweed *Eisenia bicyclis* (as *Ecklonia bicyclis*; Laminariales) could act as feed stimulants for the urchin *Strongylocentrotus intermedius* (Camarodonta). Sakata et al. (1985) reported the sea hare *Aplysia juliana* (Aplysiida) showed feed stimulatory behaviour towards glycerolipids DGTS (as DGTH) and DGDG isolated from the green seaweed *Ulva australis* (as *U. pertusa*) as low as 100 µg of the compounds. Similarly, DGDG and phosphatidylcholine from the methanolic extract of the brown alga *Undaria pinnatifida* (Laminariales) showed feed stimulatory effects on the abalone *Haliotis discus hannai* (as *H. discus*; Lepetellida) at small amounts ranging from between 30-60 µg (Sakata and Ina, 1985). Although the minimum effective concentrations of the stimulatory fractions and compounds identified in the present study was not determined, it may be suggested that the stimulatory effect of MGDG identified as the main constituent of fraction F8 was more effective than compounds identified from fraction F9 based on the low amount recovered from the fractionation process and purity of this fraction (Table **2.1**).

In the current study, urchin grazing preferences to non-polar (F1 – F3) and semi non-polar (F4 – F7) fractions may have been triggered by compensatory feeding, which is a common adaptive response of marine herbivores to low food quality (Cruz-Rivera and Hay, 2000, 2001). Prior to chemosensory trials, urchins were maintained on a mixed diet including the kelp *Ecklonia maxima* (Laminariales), fresh aquacultured *Ulva* and formulated feed consisting of 200 g.kg^{-1} dried *Ulva* (25.7% protein; Cyrus et al. 2013). However, during bioassays, starved urchins' food options were *Ulva* fractions along with microcrystalline cellulose from the Avicel® plate (low nutritional value). The palatability differences could have forced some of the urchins to carry out compensatory feeding on non-polar fractions to balance the negative effect of the low nutritional quality of food present during bioassays. This feeding response has been observed in several marine herbivores including urchins, attempting to compensate for nutritionally-deficient food or during food limitation (Stachowicz and Hay, 1996; Cruz-Rivera and Hay 2000, 2001; Hammer et al. 2004; Lyons and Scheibling, 2007; Tomas et al. 2011). Another possibility, rather than compensatory feeding, might be that the urchins were able to tolerate compounds associated with non-polar fractions that may deter feeding. Tolerance against anti-herbivory compounds from macroalgal origin has been shown against marine grazers (*e.g.* Hay et al. 1998; Jormalainen et al. 2005).

The present experimental study provided clear evidence that certain *Ulva* fractions were effective at attracting and stimulating the urchin *Tripneustes gratilla* to feed. Findings provided here further contributed to what has previously been reported about stimulants from seaweeds for marine herbivores. The chemoreceptive abilities of *T. gratilla* to detect chemical cues and discover compounds with attractant or stimulant properties could be advantageous in the formulation or supplementation of an effective artificial diet for *T. gratilla* species as well as other echinoid species. However, additional research needs to be undertaken to evaluate whether the incorporation or supplementation of these compounds in the artificial diets of this urchin species will generate similar outcomes. The development of well-balanced feed will not be beneficial if the sea urchin cannot successfully discover and does not voluntarily consume the feed.

CHAPTER 3

Evaluation of the minimum effective concentrations and effectiveness of feed stimulatory *Ulva* fractions on the sea urchin *Tripneustes gratilla*

3.1 Abstract

The minimum effective concentrations (MECs) of the polar fractions 100% ethyl acetate (F8) and 1:1 methanol-ethyl acetate (F9), derived from the green aquacultured seaweed *Ulva lacinulata* (Ulvales) as well as their effectiveness as feed stimulants (see Chapter 2) in comparison to a crude extract (CE) of fresh (FU) and dry (DU) *Ulva* were investigated on the sea urchin *Tripneustes gratilla* (Camarodonta). Determination of the MECs at which *Ulva* fractions act as feed stimulants towards *T. gratilla*, together with their effectiveness as feed stimulants, is necessary to ascertain whether using purified *Ulva* extracts over FU, DU or CE in artificial feeds for this urchin would be advantageous. To investigate the MECs of F8 and F9 on this urchin, five different concentrations for each fraction equivalent to 0.05 g FU (F8 – 0.9 mg; F9 – 13.7 mg extract), 0.5 g FU (F8 – 8.6 mg; F9 – 136.9 mg), 1.0 g FU (F8 – 17.2 mg; F9 – 273.8 mg), 2.0 g FU (F8 – 34.4 mg; F9 – 547.6 mg) and 5.0 g FU (F8 – 86.0 mg; F9 – 1369.0 mg; Table **3.1**) were tested in feeding trials. In a comparative study, the effectiveness of the polar fractions equivalent to 1.0 g FU were evaluated against 1.0 g fresh *Ulva* equivalent of CE, FU and DU in feeding trials. Results for the MECs showed that extracts equivalent to 0.05 g FU for both F8 and F9 were effective in attracting and stimulating the urchins to feed. However, the most effective treatments that attracted and stimulated the highest number of urchins to feed were F8 equivalent to 2.0 g FU and F9 equivalent to 0.5 g FU. The predicted probability of occurrence for attracting an urchin for F8 was 2.0 > 5.0 > 1.0 > 0.5 > 0.05 g FU, while for F9 was 0.5 > 1.0 ≥ 2.0 > 5.0 > 0.05 g FU. The predicted probability of occurrence for stimulating an urchin to graze for F8 was 2.0 > 1.0 > 0.5 > 5.0 > 0.05 g FU, while for F9 was 0.5 > 1.0 ≥ 2.0 ≥ 5.0 > 0.05 g FU. The comparative study showed that FU was the most effective treatment at both attracting and stimulating feeding in *T. gratilla* over the other *Ulva* treatments. The predicted probability of occurrence for attracting an urchin for this experiment was FU > F8 > CE ≥ F9 > DU, while the predicted probability for stimulating an urchin to graze was FU > DU > CE > F8 > F9. The results reported here provide evidence that polar *Ulva* fractions F8 and F9 can act as both an attractant and feeding stimulant for *T. gratilla* at low concentrations. However, findings also indicate that incorporation of F8 and/or F9 in artificial feed for this urchin may not be as effective as FU, DU or CE.

3.2 Introduction

Under intensive and semi-intensive aquaculture practices, aquafeed often constitutes the largest portion of the overall operational expenses (Azaza et al. 2009; Zhuo et al. 2016; Matulić et al. 2020). Aquafeed relies strongly on fishmeal and fish oil as the main ingredients due to their high protein content, balanced amino acid and fatty acid profiles as well as being highly palatable and easily digestible (Olsen and Hasan, 2012; Jirsa et al. 2013; Soto-Sánchez et al. 2023). However, with the predicted expansion of the aquaculture industry, the price, demand and use of fishmeal in aquafeed may be deemed unsustainable (Olsen and Hasan 2012; Estruch et al. 2018; Mitra, 2021; Gokulakrishnan et al. 2023). Recently, there has been an increase in the body of research looking at less expensive proteins sources, particularly plant proteins, for the partial or full replacement of fishmeal in aquafeeds as well as functional feed ingredients that will enhance consumption and digestibility of protein in formulated feeds and provide other nutritional benefits (Jobling, 2016; Shpigel et al. 2017; Estruch et al. 2018; Kamunde et al. 2019; Al-Souti et al. 2019; Bacchetti et al. 2020; Ferreira et al. 2021; Hossain et al. 2024).

Algae (micro- and macroalgae) have emerged as potential ingredients for aquafeed (Cyrus et al. 2013; Shpigel et al. 2017, 2018b; de Cruz et al. 2018; Valente et al. 2019; Batista et al. 2020; Santizo-Taan et al. 2020). Marine macroalgae (*i.e.* seaweeds), such as *Ulva*, can be produced at higher rates with elevated protein without the extensive need for land space, freshwater and fertilizers linked to land plant production (Robertson-Andersson et al. 2011; Lawton et al. 2013; Al-Hafedh et al. 2015; Shpigel et al. 2017, 2018a, b; Wan et al. 2019). Apart from the protein content, *Ulva* has been reported to be a great source of dietary fiber, vitamins, minerals and bioactive compounds with antiviral, antihyperlipidemic, antioxidant and immunomodulating properties (Ortiz et al. 2006; Taboada et al. 2010; Tabarsa et al. 2012; Kendel et al. 2015; Neto et al. 2018; Anjali et al. 2019; Kidgell et al. 2019; Pappou et al. 2022). Inclusion of *Ulva* at low levels (up to 14.6%) in the experimental diets of freshwater and marine finfish has shown to be a suitable alternative for the partial to full replacement of fishmeal without compromising growth performance and body composition (Marinho et al. 2013; Silva et al. 2015; Abdel-Warith et al. 2016; Shpigel et al. 2017). Dietary inclusion *Ulva*-derived polysaccharide has also shown to improve immune-related genes as well as increase the immune response of juvenile finfish and shrimp (Klongklaew et al. 2021; Akbary and Aminikhoei, 2018).

Few studies have demonstrated the stimulatory effects of seaweed incorporation in the formulated feeds of several marine organisms (*e.g.* Dworjanyn et al. 2007; Xia et al. 2012; Cyrus et al. 2015a; Sánchez et al. 2016). Dworjanyn et al. (2007) tested the stimulatory effect of incorporating 50 g.kg^{-1} dry weight of the brown seaweeds *Ecklonia radiata* (Laminariales), *Sargassum linearifolium* (Fucales) and the green alga *U. lactuca* into three separate artificial feeds on the urchin *T. gratilla* and found that the organisms consumed twice as much of the diet containing *S. linearifolium* in comparison to the control diet. The researchers suggested that the addition of small amount of palatable seaweed in the prepared feed of the urchins could act as feeding stimulant by increasing the acceptability of diets. Similarly, Cyrus et al. (2015a) also reported that the inclusion of *Ulva* at 200 g.kg^{-1} dry weight in the artificial feed of locally collected urchin *T. gratilla* could act as a feed stimulant by increasing the acceptability and palatability of the artificial feed. Under laboratory conditions, Sánchez et al. (2016) found that the inclusions 80 g.kg^{-1} dried red seaweed *Halymenia floresii* (Halymeniales) and dried microalga, *Spirulina* sp. (Spirulinales) in the diet of the fighting conch *Strombus pugilis* (Littorinimorpha) can serve as a feeding stimulant as well as increase feed intake and promote gonad maturity in broodstock. Additionally, Xia et al. (2012) suggested that the addition of the kelp *Saccharina japonica* (as *Laminaria japonica*; Laminariales) to the prepared diet of the sea cucumber *Apostichopus japonicus* (Synallactida) may contain chemical compounds that could attract and stimulate the organism to feed.

Inclusion of local aquacultured *Ulva* at 200 g.kg^{-1} dry weight in the artificial feed of *T. gratilla* as a feed stimulant has proven to be successful (Cyrus et al. 2013, 2015a, b). However, the use of crude or purified *Ulva* extracts in the diet of this species is still at a research stage. Outcomes from Chapter 2 demonstrated that polar *Ulva* fractions F8 (100% EtOAC) and F9 (1:1 MeOH:EtOAc) can act as feed stimulants towards the urchin, *T. gratilla*. However, the minimum concentrations at which these *Ulva* fractions stimulate the feeding response of this echinoid as well as a direct comparison of the stimulatory effects of these fractions to crude *Ulva* extract (CE), fresh (FU) and dried *Ulva* (DU), have not yet been determined. Therefore, the aims of this present study were to investigate the minimum effective concentrations (MECs) at which *Ulva* fractions trigger the feed stimulatory response of the urchin *T. gratilla*, as well as to evaluate the effectiveness of these *Ulva* fractions against CE, FU and DU. The main objectives were set as follows: 1) evaluate the effects of different concentrations of F8 and F9 (extract equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g FU) on the touch and feeding preferences of *T. gratilla* in separate sets of trials; and 2) evaluate the effects of F8, F9, CE, FU and DU equivalent to 1.0 g FU on the touch and feeding preferences *T. gratilla*. This study was aimed to provide information on the minimum effective doses of F8 and F9 to be stimulatory towards the urchin *T. gratilla*, as well as to access the relative effectiveness of using fractionated extracts over fresh or dry seaweed component in artificial feed for this urchin.

3.3 Materials and Methods

3.3.1. Chemicals

All solvents used were of chromatography grade (LiChrosolv®). Methanol (≥99.9%; MeOH), dichloromethane (≥99.8%; CH_2Cl_2), hexane (≥97.0%; hex) and ethyl acetate (≥99.8%; EtOAc) for fractionation and extracts resuspension were purchased from Merck® (Darmstadt, Germany). Silica gel 60 (0.040 – 0.063 mm) for column chromatography and Analtech TLC Uniplates™ microcrystalline cellulose matrix for chemosensory trials were acquired from Merck (Darmstadt, Germany).

3.3.2. Sample preparation

3.3.2.1. Extracts - CE, F8 and F9

Crude extract (CE) was obtained from alga extracted from **Section 2.3.4** of **Chapter 2** following a modified protocol used by Afolayan et al. (2008). Briefly, *U. lacinulata* (850 ± 0.01 g wet weight; Bachoo et al. 2023) roughly chopped into 2 – 3 cm pieces was steeped in MeOH (1500 mL) for 24 hrs at room temperature. The organic phase was collected through filtration, and the remaining algal biomass was re-extracted twice with CH_2Cl_2-MeOH (2:1; 1500 mL × 2) at room temperature for 24 hrs. The organic layers were combined, and sufficient de-ionised water was added until phase separation. The final organic (lower) phase was recovered and concentrated *in vacuo* (Büchi® Rotavapor R-210, Switzerland) to produce a crude extract (= 7.572 g; yield = 6.58 % *w/w* relative to DU). For the trials, extracts equivalent to 1.0 g FU in concentration was prepared by reconstituting 0.8 g (± 0.01 g) CE in 45 mL MeOH:CH_2Cl_2 (1:1; *v/v*) into a 50 mL NEST polypropylene tube. This concentration was chosen to provide a consistency between the concentration (*i.e.* extracts equivalent to 1.0 g FU) used in Chapter 2.

CE weighing 5 g (± 0.01 g) was fractionated by silica gel step gradient column chromatography using a combination of hex, EtOAc and MeOH (50 mL elution solvent per fraction for every

1.0 ± 0.01 g CE) to produce nine fractions (F1 – F9). The composition of the elution solvents were as follows: F1 (100% hex); F2 (9:1, hex:EtOAc); F3 (8:2, hex:EtOAc); F4 (7:3, hex:EtOAc); F5 (6:4, hex:EtOAc); F6 (4:6, hex:EtOAc); F7 (2:8, hex:EtOAc); F8 (100% EtOAc); F9 (1:1, MeOH:EtOAc). The nine fractions were collected, concentrated *in vacuo* (Büchi® Rotavapor R-210, Switzerland) and the weight of each fraction was measured (see Supplementary Information **3)**. For this study, fractions F8 and F9 were reconstituted in 10 mL MeOH:CH_2Cl_2 (1:1; *v*/*v*) and then divided into 50 mL NEST polypropylene tubes. A mixture of MeOH:CH_2Cl_2 (1:1; *v*/*v*) was added to the tubes to make up samples equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g FU in concentrations. The weights (mg) for F8 and F9 treatments (equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g fresh *Ulva*) are shown in Table **3.1**. Similar to **Section 2.4.1.** of **Chapter 2**, F8 represents only a smaller percentage of the crude *Ulva* extract compared to F9.

Table 3.1: Weight distribution (mg) of F8 and F9 treatments (equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g fresh *Ulva lacinulata*).

Treatments	F8 (mg)	F9 (mg)
0.05	0.9	13.7
0.5	8.6	136.9
1.0	17.2	273.8
2.0	34.4	547.6
5.0	86.0	1369.0

3.3.2.2. Seaweed components - FU and DU

Fresh *U. lacinulata* (FU) was obtained from the Department of Forestry, Fisheries and Environment (DFFE) Aquaculture Research Facility in Cape Town, South Africa (33°55'13.7"S 18°22'52.1"E), where it is cultured as an experimental material. This seaweed was initially obtained from Irvine and Johnson (I & J) Cape Abalone aquafarm, Gansbaai, South Africa (34°37'35.1"S 19°17'47.4"E) as material grown in abalone effluent. Upon receival, the alga was thoroughly cleaned of sediments, epiphytes and visible surface contaminants, and washed several times with de-ionized water. For FU bioassay plates, 1.0 g (± 0.01 g) FU were attached onto a 5 × 5 cm glass plate (length × width; 0.25 µm thickness) using fishing line as shown in Figure **3.1A**. For DU, bioassay plates were prepared by oven drying 1.0 g (± 0.01 g) FU attached onto a glass plate (5 × 5 cm; length × width; 0.25 µm thickness) at 60 °C until a constant weight was achieved as shown in Figure **3.1B**.

Figure 3.1: Example **(A)** 1.0 g of fresh *Ulva lacinulata* (FU) on a 5 × 5 cm (length × width) glass plate; **(B)** 1.0 g of FU oven-dried at 60 °C (DU) on 5 × 5 cm (length × width) glass plate.

3.3.3. Sea urchin, Tripneustes gratilla

Sea urchins were obtained from the DFFE, where they are spawned and cultured for aquaculture research purposes. At the facility, the organisms were kept as described in **Section 2.3.3.** of **Chapter 2**. For this experiment, 172 young adults ranging in size from 47 to 62 mm (53.3 ± 3.8 mm; mean ± SD; 6 – 7 months old) test diameter were used. Grown-out size urchins were chosen to increase our understanding of their feeding behaviour for *Ulva* fractions (*i.e.* F8 and F9), and therefore getting useful knowledge on their feeding preference that can be used in feed production. The organisms were starved 7 days before the start of the trials to increase their responsiveness to the stimuli.

3.3.4. Bioassay

3.3.4.1. Minimum effective concentration (MEC)

Determination of the MECs for F8 and F9 was carried out using a modified version of the ‘Avicel® plate method’ adapted from Sakata et al. (1989) as per **Section 2.3.5** of **Chapter 2**. Briefly, bioassay plates, made of glass coated microcrystalline Avicel® (5 × 5 cm; length × width; 0.25 µm Avicel® thickness), were prepared by applying extracts equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g FU in concentration onto the sample zones of the Avicel® plates (circle of 3 cm diameter). The solvent was allowed to dry for 10 min. For each single trial, five treatments of either F8 or F9 (equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g FU) were tested along with control plates containing no extracts (placed between plates containing extracts). The bioassay plates were placed in a round testing tank (80 cm diameter) positioned 13 cm apart from one another and 20 cm from the centre, containing 20 L heated seawater (depth: 9.4 cm; temperature: 24 – 25 °C; salinity: 35 PSU) as described in **Section 2.3.5** of **Chapter 2**.

3.3.4.2. Comparative study - F8 vs F9 vs CE vs FU vs DU

This section of this research focused on the direct comparison of stimulatory *Ulva* fractions F8 and F9 to crude *Ulva* extract (CE), fresh (FU) and dried *Ulva* (DU), to provide insight on whether the use of fractionated extracts over fresh or dry seaweed component in aquafeed would be suitable. The comparison and effectiveness of F8, F9, CE, FU and DU (equivalent to 1.0 g FU) were carried out using a modified version of the 'Avicel$^{®}$ plate method' adapted from Sakata et al. (1989) as per **Section 3.3.4.1**. Bioassay plates for F8, F9 and CE were prepared by applying extracts equivalent to 1.0 g FU onto the sample zones of the Avicel$^{®}$ bioassay plates. The solvent was allowed to dry for 10 min. Bioassay plates for FU and DU were prepared as per **Section 3.3.2.2**. For each single trial, the five treatments (*i.e.* F8, F9, CE, FU and DU) were tested along with control plates containing no extracts, were placed in the circular testing tank containing 20 L heated seawater (depth: 9.4 cm; temperature: 24 – 25 °C; salinity: 35 PSU) as described in **Section 3.3.4.1**.

Chemosensory trials for both **Sections 3.3.4.1** and **3.3.4.2** were performed by placing a starved urchin in the centre of the tank surrounded by the bioassay plates containing *Ulva* treatments along with the controls. The movement of the organism was monitored for 75 min. A touch preference was established when the sea urchins' tube feet touched the sample zone of the bioassay plate, and a feed preference was allocated when the sample zone of a bioassay plate was grazed with the urchin leaving its characteristic 'star marks' traces. An urchin may show touch preference to one or multiple treatments in a single trial. Likewise, an urchin may graze on one or multiple treatments in a single trial. All the touch and graze preferences of the urchin in a single trial were recorded. For MECs, 46 individual urchins were tested for both F8 and F9, while for the comparative study, 80 individual urchins were tested. Between each single trial, the tank was emptied and washed after which the water and bioassay plates were replaced. The order in which the bioassay plates were offered was randomized without repetition to avoid directional placement of specific bioassay plates.

3.3.5. Statistical analyses

Statistical analyses were performed using R (ver. 4.0.2, R Development Core Team 2020) and Microsoft® Excel® 365 as described in **Section 2.3.9** of **Chapter 2**. Non-parametric chi-square tests were used to determine significant differences in the touch preference and grazing data for both the MEC and comparative experiments. Binomial logistic regression was performed on data obtained from both the MECs and comparative experiments to assess the outcome of the predictor variable on whether the treatments had any effect on the urchins. Wald's test was used to evaluate the significance of the *Ulva* treatments in the model. Outcomes from the logistic model were reported as probabilities, lower (LCI) and upper (UCI) 95% confidence intervals, Wald z-values and p-values of an urchin to be attracted and/or to feed on a particular treatment. Significance was accepted at $p < 0.05$.

3.4 Results

3.4.1. Minimum effective concentration (MEC)

3.4.1.1. Touch preferences for F8 and F9

The touch preferences of *T. gratilla* for F8 and F9 treatments (equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g FU) are shown in Figure **3.2**. A total of 43 out of 46 urchins showed preferences to one or multiple F8 treatments and controls (C1 – C5). In contrast, 38 out of 46 urchins showed preferences to one or multiple F9 treatments and controls (C1 – C5). Touch preference data showed that the distribution of urchins among F9 treatments were significantly different ($\chi^2 = 24.980$, $df = 9$, $p < 0.01$), while for F8 treatments, no statistical difference was found ($\chi^2 = 15.443$, $df = 9$, $p > 0.05$). For F9 treatments, 0.5 g FU attracted the most urchins, with 15 observations followed by 1.0 and 2.0 g FU (13 observations), 5.0 g FU (12 observations) and 0.05 g FU (4 observations), respectively. Although no significant differences were found among F8 treatments, the animals appeared to be attracted to 2.0 g FU (19 observations) the most over the other treatments.

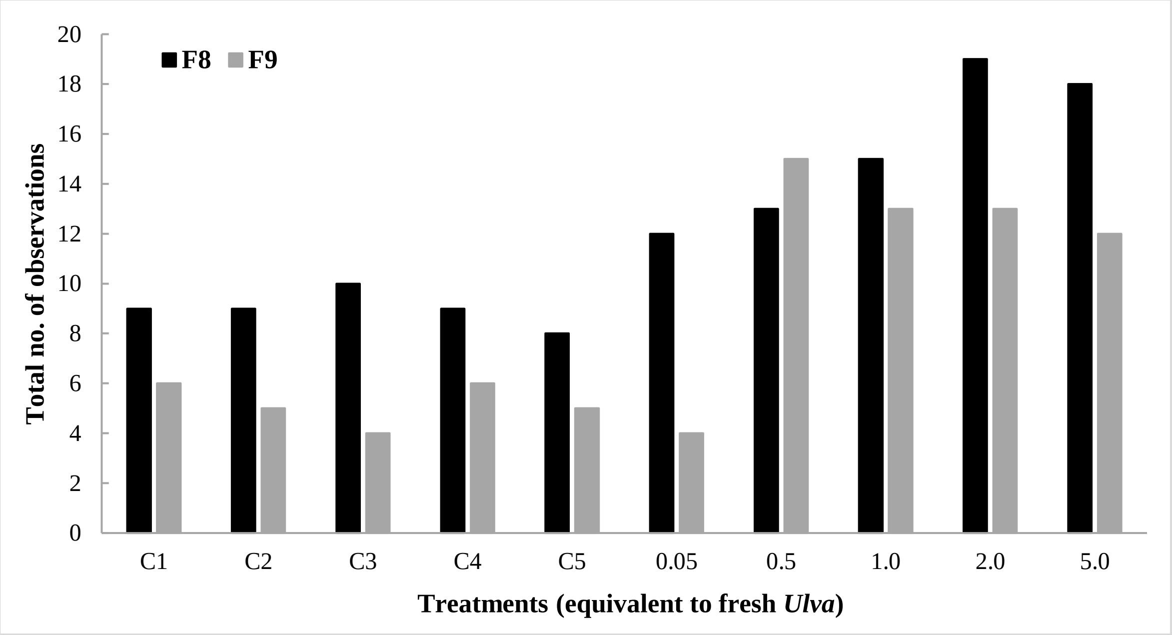


Figure 3.2: Touch preferences of *Tripneustes gratilla* for F8 and F9 treatments (equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g FU) and the controls (C1 – C5).

3.4.1.2. Grazing preferences for F8 and F9

The grazing preferences of *T. gratilla* for F8 and F9 treatments (equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g FU) are shown in Figure **3.3**. A total of 21 (F8) and 15 urchins (F9) actively grazed on one or multiple treatments and controls (C1 – C5). Grazing preference data showed that the distribution of urchins among F9 treatments were statistically significant (χ^2= 21.239, *df*= 9, p< 0.05), while for F8 treatments no statistical difference were found (χ^2= 11.844, *df*= 9, p> 0.05). For F9 treatments, 0.5 g FU stimulated the most organisms to graze (5 urchins) followed by the 1.0, 2.0 and 5.0 g FU (3 urchins) and 0.05 g FU (2 urchins) treatments, respectively. Although no significant differences were found among F8 treatments, the urchins appeared to be stimulated to grazed on 2.0 g FU the most compared (6 urchins) to the other treatments.

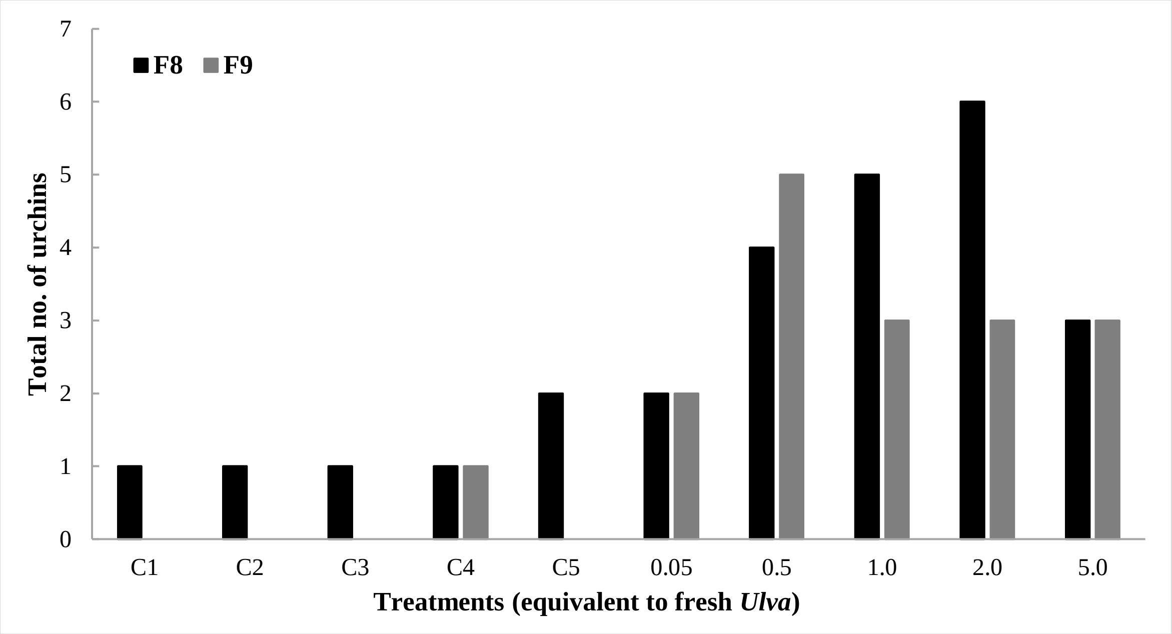


Figure 3.3: Grazing preferences of *Tripneustes gratilla* for F8 and F9 treatments (equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g FU) and the controls (C1 – C5).

3.4.1.3. Probability of occurrence - F8 and F9

3.4.1.3.1. Attraction

The probability of occurrence of *T. gratilla* for F8 and F9 treatments (equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g FU) are shown in Table **3.2** and Figure **3.4**. Results from the binomial GLM model indicated that F8 equivalent to 2.0 g FU had the highest predicted probability ($\Delta\rho$= 0.413, p< 0.05) of attracting *T. gratilla* followed by F8 equivalent to 5.0 g FU ($\Delta\rho$= 0.391, p< 0.05) and F9 equivalent to 0.5 g FU ($\Delta\rho$= 0.326, p< 0.05) in a single trial. Although not statistically significant (p> 0.05), F8 equivalent to 1.0 g FU appeared to have a greater predicted probability of attracting an urchin that the F9 equivalent to 1.0, 2.0 and 5.0 g FU in a single trial.

Table 3.2: Predicted probabilities (Δρ) for F8 and F9 treatments (equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g fresh *Ulva lacinulata*) to attract *Tripneustes gratilla* in a single trial. Lower (LCI) and upper (UCI) 95% confidence interval, Wald *z*-values and *p*-values showing significant differences.

Treatment	Probability (Δρ)	LCI	UCI	*z*- value	*p*- value
F8					
0.05	0.261	0.154	0.405	0.743	N.S
0.5	0.283	0.171	0.428	0.973	N.S
1.0	0.326	0.207	0.472	1.413	N.S
2.0	0.413	0.281	0.559	2.226	< 0.05
5.0	0.391	0.262	0.538	2.029	< 0.05
F9					
0.05	0.087	0.173	0.210	-0.666	N.S
0.5	0.326	0.173	0.473	2.173	< 0.05
1.0	0.283	0.110	0.428	1.766	N.S
2.0	0.283	0.340	0.428	1.766	N.S
5.0	0.261	0.316	0.405	1.551	N.S

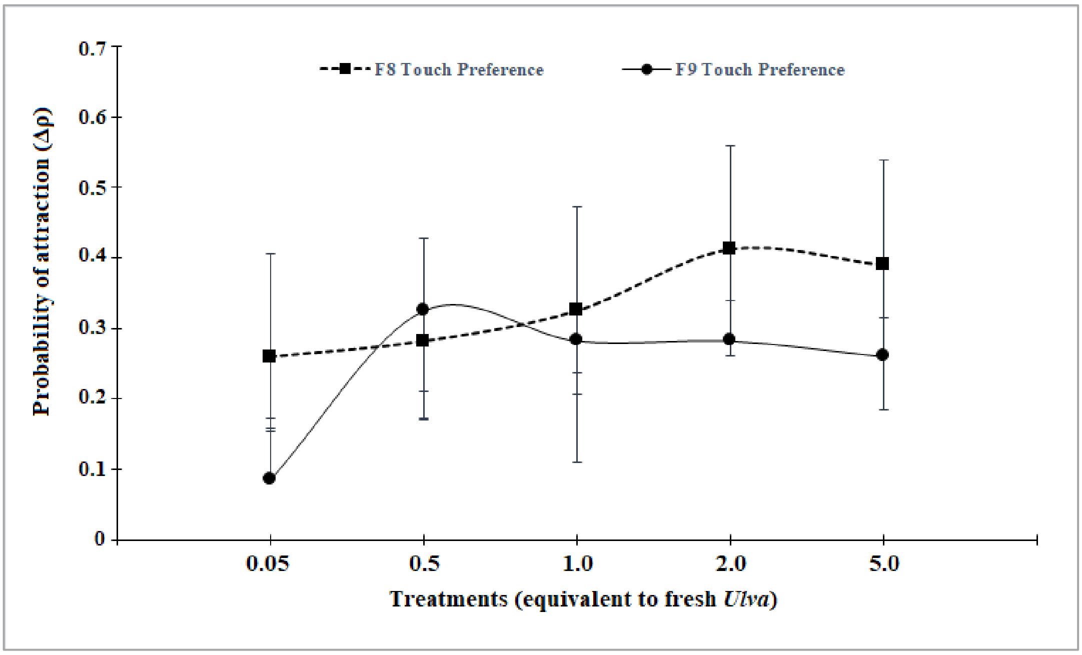


Figure 3.4: Predicted of occurrence (Δρ) for F8 and F9 treatments (equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g FU) to attract *Tripneustes gratilla* in a single trial. Error bars represent the lower (LCI) and upper (UCI) 95% confidence interval.

3.4.1.3.2. Stimulation

The probability of stimulation of *T. gratilla* for F8 and F9 treatments (equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g FU) are shown in Table **3.3** and Figure **3.5**. Although outcomes from the binomial GLM model showed no significant differences (*p*> 0.05), F8 equivalent to 2.0g FU appeared to have the highest predicted probability (Δρ= 0.130) of stimulating *T. gratilla* to graze followed by F8 equivalent to 1.0 g FU (Δρ= 0.109) and F9 equivalent to 0.5 g FU (Δρ= 0.109) in a single trial. It also appeared that F8 equivalent to 0.5 g FU had a greater predicted probability of stimulating an urchin than the F9 equivalent to 1.0, 2.0 and 5.0 g FU in single trial.

Table 3.3: Predicted probabilities (Δρ) for F8 and F9 treatments (equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g fresh *Ulva lacinulata*) to simulate *Tripneustes gratilla* in a single trial. Lower (LCI) and upper (UCI) 95% confidence interval, Wald *z*-values and *p*-values showing significant differences.

Treatment	**Probability (Δρ)**	**LCI**	**UCI**	***z*- value**	***p*- value**
F8					
0.05	0.043	0.011	0.158	0.576	0.565
0.5	0.087	0.033	0.210	1.278	0.201
1.0	0.109	0.046	0.236	1.525	0.127
2.0	0.130	0.060	0.261	1.733	0.083
5.0	0.065	0.021	0.184	0.974	0.330
F9					
0.05	0.043	0.011	0.158	0.007	0.995
0.5	0.109	0.046	0.236	0.007	0.994
1.0	0.065	0.021	0.184	0.007	0.995
2.0	0.065	0.021	0.184	0.007	0.995
5.0	0.065	0.021	0.184	0.007	0.995

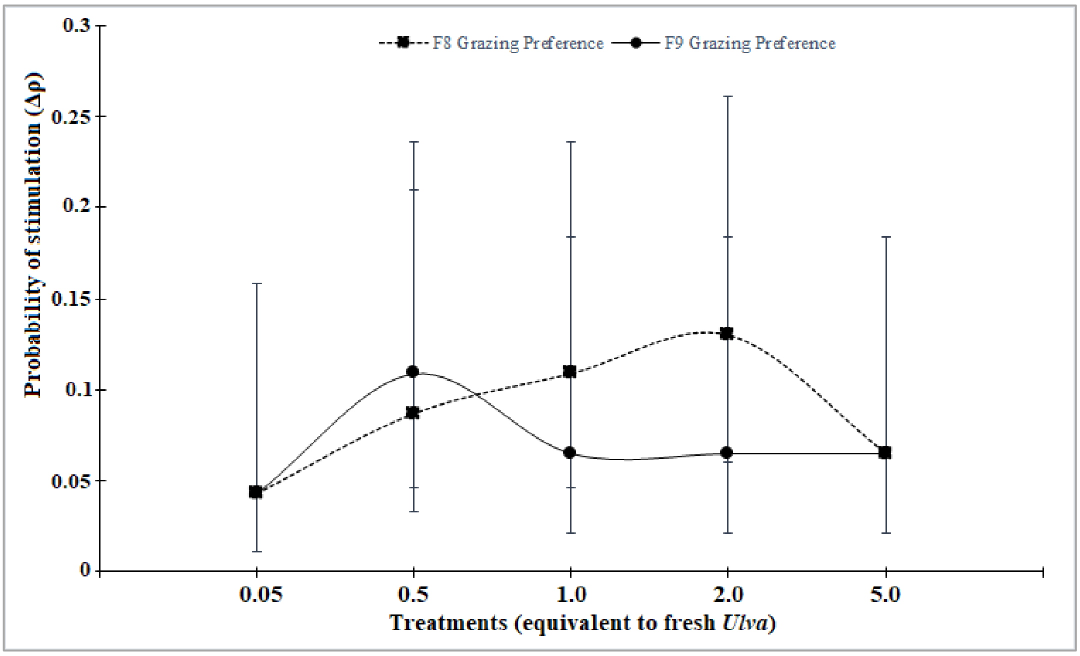


Figure 3.5: Predicted of occurrence (Δρ) for F8 and F9 treatments (equivalent to 0.05, 0.5, 1.0, 2.0 and 5.0 g FU) to stimulate *Tripneustes gratilla* in a single trial. Error bars represent the lower (LCI) and upper (UCI) 95% confidence interval.

3.4.2. Comparative study - F8 vs F9 vs CE vs FU vs DU

3.4.2.1. Touch and grazing preferences

The touch preferences of *T. gratilla* for *Ulva* treatments (F8, F9, CE, FU and DU equivalent to 1 g FU) are shown in Figure **3.6**. In total, 78 of the 80 urchins tested showed touch preferences to one or multiple *Ulva* treatments and the controls (C1 – C5) in single trials. Statistical analyses showed that the distribution of urchins among the treatments for the touch preference data was significantly different (χ^2= 86.346, *df*= 9, *p*< 0.001). FU treatment was the most effective at both attracting urchins (43 observations), followed by F8 (26 observations), F9 (22 observations), CE (22 observations) and DU (19 observations), respectively.

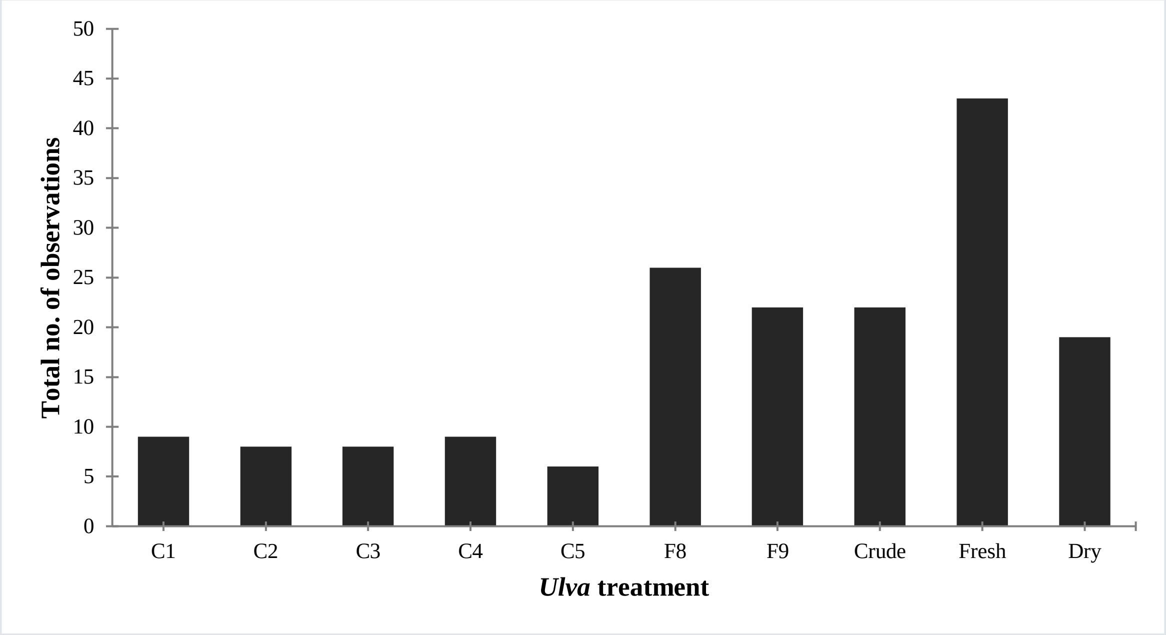


Figure 3.6: Touch preferences of *Tripneustes gratilla* for *Ulva* treatments (F8, F9, CE, FU and DU equivalent to 1g fresh *Ulva*) and controls (C1 – C5).

Only 49 of the 80 urchins tested were stimulated to feed on one or multiple *Ulva* treatments and the controls (C1 – C5) in single trials (Figure **3.7**). Graze preferences data showed that the distribution of the urchins among the treatments were statistically significant (χ^2= 112.660, *df*= 9, *p*< 0.001). FU stimulated the most urchins to graze (26 urchins), followed by DU (12 urchins), CE (9 urchins), F8 (8 urchins) and F9 (1 urchin), respectively.

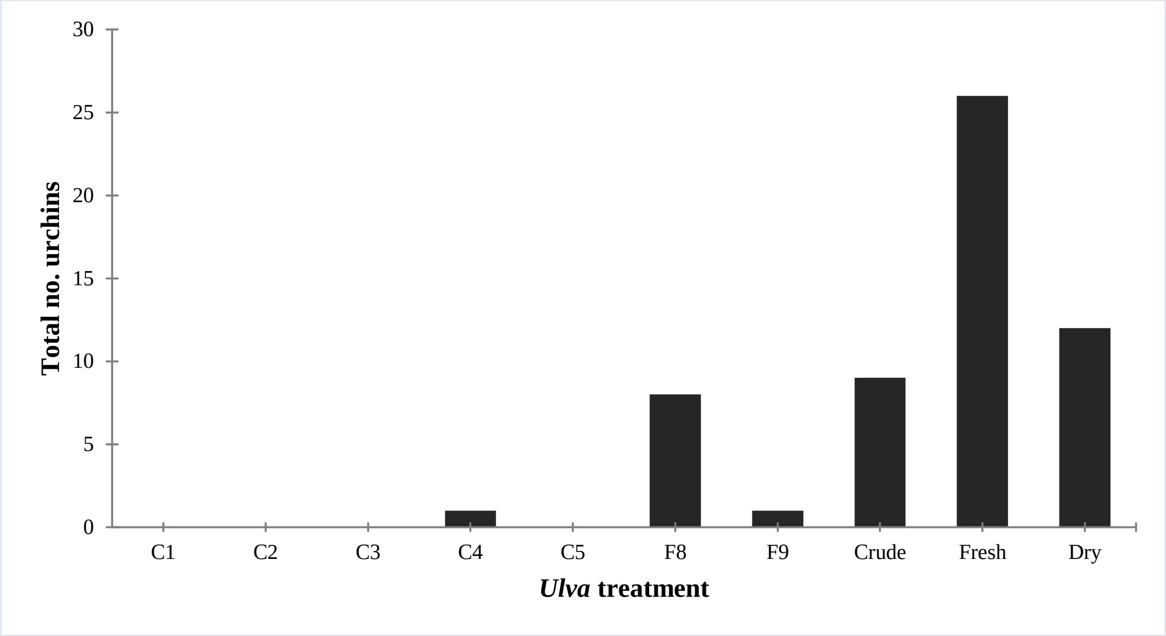


Figure 3.7: Graze preferences of *Tripneustes gratilla* for *Ulva* treatments (F8, F9, CE, FU and DU equivalent to 1 g FU) and controls (C1 – C5).

3.4.2.2. Probability of occurrence - F8 vs F9 vs CE vs FU vs DU

The probability of occurrence for the touch and grazing preferences of *T. gratilla* for the five different *Ulva* treatments (F8, F9, CE, FU and DU equivalent to 1 g FU) are shown in Table **3.4**. Outcomes from the binomial GLM showed that FU had the highest predicted probability (Δρ= 0.538, *p*< 0.05) of attracting *T. gratilla* in a single trial, followed by F8 (Δρ= 0.325, *p*< 0.05), CE (Δρ= 0.275, *p*< 0.01), F9 (Δρ= 0.275, *p*< 0.05) and DU (Δρ= 0.238, *p*< 0.001). Although no significant differences were found for the predicted probabilities for the grazing preferences, FU (Δρ= 0.325) appeared to stimulate the urchins to feed the most over DU (Δρ= 0.150), CE (Δρ= 0.125), F8 (Δρ= 0.100) and F9 (Δρ= 0.013) in single trials.

Table 3.4: Predicted probabilities (Δρ) for *Ulva* treatments (F8, F9, CE, FU and DU equivalent to 1 g *Ulva lacinulata*) to attract and stimulate *Tripneustes gratilla* in a single trial. Lower (LCI) and upper (UCI) 95% confidence interval, Wald *z*-values and *p*-values showing significant differences.

Treatment	Probability (Δρ)	LCI	UCI	*z- value*	*p-* value
Touch preference					
F8	0.325	0.232	0.435	2.529	< 0.05
F9	0.275	0.188	0.383	2.040	< 0.05
CE	0.275	0.188	0.383	3.127	< 0.01
FU	0.538	0.428	0.643	2.529	< 0.05
DU	0.238	0.157	0.343	5.289	< 0.001
Grazing preference					
F8	0.100	0.051	0.187	0.009	0.993
F9	0.013	0.002	0.083	0.009	0.992
CE	0.125	0.060	0.202	0.009	0.993
FU	0.325	0.232	0.435	0.008	0.993
DU	0.150	0.087	0.246	0.010	0.992

3.5 Discussion

It has been well established that the profitability of an aquaculture facility is directly linked to the cost of feed as well as the feed intake of the cultured species (Bolivar et al. 2006; Heinsbroek et al. 2007). In most intensive aquaculture operations, the largest returns may be achieved at the highest growth rates of the cultured organisms, which are usually associated to the feed intake and food conversion rates (Heinsbroek et al. 2007, 2008; Kankainen et al. 2012; Hirt-Chabbert et al. 2012). Aquaculturists use several strategies to increase feed consumption and overall performance of the animals (Liebert and Portz, 2005; Wang, 2007; Zheng et al. 2009; Dimitroglou et al. 2010). One is the inclusion of feed ingredients such as chemoattractants and feeding stimulants into the diets of cultured species (Papatryphon and Soares, 2000; Cyrus et al. 2015a; Nunes et al. 2019). However, the quantitative aspects of this group of chemicals are seldom considered.

Results from the current study confirmed the effectiveness of F8 and F9 as chemoattractants and feed stimulants for the urchin *T. gratilla* (see Chapter 2). However, their efficacy was shown to be dose-dependent due to the variability in rate of arrival of the urchins to different concentrations. Chemotactic responses of the urchins may also suggest that the chemical nature and potency level of the chemical stimuli may have played a role in chemoattraction. The urchins were able to perceive the chemical signals emitted through the water column as low as extracts equivalent to 0.05 g FU for both F8 and F9 (distance ≈ 14.8 – 16.3 cm). However, the F8 (equivalent to 2.0 g FU) and F9 (equivalent 0.5 g FU) treatments attracted the highest number of urchins when all five different concentrations from both F8 and F9 were tested separately in feeding trials. Outcomes from Chapter 2 showed that F8 mostly constitutes a single compound (*i.e.* monogalactosyldiacylglycerol [MGDG]), while F9 is a mixture of several glycerolipids (*i.e.* diacylglyceryl-N,N,N-trimethyl-homoserine [DGTS], digalactosyldiacylglycerol [DGDG], sulfoquinovosylmonoacylglycerol [SQMG], dialkylglycerophosphate [PA] and lysophospholipid [LPG]). Similar to Chapter 2, F8 only occurred at a very small percentage of the crude extract compared to F9 (See Supplementary Information **3**; Table **S3.1** for fractionation distribution). Therefore, the differences in activity level recorded in this study may be based on the chemical nature of the fractions as well as the concentrations of the compounds they contained.

In the present study, the potency of different treatments from F8 and F9 had to elicit a response from the urchins of either chemotaxis, chemotaxis and touch (touch preference) or chemotaxis, touch and graze (grazing preference). According to predicted probability of occurrence data, an overall trend can be observed in both the touch and grazing preferences of *T. gratilla* for the F8 and F9 treatments (Figure **3.4** and Figure **3.5**). From the predicted touch preferences of the urchins, a gradual increase in the number of observations was observed with an increase in dosage of the treatments until a peak number was reached (treatments equivalent to 2.0 g FU for F8 and 0.5 g FU for F9; Figures **3.4** and **3.5**). After the peak, a steady decline in the number of observations was noted with an increase in treatment dosage. Analogous trends were observed in predicted grazing preferences data (Figures **3.4** and **3.5**). Although the reason for the decline is not clear, it may be suggested that these treatments caused the saturation of the urchins' receptors or the compounds present in these *Ulva* fractions became inhibitory at higher concentrations. Therefore, results presented here suggest that the touch and grazing preferences of the urchin may follow a saturation curve response. Urchin responses in this study appeared to follow the same tendency as other biological responses such as bacterial growth rate or enzyme kinetics in culture, which are best described by a saturation curve rather than a straight line (Kepner, 2010). Similar observations have been reported for other marine organisms in the presence of chemical stimuli at varying concentrations (*e.g.* Carr and Thompson, 1983; Carr and Derby, 1986). For example, Carr and Thompson (1983) stated that when the marine shrimp *Palaemon pugio* (as *Palaemonetes pugio*; Decapoda) were exposed to different concentrations of the chemoattractant adenosine 5'-monophosphate in behavioural assays the responses were biphasic, increasing linearly up to a peak and then decreasing gradually at higher concentrations. In some cases, responses in the presence of chemical stimuli have been shown to follow a sigmoidal curve (*e.g.* Klinger and Lawrence, 1984).

Marine organisms use a variety of chemical cues to search for food, and single dosage of a particular stimulus may not be as important as a blend of several stimuli (Carr, 1988; Carr and Derby, 1986; Hay, 2009). In the present study, outcomes from comparative study suggest that F8 and F9 were not as effective as feed stimulants for *T. gratilla* compared to 1.0 g of FU, DU and CE (Figure **3.7**). However, their effectiveness as chemoattractants for the urchins were comparable to CE and DU based on the number of observations recorded (Figure **3.6**). In both experiments, FU proved to be the most effective chemoattractant and stimulant for *T. gratilla* compared to the other treatments. In the marine ecosystem, chemical stimuli rarely occur as isolates, rather they are released from the tissue of animals or plants in mixtures, and response to a single stimulus may not be as strong as to a mixture of stimuli (Carr, 1988; Carr and Derby, 1986; Weissburg et al. 2002; Hay, 2009, 2010). In the present study, it may be suggested that the blend of chemical stimuli emitted from 1.0 g of FU was more effective on the chemoreceptors of the urchins in comparison to the stimuli emitted by either F8 or F9. As stated above, F8 mostly constitutes a single compound, while F9 is a mixture of several glycerolipids. Therefore, results from this study confirmed that F8 may be a more potent chemoattractant and stimulant than F9 at a concentration equivalent to 1.0 g FU.

Although DU and CE were not as potent a chemoattractant and stimulant as FU, these treatments were still more effective at stimulating the urchins to graze compared to F8 and F9. *Ulva* in its unpurified state (*i.e.* FU, DU and CE) may be a more potent chemoattractant and stimulant for *T. gratilla* compared to purified extracts/fractions of *Ulva* such as F8 or F9. Even though F8 and F9 were not as potent as FU, these fractions were still effective at attracting and stimulating *T. gratilla*. Data presented in the current study showed that all treatments tested elicited, to some extent, positive chemoattraction and feed stimulation responses from the urchins. However, additional research needs to be done to evaluate the response of this organism to other types of extracts (*i.e.* mixture of F8 and F9) against *Ulva* in its unpurified state (FU, DU and CE). Findings presented here can serve as a guide to determine which form of *Ulva* to test in formulated diets for this urchin and at what levels. A possible continuation of this research would be to apply the optimal levels of F8 and F9 along with a mixture of F8 and F9 to formulated feed, and test for consumption rate and feed conversion ratio (FCR) of *T. gratilla* against the formulated diet containing dried *Ulva* developed by Cyrus et al. (2013).

CHAPTER 4

Effect of nutrient variation on the lipid and fatty acid composition of *Ulva lacinulata*: An NMR-based lipidomic approach

4.1 Abstract

The effects of varying nutrient concentrations were investigated on the total lipid (TL), lipid classes and fatty acid (FA) contents of aquacultured *Ulva lacinulata*. A culture experiment was carried out for 10 days using six different nutrient concentrations: 0 (control), 25, 50, 75, 100 and 200% NE (NE: nutrient enrichment above ambient seawater concentration; 100% NE = 8.05 nitrogen [N], 3.78 phosphorus [P] and 1.08 potassium [K] g.kg^{-1} *Ulva*) at a stocking density of 0.72 kg.m^{-2} FW *Ulva* under a volume exchange (VE.d^{-1}) rate of 30 VE.d^{-1}. Results showed that nutrient variation had no significant effect on the specific growth rate (SGR) of *Ulva* (SGR ranged from 12.92 ± 0.17 to 13.72 ± 0.25%.d^{-1}). No significant relationship was found between TL contents of *Ulva* and nutrient treatments (TL contents ranged from 3.91 ± 0.29 and 4.61 ± 0.64% DW). *Ulva* grown at low nutrient concentrations had significantly lower neutral lipid (NL) contents (0 and 25% NE; $p< 0.05$) than at higher nutrient concentrations. Glycolipid (GL) contents were significantly lower when *Ulva* was cultured at a high nutrient concentration (200% NE; $p< 0.05$). Nutrient variation had no effect on the phospholipid (PL) contents of this seaweed. A total of 25 FAs consisting mostly of saturated fatty acids (SFA; 56.40 ± 3.23 to 59.95 ± 8.28% total fatty acid [TFA]) were identified from *Ulva* by GC-MS. However, only C18:0 FA (stearic acid) was negatively impacted at high nutrient concentration ($p< 0.05$). Polyunsaturated FA (PUFA) consisted mostly of omega-3 FA (ω-3; 26.72 ± 9.21 to 30.31 ± 2.52% TFA) than ω-6 FA (2.34 ± 0.20 to 3.05 ± 0.62% TFA). ω-6:ω-3 ratios (0.08 ± 0.00 to 0.12 ± 0.06) were in the lower range to what has been reported in the literature for species of this genus. The current study provided an improved understanding of the effects of nutrient variation on the TL, lipid classes and FA profile of local aquacultured *Ulva*. This study also showed that *U. lacinulata* contains lipid and FAs (especially ω-3 PUFAs) that may be used as a suitable source of beneficial lipids and FAs for both aquafeed and human consumption.

4.2 Introduction

Marine macroalgae, commonly known as seaweeds, are considered as valuable sources of bioactive compounds characterized by a wide range of biological activities (Li et al. 2011; Holdt and Kraan, 2011; Gupta and Abu-Ghannam, 2011; Rengasamy et al. 2020). For centuries, these marine plants have been used as food and in traditional medicine in East-Asia, while their use in Western countries has been increasing mainly due to popularization of Asian cuisine and multi-nutritional attributes (Sanjeewa et al. 2018; Lopes et al. 2020). Seaweeds offer a wide selection of phytonutrients and phytochemicals including proteins, poly- and oligosaccharides, polyunsaturated fatty acids (PUFA), vitamins, micro- and macro elements, polyphenols, alkaloids and terpenoids (Dawczynski et al. 2007; Shibata et al. 2008; Güven et al. 2010; Peña-Rodríguez et al. 2011; Vera et al. 2011; Campos et al. 2012; Godlewska et al. 2016; Rengasamy et al. 2020). Recently, macroalgal lipids have gained much interest, not only for their nutritional value, but for the potential use as functional ingredients in food and feed, pharmaceutical, nutraceutical, and cosmeceutical industries (Plouguerné et al. 2014; Arafiles et al. 2014; da Costa et al. 2021; Lopes et al. 2020, 2021).

Lipid content of seaweeds varies within species, season, region and environmental factors such as availability of nutrient, light, temperature and salinity as well as the interactions between these factors (Floreto et al. 1996; Floreto and Teshima, 1998; Xu et al. 1998; Narayan et al. 2005; Gerasimenko et al. 2011; Kumari et al. 2013; Toth et al. 2020). In general, lipid content in seaweeds is relatively low (1 – 5% of dry matter), and they are largely disregarded due to their low amounts, high complexity and structural diversity (Peng et al. 2015; Lopes et al. 2021). Macroalgal lipids consist of non-polar (neutral lipids [NL]) and polar (glycolipids [GL], phospholipids [PL] and betaine lipids) lipids, with fatty acids (FA) making up a large portion of their total lipid contents (up to 50%; Mišurcová et al. 2011; Gosch et al. 2012; Kumari et al. 2013; da Costa et al. 2017). Polar lipids located in photosynthetic membranes are used as markers for cellular recognition, while NLs assist in energy storage functions (Holdt and Kraan, 2011; Kumari et al. 2013; Pérez et al. 2016). GL has been reported to be the main source of lipids in several seaweeds species, followed by NL and PL (Bhaskar et al. 2004; Narayan et al. 2005; Terme et al. 2017; Santos et al. 2019; Wang et al. 2022). Polar lipids have also been reported to be key carriers of PUFA such as omega-3 (ω-3; *e.g.* eicosapentaenoic acid (EPA) – C20:5; stearidonic acid – C18:4, and docosahexaenoic acid (DHA) – C22:6] and omega-6 [ω-6; *e.g.* arachidonic acid (AA) – C20:4] fatty acids (Wielgosz-Collin et al. 2016; Domingues and Calado, 2022).

Like most seaweeds, the lipid content of *Ulva* species is low (0.3 – 4.1% of dry weight; Ortiz et al. 2006; Tabarsa et al. 2012; Kendel et al. 2015; Magdugo et al. 2020). Contrary to other macroalgae, NL has been reported to be the major class of lipids in *Ulva* (Fleurence et al. 1994; Kendel et al. 2015; Cardoso et al. 2017; McCauley et al. 2018; Nunes et al. 2020). However, some studies have shown that polar lipids, namely GL, were the main lipid classes in *Ulva* species (Kostetsky et al. 2004; Santos et al. 2019). Species of this genus have been reported to a source of valuable long-chain PUFAs, such as docosapentaenoic acid (C22:5ω3 DPA), and more uncommon stearidonic acid (C18:4ω3) and hexadecatetraenoic acid (C16:4ω3) PUFAs as well as EPA and small amounts of docosahexaenoic acid (C22:6ω3 DHA; Holdt and Kraan, 2011; McCauley et al. 2016; Cardoso et al. 2017; Monteiro et al. 2022). The addition of *Ulva* at low levels in formulated feeds and/or as feed supplement have been shown to have beneficial effects on FA composition of a few marine organisms. Legarda et al. (2021) reported that the supplementation of IMTA-grown *Ulva* at 10 g.kg^{-1} in the formulated diet of the juvenile finfish *Seriola lalandi* (as *Seriola dorsalis*; Carangiformes) increased the level of DHA in muscular tissue that improved the quality of the fish muscle. Similarly, Sáez et al. (2020) reported that inclusion of 5% (*w/w*) the dry seaweed *U. ohnoi* in the formulated feed of the Senegalese sole (*Solea senegalensis*; Pleuronectiformes) produced desirable levels of DHA and EPA in the fish muscle. Mulvaney et al. (2015) demonstrated that the hybrid abalone *Haliotis rubra* x *Haliotis laevigata* (Lepetellida) fed with *Ulva* had higher levels of important long-chain ω-3 PUFAs in the muscle tissue as well as lower ω6:ω3 ratios. Glycerolipids such as digalactosyldiacylglycerol (DGDG) and 1, 2-diacylglycerly-4'-O-(N,N,N-trimethyl)-homoserine (DGTS; reported as DGTH) isolated from *Ulva* can act as feed stimulants for marine gastropods (Sakata et al. 1985; 1988). Similar to these studies, the compound lipids monogalactosyldiacylglycerol (MGDG), DGDG, diacylglyceryl-N,N,N-trimethyl-homoserine (DGTS), lysophospholipid (LPG), sulfoquinovosylmonoacylglycerol (SQMG) and dialkylglycerophosphate (PA), were identified from the aquacultured *U. lacinulata* as potential feed stimulants for the urchin *Tripneustes gratilla* (Camarodonta; see Chapter 2).

Despite the presence of beneficial lipids and FAs in *Ulva*, their contents are highly susceptible to nutrient changes (Floreto et al. 1993, 1996; Gómez-Pinchetti et al. 1998; Kumari et al. 2014; McCauley et al. 2018; Gao et al. 2018; Toth et al. 2020). It has been reported that *Ulva* grown in nutrient rich water can increase the total lipid content and polar lipids classes (Kumari et al. 2014). *Ulva* cultured under enriched inorganic nitrogen can contain higher levels of PUFA and lower concentrations of saturated and monounsaturated fatty acids (MUFA) compared to the alga grown in a nitrogen depleted medium, where the reverse can occur (Gómez-Pinchetti et al. 1998). McCauley et al. (2018) demonstrated that *Ulva* grown at a high nutrient level can produce biomass with desirable ω-6/ω-3 and C18:2ω6/C18:3ω3 ratios, and beneficial FA such as stearidonic acid, EPA and DPA compared to *Ulva* starved of nutrients. The authors also reported that nutrient-depleted *Ulva* samples had three times higher FA content in the neutral lipid fraction. For ca. 2 decades, the cultivation and utilization of *Ulva* as supplementary feed and for bioremediation to enable partial recirculation on South African abalone farms has been commercially successful (Bolton et al. 2009; Neveux et al. 2018). The quantification of the nutritional content of this seaweed has mainly been focused on its protein content and amino acid profile (Shuuluka et al. 2013). Knowledge on the variability of the lipid and FA content of this aquacultured alga can be beneficial considering its importance as a feed for the abalone *Haliotis midae* (Lepetellida) as well as for future feed formulation and human food application. The current study was thus aimed to investigate and characterize the effects of nutrient variations on the total lipid content, lipid classes and FA profile of local commercially aquacultured *U. lacinulata* (Bachoo et al. 2023). The main objectives were: 1) culture *Ulva* at different nutrient concentrations; 2) extract and quantify total lipid content of *Ulva*; 3) fractionate total lipid content using silica gel (Si-gel) chromatography; 4) quantify and evaluate the main lipid classes; and 5) evaluate and quantify the FA content of *Ulva*.

4.3 Materials and Methods

4.3.1. Chemicals

All solvents used were of chromatography grade (LiChrosolv®). Chloroform (≥ 99.8%; $CHCl_3$), methanol (≥ 99.8%; MeOH), dichloromethane (≥ 99.9%; CH_2Cl_2), acetone (≥ 99.8%; Ace) and hexane (≥ 98.0%; hex) were purchased from Merck® (Darmstadt, Germany). Silica gel 60 (0.040 – 0.063 mm) for column chromatography, phosphate buffered saline tablet, sodium chloride (NaCl) and sodium sulfate (Na_2SO_4) were also obtained from Merck® (Darmstadt, Germany). Granular urea, mono-ammonium phosphate (MAP) and mono-potassium phosphate (MKP) fertilizers were obtained from Omnia Holdings Limited, Brackenfell, South Africa. Boron trifluoride-methanol (BF_3) was purchased from Fisher Scientific LTD (Landsmeer, The Netherlands). Agilent Fiehn GC/MS metabolomics standards kit (400505): FAME/d^{27}-mixture, pyridine, *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA)/1% TMCS, and d^{27}-myristic acids mix was obtained from Agilent Technologies Inc. (Santa Clara, United States). Deuterated solvent: chloroform-d ($CDCl_3$, > 99.8%) and methanol-d4 (CD_3OD, > 99.8%) was purchased from Merck® (Darmstadt, Germany).

4.3.2. Algal material

Fresh *U. lacinulata* (Bachoo et al. 2023) was obtained from Irvine and Johnson (I & J) Cape Abalone aquafarm, Gansbaai, South Africa (34°37'35.1"S 19°17'47.4"E). This alga was thoroughly cleaned of sediments, epiphytes and any visible surface contaminants. The seaweed was transferred to an outdoor 1000 L stocking tank (1.2 × 1.0 × 1.16 m; length × width × depth) with natural light at the Department of Forestry, Fisheries and the Environment (DFFE) Aquaculture Research Facility in Cape Town, South Africa (33°55’13.7” S 18°22’52.1” E), and acclimated for a 7 day period under constant flow of filtered seawater and aeration. The same *Ulva* species is grown commercially at abalone farms on the west, southwest and southeast coasts of South Africa, with different temperature regimes (Bachoo et al. 2023).

4.3.3. Experimental culture system - outdoor tank

The experimental culture system was set up at the DFFE and comprised of a fibreglass raceway (3.15 × 0.98 m; length × width) holding 18 individual 40 L (0.49 × 0.34 × 0.34 m; length × width × depth [inner dimension]) white polyethylene culture tanks. A schematic representation of the culture system is shown in Figure **4.1A**. The white colour was selected to maximize reflection in the tanks as well as to lower heat adsorption. The culture tanks were modified to include an overflow located 0.34 m from the bottom of the container, making the tank total capacity 35 L (Figure **4.1B**). The overflow was covered with netting material (5 mm mesh) to prevent loss of seaweed material during the experiment.

Filtered seawater was gravity-fed from a reservoir through a 20 mm diameter PVC pipe to a holding tank (sump; 500 L) from which water was pumped (Hailea HX 6540) through a 20 mm diameter PVC ring main located 0.60 m above the culture system. Seawater was supplied to the culture tanks from the ring main through 5 mm diameter tubing. The water inflow tubing was equipped with 5 mm plastic flow valves to control the water flow. The initial volume exchange rate (VE.d^{-1}) of the culture tanks was set up at 30 VE.d^{-1} (*i.e.* 1 VE.d^{-1} = 35 L.day^{-1}). Air was supplied by a hi-blow diaphragm air pump (Hailea HAP-120) to the culture system through a 12 mm diameter PVC ring main located 0.60 m above the culture system. Culture tanks were supplied with air by 5 mm diameter silicone air tubing connected to the ring main. The silicone air tubing was attached at the bottom-centre using marine silicone sealant. Holes (± 2 mm diameter; 8 holes in total) were drilled 5 cm apart to make an air curtain in the culture tank. Each tank was tested before the start of the experiment to ensure efficient water circulation within each tank and to eliminate dead circulation spaces.

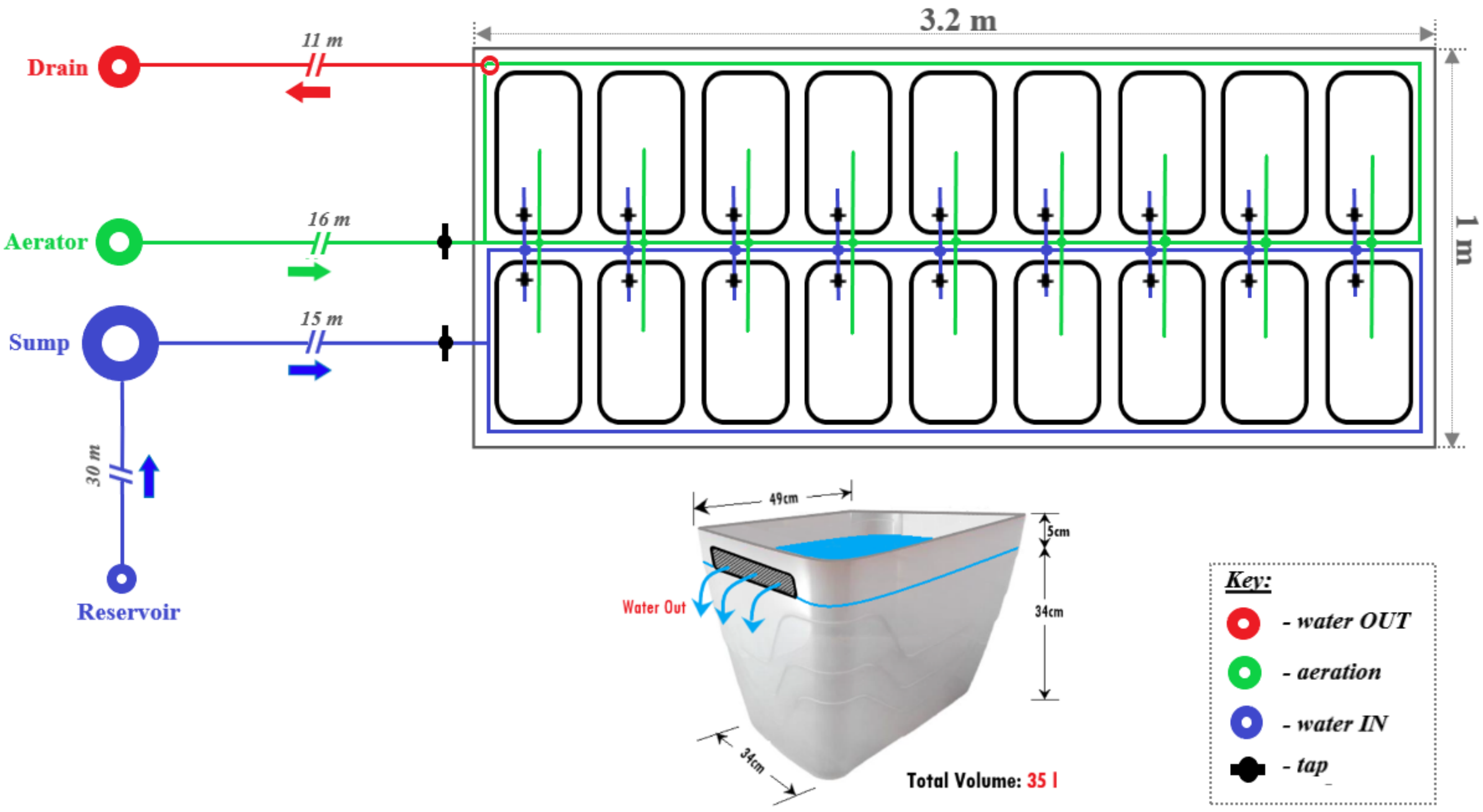


Figure 4.1: (**A**) Schematic representation of the experimental culture system; (**B**) Example of modified culture tank used to grow *Ulva lacinulata* in this study.

4.3.4. Preliminary studies

4.3.4.1. Stocking density

Optimal stocking density of *Ulva* in the system was determined over a period of 5 days. In total, six initial stocking densities were tested at ambient conditions: 50, 75, 100, 125, 150 and 175 g fresh weight (FW; ± 0.1 g) *Ulva*, which equated to 0.36, 0.54, 0.72, 0.90, 1.08 and 1.26 kg.m^{-2} FW *Ulva*. Each treatment was carried out in triplicate (*n*=3) under constant supply of filtered seawater at set at 30 volume exchange rate (VE.d^{-1}). After 5 days, the biomass yield for each treatment was recorded, and the specific growth rate (SGR; %.d^{-1}) was calculated [Equation **4.1**; Msuya and Neori (2008)].

$$SGR = 100 \times \frac{\left[ln\left(\frac{W_t}{W_o}\right)\right]}{t} \qquad \text{[Equation 4.1]}$$

Where W_o is the initial fresh biomass (g) and W_t is the final fresh biomass (g) after t is the time in culture days. The results of the short-term stocking density experiment are shown in Figure **4.2**.

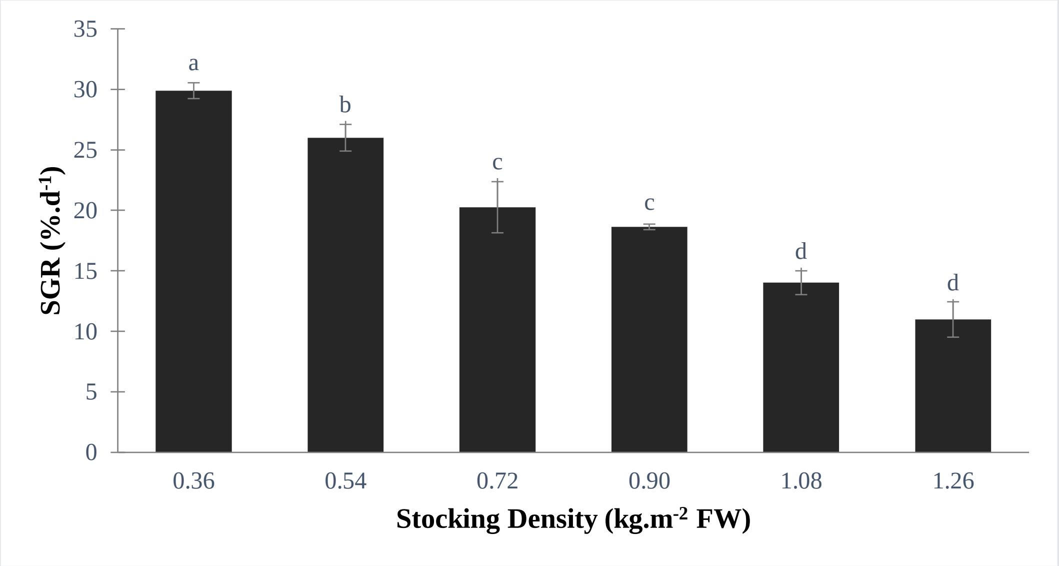


Figure 4.2: Specific growth rate (SGR; %.d^{-1}) of *Ulva lacinulata* cultured in filtered seawater at a volume exchange rate of 30 VE.d^{-1} for stocking density experiment. Error bars indicate the standard deviation of the SGR. Bars with different superscript letters (a, b, c and d) indicate significant difference (ANOVA; $p < 0.001$) between SGR treatments.

Results from this experiment showed a gradual decrease in SGR with an increase in stocking density (Figure **4.2**). In the literature, the range of stocking densities used to culture *Ulva* have been reported to vary between 0.5 and 6.0 kg.m^{-2} FW (Cohen and Neori, 1991; Neori and

Shpigel, 2000; Robertson-Andersson, 2003; Msuya and Neori, 2008; Al-Hafedh et al. 2015; Shpigel et al. 2019). However, the stocking densities used in these studies were culture system and species specific.

Stocking density of a culture directly regulates the degree of competition of individual thalli for light, nutrients and dissolved gases (Nagler et al. 2003; Pereira et al. 2006; Kim et al. 2013). Increased biomass can negatively impact the light required for maximum photosynthetic capacity (Copertino et al. 2009; Richards et al. 2011). The morphological nature of *Ulva* thalli can sometimes lead to mat formation where layers of *Ulva* thalli can shade thalli below them (Malta et al. 2003). A lower initial density may be preferred to optimize growth rate, while a higher stocking density may be chosen if the aims are either to remove nutrient or to yield biomass per area (Neori et al. 1991; Msuya, 2007; Al-Hafedh et al. 2015). Therefore, based on these findings, the initial stocking density of 0.72 kg.m^{-2} FW was chosen for the nutrient enrichment experiment, since the growth observed over the experimental period did not overload the system, leaving scope for growth when nutrient was added.

4.3.4.2. Volume exchange rate (VE.d^{-1})

The volume exchange rate (VE.d^{-1}) of the culture system was determined over a period of 5 days. In total, six VE.d^{-1} were tested: 5, 10, 15, 20, 25 and 30 (*i.e.* 1 VE.d^{-1} = 35 L.day^{-1}). Each treatment was run in triplicate (*n*=3) under constant supply of filtered seawater at ambient condition with an initial stocking density of 0.72 kg.m^{-2} FW *Ulva*. After 5 days, the *Ulva* biomass yield for each treatment was recorded, and the SGR (%.d^{-1}) was calculated. The results of the VE.d^{-1} experiment are shown in Figure **4.3.**

Results from this experiment showed a gradual increase in the SGR of *Ulva* with an increase in volume exchange rate (Figure **4.3**). It is suggested that the production of seaweed is higher under a continuous flow of nutrient-rich water (Vandermeulen and Gordin, 1990; Demetropoulos and Langdon, 2004; Mata et al. 2010; Cole et al. 2014). The renewal of water can increase the uptake of nutrient and facilitate gas exchange by reducing the diffusion of boundary layer (Hurd, 2000; 2017; Roleda and Hurd, 2019). High water exchange rate may assist with dead algal cells removal as well as reduce bacteria and epiphytes (Cole et al. 2014). A high water volume exchange rate may also increase the availability of dissolved inorganic carbon to a system that can be beneficial for biomass production (Cole et al. 2014). Robertson-

Andersson (2003) conducted an experiment on a commercial farm in South Africa with the same *Ulva* species used in this study, and found that 12 VE.d^{-1} was sufficient for growth of the *Ulva* while a 20 VE.d^{-1} resulted in the best growth rate of the seaweed. This author also reported that *Ulva* cultured 4 VE.d^{-1} resulted in poor performance since the low water exchange rate was insufficient sustain optimal temperature and pH for growth. Results from the current experiment showed that 30 VE.d^{-1} had the highest SGR when stocked with 0.72 kg.m^{-2} FW. Based on these findings, the 30 VE.d^{-1} was chosen for the nutrient enrichment experiment and to enable comparisons with similar small-scale studies.

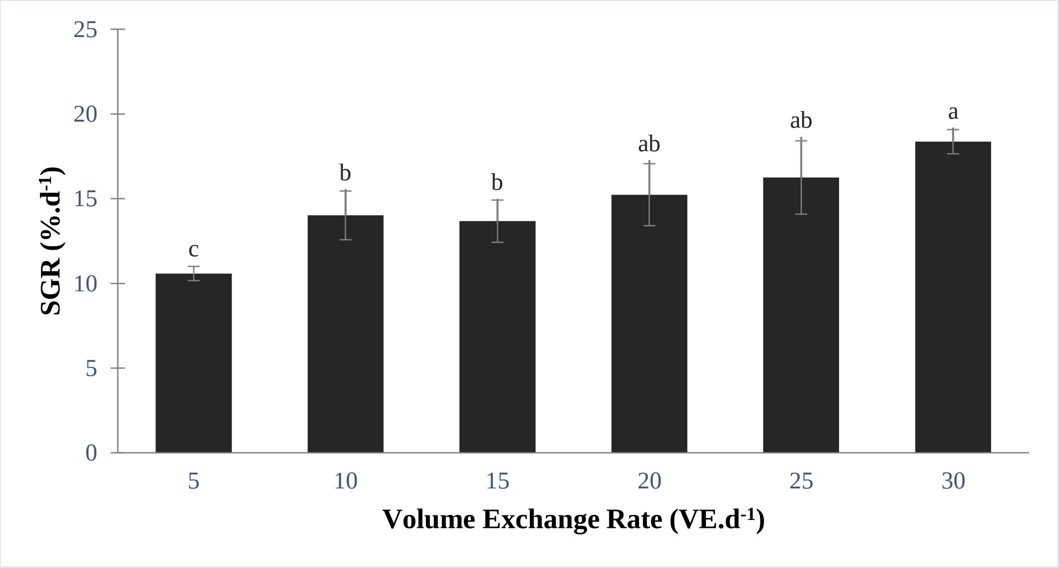


Figure 4.3: Specific growth rate (SGR; %.d^{-1}) of *Ulva lacinulata* cultured in filtered seawater for the volume exchange rate (VE.d^{-1}) experiment. Initial stocking density was 0.72 kg.m^{-2} FW *Ulva*. Error bars indicate the standard deviation of the SGR. Bars with different superscript letters (a, b and c) indicate a significant difference (ANOVA; $p< 0.001$) between SGR treatments.

4.3.5. Nutrient enrichment (NE) experiment

The culture experiment was carried out for 10 days. Six different nutrient additions were tested: 0 (control), 25, 50, 75, 100 and 200% NE (NE: nutrient enrichment; 100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva*) in the form of UREA, MAP and MKP at a ratio of 7:3:1. The 100% fertilization regime used in this experiment was equivalent to the actual fertilization regime use to grow *Ulva* at Wild Coast Abalone farm, Haga Haga, Eastern Cape, South Africa (*pers. comm*. Daphne Taylor). Each treatment was performed in triplicate (n=3) with an initial stocking density of 0.72 kg.m^{-2} FW *Ulva* at 30 VE.d^{-1}. Pulse fertilization was provided at start (D=0) and day 5 (D=5) of the experiment (Note: pulse fertilization in the current experiment was not according to farm practice). Water supplies to the culture tanks were turned off for 6 hrs when fertilization was provided, while air supply was maintained. After 10 days, the experiment was stopped and the biomass yield for each treatment was recorded. The SGR (%.d^{-1}) was calculated, and the algal material was processed for lipid extraction. Water nutrient data and environmental parameters are available in Supplementary Information **4** (Section **S4.1** and **S4.2**).

4.3.6. Moisture content

The moisture content (%; MC) of *Ulva* was determined by drying 2.00 g FW (± 0.01 g; *n*=5) of each *Ulva* sample in an oven (Labotec EcoTherm, South Africa) at 60 °C until a constant weight was obtained. The water content was determined gravimetrically according to [Equation **4.2**; Kendel et al. (2015)].

$$\% MC = \left[\frac{m_i - m_{od}}{m_i}\right] \times 100 \qquad \text{[Equation } \mathbf{4.2}\text{]}$$

where m_i = initial mass of wet *Ulva* and m_{od} = oven dry mass of *Ulva*.

4.3.7. Lipid extraction and fractionation

Total lipids (TL) were extracted using a modified Bligh and Dyer (1959) method according to Kumari et al. (2011) using $CHCl_3$-MeOH-phosphate buffer (pH-7.3). Finely chopped fresh *Ulva* sample (30 ± 0.01 g) was mixed with 95 mL of $CHCl_3$-MeOH-50 mM phosphate buffer (1:2:0.8; *v/v/v*) in a 250 mL Erlenmeyer flask and sealed with foil. The mixture was incubated on a platform shaker (Labcon FSIM SPO16, South Africa) for 45 min at room temperature. The organic phase was collected through filtration using a Büchner funnel and the biomass residue was re-extracted twice using 95 mL of $CHCl_3$-MeOH-50 mM phosphate buffer (1:1:0.8). The total collected organic phases were pooled and washed with de-ionized water in a 500 mL separatory funnel. The final organic (lower) phase was recovered and dried *in vacuo* (Büchi® Rotavapor R-210, Switzerland). The TL content was determined as dry weight percentage (%) using [Equation **4.3**; Kendel et al. (2015)].

$$\% TL = \frac{Weight\ of\ TL}{(Weight\ of\ dry\ mass + Weight\ of\ TL)} \times 100 \qquad \text{[Equation } \mathbf{4.3}\text{]}$$

Lipid extracts were stored at -20 ºC before analysis by NMR and GC-MS. One part of TL (0.05 ± 0.001 g) was fractionated into neutral lipid (NL), glycolipid (GL) and phospholipid (PL). Lipid fractions were obtained by elution on a silica gel column (2 ± 0.01 g) with 10 mL CH_2Cl_2 (NL), 10 mL Ace (GL) and 10 mL MeOH (PL). Fractions were evaporated to dryness under a stream of nitrogen gas and the amount was determined as percentage of 1 g of TL.

4.3.8. Sample preparation and NMR analyses

Lipid samples (≈10 mg) were suspended in 700 µL deuterated solvent ($CDCl_3$ for TL and NL; 1:1, $CDCl_3$:CD_3OD for GL and PL) and transferred to NMR tubes (5 mm diameter; Norell$^{®}$ Standard Series^{TM}). ^1H NMR analyses were performed at the Department of Chemistry, the University of the Western Cape, Bellville, South Africa using a Bruker 400 MHz Avance III HD (Bruker, Rheinstetten, Germany) equipped with a 5 mm BBFO probe. For each sample, 16 scans were recorded with following parameters: 30° pulse (zg 30 sequence), pulse power of 25.0 W, 64 000s data points, spectra width of 8012.82 Hz, acquisition time of 4.09 secs, fixed receiver gain of 62.96, relaxation delay of 1.00 sec, dummy scans 2, and temperature of 24.85 °C. TopSpin® (ver. 3.6.2, Bruker TopSpin$^{®}$ Inc) software was used for NMR data acquisition with a line broadening of 0.30 Hz.

MestReNova (ver. 6.0.2 Mestrelab Research) was used to process ^1H NMR data. Spectra were baseline (Whittaker Smoother) and manual phase corrected, and the chemical shifts of the data were reference to δ_H 7.260 ($CDCl_3$; for TL and NL) and δ_H 3.310 (CD_3OD; for GL and PL). The regions corresponding to solvent and interference signals were removed: 1) TL: δ_H 1.080 – 1.095, 3.460 – 3.495 (MeOH), δ_H 7.230 – 7.295 (CH_2Cl_2 and $CDCl_3$); 2) NL: δ_H 5.288 – 5.308 (CH_2Cl_2), δ_H 7.230 – 7.295 ($CDCl_3$); 3) GL: δ_H 2.140 – 2.165 (Ace), δ_H 3.305 – 3.315, 4.650 – 4.900 (CD_3OD), δ_H 7.850 – 7.920 ($CDCl_3$); and 4) PL: δ_H 3.305 – 3.315, 4.650 – 4.900 (MeOH and CD_3OD), δ_H 7.850 – 7.920 ($CDCl_3$).

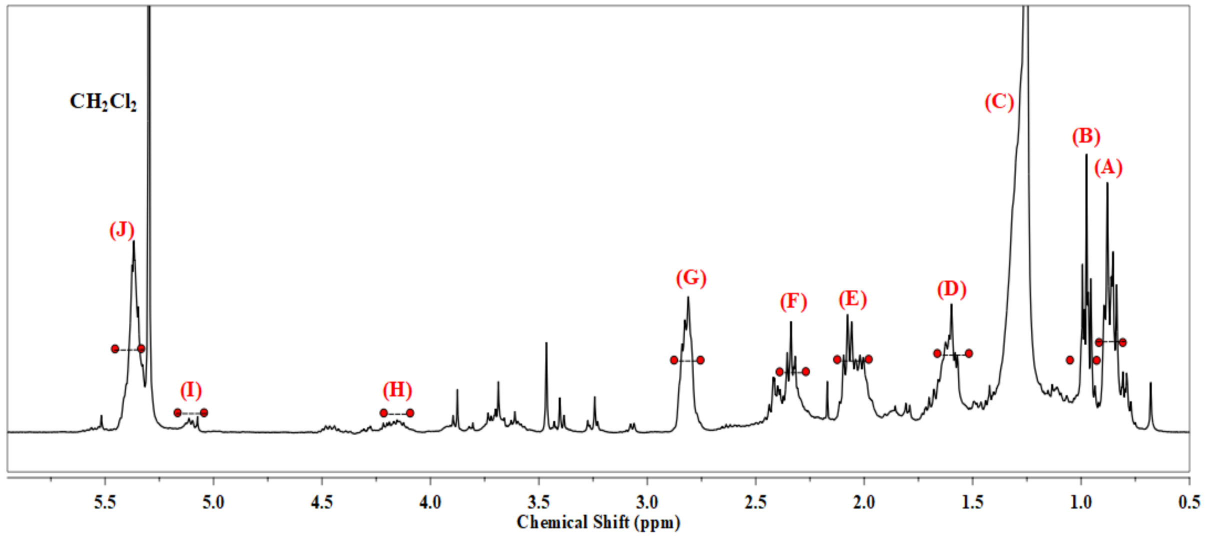


Figure 4.4: Proton nuclear magnetic resonance [^1H NMR; $CDCl_3$, 400 MHz] spectrum of total lipid (TL) sample from *Ulva lacinulata* cultured in filtered seawater at 0% NE^A (0% nutrient enrichment [NE] = control; ^{A}= replicate 1).

Spectral data were divided into bins with each integral having equal width of 0.04 ppm. The binned data were exported and rearranged in Microsoft® Excel® 365 and saved as '.csv' (comma delimited) files. Assignment of the chemical shift signals (ppm; Figure **4.4**) from the total lipid content (TL) of *U. lacinulata* are shown in Table **4.1**.

Table 4.1: Assignment of proton nuclear magnetic resonance (^1H NMR) chemical shift signals of total lipid (TL) in *Ulva lacinulata* (FA= Fatty Acids; PUFA= Polyunsaturated Fatty Acids; TAG= Triacylglycerols).

Spectral region	δ_H (ppm)	Lipid metabolites assigned
A	0.80 – 0.91	All FA ($–CH_2–C\underline{\mathbf{H}}_3$) except ω-3 FA $^{1\,2\,3}$
B	0.92 – 1.02	PUFA ω-3 ($–CH=CH–CH_2–C\underline{\mathbf{H}}_3$) $^{1\,2\,3}$
C	1.21 – 1.38	Acyl chains for all FA $–(C\underline{\mathbf{H}}_2)_n$ $^{1\,2\,3}$
D	1.54 – 1.66	β-methylene ($–OCO−CH_2−C\underline{\mathbf{H}}_2–$) $^{1\,2\,3}$
E	1.96 – 2.13	Mono-allylic methylene ($–C\underline{\mathbf{H}}_2−CH=CH–$) $^{1\,2\,3}$
F	2.26 – 2.38	α-methylene ($–OCO−C\underline{\mathbf{H}}_2–$) $^{1\,2\,3}$
G	2.73 – 2.88	Di-allylic methylene ($–CH=CH–(C\underline{\mathbf{H}}_2–CH=CH)_n–$) $^{1\,2\,3}$
H	4.10 – 4.25	TAG *sn*–1 ($–C1\underline{\mathbf{H}}_2$) and *sn*–3 ($–C3\underline{\mathbf{H}}_2$) $^{1\,2\,3}$
I	5.05 – 5.17	Unsaturated ω-1 acyl groups ($–CH=C\underline{\mathbf{H}}_2$) ^{1}
J	5.33 – 5.45	Fatty akyl chain ($–C\underline{\mathbf{H}}=C\underline{\mathbf{H}}–$; unsaturated FA) $^{1\,2\,3}$

[1]Vidal et al (2012); [2]Del Coco et al. (2018); [3]Siudem et al. (2022)

The relative ω-3 fatty acid content (%) for TL, NL, GL and PL was estimated by integrating the ^{1}H NMR peaks due to the terminal methyl protons at δ_H 0.98 (**B**) to ω-3 fatty acid and δ_H 0.87 (**A**) due to other fatty acids [Equation **4.4**; Igarashi et al. 2002].

$$\%\,\omega - 3 = 100 \times \frac{I_{(B)}}{I_{(B)} + I_{(A)}} \qquad \text{[Equation 4.4]}$$

where *I* is the integral value for the peak at the given chemical shift.

All unsaturated fatty acids are characterized by two allylic methylene protons which are observed at δ_H 2.05 (**E;** $-C\underline{\mathbf{H}}_2-CH{=}CH-$). Therefore, the total unsaturated fatty acids (UFA = monounsaturated [MUFA] + PUFA) can be estimated by the integration value at δ_H 2.05 relative to δ_H 2.34 (**F**; $-OCO-C\underline{\mathbf{H}}_2-$) [Equation **4.5;** Stabili et al. 2012].

$$\%\,UFA = 100 \times \frac{(I_{(E)}/4)}{(I_{(F)}/2)} \qquad \text{[Equation 4.5]}$$

Polyunsaturated fatty acids (PUFA) are methylene interrupted dienes and trienes, and the signal due to the bis-allylic methylene at δ_H 2.75 (**G**) can be used the estimate the amount of PUFAs [Equation **4.6**; Siudem et al. 2022].

$$\%\,PUFA = 100 \times \frac{3I_{(G)} - 2I_{(B)}}{3I_{(F)}} \qquad \text{[Equation 4.6]}$$

Saturated fatty acids (SFAs) can be estimated by subtracting the percentage of UFAs according to Siudem et al. (2022) [Equation **4.7**].

$$\%\,SFA = 100 - \%\,UFA \qquad \text{[Equation 4.7]}$$

Double bond equivalent (DBE) representing the degree of unsaturation in NL, GL and PL from ^{1}H NMR spectra can be estimated by integrating the signal at δ_H 2.34 (**F**) represents the α-methylene protons ($OCO-C\underline{H}_2$) of FA chains, to the double bonds of unsaturated fatty acyl chain at δ_H 5.37 (CH=CH; **J**) [Equation **4.8;** *pers. comm.* Professor Denzil R Beukes].

$$DBE = \frac{I_{(J)}}{I_{(F)}} \qquad \text{[Equation 4.8]}$$

Examples for % ω-3, UFA, PUFA, MUFA, SFA and DBE can be found in Supplementary Information **4** (Section **S4.7**).

4.3.9. FAME and GC-MS Analysis

Fatty acids (FA) were converted to their fatty acid methyl esters (FAMEs) by transmethylation of lipid samples following a modified protocol described by Lisec et al. (2006). Briefly, 10 µL of internal standard (2 mg.mL^{-1} – d^{27}-myristic acid in hexane) was added to 2 mg of dried lipid extract, followed by 100 µL of 10% *w/w* boron trifluoride (BF_3) in MeOH solution. The mixture was incubated at 65 ºC for 10 mins. After cooling, 500 µL hex was added and the mixture was vortexed for 30 secs. The mixture was then washed three times by adding 500 µL of a saturated NaCl solution, vortexed for another 30 secs and then centrifuged for 10 secs before removing the aqueous layer. The organic layer was dried by transferring 400 µL to a new tube and adding anhydrous sodium sulfate (Na_2SO_4). After centrifuging with a 30 secs pulse to pellet residual particulates, 300 µL of the supernatant was transferred to a 2 mL amber screw top sample vial. Derivatisation of FAME standard was carried out by transferring an aliquot of 10 µL FAME standard to a tube containing 10 µL methoxyamine HCl in pyridine (20 mg.mL^{-1}), followed by 80 µL of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide. The mixture was incubated for 30 mins at 37 ºC before allowing to cool and transferring to a 2 mL amber screw top sample vial for GC-MS analysis.

The GC-MS analyses of FAME samples were performed at the Molecular and Cell Biology Department, University Cape Town, Rondebosch, South Africa using an Agilent Model 7890A Gas Chromatograph fitted with a 7693 Autosampler interfaced with a 7000A Triple Quadrupole Mass Spectrometer (Agilent Technologies, Santa Clara, California, USA) equipped with a DB-5ms silica capillary column (30 m × 0.25 mm × 0.25 µm; length × inner diameter × film thickness). Helium at a column flow rate of 1 mL.min^{-1} was used as a carrier gas with the pre-column pressure of 0.32879 PSI. The column temperature regime was 50 ºC for 2 min, followed by 30 ºC/min increase to 180 ºC, 5 ºC/min to 260 ºC and 15 ºC/min to 310 ºC. The injection volume and temperature were 1 µL and 240 ºC, and the split ratio was 19:1. The mass spectrometer was operated in electron compact mode with an electron energy of 70 eV and the mass spectrometer ion source was maintained at 280 °C. The data was acquired using the Agilent MassHunter GC/MS Acquisition (ver. B.07.06.2704; Agilent Technologies, Santa Clara, California, USA) software. FAME peaks were identified by comparison of their retention times with authentic standards in NIST 14.L & Fiehn.L digital library, and by GC-MS post run analysis and quantified by area normalization. Agilent MassHunter Workstation Quantitative Analysis (ver. B.09.00; Agilent Technologies, Santa Clara, California, USA) and OpenChrom® Lablicate Edition (ver. 1.4.0.202102180818; Hamburg, Germany) were used for FAME data processing.

4.3.10. Data processing and statistical analyses

MetaboAnalyst 5.0 online platform (https://www.metaboanalyst.ca; Pang et al. 2021) was used to perform one-way analysis of variance (ANOVA) and principal component analysis (PCA). For TL, NL, GL and PL spectral bin data, the following parameters were used to perform ANOVA: data type= spectral bins, format of data= unpaired sample in columns, data filtering= inter-quantile range (IQR), sample normalization= none, data transformation= log transformation (base 10) and data scaling= Pareto scaling (mean-centred and divided by square root of standard deviation of each variable). For FAME data, the following parameters were used to perform PCA: data type= peak intensities, format of data= unpaired sample in columns, data filtering= inter-quantile range (IQR), sample normalization= normalization by reference feature (*i.e.* d^{27}-myristic acid), data transformation= log transformation (base 10) and data scaling= Pareto scaling (mean-centred and divided by square root of standard deviation of each variable). SGR, TL contents, lipid classes, UFA (%), PUFA (%), MUFA (%), SFA (%), ω-3 (%), DBE and FAME data were individually tested for normality and homogeneity using one-way ANOVA and post-hoc Tukey test at 95% significance level using JASP (ver. 0.15, JASP Team 2021).

4.4 Results

4.4.1. Specific Growth Rate at different nutrient concentrations

The specific growth rate (SGR; %.d^{-1}) of *U. lacinulata* grown at the six different nutrient concentrations is shown in Figure **4.5**. Nutrient variation had no significant effect on the SGR of *Ulva* (ANOVA, F= 1.141, *p*= 0.391). SGR varied from 12.92 ± 0.17 to 13.72 ± 0.25%.d^{-1}.

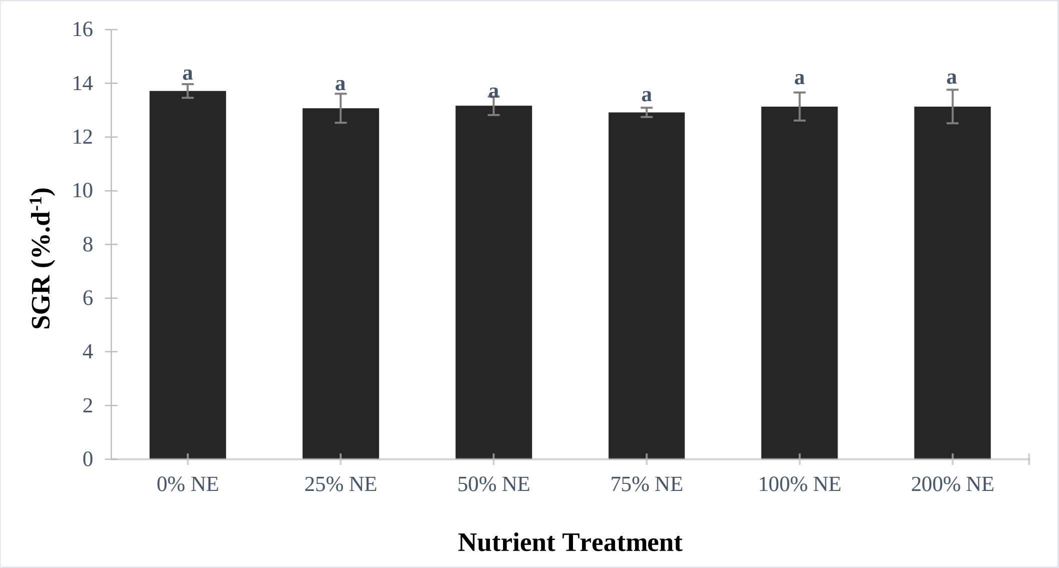


Figure 4.5: Specific growth rate (SGR; %.d^{-1}) of *Ulva lacinulata* cultured in filtered seawater for the nutrient experiment: 0 [control], 25, 50, 75, 100 and 200% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. Error bars represent the standard deviation of SGR. Bars with different superscript letter (a) within treatments indicate significant difference (ANOVA; *p*< 0.05) between SGR of *U. lacinulata*.

4.4.2. Total lipid content (TL)

The total lipid contents (TL; % DW) of *U. lacinulata* cultured at the six different nutrient concentrations is shown in Figure **4.6**. Nutrient variation had no significant effect on the TL content of *Ulva* (ANOVA, F= 2.244, *p*= 0.117). TL content of *Ulva* from the experiment varied from 3.91 ± 0.29 to 4.61 ± 0.64% DW, where 75% NE treatment had the highest TL content, while 0% NE treatment (control) had the lowest TL content.

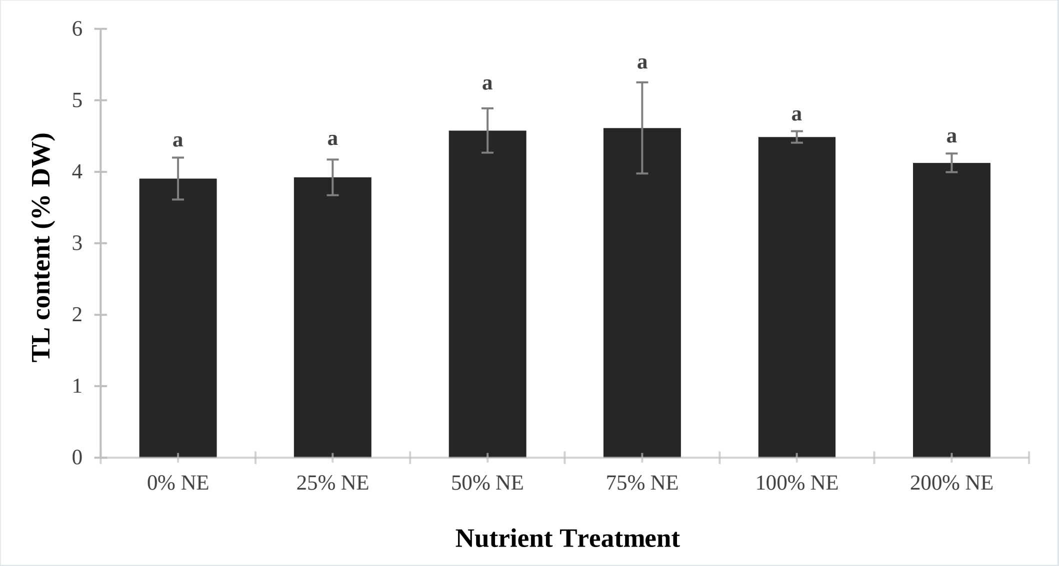


Figure 4.6: Total lipid contents (TL; % DW) of *Ulva lacinulata* cultured in filtered seawater for the nutrient experiment: 0 [control], 25, 50, 75, 100 and 200% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) $g.kg^{-1}$ *Ulva* at a ratio of 7:3:1]. Error bars represent the standard deviation of TL contents. Bars with different superscript letter (a) within treatments indicate significant difference (ANOVA; $p < 0.05$) between TL contents of *U. lacinulata*.

4.4.3. Lipid classes

The contents of the lipid classes (% TL) of *U. lacinulata* cultured at the six different nutrient concentrations are shown in Figure **4.7**. Nutrient variation had a significant effect on the neutral lipids (NL) of *Ulva* (ANOVA, F= 4.111, *p*= 0.021). NL contents of *Ulva* cultured at 0 and 25% NE were significantly lower than NL contents of seaweed from the other nutrient treatments. Nutrient variation also had a significant effect on the glycolipids (GL) of *Ulva* (ANOVA, F= 3.470, *p*= 0.036). The GL content of *Ulva* grown at 200% NE was significantly lower compared to the GL content of seaweed grown at the other nutrient treatments. No significant difference was found between the phospholipids (PL) contents of *Ulva* and the six different nutrient treatments (ANOVA, F= 1.552, *p*= 0.246). NL contents of *Ulva* ranged from 27.64 ± 6.82 to 40.30 ± 2.78% TL, GL contents from 46.63 ± 2.94 to 56.45 ± 3.64% TL while PL contents ranged 13.07 ± 0.16 to 16.25 ± 2.20% TL.

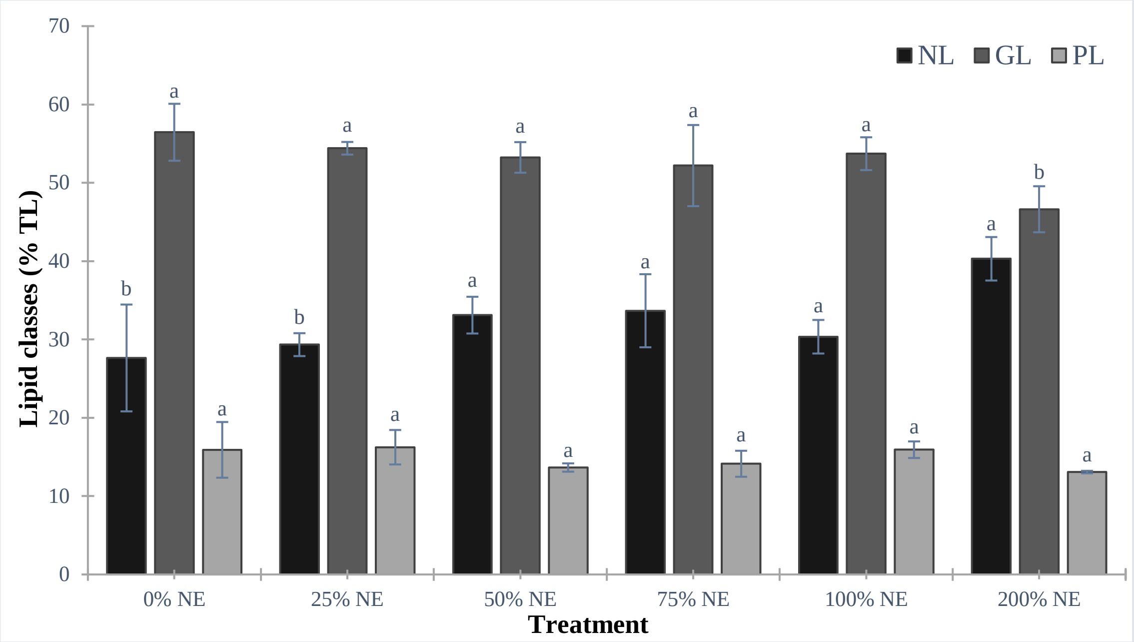


Figure 4.7: Lipid classes contents (% TL) of *Ulva lacinulata* cultured in filtered seawater for the six nutrient treatments: 0 [control], 25, 50, 75, 100 and 200% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. NL = neutral lipids (black bars), GL = glycolipids (dark grey bars) and PL = phospholipids (light grey bars). Error bars represent the standard deviation. Bars with different superscript letters (a, b) within treatments indicate significant difference (ANOVA; $p < 0.05$) between the lipid classes of *U. lacinulata*.

4.4.4. NMR - based lipid analysis

4.4.4.1. Total lipid (TL)

Total lipid (TL) content of *U. lacinulata* from this experiment was characterized by lipid metabolites such as sterols, triacylglycerols (TAG), fatty acids (FA) and phospholipids (PL). The ^{1}H NMR spectra (δ_H 0.50 – 5.60; *e.g.* Figure **4.8**) showed the presence of angular methyl proton ($C\underline{H}_3$) resonances characteristic of the CH_3-18 of a sterol (δ_H 0.65 – 0.70 [**1**]), as well as terminal FA methyl groups (δ_H 0.82 – 0.91 [**2**]) of saturated, monounsaturated, di-unsaturated, and polyunsaturated FAs (ω-3; δ_H 0.92 – 1.03 [**3**]). Resonance signals between δ_H 1.16 – 1.39 [**4**] describe non-allylic methylene protons ($C\underline{H}_2$) occurring in all FAs, while resonances between δ_H 1.92 – 2.14 [**6**] and δ_H 2.73 – 2.90 [**8**] correspond to mono-allylic ($C\underline{H}_2{-}CH{=}CH$) and di-allylic methylene protons ($CH{=}CH{-}(C\underline{H}_2{-}CH{=}CH)_n$) in unsaturated FAs (see Supplementary Information **4** Section **S4.3** for TL spectral regions assignment).

Proton signals between δ_H 1.52 – 1.72 ($OCO{-}CH_2{-}C\underline{H}_2$ [**5**]) and δ_H 2.25 – 2.45 ($OCO{-}C\underline{H}_2$ [**7**]) correspond to methylene in the carbonyl of FA chains, while proton resonances between δ_H 3.05 – 3.10 [**9**] and δ_H 3.21 – 3.29 [**10**] described regions of phospholipid resonances, such as phosphatidylethanolamine and phosphatidylcholine. Multiplet proton signals between δ_H 4.09 – 4.31 [**11**] and δ_H 5.06 – 5.16 [**12**] correspond to *sn*-1, *sn*-3 and *sn*-2 protons of glycerol moiety in TAG, while resonances between δ_H 5.31 – 5.46 [**13**] describe protons of double bonds (C$\underline{H}$=C$\underline{H}$) in the unsaturated fatty acyl chain. Nutrient variation had no main significant effect on the TL metabolites of *Ulva* (ANOVA, $p > 0.05$). However, few minor differences in intensity were present in the ^{1}H NMR spectra in spectral regions [**1**], [**2**], [**3**], [**6**] and [**11**] (see Figure **4.8** and Supplementary Information **4** Section **S4.3** for TL ^{1}H NMR spectra).

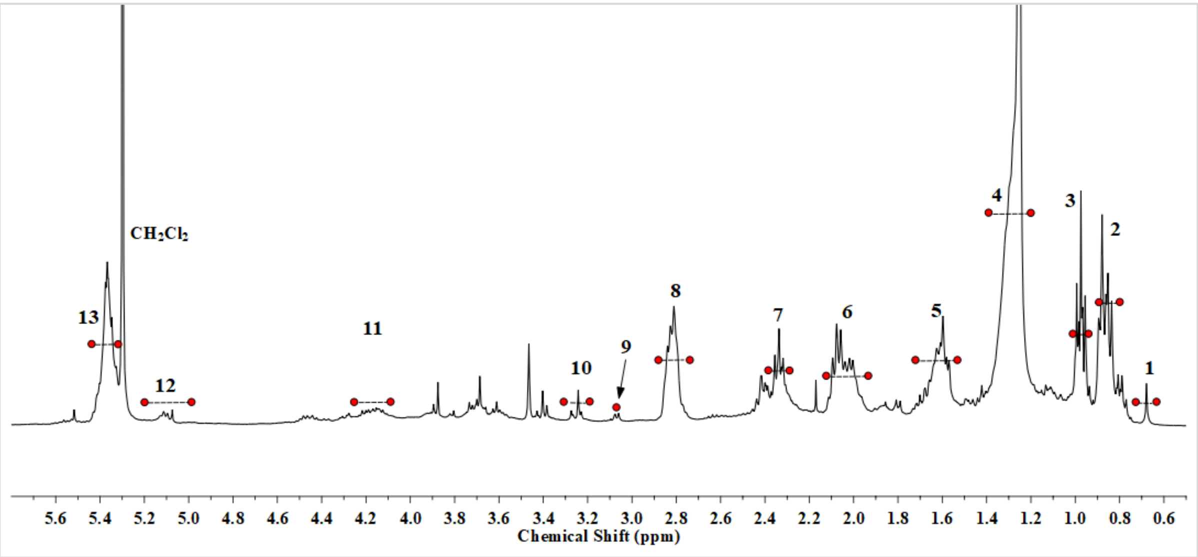


Figure 4.8: Proton nuclear magnetic resonance [^{1}H NMR; $CDCl_3$, 400 MHz] spectrum of total lipid (TL) samples from *Ulva lacinulata* cultured in filtered seawater at 0% NE^{A} (0% nutrient enrichment [NE] = control; ^{A}= replicate 1).

Nutrient variation had no significant effect on UFA (%; ANOVA, F= 0.178, p= 0.966), SFA (%; ANOVA, F= 0.178, p= 0.966), PUFA (%; ANOVA, F= 0.712, p= 0.626), MUFA (%; ANOVA, F= 1.395, p= 0.294) and ω-3 (%; ANOVA, F= 0.145, p= 0.978) fatty acid content of *U. lacinulata* cultured in this experiment (Table **4.2**). TL consisted mostly of UFA (79.92 ± 2.90 – 84.83 ± 1.28%), with MUFA content ranging from 46.92 ± 18.87 to 75.72 ± 19.70%. ω-3 constituted 39.98 ± 3.21 – 40.95 ± 0.97% of the PUFA contents.

Table 4.2: The total unsaturated (% UFA), saturated (% SFA), polyunsaturated (% PUFA), monounsaturated (% MUFA) and ω-3 (%) fatty acids of total lipid (TL) from *Ulva lacinulata* cultured at the six nutrient treatments: 0 [control], 25, 50, 75, 100 and 200% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1] calculated from proton nuclear magnetic resonance (^{1}H NMR) data. Values are represented as mean ± standard deviation (SD; *n*=3). Values with different superscript letter (^{a}) within treatments indicate significant difference (ANOVA; $p < 0.05$) between UFA (%), SFA (%), PUFA (%), MUFA (%) or ω-3 (%) of TL samples.

	0% NE	**25% NE**	**50% NE**	**75% NE**	**100% NE**	**200% NE**
% UFA	83.42 ± 7.27^a	84.00 ± 2.05^a	82.17 ± 15.13^a	79.92 ± 2.90^a	84.00 ± 4.77^a	84.83 ± 1.28^a
% SFA	16.58 ± 7.27^a	16.00 ± 2.05^a	17.83 ± 15.13^a	20.08 ± 2.90^a	16.00 ± 4.77^a	15.17 ± 1.28^a
% PUFA	10.39 ± 28.11^a	8.28 ± 21.55^a	19.94 ± 13.29^a	33.00 ± 18.65^a	13.28 ± 9.08^a	18.78 ± 12.88^a
% MUFA	73.03 ± 21.35^a	75.72 ± 19.70^a	62.22 ± 6.19^a	46.92 ± 18.87^a	70.72 ± 5.69^a	66.06 ± 11.60^a
% ω-3	40.48 ± 1.96^a	40.34 ± 0.89^a	39.98 ± 3.21^a	40.40 ± 0.71^a	40.60 ± 2.49^a	40.95 ± 0.97^a

4.4.4.2. Neutral lipid (NL)

NL content of *Ulva* from this experiment was characterized by the presence of di-allylic methylene protons ($C\underline{H}_2$) of FA (δ_H 2.75 – 2.88 [**14**]), methyl protons ($C\underline{H}_3$) relative to C-3 (δ_H 3.50 – 3.57 [**15**]), protons associated with the glycerol moiety in TAG (δ_H 4.05 – 4.33 [**16**]), and protons of double bonds in the fatty acyl chain (δ_H 5.32 – 5.40 [**17**]; Figure **4.9**; see Supplementary Information **4** Section **S4.4** for NL spectral regions assignment). Nutrient variation had no significant effect on the NL metabolite contents of *Ulva* (ANOVA, $p > 0.05$). However, minor variation in the ^{1}H NMR spectra of the NL samples was observed in the spectral region [**16**], with proton resonance from the glycerol moiety in TAG shown to be more prominent in the 50% NE treatment compared to samples of the other nutrient treatments (see Figure **4.9** and Supplementary Information **4** Section **S4.4** for NL ^{1}H NMR spectra).

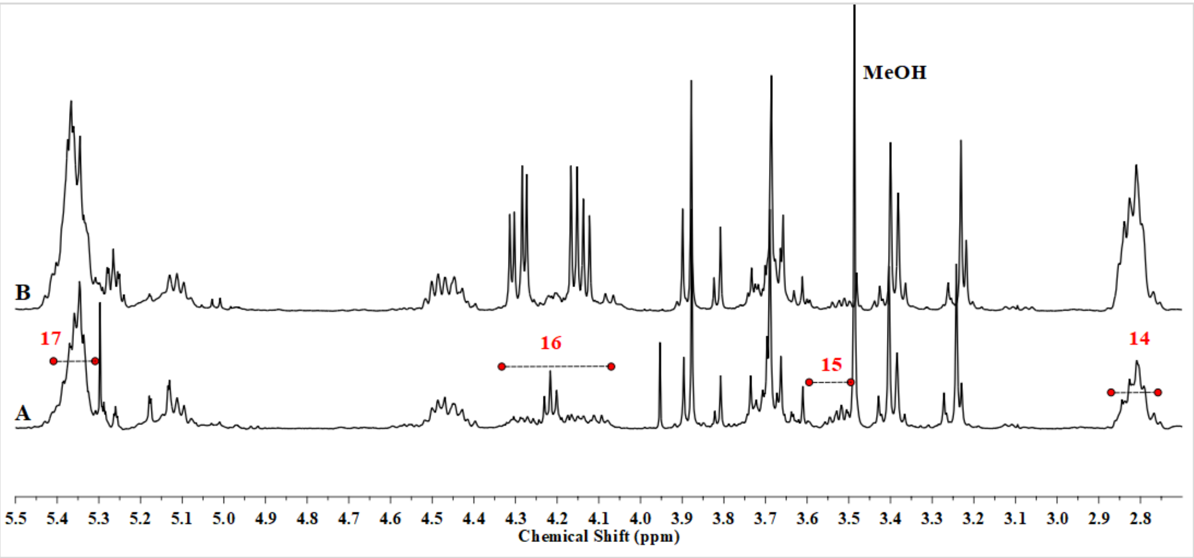


Figure 4.9: Proton nuclear magnetic resonance [^{1}H NMR; $CDCl_3$, 400 MHz] spectra of neutral lipid (NL) samples from *Ulva lacinulata* cultured at 0% NE^C (A) and 50% NE^C (B). 0% nutrient enrichment [NE] = control. 50% NE = 0.5 × (100% NE = 8.05 nitrogen [N], 3.78 phosphorus [P] and 1.08 potassium [K] g.kg^{-1} *Ulva*) at a ratio of 7:3:1. ^{C} = replicate 3.

^{1}H NMR calculated ω-3 content (%) and double-bond equivalent (DBE) of NL metabolites from *U. lacinulata* from this experiment are shown in Table **4.3**. Nutrient variations had no significant effect on the ω-3 content (%; ANOVA, F= 2.341, *p*= 0.106) and DBE (ANOVA, F= 1.488, *p*= 0.265) of NL metabolites. ω-3 content constituted 28.15 ± 9.80 – 38.51 ± 3.78%, while the DBE constituted 1.48 ± 0.87 – 2.41 ± 0.37% of the NL samples.

Table 4.3: The ω-3 (%) and double-bond equivalent (DBE) of neutral lipids (NL) from *Ulva lacinulata* cultured at six nutrient treatments: 0 [control], 25, 50, 75, 100 and 200% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1] calculated from proton nuclear magnetic resonance (^{1}H NMR) data. Values are represented as mean ± SD (*n*=3). Values with different superscript letter (^{a}) within treatments indicate significant difference (ANOVA; $p < 0.05$) between ω-3 (%) or DBE of NL samples.

	0% NE	**25% NE**	**50% NE**	**75% NE**	**100% NE**	**200% NE**
% ω-3	34.65 ± 2.11^a	36.68 ± 0.49^a	28.15 ± 9.80^a	38.51 ± 3.78^a	38.25 ± 0.83^a	37.44 ± 1.35^a
DBE	2.41 ± 0.37^a	2.29 ± 0.34^a	1.48 ± 0.87^a	2.18 ± 0.33^a	2.13 ± 0.36^a	2.09 ± 0.09^a

4.4.4.3. Glycolipid (GL)

GL content of the *Ulva* samples were characterized by protons of GL sugars (CH), with phosphatidylinositol (δ_H 3.33 – 3.39 and δ_H 3.77 – 3.82 [**21**] and [**21'**]), C-3 (δ_H 3.39 – 3.50 [**22**]), phosphatidylcholine (δ_H 3.51 – 3.58 [**23**]), (δ_H 3.60 – 3.78 [**24**]), glycerophospholipids (δ_H 3.83 – 3.88 [**25**]) and anomeric carbon protons (δ_H 4.92 – 5.12 [**26**]; Figure **4.10**; see Supplementary Information **4** Section **S4.5** for GL spectral regions assignment). Nutrient variation had no significant effect on the GL metabolite contents of *Ulva* (ANOVA, $p > 0.05$). Minor variations were detected in the GL metabolite intensities in spectral regions [**21**], [**22**], [**23**] and [**24**] for 0, 25, 75, 100 and 200 % NE samples (see Figure **4.10** and Supplementary Information **4** Section **S4.5** for GL ^{1}H NMR spectra).

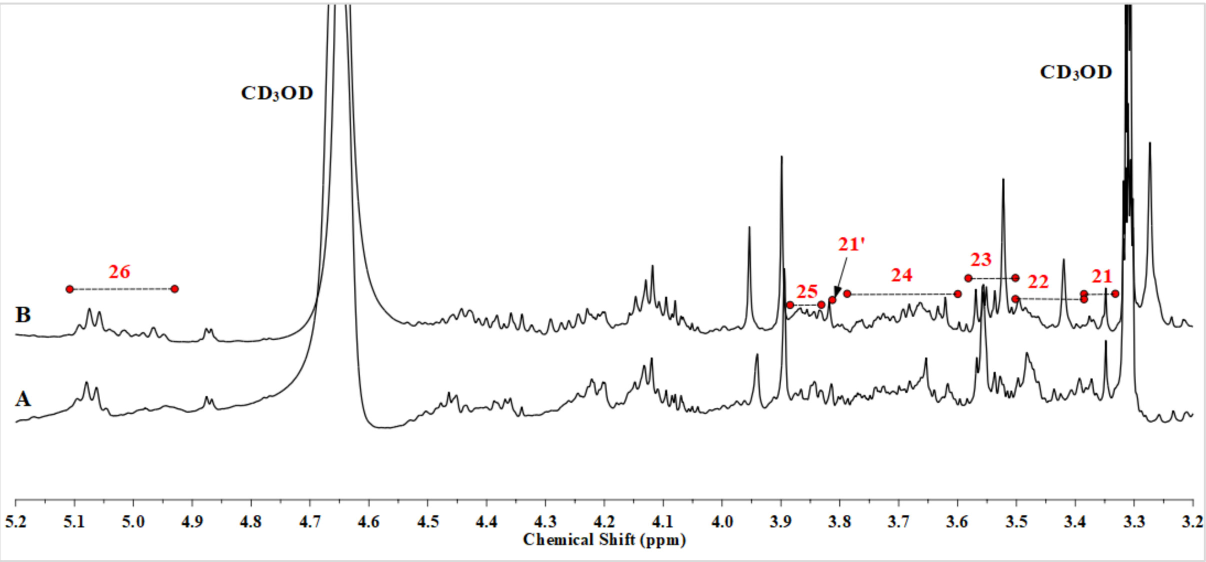


Figure 4.10: Proton nuclear magnetic resonance [^{1}H NMR; 1:1 $CDCl_3$:CD_3OD, 400 MHz] spectra of glycolipid (GL) samples of *Ulva lacinulata* cultured at 25% NE^C (A) and 200% NE^C (B). 25% nutrient enrichment [NE] = 0.25 × (100% NE = 8.05 nitrogen [N], 3.78 phosphorus [P] and 1.08 potassium [K] g.kg^{-1} *Ulva*) at a ratio of 7:3:1. 200% NE = 2 × (100% NE). ^{C} = replicate 3.

^{1}H NMR calculated ω-3 content (%), DBE and saturated fatty acid (% SFA) of GL from *Ulva* cultured at the six different nutrient treatments are summarized in Table **4.4**. Nutrient variation had no significant effect on the ω-3 content (%; ANOVA, F= 0.176, p= 0.966), DBE (ANOVA, F= 0.716, p= 0.624) and SFA (%; ANOVA, F= 1.448, p= 0.277) on the GL metabolites of the *Ulva*. ω-3 content (%) ranged from 39.18 ± 2.99 to 40.94 ± 2.36%, DBE from 2.06 ± 0.19 to 1.65 ± 0.16, and SFA (%) from 2.06 ± 3.63 to 16.50 ± 4.33%.

Table 4.4: The ω-3 (%), saturated fatty acid (% SFA) and double-bond equivalent (DBE) of glycolipid (GL) from *Ulva lacinulata* cultured at six nutrient treatments: 0 [control], 25, 50, 75, 100 and 200% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) $g.kg^{-1}$ *Ulva* at a ratio of 7:3:1] calculated from proton nuclear magnetic resonance (^1H NMR) data. Values are represented as mean ± SD (*n*=3). Values with different superscript letter (^{a}) within treatments indicate significant difference (ANOVA; $p< 0.05$) between ω-3 (%), SFA (%) or DBE of GL samples.

	0% NE	**25% NE**	**50% NE**	**75% NE**	**100% NE**	**200% NE**
% ω-3	40.01 ± 3.98^a	39.71 ± 2.71^a	40.64 ± 1.86^a	39.18 ± 2.99^a	40.94 ± 2.36^a	40.26 ± 0.70^a
% SFA	6.08 ± 7.43^a	7.69 ± 6.06^a	2.06 ± 3.63^a	5.06 ± 12.56^a	9.27 ± 4.45^a	16.50 ± 4.33^a
DBE	1.95 ± 0.44^a	1.88 ± 0.19^a	2.06 ± 0.19^a	1.98 ± 0.38^a	1.88 ± 0.21^a	1.65 ± 0.16^a

4.4.4.4. Phospholipid (PL)

PL content of *U. lacinulata* was characterized by the presence of methylene protons ($C\underline{H}_2$) in phosphatidylethanolamine (δ_H 2.95 – 3.07 [**27**]), lysophosphatidylcholine (δ_H 3.14 – 3.17 [**28**]), phosphatidylcholine (δ_H 3.18 – 3.22 and δ_H 3.49 – 3.61 [**29**] and [**29’**]) and glycerophospholipids (^{a}; δ_H 4.19 – 4.48 [**31**]), and $C\underline{H}$ protons in phosphatidylinositol (δ_H 3.39 – 3.46 and δ_H 4.09 – 4.14 [**30**] and [**30’**]) and glycerophospholipids (^{b}; δ_H 5.16 – 5.25 [**32**]; Figure **4.11**; see Supplementary Information **4** Section **S4.6** for PL spectral regions assignment). Nutrient variation had a significant effect on the PL metabolites of *Ulva* (ANOVA, $p< 0.05$). The phospholipid profile of *Ulva* was negatively affected with an increase in nutrient concentration. The intensity of the proton resonances of spectra regions [**27**], [**29**; except spectral bin δ_H 3.61], [**30**], [**31**] and [**32**] were more prominent in lower nutrient treatments (*i.e.* 25 and 50% NE) and the control than higher nutrient treatments (see Figure **4.11** and Supplementary Information **4** Section **S4.6** for PL ^1H NMR spectra).

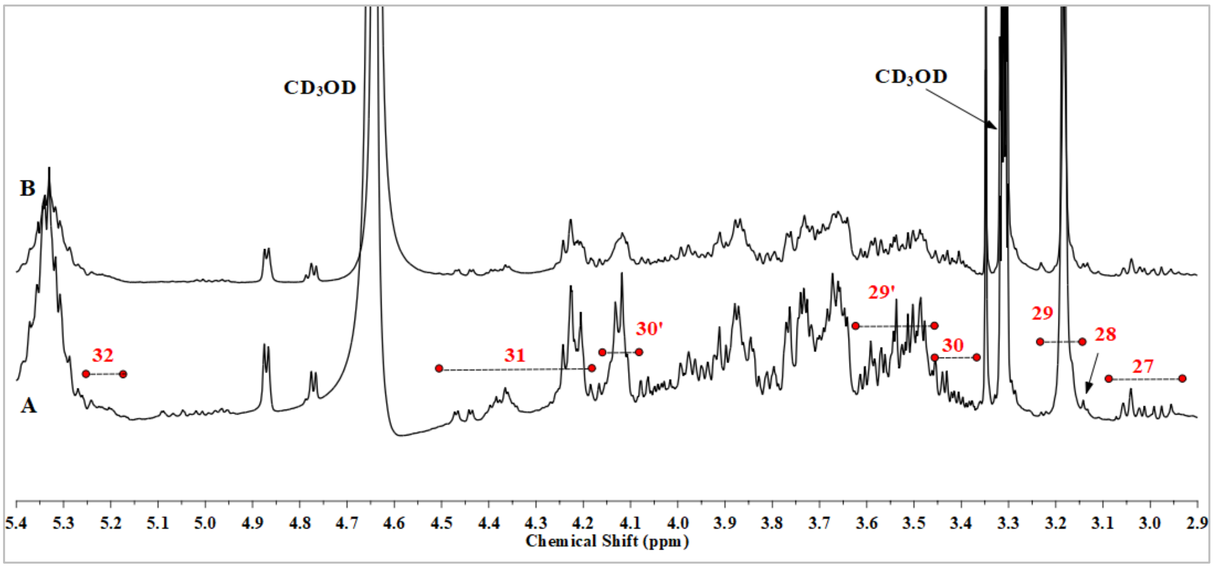


Figure 4.11: Proton nuclear magnetic resonance [^{1}H NMR; 1:1 $CDCl_3$:CD_3OD, 400 MHz] spectra of phospholipid (PL) samples of *Ulva lacinulata* cultured at 25% NE^C (A) and 100% NE^B. 25% nutrient enrichment [NE] = 0.25 × (100% NE = 8.05 nitrogen [N], 3.78 phosphorus [P] and 1.08 potassium [K] g.kg^{-1} *Ulva*) at a ratio of 7:3:1. ^{B} = replicate 2 and ^{C} = replicate 3.

^{1}H NMR calculated ω-3 content (%), DBE and SFA (%) of PL from aquacultured *Ulva* are summarized in Table **4.5**. Nutrient variation had no significant effect on the ω-3 content (%; ANOVA, F= 1.697, p= 0.210), SFA (%; ANOVA, F= 1.423, p= 0.285) and DBE (ANOVA, F= 0.847, p= 0.542) on the PL contents of this seaweed. ω-3 content (%) of PL metabolites varied from 26.93 ± 1.27 to 31.76 ± 2.53%, SFA (%) ranged from 37.17 ± 10.44 to 48.17 ± 2.40% and DBE, 1.11 ± 0.03 to 1.30 ± 0.26.

Table 4.5: The ω-3 (%), saturated fatty acid (% SFA) and double-bond equivalent (DBE) of phospholipids (PL) from *Ulva lacinulata* cultured at six nutrient treatments: 0 [control], 25, 50, 75, 100 and 200% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1] calculated from proton nuclear magnetic resonance (^{1}H NMR) data. Values are represented as mean ± SD (n=3). Values with different superscript letter (^{a}) within treatments indicate significant difference (ANOVA; p< 0.05) between ω-3 (%), SFA (%) or DBE of PL samples.

	0% NE	**25% NE**	**50% NE**	**75% NE**	**100% NE**	**200% NE**
% ω-3	28.81 ± 1.02^a	29.70 ± 3.58^a	31.76 ± 2.53^a	29.51 ± 1.40^a	26.93 ± 1.27^a	30.40 ± 1.98^a
% SFA	48.17 ± 2.40^a	45.83 ± 2.02^a	39.00 ± 7.53^a	39.83 ± 5.36^a	37.17 ± 10.44^a	41.00 ± 4.99^a
DBE	1.13 ± 0.09^a	1.23 ± 0.05^a	1.30 ± 0.26^a	1.20 ± 0.10^a	1.29 ± 0.20^a	1.11 ± 0.03^a

4.4.5. Fatty acid composition (FA)

A total of 25 FAs were detected from *U. lacinulata* cultured in this experiment (Table **4.6**). Saturated fatty acids (SFA; 56.40 ± 3.23 – 59.95 ± 8.28% total fatty acid [TFA]) were the major group of FAs identified from this seaweed, followed by polyunsaturated fatty acid (PUFA; 29.44 ± 2.43 – 33.53 ± 3.19% TFA) and monounsaturated fatty acid (MUFA; 9.75 ± 0.41 – 10.65 ± 0.65% TFA).

Nutrient variation had a significant effect on the C18:0 FA (stearic acid; ANOVA, F= 3.878, *p*= 0.025) profile of *Ulva*. C18:0 FA content was negatively affected in seaweed cultured in high nutrient concentration (*i.e.* 100 and 200% NE). No significant difference was observed in the remaining 24 FAs recorded in *U. lacinulata* among the six nutrient treatments. The main FA produced by *Ulva* in this experiment was C16:0 FA (palmitic acid), representing half of the FA (48.92 ± 1.39 – 52.23 ± 7.38% TFA) produced by *Ulva*. ω-3 was the major contributor of PUFA and constituted 26.72 ± 9.21 to 30.31 ± 2.52% of the TFA content, while ω-6 comprised 2.34 ± 0.20 to 3.05 ± 0.62% of the TFA content. The ω-6/ω-3 ratios ranged between 0.08 ± 0.00 to 0.12 ± 0.06.

Table 4.6: Fatty acids (FA) profile of *Ulva lacinulata* cultured at six nutrient treatments: 0 [control], 25, 50, 75, 100 and 200% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) $g.kg^{-1}$ *Ulva* at a ratio of 7:3:1]. Data of individual FA are expressed in relative abundance (%). Data are presented as mean ± SD (*n*=3). SFA= saturated fatty acids; MUFA= monounsaturated fatty acids; PUFA= polyunsaturated fatty acids.

Fatty acid (FA)	**0% NE**	**25% NE**	**50% NE**	**75% NE**	**100% NE**	**200% NE**
Saturated						
C12:0	0.10 ± 0.01	0.07 ± 0.02	0.15 ± 0.11	0.08 ± 0.04	0.08 ± 0.01	0.08 ± 0.01
C14:0	1.11 ± 0.04	1.12 ± 0.05	2.79 ± 3.10	1.21 ± 0.27	1.20 ± 0.20	1.06 ± 0.12
C15:0	1.47 ± 0.18	1.73 ± 0.15	1.38 ± 0.10	1.45 ± 0.40	1.54 ± 0.21	1.49 ± 0.21
C16:0	52.23 ± 7.38	51.71 ± 1.11	49.92 ± 1.39	50.19 ± 0.49	51.27 ± 4.49	49.24 ± 3.11
C17:0	0.10 ± 0.01	0.11 ± 0.01	0.11 ± 0.00	0.13 ± 0.04	0.12 ± 0.01	0.11 ± 0.01
C18:0	1.11 ± 0.31^{a}	0.88 ± 0.24^{ab}	0.65 ± 0.05^{ab}	0.71 ± 0.02^{ab}	0.64 ± 0.04^{b}	0.64 ± 0.13^{b}
C20:0	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.04	0.07 ± 0.02	0.08 ± 0.03
C22:0	3.75 ± 1.09	4.18 ± 0.44	3.70 ± 0.29	4.07 ± 0.89	3.51 ± 0.31	3.70 ± 0.18
Monounsaturated						
C16:1	0.71 ± 0.07	0.70 ± 0.02	0.63 ± 0.02	0.63 ± 0.08	0.67 ± 0.02	0.59 ± 0.02
C16:1ω7	2.19 ± 0.36	2.03 ± 0.28	2.30 ± 0.27	2.77 ± 0.58	2.22 ± 0.74	2.64 ± 0.45
C17:1ω7	0.21 ± 0.16	0.32 ± 0.01	0.28 ± 0.03	0.26 ± 0.08	0.27 ± 0.03	0.25 ± 0.03
C18:1ω9	7.25 ± 0.78	7.54 ± 0.33	6.49 ± 0.12	6.91 ± 0.49	6.94 ± 0.09	6.55 ± 0.04
C22:1	0.06 ± 0.01	0.06 ± 0.02	0.05 ± 0.02	tr	tr	tr
Polyunsaturated						
C16:2ω6	0.06 ± 0.01	0.07 ± 0.04	0.09 ± 0.03	0.09 ± 0.02	0.09 ± 0.02	0.10 ± 0.06
C16:3	0.10 ± 0.01	0.10 ± 0.03	0.11 ± 0.05	0.10 ± 0.03	0.09 ± 0.01	0.10 ± 0.03
C16:4ω3	17.42 ± 7.99	17.91 ± 2.71	20.66 ± 3.65	18.92 ± 2.82	18.86 ± 3.28	20.72 ± 1.97
C17:3ω3	0.20 ± 0.09	0.17 ± 0.05	0.16 ± 0.07	0.08 ± 0.07	0.12 ± 0.02	0.15 ± 0.07
C18:2ω6 LA	2.57 ± 0.39	2.19 ± 0.15	2.26 ± 0.24	2.47 ± 0.38	2.54 ± 0.16	2.87 ± 0.56
C18:3	0.09 ± 0.02	0.07 ± 0.02	0.07 ± 0.02	0.09 ± 0.01	0.08 ± 0.01	0.07 ± 0.02
C18:3ω3 ALA	7.04 ± 0.64	6.85 ± 0.27	7.08 ± 0.29	7.79 ± 0.58	7.67 ± 1.05	7.58 ± 0.28
C18:4ω3	tr	tr	tr	tr	tr	tr
C20:4ω3	0.53 ± 0.12	0.66 ± 0.03	0.58 ± 0.11	0.50 ± 0.08	0.59 ± 0.10	0.53 ± 0.13
C20:4ω6 ARA	0.10 ± 0.04	0.08 ± 0.01	0.07 ± 0.02	0.07 ± 0.03	0.07 ± 0.02	0.09 ± 0.03
C20:5ω3 EPA	0.31 ± 0.08	0.24 ± 0.02	0.24 ± 0.07	0.22 ± 0.04	0.21 ± 0.07	0.24 ± 0.08
C22:5ω3 DPA	1.22 ± 0.43	1.09 ± 0.30	1.14 ± 0.26	1.12 ± 0.15	1.09 ± 0.17	1.08 ± 0.47
Σ SFA	59.95 ± 8.28	59.90 ± 1.85	57.79 ± 4.35	57.91 ± 1.46	58.43 ± 5.06	56.40 ± 3.23
Σ MUFA	10.41 ± 0.63	10.65 ± 0.65	9.75 ± 0.41	10.61 ± 1.17	10.14 ± 0.77	10.07 ± 0.44
Σ PUFA	29.63 ± 8.90	29.44 ± 2.43	32.47 ± 4.68	31.48 ± 2.36	31.43 ± 4.69	33.53 ± 3.19
Σ ω-3	26.72 ± 9.21	26.93 ± 2.49	29.87 ± 4.33	28.64 ± 2.68	28.56 ± 4.68	30.31 ± 2.52
Σ ω-6	2.73 ± 0.34	2.34 ± 0.20	2.42 ± 0.29	2.64 ± 0.42	2.70 ± 0.14	3.05 ± 0.62
Σ ω-6/Σ ω-3	0.12 ± 0.06	0.09 ± 0.01	0.08 ± 0.00	0.09 ± 0.02	0.10 ± 0.02	0.10 ± 0.01

tr= trace (value < 0.05% abundance); ^{a,b} Values in a row without a common superscript are significantly different between treatments at $p < 0.05$ (ANOVA). ALA – α-linolenic acid; LA – Linoleic acid; DPA – Docosapentaenoic acid; EPA – Eicosapentaenoic acid; AA – Arachidonic acid.

4.4.5.1. Chemometric analysis of FA content

The PCA scores plot shows the distribution of FA contents of *U. lacinulata* grown in the six nutrient treatments (Figure **4.12**). The first two principal components (PC) explained 62.9% of the of the variability present among the FA data. PC 1 explained 49.4% variability present in the data, while PC 2 accounted for 13.5% of the data variance. Samples from the control (C_1 and C_3; treatment_1 = replicate 1 and treatment_3 = replicate 3), 25 (25_3), 50 (50_3), 75 (75_1), 100 (100_1) and 200 (200_1 and 200_2; treatment_2 = replicate 2) % NE were clearly separated from the rest of the samples. However, no definitive pattern was observed from the plot, since almost all the nutrient samples were grouped around the origin (0,0).

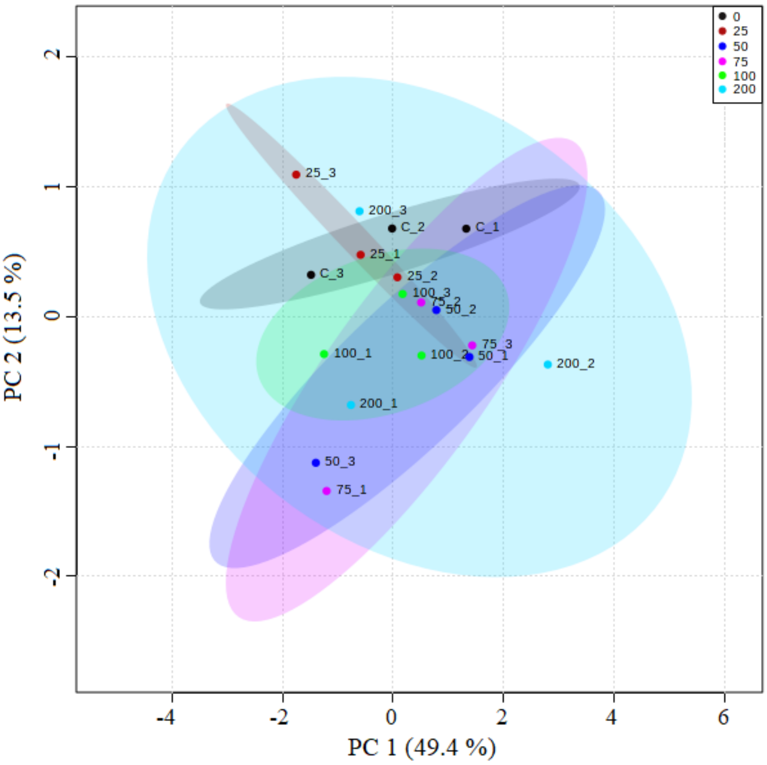


Figure 4.12: PCA scores plot of FA contents of *Ulva lacinulata* cultured at six nutrient treatments: 0 [control], 25, 50, 75, 100 and 200% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. Ellipsoids showing 95% confidence interval. C = 0% NE; 25 = 25% NE; 50 = 50% NE; 75 = 75% NE; 100 = 100% NE; 200 = 200% NE (treatment_1 = replicate 1, treatment_2 = replicate 2 and treatment_3 = replicate 3).

The eight major contributors of PC 1 in descending order for FA contents C16:3 (hexadecatrienoic acid), C20:4ω6 (arachidonic acid – AA), C17:3ω3, C20:5ω3 (eicosapentaenoic acid – EPA), C22:5ω3 (docosapentaenoic acid – DPA), C16:2ω6 (hexadienoic acid), C18:3ω3 (α-linolenic acid – ALA) and C16:4ω3 (hexadecatetraenoic acid; Figure **4.13**). All eight were PUFA including five ω-3 PUFAs and two ω-6 PUFAs. However, outcomes did not show a distinct pattern of the effect of nutrient variations on the FA contents of aquacultured *Ulva* (see Supplementary Information **4** Figure **S4.44** for Box and Whiskers plots).

4.5 Discussion

Nutrients are one of the main factors influencing the morphology, distribution, growth, development, reproduction, productivity and biochemical composition of macroalgae (Smit et al. 1996; Coelho et al. 2000; Harrison and Hurd, 2001; Gordillo et al. 2006; Roleda, 2016; Benes and Bracken, 2016; Zheng et al. 2019; Boderskov et al. 2022). Nitrogen (N) and phosphorus (P) are considered as the two most important macronutrients that limit growth and productivity of seaweeds, and in aquaculture when N and P are added, carbon (C) may become limiting (Harrison and Hurd, 2001). Limitation of one of these macronutrients may result in reduced growth and productivity, while the addition of nutrients such as N and P to nutrient-depleted medium can result in increased algal growth (Björnsäter and Wheeler, 1990; Vandermeulen and Gordin, 1990).

In the current study, results showed that growth of *Ulva* was not inhibited by the experimental nutrient variations (Figure **4.5**). SGRs recorded in the present study were comparable to values found in the literature for *Ulva* biomass cultured under laboratory-controlled conditions (*e.g.* Gómez Pinchetti et al. 1998; Luo et al. 2012; Shahar et al. 2020) and to values of *Ulva* growing in aquaculture wastewater (*e.g.* Msuya and Neori, 2010; Yokoyama and Ishihi, 2010; Brundu and Chindris, 2018). It has often been noted that addition of nutrients to cultured *Ulva* can increase growth (Luo et al. 2012; Shahar et al. 2020; Toth et al. 2020; Bews et al. 2021). *Ulva* can also efficiently take up nutrients and grow at low nutrient concentrations (Floreto et al. 1996; Pedersen and Borum, 1997; Luo et al. 2012; Bews et al. 2021). In the event of high nutrient availability, such as ‘pulse fertilization’, *Ulva* may however slow down growth to maximize the uptake of nutrients (Duke et al. 1989; Fong et al. 2004). With the experimental site of the current study located in the Benguela upwelling system and in an urban area located close to a sewage outfall, where nutrient levels are generally high (Waldron and Probyn, 1992), the seawater nutrient levels (NH_4^+ = 2.88 – 6.90 µmol.L^{-1}; NO_3^- = 0.35 – 4.82 µmol.L^{-1}; NO_2^- = 0.32 – 0.53 µmol.L^{-1} over the 10 days culture period) as well as the high water turnover rate (30 VE.d^{-1}) could have been sufficient for *Ulva* to be nutrient saturated for growth under ambient nutrient levels. Floreto et al. (1996) found no significant difference in SGR for the first

3 days when the green alga *U. australis* (as *U. pertusa*) was cultured at different levels of nitrogen (*i.e.* 0, 0.01, 1.5 and 15 μM nitrate). The authors reported that the growth of this seaweed was not affected at by either the lowest (0.01 μM) or higher (15 μM) nitrate concentrations. Therefore, in the current study, it may be suggested that the addition of nutrient through pulse fertilization could have been taken up by the alga and stored, rather than used for growth.

Total lipid contents (TL), lipid classes and fatty acid (FA) contents often fluctuate with changes in season as well as the combined effect of environmental factors such as temperature, salinity, light and nutrient availability (Floreto et al. 1993; Khotimchenko and Yakovleva, 2005; Gerasimenko et al. 2010; McCauley et al. 2018; Santos et al. 2019; Toth et al. 2020). TL contents (3.91 ± 0.29 to 4.61 ± 0.64% DW) recorded from this study were within the range reported in the literature for different *Ulva* species (3.14 – 4.14% DW; *e.g.* Rohani-Ghadikolaei et al. 2012; Khairy and El-Shafay, 2013; El Maghraby and Fakhry, 2015; Gadberry et al. 2018). Moreover, TL contents from the current study were superior (1.62 to 2.98 fold) to the values reported for *Ulva* specimens cultured in IMTA systems (0.23 – 3.40% DW; *e.g.* Mata et al. 2016; Shpigel et al. 2018b; Queirós et al. 2021). It had been reported that N is one of the main factors that may influence the lipid content in algae (Gao et al. 2018). N-limitation has on occasion been associated with enhancing the production of TL contents in *Ulva* (Floreto et al. 1996; Gordillo et al. 2001). However, in some cases, high N conditions have been shown to promote the production of lipid in *Ulva* (Floreto et al. 1996; Gao et al. 2017). It has been reported that continuous production of lipid by the cell may be possible, since the process does not involve N (Roessler, 1990). Since TL contents in the current study were not statistically affected by nutrient variation, it is plausible that lipid production similar to the SGR was unaffected due to *Ulva* being nutrient saturated under the ambient nutrient levels recorded in this study associated with the high water turnover rate (30 VE.d^{-1}).

As observed by Santos et al. (2019), glycolipids (GL; 46.63 ± 2.94 – 56.45 ± 3.64% total lipid [TL]) were the major class of lipid detected in *Ulva* in the present study, followed by neutral lipids (NL; 27.64 ± 6.82 – 40.30 ± 2.78% TL) and phospholipids (PL; 13.07 ± 0.16 – 16.25 ± 2.20% TL). The predominance of GL over NL and PL in *Ulva* from this study may be attributed to the constant presence of nutrients resulting from the high water turnover rate (30 VE.d^{-1}) which resulted in the mobilization of fatty acids from NL to GL (Kumari et al. 2014). This was also supported by the high ω-3 polyunsaturated fatty acid (PUFA; Table **4.4**) and low saturated fatty acids (SFA) contents in the GL fraction. The GL content of *Ulva* in the 200% NE treatment was shown to be significantly lower than the other nutrient treatments of this study. The high content of SFA detected in *Ulva* from the 200% NE treatment (Table **4.4**) may suggest the conversion of fatty acids from GL into NL. This notion is supported by the significantly higher NL content of *Ulva* cultured at 200% NE compared to seaweed cultured in low nutrients (*i.e.* 0 and 25% NE; Figure **4.7**). The overall low PL contents of *Ulva* observed in this experiment suggests the alga favoured the production of GL and NL, while phosphorus (P) uptake was sufficient to sustain PL production without compromising photosynthetic membranes (Kumari et al. 2014). Another plausible explanation of low levels of PL may be P budgeting, since PLs are located in non-plasmid membranes and can be replaced to an extent by GL located in thylakoid membranes (Raven, 2012, 2013; Veneklaas et al. 2012). It has been reported that lipids may undergo a series of complex processes (Gašparović et al. 2023). In the present experiment, superior ω-3 contents in GL and NL may have originated from dehydrogenation, followed by cross-linking of lipids, which could have led to the formation of more unsaturated fatty acids (*i.e.* ω-3 PUFAs; Table **4.3** and **4.4**; Gašparović et al. 2023). The lower ω-3 contents in PL could have been derived from the oxidation or hydrogenation of lipid into saturated or less unsaturated lipids (Table **4.5**; Gašparović et al. 2023). This may explain the elevated content of saturated fatty acid (SFA) and low ω-3 contents in PL overall.

The total fatty acid (TFA) compositions of *Ulva* in the current study (saturated [SFA] > polyunsaturated [PUFA] > monounsaturated FA [MUFA]) determined from GC-MS analysis were in agreement with findings reported in the literature for other *Ulva* species (*e.g.* Peña-Rodríguez et al. 2011; Ivanova et al. 2013; Kendel et al. 2015; Paiva et al. 2017). Nutrient variation had no significant effects on the SFA (except C18:0 FA), MUFA and PUFA contents. As observed with the SGR and TL contents, seawater nutrient levels coupled with the high flow rate (30 VE.d^{-1}) were adequate to saturate the nutrient level of *Ulva*, thus explaining the non-significance of nutrient variation on the fatty acid profile of this seaweed. SFA contents (56.40 ± 3.23 – 59.95 ± 8.28% TFA) were within the range reported for several other *Ulva* species (*e.g.* Gómez-Pinchetti et al. 1998; Pereira et al. 2012; El-Din, 2019; Queirós et al. 2021). C18:0 FA (stearic acid) content in the current study was significantly reduced when *Ulva* was cultured at high nutrient concentrations (*i.e.* 100 and 200% NE; Table **4.6**). The higher level of C18:0 FA in *Ulva* cultured at 0% NE (control) may indicate the formation and accumulation of triglycerides (consist mostly of SFA), since this treatment did not receive additional fertilization (*i.e.* pulse fertilization), which could have limited the availability of additional nitrogen (Sharma et al. 2012). Comparable to other studies, C16:0 FA (palmitic acid) was the most abundant SFA recorded from *Ulva* in this study (*e.g.* Mata et al. 2016; Santos et al. 2019; Queirós et al. 2021). However, the levels recorded in this study were superior to the levels recorded from *Ulva* species cultured in laboratory-based experiments using different nutrient levels (*e.g.* Floreto et al. 1996; Gómez Pinchetti et al. 1998; McCauley et al. 2016) and land-based culture system (*e.g.* Mata et al. 2016).

Monounsaturated fatty acid contents (MUFA; 9.75 ± 0.41 – 10.65 ± 0.65% TFA) in this study were on the lower range to those reported in the literature (*e.g.* McCauley et al. 2015; Kendel et al. 2015; Paiva et al. 2017; Pirian et al. 2018). C18:1ω9 FA (oleic acid) was the most prominent MUFA observed in this study and the levels were in agreement with some reports on the MUFA content of *Ulva* species (*e.g.* Rohani-Ghadikolaei et al. 2012; Ivanova et al. 2013; Magdugo et al. 2020). Conversely, other studies have reported C16:1ω7 FA (palmitoleic acid) to be the most prominent MUFA in *Ulva* (*e.g.* Durmaz et al. 2008; Pereira et al. 2012). Floreto et al. (1996) reported a significant increase in C18:1ω9 FA levels in *U. australis* (as *U. pertusa*) when cultured in high N media compared to a control, whereas McCauley et al. (2016) found no significant difference in C18:1ω9 levels in *Ulva* cultured in both high and low nutrient media.

Polyunsaturated fatty acid contents (PUFA; 29.44 ± 2.43 – 33.53 ± 3.19% TFA) in this study were within the range previously reported for *Ulva* species (*e.g.* Durmaz et al. 2008; Peña-Rodríguez et al. 2011; Kendel et al. 2015; Paiva et al. 2017). Findings from the current study showed that C16:4ω3 (hexadecatetraenoic acid) was the most prominent PUFA produced by *U. lacinulata*, unlike C18:3ω3 (α-linolenic acid ALA) and C18:4ω3 (stearidonic acid) documented in other *Ulva* species (*e.g.* Durmaz et al. 2008; Kendel et al. 2015; Serviere-Zaragoza et al. 2015; McCauley et al. 2016). C16:4ω3, C18:3ω3 and C18:4ω3 FAs have been reported to be among the major contributors of plastidic galactolipids, such as monogalactosyldiacylglycerol (MGDG; Arisz et al. 2000; Khotimchenko, 2003). It was reported that C16:3ω3 (hexadecadienoic acid), C16:4ω3, C18:3ω3 and C18:4ω3 FAs located in MGDG of *U. fenestrata* constituted 88.1% of its total fatty acid content (TFA) compared to 27.7% TFA of digalactosyldiacylglycerol (DGDG) and 14.3% TFA of sulfoquinovosyldiacylglycerols (SQDG; Khotimchenko, 2003). Therefore, in the current study, it may plausible that the high levels of C16:4ω3 and C18:3ω3 FAs present in the aquacultured *Ulva* could be related to the high levels of glycolipids (GL). The ω-6:ω-3 ratios in this study calculated from the PUFA data were on the lower range compared to what has been reported in the literature for *Ulva* species collected from the wild (*e.g.* Pereira et al. 2012; Schmid et al. 2014; Kendel et al. 2015) and from laboratory-based experiments growing *Ulva* under different nutrient concentrations (*e.g.* McCauley et al. 2016; Gao et al. 2018). Contrary to this study, McCauley et al. (2016) reported that *Ulva* grown in high nutrients had significantly higher ω-3 PUFA contents and lower ω-6:ω-3 ratio compared to seaweed cultured in low nutrient levels. FAO/WHO (1994) recommends that the ω-6:ω-3 ratio should not exceed 10:1 in diet. Therefore, the low ω-6:ω-3 ratios recorded in *Ulva* from this study makes it suitable for human and animal health applications.

Although the results revealed limited and low significant differences, data collected from this study provided an understanding on how nutrient enrichment above ambient seawater concentration following an actual fertilization regime use by an aquaculture farm, affect the total lipid content, lipid classes and fatty acid composition of *U. lacinulata*. Findings showed that this seaweed consists essential and non-essential long-chain PUFA, including C18:3ω3 (α-linolenic acid – ALA), C18:2ω6 (linoleic acid – LA), C16:4ω3 (hexadecatetraenoic acid), C22:5ω3 (docosapentaenoic acid – DPA), C20:5ω3 (eicosapentaenoic acid – EPA) and C20:4ω6 (arachidonic acid – AA), which can provide a sustainable source of biomass with beneficial PUFAs for inclusion in aquafeeds and/or for human consumption. However, the cultivation of *Ulva* under nutrient enrichment conditions above seawater concentration is not recommended to produce biomass intended to be used as a functional feed ingredient (*i.e.* as feed attractant and stimulant; see Chapter 2) for the sea urchin *Tripneustes gratilla*, as it hinders glycolipids production. Therefore, based on data collected, the optimal culture condition to grow *Ulva* would be at ambient seawater nutrient concentration to maximize glycolipid production.

CHAPTER 5

Evaluation of volatile organic compounds from aquacultured *Ulva lacinulata*

5.1 Abstract

In this study, the volatile organic compounds (VOCs) produced by commercially aquacultured *Ulva lacinulata* were investigated for the first time. This seaweed has been a fundamental part of the South African abalone industry as supplementary feed and biofilter, however, very little is known about its volatile compound composition that contributes to its typical aroma. VOCs from this alga were extracted using solvent extraction (SE), steam distillation-liquid/liquid extraction (SD-LLE) and headspace solid-phase microextraction (HS-SPME) and analysed using gas chromatography-mass spectrometry (GC-MS). Results showed that aquacultured *U. lacinulata* produces a mixture of volatile compounds ranging from simple to complex volatile compounds that can contribute to its typical aroma. A total of 63 compounds including 18 aldehydes, 15 hydrocarbons, 10 ketones, 9 esters, 6 free fatty acids, 4 alcohols and 1 aromatic compound, were identified. However, based on the relative content (%) and information available from the literature, only aldehydes, hydrocarbons, ketones, esters and alcohol are considered the most important classes of compounds associated with the aroma of this seaweed. Findings also showed that aquacultured *Ulva* produces VOCs of interest such as α- and β-Ionone, which have commercial application as well as benzaldehyde and 6-methyl-5-hepten-2-one that have been shown to be flavour enhancers in marine organisms. Aquacultured *Ulva* has already been shown to be a suitable supplementary feed for the South African abalone *Haliotis midae* (Lepetellida) and sea urchin *Tripneustes gratilla* (Camarodonta). Enhancing the flavours of these animals may increase their marketability and financial return on the products. Therefore, further research needs to be done to investigate the effect of using *Ulva* as feed on the organoleptic properties of *H. midae* and *T. gratilla*, and how they can be manipulated to enhance flavours of the marketed products.

5.2 Introduction

‘Flavour’ is a unitary perception involving a combination of olfaction and taste (Francezon et al. 2021). For seafood, it may be the critical factor in the choice of a specific product by the consumer, while the odour or aroma may be the decisive factor in the decision-making process (Moreira et al. 2022). For seaweeds, ‘taste’ may be derived from non-volatile compounds in food such as amino acids, polysaccharides, lipids and carbohydrates (Urlass et al. 2023). On the contrary, the typical aroma of seaweeds is derived from the complex interactions of different types of volatile organic compounds (VOCs; Hu et al. 2021; Moreira et al. 2022). VOCs from seaweeds are mainly composed of hydrocarbons, aldehydes, ketones, alcohols, free fatty acids, esters, terpenes, phenols, halogenated and sulphonated compounds, a variety of which are odour active (de Alencar et al. 2017; López-Pérez et al. 2017; Maruti et al. 2018; Keng et al. 2020; Urlass et al. 2023). Odours such as ‘seafood’, ‘fish’, ‘marine’, ‘spices’, ‘licorice’, ‘green grass’, ‘fatty’ and ‘honey’ have been associated with the VOCs present in seaweeds (López-Pérez et al. 2017; Moreira et al. 2022). However, the compositions of VOCs can vary between species, while their levels may be related to the physiological state of the species (Gressler et al. 2009; de Alencar et al. 2017; López-Pérez et al. 2017; Garicano Vilar et al. 2020).

Green seaweeds from the genus *Ulva* Linnaeus (Phylum: Chlorophyta; Class: Ulvophyceae; Order: Ulvales), commonly known as ‘sea lettuce’, are among the most abundant macroalgae species, being omnipresent on marine rocky shores and in lagoons and estuaries around the world (Wichard et al. 2015; Miladi et al. 2018). They are also known to form large, sometimes problematic, free-floating masses, known as ‘green tides’, usually in eutrophic conditions (Mantri et al. 2020). *Ulva* species has been identified as ideal candidates for bioremediation applications and biomass production in land-based integrated multi-tropic aquaculture systems (IMTA) due to their high growth rates and high nutrient uptake capacity (Robertson-Andersson et al. 2008; Lawton et al. 2013; Al-Hafedh et al. 2015; Shpigel et al. 2018a). In recent years, these green seaweeds have gained much attention as promising alternatives for animal-based feed additives and as supplementary feed for several high value aquacultured animals (Naidoo et al. 2006; Mulvaney et al. 2013; Cyrus et al. 2013, 2015a, b; Laramore et al. 2018). *Ulva* grown in animal wastewater can have higher protein content (up to 40% crude protein) than species collected from nature and can have similar amino acid profiles to animal and other plant protein, making aquacultured *Ulva* a suitable ingredient for partial replacement of fishmeal in aquafeed (Robertson-Andersson et al. 2011; Fleurence et al. 2012; Shuuluka et al. 2013; Cyrus et al. 2013, 2015a, b; Angell et al. 2015, 2017; Bolton et al. 2016; Shpigel et al. 2018a, b). Dietary supplementation with *Ulva* at low levels (5 – 10%) into the experimental diets of a few freshwater and marine finfish have been shown to promote growth, increase nutrient utilization, feed efficiency and body composition (Ergün et al. 2009; Güroy et al. 2013; Wassef et al. 2013; Silva et al. 2015; Valente et al. 2016; Madibana et al. 2017). The dietary supplementation with *Ulva* can also enhance the flavour of farm finfish as well as increase the colouration of cultured shrimp and sea urchin gonads, which can increase the marketability of the product and financial return (Cruz-Suarez et al. 2010; Cyrus et al. 2013, 2015b; Jones et al. 2016; Shpigel et al. 2018b).

Like any other seaweeds, members from the *Ulva* genus have been reported to produce a wide collection of volatile organic compounds (VOCs; *e.g.* Yamamoto et al. 2014; López-Pérez et al. 2017; Sánchez-García et al. 2021). Aldehydes have been reported to be the most prominent group of VOCs produced by the Ulvales (Fujimura et al. 1990; Kajiwara et al. 1992; Roussis et al. 2000; López-Pérez et al. 2017). This group of organic compounds has been reported to originate through a few different pathways and have a low odour threshold that may influence flavour (Akakabe et al. 1999, 2003; Varlet et al. 2007; Yamamoto et al. 2014; Jeleń and Gracka, 2016; Cui et al. 2023). Aldehydes have been suggested to be among the main group of VOCs responsible for the sensory characteristic aroma of *Ulva* (Sugisawa et al. 1990). Species from this genus are also known to produce high concentrations of the osmolyte compound, dimethylsulfoniopropionate (up to 6977 $\mu g.g^{-1}$ fresh *Ulva*; DMSP; Van Alstyne et al. 2001; Smit et al. 2007; Van Alstyne et al. 2007; Kerrison et al. 2012). Studies have found that this compound could act as a feed attractant for marine organisms, including sea urchins (Van Alstyne et al. 2001; Kiehn and Morris, 2010). However, dimethylsulfide (DMS) and acrylic acid formed from DMSP cleavage have been shown to deter feeding in several marine grazers (Van Alstyne et al. 2001; Van Alstyne and Houser 2003; Lyons et al. 2007; Wiesemeier et al. 2007). DMS also produces a highly noticeable and unpleasant smell during the canning process of the aquacultured abalone *Haliotis midae* (Lepetellida) that have been fed on *Ulva* (Smit et al. 2007). On the other hand, consumers perceptions differed regarding the taste of fresh and cooked *Ulva*-fed abalone compared to those raised on formulated feed without seaweed (Smit et al. 2010).

The production of *Ulva* in integrated systems with the South African abalone has proven to be commercially viable, with a continuous and increasing production of this seaweed since 2002 (Bolton et al. 2016). *Ulva* is grown in abalone effluent as a bioremediation tool to enable partial water recirculation and is often used as a primary or supplementary feed for the cultured abalone *H. midae*, with the current annual production of *Ulva* estimated to be around 2000 t fresh weight (Bolton et al. 2009, 2016; Neveux et al. 2018; Rothman et al. 2020). Until now, no research has been done to elucidate the VOCs composition of this seaweed. The identification of VOCs from *Ulva* that may influence the aroma and flavour of the aquacultured species such as the abalone *H. midae* and sea urchin *Tripneustes gratilla* (Camarodonta) can provide local aquaculturists with data that can be used to tail make their products to consumers sensory preference which in term may increase the financial return on product. Therefore, the aim of this study was to evaluate the VOCs produced by aquacultured *U. lacinulata* (Bachoo et al. 2023) using gas chromatography coupled with mass spectrometry (GC-MS). The main objectives were set as follows: 1) extract VOCs from *Ulva* using solvent extraction [SE], steam distillation-liquid/liquid extraction [SD-LLE] and headspace solid-phase microextraction [HS-SPME]; 2) analyse VOC extracts using GC-MS; 3) identity VOCs by matching detected mass spectra with spectra of authentic standards present in the Wiley 275.L library; and 4) quantitate the relative content of identified VOCs from *Ulva*.

5.3 Materials and Methods

5.3.1. Chemicals

All solvents used were of chromatography grade (LiChrosolv®). Methanol (≥ 99.9%; MeOH), dichloromethane (≥ 99.8%; CH_2Cl_2), ethyl acetate (≥ 99.8%; EtOAc) and ethanol (≥ 99.2%; EtOH) were purchased from Merck® (Darmstadt, Germany). Sodium chloride (NaCl) and SPME fiber assembly Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) were obtained from Merck (Darmstadt, Germany).

5.3.2. Algal material

Fresh *U. lacinulata* (Bachoo et al. 2023) was obtained from the Department of Forestry, Fisheries and the Environment (DFFE) Aquaculture Research Facility in Cape Town, South Africa (33°55'13.7"S 18°22'52.1"E), where it was cultured as an experimental material. This alga was initially obtained from the Irvine and Johnson (I & J) Cape Abalone aquafarm, Gansbaai, South Africa (34°37'35.1"S 19°17'47.4"E) as material grown in abalone effluent. Upon receival, the alga was thoroughly cleaned of sediments, epiphytes and visible surface contaminants, and washed several times with de-ionized water. The algal material was then blotted-dried using paper towel to remove excess moisture before VOCs extraction.

5.3.3. Solvent extraction (SE)

Ulva solvent extract was prepared by extracting fresh material using MeOH and CH_2Cl_2 (1:2, *v*/*v*) following a modified protocol by Afolayan et al. (2008). Alga samples (2.0 ± 0.01 g; n=5) were placed in test tubes containing 3 mL MeOH. The test tubes were sonicated (Cole-Parmer Ultrasonic Bath, Chicago, Illinois, USA) in a water bath set at 35 ºC for 10 min. CH_2Cl_2 (6 mL) was added to the test tubes and sonicated for another 10 min. The resulting organic phases were collected through filtration and the remaining algal biomass were re-extracted twice using 9 mL MeOH: CH_2Cl_2 (1:2, *v*/*v*). The total organic phases were combined, and phase separated. The final organic (lower) phase was collected and concentrated *in vacuo* (Büchi® Rotavapor R-210, Switzerland) to produce the crude *Ulva* extracts. The extracts were re-constituted in 1 mL EtOAc and stored in 2 mL amber autosampler vials at -20 ºC until GC-MS analyses.

5.3.4. Steam distillation-liquid/liquid extraction (SD-LLE)

Ulva essential oil was obtained using a Clevenger apparatus following a modified protocol described by Gu et al. (2009). Finely chopped seaweed (800 ± 0.1 g) placed in a round bottom flask containing 2.5 L of distilled water was extracted for 3 hrs at 75 ºC. The resultant effluent liquid was collected and extracted thrice using CH_2Cl_2 (30 mL x 3). The organic phases were combined in an Erlenmeyer flask and concentrated at 30 ºC under a stream of nitrogen. *Ulva* essential oil was re-constituted in 1 mL EtOAc and stored in a 2 mL amber autosampler vial at -20 ºC until GC-MS analyses.

5.3.5 Headspace solid-phase microextraction (HS-SPME)

HS-SPME was performed using Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS; 50/30 µm thickness) fiber. The SPME fiber was conditioned for 30 min at 270 °C following the manufacturer’s instructions prior to use. Several preliminary extractions were conducted to improve the efficiency of the HS-SPME by varying experimental parameters such as extraction temperature (50 and 60 °C), incubation time (20 and 30 min) and extraction time (20 and 30 min), to find the optimal extraction conditions. Fresh *Ulva* samples (2 ± 0.01 g; *n*=5) were placed in 20 mL clear SPME glass vials containing 5 mL NaCl solution (0.1 M; 5% EtOH) and sealed with 20 mm PTFE/silicone headspace crew caps. The vials were treated according to the conditions mentioned above followed by the introduction of SPME fibers that were exposed to the headspace volatiles. The fibers were retracted and subsequently inserted into the injector port of the GC-MS followed by 10 min desorption. After comparing the different detector responses, the optimal extraction conditions were established as follows: extraction temperature of 60 °C; incubation time of 30 min; and extraction time of 30 min.

5.3.6 Gas chromatography-mass spectrometry analysis (GC-MS) conditions

GC-MS analysis of SE, SD-LLE and HS-SPME samples were performed at the Central Analytical Facilities (CAF), Stellenbosch University, South Africa using an Agilent Model 6890 Gas Chromatograph fitted with a PAL COMBI-*xt* autosampler interfaced with a 5975B Mass Spectrometer (Agilent Technologies, Santa Clara, California, USA). Chromatographic separation was achieved on a Restek 10626 Stabilwax capillary column (60 m x 0.25 mm x 0.25 µm). Helium at a column flow rate of 2 mL.min^{-1} was used as a carrier gas at a nominal initial pressure of 191.9 kPa. The GC oven program was as follows: initial temperature of 35 °C for 5 min followed by incremental increases in temperature of 3 °C.min^{-1} to 120 °C and thereafter 8 °C.min^{-1} to 240 °C, and a final incubation at 240 °C for 40 min. The injection volume and temperature for SE and SD-LLE was 1 µL and 240 ºC. For HS-SPME, the vial needle penetration and fiber exposure were 12 mm, while the injection fiber exposure was 22 mm and the injection temperature of 240 ºC after following the optimal extraction conditions as per Section **5.3.5**. SE, SD-LLE and HS-SPME were analysed in splitless mode. Detection was performed in full scan mode, with a mass range of 30 – 500 m/z. Electron ionization was employed with collision energy of 70 eV and the mass spectrometer ion source was maintained at 230 °C. Data acquisitions were performed using Agilent MSD ChemStation (E.02.01.1177; Agilent Technologies, Santa Clara, California, USA) software. Visualization and data processing was performed using OpenChrom® Lablicate Edition (ver. 1.4.0.202102180818; Hamburg, Germany).

5.3.7 Qualitative and quantitative analysis

Tentative identification of VOCs was performed by matching mass spectra with spectra of authentic standards present in the Wiley 275.L (Wiley, New York, USA) and Mass Spectral Library and National Institute of Standards and Technology (NIST) Chemistry WebBook SRD 69 (https://webbook.nist.gov/). When no spectrum for comparison was available, no identification was proposed. Mass spectra of peaks identified as common contaminants were manually inspected and removed from the dataset. The relative content of identified VOC was expressed as percentage peak area relative to the total peak area ± standard error. Number of replicates (*n*) for HS-SPME= 5, SD-LLE= 3 and SE= 3.

5.4 Results

Sixty-three VOCs classed as hydrocarbons (alkanes and alkenes), aldehydes, ketones, esters, free fatty acids, alcohols and aromatic compound were identified from aquacultured *U. lacinulata* using solvent extraction (SE), steam distillation-liquid/liquid extraction (SD-LLE) and headspace solid-phase microextraction (HS-SPME; Figure **5.1**). A total of 48 VOCs were identified from HS-SPME extracts followed by 30 compounds from SD-LLE and 9 VOCs from SE extracts of *Ulva*. Outcomes from the GC-MS analysis showed that aldehydes were the most prominent group of VOCs associated with aquacultured *Ulva* with 18 compounds, followed by 15 hydrocarbons, 10 ketones, 9 esters, 6 free fatty acids, 4 alcohols and 1 aromatic compound.

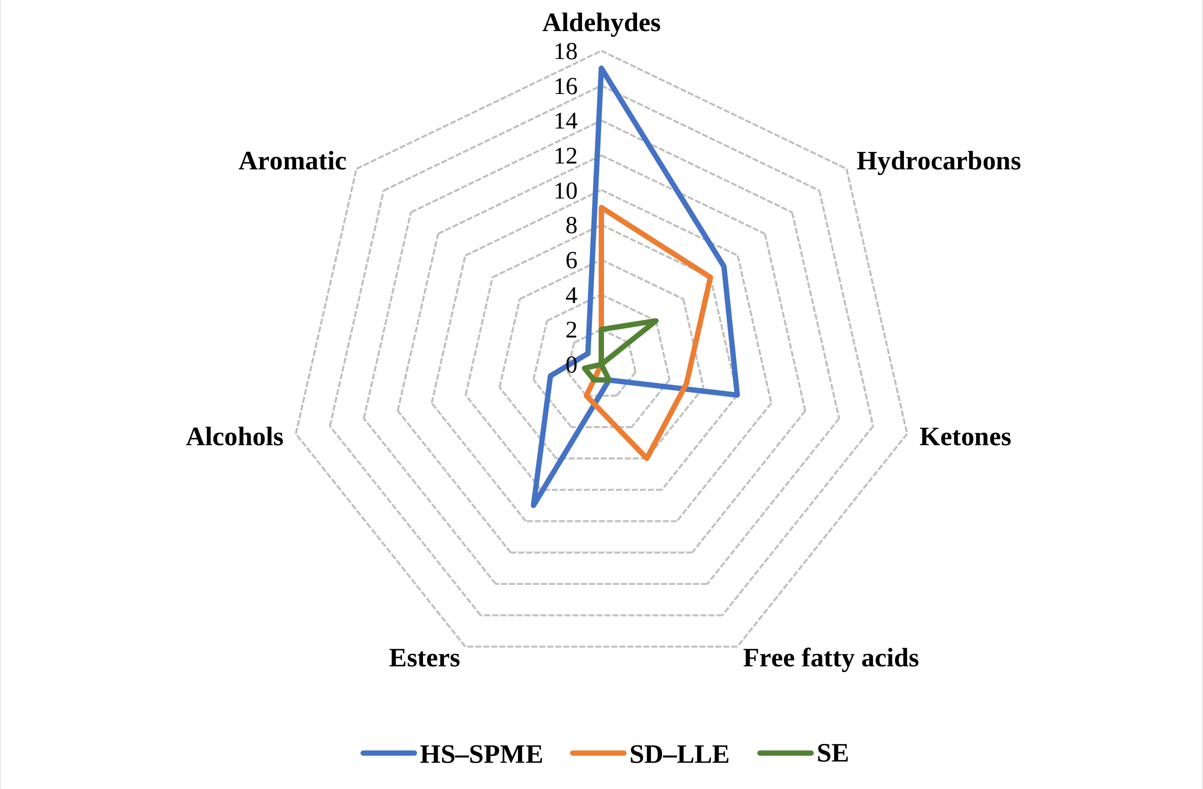


Figure 5.1: Chemical classification of VOCs identified from aquacultured *Ulva lacinulata* (SE= solvent extraction; SD-LLE= steam distillation-liquid/liquid extraction; HS-SPME= headspace solid-phase microextraction).

Table 5.1: VOCs identified from *Ulva lacinulata* from headspace solid-phase microextraction [HS-SPME; *n*=5], steam distillation-liquid/liquid extraction [SD-LLE; *n*=3] and solvent extraction [SE; *n*=3]. Values are presented as % relative content ± standard error. RT= retention time. ND= not detected.

No.	Compounds	Molecular Formula	RT (min)	HS-SPME (%)	SD-LLE (%)	SE (%)
Aldehydes						
1	n-Hexanal	$C_6H_{12}O$	13.22	0.55 ± 0.06	ND	ND
2	(*E*)-2-Pentenal	C_5H_8O	15.98	0.24 ± 0.02	ND	ND
3	n-Heptanal	$C_7H_{14}O$	18.92	0.55 ± 0.06	ND	ND
4	(*E*)-2-Hexenal	$C_6H_{10}O$	20.95	0.56 ± 0.23	ND	ND
5	n-Nonanal	$C_9H_{18}O$	29.90	0.30 ± 0.05	ND	ND
6	(*E*)-2-Octenal	$C_8H_{14}O$	31.61	0.68 ± 0.12	0.20 ± 0.05	ND
7	(*E*,*E*)-2,4-Heptadienal	$C_7H_{10}O$	34.49	1.56 ± 0.49	1.39 ± 0.38	ND
8	Benzaldehyde	C_7H_6O	35.64	1.55 ± 0.37	ND	ND
9	(*E*,*Z*)-2,6-Nonadienal	$C_9H_{14}O$	38.60	0.63 ± 0.09	0.53 ± 0.17	ND
10	1-Cyclohexene-1-carboxaldehyde, 2,6,6-trimethyl-	$C_{10}H_{16}O$	40.33	4.52 ± 0.94	ND	ND
11	n-Dodecanal	$C_{12}H_{24}O$	44.55	0.12 ± 0.10	ND	ND
12	(*E*,*E*)-2,4-Decadienal	$C_{10}H_{16}O$	47.40	0.65 ± 0.15	1.99 ± 0.25	ND
13	n-Tetradecanal	$C_{14}H_{28}O$	47.56	1.54 ± 0.61	ND	ND
14	n-Tridecanal	$C_{13}H_{26}O$	47.58	ND	1.86 ± 0.21	ND
15	n-Hexadecanal	$C_{16}H_{32}O$	49.80[a]	1.69 ± 0.47	20.08 ± 3.76	ND
16	n-Pentadecanal	$C_{15}H_{30}O$	51.61[b]	9.85 ± 2.82	2.03 ± 0.53	12.99 ± 3.81
17	(*Z*)-13-Octadecenal	$C_{18}H_{34}O$	52.07	2.28 ± 0.70	2.14 ± 0.47	ND
18	(*Z*)-Octadec-9-enal	$C_{18}H_{34}O$	54.95	2.31 ± 0.30	5.14 ± 0.98	8.10 ± 1.92
Total (%)				29.58 ± 5.35	28.36 ± 1.16	21.08 ± 5.65
Hydrocarbons						
19	Nonane, 2-methyl-	$C_{10}H_{22}$	8.53	ND	0.89 ± 0.60	ND
20	n-Decane	$C_{10}H_{22}$	9.80	ND	1.68 ± 0.32	2.42 ± 3.42

[a] n-Hexadecanal eluted at 51.74 min for SD-LLE samples; [b] n-Pentadecanal eluted at 49.83 min for SD-LLE samples.

Table 5.1 (Continues)

21	Undecane, 5-methyl-	$C_{12}H_{26}$	11.20	ND	ND	1.73 ± 2.73
22	(3*E*,5*Z*)-octa-1,3,5-triene	C_8H_{12}	14.27	0.20 ± 0.15	ND	ND
23	n-Undecane	$C_{11}H_{24}$	14.90	ND	0.52 ± 0.09	ND
24	1,3-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-	$C_{10}H_{16}$	16.35	0.52 ± 0.16	ND	ND
25	n-Dodecane	$C_{12}H_{26}$	19.09[c]	0.20 ± 0.20	0.37 ± 0.09	2.57 ± 3.62
26	n-Tridecane	$C_{13}H_{28}$	25.75	ND	0.29 ± 0.05	ND
27	n-Tetradecane	$C_{14}H_{30}$	30.34	ND	0.55 ± 0.08	ND
28	n-Pentadecane	$C_{15}H_{32}$	34.84	0.38 ± 0.32	ND	ND
29	n-Hexadecane	$C_{16}H_{34}$	39.33	0.37 ± 0.05	ND	ND
30	8-Heptadecene	$C_{17}H_{34}$	44.95	1.42 ± 0.36	5.27 ± 0.67	24.25 ± 3.22
31	(*E*)-5-Octadecene	$C_{18}H_{36}$	45.78	0.67 ± 0.17	ND	ND
32	n-Butylcyclopentane	C_9H_{18}	46.39	0.54 ± 0.12	ND	ND
33	n-Tetradec-1-ene	$C_{14}H_{28}$	53.64	10.78 ± 2.44	1.78 ± 0.32	ND
Total (%)				15.09 ± 3.74	11.36 ± 0.25	30.96 ± 11.27
Ketones						
34	2-Heptanone, 6-methyl-	$C_8H_{16}O$	22.05	0.22 ± 0.06	ND	ND
35	3-Octanone	$C_8H_{16}O$	22.90	0.24 ± 0.12	ND	ND
36	Cyclohexanone, 2,2,6-trimethyl-	$C_9H_{16}O$	25.97	0.83 ± 0.16	0.19 ± 0.05	ND
37	5-Hepten-2-one, 6-methyl-	$C_8H_{14}O$	27.34	0.26 ± 0.07	ND	ND
38	(*E*,*E*)-3,5-Octadien-2-one	$C_8H_{12}O$	37.79	ND	0.80 ± 0.20	ND
39	2-Undecanone, 6,10-dimethyl-	$C_{13}H_{26}O$	43.80	0.68 ± 0.21	ND	ND
40	α-Ionone	$C_{13}H_{22}O$	47.96[d]	6.12 ± 2.23	1.40 ± 0.38	ND
41	(*E*)-6,10-dimethyl-5,9- undecadien-2-one	$C_{13}H_{22}O$	48.46	0.68 ± 0.15	ND	ND
42	β-Ionone	$C_{13}H_{20}O$	50.16	5.79 ± 1.53	8.71 ± 2.29	ND
43	6,10,14-Trimethylpentadecan-2-one	$C_{18}H_{36}O$	53.02	ND	0.83 ± 0.10	ND
Total (%)				14.81 ± 4.38	11.94 ± 0.95	-

[c] n-Dodecane eluted at 20.28 min for SE and 20.47 min for SD-LLE samples; [d] α-Ionone eluted at 48.34 min SD-LLE samples.

Table 5.1 (Continues)

Esters						
44	Octanoic acid, ethyl ester	$C_{10}H_{20}O_2$	31.94	0.08 ± 0.12	ND	ND
45	Tetradecanoic acid, ethyl ester	$C_{16}H_{32}O_2$	51.90	2.16 ± 0.45	ND	ND
46	Pentadecanoic acid, ethyl ester	$C_{17}H_{34}O_2$	53.37	1.73 ± 0.75	ND	ND
47	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	54.71[e]	20.87 ± 7.69	0.54 ± 0.05	3.20 ± 0.95
48	9-Hexadecenoic acid, ethyl ester	$C_{18}H_{34}O_2$	55.04	3.24 ± 0.38	ND	ND
49	Heptadecanoic acid, ethyl ester	$C_{19}H_{38}O_2$	55.91	0.32 ± 0.06	ND	ND
50	Octadecanoic acid, ethyl ester	$C_{20}H_{40}O_2$	57.09	0.38 ± 0.07	ND	ND
51	(*E*)-9-Octadecenoic acid ethyl ester	$C_{20}H_{38}O_2$	57.47	3.73 ± 1.00	ND	ND
52	Linoleic acid ethyl ester	$C_{20}H_{36}O_2$	58.92[f]	0.57 ± 0.20	2.21 ± 0.42	ND
Total (%)				33.07 ± 11.38	2.76 ± 0.26	3.20 ± 0.95
Free fatty acids						
53	n-Pentadecanoic acid	$C_{15}H_{30}O_2$	51.31	ND	3.02 ± 0.63	ND
54	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	56.34	1.37 ± 0.50	21.67 ± 7.33	38.69 ± 15.58
55	Octadec-9-enoic acid	$C_{18}H_{34}O_2$	57.03	ND	1.74 ± 1.74	ND
56	n-Dodecanoic acid	$C_{12}H_{24}O_2$	57.33	ND	9.04 ± 6.30	ND
57	n-Tridecanoic acid	$C_{13}H_{26}O_2$	58.69	ND	2.30 ± 1.41	ND
58	n-Tetradecanoic acid	$C_{14}H_{28}O_2$	60.36	ND	7.82 ± 1.80	ND
Total (%)				1.37 ± 0.50	45.58 ± 2.85	38.69 ± 15.58
Alcohols						
59	Octan-3-ol	$C_8H_{18}O$	30.48	4.53 ± 0.42	ND	ND
60	1-Octen-3-ol	$C_8H_{16}O$	32.98	0.05 ± 0.06	ND	ND
61	n-Dodecan-1-ol	$C_{12}H_{26}O$	50.65	0.68 ± 0.11	ND	ND
62	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	$C_{20}H_{40}O$	59.03	ND	ND	6.06 ± 1.48
Total (%)				5.26 ± 0.55	-	6.06 ± 1.48
Aromatic compound						
63	Phenol, 3-ethyl-	$C_8H_{10}O$	20.80	0.83 ± 0.40	ND	ND
Total (%)				0.83 ± 0.40	-	-

[e] Hexadecanoic acid, methyl ester eluted at 54.22 min for SD-LLE and SE samples; [f] Linoleic acid ethyl ester eluted at 55.49 min for SD-LLE samples.

The VOCs identified from HS-SPME, SD-LLE and SE extracts (SE) of *U. lacinulata* are shown in **Table 5.1**. Esters were the most prominent group of VOC identified from the HS-SPME samples (33.07 ± 11.38%) of aquacultured *Ulva*, while free fatty acids were the main contributed of VOC for SD-LLE (45.58 ± 2.85%) and SE (38.69 ± 15.58%) samples. Hexadecanoic acid, methyl ester (20.87 ± 7.69%) was identified as the main VOC from the HS-SPME samples while n-hexadecanoic acid was identified the main VOC from SD-LLE (21.67 ± 7.33%) and SE (38.69 ± 15.58%) samples of *U. lacinulata*. The total ion chromatograms (TIC) of HS-SPME, SD-LLE and SE are available in Supplementary Information **5**.

5.5 Discussion

Volatile organic compounds (VOCs) are the most significant factor of food flavour and freshness (Ferraces-Casais et al. 2013). For seaweed, aroma can be the decisive factor in the overall preference of a species or foodstuffs containing a specific species (Ferraces-Casais et al. 2013; Urlass et al. 2023). In the present study, a total of 63 VOCs were identified from aquacultured *U. lacinulata* using 3 different extraction methods namely solvent extraction (SE), steam distillation-liquid/liquid extraction (SD-LLE) and headspace solid-phase microextraction (HS-SPME). This number was higher than the 41 VOCs identified previously from the headspace extracts of fresh, boiled, vacuumed and steamed *U. rigida* (Sánchez-García et al. 2021) and lower than the 127 VOCs identified previously from dried *U. lactuca* (López-Pérez et al. 2017).

In the current study, aldehydes were identified the main class of VOCs produced by aquacultured *U. lacinulata*. This group of compounds can originate from long-chain fatty acids (polyunsaturated fatty acid; PUFA) through the oxygenation or enzymatic reaction of lipoxygenase (Akakabe et al. 1999, 2003; Varlet et al. 2007). Aldehydes, such as benzaldehyde, can also be formed from the amino acid biosynthesis pathway (Yamamoto et al. 2014). This group of compounds has a low odour threshold, with aromas ranging from unpleasant to sweet and fruity, and thus largely influence the flavour of seafood (Jeleń and Gracka, 2016; Cui et al. 2023). In the current study, a total of 18 aldehydes ranging from straight chain (*e.g.* n-hexanal), branched chain (*e.g.* (*Z*)-13-octadecenal) and aromatic (*e.g.* benzaldehyde) aldehydes were identified from aquacultured *U. lacinulata*. It has been reported that aldehydes such as n-hexanal, (*E*)-2-octenal, (*E*,*Z*)-2,6-nonadienal and (*E*,*E*)-2,4-decadienal may be responsible for the sensory characteristic aroma in *Ulva* (Sugisawa et al. 1990). Aromatic benzaldehyde, also identified in the current study, has been reported to have an ‘almond nutty’ and ‘stone fruit’ aroma (Narain et al. 1990). This compound is also reported to be associated with the desirable aroma of urchin gonad (Rodríguez-Bernaldo De Quirós et al. 2001). Sato et al. (2019) suggested that the presence of compounds such as benzaldehyde, n-decanal and n-nonanal in the gonads of the urchin *Mesocentrotus nudus* (Camarodonta) can be derived from the seaweeds that the urchin consumes in nature. It has been reported that n-pentanal can give boiled shrimp (*Penaeus vannamei* [Decapoda]) a ‘fruity’ flavour (Hu et al. 2021). Cui et al. (2023) reported that when the dietary supplementation of phospholipid for the abalone, *Haliotis discus hannai* (Lepetellida) was higher than 1.75%, there was an increase in the levels of n-propanal, acetaldehyde diethyl acetal, (*E*)-2-pentenal and n-heptanal which resulted in an increase of the ‘fruity’ aroma in the abalone muscle. In the current study, it may be suggested that aldehydes largely contribute to the overall aroma profile of aquacultured *Ulva* mainly due to their abundance in terms of individual compounds and contents within the algal samples.

Hydrocarbons were identified as another main class of VOCs produced by aquacultured *U. lacinulata* in the current study. Aliphatic hydrocarbons such n-alkanes and n-alkenes, can be derived from the degradations of hydroperoxides of unsaturated fatty acids (Pohnert and Boland, 2002; López-Pérez et al. 2017). This group of compounds has previously been documented to be among the most prominent VOCs in *Ulva* species (*e.g*. Yamamoto et al. 2014; López-Pérez et al. 2017). In the current study, 8-heptadecene was one of the most abundant unsaturated hydrocarbons. This compound has been reported to be released from the green seaweed *Bryopsis maxima* (Bryopsidales) after mechanical wounding (Akakabe et al. 2007). Aliphatic hydrocarbons such as n-decane and n-dodecane, detected from the current study, have high odour threshold and therefore, have low significant aroma contribution (Wang et al. 2020; Zhu et al. 2020). Conversely, unsaturated hydrocarbons such as n-tetradec-1-ene and 8-heptadecene, identified from *Ulva* in the current study, may contribute to aroma (Wang et al. 2020). Takagi et al. (2020a) reported that n-decane and n-undecane from the gonads of the urchin *M. nudus* collected from an *Eisenia bicyclis* (Laminariales) kelp bed were described by panelists in gas-chromatography (GC) sniffing analyses as a fishy odour and as the smell of oxidized seaweed and hay, respectively. n-Tridecane identified from the gonads of *M. nudus,* was characterized as ozone odour in GC-sniffing analysis (Sato et al. 2019). In the current study, the presence of unsaturated hydrocarbons such as n-tetradec-1-ene and 8-

heptadecene, present in moderate amounts, can be suggested that hydrocarbons produced by aquacultured *Ulva* can positively contribute to the overall aroma profile of this seaweed.

Ketones were another important aroma contributing class of VOCs identified from aquacultured *U. lacinulata*. Similar to aldehydes, ketones can be generated from the oxidation as well as from the degradation of carotenoids (Yamamoto et al. 2014; Balbas et al. 2015; López-Pérez et al. 2017). Compounds such as α-ionone, β-ionone, cyclohexanone, 2,2,6-trimethyl- and 2-heptanone, 6-methyl-5-hepten-2-one, have been reported to be dominant representatives of ketones found in *Ulva* (Fujimura et al. 1990; Sugisawa et al. 1990; Yamamoto et al. 2014; López-Pérez et al. 2017; Sánchez-García et al. 2019). Ketones have been reported to be important aroma contributors in seaweeds such as *Ulva*, due to their low odour thresholds (Yamamoto et al. 2014; Balbas et al. 2015). Volatiles such as α-ionone and β-ionone have been reported to be valued in the cosmetic and perfume industries due to their 'floral-violet' scents (Rodríguez-Bustamante and Sánchez, 2007; Silva et al. 2010; Lukin et al. 2019; Francezon et al. 2021). Takagi et al. (2020a) characterized 6-methyl-5-hepten-2-one from the gonads of the cultured sea urchin *M. nudus* to a 'citrus-like and sweet' aroma. This VOC along with others have been reported to contribute towards the sweet taste of the gonads of the cultured sea urchin, *M. nudus* (Takagi et al. 2020a). Cui et al. (2023) reported that when the abalone *H. discus hannai* was fed with diets supplemented with 1.75 and 2.25% phospholipids, there was an increase in the acetone and 4-methyl-2-pentanone content, which resulted in a positive effect on the flavour of abalone muscle. In the current study, it may be suggested that ketones can also positively influence to the aroma profile of *U. lacinulata*. However, their contribution may not be as effective as aldehydes due to their relative content.

In the current study, esters were identified among the main contributor of VOC in terms of relative content (33.07 ± 11.38% of HS-SPME samples) in aquacultured *Ulva*. Esters are products of esterification and are derived from carboxylic acids and alcohols (Wang et al. 2013). Similar to the current study, hexadecanoic acid methyl ester has been reported to be among the main VOC produced by seaweeds including *Ulva* (Kamenarska et al. 2006; Yamamoto et al. 2014; Sánchez-García et al. 2019; Nazarudin et al. 2020). This metabolite was identified in Chapter 4 as being the most prominent fatty acid in its fatty acid methyl ester (FAME) form. Esters are important contributors of flavour, which are characterized by an overall 'fruity' aroma (Urlass et al. 2023). Takagi et al. (2020b) reported that the presence of butyl acetate in the gonads of the urchin *M. nudus* fed with the laminarian seaweeds *Saccharina* *japonica* and *Undaria pinnatifida* (Laminariales), can be an important indicator of good flavour from high-quality gonads. The supplementation of the diet of the abalone *H. discus hannai* with phospholipid may enhance the levels of ethyl acetate and butyl propionate and thus improve the fruity aroma of abalone muscle (Cui et al. 2023). Due to the high relative content of esters detected from aquacultured *Ulva*, it may be suggested that this group of VOCs can positively influence the aroma profile of this alga.

Volatile free fatty acids were among the most abundant VOC in terms of relative content (45.58 ± 2.85% of SD-LLE samples) from aquacultured *Ulva*. These compounds can be derived from linoleic and linolenic acid through deoxygenation and catalysis of lipoxygenases, that can further degrade to form small molecular volatile compounds such as aldehydes (Chen et al. 2021; Murali-Baskaran et al. 2022). Similar to the current study, n-hexadecanoic acid has been reported to be among the most prominent volatile fatty acids produced in seaweeds (Kamenarska et al. 2006; Sun et al. 2012; Patra et al. 2017; Yuan et al. 2019; Nazarudin et al. 2020). Volatile free fatty acid such as α-linolenic, linoleic and trihydroxyoctadecenoic acids have been linked to bitterness pea-protein (Gläser et al. 2021). However, due to the high odour threshold and low volatility of free fatty acids, they may only be minor contributor to algal flavour (Sun et al. 2012).

Alcohols were the least prominent group of VOC detected from *U. lacinulata*. Alcohols can be derived from the secondary breakdown of hydroperoxides of unsaturated fatty acids; however some of them can also be produced from carbohydrates by glycolysis and/or from amino acids via the Ehrlich pathway (Girard and Durance, 2000; Giri et al. 2010; Peinado et al. 2014). The odour threshold of volatile alcohols is generally high; therefore they are not considered as major contributors of aroma (Giri et al. 2010). However, branched alcohols such as 1-octen-3-ol, detected in the current study, can contribute to the aroma due to its low odour threshold (Giri et al. 2010). Compounds 2-butanol and 2-ethylhexanol detected from the gonads of the cultured urchin *M. nudus* were described by panelists as a ‘sea urchin-seafood’ aroma in GC-sniffing analyses (Takagi et al. 2020a). Hu et al. (2021) reported that 1-octen-3-ol was described as a ‘fishy-grassy’ like aroma, and was one of the major contributors to the flavour of dried shrimp (*P. vannamei*). Rodríguez-Bernaldo De Quirós et al. (2001) reported that the volatile alcohols produced by the fresh and canned gonad of the urchin *Paracentrotus lividus* (Camarodonta) did not contribute to the overall odour of the gonads. Similarly, Sato et al. (2019) reported that alcohols identified from the gonads of the wild caught urchin *M. nudus*

(Camarodonta) were negligible to the aroma of the gonads. In the current study, it may be suggested that alcohols, depending on the concentration, can contribute to the aroma of aquacultured *Ulva* due to the presence of branched alcohols.

The current study presents preliminary results on the VOCs of aquacultured *U. lacinulata* and final identification can only be done by direct comparison with authentic standards. Findings from this study showed that this alga produces mixture ranging from simple to complex volatile compounds that may contribute to its typical smell. Aldehydes, unsaturated hydrocarbons, ketones, esters and alcohol may be the most important classes of VOCs associated with aroma this alga based on their relative contents. Findings also showed that *U. lacinulata* produces VOCs such as benzaldehyde and 6-methyl-5-hepten-2-one that have been shown to be flavour enhancers for sea urchin gonad (*e.g.* Takagi et al. 2020a). Aquacultured *Ulva* has already been shown to be a suitable supplementary feed for the South African abalone *H. midae* and sea urchin *T. gratilla* (Naidoo et al. 2006; Cyrus et al. 2013, 2015a, b). However, the effect of using this seaweed on the flavour of the marketed products remains to be investigated. Therefore, future studies should focus on the effect of using aquacultured *Ulva* supplementary feed on the organoleptic properties of the aquacultured abalone *H. midae* and sea urchin *T. gratilla,* and determine how they can be manipulated to enhance flavours that will increase their marketability and the financial return on products.

CHAPTER 6

General discussion

6.1 Context

One of the major limitations for commercial aquafeed production is the limited availability of fish meal and fish oil (Salin et al. 2018; Zare et al. 2023). Due to their unsustainability as well as high price, feed materials from captured fisheries are being partially replaced in aquafeed by terrestrial plant-based materials (Naylor et al. 2021; Fernandes et al. 2022). However, this shift has raised concerns on the carbon footprint of aquafeed production as well as on increasing competition with human food production for freshwater, food and land usage (Pahlow et al. 2015; Fry et al. 2016; Wan et al. 2019; Fernandes et al. 2022).

Novel ingredient such as seaweeds have emerged as a potential contender to be used in aquafeed (Cyrus et al. 2013, 2015a, b; Shpigel et al. 2017, 2018b; Anh et al. 2018; Kamunde et al. 2019; Santizo-Taan et al. 2020; Jeong et al. 2023). Seaweeds, such as *Ulva*, can be produced at higher rates with elevated protein contents in land-based IMTA systems, without the need of expensive fertilizers associated with terrestrial plant production (Robertson-Andersson et al. 2011; Lawton et al. 2013; Al-Hafedh et al. 2015; Shpigel et al. 2017, 2018a, b; Wan et al. 2019). Apart from protein content, species from this genus can be good sources of dietary fiber, vitamins, minerals and bioactive compounds, therefore making *Ulva* useful as a functional ingredient in aquafeed (Ortiz et al. 2006; Taboada et al. 2010; Tabarsa et al. 2012; Kendel et al. 2015; Neto et al. 2018; Anjali et al. 2019; Kidgell et al. 2019; Pappou et al. 2022). Formulation of aquafeeds with *Ulva* (up to 20%) have shown favourable results for a number aquacultured species while the inclusion of *Ulva* as a feed supplement at low levels (5 – 10%) in practical feed of a few freshwater and marine finfish have been tested without negative effects (Ergün et al. 2009; Güroy et al. 2013; Wassef et al. 2013; Cyrus et al. 2013, 2015a, b; Silva et al. 2015; Valente et al. 2016; Madibana et al. 2017). In South Africa, *Ulva* produced from abalone effluent is mainly used as a primary or supplementary feed for the abalone *Haliotis midae* (Lepetellida; Bolton et al. 2009, 2016; Neveux et al. 2018). This farmed seaweed has been shown to have a higher protein content that material collected from nature and can have similar amino acid profiles to animal and other plant protein (Robertson-Andersson et al. 2011; Shuuluka et al. 2013). Effluent-grown *Ulva* has also been found to be a suitable supplementary feed for high commercial value macroalgivores such as abalone and sea urchin (Naidoo et al. 2006; Cyrus et al. 2013, 2015a, b; Onomu et al. 2020). Laboratory work performed by (Cyrus et al. 2015a) demonstrated that the incorporation of dried aquacultured *Ulva* in the formulated feed for sea urchin *Tripneustes gratilla* (Camarodonta) at

200 g.kg^{-1} (25.7% protein) was also found to increase the chemosensory properties of the feed acting as a feed stimulant. Up to now, our knowledge on the chemistry and chemical compounds from this seaweed and how they may influence feeding behaviour, was lacking. Identification of compounds that may influence feeding behaviour could be beneficial for the South African aquaculture industry.

Although some terrestrial plant-based feed ingredients, such as soybean meal, have been shown to be effective as alternative protein sources for aquafeed, inclusion at high dietary levels have often raised concerns in terms of poor attractability and palatability (Tantikitti, 2014; Guo et al. 2018; Jannathulla et al. 2021; Peixoto et al. 2022). Novel aquafeed ingredients such as seaweeds can overcome this issue by improving the chemosensory properties of the feed, by acting as stimulants and, therefore, increase feed consumption and daily protein intake (*e.g.* Cyrus et al. 2015a). However, studies to date have only allowed the identification of a few compounds from seaweeds that may act as feed stimulants (*e.g.* Sakata and Ina; 1985; Sakata et al. 1985, 1988, 1989). Chapter 2 of this thesis was aimed at identifying the fractions or compounds responsible for the attractant activity in aquacultured *Ulva*. To achieve this, a multidisciplinary approach comprising behavioural biology and analytical chemistry techniques was adopted to prepare fractions from the crude extract of aquacultured *Ulva* and evaluated their efficacy as chemoattractants for the sea urchin *T. gratilla* in chemosensory trials. Thereafter, compounds and chemical classes in stimulant fractions were further investigated. Findings from the current study showed clear evidence that the sea urchin *T. gratilla* can detect and discriminate chemical cues emitted by different food items (*i.e. Ulva* fractions). This was evident from urchin arrival frequencies in the chemosensory trials, where the animals elicited clear chemical preferences to certain *Ulva* fractions over others. *T. gratilla* demonstrated strong positive chemotactic responses for polar *Ulva* fractions (F8 and F9) compared to semi non-polar (F4 – F7) and non-polar (F1 – F3) fractions, showing that the chemical characteristics of the fractions influenced the preferences of the animals. These results are comparable to the research of Erickson et al. (2006), where a clear feeding preference for the polar extracts of the green alga *Ulva lactuca* was shown by the sea urchin *Echinometra lucunter* (Camarodonta) compared to its non-polar extract that acted as a deterrent. Findings from the current study were also comparable to the work of Bianco et al. (2010), where the polar extract of the brown alga *Canistrocarpus cervicornis* (Dictyotales) was found to significantly attract the urchin, *Lytechinus variegatus* (Camarodonta) compared to its lipophilic extract. In the current study, the chemotactic response of *T. gratilla* to different fractions suggested that solubility and polarity of the fractions played a role in either the attraction or inhibition of the animals. Chemicals emitted from polar fractions (F8 and F9) were mostly likely detected from a distance due to their high-water solubility. Monogalactosyldiacylglycerol (MGDG) was identified as the main compound from fraction F8 while a mixture of compounds including diacylglyceryl-N,N,N-trimethyl-homoserine (DGTS), digalactosyldiacylglycerol (DGDG), sulfoquinovosylmonoacylglycerol (SQMG) dialkylglycerophosphate (PA), and lysophospholipid (LPG) were identified from fraction F9 that could act as feed stimulants for *T. gratilla*. These findings were comparable to those of Sakata et al. (1985) and Sakata et al. (1988) for *U. australis* (as *U. pertusa*). Sakata et al. (1985) found that 1,2-diacylglycerly-4'-O-(N,N,N-trimethyl)-homoserine (DGTS; reported as DGTH) and DGDG found in the ether extract of *U. australis* stimulated the feeding response of the sea hare, *Aplysia juliana* (Aplysiida). Similarly, Sakata et al. (1988) found that the extracts of *U. australis* contained four types of complex lipids namely 6-sulfoquinovosyldiacylglycerol (SQDG), DGDG, DGTS (as DGTH) and 1-monoacylglyceryl-4'-O-(N,N,N-trimethyl)-homoserine (MGTS; reported as MGTH), that can have feed stimulatory effects on the marine gastropods *Haliotis discus hannai* (as *Haliotis discus*; Lepetellida), *Turbo cornutus* (Trochida), *Tegula pfeifferi carpenteri* (as O*mphalius pfeifferi*; Trochida) and the sea hare *A. juliana*. Compounds identified from the current study were also comparable to findings from Sakata and Ina (1985) and Sakata et al. (1989), where glycolipids from other types of seaweeds were found to influence feeding. DGDG and phospholipid phosphatidylcholine found in the methanolic extract of the brown kelp *Undaria pinnatifida* (Laminariales) were reported to have a feed stimulatory effect on the abalone *H. discus hannai* (as *H. discus*; Sakata and Ina, 1985). DGDG, MGDG, SQDG and phosphatidylcholine identified from the brown seaweed *Eisenia bicyclis* (as *Ecklonia bicyclis*; Laminariales), could act as feed stimulant for the sea urchin *Strongylocentrotus intermedius* (Camarodonta; Sakata et al. 1989).

Polar *Ulva* fractions F8 and F9 were further investigated for their minimum effective concentration (MEC) required to stimulate the sea urchin *T. gratilla* to feed as well as to directly compare their effectiveness against crude *Ulva* extract (CE), fresh (FU) and dry *Ulva* in chemosensory trials (Chapter 3). This study was formulated to determine whether it would advantageous and practical to use *Ulva* extracts over fresh or dry seaweed. Findings showed that all the treatments that includes fractions F8 and F9, to some extent, elicited positive chemoattraction and feed stimulation responses from *T. gratilla*. However, based on the data collected from this study and the findings from Chapter 2, fraction F8 appears to be a more potent feed stimulant than F9. Findings from this study may be compared to the research of Sakata et al. (1988), where the organisms under investigation showed marked feed stimulatory effects to the dosage of the individual glycerolipids isolated from *U. australis* (as *U. pertusa*). Sakata et al. (1988) reported that the gastropods *T. cornutus* and *T. pfeifferi carpenteri* (as *O. pfeifferi*) showed marked sensitivity towards the dosages of the glycerolipids DGDG, DGTS (as DGTH), MGTS (as MGTH) and SQDG identified from the extracts of *U. australis* (as *U. pertusa*). The researchers found that for *T. cornutus*, DGDG was stimulatory between 15-25 µg, DGTS at 24 µg, MGTS between 25-50 µg and SGDG between 20-40, while for *T. pfeifferi carpenteri*, DGDG was stimulatory between 18-23 µg, DGTS at < 10 µg, MGTS at < 23 µg and SGDG at < 20 µg. Outcomes from this experiment may also be compared to findings from Sakata et al. (1989), where the sea urchin *S. intermedius* showed different stimulatory levels towards the glycerolipids isolated from the brown seaweed *E. bicyclis* (as *E. bicyclis*). Findings from the current study showed that *Ulva* in its fractionated state (*i.e. Ulva* fractions) may not be as effective as *Ulva* in its unpurified state (*i.e.* crude extract, fresh and dried *Ulva*). It has been suggested that in the aquatic environment, chemical stimuli seldom occur as isolates, instead they are released from plants or animals tissues in blends and feeding stimulation may occur by a mixture of compounds rather than one dominant stimulus (Carr, 1988; Carr and Derby, 1986; Weissburg et al. 2002; Hay, 2009, 2010). In the current study, it was concluded that the mixture of chemical stimuli released from the fresh *Ulva* treatment was more potent than the compounds emitted by polar fractions F8 and F9.

Although the lipid contents of seaweeds (*Ulva* species 0.3 – 4.1% of dry weight) are low, they are considered as an important source of omega-3 polyunsaturated fatty acids (ω-3 PUFA) that are known to have health benefits (Ortiz et al. 2006; Tabarsa et al. 2012; Swanson et al. 2012; Kendel et al. 2015; Sakamoto et al. 2019; Magdugo et al. 2020). However, lipid contents and fatty acids are known to vary with seasonal changes and environmental factors such as temperature, salinity, light and nutrient availability (Floreto et al. 1993; Khotimchenko and Yakovleva, 2005; Gerasimenko et al. 2010; McCauley et al. 2018; Santos et al. 2019; Toth et al. 2020). Therefore, knowledge on the variability of the lipid content, lipid classes and fatty acid contents of aquacultured *Ulva* is crucial considering its importance to the South African aquaculture industry and its potential future use in the food application industry (Chapter 4). Findings from the current study showed that nutrient concentrations did not have a significant effect on the specific growth rates (SGR) and total lipid (TL) contents of aquacultured *Ulva*. A potential explanation for this indifference was that the seawater nutrient levels coupled with high-water turnover rate of the culture system utilized in this study were sufficient for *Ulva* to be nutrient saturated for growth under ambient nutrient levels. TL contents of *Ulva* recorded from this study (3.91 ± 0.29 to 4.61 ± 0.64% DW) were within the range documented in the literature for different *Ulva* species (3.14 – 4.14% DW; *e.g.* Rohani-Ghadikolaei et al. 2012; Khairy and El-Shafay, 2013; El Maghraby and Fakhry, 2015; Gadberry et al. 2018) and were higher than *Ulva* materials cultured in IMTA systems elsewhere (0.23 – 3.40% DW; *e.g.* Mata et al. 2016; Shpigel et al. 2018b; Moreira et al. 2020; Queirós et al. 2021). *Ulva* from this study was characterized by higher amounts of glycolipids (GL) over neutral lipids (NL) and phospholipids (PL), which were comparable to the findings from Santos et al. (2019) for *Ulva* collected from the wild. *Ulva* grown in the highest nutrient concentration (*i.e.* 200% NE) had significantly lower GL and significantly higher NL content than *Ulva* cultured in lower nutrient levels (*i.e.* 0 and 25% NE). Therefore, it is not recommended to cultivate *Ulva* under nutrient enrichment above seawater concentrations (NH_4^+ = 2.43 – 8.96 μmol.L^{-1}; NO_3^- = 0.35 – 4.84 μmol.L^{-1}; NO_2^- = 0.28 – 0.49 μmol.L^{-1} in this experiment) if the purpose of biomass is intended to be used as a functional feed ingredient (*i.e.* attractant and stimulant) for the sea urchin *T. gratilla*, since it inhibits the production of glycolipids. Nutrient variation did not have a significant effect on the PL content of aquacultured *Ulva*.

Total fatty acid (TFA) compositions of *Ulva* cultured in this study was in agreement with other species from the literature (saturated FA [SFA]> polyunsaturated FA [PUFA] > monounsaturated FA [MUFA]; *e.g.* Kendel et al. 2015; Paiva et al. 2017; García-Poza et al. 2022). Similar to Kendel et al. (2015), McCauley et al. (2016) and Trentin et al. (2020) findings for other *Ulva* species, C16:0 (palmitic acid) was the most prominent SFA in aquacultured *Ulva* from this study. PUFAs contents of *Ulva* were characterized by higher amount of omega-3 FA (ω-3; 26.7 – 30.31% TFA) compared to ω-6 FA (2.34 – 3.05% TFA), while MUFA ranged from 9.75% to 10.65% TFA. Palmitic acid (C16:0 FA) is the main SFA in vegetable oils (Shi et al. 2022). Overloading of this FA has been reported to induce inflammatory response of macrophages at the intestine level in mammals (Korbecki and Bajdak-Rusinek, 2019; Gori et al. 2020; Ghezzal et al. 2020). In finfish, the use of vegetable oils, such as palm oil, at high dietary levels as a substitute for fish oil in aquafeeds, can reduce growth, antioxidant capacity and induce inflammation in finfish (Li et al. 2019; Zhang et al. 2020). On the other hand, PUFAs in general have been recognized for their numerous health benefits while there has been a growing interest in the potential health benefits of MUFA (McCauley et al. 2018; Gammone et al. 2019; Saini et al. 2021; Kapoor et al. 2021; Wang et al. 2023). α-linolenic acid (C18:3ω3 – ALA) and linoleic acid (C18:2ω6 – LA) are precursor for the synthesis of other PUFAs including arachidonic acid (C20:4ω6 – AA), eicosapentaenoic acid (C20:5ω3 – EPA) and docosahexaenoic acid (C22:6ω3 – DHA; van Ginneken et al. 2011; Calder, 2017). These two fatty acids are considered essential to humans since they can only be obtained via dietary supplementation (Calder, 2017; Soares et al. 2021). Fatty/oily fish, such as sardine and salmon, represent the primary dietary sources of long-chain PUFAs such as DHA and EPA (Kapoor et al. 2021). However, these fatty/oily fish themselves cannot synthesize DHA and EPA, and these FAs are acquired by feeding down the food chain (Soares et al. 2021). Through aquaculture, there is the potential of increasing and/or retaining endogenous long-chain PUFAs in cultured species either by adapting a transgenic or nutritional/pharmacological/biochemical approach (Tocher, 2015). The use of seaweed such as *Ulva* either as an ingredient in formulated feeds or as a feed supplement can increase the levels long-chain PUFAs in aquacultured species (*e.g.* Mulvaney et al. 2015; Legarda et al. 2021), which can in turn be used as a source of PUFAs to supplement the human diet. In the current study, PUFA together with MUFA contents represented 40.0 – 43.6% of the TFA. These amounts particularly with the presence of high levels of C16:4ω3 (hexadecatetraenoic acid) and C18:3ω3 (α-linolenic acid – ALA) FAs and the low ω-6:ω-3 ratios, which are below the FAO/WHO (1994) maximum recommended ratio of 10:1, makes aquacultured *Ulva* suitable for human and animal health applications

According to the International Standard Organization (ISO 5492), flavour is defined as "*complex combination of the olfactory, gustatory and trigeminal sensations perceived during tasting. The flavour may be influenced by tactile, thermal, painful and/or kinaesthetic effects*", while aroma is defined as "*sensory attribute perceptible by the olfactory organ via the back of the nose when tasting*" (ISO, 2008). Flavour and aroma along with texture are regarded as the main driving factors of the consumer choice and preference for a particular algae and algae-containing products (Moreira et al. 2022; Urlass et al. 2023). The flavour of seaweeds is often associated with the '*umami'* taste which may be derived from water soluble components such as free amino acids and nucleotides (Mouritsen, 2013; Mouritsen et al. 2019; Figueroa et al. 2021). In contrast, aroma such as 'seafood-like', 'fishy', 'marine', 'green grass', 'fatty' or 'honey-like', are derived from volatile organic compounds (VOCs), where some compounds can also have major influence on the flavour (Sun et al. 2012; López-Pérez et al. 2017; Figueroa et al. 2021; Moreira et al. 2022; Li et al. 2023).

In Chapter 5, the volatile organic compounds (VOCs) produced by local aquacultured *Ulva* was evaluated. A total of sixty-three compounds classed as hydrocarbons, aldehydes, ketones, esters, free fatty acids, alcohols and phenol, were tentatively identified. However, based on the relative content (%) and information available from the literature, only the aldehydes, hydrocarbons, ketones, esters and alcohol may contribute to the typical aroma of this seaweed. For *Ulva*, 'seaweed-like', 'marine-like', and 'seafood-like' are among the main terms used to characterize the aroma of species from this genus (Yamamoto et al. 2014; López-Pérez et al. 2017; Sánchez-García et al. 2021). Compounds such as aldehydes, ketones, hydrocarbons, esters, and carboxylic acids, have been reported to be the main contributors of VOCs that can influence aroma in *Ulva* (Sugisawa et al. 1990; Yamamoto et al. 2014; López-Pérez et al. 2017, 2021; Sánchez-García et al. 2019). Utilization of *Ulva* as an ingredient in human food products has so far produced mixed reviews on their texture and sensory attributes by panellists (Castillejo et al. 2017; Nuñez and Picon, 2017; del Olmo et al. 2018; Jannat-Alipour et al. 2019; Oh et al. 2020).

Aquatic organisms are characterized by distinctive flavours that set them apart from terrestrial animals (Jones et al. 2022). The organoleptic properties of seafood, whether wild-caught or aquacultured, are influenced by diet before harvest (Boyle et al. 1993; Whitfield et al. 1997; 2002; Ma et al. 2005; Jones et al. 2016). Thought aquaculture products have been found to be highly desirable, consumers have sometimes reported the flavour differences between aquacultured and wild-caught organisms (Hall and Amberg, 2013; Claret et al. 2014; Jones et al. 2016; Parma et al. 2019; Calanche et al. 2020). Traditional aquaculture systems are designed to enhance somatic growth through the use of formulated feeds, which lack the diversity of feed types the wild-caught organisms consume that may contribute to characteristic seafood flavours (Jones et al. 2016). However, through the manipulation of diet and environment, modern aquaculture systems may have the potential of modifying flavour (Jones et al. 2022). *Ulva* species are known to produce olfactory compounds such as bromophenols (BPs), dimethylsulfoniopropionate (DMSP; up to 6977 $\mu g.g^{-1}$ fresh *Ulva*) and dimethylsulfide (DMS; Flodin et al. 1999, Sugisawa et al. 1990, Smit et al. 2007, 2010). It has been demonstrated that supplementation of diets with BPs and DMS-containing seaweeds during the pre-harvest can enhance the characteristic seafood flavour in aquacultured finfish (Ma et al. 2005; Kim et al. 2007; Jones et al. 2016). Flavour enhancement has also been shown in sea urchin, where supplementation of diets with a specific seaweed can enhance the flavour of the end product (Takagi et al. 2020a). With the rise of the use of terrestrial plant-based products in aquafeed, the organoleptic qualities of aquacultured species can become an issue with the lack of key flavours and aromas (Jones et al. 2016). This problem can be overcome with the use of seaweed, such as *Ulva*, as ingredients in aquafeeds.

6.2 Conclusion and future perspectives

Findings from this study clearly showed that aquacultured *Ulva lacinulata* can be an effective chemoattractant and feed stimulant for the sea urchin *Tripneustes gratilla*. The chemoreceptive ability of *T. gratilla* to detect and discriminate chemical cues emitted by specific *Ulva* fractions (*i.e.* F8 and F9) with chemoattractant and feed stimulant properties can be advantageous in the formulation and supplementation of their diet to promote consumption. In the eventuality of the use of F8 and F9 fractions in formulated feed to increase its chemosensory properties or act as a feed stimulant, much consideration should be given to their concentrations. It was shown in this study that chemoattractant and feed stimulant properties of these fractions are dose-dependent and are not as effective as fresh *Ulva* as a dietary supplement. However, their use may be more practical and cost effective that using fresh *Ulva*. Findings from this study also showed that aquacultured *Ulva* is rich in polar lipids (glycolipids and phospholipids) and the important fatty acid profile of *Ulva*, consisting of essential PUFAs C18:3ω3 (α-linolenic acid – ALA) and linoleic acid (C18:2ω6 – LA) and non-essential long chain PUFAs C16:4ω3 (hexadecatetraenoic acid), arachidonic acid (C20:4ω6 – AA) and C20:5ω3 (eicosapentaenoic acid – EPA), suggest that it can be used as a sustainable source of biomass for human and animal food health applications. Lastly, this research showed that aquacultured *Ulva* produces an array of volatile organic compounds which may contribute to its typical aroma. With the growth in sea urchin production around the world, the use of aquafeed is expected to increase. It is of upmost importance that these formulated feeds promote a good growth rate of the target organism without hindering the health of the animals and the quality of the end product intended for human consumption. With the progress in modern aquaculture and feed manufacturing technologies, there is the potential of tail making products with the desired appearance, specific nutritional traits and flavour through the manipulation of the diets. There is clear evidence on the multiple benefits of using novel ingredients such as seaweeds in formulated feeds. With the growing interest looking at the commercial application of *Ulva* worldwide, findings from this research can be of particular interest for the aquaculture, animal feed and human food industries. This thesis can serve as a guide when considering the chemosensory properties during aquafeed formulation, as the chemosensory properties during the development of feeds containing seaweeds are seldom considered. Findings from this thesis can also provide aquaculturists with valuable information on how the use of fertilizers during *Ulva* culture affects its lipid profile and fatty acid composition, especially if the biomass produced is intended for human and animal feed. With *Ulva* being an important crop in the South African and global aquaculture industry, future research should focus on further evaluating the chemoattractant and stimulant properties of *Ulva* fraction F8 and F9 in formulated feed for *T. gratilla*, to test whether it would have the same effects. It would also be worth investigating the effects of *Ulva* diets against commercially used aquafeeds on the lipid and fatty profiles as well as organoleptic properties of the aquacultured abalone *H. midae* and sea urchin *T. gratilla*. With the increase in demand for aquaculture products, the overall nutritional quality as well as the chemosensory properties of the products can become the main deciding factor for market acceptance.

REFERENCES

Abdel-Warith, A.A-W., Younis, M.I.E. & Al-Asgah, A.N. 2016. Potential use of green macroalgae *Ulva lactuca* as a feed supplement in diets on growth performance, feed utilization and body composition of the African catfish, *Clarias gariepinus*. *Saudi Journal of Biological Sciences.* 23:404–9. DOI: 10.1016/j.sjbs.2015.11.010

Addis, P., Pasquini, V., Angioni, A., Malavasi, V., Moccia, D. & Solari, P. 2023. *Ulva* as potential stimulant and attractant for a valuable sea urchin species: a chemosensory study. *Journal of Applied Phycology*. 35:1407–15. DOI: 10.1007/s10811-023-02925-0

Adeleke, B., Robertson-Andersson, D., Moodley, G. & Taylor, S. 2020. Aquaculture in Africa: a comparative review of Egypt, Nigeria, and Uganda Vis-À-Vis South Africa. *Reviews in Fisheries Science & Aquaculture.* 29:167–97. DOI: 10.1080/23308249.2020.1795615

Afolayan, A.F., Bolton, J.J., Lategan, C.A., Smith, P.J., & Beukes, D.R. 2008. Fucoxanthin, tetraprenylated toluquinone and toluhydroquinone metabolites from *Sargassum heterophyllum* inhibit the *in vitro* growth of the malaria parasite *Plasmodium falciparum*. *Zeitschrift für Naturforschung C.* 63:848–52. DOI: 10.1515/znc-2008-11-1211

AgriSETA (Agriculture Sector Training Authority). 2020. Aquaculture sector sub-sector skills plan 2020-2021. Pretoria, South Africa.

Akakabe, Y., Matsui, K. & Kajiwara, T. 1999. Enantioselective α-hydroperoxylation of long-chain fatty acids with crude enzyme of marine green alga *Ulva pertusa. Tetrahedron Letters.* 40:1137–40. DOI: 10.1016/S0040-4039(98)02547-7

Akakabe, Y., Matsui, K. & Kajiwara, T. 2003. 2,4-Decadienals are produced via (*R*)-11-HPITE from arachidonic acid in marine green alga *Ulva conglobata*. *Bioorganic & Medicinal Chemistry.* 11:3607–9. DOI: 10.1016/s0968-0896(03)00364-x

Akakabe, Y., Iwamoto, S., Miyamura, S. & Kajiwara, T. 2007. Induction of (Z)-8-heptadecene in marine green alga *Bryopsis maxima* by mechanical wounding. *Fisheries Science.* 73:1057–60. DOI: 10.1111/j.1444-2906.2007.01436.x

Akakabe, Y. & Kajiwara, T. 2008. Bioactive volatile compounds from marine algae: feeding attractants. *Journal of Applied Phycology.* 20:661–4. DOI: 10.1007/978-1-4020-9619-8_26

Akbary, P. & Aminikhoei, Z. 2018. Effect of water-soluble polysaccharide extract from the green alga *Ulva rigida* on growth performance, antioxidant enzyme activity, and immune stimulation of grey mullet *Mugil cephalus*. *Journal of Applied Phycology.* 30:1345–53. DOI: 10.1007/s10811-017-1299-8

Al-Hafedh, Y.S., Alam, A. & Buschmann, A.H. 2015. Bioremediation potential, growth and biomass yield of the green seaweed, *Ulva lactuca* in an integrated marine aquaculture system at the Red

Sea coast of Saudi Arabia at different stocking densities and effluent flow rates. *Reviews in Aquaculture*. 7:161–71. DOI: 10.1111/raq.12060

Al-Souti, A., Gallardo, W., Claereboudt, M. & Mahgoub, O. 2019. Attractability and palatability of formulated diets incorporated with chicken feather and algal meals for juvenile gilthead seabream, *Sparus aurata*. *Aquaculture Reports*. 14:100199. DOI: 10.1016/j.aqrep.2019.100199

Amiel, A., Tremblay-Franco, M., Gautier, R., Ducheix, S., Montagner, A., Arnaud Polizzi, A., Debrauwer, L., Guillou, H., Bertrand-Michel, J. & Canlet, C. 2020. Proton NMR enables the absolute quantification of aqueous metabolites and lipid classes in unique mouse liver samples. *Metabolites*. 10:9 DOI: 10.3390%2Fmetabo10010009

Amsler, C.D., McClintock, J.B. & Baker, B.J. 1998. Chemical defense against herbivory in the antarctic marine macroalgae *Iridaea cordata* and *Phyllophora antarctica* (Rhodophyceae). *Journal of Phycology*. 34:53–9. DOI: 10.1046/j.1529-8817.1998.340053.x

Angell, A.R., Pirozzi, I., de Nys, R. & Paul, N.A. 2012. Feeding preferences and the nutritional value of tropical algae for the abalone *Haliotis asinina*. *PLoS ONE*. 7:e38857. DOI: 10.1371/journal.pone.0038857

Angell, A.R., Mata, L., de Nys, R. & Paul, N.A. 2015. Indirect and direct effects of salinity on the quantity and quality of total amino acids in *Ulva ohnoi* (Chlorophyta). *Journal of Phycology*. 51:536–45. DOI: 10.1111/jpy.12300

Angell, A.R., Paul, N.A. & de Nys, R. 2017. A comparison of protocols for isolating and concentrating protein from the green seaweed *Ulva ohnoi*. *Journal of Applied Phycology*. 29:1011–26. DOI: 10.1007/s10811-016-0972-7

Anh, N.T.N., Hai, T.N. & Hien, T.T.T. 2018. Effects of partial replacement of fishmeal protein with green seaweed (*Cladophora* spp.) protein in practical diets for the black tiger shrimp (*Penaeus monodon*) postlarvae. *Journal of Applied Phycology*. 30:2649–58. DOI: 10.1007/s10811-018-1457-7

Anjali, K.P., Sangeetha, B.M., Devi, G., Raghunathan, R. & Dutta, S. 2019. Bioprospecting of seaweeds (*Ulva lactuca* and *Stoechospermum marginatum*): The compound characterization and functional applications in medicine - a comparative study. *Journal of Photochemistry and Photobiology B: Biology*. 200:111622. DOI: 10.1016/j.jphotobiol.2019.111622

Arafiles, K.H.V., Iwasaka, H., Eramoto, Y., Okamura, Y., Tajima, T., Matsumura, Y., Nakashimada, Y & Aki, T. 2014. Value-added lipid production from brown seaweed biomass by two-stage fermentation using acetic acid bacterium and thraustochytrid. *Applied Microbiology and Biotechnology*. 98:9207–16. DOI: 10.1007/s00253-014-5980-4

Arisz, S.A., van Himbergen, J.A., Musgrave, A., van den Ende, H. & Munnik, T. 2000. Polar glycerolipids of *Chlamydomonas moewusii*. *Phytochemistry*. 53:265–70. DOI: 10.1016/s0031-9422(99)00505-1

Azaza, M.S., Mensi, F., Kammoun, W., Abdelouaheb, A., Brini, B. & Kraiem, M. 2009. Nutritional evaluation of waste date fruit as partial substitute for soybean meal in practical diets of juvenile Nile tilapia, *Oreochromis niloticus* L. *Aquaculture Nutrition.* 15:262–72. DOI: 10.1111/j.1365-2095.2008.00591.x

Bacchetti, T., Annibaldi, A., Comitini, F., Ciani, M., Damiani, E., Norici, A., Tiano, L., Truzzi, C. & Olivotto, I. 2020. Alternative ingredients for feed and food. *In* Longhi, S., Monteriù, A., Freddi, A., Aquilanti, L., Ceravolo, M.G., Carnevali, O., Giordano, M. & Moroncini, G. [Eds.] *Outstanding 50 Years of "Università Politecnica delle Marche": Research Achievements in Life Sciences.* Springer, Cham, Switzerland, pp. 529–45. DOI: 10.1007/978-3-030-33832-9_34

Bachoo, T., Bolton, J.J., Macey, B.M., Kandjengo, L. & Reddy, M.M. 2023. Resolving the identity of commercially cultivated *Ulva* (Ulvaceae, Chlorophyta) in integrated seaweed-abalone aquaculture farms in South Africa. *Journal of Phycology*. 59:1272–83. DOI: 10.1111/jpy.13391

Baker, C.F., Montgomery, J.C. & Dennis, T.E. 2002. The sensory basis of olfactory search behavior in banded kokopu (*Galaxias fasciatus*). *Journal of Comparative Physiology A.* 188:553–60. DOI: 10.1007/s00359-002-0329-3

Balbas, J., Hamid, N., Liu, T., Kantono, K., Robertson, J., White, W.L., Ma, Q. & Lu, J. 2015. Comparison of physicochemical characteristics, sensory properties and volatile composition between commercial and New Zealand made wakame from *Undaria pinnatifida. Food Chemistry*. 186:168–75. DOI: 10.1016/j.foodchem.2015.03.079

Barbosa, J.P., Teixeira, V.L. & Pereira, R.C. 2004. A dolabellane diterpene from the brown alga *Dictyota pfaffii* as chemical defense against herbivores. *Botanica Marina*. 47:147–51. DOI: 10.1515/BOT.2004.015

Barlow, L.A. 1990. Electrophysiological and behavioral responses of larvae of the red abalone (*Haliotis rufescens*) to settlement-inducing substances. *Bulletin of Marine Science*. 46:537–54.

Barrilero, R., Gil, M., Amigó, N., Dias, C.B., Wood, L.G., Garg, M.L., Ribalta, J., Heras, M., Vinaixa, M. & Correig, X. 2018. LipSpin: A new bioinformatics tool for quantitative ^{1}H NMR lipid profiling. *Analytical Chemistry*. 90:2031–40 DOI: 10.1021/acs.analchem.7b04148

Batista, S., Pereira, R., Oliveira, B., Baião, L.F., Jessen, F., Tulli, F., Messina, M., Silva, J.L., Abreu, H. & Valente, L.M.P. 2020. Exploring the potential of seaweed *Gracilaria gracilis* and microalga *Nannochloropsis oceanica*, single or blended, as natural dietary ingredients for European seabass *Dicentrarchus labrax*. *Journal of Applied Phycology*. 32:2041–59. DOI: 10.1007/s10811-020-02118-z

Benes, K.M. & Bracken, M.E.S. 2016. Nitrate uptake varies with tide height and nutrient availability in the intertidal seaweed *Fucus vesiculosus*. *Journal of Phycology*. 52:863–76. DOI: 10.1111/jpy.12454

Bews, E., Booher, L., Polizzi, T., Long, C., Kim, J-H., Edwards, M.S. 2021. Effects of salinity and nutrients on metabolism and growth of *Ulva lactuca*: Implications for bioremediation of coastal watersheds. *Marine Pollution Bulletin.* 166:112199. DOI: 10.1016/j.marpolbul.2021.112199

Bhaskar, N., Kinami, T., Miyashita, K., Park, S-B., Endo, Y. & Fujimoto, K. 2004. Occurrence of conjugated polyenoic fatty acids in seaweeds from the Indian Ocean. *Zeitschrift für Naturforschung C.* 59:310–4. DOI: 10.1515/znc-2004-5-602

Bianco, E.M., Teixeira, V.L. & Pereira, R.C. 2010. Chemical defenses of the tropical marine seaweed *Canistrocarpus cervicornis* against herbivory by sea urchin. *Brazilian Journal of Oceanography.* 58:213–8.

Björnsäter, B.O. & Wheeler, P.A. 1990. Effect of nitrogen and phosphorus supply on growth and tissue composition of *Ulva fenestrata* and *Enteromorpha intestinalis* (Ulvales, Chlorophyta). *Journal of Phycology.* 26:603–11. DOI: 10.1111/j.0022-3646.1990.00603.x

Bligh, E.G. & Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology.* 37:911–5. DOI: 10.1139/o59-099

Boderskov, T., Rasmussen, M.B., Cassard, C.H., Svensgaard, J., Enevoldsen, L.N. & Bruhn, A. 2022. Comparing effects of nutrient sources approved for organic seaweed production on hatchery stage development of sugar kelp, *Saccharina latissimi. Algal Research.* 61:102602. DOI: 10.1016/j.algal.2021.102602

Bolivar, R.B., Jimenez, E.B.T. & Brown, C.L. 2006. Alternate-day feeding strategy for Nile tilapia grow out in the Philippines: Marginal Cost-Revenue Analyses. *North American Journal of Aquaculture.* 68:192–7. DOI: 10.1577/A05-012.1

Bolker, B.M., Brooks, M.E., Clark, C.J., Geange, S.W., Poulsen, J.R., Stevens, H.H. & White, J.S.S. 2009. Generalized linear mixed models: a practical guide for ecology and evolution. *Trends in Ecology and Evolution.* 24:127–35. DOI: 10.1016/j.tree.2008.10.008

Bolser, R.C. & Hay, M.E. 1996. Are tropical plants better defended? Palatability and defenses of temperate vs. tropical seaweeds. *Ecology.* 77:2269–86. DOI: 10.2307/2265730

Bolton, J.J., Robertson-Andersson, D.V., Shuuluka, D. & Kandjengo, L. 2009. Growing *Ulva* (Chlorophyta) in integrated systems as a commercial crop for abalone feed in South Africa: A swot analysis. *Journal of Applied Phycology.* 21:575–83. DOI: 10.1007/s10811-008-9385-6

Bolton, J.J., Davies-Coleman, M.T. & Coyne, V.E. 2013. Innovative processes and products involving marine organisms in South Africa. *African Journal of Marine Science.* 35:449–64. DOI: 10.2989/1814232X.2013.830990

Bolton, J.J., Cyrus, M.D., Brand, M.J., Joubert, M. & Macey, B.M. 2016. Why grow *Ulva*? Its potential role in the future of aquaculture. *Perspectives in Phycology.* 3:113–20. DOI: 10.1127/pip/2016/0058

Bolton, J.J. 2020. The problem of naming commercial seaweeds. *Journal of Applied Phycology.* 32:751–8. DOI: 10.1007/s10811-019-01928-0

Boriss, H., Boersma, M. & Wiltshire, K. 1999. Trimethylamine induces migration of waterfleas. *Nature*. 398:382. DOI: 10.1038/18796

Boyle, J.L., Lindsay, R.C. & Stuiber, D.A. 1993. Occurrence and properties of flavor-related bromophenols found in the marine environment: a review. *Journal of Aquatic Food Product Technology*. 2:75–112. DOI: 10.1300/J030v02n02_06

Brand, M.J. 2023. *Ulva* as a functional feed: A practical investigation into the effects of *Ulva lacinulata* on the growth, consumption, health and gut microbiota of the farmed abalone *Haliotis midae*. PhD Thesis, University of Cape Town, South Africa, 234 pp.

Brundu, G. & Chindris, A. 2018. Nutrients uptake and growth of *Ulva lactuca* (Linnaeus, 1753) in grey mullet (*Mugil cephalus*) wastewater versus natural estuarine water. *Chemistry and Ecology*. 34:495–505: DOI: 10.1080/02757540.2018.1452918

Buck, B.H. & Shpigel, M. 2023. ULVA: Tomorrow's "Wheat of the sea", a model for an innovative mariculture. *Journal of Applied Phycology*. 35:1967–70. DOI: 10.1007/s10811-023-03003-1

Burdett-Coutts, V. & Metaxas, A. 2004. The effect of the quality of food patches on larval vertical distribution of the sea urchins *Lytechinus variegatus* (Lamarck) and *Strongylocentrotus droebachiensis* (Mueller). *Journal of Experimental Marine Biology and Ecology*. 308:221–36. DOI: 10.1016/j.jembe.2004.02.023

Cai, J., Lovatelli, A., Aguilar-Manjarrez, J., Cornish, L., Dabbadie, L., Desrochers, A., Diffey, S., Garrido Gamarro, E., Geehan, J., Hurtado, A., Lucente, D., Mair, G., Miao, W., Potin, P., Przybyla, C., Reantaso, M., Roubach, R., Tauati, M. & Yuan, X. 2021. *Seaweeds and microalgae: an overview for unlocking their potential in global aquaculture development*. Food and Agriculture Organization Fisheries and Aquaculture (FAO) Circular No. 1229, Rome, Italy, 48 pp. DOI: 10.4060/cb5670en

Calanche, J.B., Beltrán, J.A. & Hernández Arias, A.J. 2020. Aquaculture and sensometrics: the need to evaluate sensory attributes and the consumers' preferences. *Reviews in Aquaculture*. 12:805–21. DOI: 10.1111/raq.12351

Calder, P.C. 2017. Omega-3 fatty acids and inflammatory processes: from molecules to man. *Biochemical Society Transactions*. 45:1105–15. DOI: 10.1042/BST20160474

Campos, A., Souza, C.B., Lhullier, C., Falkenberg, M., Schenkel, E.P., Ribeiro-do-Valle, R.M. & Siqueira, J.M. 2012. Anti-tumour effects of elatol, a marine derivative compound obtained from red algae *Laurencia microcladia*. *Journal of Pharmacy and Pharmacology*. 64:1146–54 DOI: 10.1111/j.2042-7158.2012.01493.x

Cardoso, C., Ripol, A., Afonso, C., Freire, M., Varela, J., Quental-Ferreira, H., Pousão-Ferreira, P. & Bandarra, N. 2017. Fatty acid profiles of the main lipid classes of green seaweeds from fish pond aquaculture. *Food Science and Nutrition*. 5:1186–94. DOI: 10.1002/fsn3.511

Carr, W.E.S. & Thompson, H.W. 1983. Adenosine 5′-monophosphate, an internal regulatory agent, is a potent chemoattractant for a marine shrimp. *Journal of Comparative Physiology*. 153:47–53. DOI: 10.1007/BF00610341

Carr, W.E.S. & Derby, C.D. 1986. Chemically stimulated feeding behavior in marine animals. *Journal of Chemical Ecology*. 12:989–1011. DOI: 10.1007/BF01638992

Carr, W.E.S. 1988. The Molecular Nature of Chemical Stimuli in the Aquatic Environment. *In* Atema, J., Fay, R.R., Popper, A.N. & Tavolga, W.N. [Eds.] *Sensory Biology of Aquatic Animals*. Springer, New York, NY, pp. 3–27. DOI: 10.1007/978-1-4612-3714-3_1

Carr, W.E.S., Netherton III, J.C., Gleeson, R.A. & Derby, C.D. 1996. Stimulants of feeding behavior in fish: analyses of tissues of diverse marine organisms. *The Biological Bulletin*. 190:149–60. DOI: 10.2307/1542535

Casal-Porras, I., Jiménez-Ramos, R., Zubía, E. & Brun, F.G. 2021. Importance of the chemical defenses and sugars in the feeding preference of *Paracentrotus lividus* over two sympatric template seagrass species. *Estuarine, Coastal and Shelf Science*. 259:107466. DOI: 10.1016/j.ecss.2021.107466

Castillejo, N., Martínez-Hernández, G.B., Goffi, V., Gómez, P. A., Aguayo, E., Artés, F. & Artés-Hernández, F. 2017. Natural vitamin B12 and fucose supplementation of green smoothies with edible algae and related quality changes during their shelf life. *Journal of the Science of Food and Agriculture*. 98:2411–21. DOI: 10.1002/jsfa.8733

Chang, Y.-Q., Lawrence, J.M., Cao, X.-B. & Lawrence, A. 2005. Food consumption, absorption, assimilation and growth of the sea urchin *Strongylocentrotus intermedius* fed a prepared feed and the alga *Laminaria japonica*. *Journal of the World Aquaculture Society*. 36:68–75. DOI: 10.1111/j.1749-7345.2005.tb00132.x

Chen, X., Luo, J., Lou, A., Wang, Y., Yang, D. & Shen, Q.W. 2021. Duck breast muscle proteins, free fatty acids and volatile compounds as affected by curing methods. *Food Chemistry*. 338:128138. DOI: 10.1016/j.foodchem.2020.128138

Chopin T., Troell, M., Reid, G.K., Knowler, D., Robinson, S.M.C., Neori, A., Buschmann, A.H., Pang, S.J. & Fang, J.-C. 2010. Integrated Multi-Trophic Aquaculture (IMTA)– a responsible practice providing diversified seafood products while rendering biomitigating services through its extractive components. *In* Franz, N. & Schmidt, C-C. [Eds.] *Proceedings OECD Workshop 'Advancing the Aquaculture Agenda: Policies to Ensure a Sustainable Aquaculture Sector.* Organization for Economic Co-operation and Development, Paris, France, pp. 195–217. DOI: 10.1787/9789264088726-15-en

Chopin, T., Cooper, J.A., Reid, G., Cross, S. & Moore, C. 2012. Open-water integrated multi-trophic aquaculture: environmental biomitigation and economic diversification of fed aquaculture by extractive aquaculture. *Reviews in Aquaculture*. 4:209–20. DOI: 10.1111/j.1753-5131.2012.01074.x

Claret, A., Guerrero, L., Ginés, R., Grau, A., Hernández, M.D., Aguirre, E., Peleteiro, J.B., Fernández-Pato, C. & Rodríguez-Rodríguez, C. 2014. Consumer beliefs regarding farmed versus wild fish. *Appetite*. 79:25–31. DOI: 10.1016/j.appet.2014.03.031

Coelho, S.M., Rijstenbil, J.W. & Brown, M.T. 2000. Impacts of anthropogenic stresses on the early development stages of seaweeds. *Journal of Aquatic Ecosystem Stress and Recovery*. 7: 317–33. DOI: 10.1023/A:1009916129009

Cohen, I. & Neori, A. 1991. *Ulva lactuca* biofilters for marine fishpond effluents. I. Ammonia uptake kinetics and nitrogen content. *Botanica Marina*. 34:475–82. DOI: 10.1515/botm.1991.34.6.475

Cole, A.J., de Nys, R. & Paul, N.A. 2014. Removing constraints on the biomass production of freshwater macroalgae by manipulating water exchange to manage nutrient flux. *PLoS ONE* 9:e101284. DOI: 10.1371/journal.pone.0101284

Coleman, R.A., Ramchunder, S.J., Davies, K.M., Moody, J. & Foggo, A. 2007. Herbivore-induced infochemicals influence foraging behaviour in two intertidal predators. *Oecologia*. 151:454–63. DOI: 10.1007/s00442-006-0601-2

Copertino, M.S., Cheshire, A. & Kildea, T. 2009. Photophysiology of a turf algal community: integrated responses to ambient light and standing biomass. *Journal of Phycology*. 45:324–36. DOI: 10.1111/j.1529-8817.2009.00664.x

COST (European Cooperative in Science and Technology). 2022. CA20106 - Tomorrow's 'wheat of the sea': *Ulva*, a model for an innovative mariculture (seawheat). Available at: https://www.cost.eu/actions/CA20106/

Cronin, G. & Hay, M.E. 1996. Susceptibility to herbivores depends on recent history of both the plant and animal. *Ecology*. 77:1531–43. DOI: 10.2307/2265549

Cruz-Rivera, E. & Hay, M.E. 2000. Can quantity replace quality? Food choice, compensatory feeding, and fitness of marine mesograzers. *Ecology*. 81:201–19. DOI: 10.1890/0012-9658(2000)081[0201:CQRQFC]2.0.CO;2

Cruz-Rivera, E. & Hay, M.E. 2001. Macroalgal traits and the feeding and fitness of an herbivorous amphipod: the roles of selectivity, mixing, and compensation. *Marine Ecology Progress Series*. 218:249–66. DOI: 10.3354/meps218249

Cruz-Suárez, L.E., León, A., Peña-Rodríguez, A., Rodríguez-Peña, G., Moll, B. & Ricque-Marie, D. 2010. Shrimp/*Ulva* co-culture: A sustainable alternative to diminish the need for artificial feed and improve shrimp quality. *Aquaculture*. 301:64–8. DOI: 10.1016/j.aquaculture.2010.01.021

Cui, Z., Liu, C., Rao, W., Chen, P., Lei, K., Mai, K. & Zhang, W. 2023. Dietary phospholipids improve growth performance and change the lipid composition and volatile flavor compound profiles in the muscle of abalone *Haliotis discus hannai* by affecting the glycerophospholipid metabolism. *Aquaculture Reports*. 30:101567. DOI: 10.1016/j.aqrep.2023.101567

Cyrus, M.D., Bolton, J.J., Macey, B.M. & De Wet, L. 2013. The development of a formulated feed containing *Ulva* (Chlorophyta) to promote rapid growth and enhanced production of high quality roe in the sea urchin *Tripneustes gratilla* (Linnaeus). *Aquaculture Research.* 45:159–76. DOI: 10.1111/j.1365-2109.2012.03219.x

Cyrus, M.D., Bolton, J.J, Scholtz, R. & Macey, B.M. 2015a. The advantages of *Ulva* (Chlorophyta) as an additive in sea urchin formulated feeds: effects on palatability, consumption and digestibility. *Aquaculture Nutrition.* 21:578–91. DOI: 10.1111/anu.12182

Cyrus, M.D., Bolton, J.J. & Macey, B.M. 2015b. The role of the green seaweed *Ulva* as a dietary supplement for full life-cycle grow-out of *Tripneustes gratilla*. *Aquaculture.* 446:187–97. DOI: 10.1016/j.aquaculture.2015.05.002

Cyrus, M.D., Bolton, J.J. & Macey, B.M. 2020. The use of stable isotope ratios $\delta^{13}C$ and $\delta^{15}N$ to track the incorporation of *Ulva* and other important dietary ingredients into the gonads of the sea urchin *Tripneustes gratilla*. *Aquaculture Nutrition.* 26:174–85. DOI: 10.1111/anu.12979

da Costa, E., Melo, T., Moreira, A.S.P., Bernardo, C., Helguero, L., Ferreira, I., Cruz, M.T., Rego, A.M., Domingues, P., Calado, R., Abreu, M.H. & Domingues, M.R. 2017. Valorization of lipids from *Gracilaria* sp. through lipidomics and decoding of antiproliferative and anti-inflammatory activity. *Marine Drugs*. 15:62. DOI: 10.3390/md15030062

da Costa, E., Melo, T., Reis, M., Domingues, P., Calado, R., Abreu, M.H. & Domingues, M.R. 2021. Polar lipids composition, antioxidant and anti-inflammatory activities of the Atlantic red seaweed *Grateloupia turuturu*. *Marine Drugs.* 19:414. DOI: 10.3390/md19080414

Dawczynski, C., Schubert, R. & Jahreis, G. 2007. Amino acids, fatty acids, and dietary fibre in edible seaweed products. *Food Chemistry.* 103:891–9. DOI: 10.1016/j.foodchem.2006.09.041

de Alencar, D.B., Diniz, J.C., Rocha, S.A.S., dos Santos Pires-Cavalcante, K.M., Freitas, J.O., Nagano, C.S., Sampaio, A.H. & Saker-Sampaio, S. 2017. Chemical composition of volatile compounds in two red seaweeds, *Pterocladiella capillacea* and *Osmundaria obtusiloba*, using static headspace gas chromatography mass spectrometry. *Journal of Applied Phycology.* 29:1571–6. DOI: 10.1007/s10811-016-1020-3

de Cruz, C.R., Lubrano, A. & Gatlin, D.M. 2018. Evaluation of microalgae concentrates as partial fishmeal replacements for hybrid striped bass *Morone* sp. *Aquaculture.* 493:130–6. DOI: 10.1016/j.aquaculture.2018.04.060

De Prisco, J.A. 2019. An investigation of some key physico-chemical water quality parameters of an Integrated Multi-Trophic Aquaculture (IMTA) system operating recirculation methodology in the Western Cape of South Africa. MSc Thesis, University of Cape Town, South Africa, 66 pp. Available at: https://open.uct.ac.za/handle/11427/32653

De Vos, B.C., Cyrus, M.D., Bolton, J.J. & Macey, B.M. 2024. Effect of basket height and stocking density on production of the sea urchin *Tripneustes gratilla*: insights and recommendations. *Aquaculture International*. DOI: 10.1007/s10499-024-01412-8

De Vos, B.C., Cyrus, M.D., Macey, B.M., Batik, T. & Bolton, J.J. 2023. Combining computer vision and standardised protocols for improved measurement of live sea urchins for research and industry. *Aquaculture, Fish and Fisheries*. 3:507–17. DOI: 10.1002/aff2.137

Deal, M.S., Hay, M.E., Wilson, D. & Fenical, W. 2003. Galactolipids rather than phlorotannins as herbivore deterrents in the brown seaweed *Fucus vesiculosus*. *Oecologia*. 136:107–14. DOI: 10.1007/s00442-003-1242-3

Del Coco, L., Felline, S., Girelli, C.R., Angilè, F., Magliozzi, L., Almada, F., D'Aniello, B., Mollo, E., Terlizzi, A. & Fanizzi, F.P. 2018. ^{1}H NMR spectroscopy and MVA to evaluate the effects of caulerpin-based diet on *Diplodus sargus* lipid profiles. *Marine Drugs*. 16:390 DOI: 10.3390%2Fmd16100390

del Olmo, A., Picon, A. & Nuñez, M. 2018. Cheese supplementation with five species of edible seaweeds: Effect on microbiota, antioxidant activity, colour, texture and sensory characteristics. *International Dairy Journal*. 84:36–45. DOI: 10.1016/j.idairyj.2018.04.004

Demetropoulos, C.L. & Langdon, C.J. 2004. Enhanced production of Pacific dulse (*Palmaria mollis*) for coculture with abalone in a land-based system: nitrogen, phosphorus, and trace metal nutrition. *Aquaculture*. 235:433–55. DOI: 10.1016/j.aquaculture.2003.09.010

Derby, C.D. & Ache, B.W. 1984. Electrophysiological identification of the stimulatory and interactive components of a complex odorant. *Chemical Senses*. 9:201 – 18. DOI: 10.1093/chemse/9.3.201

Derby, C.D. & Sorensen, P.W. 2008. Neural processing, perception, and behavioral responses to natural chemical stimuli by fish and crustaceans. *Journal of Chemical Ecology*. 34:898–914. DOI: 10.1007/s10886-008-9489-0

Devine, D.V. & Atema, J. 1982. Function of chemoreceptor organs in spatial orientation of the lobster, *Homarus americanus*: differences and overlap. *The Biological Bulletin*. 163:144–53. DOI: 10.2307/1541504

DFFE (Department of Forestry, Fisheries and the Environment). 2016. *Aquaculture yearbook South Africa 2016*. Cape Town, South Africa.

DFFE (Department of Forestry, Fisheries and the Environment). 2020. *Aquaculture yearbook South Africa 2020*. Cape Town, South Africa.

Dimitroglou, A., Merrifield, D.L., Spring, P., Sweetman, J., Moate, R. & Davies, S.J. 2010. Effects of mannan oligosaccharide (MOS) supplementation on growth performance, feed utilisation, intestinal histology and gut microbiota of gilthead sea bream (*Sparus aurata*). *Aquaculture*. 300:182–8. DOI: 10.1016/j.aquaculture.2010.01.015

Domingues, M.R. & Calado, R. 2022. Lipids of Marine Algae-Biomolecules with High Nutritional Value and Important Bioactive Properties. *Biomolecules*. 12:134. DOI: 10.3390/biom12010134

Duffy, J.E. & Hay, M.E. 1994. Herbivore resistance to seaweed chemical defense: the role of mobility and predator risk. *Ecology*. 75:1304–19. DOI: 10.2307/1937456

Duke, C.S., Litaker, W. & Ramus, J. 1989. Effect of temperature on nitrogen-limited growth rate and chemical composition of *Ulva curvata* (Ulvales: Chlorophyta). *Marine Biology*. 100:143–50. DOI: 10.1007/BF00391953

Durmaz, Y., Duyar, H., Gökpınar, Ş., Taşkaya, L., Öğretmen, Y.Ö., Bandarra, N. & Nunes, M. 2008. Fatty acids, α-tocopherol and total pigment contents of *Cystoseira* spp., *Ulva* spp. and *Zostera* spp. from Sinop Bay (Turkey). *International Journal of Natural and Engineering Sciences*. 2:111–4.

Dworjanyn, S.A., Pirozzi, I. & Liu, W. 2007. The effect of the addition of algae feeding stimulants to artificial diets for the sea urchin *Tripneustes gratilla*. *Aquaculture*. 273:624–33. DOI: 10.1016/j.aquaculture.2007.08.023

El Maghraby, D.M. & Fakhry, E.M. 2015. Lipid content and fatty acid composition of Mediterranean macro-algae as dynamic factors for biodiesel production. *Oceanologia*. 57:86–92. DOI: 10.1016/j.oceano.2014.08.001

El-Din, S.M.M. 2019. Temporal variation in chemical composition of *Ulva lactuca* and Corallina mediterranea. *International Journal of Environmental Science and Technology*. 16:5783–96. DOI: 10.1007/s13762-018-2128-6

Elizondo-González, R., Quiroz-Guzmán, E., Escobedo-Fregoso, C., Magallón-Servín, P. & Peña-Rodríguez, A. 2018. Use of seaweed *Ulva lactuca* for water bioremediation and as feed additive for white shrimp *Litopenaeus vannamei*. *PeerJ*. 6:e4459. DOI: 10.7717/peerj.4459

Ergün, S., Soyutürk, M., Güroy, B., Güroy, D. & Merrifield, D. 2009. Influence of *Ulva* meal on growth, feed utilization, and body composition of juvenile Nile tilapia (*Oreochromis niloticus*) at two levels of dietary lipid. *Aquaculture International*. 17:355–61. DOI: 10.1007/s10499-008-9207-5

Erickson, A.A., Paul, V.J., Van Alstyne, K.L. & Kwiatkowski, L.M. 2006. Palatability of macroalgae that use different types of chemical defenses. *Journal of Chemical Ecology*. 32:1883–95. DOI: 10.1007/s10886-006-9116-x

Estruch, G., Tomás-Vidal, A., El Nokrashy, A.M., Monge-Ortiz, R., Godoy-Olmos, S., Cerdá, M.J. & Martínez-Llorens, S. 2018. Inclusion of alternative marine by-products in aquafeeds with different levels of plant-based sources for on-growing gilthead sea bream (*Sparus aurata*, L.): effects on digestibility, amino acid retention, ammonia excretion and enzyme activity. *Archives of Animal Nutrition*. 72:321–39, DOI: 10.1080/1745039X.2018.1472408

FAO/WHO (Food and Agriculture Organization of the United Nations and the World Health Organization Rome). 1994. *Fats and oils in human nutrition*. Rome, Italy.

FAO (Food and Agriculture Organization of the United Nations). 2018. *The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable development goals*. Rome, Italy, pp 1–210.

FAO (Food and Agriculture Organization of the United Nations). 2022. *The State of World Fisheries and Aquaculture 2022 – Towards Blue Transformation.* Rome, Italy, pp 1–236. DOI: 10.4060/cc0461en

Fernandes, H., Martins, N., Vieira, L., Salgado, J.M., Castro, C., Oliva-Teles, A., Belo, I. & Peres, H. 2022. Pre-treatment of *Ulva rigida* improves its nutritional value for European seabass (*Dicentrarchus labrax*) juveniles. *Algal Research.* 66:102803. DOI: 10.1016/j.algal.2022.102803

Fernando, H., Kondraganti, S., Bhopale, K.K., Volk, D.E., Neerathilingam, M., Kaphalia, B.S., Luxon, B.A., Boor, P.J. & Ansari, G.A.S. 2010. ^{1}H and ^{31}P NMR lipidome of ethanol-induced fatty liver. *Alcoholism: Clinical and Experimental Research.* 34:1937–47 DOI: 10.1111%2Fj.1530-0277.2010.01283.x

Fernando, H., Bhopale, K.K., Boor, P.J., Ansari, G.A.S. & Kaphalia, B.S. 2012. Hepatic lipid profiling of deer mice fed ethanol using ^{1}H and ^{31}P NMR spectroscopy: a dose-dependent subchronic study. *Toxicology and Applied Pharmacology.* 264:361–9 DOI: 10.1016/j.taap.2012.07.026

Ferraces-Casais, P., Lage-Yusty, M.A, Rodríguez-Bernaldo de Quirós, A. & López-Hernández, J. 2013. Rapid identification of volatile compounds in fresh seaweed. *Talanta.* 115:798–800. DOI: 10.1016/j.talanta.2013.06.049

Ferreira, M., Teixeira, C., Abreu, H., Silva, J., Costas, B., Kiron, V. & Valente, L.M.P. 2021. Nutritional value, antimicrobial and antioxidant activities of micro- and macroalgae, single or blended, unravel their potential use for aquafeeds. *Journal of Applied Phycology.* 33:3507–18. DOI: 10.1007/s10811-021-02549-2

Fields, D.M., Weissburg, M.J., Browman, H.I. 2007. Chemoreception in the salmon louse *Lepeophtheirus salmonis*: an electrophysiology approach. *Diseases of Aquatic Organisms.* 78:161–8. DOI: 10.3354/dao01870

Figueroa, V., Farfán, M. & Aguilera, J.M. 2021. Seaweeds as novel foods and source of culinary flavors. *Food Reviews International.* 39:1–26. DOI: 10.1080/87559129.2021.1892749

Fink, P., von Elert, E. & Jüttner, F. 2006. Volatile foraging kairomones in the littoral zone: attraction of an herbivorous freshwater gastropod to algal odors. *Journal of Chemical Ecology.* 32:1867–81. DOI: 10.1007/s10886-006-9115-y

Fleurence, J., Gutbier, G., Mabeau, S. & Leray, C. 1994. Fatty acids from 11 marine macroalgae of the French Brittany coast. *Journal of Applied Phycology.* 6:527–32. DOI: 10.1007/BF02182406

Fleurence, J., Morançais, M., Dumay, J., Decottignies, P., Turpin. V., Munier, M., Garcia-Bueno, N. & Jaouen, P. 2012. What are the prospects for using seaweed in human nutrition and for marine animals raised through aquaculture? – *Trends in Food Science & Technology.* 27:57–61. DOI: 10.1016/j.tifs.2012.03.004

Fleurence, J., Morançais, M. & Dumay, J. 2018. Chapter 9 – Seaweed proteins. *In* Yada, R.Y. [Eds.] *Proteins in food processing*. Woodhead Publishing, Sawston, United Kingdom, pp. 245–262. DOI: 10.1016/B978-0-08-100722-8.00010-3

Flodin, C., Helidoniotis, F. & Whitfield, F.B. 1999. Seasonal variation in bromophenol content and bromoperoxidase activity in *Ulva lactuca*. *Phytochemistry*. 51:135–8. DOI: 10.1016/S0031-9422(98)00668-2

Floreto, E.A.T., Hirata, H., Ando, S. & Yamasaki, S. 1993. Effects of temperature, light intensity, salinity and source of nitrogen on the growth, total lipid and fatty acid composition of *Ulva pertusa* Kjellman (Chlorophyta). *Botanica Marina*. 36:149–58. DOI: 10.1515/botm.1993.36.2.149

Floreto, E.A.T., Teshima, S. & Ishikawa, M. 1996. Effects of nitrogen and phosphorus on the growth and fatty acid composition of *Ulva pertusa* Kjellman (Chlorophyta). *Botanica Marina*. 39:69–74. DOI: 10.1515/botm.1996.39.1-6.69

Floreto, E.A.T. & Teshima, S. 1998. The fatty acid composition of seaweeds exposed to different levels of light intensity and salinity. *Botanica Marina*. 41:467–81. DOI: 10.1515/botm.1998.41.1-6.467

Fong, P., Fong, J.J. & Fong, C.R. 2004. Growth, nutrient storage, and release of dissolved organic nitrogen by *Enteromorpha intestinalis* in response to pulses of nitrogen and phosphorus. *Aquatic Botany*. 78:83–95. DOI: 10.1016/j.aquabot.2003.09.006

Fort, A., McHale, M., Cascella, K., Potin, P., Perrineau, M.-M., Kerrison, P.D., da Costa, E., Calado, R., Domingues, M.D.R., Costa Azevedo, I., Sousa-Pinto, I., Gachon, C., van der Werf, A., de Visser, W., Beniers, J.E., Jansen, H., Guiry, M.D. & Sulpice, R. 2022. Exhaustive reanalysis of barcode sequences from public repositories highlights ongoing misidentifications and impacts taxa diversity and distribution. *Molecular Ecology Resources*. 22:86–101. DOI: 10.1111/1755-0998.13453

Francezon, N., Tremblay, A., Mouget, J-L., Pasetto, P. & Beaulieu, L. 2021. Algae as a source of natural flavors in innovative foods. *Journal of Agricultural and Food Chemistry*. 69:11753–72. DOI: 10.1021/acs.jafc.1c04409

Fry, J.P., Love, D.C., MacDonald, G.K., West, P.C., Engstrom, P.M., Nachman, K.E. & Lawrence, R.S. 2016. Environmental health impacts of feeding crops to farmed fish. *Environment International*. 91:201–14. DOI: 10.1016/j.envint.2016.02.022

Fujimura, T., Kawai, T., Shiga, M., Kajiwara, T. & Hatanaka, A. 1990. Long-chain aldehyde production in thalli culture of the marine green alga *Ulva pertusa*. *Phytochemistry*. 29:745–7. DOI: 10.1016/0031-9422(90)80011-5

Fuke, S., Konsu, S. & Ina, K. 1981. Identification of feeding stimulants for red sea bream in the extract of marine worm *Perinereis brevicirrus*. *Bulletin of the Japanese Society of Scientific Fisheries*. 47:1631–5. DOI: 10.2331/suisan.47.1631

Gadberry, B.A., Colt, J., Maynard, D., Boratyn, D.C., Webb, K., Johnson, R.B., Saunders, G.W. & Boyer, R.H. 2018. Intensive land-based production of red and green macroalgae for human consumption in the Pacific Northwest: an evaluation of seasonal growth, yield, nutritional composition, and contaminant levels. *Algae*. 33:109–25. DOI: 10.4490/algae.2018.33.2.21

Gammone, M.A., Riccioni, G., Parrinello, G. & D’Orazio, N. 2019. Omega-3 polyunsaturated fatty acids: benefits and endpoints in sport. *Nutrients.* 11:46. DOI: 10.3390/nu11010046

Gao, G., Clare, A.S., Rose, C. & Gary S. Caldwell, G.S. 2017. Eutrophication and warming driven green tides (*Ulva rigida*) are predicted to increase under future climate change scenarios. *Marine Pollution Bulletin.* 114:439–47. DOI: 10.1016/j.marpolbul.2016.10.003

Gao, G., Clare, A.S., Chatzidimitriou, E., Rose, C. & Caldwell, G. 2018. Effects of ocean warming and acidification, combined with nutrient enrichment, on chemical composition and functional properties of *Ulva rigida*. *Food Chemistry*. 258:71–8. DOI: 10.1016/j.foodchem.2018.03.040

García–Casal, M.N., Pereira, A.C., Leets, I., Ramírez, J. & Quiroga, M.F. 2007. High iron content and bioavailability in humans from four species of marine algae. *Journal of Nutrition.* 137:2691–5. DOI: 10.1093/jn/137.12.2691

Garcia-Jimenez, P., Brito-Romano, O. & Robaina, R.R. 2013. Production of volatiles by the red seaweed *Gelidium arbuscula* (Rhodophyta): emission of ethylene and dimethyl sulfide. *Journal of Phycology*. 49:661–9. DOI: 10.1111/jpy.12083

García-Poza, S., Morais, T., Leandro, A., Cotas, J., Marques, J.C., Pereira, L. & Gonçalves, A.M.M. 2022. A comparative study of the fatty acids and monosaccharides of wild and cultivated *Ulva* sp. *Journal of Marine Science and Engineering.* 10:233. DOI: 10.3390/jmse10020233

Garicano Vilar, E., O'Sullivan, M.G., Kerry, J.P. & Kilcawley, K.N. 2020. Volatile compounds of six species of edible seaweed: a review. *Algal Research.* 45:101740. DOI: 10.1016/j.algal.2019.101740

Garnick, E. 1978. Behavioral ecology of *Strongylocentrotus droebachiensis* (Muller) (Echinodermata: Echinoidea). *Oecologia*. 37:77–84. DOI: 10.1007/BF00349993

Gašparović, B., Lampitt, R.S., Sudasinghe, N. & Schaub, T. 2023. Molecular-level evidence of early lipid transformations throughout oceanic depths. *Geochimica et Cosmochimica Acta.* 343:49–63. DOI: 10.1016/j.gca.2022.12.021

Gerasimenko, N.I., Busarova, N.G. & Moiseenko, O.P. 2010. Seasonal changes in the content of lipids, fatty acids, and pigments in brown alga *Costaria costata*. *Russian Journal of Plant Physiology* 57:205–11. DOI: 10.1134/S102144371002007X

Gerasimenko, N.I., Skriptsova, A.V., Busarova, N.G. & Moiseenko, O.P. 2011. Effects of the season and growth stage on the contents of lipids and photosynthetic pigments in brown alga *Undaria pinnatifida. Russian Journal of Plant Physiology.* 58:885–91. DOI: 10.1134/S1021443711050086

Ghezzal, S., Postal, B.G., Quevrain, E., Brot, L., Seksik, P., Leturque, A., Thenet, S. & Carrière, V. 2020. Palmitic acid damages gut epithelium integrity and initiates inflammatory cytokine production. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids.* 1865:158530. DOI: 10.1016/j.bbalip.2019.158530

Girard, B. & Durance, T. 2000. Headspace volatiles of sockeye and pink salmon as affected by retort process. *Journal of Food Science*. 65:34–9. DOI: 10.1111/j.1365-2621.2000.tb15952.x

Giri, A., Osako, K. & Ohshima, T. 2010. Identification and characterisation of headspace volatiles of fish miso, a Japanese fish meat based fermented paste, with special emphasis on effect of fish species and meat washing. *Food Chemistry*. 120:621–31. DOI: 10.1016/j.foodchem.2009.10.036

Gläser, P., Mittermeier-Kleßinger, V.K., Spaccasassi, A., Hofmann, T. & Dawid, C. 2021. Quantification and bitter taste contribution of lipids and their oxidation products in pea-protein isolates (*Pisum sativum* L.). *Journal of Agricultural and Food Chemistry*. 69:8768–76. DOI: 10.1021/acs.jafc.1c02889

Godlewska, K., Michalak, I., Tuhy, L. & Chojnacka, K. 2016. Plant growth biostimulants based on different methods of seaweed extraction with water. *BioMed Research International*. 5973760. DOI: 10.1155/2016/5973760

Gokulakrishnan, M., Kumar, R., Ferosekhan, S., Siddaiah, G.M., Nanda, S., Pillai, B.R. & Swain, S.K. 2023 Bio-utilization of brewery waste (Brewer's spent yeast) in global aquafeed production and its efficiency in replacing fishmeal: From a sustainability viewpoint. *Aquaculture*. 565:739161. DOI: 10.1016/j.aquaculture.2022.739161

Gómez-Pinchetti, J.L., del Campo Fernández, E., Moreno Díez, P. & Garcia Reina, G. 1998. Nitrogen availability influences the biochemical composition and photosynthesis of tank cultivated *Ulva rigida* (Chlorophyta). *Journal of Applied Phycology*. 10:383–9. DOI: 10.1023/A:1008008912991

Gordillo, F.J.L., Jiménez, C., Goutx, M. & Niell, X. 2001. Effects of CO_2 and nitrogen supply on the biochemical composition of *Ulva rigida* with especial emphasis on lipid class analysis. *Journal of Plant Physiology*. 158:367–73. DOI: 10.1078/0176-1617-00209

Gordillo, F.J.L., Aguilera, J. & Jiménez, C. 2006. The response of nutrient assimilation and biochemical composition of Arctic seaweeds to a nutrient input in summer. *Journal of Experimental Botany*. 57:2661–71. DOI: 10.1093/jxb/erl029

Gori, M., Altomare, A., Cocca, S., Solida, E., Ribolsi, M., Carotti, S., Rainer, A., Francesconi, M., Morini, S., Cicala, M. & Guarino, M.P.L. 2020. Palmitic acid affects intestinal epithelial barrier integrity and permeability *in vitro*. *Antioxidants*. 9:417. DOI: 10.3390/antiox9050417

Gosch, B.J., Magnusson, M., Paul, N.A. & de Nys, R. 2012. Total lipid and fatty acid composition of seaweeds for the selection of species for oil-based biofuel and bioproducts. *GCB Bioenergy*. 4: 919–30. DOI: 10.1111/j.1757-1707.2012.01175.x

Gressler, V., Colepicolo, P. & Pinto, E. 2009. Useful strategies for algal volatile analysis. *Current Analytical Chemistry*. 5:271–92. DOI: 10.2174/157341109788680255

Gu, X., Zhang, Z., Wan, X., Ning, J., Yao, C. & Shao, W. 2009. Simultaneous distillation extraction of some volatile flavor components from Pu-erh tea samples – comparison with steam distillation-liquid/liquid extraction and Soxhlet extraction. *International Journal of Analytical Chemistry*. DOI: 10.1155/2009/276713

Guiry, M.D. & Guiry, G.M. 2023. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. Available at: http://www.algaebase.org (Accessed: 10th November 2023).

Guo, J., Gao, W., Guo, B., Xu, W., Zhang, W. & Mai, K. 2018. Using a selectively bred nongenetically modified soybean meal to replace fishmeal in practical diets for the Pacific white shrimp *Litopenaeus vannamei*. *Aquaculture Nutrition*. 24:1689–97. DOI: 10.1111/anu.12803

Gupta, S. & Abu-Ghannam, N. 2011. Bioactive potential and possible health effects of edible brown seaweeds. *Trends in Food Science & Technology*. 22:315–26. DOI: 10.1016/j.tifs.2011.03.011

Güroy, B., Ergün, S., Merrifield, D.L. & Güroy, D. 2013. Effect of autoclaved *Ulva* meal on growth performance, nutrient utilization and fatty acid profile of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture International*. 21:605–15. DOI: 10.1007/s10499-012-9592-7

Güven, K.C., Percot, A. & Sezik, E. 2010. Alkaloids in Marine Algae. *Marine Drugs*. 8:269–84. DOI: 10.3390/md8020269

Hall, T.E. & Amberg, S.M. 2013. Factors influencing consumption of farmed seafood products in the Pacific northwest. *Appetite*. 66:1–9. DOI: 10.1016/j.appet.2013.02.012

Hammer, B.W., Hammer, H.S., Watts, S.A., Desmond, R.A., Lawrence, J.M. & Lawrence, A.L. 2004. The effects of dietary protein concentration on feeding and growth of small *Lytechinus variegatus* (Echinodermata: Echinoidea). *Marine Biology*. 145:1143–57. DOI: 10.1007/s00227-004-1391-x

Hara, T.J. 1994a. The diversity of chemical stimulation in fish olfaction and gustation. *Reviews in Fish Biology and Fisheries*. 4:1–35. DOI: 10.1007/BF00043259

Hara, T.J. 1994b. Olfaction and gustation in fish: an overview. *Acta Physiologica Scandinavica*. 152:207–17. DOI: 10.1111/j.1748-1716.1994.tb09800.x

Hardege, J.D., Bartels-Hardege, H.D., Fletcher, N., Terschak, J.A., Harley, M., Smith, M.A., Davidson, L., Hayden, D., Müller, C.T., Lorch, M., Welham, K., Walther, T. & Bublitz, R. 2011. Identification of a female sex pheromone in *Carcinus maenas*. *Marine Ecology Progress Series*. 436:177–189. DOI: 10.3354/meps09226

Harrison, P.J. & Hurd, C.L. 2001. Nutrient physiology of seaweeds: application of concepts to aquaculture. *Cahiers de Biologie Marine*. 42:71–82.

Hay, M.E., Lee Jr, R.R., Guieb, R.E.A. & Bennett, M.M. 1986. Food preference and chemotaxis in the sea urchin *Arbacia punctulata* (Lamarck) Philippi. *Journal of Experimental Marine Biology and Ecology*. 96:147–53. DOI: 10.1016/0022-0981(86)90239-X

Hay, M.E., Pawlik, J.R., Duffy, J.E. & Fenical, W. 1989. Seaweed-herbivore-predator interactions: host-plant specialization reduces predation on small herbivores. *Oecologia*. 81:418–27. DOI: 10.1007/BF00377093

Hay, M. E., Piel, J., Boland, W. and Schnitzler, I. 1998. Seaweed sex pheromones and their degradation products frequently suppress amphipod feeding but rarely suppress sea urchin feeding. *Chemoecology*. 8:91–8. DOI: 10.1007/PL00001809

Hay, M.E. 2009. Marine chemical ecology: chemical signals and cues structure marine populations, communities, and ecosystems. *Annual Review of Marine Science*. 1:193–212. DOI: 10.1146/annurev.marine.010908.163708

Hay, M.E. 2010. Chapter 3 – Crustaceans as powerful models in aquatic chemical ecology. *In*: Breithaupt, T. & Thiel, M. [Eds.] *Chemical communication in crustaceans*. Springer, New York, United States of America, pp. 41–62. DOI: 10.1007/978-0-387-77101-4_3

Hayden, H.S., Blomster, J., Maggs, C.A., Silva, P.A., Stanhope, M.J. & Waaland, J.R. 2003. Linnaeus was right all along: *Ulva* and *Enteromorpha* are not distinct genera. *European Journal of Phycology*. 38:277–94. DOI: 10.1080/1364253031000136321

Hayden, H. S. & Waaland, J. R. 2004. A molecular systematic study of *Ulva* (Ulvaceae, Ulvales) from the northeast Pacific. *Phycologia*. 43:364–82. DOI: 10.2216/i0031-8884-43-4-364.1

Heesch, S., Broom, J.E., Neill, K.F., Farr, T.J., Dalen, J.L. & Nelson, W.A. 2009. *Ulva*, *Umbraulva* and *Gemina*: genetic survey of New Zealand taxa reveals diversity and introduced species. *European Journal of Phycology*. 44:143–54, DOI: 10.1080/09670260802422477

Heinsbroek, L.T.N., Van Hooff, P.L.A., Swinkels, W., Tanck, M.W.T., Schrama, J.W. & Verreth, J.A.J. 2007. Effects of feed composition on life history developments in feed intake, metabolism, growth and body composition of European eel, *Anguilla anguilla*. *Aquaculture*. 267:175–87. DOI: 10.1016/j.aquaculture.2007.03.028

Heinsbroek, L.T.N., Goedegebuur, B.J., Bloemhof, G., Flach, R.B. & de Jong, G.B.C. 2008. Gastrointestinal and metabolic effects of feeding schedule on voluntary feed intake and growth of European eel, *Anguilla anguilla*. *Aquaculture International*. 16:93–108. DOI: 10.1007/s10499-007-9128-8

Hinrichsen, E., Walakira, J.K., Langi, S., Ibrahim, N.A., Tarus, V., Badmus, O. & Baumüller, H. 2022. Prospects for aquaculture development in Africa: a review of past performance to assess future potential. *In* Bonn: Center for Development Research (ZEF) Working Paper 211. University of Bonn, Germany. DOI: 10.48565/bonndoc-17

Hirt-Chabbert, J.A., Skalli, A., Young, O.A. & Gisbert, E. 2012. Effects of feeding stimulants on the feed consumption, growth and survival at glass eel and elver stages in the European eel (*Anguilla anguilla*). *Aquaculture Nutrition*. 18:152–66. DOI: 10.1111/j.1365-2095.2011.00883.x

Hofmann, L.C., Nettleton, J.C., Neefus, C.D. & Mathieson, A.C. 2010. Cryptic diversity of *Ulva* (Ulvales, Chlorophyta) in the Great Bay Estuarine System (Atlantic USA): introduced and indigenous distromatic species. *European Journal of Phycology*. 45:230–9. DOI: 10.1080/09670261003746201

Holdt, S.L. & Kraan, S. 2011. Bioactive compounds in seaweed: functional food applications and legislation. *Journal of Applied Phycology*. 23:543–97. DOI: 10.1007/s10811-010-9632-5

Holdt, S.L. & Edwards, M.D. 2014. Cost-effective IMTA: a comparison of the production efficiencies of mussels and seaweed. *Journal of Applied Phycology*. 26:933–45. DOI: 10.1007/s10811-014-0273-y

Holland, K. 1978. Chemosensory orientation to food by a Hawaiian goatfish (*Parupeneus porphyreus*, Mullidae). *Journal of Chemical Ecology*. 4:173–86. DOI: 10.1007/BF00988053

Hossain, M.S., Small, B.C., Kumar, V. & Hardy, R. 2024. Utilization of functional feed additives to produce cost-effective, ecofriendly aquafeeds high in plant-based ingredients. *Reviews in Aquaculture*. 16:121–53. DOI: 10.1111/raq.12824

Hu, M., Wang, S., Liu, Q., Cao, R. & Xue, Y. 2021. Flavor profile of dried shrimp at different processing stages. *LWT-Food Science and Technology*. 146:111403. DOI: 10.1016/j.lwt.2021.111403

Hughey, J.R., Maggs, C.A., Mineur, F., Jarvis, C., Miller, K.A., Shabaka, S.H. & Gabrielson, P.W. 2019. Genetic analysis of the Linnaean *Ulva lactuca* (Ulvales, Chlorophyta) holotype and related type specimens reveals name misapplications, unexpected origins, and new synonymies. *Journal of Phycology*. 55:503–8. DOI: 10.1111/jpy.12860

Hurd, C.L. 2000. Water motion, marine macroalgal physiology, and production. *Journal of Phycology*. 36:453–72. DOI: 10.1046/j.1529-8817.2000.99139.x

Hurd, C. 2017. Shaken and stirred: the fundamental role of water motion in resource acquisition and seaweed productivity. University Of Tasmania. Journal contribution. Available from: https://hdl.handle.net/102.100.100/566066

Igarashi, T., Aursand, M., Sacchi, R., Paolillo, L., Nonaka, M. & Wada, Y. 2002. Determination of docosahexaenoic acid and n-3 fatty acids in refined fish oils by H-NMR spectroscopy: IUPAC Interlaboratory Study. *Journal of AOAC International*. 85:1341–54. DOI: 10.1093/jaoac/85.6.1341

Ishii, T., Okino, T., Suzuki, M. & Machiguchi, Y. 2004. Tichocarpols A and B, two novel phenylpropanoids with feeding-deterrent activity from the red alga *Tichocarpus crinitus*. *Journal of Natural Products* 67: 1764–1766. DOI: 10.1021/np0498509

ISO (International Organization for Standardization). 2008. ISO 5492:2008 Sensory analysis Vocabulary. Geneva, Switzerland. Available at: https://www.iso.org/obp/ui/#iso:std:iso:5492:ed-2:v1:en [Accessed on 10th December 2023]

Ivanova, V., Stancheva, M. & Petrova, D. 2013. Fatty acid composition of Black Sea *Ulva rigida* and *Cystoseira crinita*. *Bulgarian Journal of Agricultural Science*. 19:42–7.

Jacobsen, H.P. & Stabell, O.B. 2004. Antipredator behaviour mediated by chemical cues: the role of conspecific alarm signalling and predator labelling in the avoidance response of a marine gastropod. *Oikos*. 104:43–50. DOI: 10.1111/j.0030-1299.2004.12369.x

Jannat-Alipour, H., Rezaei, M., Shabanpour, B., Tabarsa, M. & Rafipour, F. 2019. Addition of seaweed powder and sulphated polysaccharide on shelf-life extension of functional fish surimi restructured product. *Journal of Food Science and Technology*. 56:3777–89. DOI: 10.1007/s13197-019-03846-y

Jannathulla, R., Sravanthi, O., Imran Khan, H., Syed Moomeen, H., Gomathi, A. & Syama Dayal, J. 2021. Chemoattractants: their essentiality and efficacy in shrimp aquaculture. *Indian Journal of Fisheries*. 68:151–9. DOI: 10.21077/ijf.2021.68.1.95994-20

JASP Team. 2021. JASP (Version 0.15) [Computer software]. Available at: https://jasp-stats.org/download/

Jeleń, H. & Gracka, A. 2016. Chapter 6: Characterization of aroma compounds: structure, physico-chemical and sensory properties. *In* Guichard, E., Salles, C., Morzel M. & Le Bon, A.-M. [Eds.] *Flavour: From Food to Perception*. John Wiley & Sons Ltd, United Kingdom, pp. 126–53. DOI: 10.1002/9781118929384.ch6

Jeong, S-M., Gunathilaka, B.E., Khosravi, S., Cho, M-U., Kwak, I., Han, Y-U. & Lee, S-M. 2023. Effects of *Sargassum horneri* as a substitute for *Undaria pinnatifida* in high plant protein diet for juvenile Pacific abalone (*Haliotis discus discus*, Reeve 1846). *Aquaculture Reports*. 30:101607. DOI: 10.1016/j.aqrep.2023.101607

Jirsa, D., Salze, G.P., Barrows, F.T., Davis, D.A. & Mark Drawbridge, M. 2013. First-limiting amino acids in soybean-based diets for white seabass *Atractoscion nobilis*. *Aquaculture*. 414–415:167–72. DOI: 10.1016/j.aquaculture.2013.07.050

Jobling, M. 2016. Fish nutrition research: past, present and future. *Aquaculture International*. 24:767–86. DOI: 10.1007/s10499-014-9875-2

Jones, B., Smullen, R. & Carton, A.G. 2016. Flavour enhancement of freshwater farmed barramundi (*Lates calcarifer*), through dietary enrichment with cultivated sea lettuce, *Ulva ohnoi*. *Aquaculture*. 454:192–8. DOI: 10.1016/j.aquaculture.2015.12.017

Jones, B.C., Rocker, M.M., Keast, R.S.J., Callahan, D.L., Redmond, H.J., Smullen, R.P. & Francis, D.S. 2022. Systematic review of the odorous volatile compounds that contribute to flavour profiles of aquatic animals. *Reviews in Aquaculture*. 14:1418–77. DOI: 10.1111/raq.12657

Jormalainen, V., Honkanen, T., Vesakoski, O. & Koivikko, R. 2005. Polar extracts of the brown alga *Fucus vesiculosus* (L.) reduce assimilation efficiency but do not deter the herbivorous isopod *Idotea baltica* (Pallas). *Journal of Experimental Marine Biology and Ecology*. 317:143–57. DOI: 10.1016/j.jembe.2004.11.021

Kajiwara, T., Hatanaka, A., Matsui, K., Tomoi, T., Fujimura, T. & Kawai, T. 1992. Distribution of long chain aldehyde-forming activity from fatty acids and changes in its activity with growth in marine algae. *Phytochemistry*. 31:2635–9. DOI: 10.1016/0031-9422(92)83601-T

Kamenarska, Z., Ivanova, A., Stancheva, R., Stoyneva, M., Stefanov, K., Dimitrova-Konaklieva, S. & Popov, S. 2006. Volatile compounds from some Black Sea red algae and their chemotaxonomic application. *Botanica Marina*. 49:47–56. DOI: 10.1515/BOT.2006.006

Kamio, M. & Derby C.D. 2017. Finding food: how marine invertebrates use chemical cues to track and select food. *Natural Product Reports.* 34:514–28. DOI: 10.1039/C6NP00121A

Kamio, M., Yambe, H. & Fusetani, N. 2022. Chemical cues for intraspecific chemical communication and interspecific interactions in aquatic environments: applications for fisheries and aquaculture. *Fisheries Science.* 88:203–39. DOI: 10.1007/s12562-021-01563-0

Kamunde, C., Sappal, R. & Melegy, T.M. 2019. Brown seaweed (AquaArom) supplementation increases food intake and improves growth, antioxidant status and resistance to temperature stress in Atlantic salmon, *Salmo salar*. *PLoS ONE.* 14:e0219792. DOI: 10.1371/journal. pone.0219792

Kang, J.H., Jang, J.E., Kim, J.H., Byeon, S.Y., Kim, S., Choi, S.K., Kang, Y.H., Park, S.R. & Lee, H.J. 2019. Species composition, diversity, and distribution of the genus *Ulva* along the coast of Jeju Island, Korea based on molecular phylogenetic analysis. *PLoS ONE.* 14:e0219958. DOI: 10.1371/journal.pone.0219958

Kankainen, M., Setälä, J., Berrill, I.K., Ruohonen, K., Noble, C. & Schneider, O. 2012. How to measure the economic impacts of changes in growth, feed efficiency and survival in aquaculture. *Aquaculture Economics & Management.* 16:341–64. DOI: 10.1080/13657305.2012.729247

Kapoor, B., Kapoor, D., Gautam, S., Singh, R. & Bhardwaj, S. 2021. Dietary polyunsaturated fatty acids (PUFAs): Uses and potential health benefits. *Current Nutrition Reports.* 10:232–42. DOI: 10.1007/s13668-021-00363-3

Kazi, M.A., Kavale, M.G. & Singh, V.V. 2016. Morphological and molecular characterization of *Ulva chaugulii sp. nov.*, *U. lactuca* and *U. ohnoi* (Ulvophyceae, Chlorophyta) from India. *Phycologia.* 55:45–54. DOI: 10.2216/15-11.1

Kendel, M., Wielgosz-Collin, G., Bertrand, S., Roussakis, C., Bourgougnon, N. & Bedoux, G. 2015. Lipid composition, fatty acids and sterols in the seaweeds *Ulva armoricana*, and *Solieria chordalis* from Brittany (France): an analysis from nutritional, chemotaxonomic, and antiproliferative activity perspectives. *Marine Drugs.* 13:5606–28. DOI: 10.3390/md13095606

Keng, F.S-L., Phang, S-M., Abd Rahman, N., Leedham Elvidge, E.C., Malin, G. & Sturges, W.T. 2020. The emission of volatile halocarbons by seaweeds and their response towards environmental changes. *Journal of Applied Phycology.* 32:1377–94. DOI: 10.1007/s10811-019-02026-x

Kepner, G.R. 2010. Saturation Behavior: a general relationship described by a simple second-order differential equation. *Theoretical Biology and Medical Modelling.* 7:11. DOI: 10.1186/1742-4682-7-11

Kerrison, P., Suggett, D.J., Hepburn, L.J. & Steinke, M. 2012. Effect of elevated pCO_2 on the production of dimethylsulphoniopropionate (DMSP) and dimethylsulphide (DMS) in two species of *Ulva* (Chlorophyceae). *Biogeochemistry.* 110:5–16. DOI: 10.1007/s10533-012-9707-2

Khairy, H.M. & El-Shafay. S.M. 2013. Seasonal variations in the biochemical composition of some common seaweed species from the coast of Abu Qir Bay, Alexandria, Egypt. *Oceanologia.* 55:435–52. DOI: 10.5697/oc.55-2.435

Khotimchenko, S.V. 2003. The fatty acid composition of glycolipids of marine macrophytes. *Russian Journal of Marine Biology*. 29:126–8. DOI: 10.1023/A:1023960825983

Khotimchenko, S.V. & Yakovleva, I.M. 2005. Lipid composition of the red alga *Tichocarpus crinitus* exposed to different levels of photon irradiance. *Phytochemistry*. 66:73–9. DOI: 10.1016/j.phytochem.2004.10.024

Kidawa, A. 2005a. The role of amino acids in phagostimulation in the shallow-water omnivorous Antarctic sea star *Odontaster validus*. *Polar Biology*. 28:147–55. DOI: 10.1007/s00300-004-0664-7

Kidawa, A. 2005b. Behavioural and metabolic responses of the Antarctic sea star *Odontaster validus* to food stimuli of different concentration. *Polar Biology*. 28:449–55. DOI: 10.1007/s00300-004-0705-2

Kidgell, J.T., Magnusson, M., de Nys, R. & Glasson, C.R.K. 2019. Ulvan: A systematic review of extraction, composition and function. *Algal Research*. 39:101422. DOI: 10.1016/j.algal.2019.101422

Kiehn, W.M. & Morris, J.T. 2010. Variability in dimethylsulfoniopropionate (DMSP) concentrations in *Spartina alterniflora* and the effect on *Littoraria irrorata*. *Marine Ecology Progress Series*. 406:47–55. DOI: 10.3354/meps08548

Kim, J-S., Ma, W.C.J. & Chung, H.Y. 2007. Enhancement of bromophenol content in cultivated green grouper (*Epinephelus coioides*). *Fisheries and Aquatic Sciences*. 10:113–8. DOI: 10.5657/fas.2007.10.3.113

Kim, S-K., Pangestuti, R. & Rahamadi, P. 2011. Chapter 5 - Sea lettuces: culinary uses and nutritional value. *In* Kim, S-K. [Ed.] *Advances in food and nutrition research. Marine medicinal foods: implications and applications, macro and microalgae*. Academic Press, Waltham, pp 57–68. DOI: 10.1016/B978-0-12-387669-0.00005-3

Kim, J.K., Duston, J., Corey, P. & Garbary, D.J. 2013. Marine finfish effluent bioremediation: effects of stocking density and temperature on nitrogen removal capacity of *Chondrus crispus* and *Palmaria palmata* (Rhodophyta). *Aquaculture*. 414–415:210-6. DOI: 10.1016/j.aquaculture.2013.08.008.

Kintzing, M.D. & Butler, M.J. 2014. The influence of shelter, conspecifics, and threat of predation on the behavior of the long-spined sea urchin (*Diadema antillarum*). *Journal of Shellfish Research*. 33:781–5. DOI: 10.2983/035.033.0312

Klinger, T.S. & Lawrence, J.M. 1984. Phagostimulation of *Lytechinus variegatus* (Lamarck) (Echinodermata: Echinoidea). *Marine Behaviour and Physiology*. 11:49–67. DOI: 10.1080/10236248409387034

Klongklaew, N., Praiboon, J., Tamtin, M. & Srisapoome, P. 2021. Chemical composition of a hot water crude extract (HWCE) from *Ulva intestinalis* and its potential effects on growth performance, immune responses, and resistance to white spot syndrome virus and yellowhead virus in Pacific white shrimp (*Litopenaeus vannamei*). *Fish & Shellfish Immunology*. 112:8–22. DOI: 10.1016/j.fsi.2021.02.004

Korbecki, J. & Bajdak-Rusinek, K. 2019. The effect of palmitic acid on inflammatory response in macrophages: an overview of molecular mechanisms. *Inflammation Research*. 68:915–32. DOI: 10.1007/s00011-019-01273-5

Kostetsky, E.Y., Goncharova, S.N., Sanina, N.M. & Shnyrov, V.L. 2004. Season influence on lipid composition of marine macrophytes. *Botanica Marina*. 47:134–9. DOI: 10.1515/BOT.2004.013

Kraft, L.G., Kraft, G.T. & Waller, R.F. 2010. Investigations into southern Australian *Ulva* (Ulvophyceae, Chlorophyta) taxonomy and molecular phylogeny indicate both cosmopolitanism and endemic cryptic species. *Journal of Phycology*. 46:1257–77. DOI: 10.1111/j.1529-8817.2010.00909.x

Kubanek, J., Lester, S.E., Fenical, W. & Hay, M.E. 2004. Ambiguous role of phlorotannins as chemical defenses in the brown alga *Fucus vesiculosus*. *Marine Ecology Progress Series*. 277:79–93. DOI: 10.3354/meps277079

Kumari, P., Reddy, C.R.K. & Jha, B. 2011. Comparative evaluation and selection of a method for lipid and fatty acid extraction from macroalgae. *Analytical Biochemistry*. 415:134–44. DOI: 10.1016/j.ab.2011.04.010

Kumari, P., Kumar, M., Reddy, C.R.K. & Jha, B. 2013. Chapter 3 - Algal lipids, fatty acids and sterols. *In* Domínguez, H. [Ed.] *Functional Ingredients from Algae for Foods and Nutraceuticals*. Woodhead Publishing, Cambridge, United Kingdom, pp. 87–134. DOI: 10.1533/9780857098689.1.87

Kumari, P., Kumar, M., Reddy, C.R.K. & Bhavanath Jha, B. 2014. Nitrate and phosphate regimes induced lipidomic and biochemical changes in the intertidal macroalga *Ulva lactuca* (Ulvophyceae, Chlorophyta). *Plant and Cell Physiology*. 55:52–63. DOI: 10.1093/pcp/pct156

Kupfermann, I. & Carew, T.J. 1974. Behavior patterns of *Aplysia californica* in its natural environment. *Behavioral Biology*. 12:317–37. DOI: 10.1016/S0091-6773(74)91503-X

Laramore, S., Baptiste, R., Wills, P.S. & Hanisak, D.M. 2018. Utilization of IMTA-produced *Ulva lactuca* to supplement or partially replace pelleted diets in shrimp (*Litopenaeus vannamei*) reared in a clear water production system. *Journal of Applied Phycology*. 30:3603–10. DOI: 10.1007/s10811-018-1485-3

Laverack, M.S. 1963. Aspects of chemoreception in crustacea. *Comparative Biochemistry and Physiology*. 8:141–51. DOI: 10.1016/0010-406X(63)90078-1

Lawrence, J.M., Lawrence, A.L. & Watts, S.A. 2013. Chapter 9 - Feeding, Digestion and Digestibility of Sea Urchins. *In* Lawrence, J.M. (Ed.) *Sea Urchins: Biology and Ecology*. Elsevier B.V., London, United Kingdom, pp. 135–158. DOI: 10.1016/B978-0-12-396491-5.00009-5

Lawton, R.J., Mata L., de Nys, R. & Paul, N.A. 2013. Algal bioremediation of waste waters from land-based aquaculture using *Ulva*: selecting target species and strains. *PLoS ONE* 8:e77344. DOI: 10.1371/journal.pone.0077344

Lecchini, D., Dixson, D.L., Lecellier, G., Roux, N., Frédérich, B., Besson, M., Tanaka, Y., Banaigs, B. & Nakamura, Y. 2017. Habitat selection by marine larvae in changing chemical environments. *Marine Pollution Bulletin.* 114:210–7. DOI: 10.1016/j.marpolbul.2016.08.083

Lee, P.G. & Meyers, S.P. 1996. Chemoattraction and feeding stimulation in crustaceans. *Aquaculture Nutrition.* 2:157–64. DOI: 10.1111/j.1365-2095.1996.tb00055.x

Legarda, E.C., Viana, M.T., Del Rio Zaragoza, O.B., Skrzynska, A.K., Braga, A., de Lorenzo, M.A. & Vieira, F.N. 2021. Effects on fatty acids profile of *Seriola dorsalis* muscle tissue fed diets supplemented with different levels of *Ulva fasciata* from an Integration Multi Trophic Aquaculture system. *Aquaculture.* 535:736414. DOI: 10.1016/j.aquaculture.2021.736414

Li, Y-X., Wijesekara, I., Li, Y. & Kim, S.-K. 2011. Phlorotannins as bioactive agents from brown algae. *Process Biochemistry.* 46:2219–24. DOI: 10.1016/j.procbio.2011.09.015

Li, X., Ji, R., Cui, K., Chen, Q., Chen, Q., Fang, W., Mai, K., Zhang, Y., Xu, W. & Ai, Q. 2019. High percentage of dietary palm oil suppressed growth and antioxidant capacity and induced the inflammation by activation of TLR-NF-κB signaling pathway in large yellow croaker (*Larimichthys crocea*). *Fish & Shellfish Immunology.* 87:600–8. DOI: 10.1016/j.fsi.2019.01.055

Li, S., Hu, M., Tong, Y., Xia, Z., Tong, Y., Sun, Y., Cao, J., Zhang, J., Liu, J., Zhao, S. & He, P. 2023. A review of volatile compounds in edible macroalgae. *Food Research International.* 165:112559. DOI: 10.1016/j.foodres.2023.112559

Liebert, F. & Portz, L. 2005. Nutrient utilization of Nile tilapia *Oreochromis niloticus* fed plant based low phosphorus diets supplemented with graded levels of different sources of microbial phytase. *Aquaculture.* 248:111–9. DOI: 10.1016/j.aquaculture.2005.04.009

Lim, L.-S., Lai, S.-K.J., Yong, A.S.-K., Shapawi, R. & Kawamura, G. 2016. Evaluation on the potential of betaine, taurine, nucleotide and nucleoside as feeding stimulant for juvenile marble goby *Oxyeleotris marmoratus* through behavioural assays. *International Aquatic Research.* 8:161–7. DOI: 10.1007/s40071-016-0131-4

Lindstedt, K.J. 1971. Chemical control of feeding behavior. *Comparative Biochemistry and Physiology Part A: Physiology.* 39:553–81. DOI: 10.1016/0300-9629(71)90319-7

Linstrom, P.J. & Mallard, W.G. [Eds.] NIST Chemistry WebBook, NIST Standard Reference Database Number 69. National Institute of Standards and Technology, Gaithersburg, United States. DOI: 10.18434/T4D303

Lisec, J., Schauer, N., Kopka, J., Willmitzer, L. & Fernie, A.R. 2006. Gas chromatography mass spectrometry-based metabolite profiling in plants. *Nature Protocols.* 1:387–96. DOI: 10.1038/nprot.2006.59

Lonsdale, D.J., Frey, M.A. & Snell, T.W. 1998. The role of chemical signals in copepod reproduction. *Journal of Marine Systems*. 15:1–12. DOI: 10.1016/S0924-7963(97)00044-4

Lopes, D., Moreira, A.S.P., Rey, F., da Costa, E., Melo, T., Maciel, E., Rego, A., Abreu, M.H., Domingues, P., Calado, R., Lillebø, A.I. & Domingues, M.R. 2019. Lipidomic signature of the green macroalgae *Ulva rigida* farmed in a sustainable integrated multi-trophic aquaculture. *Journal of Applied Phycology*. 31:1369–81. DOI: 10.1007/s10811-018-1644-6

Lopes, D., Melo, T., Rey, F., Meneses, J., Monteiro, F.L., Helguero, L.A., Abreu, M.H., Lillebø, A.I., Calado, R. & Domingues, M.R. 2020. Valuing bioactive lipids from green, red and brown macroalgae from aquaculture, to foster functionality and biotechnological applications. *Molecules*. 25:3883. DOI: 10.3390/molecules25173883

Lopes, D., Rey, F., Leal, M.C., Lillebø, A.I., Calado, R. & Domingues, M.R. 2021. Bioactivities of lipid extracts and complex lipids from seaweeds: current knowledge and future prospects. *Marine Drugs*. 19:686. DOI: 10.3390/md19120686

López-Pérez, O., Picon, A. & Nuñez, M. 2017. Volatile compounds and odour characteristics of seven species of dehydrated edible seaweeds. *Food Research International*. 99:1002–10. DOI: 10.1016/j.foodres.2016.12.013

López-Pérez, O., del Olmo, A., Picon, A. & Nuñez, M. 2021. Volatile compounds and odour characteristics of five edible seaweeds preserved by high pressure processing: Changes during refrigerated storage. *Algal Research*. 53:102137. DOI: 10.1016/j.algal.2020.102137

Lotze, H.K. & Worm, B. 2002. Complex interactions of climatic and ecological controls on macroalgal recruitment. *Limnology and Oceanography*. 47:1734–41. DOI: 10.4319/lo.2002.47.6.1734

Loughnane, C.J., McIvor, L.M., Rindi, F., Stengel, D.B. & Guiry, M.D. 2008. Morphology, *rbc*L phylogeny and distribution of distromatic *Ulva* (Ulvophyceae, Chlorophyta) in Ireland and southern Britain. *Phycologia*. 47:416–29. DOI: 10.2216/PH07-61.1

Luis, O.J. & Gago, J.M. 2021. Perception of dissolved food-related compounds by the sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea). *Frontiers in Marine Science*. 8:719670. DOI: 10.3389/fmars.2021.719670

Lukin, I., Jach, G., Wingartz, I., Welters, P. & Schembecker, G. 2019. Recovery of natural α-ionone from fermentation broth. *Journal of Agricultural and Food Chemistry*. 67:13412–9. DOI: 10.1021/acs.jafc.8b07270

Luo, M.B., Liu, F. & Li Xu, Z.L. 2012. Growth and nutrient uptake capacity of two co occurring species, *Ulva prolifera* and *Ulva linza*. *Aquatic Botany*. 100:18–24. DOI: 10.1016/j.aquabot.2012.03.006

Lyons, D.A. & Scheibling, R.E. 2007. Effect of dietary history and algal traits on feeding rate and food preference in the green sea urchin *Strongylocentrotus droebachiensis*. *Journal of Experimental Marine Biology and Ecology*. 349:194–204. DOI: 10.1016/j.jembe.2007.05.012

Lyons, D.A., Van Alstyne, K.L. & Scheibling, R.E. 2007. Anti-grazing activity and seasonal variation of dimethylsulfoniopropionate-associated compounds in the invasive alga *Codium fragile* ssp. *tomentosoides. Marine Biology*. 153:179–88. DOI: 10.1007/s00227-007-0795-9

Ma, W.C.J., Chung, H.Y., Ang, P.O. & Kim, J-S. 2005. Enhancement of bromophenol levels in aquacultured silver seabream (*Sparus sarba*). *Journal of Agriculture and Food Chemistry*. 53:2133–9. DOI: 10.1021/jf048390m

Macey, B.M., Brink-Hull, M., Makhahlela, N.B., Brand, M.J., Cyrus, M.D. & Bolton, J.J. 2022. Impact of *Ulva lacinulata* on the microbiome of abalone-*Ulva* IMTA systems. *In* Abstract. Aquaculture Europe 2022, Rimini, Italy, 27–30 September 2022, Paper 758. Retrieved from URL: https://eposters.blob.core.windows.net/eas-eposters/AE2022AbstractBook.pdf

Madibana, M.J., Mlambo, V., Lewis, B. & Fouché, C. 2017. Effect of graded levels of dietary seaweed (*Ulva* sp.) on growth, hematological and serum biochemical parameters in dusky kob, *Argyrosomus japonicus*, Sciaenidae. *The Egyptian Journal of Aquatic Research*. 43:249–54. DOI: 10.1016/j.ejar.2017.09.003

Magdugo, R.P., Terme, N., Lang, M., Pliego-Cortés, H., Marty, C., Hurtado, A.Q., Bedoux, G. & Bourgougnon, N. 2020. An analysis of the nutritional and health values of *Caulerpa racemosa* (Forsskål) and *Ulva fasciata* (Delile)-two Chlorophyta collected from the Philippines. *Molecules*. 25:2901. DOI: 10.3390%2Fmolecules25122901

Malta, E.-J., Draisma, S.G.A. & Kamermans, P. 1999. Free-floating *Ulva* in the southwest Netherlands: species or morphotypes? A morphological, molecular and ecological comparison. *European Journal of Phycology*. 34:443–54. DOI: 10.1080/09541449910001718801

Malta, E., Rijstenbil, J.W., Brouwer, P.E.M., & Kromkamp, J.C. 2003. Vertical heterogeneity in physiological characteristics of *Ulva* spp. mats. *Marine Biology*. 143:1029–38. DOI: 10.1007/s00227-003-1134-4

Mann, K.H., Wright, J.L.C., Welsford, B.E. & Hatfield, E. 1984. Responses of the sea urchin *Strongylocentrotus droebachiensis* (O.F. Müller) to water-borne stimuli from potential predators and potential food algae. *Journal of Experimental Marine Biology and Ecology*. 79:233–44. DOI: 10.1016/0022-0981(84)90197-7

Mantri, V.A., Kazi, M.A., Balar, N.B., Gupta, V. & Gajaria, T. 2020. Concise review of green algal genus *Ulva* Linnaeus. *Journal of Applied Phycology*. 32:2725–41. DOI: 10.1007/s10811-020-02148-7

Marinho, G., Nunes, C., Sousa-Pinto, I., Pereira, R., Rema, P. & Valente, L.M.P. 2013. The IMTA-cultivated Chlorophyta *Ulva* spp. as a sustainable ingredient in Nile tilapia (*Oreochromis niloticus*) diets. *Journal of Applied Phycology*. 25:1359–67. DOI: 10.1007/s10811-012-9965-3

Maruti, A., Durán-Guerrero, E., Barroso, C. G., & Castro, R. 2018. Optimization of a multiple headspace sorptive extraction method coupled to gas chromatography-mass spectrometry for the determination of volatile compounds in macroalgae. *Journal of Chromatography A.* 1551:41–51. DOI: 10.1016/j.chroma.2018.04.011

Mata, L., Schuenhoff, A. & Santos, R. 2010. A direct comparison of the performance of the seaweed biofilters, *Asparagopsis armata* and *Ulva rigida. Journal of Applied Phycology.* 22:639– 44. DOI: 10.1007/s10811-010-9504-z

Mata, L., Magnusson, M., Paul, N.A. & de Nys, R. 2016. The intensive land-based production of the green seaweeds *Derbesia tenuissima* and *Ulva ohnoi*: biomass and bioproducts. *Journal of Applied Phycology*. 28:365–75. DOI: 10.1007/s10811-015-0561-1

Matulić, D., Barišić, J., Aničić, I., Tomljanović, T., Safner, R., Treer, T., Gao, G., Glojnarić, I. & Čož-Rakovac, R. 2020. Growth, health aspects and histopathology of brown bullhead (*Ameiurus nebulosus* L.): replacing fishmeal with soybean meal and brewer's yeast. *Scientific Reports.* 10:1104. DOI: 10.1038/s41598-020-57722-3

McCauley, J.I., Meyer, B.J., Winberg, P.C., Ranson, M. & Skropeta, D. 2015. Selecting Australian marine macroalgae based on the fatty acid composition and anti-inflammatory activity. *Journal of Applied Phycology.* 27:2111–21. DOI: 10.1007/s10811-014-0465-5

McCauley, J.I., Meyer, B.J., Winberg, P.C. & Skropeta, D. 2016. Parameters affecting the analytical profile of fatty acids in the macroalgal genus *Ulva*. *Food Chemistry*. 209:332–40. DOI: 10.1016/j.foodchem.2016.04.039

McCauley, J.I., Winberg, P.C., Meyer, B.J. & Skropeta, D. 2018. Effects of nutrients and processing on the nutritionally important metabolites of *Ulva* sp. (Chlorophyta). *Algal Research.* 35:586–94. DOI: 10.1016/j.algal.2018.09.016

McHugh, D.J. 2003. A guide to the seaweed industry. Fisheries Technical Paper. Food and Agriculture Organization of the United Nations, Rome, Italy 441:1–105.

MestReNova (ver. 6.0.2). Mestrelab Research, Santiago de Compostela, Spain.

Microsoft® Corporation. Microsoft® Excel® 365 MSO. Available from: https://office.microsoft.com/excel

Miladi, R., Manghisi, A., Minicante, S. A., Genovese, G., Abdelkafi, S. & Morabito, M. 2018. A DNA barcoding survey of *Ulva* (Chlorophyta) in Tunisia and Italy reveals the presence of the overlooked alien *U. ohnoi*. *Cryptogamie, Algologie.* 39:85–107. DOI: 10.7872/crya/v39.iss1.2018.85

Mišurcová, L., Ambrožová, J., & Samek, D. 2011. Seaweed Lipids as Nutraceuticals. *Advances in Food and Nutrition Research.* 64:339–55. DOI: 10.1016/B978-0-12-387669-0.00027-2

Mitra, A. 2021. Thought of alternate aquafeed: conundrum in aquaculture sustainability? *Proceedings of the Zoological Society*. 74:1–18. DOI: 10.1007/s12595-020-00352-4

Mollo, E., Garson, M.J., Polese, G., Amodeo, P. & Ghiselin, M.T. 2017. Taste and smell in aquatic and terrestrial environments. *Natural Product Reports.* 34:496–513. DOI: 10.1039/C7NP00008A

Monteiro, J.P., Costa, E., Melo, T., Domingues, P., Fort, A., Domingues, M.R. & Sulpice, R. 2022. Lipidome in-depth characterization highlights the nutritional value and species specific idiosyncrasies of different *Ulva* species. *Algal Research.* 64:102694. DOI: 10.1016/j.algal.2022.102694

Moore, P.A. & Lepper, D.M.E. 1997. Role of chemical signals in the orientation behavior of the sea star *Asterias forbesi*. *The Biological Bulletin.* 192:410–7. DOI: 10.2307/1542750

Moreira, A.S.P., da Costa E., Melo, T., Sulpice, R., Cardoso, S.M., Pitarma, B., Pereira, R., Abreu, M.H., Domingues, P., Calado, R. & Domingues, M.R. 2020. Seasonal plasticity of the polar lipidome of *Ulva rigida* cultivated in a sustainable integrated multi-trophic aquaculture. *Algal Research.* 49:101958. DOI: 10.1016/j.algal.2020.101958

Moreira, C., Ferreira-Santos, P., Teixeira, J.A. & Rocha, C.M.R. 2022. Active aroma compounds assessment of processed and non-processed micro- and macroalgae by solid-phase microextraction and gas chromatography/mass spectrometry targeting seafood analogs. *Frontiers in Sustainable Food Systems.* 6:1011020. DOI: 10.3389/fsufs.2022.1011020

Morishita, V.R. & Barreto, R.E. 2011. Black sea urchins evaluate predation risk using chemical signals from a predator and injured con- and heterospecific prey. *Marine Ecology Progress Series.* 435:173–81. DOI: 10.3354/meps09253

Mouritsen, O.G. 2013. *Seaweeds: Edible, Available, and Sustainable*. The University of Chicago Press, London, pp. 96.

Mouritsen, O.G., Duelund, L., Petersen, M.A., Hartmann, A.L. & Frøst, M.B. 2019. Umami taste, free amino acid composition, and volatile compounds of brown seaweeds. *Journal of Applied Phycology.* 31:1213–32. DOI: 10.1007/s10811-018-1632-x

Msuya, F.E. 2007. The effect of stocking density on the performance of the seaweed *Ulva reticulata* as a biofilter in earthen pond channels, Zanzibar, Tanzania. *Western Indian Ocean Journal of Marine Science.* 6:65–72. DOI: 10.4314/wiojms.v6i1.48227

Msuya, F.E. & Neori, A. 2008. Effect of water aeration and nutrient load level on biomass yield, N uptake and protein content of the seaweed *Ulva lactuca* culture in seawater tanks. *Journal of Applies Phycology.* 20:1021–31. DOI: 10.1007/s10811-007-9300-6

Msuya, F.E. & Neori, A. 2010. The performance of spray-irrigated *Ulva lactuca* (Ulvophyceae, Chlorophyta) as a crop and as a biofilter of fishpond effluents. *Journal of Phycology.* 46:813–7. DOI: 10.1111/j.1529-8817.2010.00843.x

Msuya, F.E., Bolton, J., Pascal, F., Narrain, K., Nyonje, B. & Cottier-Cook, E.J. 2022. Seaweed farming in Africa: current status and future potential. *Journal of Applied Phycology.* 34:985–1005. DOI: 10.1007/s10811-021-02676-w

Mulvaney, W.J., Winberg, P.C. & Adams, L. 2013. Comparison of macroalgal (*Ulva* and *Grateloupia* spp.) and formulated terrestrial feed on the growth and condition of juvenile abalone. *Journal of Applied Phycology.* 25:815–24 DOI: 10.1007/s10811-013-9998-2

Mulvaney, W.J., Jahangard, S., Ingram, B.A., Turchini, G.M. & Winberg, P.C. 2015. Recovery of omega-3 profiles of cultivated abalone by dietary macroalgae supplementation. *Journal of Applied Phycology*. 27:2163–71. DOI: 10.1007/s10811-015-0530-8

Murali-Baskaran, R.K., Mooventhan, P., Das, D., Dixit, A., Sharma, K.C., Senthil-Nathan, S., Kaushal, P. & Ghosh, P.K. 2022. The future of plant volatile organic compounds (*p*VOCs) research: Advances and applications for sustainable agriculture. *Environmental and Experimental Botany*. 200:104912. DOI: 10.1016/j.envexpbot.2022.104912

Nagler, P.L., Glenn, E.P., Nelson, S.G. & Napolean, S. 2003. Effects of fertilization treatment and stocking density on the growth and production of the economic seaweed *Gracilaria parvispora* (Rhodophyta) in cage culture at Molokai, Hawaii. *Aquaculture*. 219:379–91. DOI: 10.1016/S0044-8486(02)00529-X

Naidoo, K., Maneveldt, G., Ruck, K. & Bolton, J.J. 2006. A comparison of various seaweed-based diets and formulated feed on growth rate of abalone in a land-based aquaculture system. *Journal of Applied Phycology*. 18:437–43. DOI: 10.1007/s10811-006-9045-7

Narain, N., Hsieh, T.C.Y. & Johnson, C.E. 1990. Dynamic Headspace Concentration and Gas Chromatography of volatile flavor components in peach. *Journal of Food Science*. 55:1303–7. DOI: 10.1111/j.1365-2621.1990.tb03921.x

Narayan, B., Miyashita, K. & Hosakawa, M. 2005. Comparative evaluation of fatty acid composition of different *Sargassum* (Fucales, Phaeophyta) species harvested from temperate and tropical waters. *Journal of Aquatic Food Product Technology*. 13:53–70. DOI: 10.1300/J030v13n04_05

Naylor, R.L., Hardy, R.W., Buschmann, A.H., Bush, S.R., Cao, L., Klinger, D.H., Little, D.C., Lubchenco, J., Shumway, S.E. & Troell, M. 2021. A 20-year retrospective review of global aquaculture. *Nature*. 591:551–63. DOI: 10.1038/s41586-021-03308-6

Nazarudin, M.F., Isha, A., Mastuki, S.N., Ain, N.M., Mohd Ikhsan, N.F., Abidin, A.Z. & Aliyu-Paiko M. 2020. Chemical composition and evaluation of the α-glucosidase inhibitory and cytotoxic properties of marine algae *Ulva intestinalis*, *Halimeda macroloba*, and *Sargassum ilicifolium*. *Evidence-Based Complementary and Alternative Medicine*. 2020:2753945. DOI: 10.1155%2F2020%2F2753945

Neori, A., Cohen, I. & Gordin, H. 1991. *Ulva lactuca* biofilters for marine fishpond effluents. II. Growth rate, yield and C: N ratio. *Botanica Marina*. 34:483–9. DOI: 10.1515/botm.1991.34.6.483

Neori, A. & Shpigel, M. 2000. A sustainable integrated system for culture of fish, seaweed and abalone. *Aquaculture*. 186:279–91. DOI: 10.1016/S0044-8486(99)00378-6

Neori, A., Chopin, T., Troell, M., Buschmann, A.H., Kraemer, G.P., Halling, C., Shpigel, M. & Yarish, C. 2004. Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. *Aquaculture*. 231:361–91. DOI: 10.1016/j.aquaculture.2003.11.015

Neto, R.T., Marçal, C., Queirós, A.S., Abreu, H., Silva, A. & Cardoso, S.M. 2018. Screening of *Ulva rigida*, *Gracilaria* sp., *Fucus vesiculosus* and *Saccharina latissima* as functional ingredients. *International Journal of Molecular Sciences.* 19:2987. DOI: 10.3390/ijms19102987

Neveux, N, Bolton, J.J., Bruhn, A., Roberts, D.A. & Ras, M. 2018. Chapter 7 - The bioremediation potential of seaweeds: recycling nitrogen, phosphorus and other waste products. *In* La Barre, S. & Bate, S.S. [Eds] *Blue technologies – production and use of marine molecules*. Wiley, VCH-Verlag GmbH & Co KgaA, Weinheim, Germany, pp. 217–239. DOI: 10.1002/9783527801718.ch7

Nisizawa, K., Noda, H., Kikuchi, R. & Watanabe, T. 1987. The main seaweed foods in Japan. *Hydrobiologia.* 151:5–29. DOI: 10.1007/BF00046102

Nunes, A.J.P, Sabry-Neto, H., Oliveira-Neto, S. & Burri, L. 2019. Feed preference and growth response of juvenile *Litopenaeus vannamei* to supplementation of marine chemoattractants in a fishmeal-challenged diet. *Journal of World Aquaculture Society.* 50:1048–63. DOI: 10.1111/jwas.12648

Nunes, N., Rosa, G.P., Ferraz, S., Barreto, M.C. & de Carvalho, M.M.A.P. 2020. Fatty acid composition, TLC screening, ATR-FTIR analysis, anti-cholinesterase activity, and *in vitro* cytotoxicity to A549 tumor cell line of extracts of 3 macroalgae collected in Madeira. *Journal of Applied Phycology.* 32:759–71. DOI: 10.1007/s10811-019-01884-9

Nuñez, M. & Picon, A. 2017. Seaweeds in yogurt and quark supplementation: influence of five dehydrated edible seaweeds on sensory characteristics. *International Journal of Food Science + Technology*. 52:431–8. DOI: 10.1111/ijfs.13298

Nuzzo, G., Gallo, C., d'Ippolito, G., Cutignano, A., Sardo, A. & Fontana, A. 2013. Composition and quantitation of microalgal lipids by ERETIC ^{1}H NMR method. *Marine Drugs.* 11:3742–53 DOI: 10.3390%2Fmd11103742

O'Kelly, C.J., Kurihara, A., Shipley, T.C. & Sherwood, A.R. 2010 Molecular assessment of *Ulva* spp. (Ulvophyceae, Chlorophyta) in the Hawaiian Islands. *Journal of Phycology.* 46:728–35. DOI: 10.1111/j.1529-8817.2010.00860.x

Oh, H., Lee, P., Kim, S.Y. & Kim, Y-S. 2020. Preparation of cookies with various native seaweeds found on the Korean coast. *Journal of Aquatic Food Product Technology.* 29:167–74, DOI: 10.1080/10498850.2019.1707925

Olsen, R.L. & Hasan, M.R. 2012. A limited supply of fishmeal: Impact on future increases in global aquaculture production. *Trends in Food Science & Technology.* 27:120–8. DOI: 10.1016/j.tifs.2012.06.003

Onomu, A.J., Vine, N.G., Cyrus, M.D., Macey, B.M. & Bolton, J.J. 2020. The effect of fresh seaweed and a formulated diet supplemented with seaweed on the growth and gonad quality of the collector sea urchin, *Tripneustes gratilla*, under farm conditions. *Aquaculture Research.* 51:4087–4102. DOI: 10.1111/are.14752

Ortiz, J., Romero, N., Robert, P., Araya, J., Lopez-Hernández, J., Bozzo, C., Navarrete, E., Osorio, A. & Rios, A. 2006. Dietary fiber, amino acid, fatty acid and tocopherol contents of the edible seaweeds *Ulva lactuca* and *Durvillaea antarctica. Food Chemistry*. 99:98–104. DOI: 10.1016/j.foodchem.2005.07.027

Pagès, J.F., Bartumeus, F., Romero, J. & Alcoverro, T. 2021. The scent of fear makes sea urchins go ballistic. *Movement Ecology*. 9:50. DOI: 10.1186/s40462-021-00287-1

Pahlow, M., van Oel, P.R., Mekonnen, M.M. & Hoekstra, A.Y. 2015. Increasing pressure on freshwater resources due to terrestrial feed ingredients for aquaculture production. *Science of The Total Environment.* 536:847–57. DOI: 10.1016/j.scitotenv.2015.07.124

Paiva, L., Lima, E., Neto, A.I., Marcone, M. & Baptista, J. 2017. Nutritional and functional bioactivity value of selected Azorean macroalgae: *Ulva compressa*, *Ulva rigida*, *Gelidium microdon* and *Pterocladiella capillacea*. *Journal of Food Science.* 82:1757–64. DOI: 10.1111/1750-3841.13778

Pang, Z., Chong, J., Zhou, G., de Lima Morais, D.A., Chang, L., Barrette, M., Gauthier, C., Jacques, P-É., Li, S. & Xia, J. 2021. MetaboAnalyst 5.0: narrowing the gap between raw spectra and functional insights, *Nucleic Acids Research*. 49: 388 – 396. DOI: 10.1093/nar/gkab382

Papatryphon, E. & Soares, J.H. 2000. The effect of dietary feeding stimulants on growth performance of striped bass, *Morone saxatilis*, fed-a-plant feedstuff-based diet. *Aquaculture.* 185:329–38. DOI: 10.1016/S0044-8486(99)00348-8

Pappou, S., Dardavila, M.M., Savvidou, M.G., Louli, V., Magoulas, K. & Voutsas, E. 2022. Extraction of bioactive compounds from *Ulva lactuca*. *Applied Sciences.* 12:2117. DOI: 10.3390/app12042117

Parker, D.A. & M.J. Shulman, M.J. 1986. Avoiding predation: alarm responses of Caribbean sea urchins to simulated predation on conspecific and heterospecific sea urchins. *Marine Biology*. 93:201–8. DOI: 10.1007/BF00508257

Parma, L., Badiani, A., Bonaldo, A., Viroli, C., Farabegoli, F., Silvi, M., Bonvini, E., Pirini, & M. & Gatta, P.P. 2019. Farmed and wild common sole (*Solea solea* L.): Comparative assessment of morphometric parameters, processing yields, selected nutritional traits and sensory profile. *Aquaculture.* 502:63–71. DOI: 10.1016/j.aquaculture.2018.12.029

Patra, J.K., Lee, S-W., Kwon, Y-S., Park, J.G. & Baek, K-H. 2017. Chemical characterization and antioxidant potential of volatile oil from an edible seaweed *Porphyra tenera* (Kjellman, 1897). *Chemistry Central Journal.* 11:34. DOI: 10.1186/s13065-017-0259-3

Pedersen, M.F. & Borum, J. 1997. Nutrient control of estuarine macroalgae: growth strategy and the balance between nitrogen requirements and uptake. *Marine Ecology Progress Series.* 161:155–63. DOI: 10.3354/meps161155

Peinado, I., Girón, J., Koutsidis, G. & Ames, J.M. 2014. Chemical composition, antioxidant activity and sensory evaluation of five different species of brown edible seaweeds. *Food Research International*. 66:36–44. DOI: 10.1016/j.foodres.2014.08.035

Peixoto, S., Strebel, L., Soares, R. & Davis, D.A. 2022. Acoustic feeding responses using marine chemoattractants in plant-based diets for naive and non-naive *Litopenaeus vannamei*. *Applied Animal Behaviour Science*. 257:105792. DOI: 10.1016/j.applanim.2022.105792

Pelletreau, K. & Muller-Parker, G. 2002. Sulfuric acid in the phaeophyte alga *Desmarestia munda* deters feeding by the sea urchin *Strongylocentrotus droebachiensis*. *Marine Biology*. 141:1–9. DOI: 10.1007/s00227-002-0809-6

Peña-Rodríguez, A., Mawhinney, T.P., Ricque-Marie, D. & Cruz-Suárez, L.E. 2011. Chemical composition of cultivated seaweed *Ulva clathrata* (Roth) C. Agardh. *Food Chemistry*. 129:491–8. DOI: 10.1016/j.foodchem.2011.04.104

Peng, Y., Hu, J., Yang, B., Lin, X-P., Zhou, X-F., Yang, X-W. & Liu, Y. 2015. Chemical composition of seaweeds. *In* Tiwari, B.K. & Troy, D.J. [Eds.] *Seaweed Sustainability*. Academic Press, pp 79–124. DOI: 10.1016/B978-0-12-418697-2.00005-2

Pereira, R., Yarish, C. & Sousa-Pinto, I. 2006. The influence of stocking density, light and temperature on the growth, production and nutrient removal capacity of *Porphyra dioica* (Bangiales, Rhodophyta). *Aquaculture*. 252:66–78. DOI: 10.1016/j.aquaculture.2005.11.050

Pereira, H., Barreira, L., Figueiredo, F., Custódio, L., Vizetto-Duarte, C., Polo, C., Rešek, E., Engelen, A. & Varela, J. 2012. Polyunsaturated fatty acids of marine macroalgae: potential for nutritional and pharmaceutical applications. *Marine Drugs*. 10:1920–35. DOI: 10.3390/md10091920

Pereira, R.C., Nocchi, N., Souto, M.L., Fernández, J.J., Norte, M., Duarte, H.M. & Soares, A.R. 2020. The sea-hare *Aplysia brasiliana* promotes induction in chemical defense in the seaweed *Laurencia dendroidea* and in their congeneric neighbors. *Plant Physiology and Biochemistry*. 154:295–303. DOI: 10.1016/j.plaphy.2020.05.020

Pérez, M.J., Falqué, E. & Domínguez, H. 2016. Antimicrobial Action of Compounds from Marine Seaweed. *Marine Drugs*. 14:52. DOI: 10.3390/md14030052

Pirian, K., Piri, K., Sohrabipour, J. & Blomster, J. 2018. Three species of *Ulva* (Ulvophyceae) from the Persian Gulf as potential sources of protein, essential amino acids and fatty acids. *Phycological Research*. 66:149–54. DOI: 10.1111/pre.12212

Plouguerné, E., da Gama, B.A.P., Pereira, R.C. & Barreto-Bergter, E. 2014. Glycolipids from seaweeds and their potential biotechnological applications. *Frontiers in Cellular and Infection Microbiology*. 4:174. DOI: 10.3389/fcimb.2014.00174

Pluskal, T., Castillo, S., Villar-Briones, A. & Orešič, M. 2010. MZmine 2: Modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data. *BMC Bioinformatics*. 11:395. DOI: 10.1186/1471-2105-11-395

Pohnert, G. & Bolanda, W. 2002. The oxylipin chemistry of attraction and defense in brown algae and diatoms. *Natural Product Reports.* 19:108–22. DOI: 10.1039/A806888G

Prince, J.S. & LeBlanc, W.G. 1992. Comparative feeding preference of *Strongylocentrotus droebachiensis* (Echinoidea) for the invasive seaweed *Codium fragile* ssp. *Tomentosoides* (Chlorophyceae) and four other seaweeds. *Marine Biology.* 113:159–63. DOI: 10.1007/BF00367649

Qiu, X., Neori, A., Kim, J.K., Yarish, C., Shpigel, M., Guttman, L., Ben Ezra, D., Odintsov, V. & Davis, D.A. 2018. Evaluation of green seaweed *Ulva* sp. as a replacement of fish meal in plant-based practical diets for Pacific white shrimp, *Litopenaeus vannamei*. *Journal of Applied Phycology.* 30:1305–16. DOI: 10.1007/s10811-017-1278-0

Queirós, A.S., Circuncisão, A.R., Pereira, E., Válega, M., Abreu, M.H., Silva, A.M.S. & Cardoso, S.M. 2021. Valuable nutrients from *Ulva rigida*: modulation by seasonal and cultivation factors. *Applied Sciences.* 11:6137. DOI: 10.3390/app11136137

R Core Team. 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Vienna, Austria.

Rasher, D.B., Stout, P.E., Engel, S., Shearer, T.L., Kubanek, J. & Hay, M.E. 2015. Marine and terrestrial herbivores display convergent chemical ecology despite 400 million years of independent evolution. *Proceedings of the National Academy of Sciences of the United States of America.* 112:12110–5. DOI: 10.1073/pnas.1508133112

Raven, J.A. 2012. Protein turnover and plant RNA and phosphorus requirements in relation to nitrogen fixation. *Plant Science.* 188–189:25–35. DOI: 10.1016/j.plantsci.2012.02.010

Raven, J.A. 2013. The evolution autotrophy in relation to phosphorus requirements. *Journal of Experimental Botany.* 64:4023–46. DOI: 10.1093/jxb/ert306

Rengasamy, K.R.R., Mahomoodally, M.F., Aumeeruddy, M.Z., Zengin, G., Xiao, J. & Kim, D.H. 2020. Bioactive compounds in seaweeds: An overview of their biological properties and safety. *Food and Chemical Toxicology.* 135:111013. DOI: 10.1016/j.fct.2019.111013

Restrepo, D. & Boyle, A.G. 1991. Stimulation of olfactory receptors alters regulation of [Ca_i] in olfactory neurons of the catfish (*Ictalurus punctatus*). *Journal of Membrane Biology.* 120:223–32. DOI: 10.1007/BF01868533

Richards, D.K., Hurd, C.L., Pritchard, D.W., Wing, S.R. & Hepburn, C.D. 2011. Photosynthetic response of monospecific macroalgal stands to density. *Aquatic Biology.* 13:41–9. DOI: 10.3354/ab00349

Robertson-Andersson, D.V. 2003. The cultivation of *Ulva lactuca* (Chlorophyta) in an integrated aquaculture system, for the production of abalone feed and the bioremediation of aquaculture effluent. MSc Thesis, University of Cape Town, South Africa. Available at: http://hdl.handle.net/11427/6175

Robertson-Andersson, D.V., Potgieter, M., Hansen, J., Bolton, J.J., Troell, M., Anderson, R.J., Halling, C. & Probyn, T. 2008. Integrated seaweed cultivation on an abalone farm in South Africa. *Journal of Applied Phycology*. 20:579–95. DOI: 10.1007/s10811-007-9239-7

Robertson-Andersson, D.V., Maneveldt, G.W. & Naidoo, K. 2011. Effects of wild and farm-grown macroalgae on the growth of juvenile South African abalone *Haliotis midae* Linnaeus. *African Journal of Aquatic Science*. 36:331–7. DOI: 10.2989/16085914.2011.636910

Rodríguez-Bernaldo De Quirós, A., López-Hernández, J., González-Castro, M., de la Cruz-García, C. & Simal-Lozano, J. 2001. Comparison of volatile components in fresh and canned sea urchin (*Paracentrotus lividus*, Lamarck) gonads by GC–MS using dynamic headspace sampling and microwave desorption. *European Food Research and Technology*. 212:643–7. DOI: 10.1007/s002170100315

Rodríguez-Bustamante, E. & Sánchez, S. 2007 Microbial production of C_{13}-norisoprenoids and other aroma compounds via carotenoid cleavage. *Critical Reviews in Microbiology*. 33:211–30. DOI: 10.1080/10408410701473306

Roessler, P.G. 1990. Environmental control of glycerolipid metabolism in microalgae: commercial implications and future research directions. *Journal of Phycology*. 26:393–9. DOI: 10.1111/j.0022-3646.1990.00393.x

Rohani-Ghadikolaei, K., Abdulalian, E. & Ng, W.-K. 2012. Evaluation of the proximate, fatty acid and mineral composition of representative green, brown and red seaweeds from the Persian Gulf of Iran as potential food and feed resources. *Journal of Food Science and Technology*. 49:774–80. DOI: 10.1007/s13197-010-0220-0

Roleda, M.Y. & Hurd, C.L. 2019. Seaweed nutrient physiology: application of concepts to aquaculture and bioremediation. *Phycologia*. 58:552–62, DOI: 10.1080/00318884.2019.1622920

Roleda, M.Y. 2016. Stress physiology and reproductive phenology of Arctic endemic kelp *Laminaria solidungula* J. Agardh. *Polar Biology*. 39:1967–77. DOI: 10.1007/s00300-015-1813-x

Rolen, S.H., Sorensen, P.W., Mattson, D. & Caprio, J. 2003. Polyamines as olfactory stimuli in the goldfish *Carassius auratus*. *Journal of Experimental Biology*. 206:1683–96. DOI: 10.1242/jeb.00338

Rothman, M.D., Anderson, R.J., Kandjengo, L. & Bolton, J.J. 2020. Trends in seaweed resource use and aquaculture in South Africa and Namibia over the last 30 years. *Botanica Marina*. 63:315–25. DOI: 10.1515/bot-2019-0074

Roussis, V., Rapti, T., Vagias, C., Harvala, C. & Caberi, H. 2000. Volatile metabolites of the green alga *Ulva rigida* (C.Ag). *Journal of Essential Oil Research*. 12:201–4. DOI: 10.1080/10412905.2000.9699497

Ruff, O.E., Gentry, R.R. & Lester, E.S. 2020. Understanding the role of socioeconomic and governance conditions in country-level marine aquaculture production. *Environmental Research Letters*. 15:1040a8. DOI: 10.1088/1748-9326/abb908

Sáez, M.I., Vizcaíno, A., Galafat, A., Anguís, V., Fernández-Díaz, C., Balebona, M.C., Alarcón, F.J. & Martínez, T.F. 2020. Assessment of long-term effects of the macroalgae *Ulva ohnoi* included in diets on Senegalese sole (*Solea senegalensis*) fillet quality. *Algal Research*. 47:101885. DOI: 10.1016/j.algal.2020.101885

Saha, M. & Fink, P. 2022. Algal volatiles – the overlooked chemical language of aquatic primary producers. *Biological Reviews*. 97:2162–73. DOI: 10.1111/brv.12887

Saini, R.K., Prasad, P., Sreedhar, R.V., Akhilender Naidu, K., Shang, X. & Keum, Y.-S. 2021. Omega-3 polyunsaturated fatty acids (PUFAs): emerging plant and microbial sources, oxidative stability, bioavailability, and health benefits – A review. *Antioxidants*. 10:1627. DOI: 10.3390/antiox10101627

Sakamoto, A., Saotome, M., Iguchi, K. & Maekawa, Y. 2019. Marine-derived omega-3 polyunsaturated fatty acids and heart failure: current understanding for basic to clinical relevance. *International Journal of Molecular Sciences*. 20:4025. DOI: 10.3390/ijms20164025

Sakata, K. & Ina, K. 1985. Digalactosyldiacylglycerols and phosphatidylcholines isolated from a brown alga as effective phagostimulants for a young abalone. *Bulletin of the Japanese Society of Scientific Fisheries*. 51:659–65. DOI: 10.2331/suisan.51.659

Sakata, K., Tsuge, M., Kamiya, Y. & Ina, K. 1985. Isolation of a glycerolipid (DGTH) as a phagostimulant for a seahare, *Aplysia Juliana*, from a green alga, *Ulva pertusa*. *Agricultural and Biological Chemistry*. 49:1905–7. DOI: 10.1271/bbb1961.49.1905

Sakata, K., Sakura, T. & Ina, K. 1988. Algal phagostimulants for marine herbivorous gastropods. *Journal of Chemical Ecology*. 14:1405–16. DOI: 10.1007/BF01020144

Sakata, K., Kato, K., Ina, K. & Machiguchi, Y. 1989. Glycerolipids as potent feeding stimulants for the sea urchin, *Strongylocentrotus intermedius*. *Agricultural and Biological Chemistry*. 53:1457–9. DOI: 10.1080/00021369.1989.10869459

Salin, K.R., Arun, V.V., Mohanakumaran Nair, C. & Tidwell, J.H. 2018. Chapter 4 - Sustainable Aquafeed. *In* Hai, F., Visvanathan, C. & Boopathy, R. [Eds.] *Sustainable Aquaculture. Applied Environmental Science and Engineering for a Sustainable Future*. Springer, Cham, pp. 123–151. DOI: 10.1007/978-3-319-73257-2_4

Sánchez, F.C., Díaz, M.E., Morales, I.M. & Aranda, D.A. 2016. Formulated feed for *Strombus pugilis* (Mollusca, Gastropoda) allowed effective gonad maturity. *Journal of Aquaculture Research and Development*. 7:1–8. DOI: 10.4172/2155-9546.1000453

Sánchez-García, F., Mirzayeva, A., Roldán, A., Castro, R., Palacios, V., García-Barroso, C. & Durán-Guerrero, E. 2019. Evolution of volatile compounds and sensory characteristics of edible green seaweed (*Ulva rigida*) during storage at different temperatures. *Journal of the Science of Food and Agriculture*. 99:5475–82. DOI: 10.1002/jsfa.9808

Sánchez-García, F., Mirzayeva, A., Roldán, A., Castro, R., Palacios, V., G Barroso, C. & Durán-Guerrero, E. 2021. Effect of different cooking methods on sea lettuce (*Ulva rigida*) volatile compounds and sensory properties. *Journal of the Science of Food and Agriculture*. 101:970–80. 10.1002/jsfa.10705

Sanjeewa, K., Lee, W. & Jeon, Y.J. 2018. Nutrients and bioactive potentials of edible green and red seaweed in Korea. *Fisheries and Aquatic Sciences*. 21:19. DOI: 10.1186/s41240-018-0095-y

Santizo-Taan, R., Bautista-Teruel, M. & Maquirang, J.R.H. 2020. Enriched *Ulva pertusa* as partial replacement of the combined fish and soybean meals in juvenile abalone *Haliotis asinina* (Linnaeus) diet. *Journal of Applied Phycology*. 32:741–9. DOI: 10.1007/s10811-019-01977-5

Santos, J.P., Guihéneuf, F., Fleming, G., Chow, F. & Stengel, D.B. 2019. Temporal stability in lipid classes and fatty acid profiles of three seaweed species from the north-eastern coast of Brazil. *Algal Research*. 41:101572. DOI: 10.1016/j.algal.2019.101572

Sato, Y., Takagi, S., Inomata, E. & Agatsuma, Y. 2019. Odor-active volatile compounds from the gonads of the sea urchin *Mesocentrotus nudus* in the wild in Miyagi Prefecture, Tohoku, Japan. *Food and Nutrition Sciences*. 10:860–75. DOI: 10.4236/fns.2019.107062

Scheibling, R.E. & Hamm, J. 1991. Interactions between sea urchins (*Strongylocentrotus droebachiensis*) and their predators in field and laboratory experiments. *Marine Biology*. 110:105–116. DOI: 10.1007/BF01313097

Schmid, M., Guihéneuf, F. & Stengel, D.B. 2014. Fatty acid contents and profiles of 16 macroalgae collected from the Irish Coast at two seasons. *Journal of Applied Phycology*. 26:451–63. DOI: 10.1007/s10811-013-0132-2

Schnitzler, I., Pohnert, G., Hay, M. & Boland, W. 2001. Chemical defense of brown algae (*Dictyopteris* spp.) against the herbivorous amphipod *Ampithoe longimana*. *Oecologia*. 126:515–21. DOI: 10.1007/s004420000546

Scholtz R., Bolton J.J. & Macey B.M. 2013. Effects of different microalgal feeds and their influence on larval development in the white-spined sea urchin *Tripneustes gratilla*. *African Journal of Marine Science*. 35:25–34. DOI: 10.2989/1814232X.2013.769902

Serviere-Zaragoza, E., Hurtado, M.A., Manzano-Sarabia, M., Mazariegos-Villarreal, A., Reza, M., Arjona, O. & Palacios, E. 2015. Seasonal and interannual variation of fatty acids in macrophytes from the Pacific coast of Baja California Peninsula (Mexico). *Journal of Applied Phycology*. 27:1297–1306. DOI: 10.1007/s10811-014-0415-2

Seymour, S., Paul, N.A., Dworjanyn, S.A. & de Nys, R. 2013. Feeding preference and performance in the tropical sea urchin *Tripneustes gratilla*. *Aquaculture*. 400–401:6–13. DOI: 10.1016/j.aquaculture.2013.02.030

Shahar, B., Shpigel, M., Barkan, R., Masasa, M., Neori, A., Chernov, H., Salomon, E., Kiflawi, M. & Guttman, L. 2020. Changes in metabolism, growth and nutrient uptake of *Ulva fasciata* (Chlorophyta) in response to nitrogen source. *Algal Research*. 46:101781. DOI: 10.1016/j.algal.2019.101781

Sharma, K.K., Schuhmann, H., Schenk, P.M. 2012. High lipid induction in microalgae for biodiesel production. *Energies*. 5:1532–53. DOI: 10.3390/en5051532

Sheybani, A., Nusnbaum, M., Caprio, J. & Derby, C.D. 2009. Responses of the sea catfish *Ariopsis felis* to chemical defenses from the sea hare *Aplysia californica*. *Journal of Experimental Marine Biology and Ecology*. 368:153–60. DOI: 10.1016/j.jembe.2008.09.024

Shi, P., Liao, K., Xu, J., Wang, Y., Xu, S. & Yan, X. 2022. Eicosapentaenoic acid mitigates palmitic acid-induced heat shock response, inflammation and repair processes in fish intestine. *Fish & Shellfish Immunology*. 124:362–71. DOI: 10.1016/j.fsi.2022.04.011

Shibata, T., Ishimaru, K., Kawaguchi, S., Yoshikawa, H. & Hama, Y. 2008. Antioxidant activities of phlorotannins isolated from Japanese Laminariaceae. *Journal of Applied Phycology*. 20:705. DOI: 10.1007/s10811-007-9254-8

Shipeng, Y., Hee-Chul, W., Jae-Hyung, C., Yong-Beom, P. & Byung-Soo, C. 2015. Measurement of antioxidant activities and phenolic and flavonoid contents of the brown seaweed *Sargassum horneri*: comparison of supercritical CO_2 and various solvent extractions. *Fisheries and Aquatic Sciences*. 18:123–30. DOI: 10.5657/FAS.2015.0123

Shpigel, M., Guttman, L., Shauli, L., Odintsov, V., Ben-Ezra, D. & Harpaz, S. 2017. *Ulva lactuca* from an Integrated Multi-Trophic Aquaculture (IMTA) biofilter system as a protein supplement in gilthead seabream (*Sparus aurata*) diet. *Aquaculture*. 481:112–8. DOI: 10.1016/j.aquaculture.2017.08.006

Shpigel, M., Shauli, L., Odintsov, V., Ben-Ezra, D., Neori, A. & Guttman, L. 2018a. The sea urchin, *Paracentrotus lividus*, in an Integrated Multi-Trophic Aquaculture (IMTA) system with fish (*Sparus aurata*) and seaweed (*Ulva lactuca*): Nitrogen partitioning and proportional configurations. *Aquaculture*. 490:260–9. DOI: 10.1016/j.aquaculture.2018.02.051

Shpigel, M., Shauli, L., Odintsov, V., Ashkenazi, N. & Ben-Ezra, D. 2018b. *Ulva lactuca* biofilter from a land-based integrated multi trophic aquaculture (IMTA) system as a sole food source for the tropical sea urchin *Tripneustes gratilla elatensis*. *Aquaculture*. 496:221–31. DOI: 10.1016/j.aquaculture.2018.06.038

Shpigel, M., Guttman, L., Ben-Ezra, D., Yu, J. & Chen, S. 2019. Is *Ulva* sp. able to be an efficient biofilter for mariculture effluents? *Journal of Applied Phycology*. 31:2449–59. DOI: 10.1007/s10811-019-1748-7

Shuuluka, D., Bolton, J.J. & Anderson, R.J. 2013. Protein content, amino acid composition and nitrogen-to-protein conversion factors of *Ulva rigida* and *Ulva capensis* from natural populations and *Ulva lactuca* from an aquaculture system, in South Africa. *Journal of Applied Phycology*. 25:677–85. DOI: 10.1007/s10811-012-9902-5

Silva, I., Rocha, S.M. & Coimbra, M.A. 2010. Quantification and potential aroma contribution of β-ionone in marine salt. *Flavour Fragrance Journal*. 25:93–7. DOI: 10.1002/ffj.1975

Silva, D.M., Valente, L.M.P., Sousa-Pinto, I., Pereira, R., Pires, M.A., Seixas, F. & Rema, P. 2015. Evaluation of IMTA-produced seaweeds (*Gracilaria*, *Porphyra*, and *Ulva*) as dietary ingredients in Nile tilapia, *Oreochromis niloticus* L., juveniles. Effects on growth performance and gut histology. *Journal of Applied Phycology*. 27:1671–80. DOI: 10.1007/s10811-014-0453-9

Siudem, P., Zielińska, A. & Paradowska, K. 2022. Application of ^{1}H NMR in the study of fatty acids composition of vegetable oils. *Journal of Pharmaceutical and Biomedical Analysis.* 212:114658. DOI: 10.1016/j.jpba.2022.114658

Smee, D.L. & Weissburg, M.J. 2006. Hard clams (*Mercenaria mercenaria*) evaluate predation risk using chemical signals from predators and injured conspecifics. *Journal of Chemical Ecology.* 32:605–19. DOI: 10.1007/s10886-005-9021-8

Smit, A.J., Robertson, B.L. & du Preez, D.R. 1996. Influence of ammonium-N pulse concentrations and frequency, tank condition and nitrogen starvation on growth rate and biochemical composition of *Gracilaria gracilis. Journal of Applied Phycology.* 8:473–81. DOI: 10.1007/BF02186325

Smit A.J., Robertson-Andersson D.V., Peall S. & Bolton J.J. 2007. Dimethylsulfoniopropionate (DMSP) accumulation in abalone *Haliotis midae* (Mollusca: Prosobranchia) after consumption of various diets, and consequences for aquaculture. *Aquaculture.* 269:377–89. DOI: 10.1016/j.aquaculture.2007.03.034

Smit, A.J., Robertson-Andersson, D.V. & Bolton, J.J. 2010. The effect of macroalgal and compound feeds on the sensory quality of cultivated South African abalone, *Haliotis midae* Linnaeus (Mollusca, Gastropoda). *Aquaculture Nutrition.* 16:590–603. DOI: 10.1111/j.1365-2095.2009.00696.x

Soares, C., Sousa, S., Machado, S., Vieira, E., Carvalho, A.P., Ramalhosa, M.J., Morais, S., Correia, M., Oliva-Teles, T., Domingues, V.F. & Delerue-Matos, C. 2021. Bioactive lipids of seaweeds from the Portuguese north coast: Health benefits versus potential contamination. *Foods.* 10:1366. DOI: 10.3390/foods10061366

Sola, C., Spampanato, A. & Tosi, L. 1993. Behavioural responses of glass eels (*Anguilla anguilla*) towards amino acids. *Journal of Fish Biology.* 42:683–91. DOI: 10.1111/j.1095-8649.1993.tb00377.x

Solari, P., Pasquini, V., Secci, M., Giglioli, A., Crnjar, R. & Addis, P. 2021. Chemosensitivity in the sea urchin *Paracentrotus lividus* (Echinodermata: Echinoidea) to food-related compounds: an innovative behavioral bioassay. *Frontier in Ecology and Evolution.* 9:749493. DOI: 10.3389/fevo.2021.749493

Soto-Sánchez, O., Hidalgo, P., González, A., Oliveira, P.E., Arias, A.J.H. & Dantagnan, P. 2023. Microalgae as raw materials for aquafeeds: growth kinetics and improvement strategies of polyunsaturated fatty acids production. *Aquaculture Nutrition.* 5110281. DOI: 10.1155/2023/5110281

Sousa, A.I., Martins, I., Lillebø, A.I., Flindt, M.R. & Pardal, M.A. 2007. Influence of salinity, nutrients and light on the germination and growth of *Enteromorpha* sp. spores. *Journal of Experimental Marine Biology and Ecology*. 341:142–50. DOI: 10.1016/j.jembe.2006.09.020

Spiers, L.J., Harrison, S.J., Deutsch, J.M., Garg, N. & Paul, V.J. 2021. The role of algal chemical defenses in the feeding preferences of the long-spined sea urchin *Diadema antillarum*. *Aquatic Ecology*. 55:941–53. DOI: 10.1007/s10452-021-09873-2

Stabili, L., Acquaviva, M.I., Biandolino, F., Cavallo, R.A., De Pascali, S.A., Fanizzi, F.P., Narracci, M., Petrocelli, A. & Cecere, E. 2012. The lipidic extract of the seaweed *Gracilariopsis longissima* (Rhodophyta, Gracilariales): a potential resource for biotechnological purposes? *New Biotechnology*. 29:443–50. DOI: 10.1016/j.nbt.2011.11.003

Stachowicz, J.J. & Hay, M.E. 1996. Facultative mutualism between an herbivorous crab and a coralline alga: advantages of eating noxious seaweeds. *Oecologia*. 105:377–87. DOI: 10.1007/BF00328741

Steinberg, P.D. & van Altena, I. 1992. Tolerance of marine invertebrate herbivores to brown algal phlorotannins in temperate Australasia. *Ecological Monographs*. 62:189–222. DOI: 10.2307/2937093

Stimson, J., Cunha, T. & Philippoff, J. 2007. Food preferences and related behavior of the browsing sea urchin *Tripneustes gratilla* (Linnaeus) and its potential for use as a biological control agent. *Marine Biology*. 151:1761–72. DOI: 10.1007/s00227-007-0628-x

Subramanian, A., Joshi, B.S., Roy, A.D., Roy, R., Gupta, V. & Dang, R.S. 2008. NMR spectroscopic identification of cholesterol esters, plasmalogen and phenolic glycolipids as fingerprint markers of human intracranial tuberculomas. *NMR in Biomedicine*. 21:272–88. DOI: 10.1002/nbm.1191

Sudatti, D.B., Fujii, M.T., Rodrigues, S.V., Turra, A. & Pereira, R.C. 2018. Prompt induction of chemical defenses in the red seaweed *Laurencia dendroidea*: The role of herbivory and epibiosis. *Journal of Sea Research*. 138:48–55. DOI: 10.1016/j.seares.2018.04.007

Sugisawa, H., Nakamura, K. & Tamura, H. 1990. The aroma profile of the volatiles in marine green algae (*Ulva pertusa*). *Food Reviews International*. 6:573–89. DOI: 10.1080/87559129009540893

Sun, S-M., Chung, G-H. & Shin, T-S. 2012. Volatile compounds of the green alga, *Capsosiphon fulvescens* . *Journal of Applied Phycology*. 24:1003–13. DOI: 10.1007/s10811-011-9724-x

Swanson, R.L., Williamson, J.E., De Nys, R., Kumar, N., Bucknall, M.P. & Steinberg, P.D. 2004. Induction of settlement of larvae of the sea urchin *Holopneustes purpurascens* by histamine from a host alga. *The Biological Bulletin*. 206:161–72. DOI: 10.2307/1543640

Swanson, D., Block, R. & Mousa, S.A. 2012. Omega-3 Fatty Acids EPA and DHA: Health Benefits Throughout Life. *Advances in Nutrition*. 3:1–7. DOI: 10.3945/an.111.000893

Tabarsa, M., Rezaei, M., Ramezanpour, Z. & Waaland, J.R. 2012. Chemical compositions of the marine algae *Gracilaria salicornia* (Rhodophyta) and *Ulva lactuca* (Chlorophyta) as a potential food source. *Journal of the Science of Food and Agriculture*. 92:2500–6. DOI: 10.1002/jsfa.5659

Taboada, C., Millán, R. & Míguez, I. 2010. Composition, nutritional aspects and effect on serum parameters of marine algae *Ulva rigida*. *Journal of the Science of Food and Agriculture*. 90:445–9. DOI: 10.1002/jsfa.3836

Takagi, S., Sato, Y., Kokubun, A., Inomata, E. & Agatsuma, Y. 2020a. Odor-active compounds from the gonads of *Mesocentrotus nudus* sea urchins fed *Saccharina japonica* kelp. *PLoS ONE*. 15:e0231673. DOI: 10.1371/journal.pone.0231673

Takagi, S., Sato, Y., Murata, Y., Kokubun, A., Touhata, K., Ishida, N. & Agatsuma, Y. 2020b. Quantification of the flavor and taste of gonads from the sea urchin *Mesocentrotus nudus* using GC–MS and a taste-sensing system. *Sensors*. 20:7008. DOI: 10.3390/s20247008

Takaoka, O., Takii, K., Nakamura, M., Kumai, H. & Takeda, M. 1995. Identification of feeding stimulants for tiger puffer. *Fisheries Science*. 61:833–6. DOI: 10.2331/fishsci.61.833

Tanner, C.E. 1986. Investigations of the taxonomy and morphological variation of *Ulva* (Chlorophyta): *Ulva californica* Wille. *Phycologia*. 25:510–20. DOI: 10.2216/i0031-8884-25-4-510.1

Tantikitti, C. 2014. Feed palatability and the alternative protein sources in shrimp feed. *Songklanakarin Journal of Science and Technology*. 36:51–5.

Terme, N., Boulho, R., Kendel, M., Kucma, J-P., Wielgosz-Collin, G., Bourgougnon, N. & Bedoux, G. 2017. Selective extraction of lipid classes from *Solieria chordalis* and *Sargassum muticum* using supercritical carbon dioxide and conventional solid–liquid methods. *Journal of Applied Phycology*. 29:2513–9. DOI: 10.1007/s10811-017-1084-8

Tocher, D.R. 2015. Omega-3 long-chain polyunsaturated fatty acids and aquaculture in perspective. *Aquaculture*. 449:94–107. DOI: 10.1016/j.aquaculture.2015.01.010

Tomas, F., Box, A. & Terrados, J. 2011. Effects of invasive seaweeds on feeding preference and performance of a keystone Mediterranean herbivore. *Biological Invasions*. 13:1559–70. DOI: 10.1007/s10530-010-9913-6

Toth, G.B. & Pavia, H. 2000. Water-borne cues induce chemical defense in a marine alga (*Ascophyllum nodosum*). *Proceedings of the National Academy of Sciences of the United States of America*. 97:14418–20. DOI: 10.1073/pnas.250226997

Toth, G.B., Harrysson, H., Wahlström, N., Olsson, J., Oerbekke, A., Steinhagen, S., Kinnby, A., White, J., Albers, E., Edlund, U., Undeland, I. & Pavia, H. 2020. Effects of irradiance, temperature, nutrients, and pCO2 on the growth and biochemical composition of cultivated *Ulva fenestrata*. *Journal of Applied Phycology*. 32:3243–54. DOI: 10.1007/s10811-020-02155-8

Trentin, R., Custódio, L., Rodrigues, M.J., Moschin, E., Sciuto, K., da Silva, J.P. & Moro, I. 2020. Exploring *Ulva australis* Areschoug for possible biotechnological applications: In vitro antioxidant and enzymatic inhibitory properties, and fatty acids contents. *Algal Research.* 50:101980. DOI: 10.1016/j.algal.2020.101980

Troell, M., Joyce, A., Chopin, T., Neori, A., Buschmann, A.H., & Fang, J.-G. 2009. Ecological engineering in aquaculture – potential for integrated multi-trophic aquaculture (IMTA) in marine offshore systems. *Aquaculture.* 297:1–9. DOI: 10.1016/j.aquaculture.2009.09.010

Tugnoli, V., 1, Bottura, G., Fini, G., Reggiani, A., Tinti, A., Trinchero, A. & Tosi, M.R. 2003. ^{1}H-NMR and ^{13}C-NMR lipid profiles of human renal tissues. *Biopolymers.* 72:86–95 DOI: 10.1002/bip.10299

Tuya, F., Martín, J.A., Reuss, G.M. & Luque, A. 2001. Food preferences of the sea urchin *Diadema antillarum* in Gran Canaria (Canary Islands, central-east Atlantic Ocean). *Journal of the Marine Biological Association of the United Kingdom.* 81:845–9. DOI: 10.1017/S0025315401004672

Urlass, S., Wu, Y., Nguyen, T.T.L., Winberg, P., Turner, M.S. & Smyth, H. 2023. Unravelling the aroma and flavour of algae for future food applications. *Trends in Food Science & Technology.* 138:370–81. DOI: 10.1016/j.tifs.2023.06.018

Vadas, R.L. 1977. Preferential feeding: an optimization strategy in sea urchins. *Ecological Monographs.* 47:337–71. DOI: 10.2307/1942173

Valente, L.M.P., Gouveia, A., Rema, P., Matos, J., Gomes, E.F. & Pinto, I.S. 2006. Evaluation of three seaweeds *Gracilaria bursa-pastoris*, *Ulva rigida* and *Gracilaria cornea* as dietary ingredients in European sea bass (*Dicentrarchus labrax*) juveniles. *Aquaculture.* 252:85–91. DOI: 10.1016/j.aquaculture.2005.11.052

Valente, L.M.P., Araújo, M., Batista, S., Peixoto, M.J., Sousa-Pinto, I., Brotas, V., Cunha, L.M. & Rema, P. 2016. Carotenoid deposition, flesh quality and immunological response of Nile tilapia fed increasing levels of IMTA-cultivated *Ulva* spp.. *Journal of Applied Phycology.* 28:691–701. DOI: 10.1007/s10811-015-0590-9

Valente, L.M.P., Custódio, M., Batista, S., Fernandes, H. & Kiron, V. 2019. Defatted microalgae (*Nannochloropsis* sp.) from biorefinery as a potential feed protein source to replace fishmeal in European sea bass diets. *Fish Physiology and Biochemistry.* 45:1067–81. DOI: 10.1007/s10695-019-00621-w

Van Alstyne, K., Wolfe, G., Freidenburg, T., Neill, A. & Hicken, C. 2001. Activated defense systems in marine macroalgae: evidence for an ecological role for DMSP cleavage. *Marine Ecology Progress Series.* 213:53–65. DOI: 10.3354/meps213053

Van Alstyne, K.L. & Houser, L.T. 2003. Dimethylsulfide release during macroinvertebrate grazing and its role as an activated chemical defense. *Marine Ecology Progress Series.* 250:175–81. DOI: 10.3354/meps250175

Van Alstyne, K.L., Nelson. A.V., Vyvyan, J.R. & Cancilla, D.A. 2006. Dopamine functions as an antiherbivore defense in the temperate green alga *Ulvaria obscura. Oecologia.* 148:304–11. DOI: 10.1007/s00442-006-0378-3

Van Alstyne, K.L., Koellermeier, L. & Nelson, T.A. 2007. Spatial variation in dimethylsulfoniopropionate (DMSP) production in *Ulva lactuca* (Chlorophyta) from the Northeast Pacific. *Marine Biology*. 150:1127–35. DOI: 10.1007/s00227-006-0448-4

van Ginneken, V.J., Helsper, J.P., de Visser, W., van Keulen, H. & Brandenburg, W.A. 2011. Polyunsaturated fatty acids in various macroalgal species from north Atlantic and tropical seas. *Lipids in Health and Disease*. 10:104. DOI: 10.1186/1476-511X-10-104

Vandermeulen, H. & Gordin, H. 1990. Ammonium uptake using *Ulva* (Chlorophyta) in intensive fishpond systems: mass culture and treatment of effluent. *Journal of Applied Phycology*. 2:363–74. DOI: 10.1007/BF02180927

Varlet, V., Prost, C. & Serot, T. 2007. Volatile aldehydes in smoked fish: analysis methods, occurrence and mechanisms of formation. *Food Chemistry*. 105:1536–56. DOI: 10.1016/j.foodchem.2007.03.041

Velez, Z., Hubbard, P.C., Hardege, J.D., Barata, E.N. & Canário, A.V.M. 2007. The contribution of amino acids to the odour of a prey species in the Senegalese sole (*Solea senegalensis*). *Aquaculture*. 265:336–42. DOI: 10.1016/j.aquaculture.2007.02.029

Veneklaas, E.J., Lambers, H., Bragg, J., Finnegan, P.M., Lovelock, C.E., Plaxton, W.C., Price, C.A., Scheible, W.-R., Shane, M.W., White, P.J. & Raven, J.A. 2012. Opportunities for improving phosphorus-use efficiency in crop plants. *New Phytologist*. 195:306–20. DOI: 10.1111/j.1469-8137.2012.04190.x

Vera, J., Castro, J., Gonzalez, A. & Moenne, A. 2011. Seaweed polysaccharides and derived oligosaccharides stimulate defense responses and protection against pathogens in plants. *Marine Drugs*. 9:2514–25. DOI: 10.3390/md9122514

Vidal, N.P., Manzanos, M.J., Goicoechea, E. & Guillén, M.D. 2012. Quality of farmed and wild sea bass lipids studied by ^{1}H NMR: Usefulness of this technique for differentiation on a qualitative and a quantitative basis. *Food Chemistry*. 135:1583–91. DOI: 10.1016/j.foodchem.2012.06.002

Voigt, R. & Atema, J. 1992. Tuning of chemoreceptor cells of the second antenna of the American lobster (*Homarus americanus*) with a comparison of four of its other chemoreceptor organs. *Journal of Comparative Physiology A*. 171:673–83. DOI: 10.1007/BF00194115

Waldron, H.N. & Probyn, T.A. 1992. Nitrate supply and potential new production in the Benguela upwelling system. *South African Journal of Marine Science*. 12:29–39. DOI: 10.2989/02577619209504688

Wan, A.H.L., Davies, S.J., Soler-Vila, A., Fitzgerald, R. & Johnson, M.P. 2019. Macroalgae as a sustainable aquafeed ingredient. *Reviews in Aquaculture*. 11:458–92. DOI: 10.1111/raq.12241

Wang, Y.-B. 2007. Effect of probiotics on growth performance and digestive enzyme activity of the shrimp *Penaeus vannamei*. *Aquaculture*. 269:259–64. DOI: 10.1016/j.aquaculture.2007.05.035

Wang, S., Wang, Q., Jiang, X., Han, X. & Ji, H. 2013. Compositional analysis of bio-oil derived from pyrolysis of seaweed. *Energy Conversion and Management.* 68:273–80. DOI: 10.1016/j.enconman.2013.01.014

Wang, Y., Gao, Y., Liang, W., Liu, Y. & Gao, H. 2020. Identification and analysis of the flavor characteristics of unfermented stinky tofu brine during fermentation using SPME-GC–MS, e-nose, and sensory evaluation. *Food Measure.* 14:597–612. DOI: 10.1007/s11694-019-00351-w

Wang, H., Yang, L., Wang, X., Cong, P., Xu, J. & Xue, C. 2022. Comprehensive lipidomic analysis of three edible brown seaweeds based on reversed-phase liquid chromatography coupled with quadrupole time-of-flight mass spectrometry. *Journal of Agricultural and Food Chemistry.* 70:4138–51. DOI: 10.1021/acs.jafc.1c07513

Wang, Y., Liu, R., Chang, M., Wei, W., Guo, Y., Jin, Q. & Wang, X. 2023 Does omega-3 PUFA-enriched oral nutritional intervention benefit cancer patients receiving chemo (radio) therapy? A systematic review and meta-analysis of randomized controlled trials. *Critical Reviews in Food Science and Nutrition.* 63:3081–96. DOI: 10.1080/10408398.2021.1984199

Wassef, E.A., El-Sayed, AF.M. & Sakr, E.M. 2013 *Pterocladia* (Rhodophyta) and *Ulva* (Chlorophyta) as feed supplements for European seabass, *Dicentrarchus labrax* L., fry. *Journal of Applied Phycology*. 25:1369–76. DOI: 10.1007/s10811-013-9995-5

Webster, D.R. & Weissburg, M.J. 2009. The hydrodynamics of chemical cues among aquatic organisms. *Annual Review of Fluid Mechanics.* 41:73–90. DOI: 10.1146/annurev.fluid.010908.165240

Weissburg, M.J., Ferner, M.C., Pisut, D.P. & Smee, D.L. 2002. Ecological consequences of chemically mediated prey perception. *Journal of Chemical Ecology.* 28:1953–70. DOI: 10.1023/A:1020741710060

Wessels, H., Hagen, W., Molis, M., Wiencke, C. & Karsten, U. 2006. Intra- and interspecific differences in palatability of Arctic macroalgae from Kongsfjorden (Spitsbergen) for two benthic sympatric invertebrates. *Journal of Experimental Marine Biology and Ecology.* 329:20–33. DOI: 10.1016/j.jembe.2005.08.006

Whitfield, F.B., Helidoniotis, F., Shaw, K.J. & Svoronos, D. 1997. Distribution of bromophenols in Australian wild-harvested and cultivated prawns (shrimp). *Journal of Agriculture and Food Chemistry.* 45:4398–405. DOI: 10.1021/jf970363r

Whitfield, F.B., Helidoniotis, F. & Smith, D. 2002. Role of feed ingredients in the bromophenol content of cultured prawns. *Food Chemistry.* 79:355–65. DOI: 10.1016/S0308-8146(02)00187-5

Wichard, T., Charrier, B., Mineur, F., Bothwell, J.H., De Clerck, O. & Coates, J.C. 2015. The green seaweed *Ulva*: a model system to study morphogenesis. *Frontiers in Plant Science.* 6:72. DOI: 10.3389/fpls.2015.00072

Wielgosz-Collin, G., Kendel, M. & Couzinet-Mossion, A. 2016. Chapter 7 - Lipids, Fatty Acids, Glycolipids, and Phospholipids. Fleurence, J. & Levine, I. [Eds.] *Seaweed in Health and Disease Prevention*. Academic Press, pp. 185–221. DOI: 10.1016/B978-0-12-802772-1.00007-5

Wiesemeier, T., Hay, M. & Pohnert, G. 2007. The potential role of wound-activated volatile release in the chemical defence of the brown alga *Dictyota dichotoma*: Blend recognition by marine herbivores. *Aquatic Sciences.* 69:403–12. DOI: 10.1007/s00027-007-0889-y

Williams, K.C. 2007. Nutritional requirements and feeds development for post-larval spiny lobster: A review. *Aquaculture.* 263:1–14. DOI: 10.1016/j.aquaculture.2006.10.019

Williamson, J.E., Carson, D.G., de Nys, R. & Steinberg, P.D. 2004. Demographic consequences of an ontogenetic shift by a sea urchin in response to host plant chemistry. *Ecology.* 85:1355–71. DOI: 10.1890/02-4083

Williamson, J.E., Gleeson, C., Bell, J.E. & Vaïtilingon, D. 2012. The role of visual and chemical cues in host detection by the symbiotic shrimp *Gnathophylloides mineri*. *Journal of Experimental Marine Biology and Ecology.* 414–415:38–43. DOI: 10.1016/j.jembe.2012.01.009

Wolf, M.A., Sciuto, K., Andreoli, C. & Moro, I. 2012. *Ulva* (Chlorophyta, Ulvales) biodiversity in the North Adriatic Sea (Mediterranean, Italy): cryptic species and new introductions. *Journal of Phycology*. 48:1510–21. DOI: 10.1111/jpy.12005

Xia, S., Zhao, P., Chen, K., Li, Y., Liu, S., Zhang, L. & Yang, H. 2012. Feeding preferences of the sea cucumber *Apostichopus japonicus* (Selenka) on various seaweed diets. *Aquaculture.* 344–349:205–9. DOI: 10.1016/j.aquaculture.2012.03.022

Xu, X-Q., Tran, V.H., Kraft, G. & Beardall, J. 1998. Fatty acids of six *Codium* species from southeast Australia. *Phytochemistry*. 48:1335–9. DOI: 10.1016/S0031-9422(98)00115-0

Yamamoto, M., Baldermann, S., Yoshikawa, K., Fujita, A., Mase, N. & Watanabe, N. 2014. Determination of volatile compounds in four commercial samples of Japanese green algae using solid phase microextraction gas chromatography mass spectrometry. *The Scientific World Journal.* 289780. DOI: 10.1155/2014/289780

Yang, K.M., Jeon, B.H., Kim, H.G. & Kim, J.H. 2021. Feeding behaviors of a sea urchin, *Mesocentrotus nudus*, on six common seaweeds from the east coast of Korea. *Algae.* 36:51–60. DOI: 10.4490/algae.2021.36.3.5

Yangthong, M. & Ruensirikul, J. 2020. Feed intake stimulation of juvenile spotted scat (*Scatophagus argus* Linnaeus, 1766) using dietary seaweed supplementation (*Ulva* sp.). *Aquaculture.* 529:735626. DOI: 10.1016/j.aquaculture.2020.735626

Yokoyama, H. & Ishihi, Y. 2010. Bioindicator and biofilter function of *Ulva* spp. (Chlorophyta) for dissolved inorganic nitrogen discharged from a coastal fish farm – potential role in integrated multi-trophic aquaculture. *Aquaculture.* 310:74–83. DOI: 10.1016/j.aquaculture.2010.10.018

Yu, C.E., Kim, Y.R., Noh, G.E. & Kim, J.M. 2023. Behavioral analysis of Pacific abalone, *Haliotis discus hannai*, reveals its feeding preference and attraction potential for brown alga, *Sargassum horneri*. *Fisheries and Aquatic Sciences.* 26:355–65. DOI: 10.47853/FAS.2023.e30

Yuan, S., Wu, K., Duan, Z., Huang, Y., Lu, Y. & Ma, X. 2019. A sustainable process for the recovery of volatile constituents from *Gracilaria lemaneiformis* in agar production and evaluation of their antioxidant activities. *BMC Chemistry.* 13:74. DOI: 10.1186/s13065-019-0590-y

Zare, M., Kazempour, M., Hosseini, H., Hosseini Choupani, S.M., Akhavan, S.R., Rombenso, A. & Esmaeili, N. 2023. Fish meal replacement and early mild stress improve stress responsiveness and survival of fish after acute stress. *Animals.* 13:1314. DOI: 10.3390/ani13081314

Zhang, J., Liu, Q., Pang, Y., Xu, X., Cui, K., Zhang, Y., Mai, K. & Ai, Q. 2020. Molecular cloning and the involvement of IRE1α-XBP1s signaling pathway in palmitic acid induced - Inflammation in primary hepatocytes from large yellow croaker (*Larimichthys crocea*). *Fish & Shellfish Immunology.* 98:112–21. DOI: 10.1016/j.fsi.2019.12.089

Zheng, Z.L., Tan, J.Y.W., Liu, H.Y., Zhou, X.H., Xiang, X. & Wang, K.Y. 2009. Evaluation of oregano essential oil (*Origanum heracleoticum* L.) on growth, antioxidant effect and resistance against *Aeromonas hydrophila* in channel catfish (*Ictalurus punctatus*). *Aquaculture.* 292:214–8. DOI: 10.1016/j.aquaculture.2009.04.025

Zheng, Y., Jin, R., Zhang, X., Wang, Q. & Wu, J. 2019. The considerable environmental benefits of seaweed aquaculture in China. *Stochastic Environmental Research and Risk Assessment.* 33:1203–21. DOI: 10.1007/s00477-019-01685-z

Zhu, Y., Chen, J., Chen, X., Chen, D. & Deng, S. 2020. Use of relative odor activity value (ROAV) to link aroma profiles to volatile compounds: application to fresh and dried eel (*Muraenesox cinereus*), *International Journal of Food Properties.* 23:2257–70, DOI: 10.1080/10942912.2020.1856133

Zhuo, L.-C., Liu, K. & Lin, Y.-H. 2016. Apparent digestibility of soybean meal and *Lactobacillus* spp. fermented soybean meal in diets of grouper, *Epinephelus coioides*. *Aquaculture Research.* 47:1009–12. DOI: 10.1111/are.12543

Zimmer-Faust, R.K., Gleeson, R.A. & Carr, W.E.S. 1988. The behavioral response of spiny lobsters to ATP: evidence for mediation by P_2-like chemosensory receptors. *The Biological Bulletin.* 175:167–74. DOI: 10.2307/1541904

Supplementary Information 2 (Chapter 2)

S2.1: ^{1}H NMR of crude extract and *Ulva* fractions

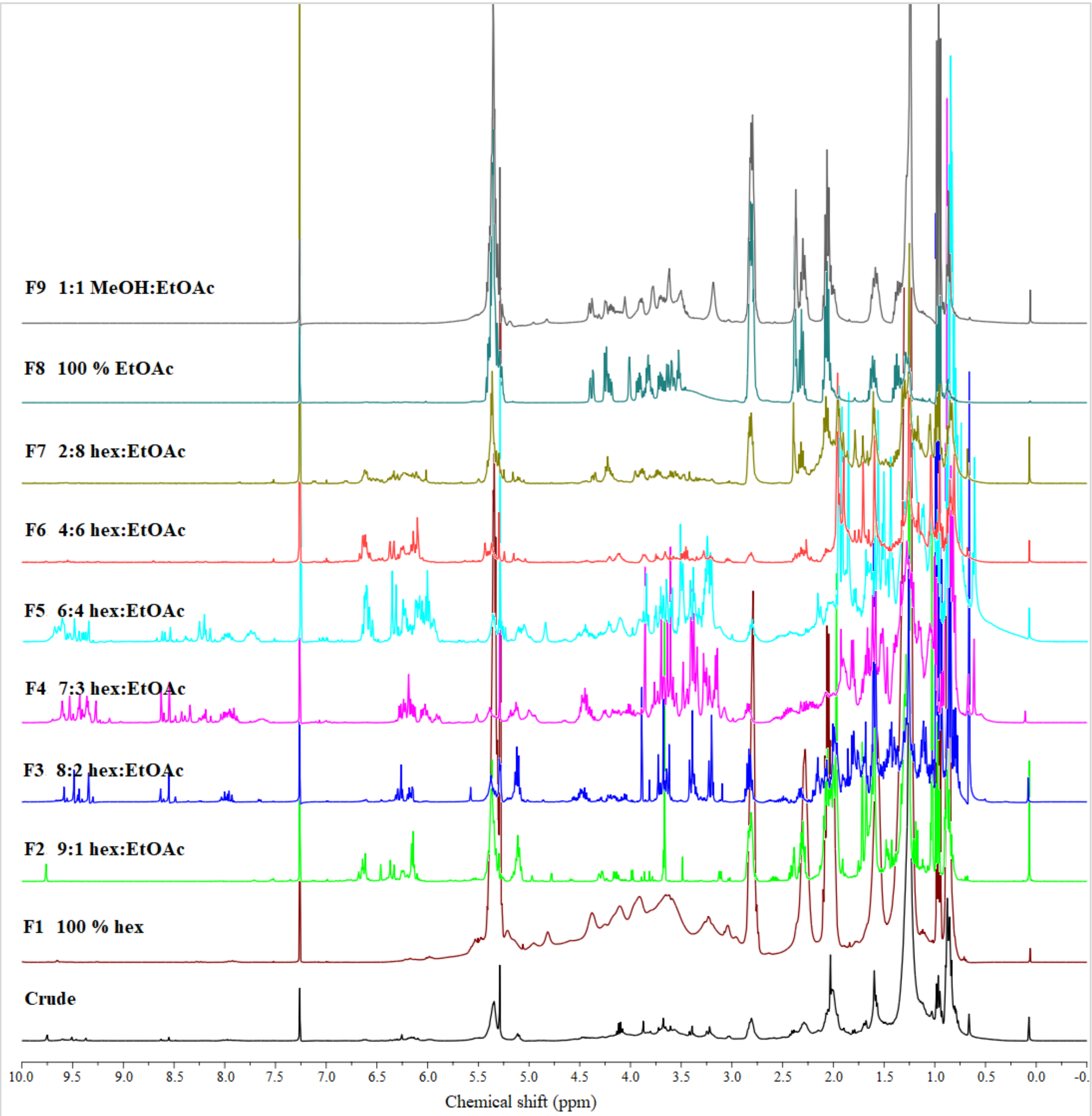


Figure S2.1: ^{1}H NMR spectra ($CDCl_3$, 400 MHz) of the crude organic extract of *Ulva lacinulata* and the step gradient column fractions from δ_H -0.20 to δH 10.00 with F1 (100% hexane), F2 (9:1 hexane:ethyl acetate), F3 (8:2 hexane:ethyl acetate), F4 (7:3 hexane:ethyl acetate), F5 (6:4 hexane:ethyl acetate), F6 (4:6 hexane:ethyl acetate), F7 (2:8 hexane:ethyl acetate), F8 (100% ethyl acetate) and F9 (1:1 methanol:ethyl acetate).

S2.2: HPLC chromatogram of F8

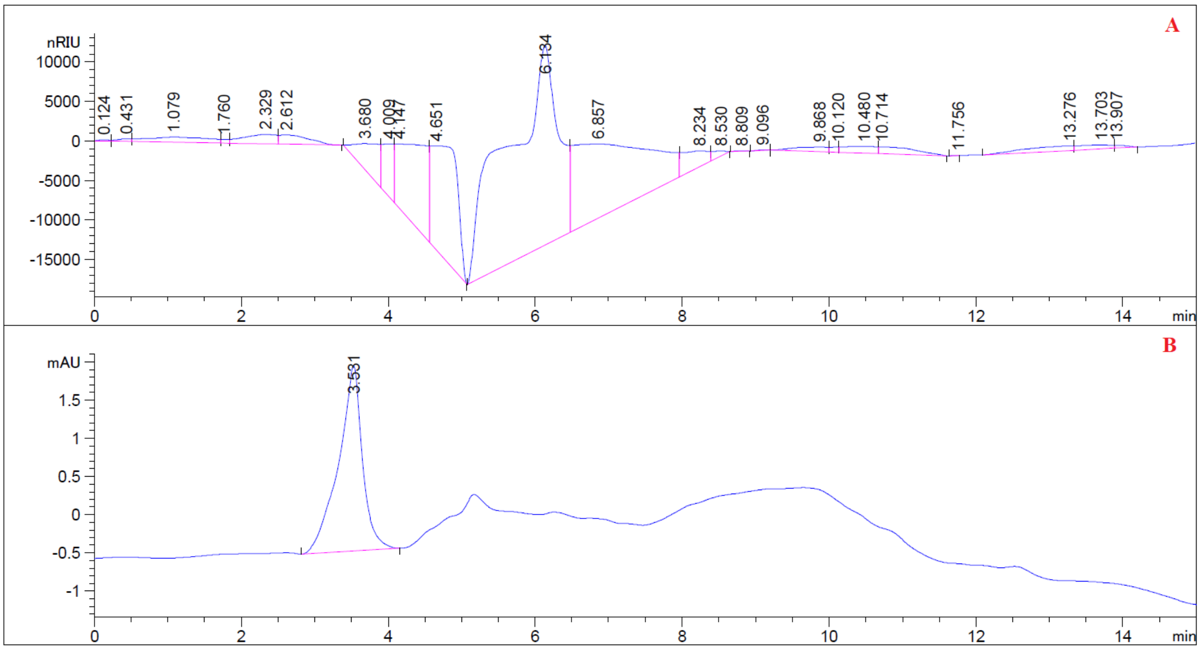


Figure S2.2: HPLC chromatogram of *Ulva lacinulata* F8 (A) refractive index signal (RI); and (B) variable wavelength detectors (VWD; 254 nm).

S2.3: COSY spectra of compound 2.1

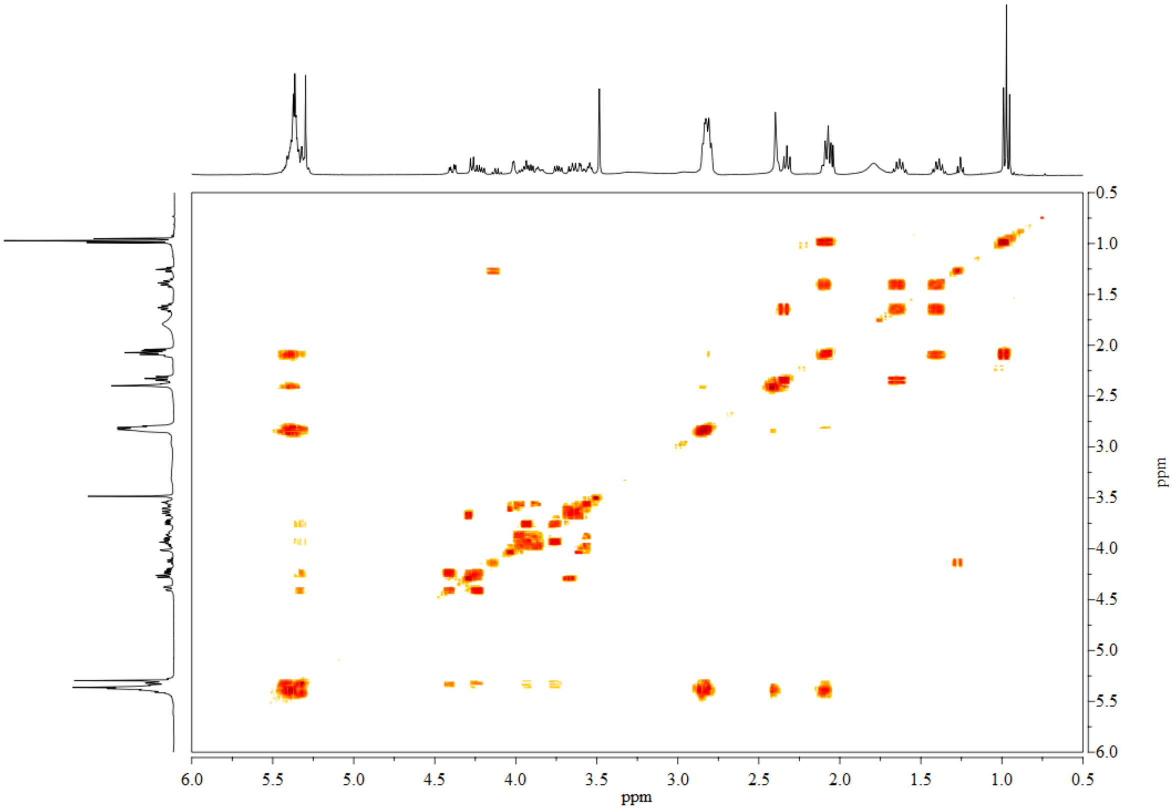


Figure S2.3: ^{1}H-^{1}H homonuclear correlation spectroscopy spectrum (COSY; 400 MHz $CDCl_3$) of compound **2.1**.

S2.4: HSQC spectra of compound 2.1

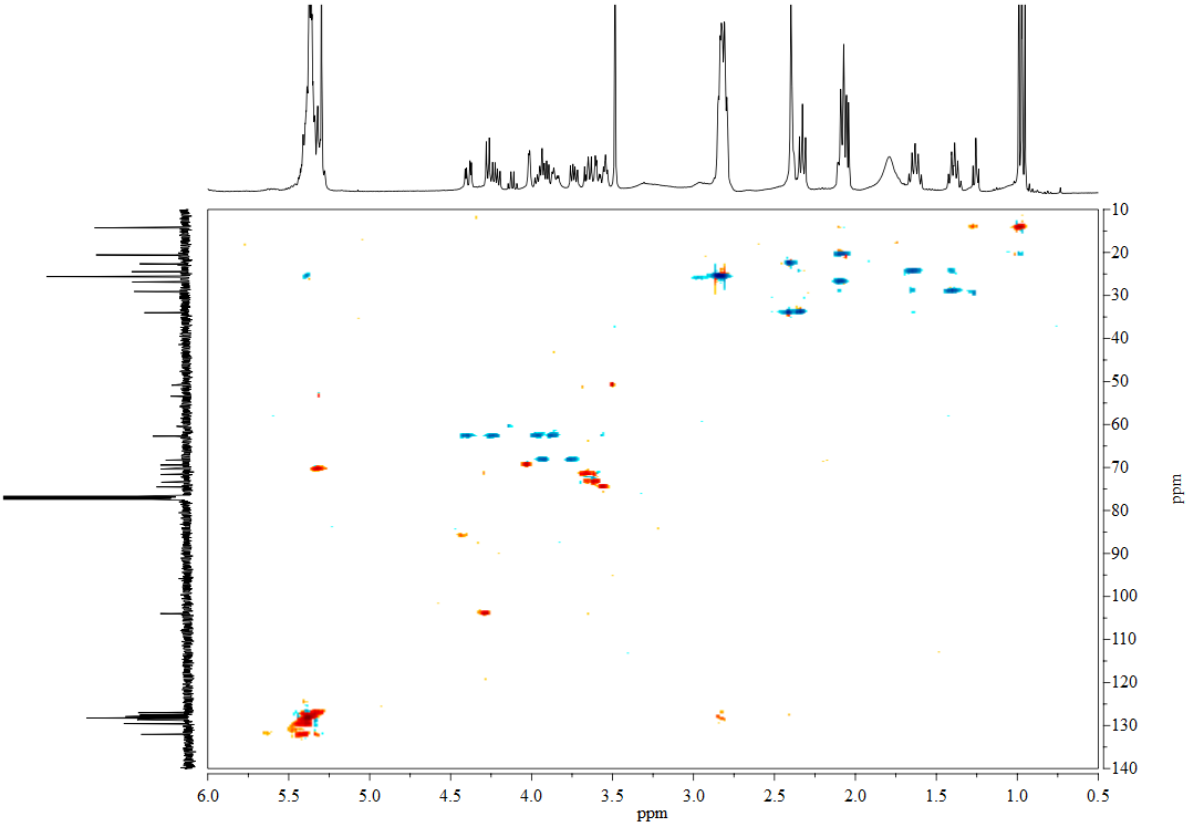


Figure S2.4: ^{1}H-^{13}C heteronuclear single quantum correlation (HSQC; 400 Hz $CDCl_3$) of compound **2.1**.

S2.5: DEPT – 135 spectra of compound 2.1

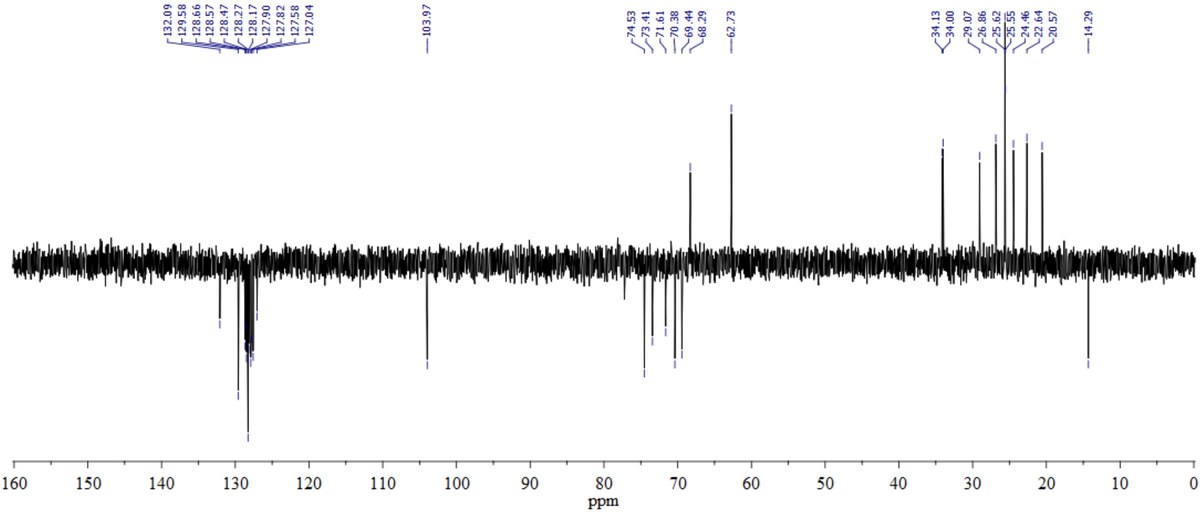


Figure S2.5: Distortionless enhancement by polarization transfer-135 (DEPT; 100 Hz) spectra of compound **2.1**.

S2.6: LC-MS chromatogram of F8

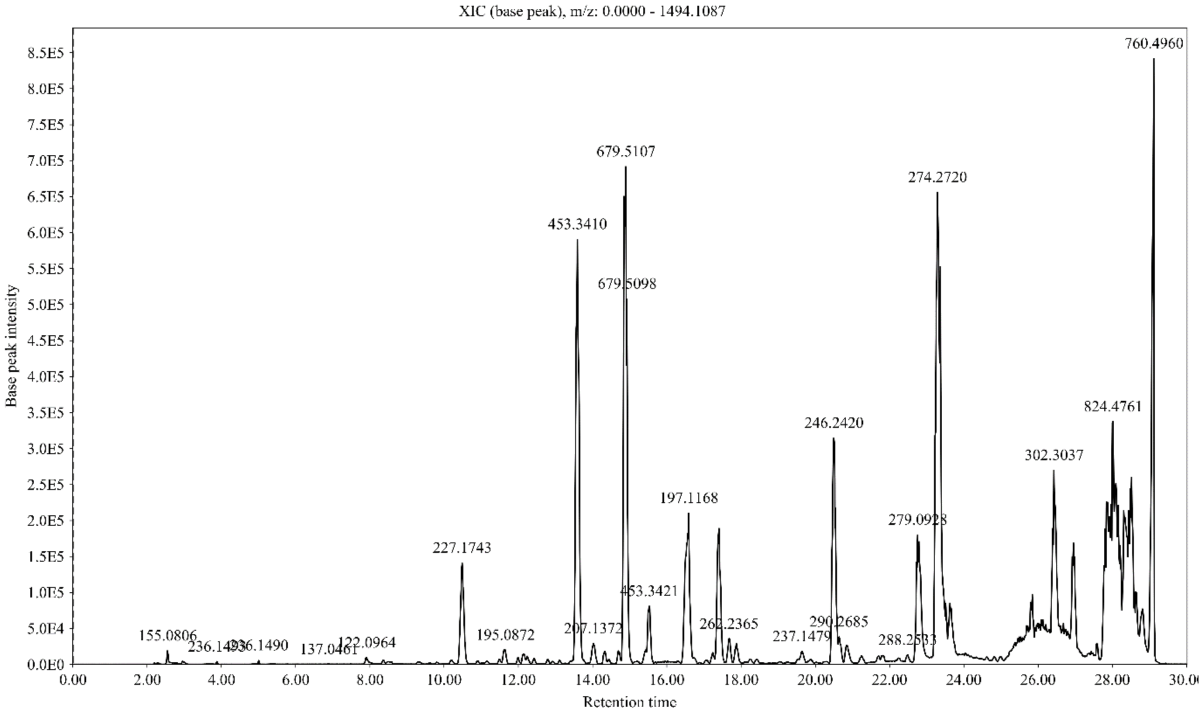


Figure S2.6: Chromatogram of *Ulva lacinulata* F8 from liquid chromatography-mass spectrometry (LC-MS) in positive ESI mode.

S2.7: Mass spectra of compound 2.1

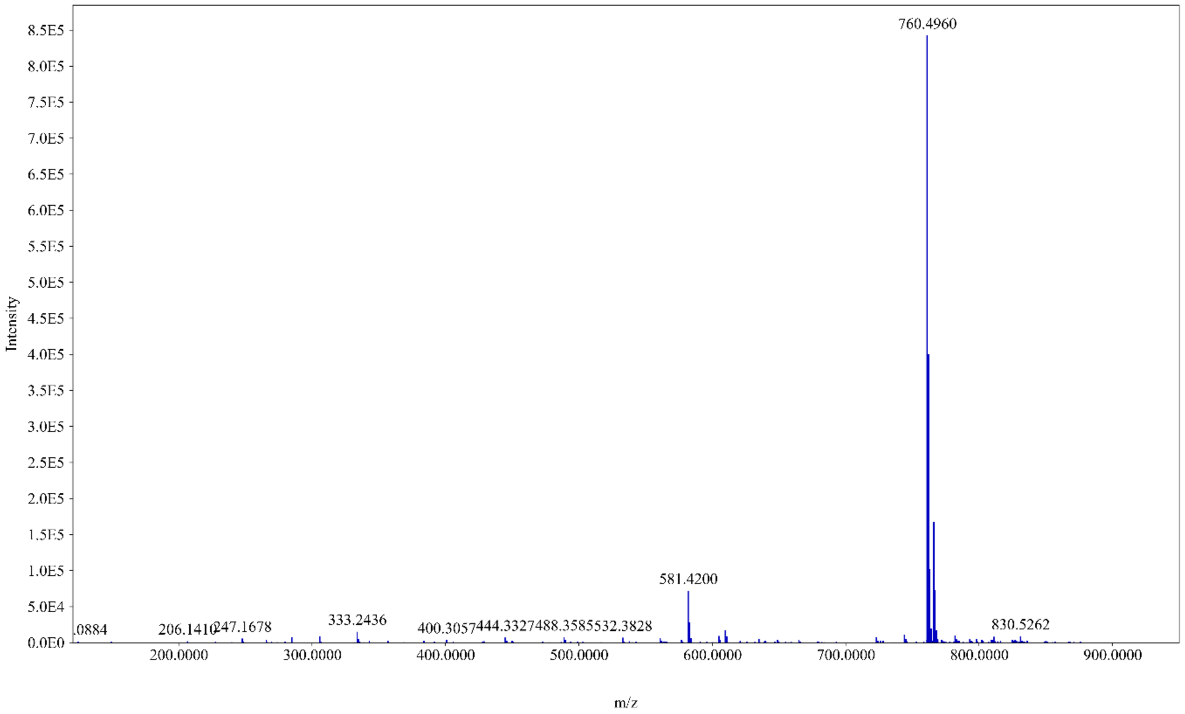


Figure S2.7: Mass spectra of compound **2.1** from mass spectra 1 (MS1).

Table S2.1: Compound **2.1** detected from *Ulva lacinulata* fraction F8 in positive ESI mode.

RT (min)	Compound	Chemical formula	Theoretical m/z (T)	Detected m/z (D)	Difference (T – D)	Fragment m/z
$[M+NH_4]^+$						
28.98	**MGDG**	$C_{43}H_{70}O_{10}N$	760.4994	760.4960	0.0034	581.4

S2.8: HPLC chromatogram of F9

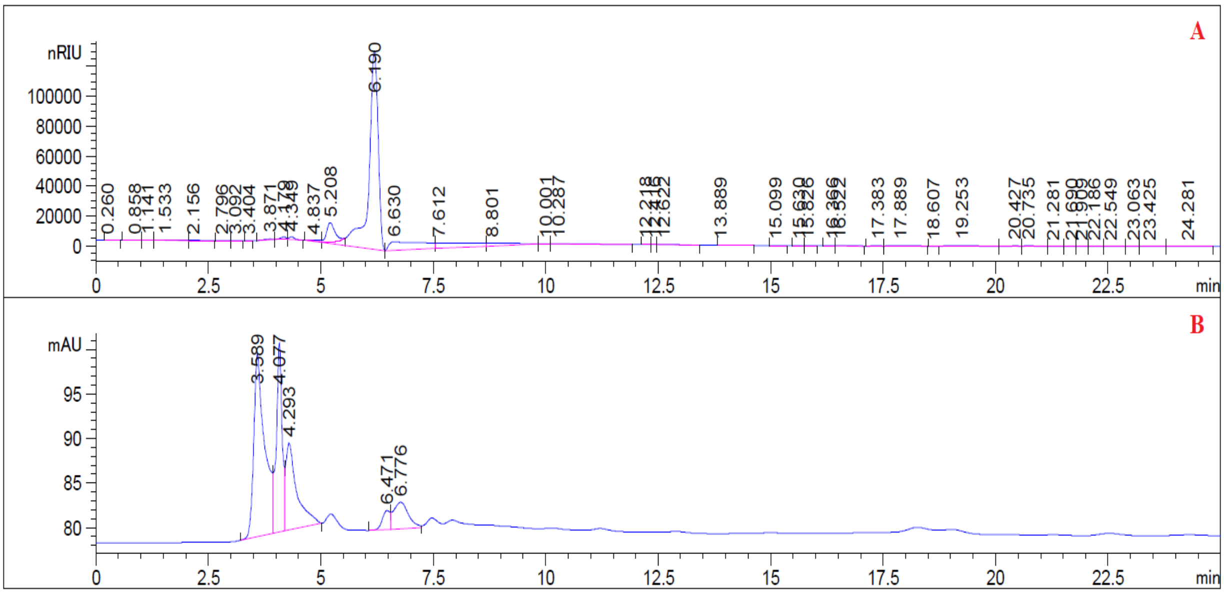


Figure S2.8: HPLC chromatogram of *Ulva lacinulata* F9 (A) refractive index signal (RI); and (B) variable wavelength detectors (VWD; 254 nm).

S2.9: LC-MS chromatogram of F9 positive ESI mode

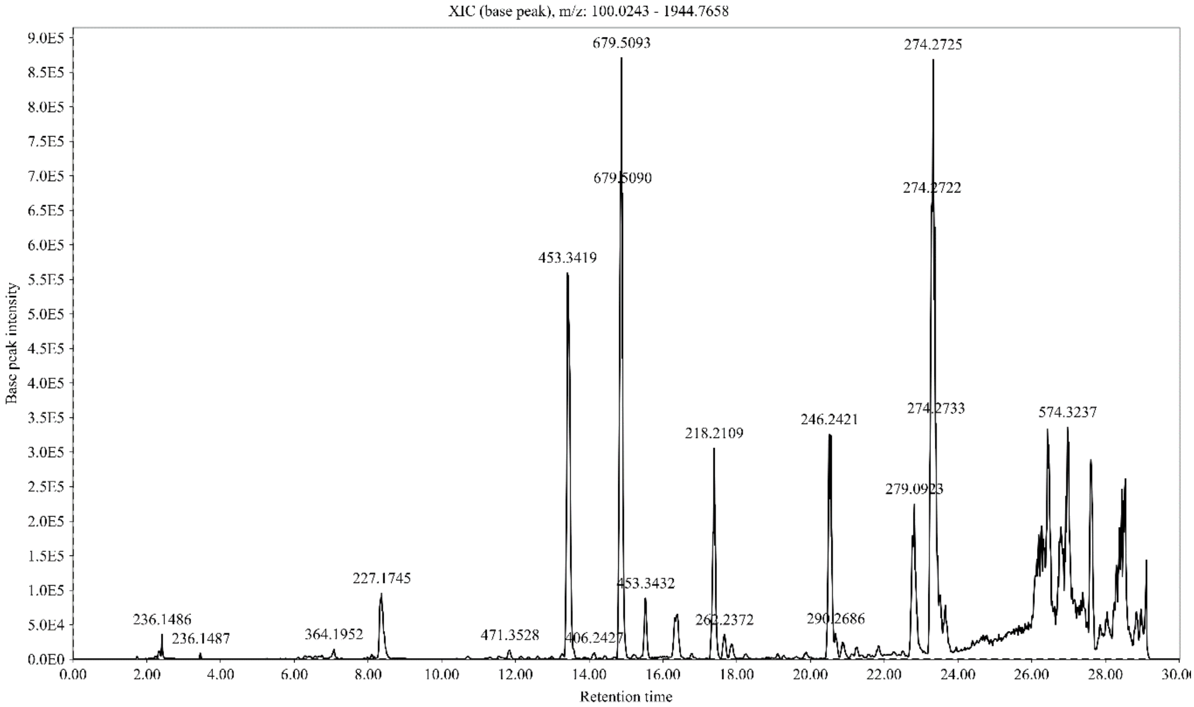


Figure S2.9: Chromatogram of *Ulva lacinulata* F9 from liquid chromatography–mass spectrometry (LC–MS) in MS level 1.

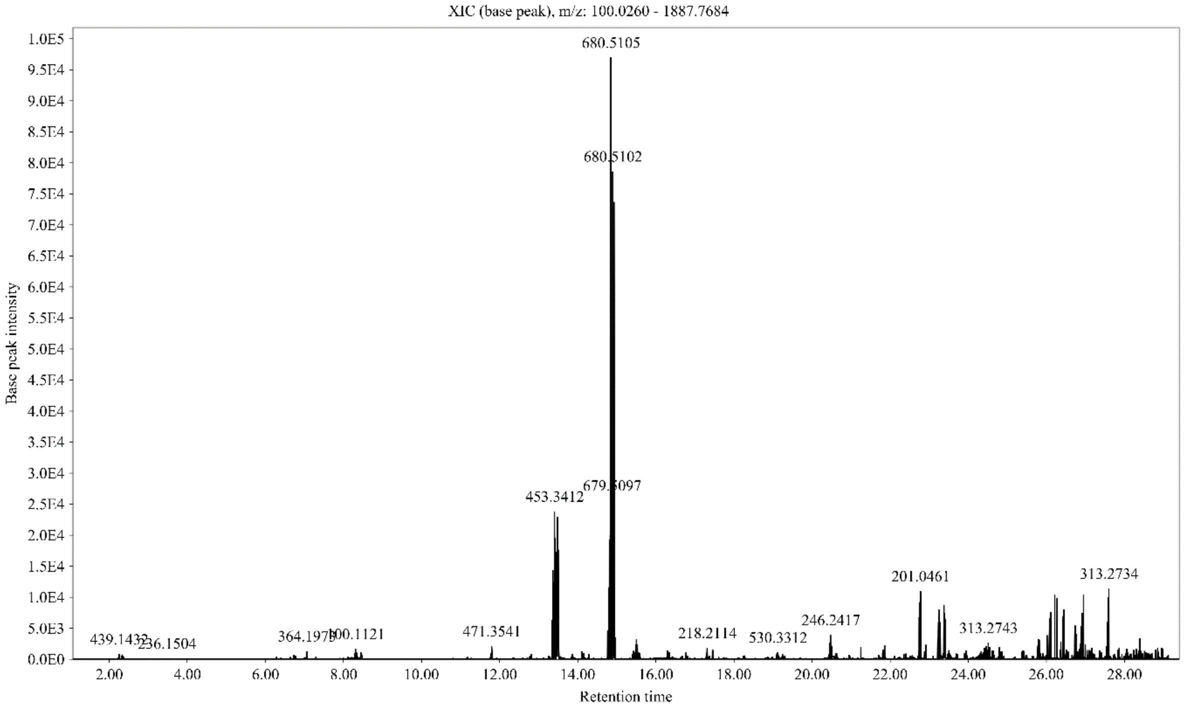


Figure S2.10: Chromatogram of *Ulva lacinulata* F9 from liquid chromatography–mass spectrometry (LC–MS) in MS level 2.

S2.10: Mass spectra of compounds from F9

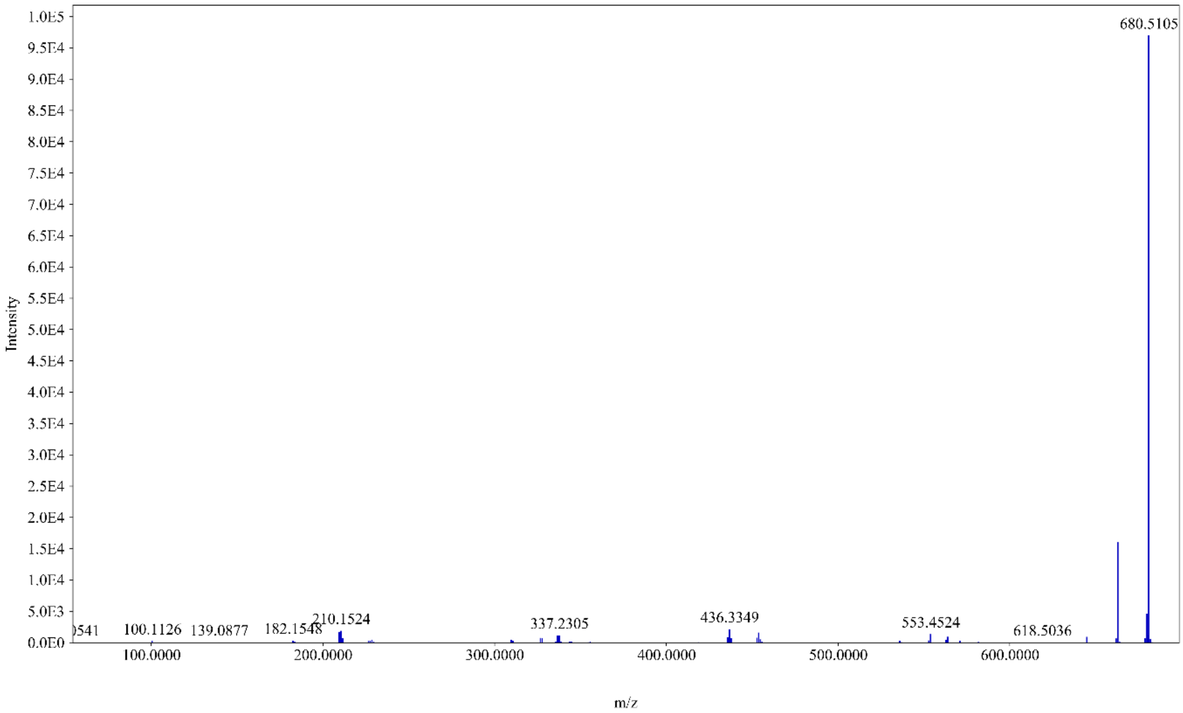


Figure S2.11: Mass spectra of diacylglyceryl-N,N,N-trimethyl-homoserine (DGTS) from *Ulva lacinulata* fraction F9 (MS level 2).

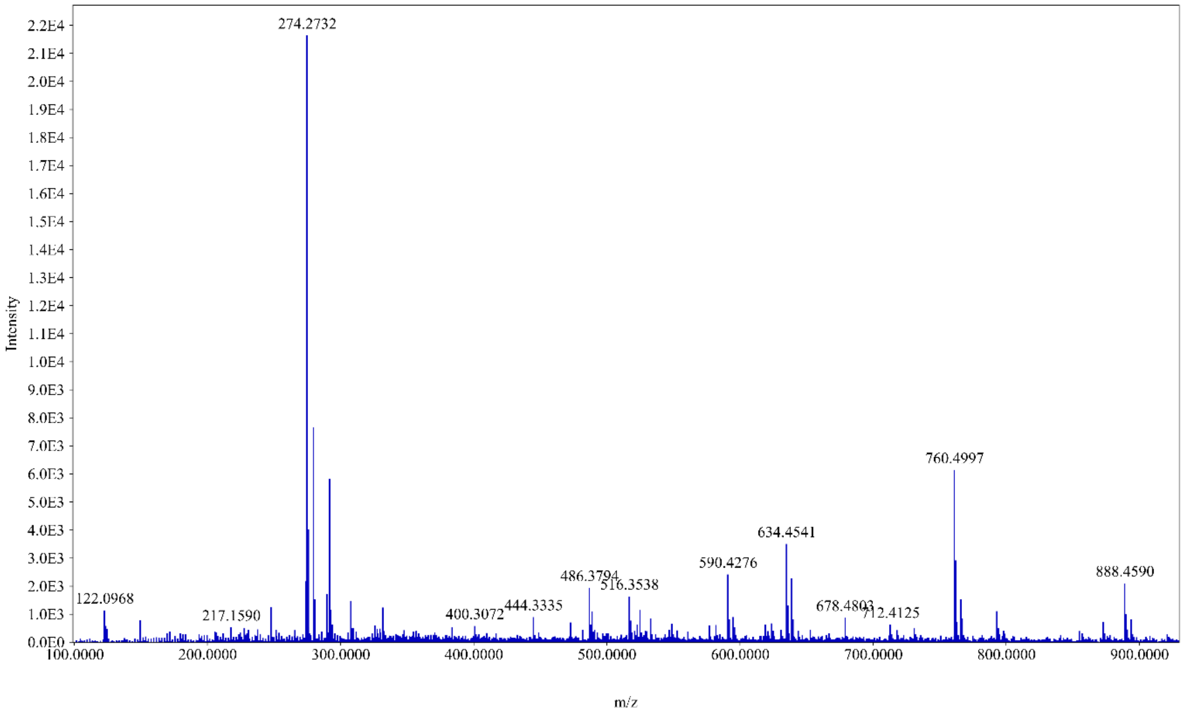


Figure S2.12: Mass spectra of digalactosyldiacylglycerol (DGDG) from *Ulva lacinulata* fraction F9 (MS level 1).

S2.11: LC-MS chromatogram of F9 negative ESI mode

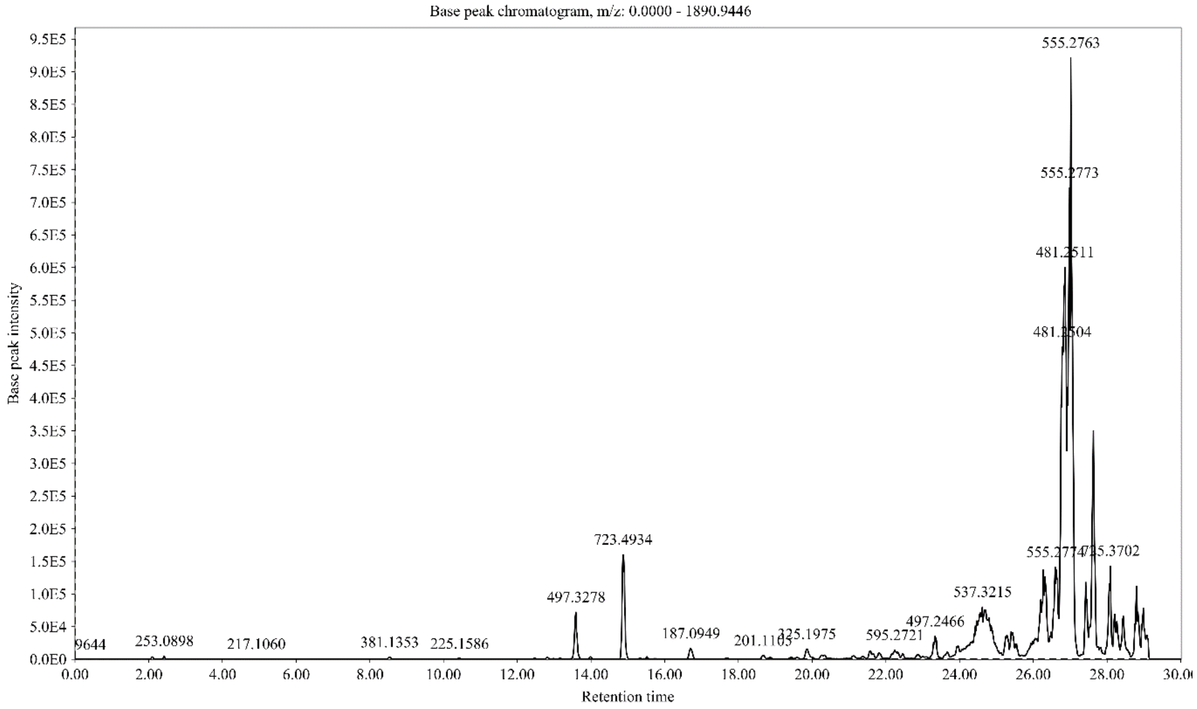


Figure S2.13: Chromatogram of *Ulva lacinulata* F9 from liquid chromatography–mass spectrometry (LC–MS) in negative ESI mode.

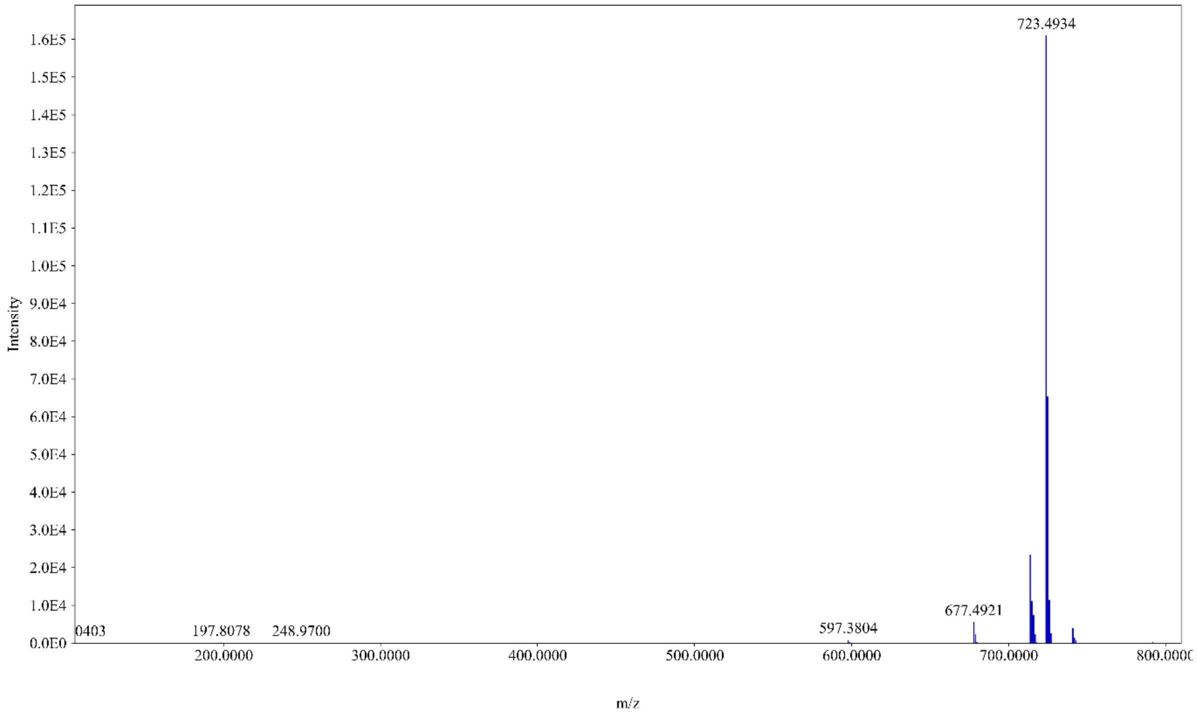


Figure S2.14: Mass spectra of dialkylglycerophosphate (PA) from *Ulva lacinulata* fraction F9 (MS level 1).

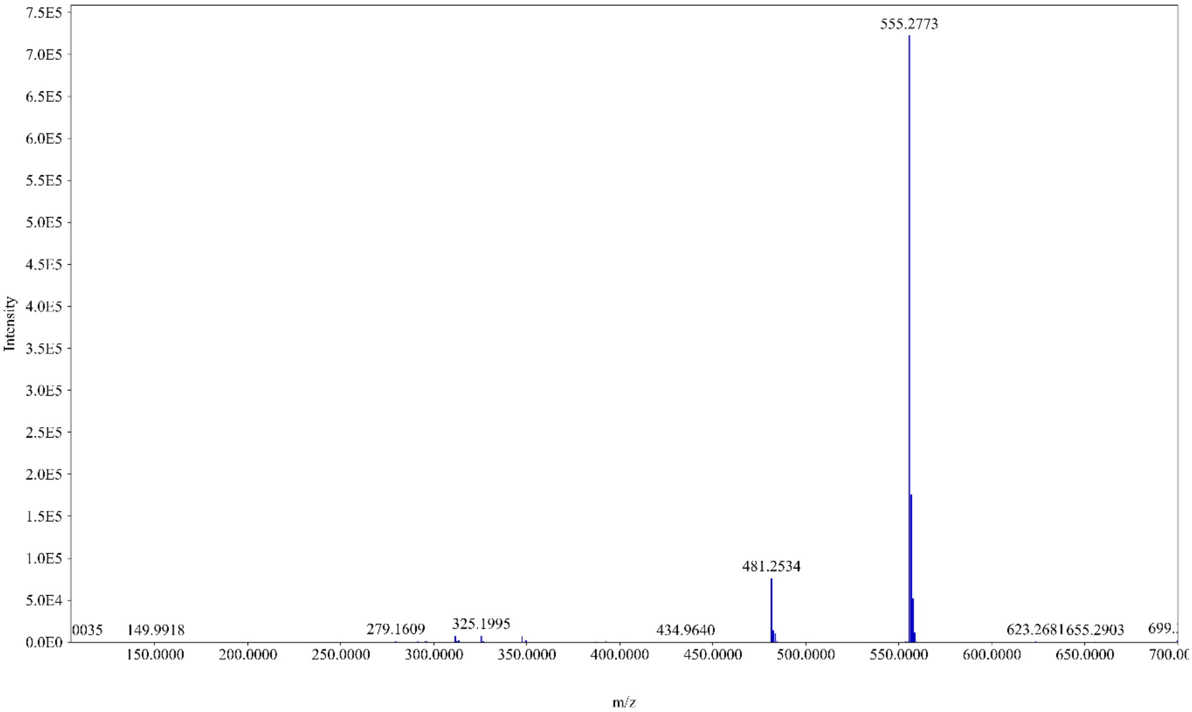


Figure S2.15: Mass spectra of sulfoquinovosylmonoacylglycerol (SQMG) from *Ulva lacinulata* fraction F9 (MS level 1).

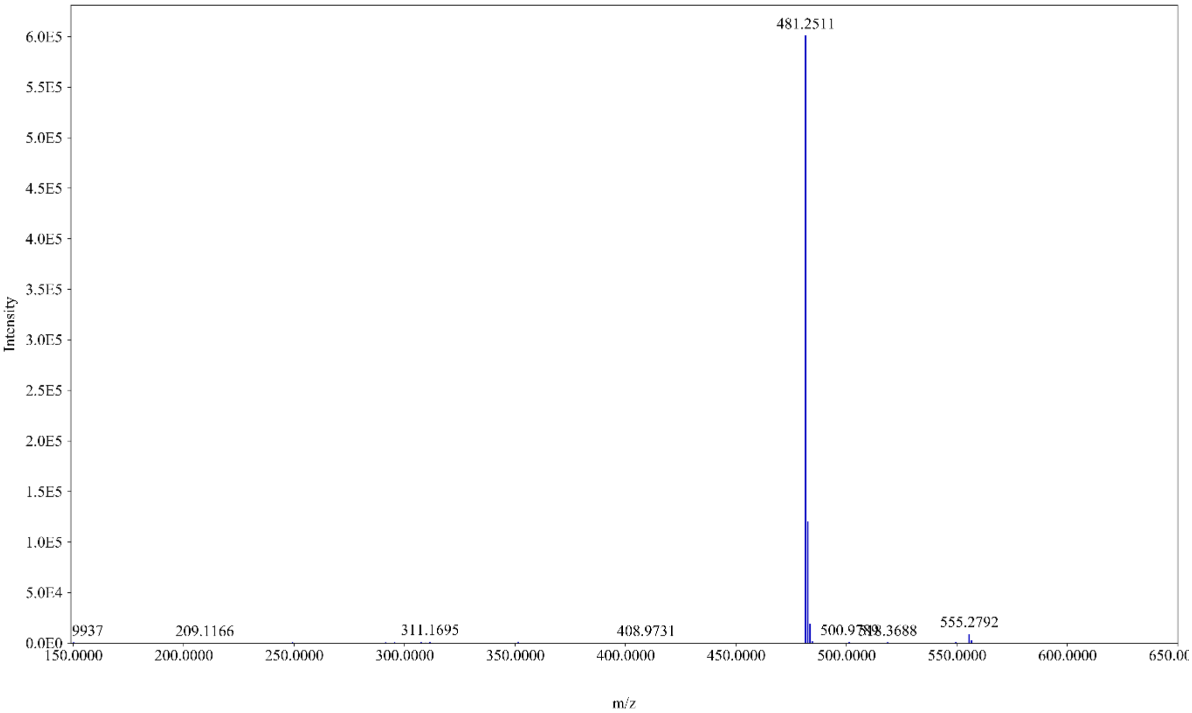


Figure S2.16: Mass spectra of lysophospholipid (LPG) from *Ulva lacinulata* fraction F9 (MS level 1).

Table S2.2: Compound detected from *Ulva lacinulata* fraction F9 in positive and negative ESI mode.

RT (min)	Compound	Chemical formula	Theoretical m/z (T)	Detected m/z (D)	Difference (T – D)	Fragment m/z
$[M+H]^+$						
14.77	**DGTS**	$C_{44}H_{70}O7N$	680.5460	680.5105	0.0355	-
$[M+NH_4]^+$						
23.20	**DGDG**	$C_{47}H_{66}O_{15}N$	888.4740	888.4590	0.0150	-
$[M-H]^-$						
14.82	**PA**	$C_{41}H_{72}O_8P$	723.4970	723.4934	0.0036	-
26.70	**LPG**	$C_{22}H_{42}O_9P$	481.2572	481.2539	0.0033	-
26.92	**SQMG**	$C_{25}H_{47}O_{11}S$	555.2845	555.2773	0.0072	-

Supplementary Information 3 (Chapter 3)

Table S3.1: *Ulva lacinulata* fractions distributions from 5 g (± 0.01 g) crude extract (mean ± SD).

	Weight (g)
F1 (100 % Hex)	125.6 ± 2.07
F2 (9:1 hex:EtOAc)	93.8 ± 3.27
F3 (8:2 hex:EtOAc)	167.6 ± 17.11
F4 (7:3 hex:EtOAc)	94.0 ± 5.57
F5 (6:4 hex:EtOAc)	72.6 ± 2.70
F6 (4:6 hex:EtOAc)	54.2 ± 4.49
F7 (2:8 hex:EtOAc)	21.8 ± 1.92
F8 (100 % EtOAc)	17.2 ± 1.92
F9 (1:1 EtOAc:MeOH)	273.8 ± 19.19
Percentage Recovery	92.1 ± 1.38

Supplementary Information 4 (Chapter 4)

S4.1: Water nutrients

Please note: As per the Declaration pg. i of this thesis, analyses of water nutrient (Ammonium [NH_4^+], Nitrate [NO_3^-] and Nitrite [NO_2^-]) were performed by Mr. Dhiren Vanmari of the Department of Biodiversity and Conservation Biology, University of the Western Cape, South Africa. Charts shown in Figures **S4.1**, **S4.2** and **S4.3** were calculated from the raw data provided by Mr. Dhiren Vanmari and are expressed as average (*n*=3).

(i) Ammonium (NH_4^+)

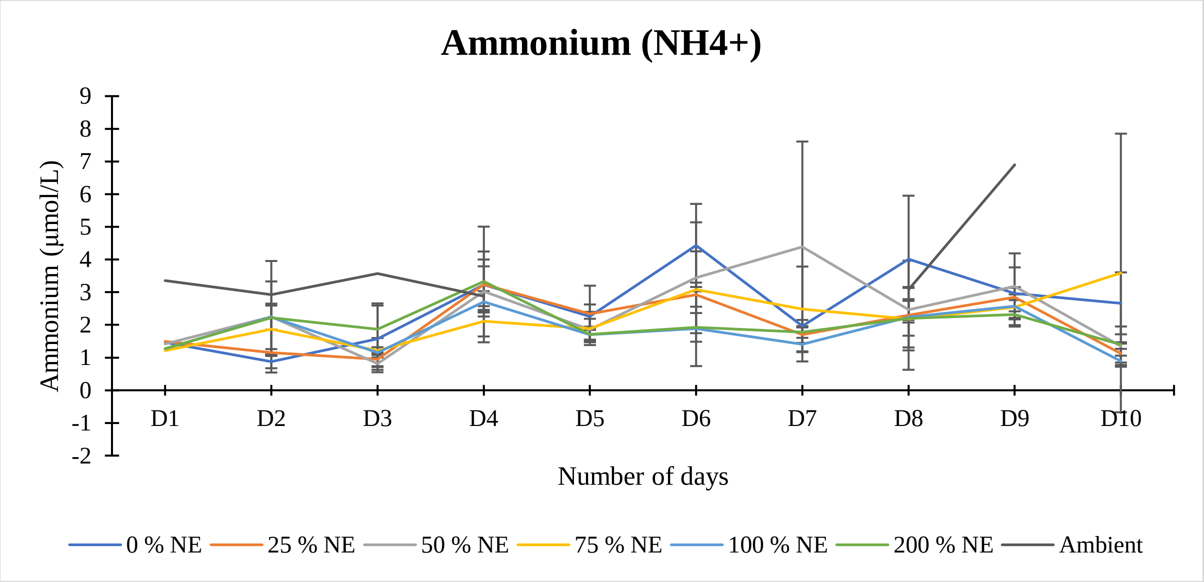


Figure S4.1: Average ammonium concentration (NH_4^+ [µmol/L]) monitored over 10 days of trials. D= day.

(ii) Nitrate – NO_3^-

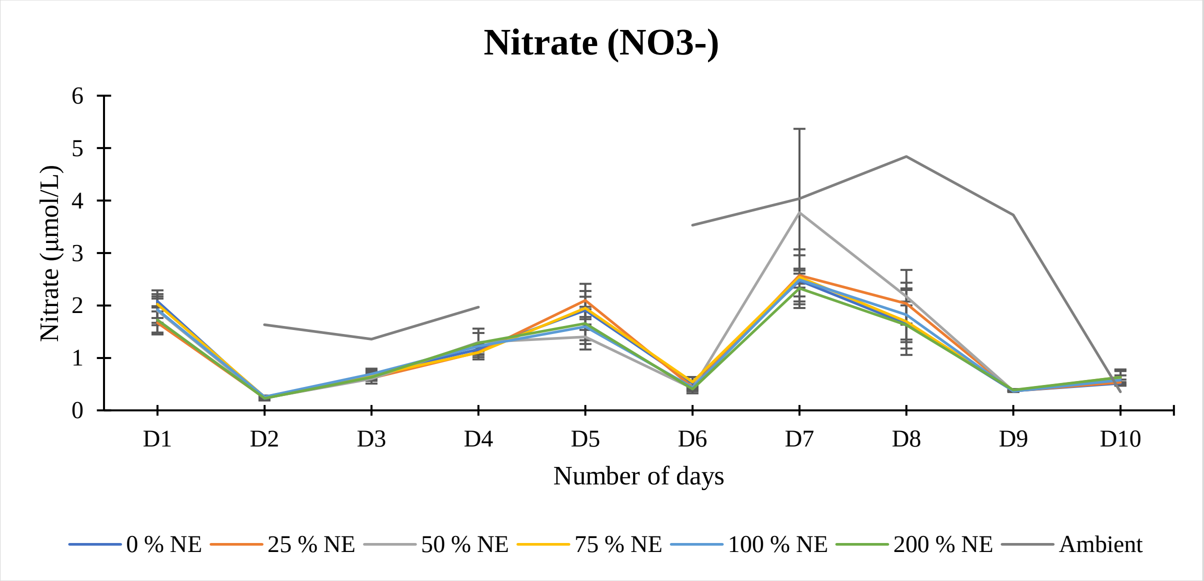


Figure S4.2: Average nitrate concentration (NO_3^- [µmol/L]) monitored over 10 days of trials. D= day.

(iii) Nitrite – NO_2^-

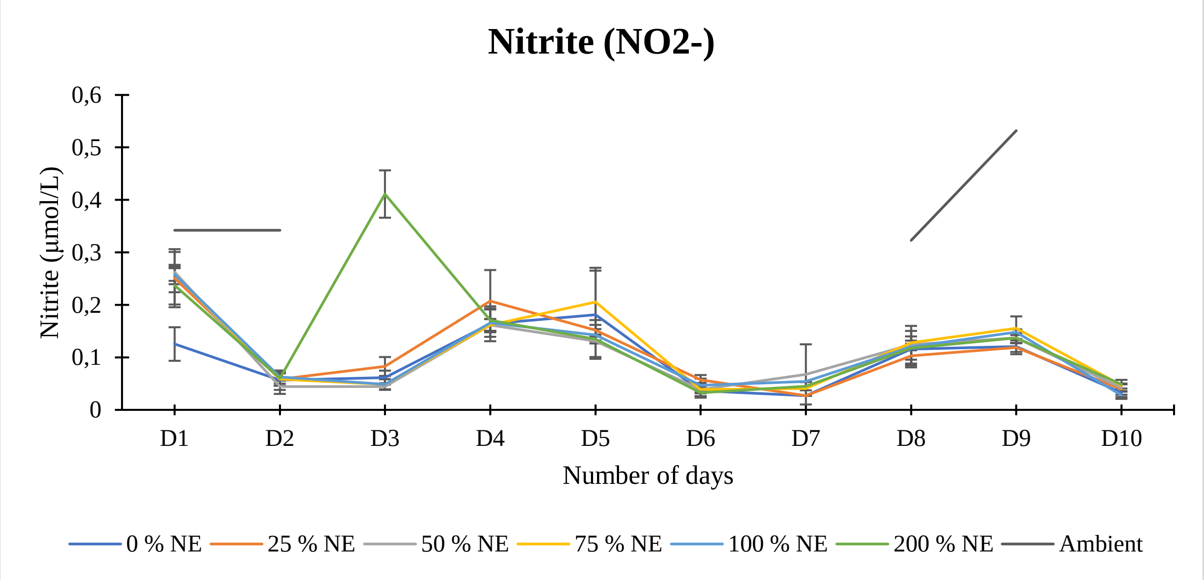


Figure S4.3: Average nitrite concentration (NO_2^- [µmol/L]) monitored over 10 days of trials. D= day.

S4.2: Environmental parameters

Please note: As per the Declaration pg. i of this thesis, environmental parameters (temperature, salinity, dissolved oxygen and pH) were collected by Mr. Dhiren Vanmari of the Department of Biodiversity and Conservation Biology, University of the Western Cape, South Africa. Charts shown in Figures **S4.4**, **S4.5**, **S4.6** and **S4.7** the averages (*n*=3) of environmental parameters recorded during the nutrient culture experiment provided by Mr. Dhiren Vanmari.

(i) Temperature

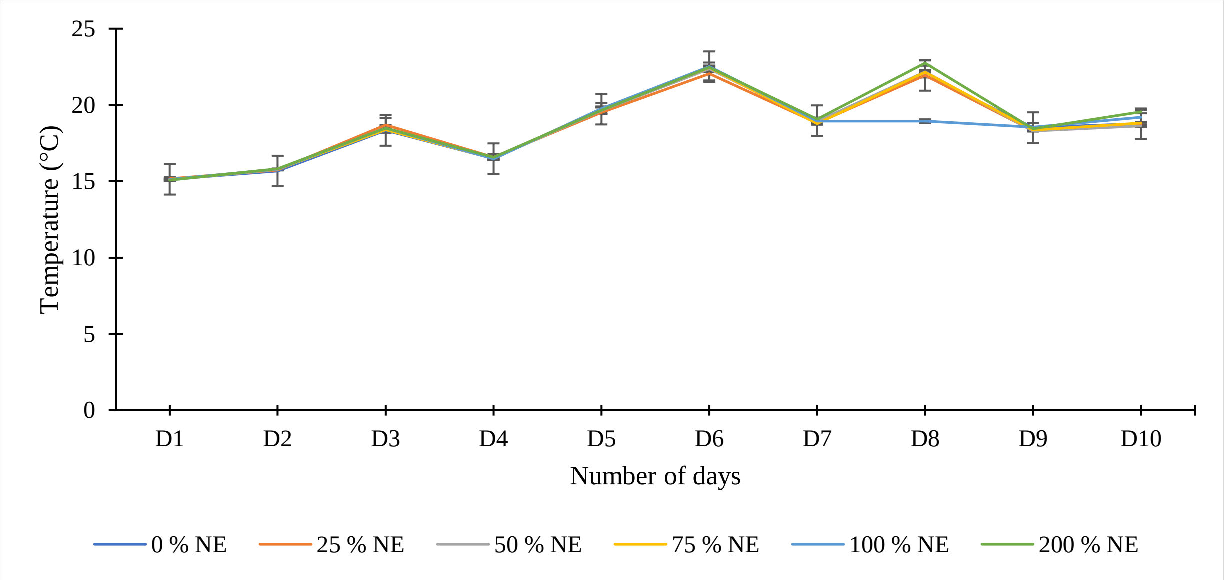


Figure S4.4: Average temperature (°C) recorded over 10 days of trials. D= day.

(ii) Salinity

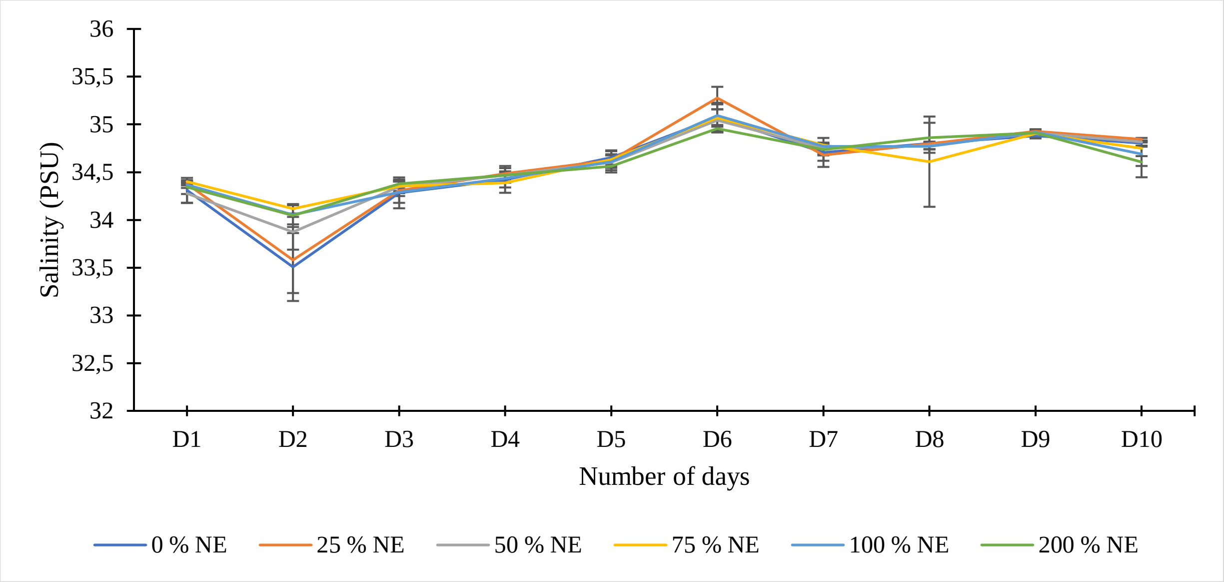


Figure S4.5: Average salinity (PSU) recorded over 10 days of trials. D= day.

(iii) Dissolved oxygen

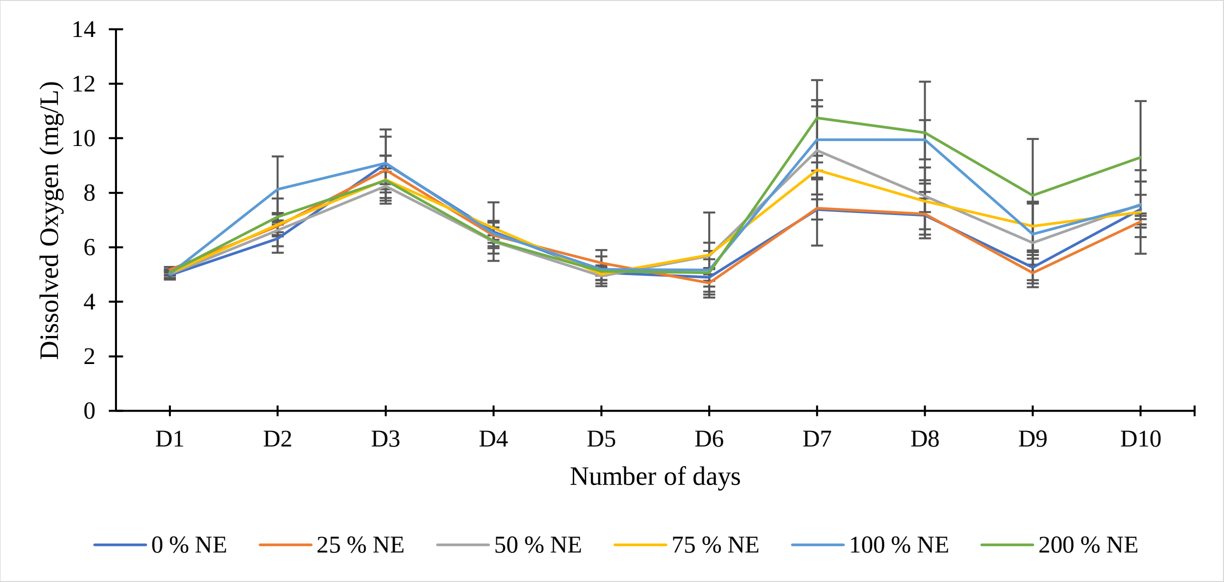


Figure S4.6: Average dissolved oxygen level (mg/L) recorded over 10 days trials. D= day

(iv) pH

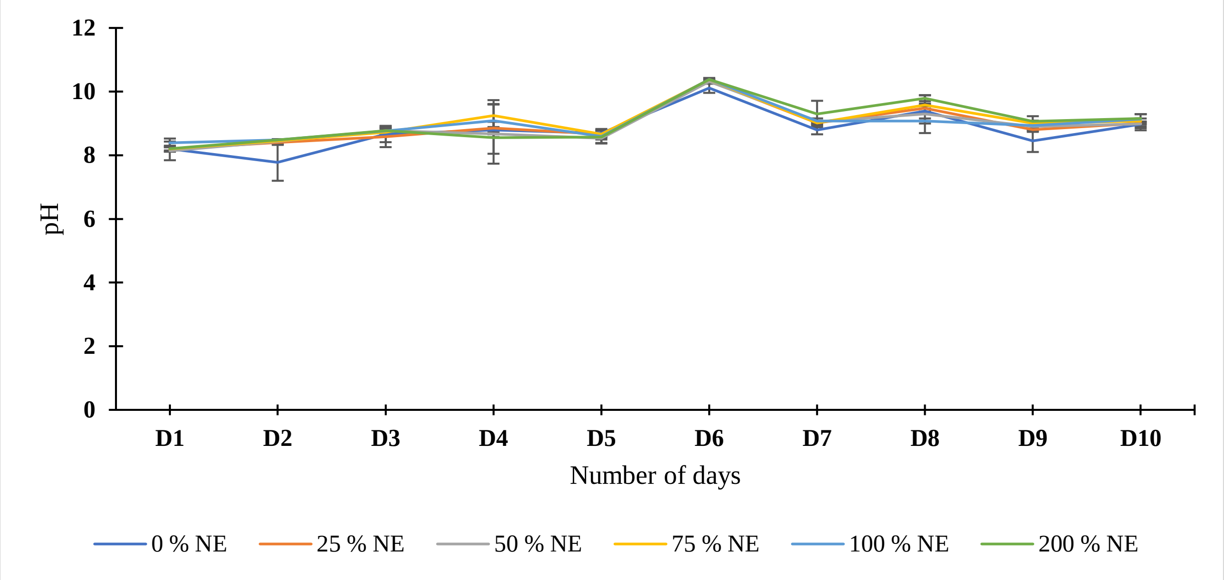


Figure S4.7: Average pH level recorded over 10 days trials. D= day.

S4.3: ^{1}H NMR Total lipid (TL) of *Ulva lacinulata*

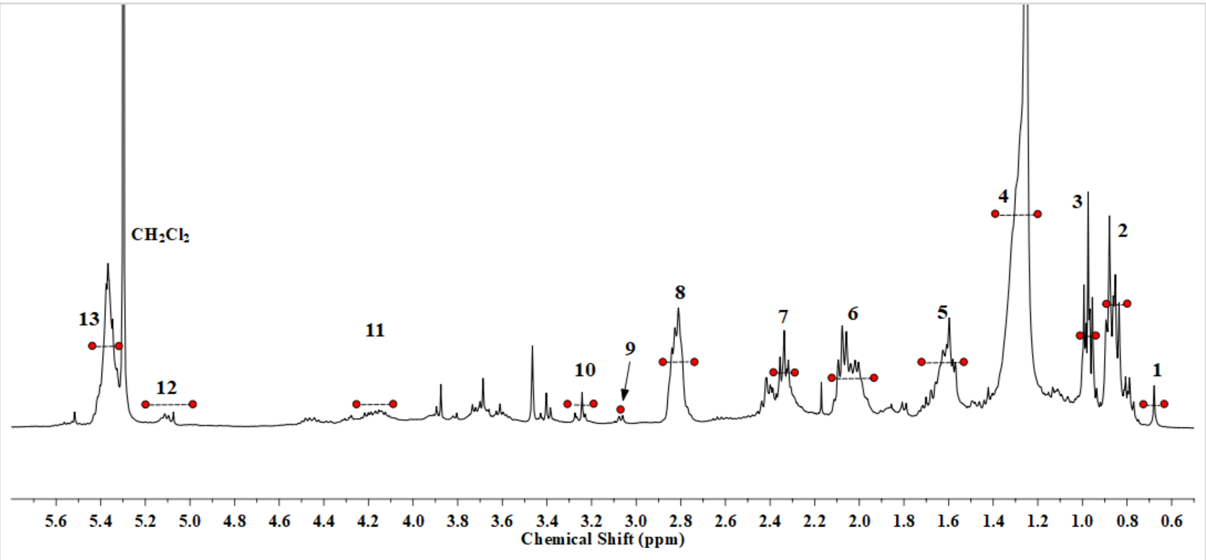


Figure S4.8: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of total lipid from *Ulva lacinulata*.

Table S4.1: Assignment of total lipid (TL) metabolites resonances (ppm) in ^{1}H NMR spectra of *Ulva lacinulata* (FA – Fatty Acids; PUFA – Polyunsaturated Fatty Acids; TAG – Triglycerides; PL – Phospholipid).

Spectral region No.	Chemical shift bins (ppm)	Lipid metabolites assigned
1	0.65 – 0.70	(–C$\underline{\mathbf{H}}_3$) attached to C13 [1,2,4]
2	0.82 – 0.91	All FA (–C$\underline{\mathbf{H}}_3$) except ω–3 FA [1,2,3]
3	0.92 – 1.03	PUFA (–C$\underline{\mathbf{H}}_3$; ω–3) [1,2,3]
4	1.16 – 1.39	All FA –(C$\underline{\mathbf{H}}_2$)$_n$ [1,2,3]
5	1.52 – 1.72	β-methylene (–OCO–CH$_2$–C$\underline{\mathbf{H}}_2$–) [1,2,3]
6	1.92 – 2.14	Mono-allylic methylene (–C$\underline{\mathbf{H}}_2$–CH=CH–) [1,2,3]
7	2.25 – 2.45	α-methylene (–OCO–C$\underline{\mathbf{H}}_2$–) [1,2,3]
8	2.73 – 2.90	Di-allylic methylene (–CH=CH–(C$\underline{\mathbf{H}}_2$–CH=CH)$_n$–) [1,2,3]
9	3.05 – 3.10	PL (–C$\underline{\mathbf{H}}_2$–N–) [2,4]
10	3.21 – 3.29	PL (–C$\underline{\mathbf{H}}_2$–N$^+$(CH$_3$)$_3$–) [2,4]
11	4.09 – 4.31	TAG *sn*–1 (–C1$\underline{\mathbf{H}}_2$) and *sn*–3 (–C3$\underline{\mathbf{H}}_2$) [1,2,3]
12	5.06 – 5.16	TAG *sn*–2 (–C2$\underline{\mathbf{H}}$) [1]
13	5.31 – 5.46	Fatty acyl chain (–C$\underline{\mathbf{H}}$=C$\underline{\mathbf{H}}$–; unsaturated FA) [1,2,3]

[1]Vidal et al. (2012); [2]Del Coco et al. (2018); [3]Siudem et al. (2022); [4]Subramanian et al. (2008)

(i) 0 % NE [control] treatment

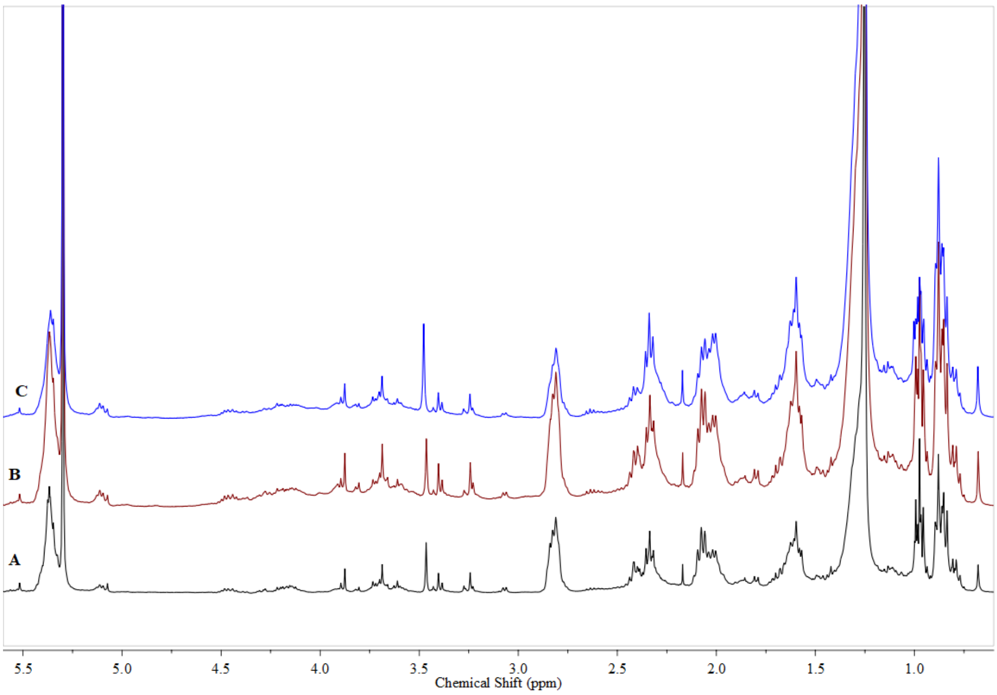


Figure S4.9: ^{1}H NMR spectrum (CDCl$_3$, 400 MHz) of total lipid for *Ulva lacinulata* cultured at 0% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(ii) 25 % NE treatment

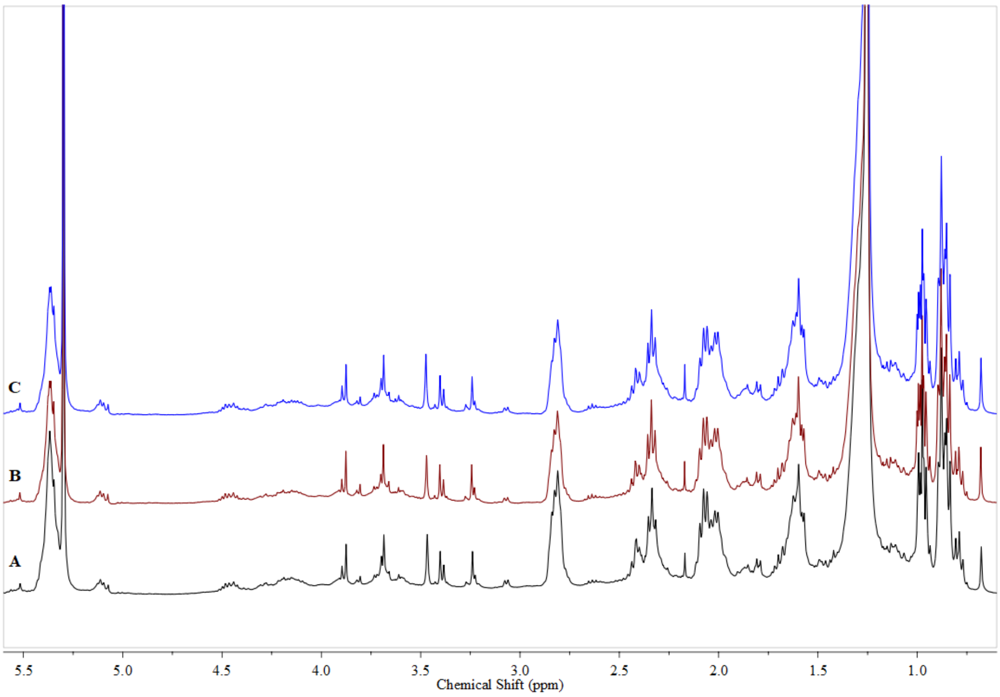


Figure S4.10: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of total lipid for *Ulva lacinulata* cultured at 25% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(iii) 50 % NE treatment

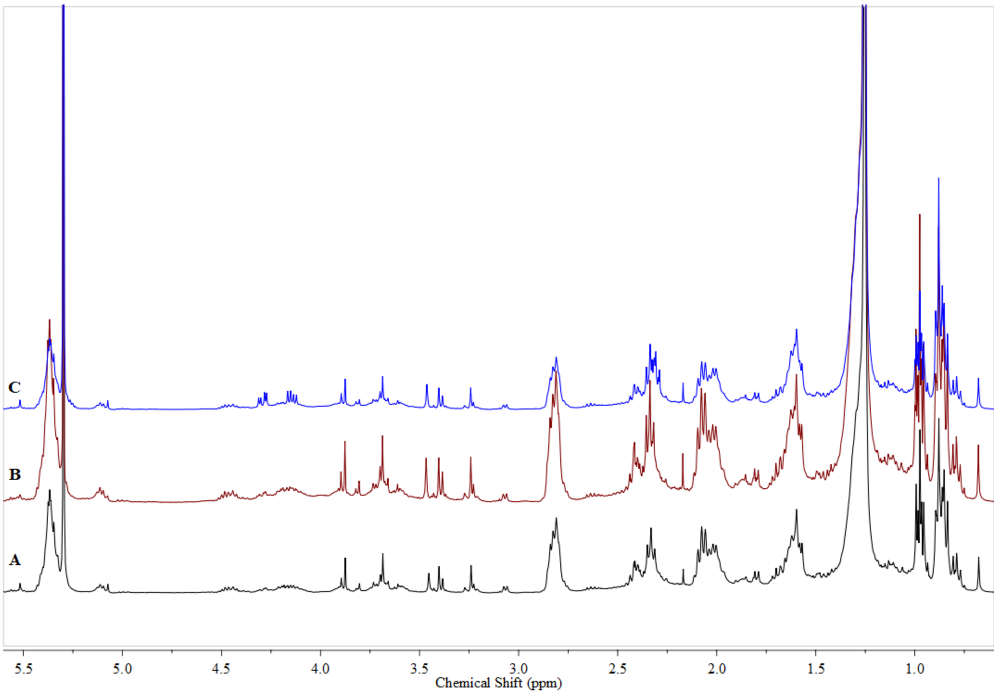


Figure S4.11: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of total lipid for *Ulva lacinulata* cultured at 50% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(iv) 75 % NE treatment

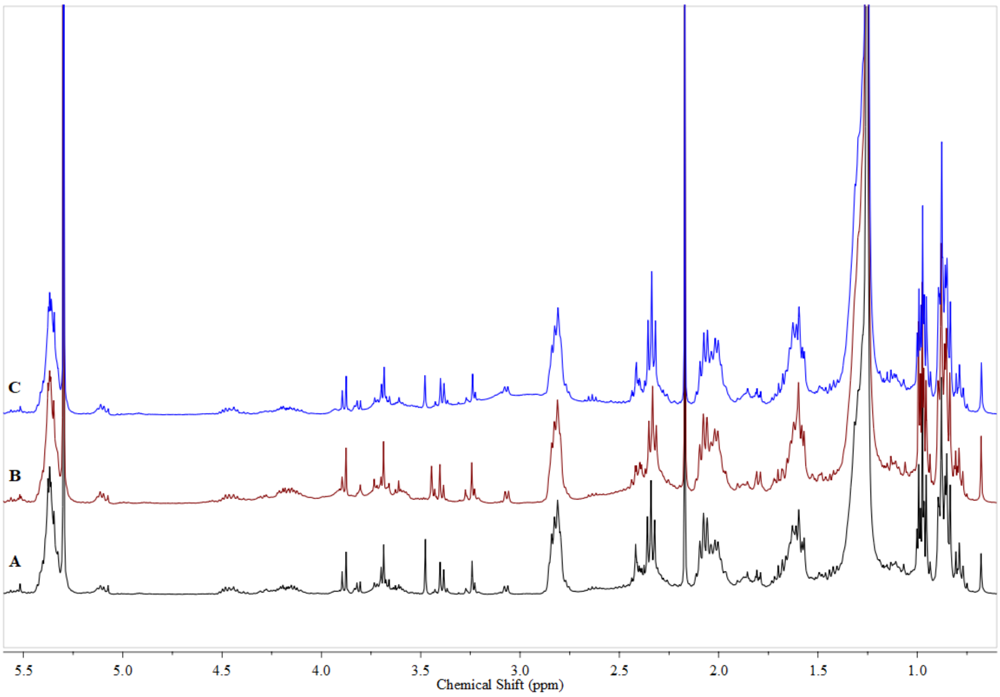


Figure S4.12: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of total lipid for *Ulva lacinulata* cultured at 75% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(v) 100 % NE treatment

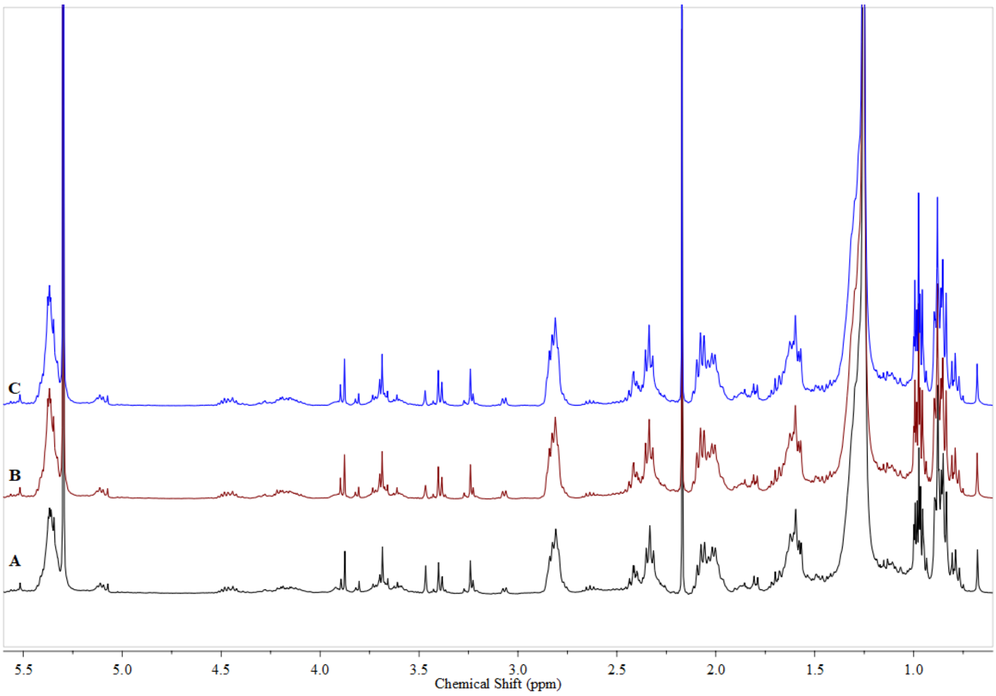


Figure S4.13: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of total lipid for *Ulva lacinulata* cultured at 100% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(vi) 200 % NE treatment

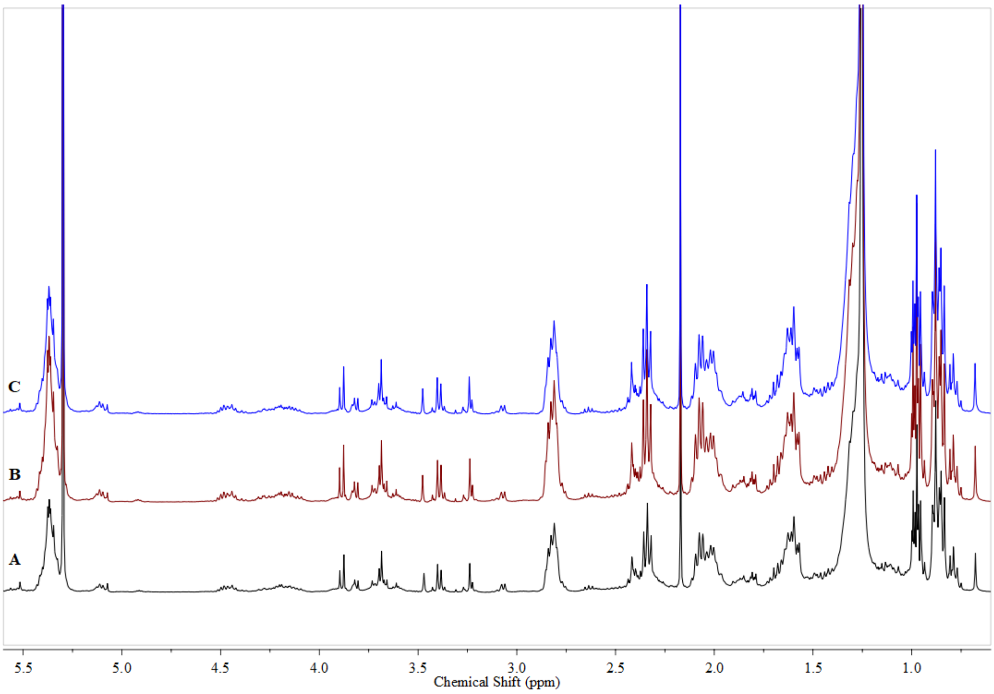


Figure S4.14: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of total lipid for *Ulva lacinulata* cultured at 200% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

S4.4: ^{1}H NMR Neutral lipids (NL) of *Ulva lacinulata*

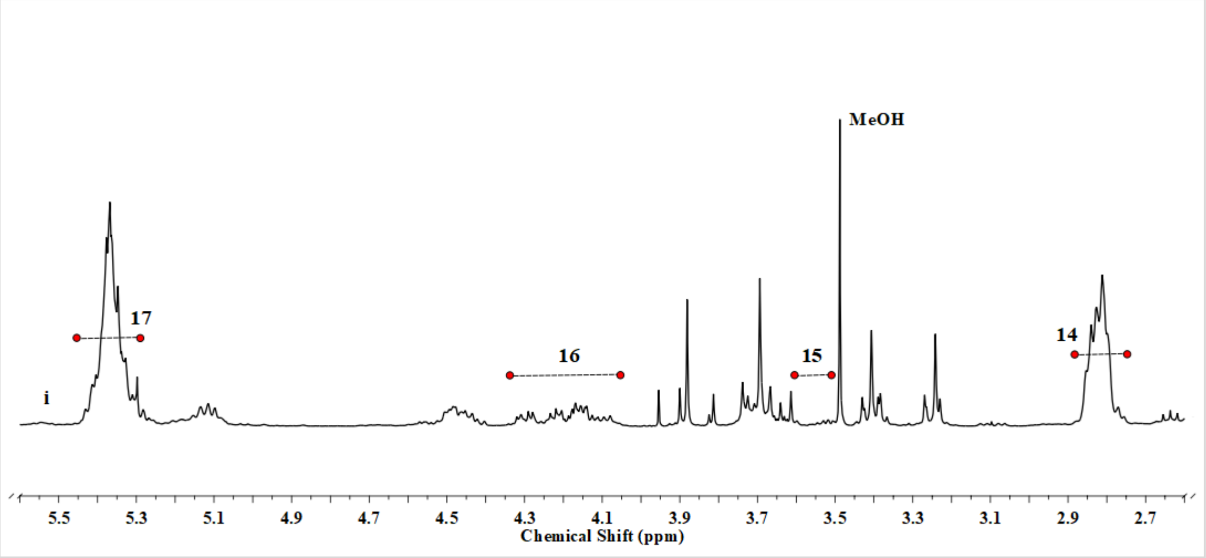


Figure S4.15: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of neutral lipid (NL) sample from *Ulva lacinulata*.

Table S4.2: Assignment of neutral lipid (NL) metabolites resonances (ppm) in ^{1}H NMR spectra of *Ulva lacinulata* (TAG – Triglycerides; FA – Fatty Acids).

Spectral region No.	Chemical shift bins (ppm)	Lipid metabolites assigned
14	2.76 – 2.88	Di-allylic (=CH–C**H$_2$**–CH=) [1,3]
15	3.53, 3.57	C-3 proton[1]
16	4.06 – 4.30	TAG glycerol [1,3]
17	5.36, 5.40	Fatty acyl chain (–C**H**=C**H**; unsaturated FA) [2,3,4]

[1] Tugnoli et al. 2003; [2] Fernando et al. 2010; [3] Fernando et al 2012; [4] Del Coco et al 2018

(i) 0 % NE [control] treatment

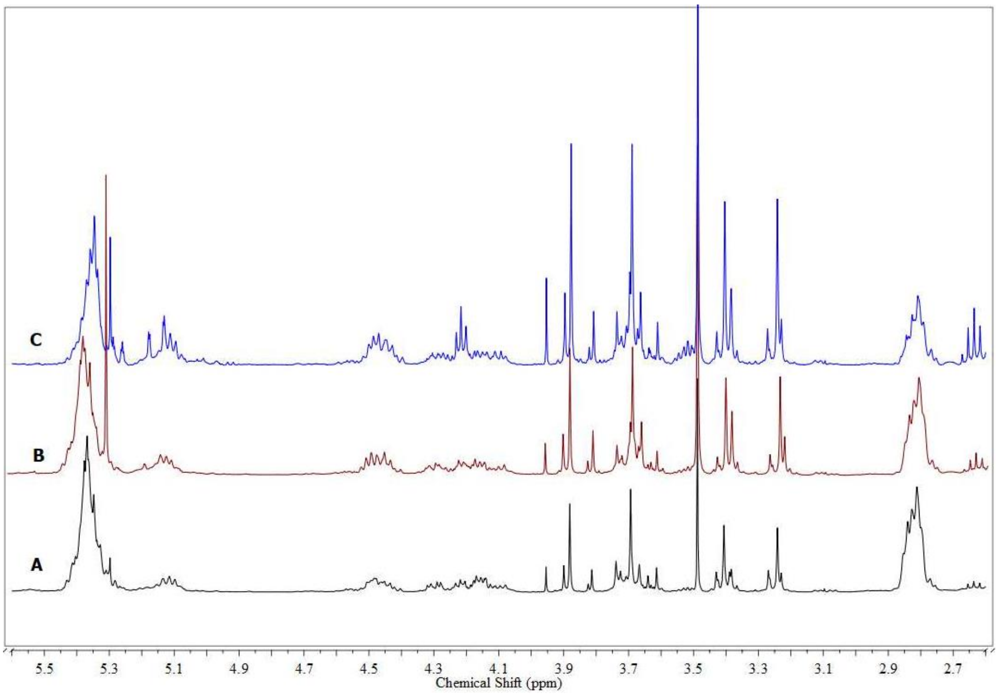


Figure S4.16: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of neutral lipid for *Ulva lacinulata* cultured at 0% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(ii) 25 % NE treatment

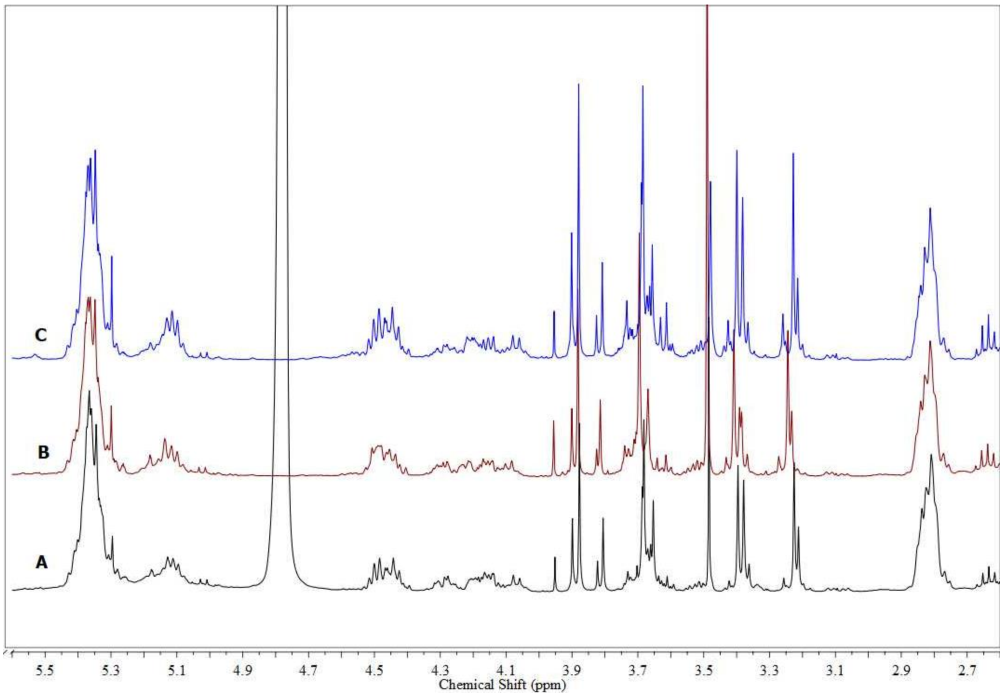


Figure S4.17: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of neutral lipid for *Ulva lacinulata* cultured at 25% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(iii) 50 % NE treatment

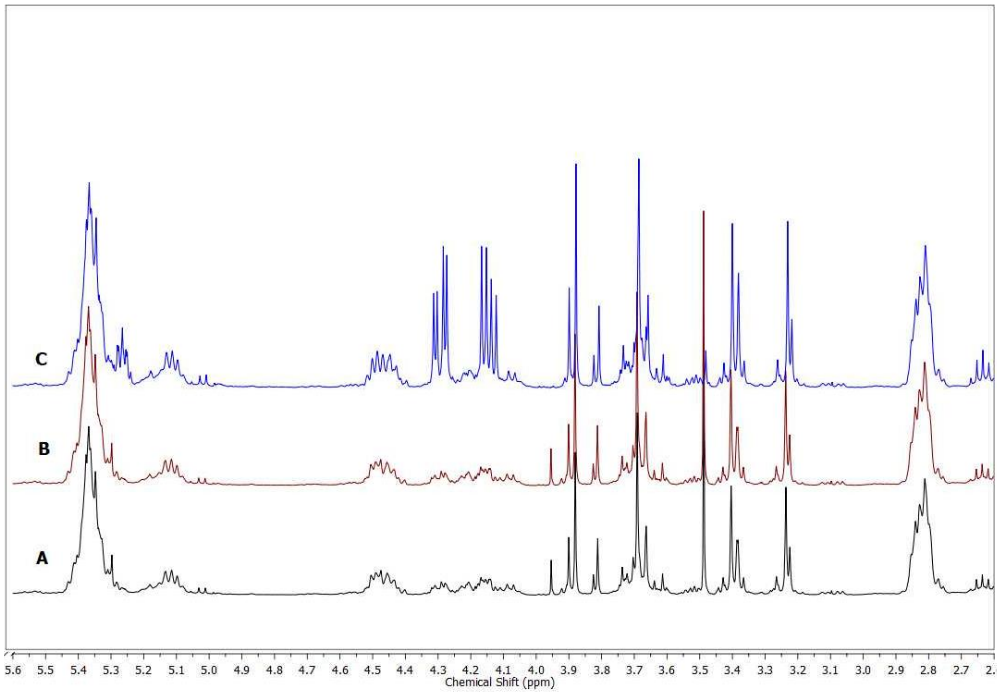


Figure S4.18: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of neutral lipid for *Ulva lacinulata* cultured at 50% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(iv) 75 % NE treatment

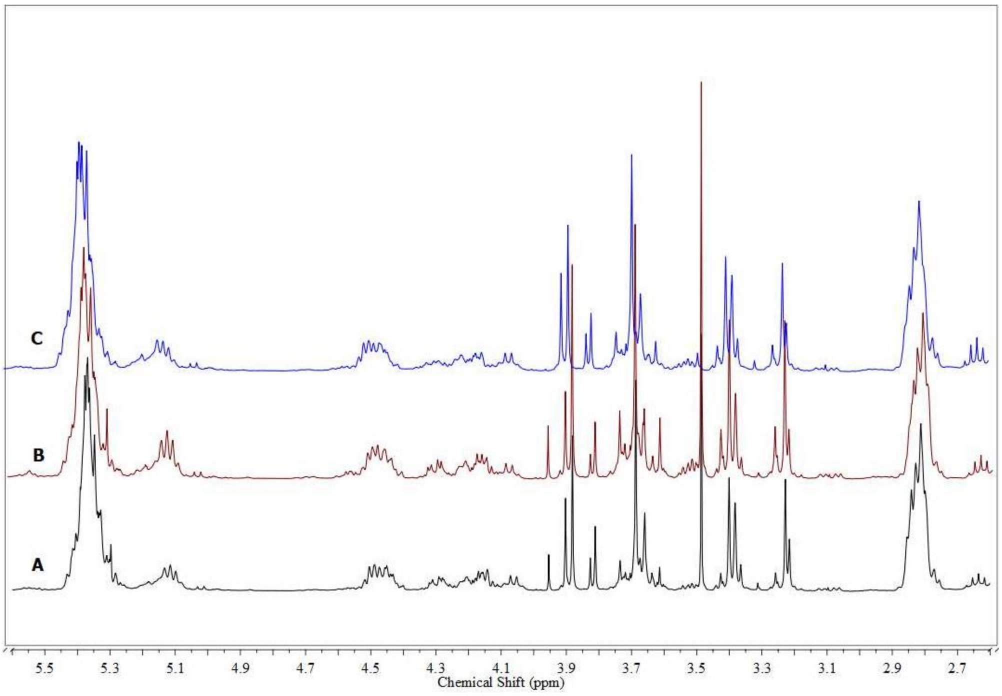


Figure S4.19: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of neutral lipid for *Ulva lacinulata* cultured at 75% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(v) 100 % NE treatment

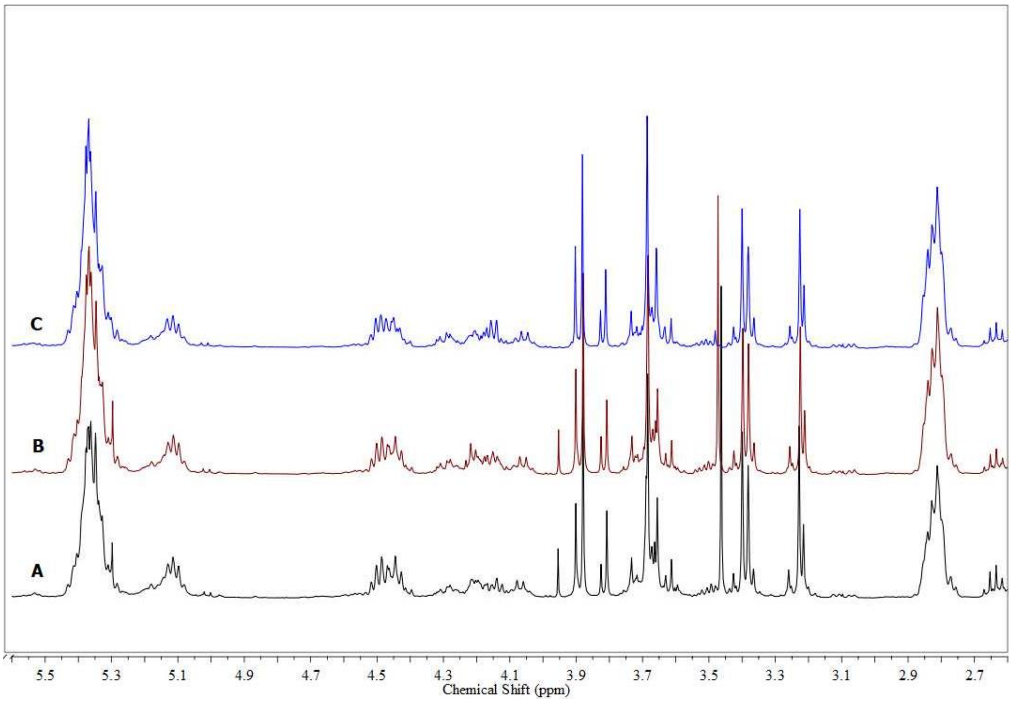


Figure S4.20: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of neutral lipid for *Ulva lacinulata* cultured at 100% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(vi) 200 % NE treatment

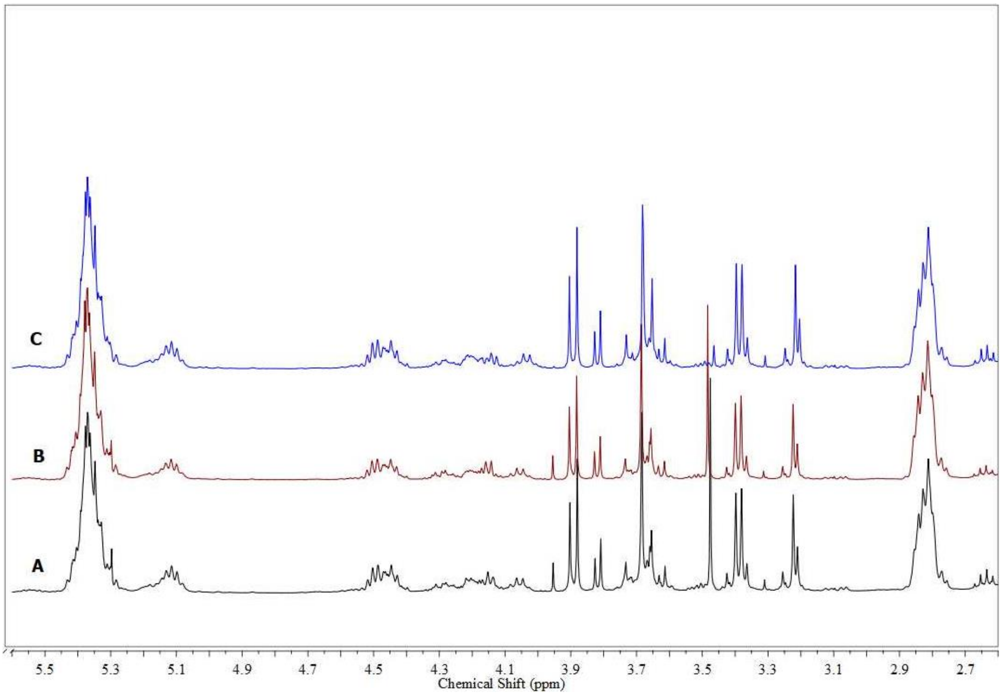


Figure S4.21: ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of neutral lipid for *Ulva lacinulata* cultured at 200% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

S4.5: ^{1}H NMR Glycolipids (GL) of *Ulva lacinulata*

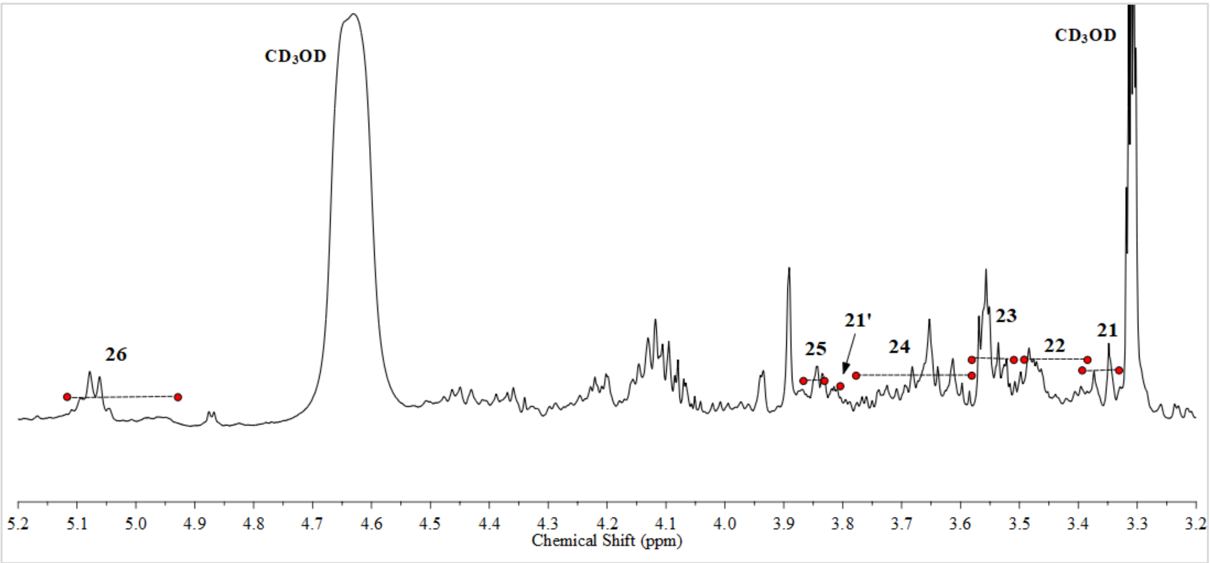


Figure S4.22: ^{1}H NMR spectrum (1:1 $CDCl_3$:CD_3OD, 400 MHz) of glycolipid (GL) sample from *Ulva lacinulata*.

Table S4.3: Assignment of glycolipid (GL) metabolites resonances (ppm) in ^{1}H NMR spectra of *Ulva lacinulata* (GL – Glycolipid).

Spectral region No.	Chemical shift bins (ppm)	Lipid metabolites assigned
21	3.36, 3.40 3.77	GL sugars (–C**H**) with phosphatidylinositol [1]
22	3.44	GL sugars (–C**H**) with C3 proton[1]
23	3.49 – 3.57	GL sugars (–C**H**) with phosphatidylcholine [1]
24	3.61 – 3.73	GL sugars (–C**H**) [1]
25	3.81	GL sugars (–C**H**) with glycerophospholipids [1]
26	4.91 – 5.08	Anomeric –C**H** [1] [2]

[1] Subramanian et al. 2008; [2] Nuzzo et al. 2013

(i) 0 % NE [control] treatment

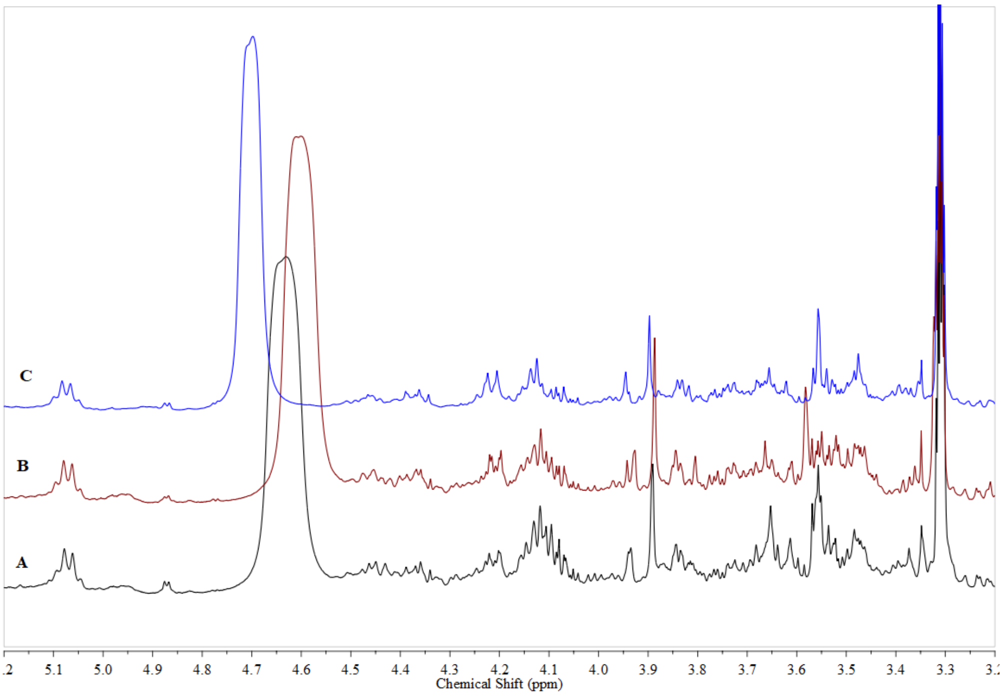


Figure S4.23: ^{1}H NMR spectrum (1:1 $CDCl_3$:CD_3OD, 400 MHz) of glycolipid for *Ulva lacinulata* cultured at 0% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(ii) 25 % NE treatment

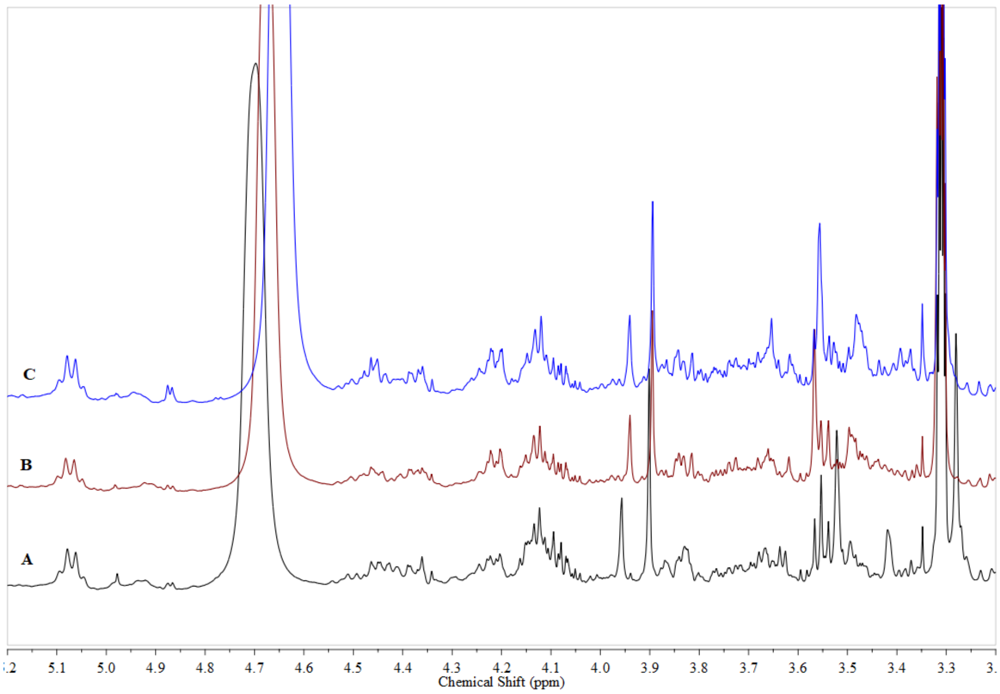


Figure S4.24: ^{1}H NMR spectrum (1:1 $CDCl_3$:CD_3OD, 400 MHz) of glycolipid for *Ulva lacinulata* cultured at 25% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(iii) 50 % NE treatment

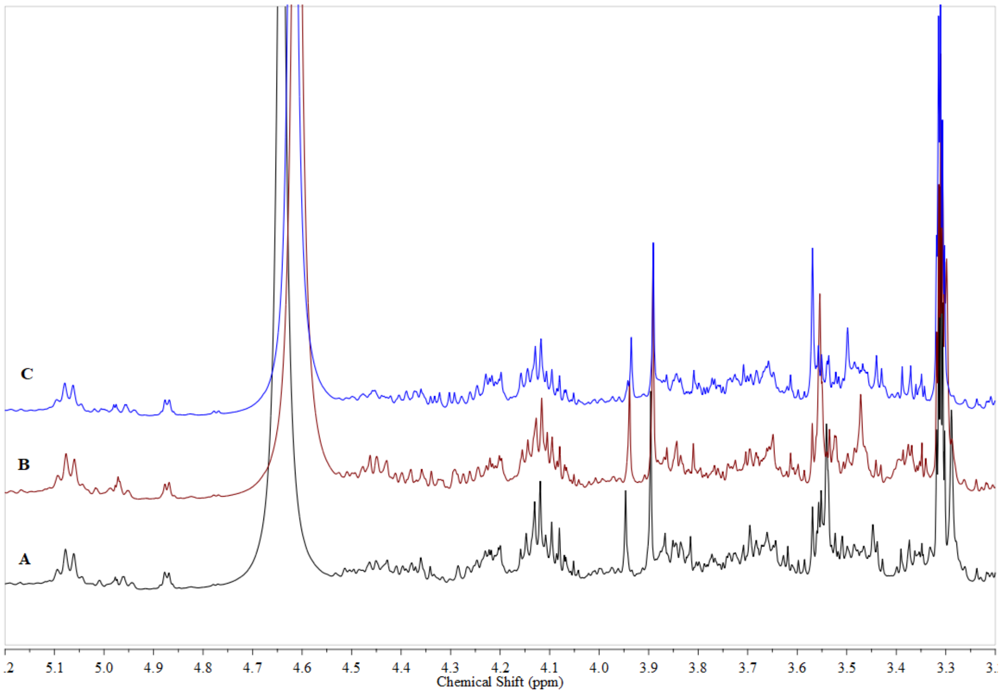


Figure S4.25: ^{1}H NMR spectrum (1:1 $CDCl_3$:CD_3OD, 400 MHz) of glycolipid for *Ulva lacinulata* cultured at 50% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(iv) 75 % NE treatment

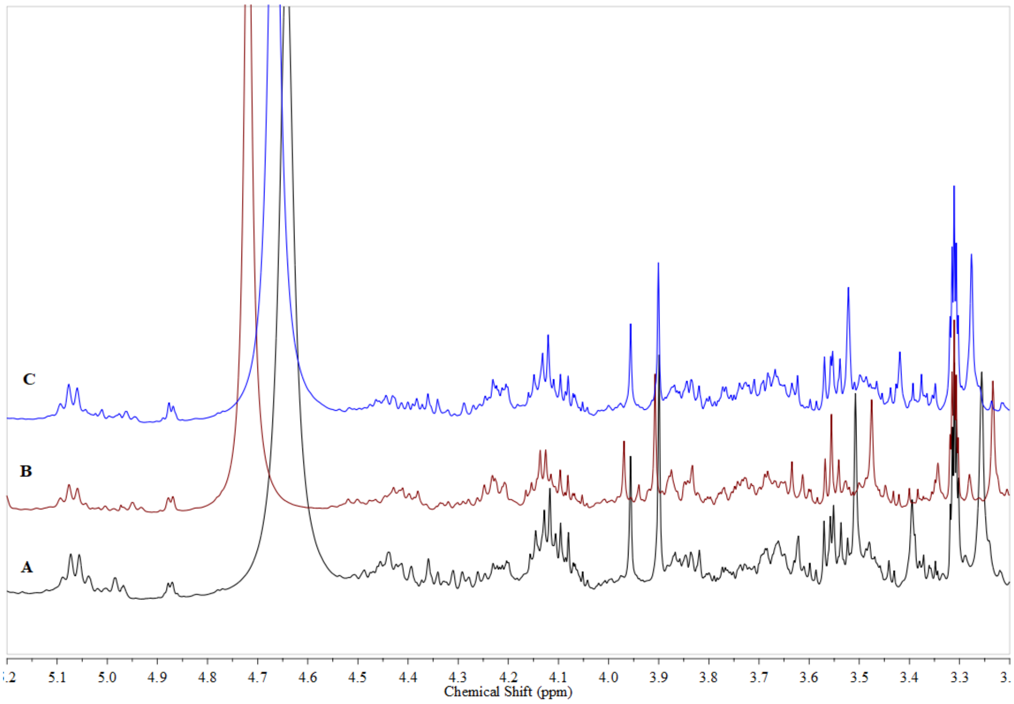


Figure S4.26: ^{1}H NMR spectrum (1:1 $CDCl_3$:CD_3OD, 400 MHz) of glycolipid for *Ulva lacinulata* cultured at 75% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(v) 100 % NE treatment

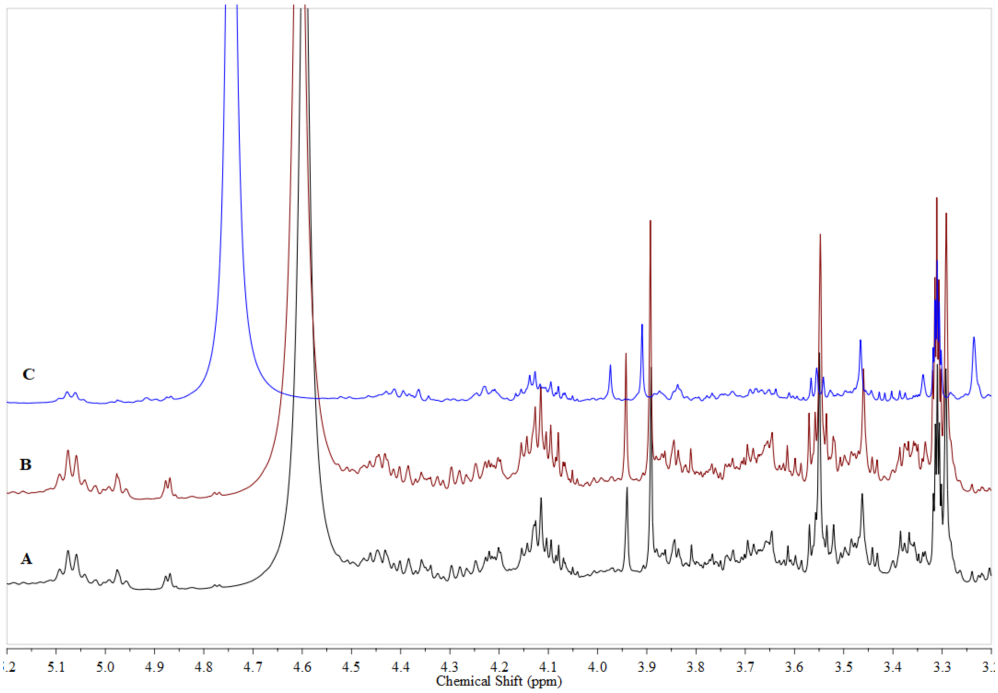


Figure S4.27: ^{1}H NMR spectrum (1:1 $CDCl_3$:CD_3OD, 400 MHz) of glycolipid for *Ulva lacinulata* cultured at 100% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(vi) 200 % NE treatment

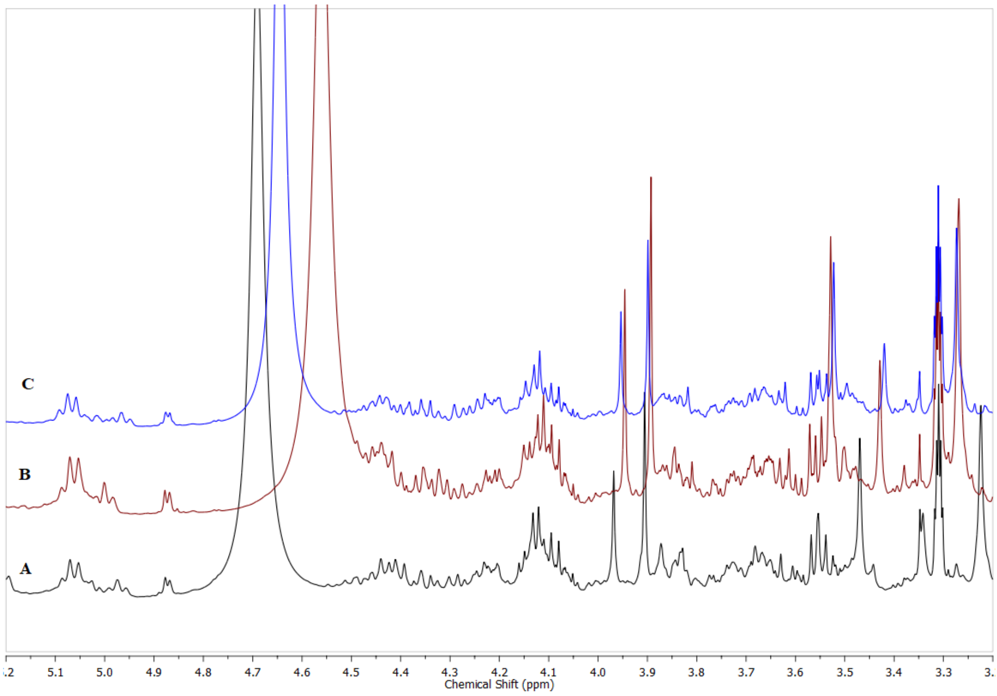


Figure S4.28: ^{1}H NMR spectrum (1:1 $CDCl_3$:CD_3OD, 400 MHz) of glycolipid for *Ulva lacinulata* cultured at 200% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

S4.6: ^{1}H NMR Phospholipids (PL) of *Ulva lacinulata*

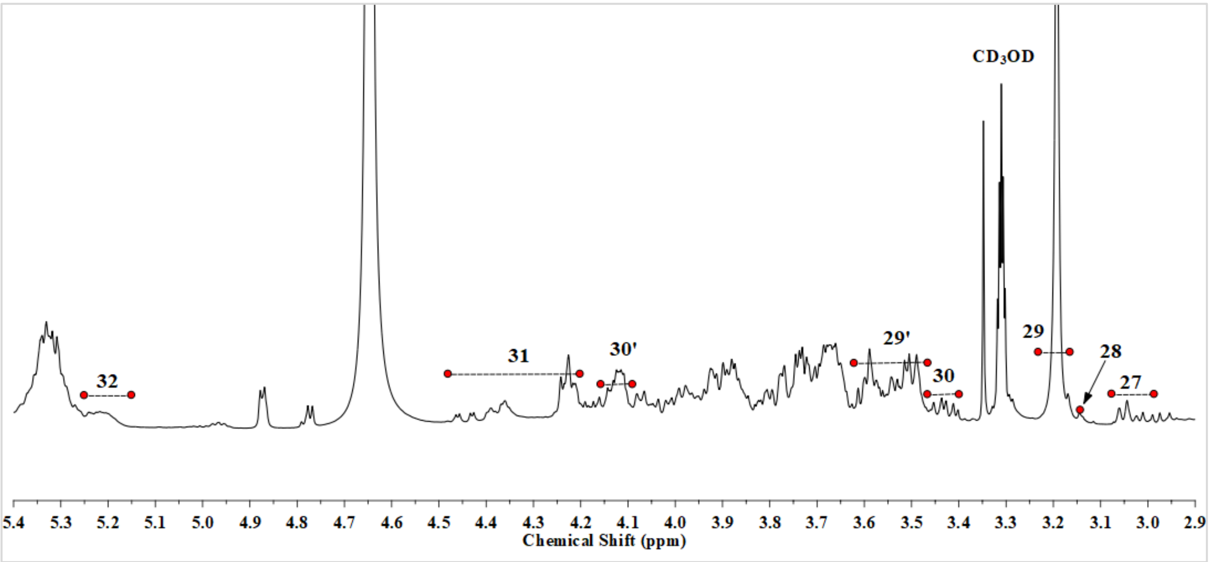


Figure S4.29: ^{1}H NMR spectrum (1:1 $CDCl_3$:CD_3OD, 400 MHz) of phospholipid (PL) sample from *Ulva lacinulata*.

Table S4.4: Assignment of phospholipid (PL) metabolites resonances (ppm) in ^{1}H NMR spectra of *Ulva lacinulata.*

Spectral region No.	Chemical shift bins (ppm)	PL metabolites assigned
27	3.00, 3.04	Phosphatidylethanolamine (–C$\mathbf{H}_2$–N) [1,2,4]
28	3.17	Lysophosphatidylcholine (–C$\mathbf{H}_2$–N$^+$(CH$_3$)$_3$ [3,4]
29	3.21	Phosphatidylcholine (–C$\mathbf{H}_2$–N$^+$(CH$_3$)$_3$) [1,2,3,4]
	3.57, 3.61	
30	3.41, 3.45	Phosphatidylinositol (–C$\mathbf{H}$) [1]
	4.10, 4.14	
31	4.22–4.47	Glycerophospholipids[a] (CH$_2$OR1–CHOR2–C$\mathbf{H}_2$–) [1,3]
32	5.20, 5.24	Glycerophospholipids[b] (CH$_2$OR1–C$\mathbf{H}$OR2–CH$_2$–) [1,3]

[1] Subramanian et al. 2008; [2] Del Coco et al. 2018; [3] Barrilero et al. 2018; [4] Amiel et al. 2020

(i) 0 % NE [control] treatment

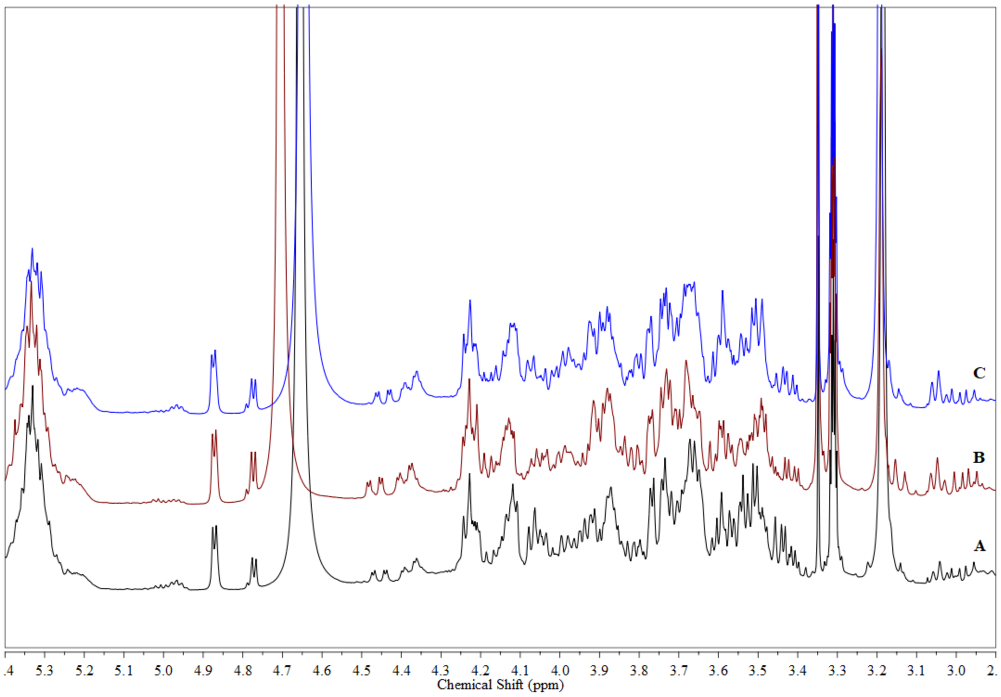


Figure S4.30: ^{1}H NMR spectrum (1:1 CDCl$_3$:CD$_3$OD, 400 MHz) of phospholipid for *Ulva lacinulata* cultured at 0% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(ii) 25 % NE treatment

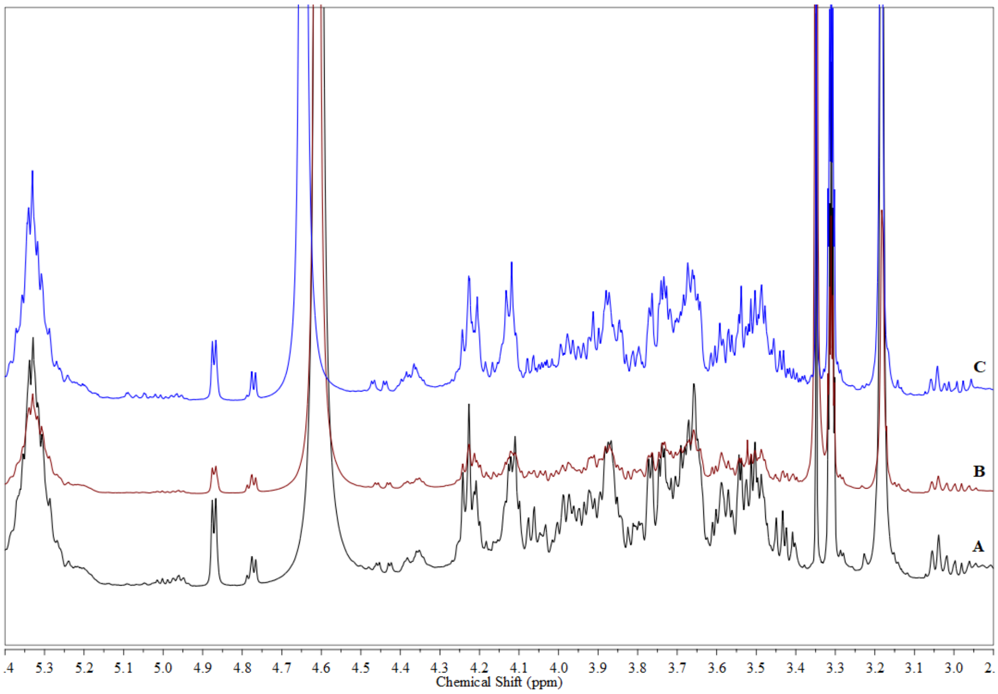


Figure S4.31: ^{1}H NMR spectrum (1:1 $CDCl_3$:CD_3OD, 400 MHz) of phospholipid for *Ulva lacinulata* cultured at 25% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(iii) 50 % NE treatment

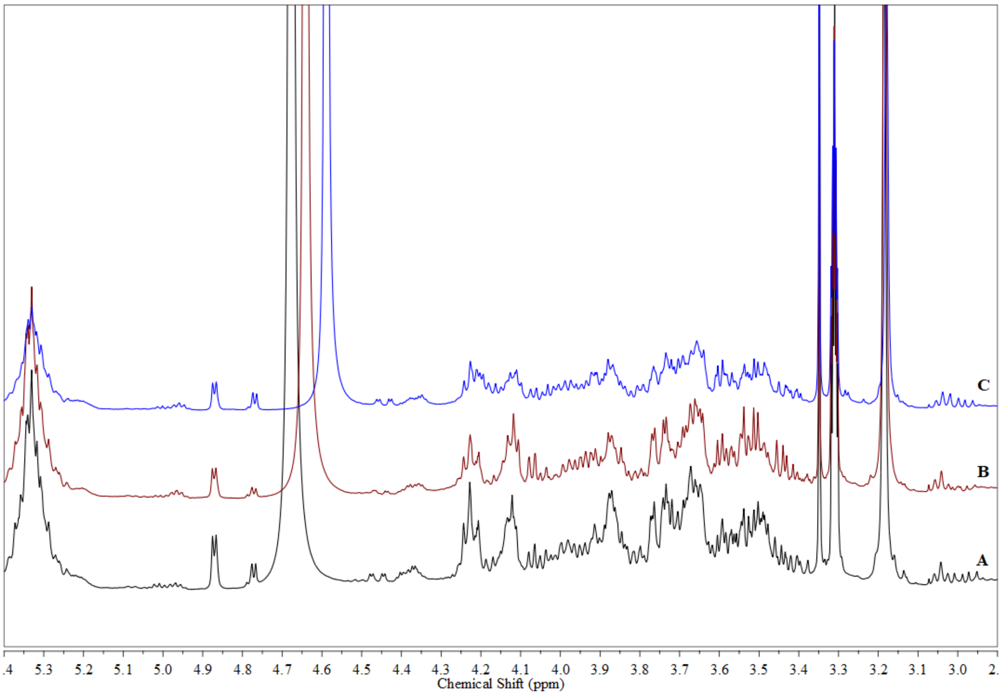


Figure S4.32: ^{1}H NMR spectrum (1:1 $CDCl_3$:CD_3OD, 400 MHz) of phospholipid for *Ulva lacinulata* cultured at 50% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(iv) 75 % NE treatment

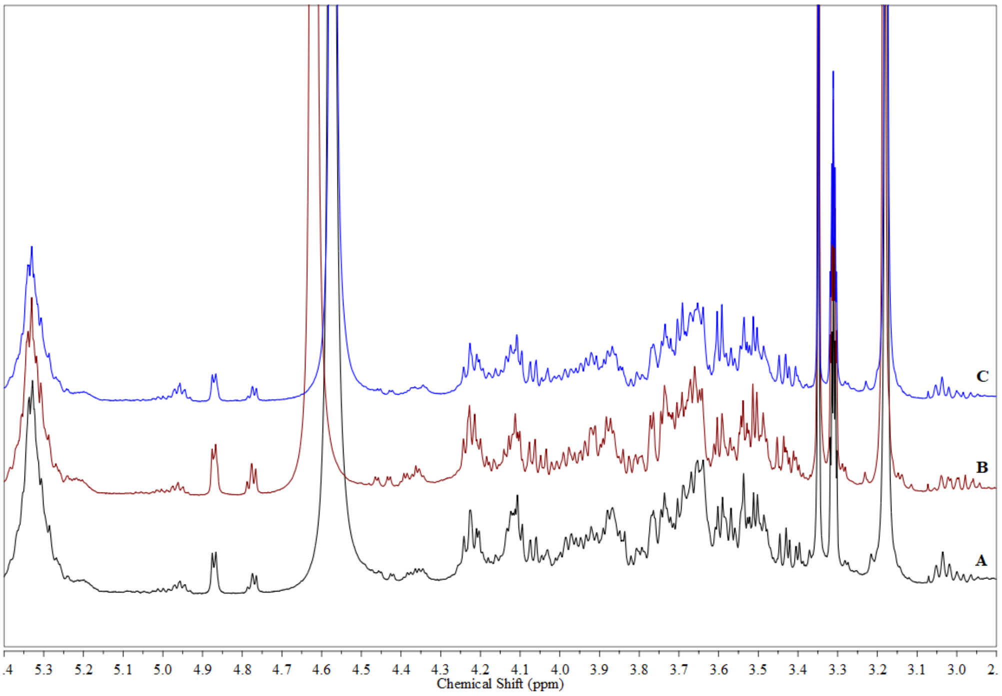


Figure S4.33: ^{1}H NMR spectrum (1:1 $CDCl_3$:CD_3OD, 400 MHz) of phospholipid for *Ulva lacinulata* cultured at 75% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(v) 100 % NE treatment

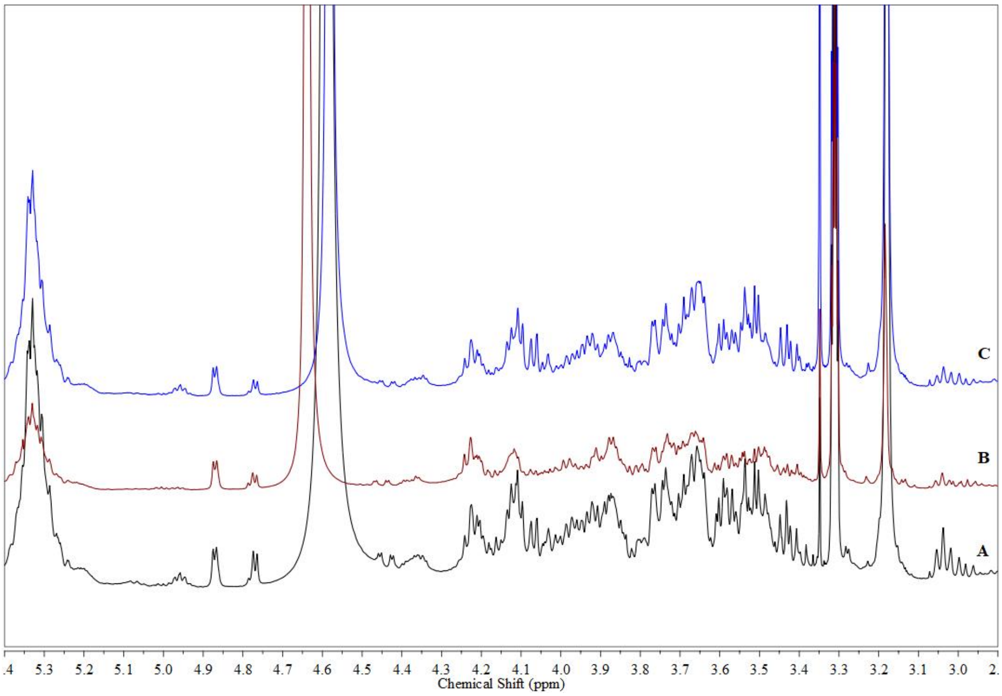


Figure S4.34: ^{1}H NMR spectrum (1:1 $CDCl_3$:CD_3OD, 400 MHz) of phospholipid for *Ulva lacinulata* cultured at 100% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

(vi) 200 % NE treatment

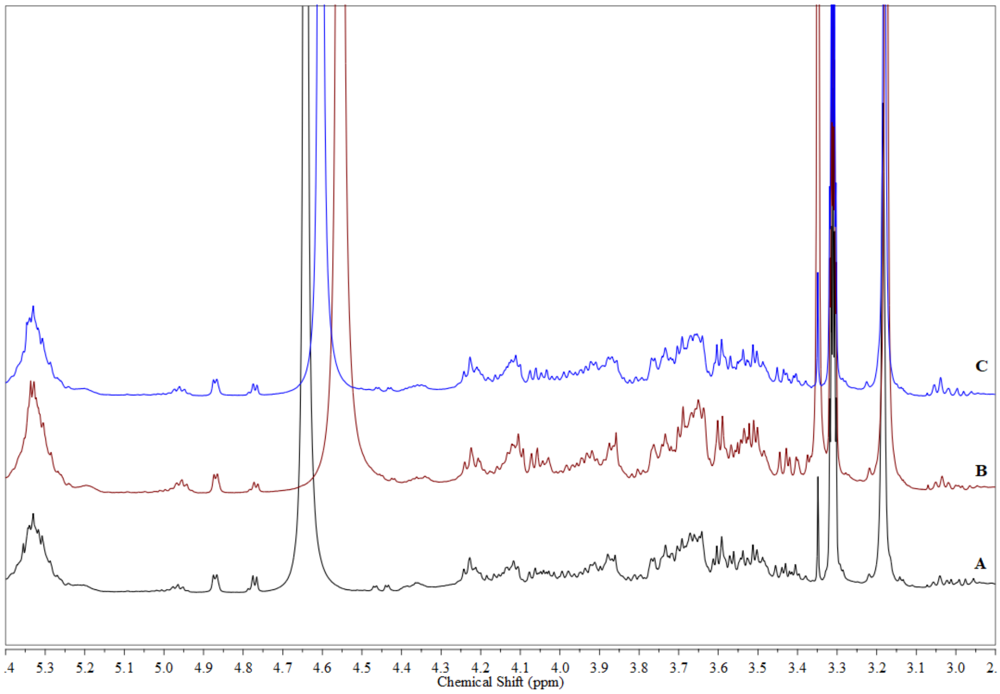


Figure S4.35: ^{1}H NMR spectrum (1:1 $CDCl_3$:CD_3OD, 400 MHz) of phospholipid for *Ulva lacinulata* cultured at 200% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. A, B and C are replicates.

S4.7: ^{1}H NMR integration for ω-3 content (%), double bond equivalent and saturated fatty acid (SFA)

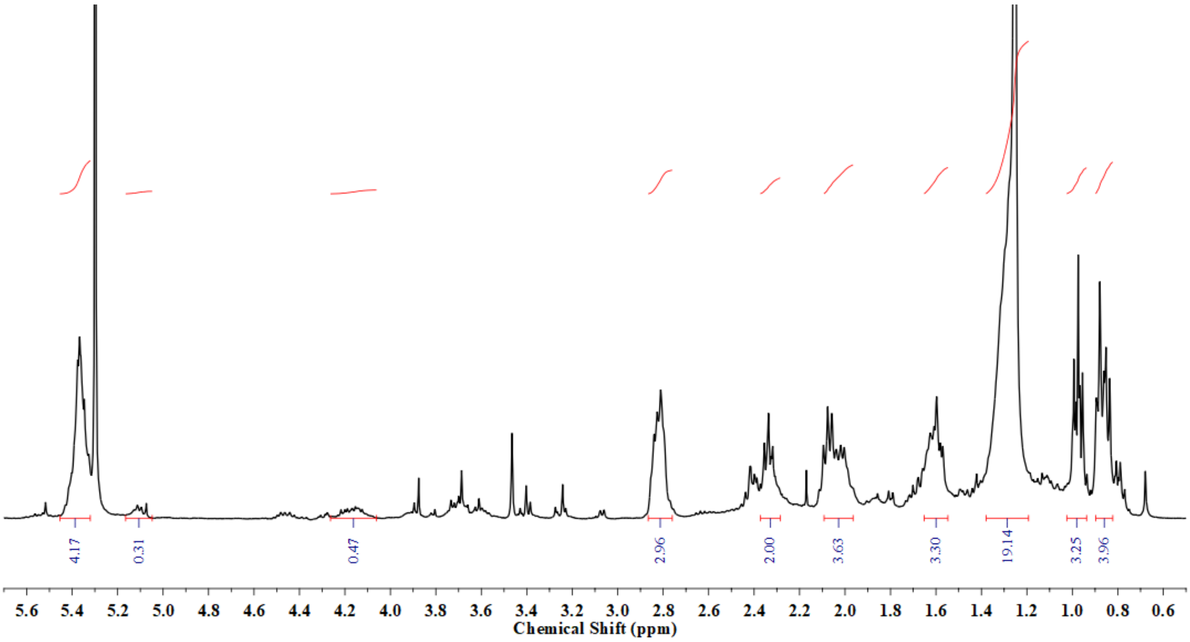


Figure S4.36: Example of ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) integration (δ_H 0.50 – 5.60).

ω-3 content = 45.08%

Unsaturated fatty acid (UFA) content = 90.75%

Polyunsaturated fatty acid (PUFA) content = 39.67%

Saturated fatty acid (SFA) content = 9.25%

Monounsaturated fatty acid (MUFA) content = 51.08%

Double bond equivalent = 2.09

S4.8: Fatty acids (FA) of *Ulva lacinulata*

(i) 0 % NE [control] treatment

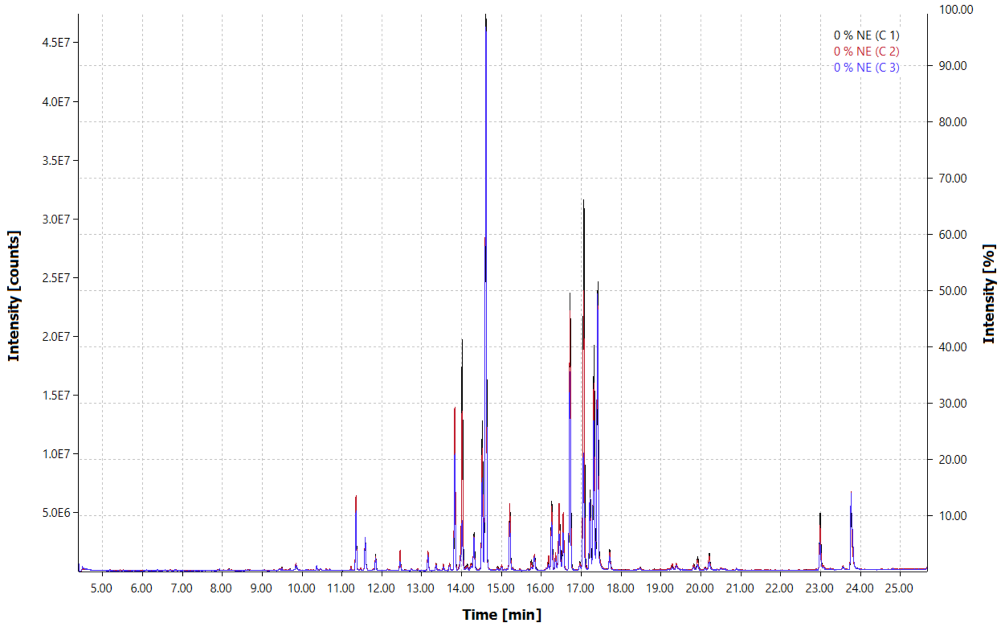


Figure S4.37: Chromatogram of fatty acid methyl esters (FAMEs) obtained from *Ulva lacinulata* cultured at 0% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. C1, C 2 and C 3 are replicates.

(ii) 25 % NE treatment

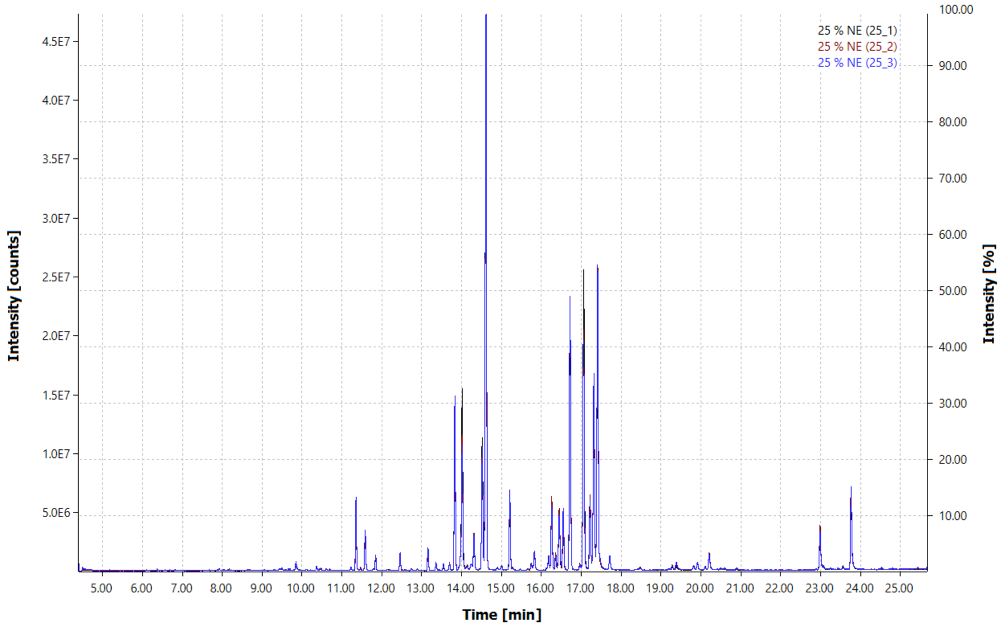


Figure S4.38: Chromatogram of fatty acid methyl esters (FAMEs) obtained from *Ulva lacinulata* cultured at 25% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. 25_1, 25_2 and 25_3 are replicates.

(iii) 50 % NE treatment

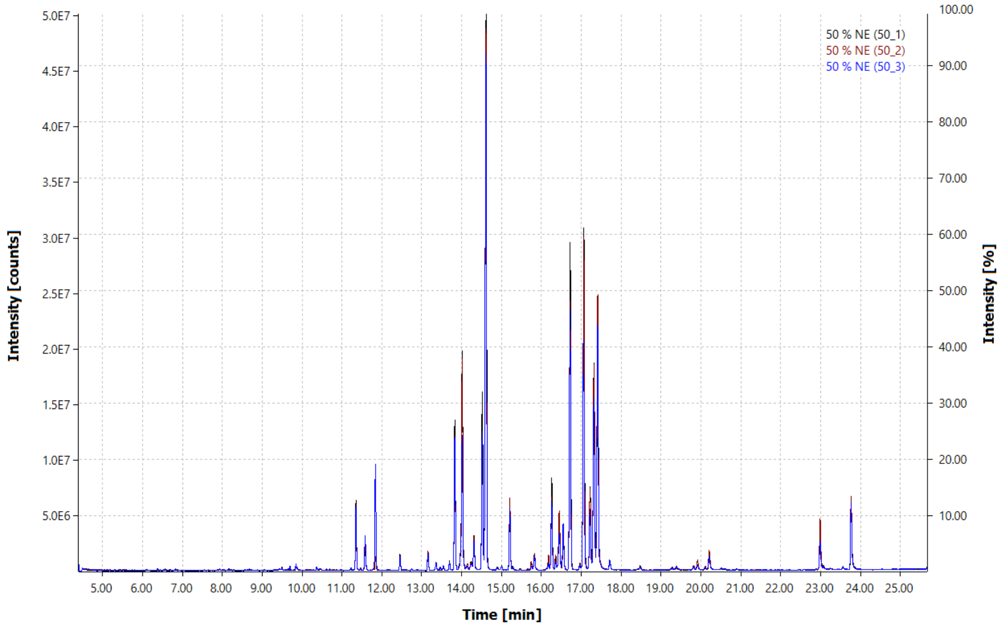


Figure S4.39: Chromatogram of fatty acid methyl esters (FAMEs) obtained from *Ulva lacinulata* cultured at 50% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. 50_1, 50_2 and 50_3 are replicates.

(iv) 75 % NE treatment

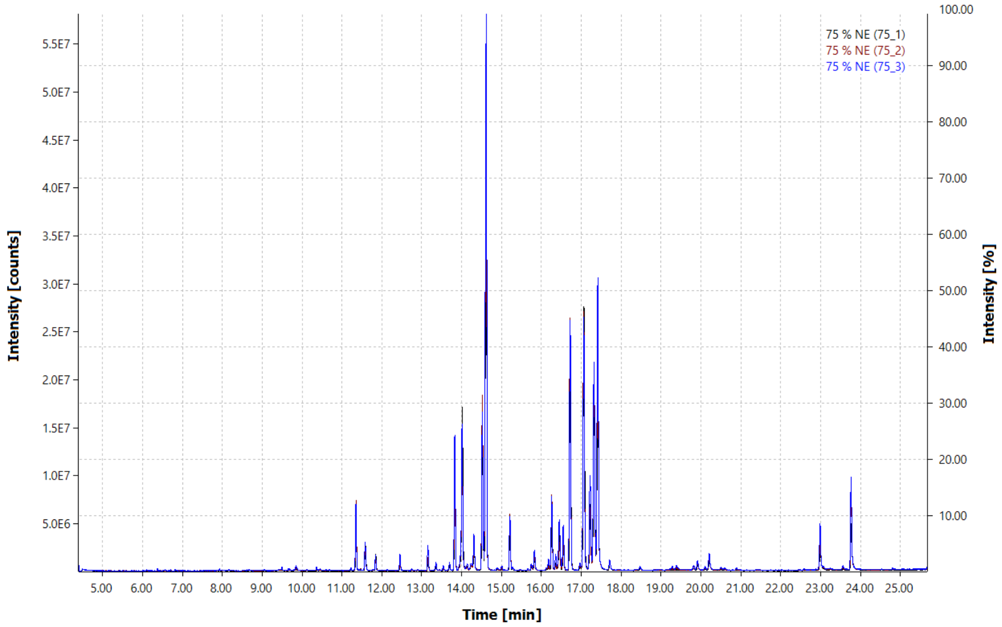


Figure S4.40: Chromatogram of fatty acid methyl esters (FAMEs) obtained from *Ulva lacinulata* cultured at 75% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. 75_1, 75_2 and 75_3 are replicates.

(v) 100 % NE treatment

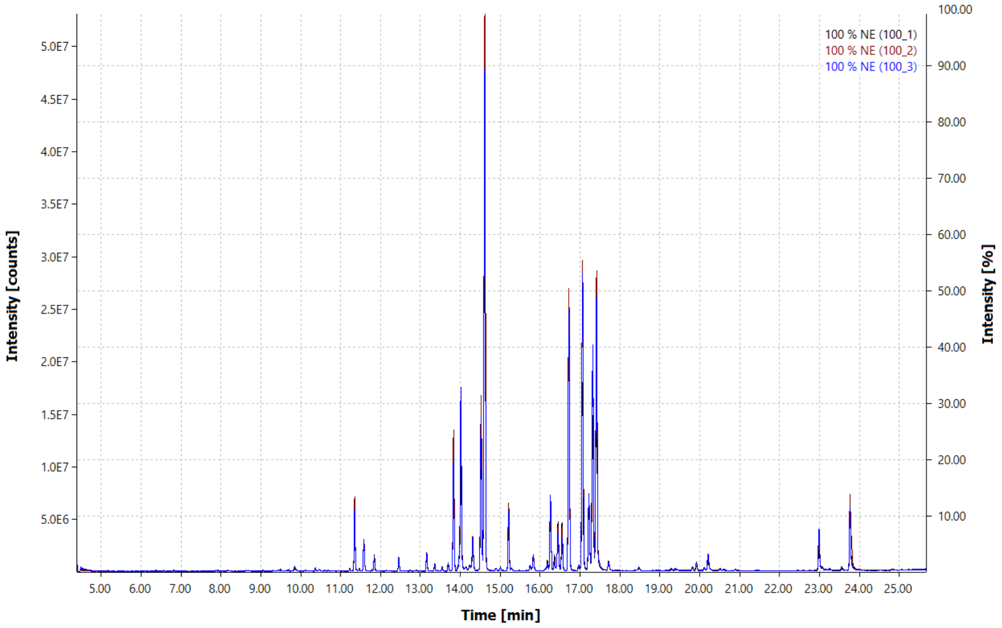


Figure S4.41: Chromatogram of fatty acid methyl esters (FAMEs) obtained from *Ulva lacinulata* cultured at 100% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. 100_1, 100_2 and 100_3 are replicates.

(vi) 200 % NE treatment

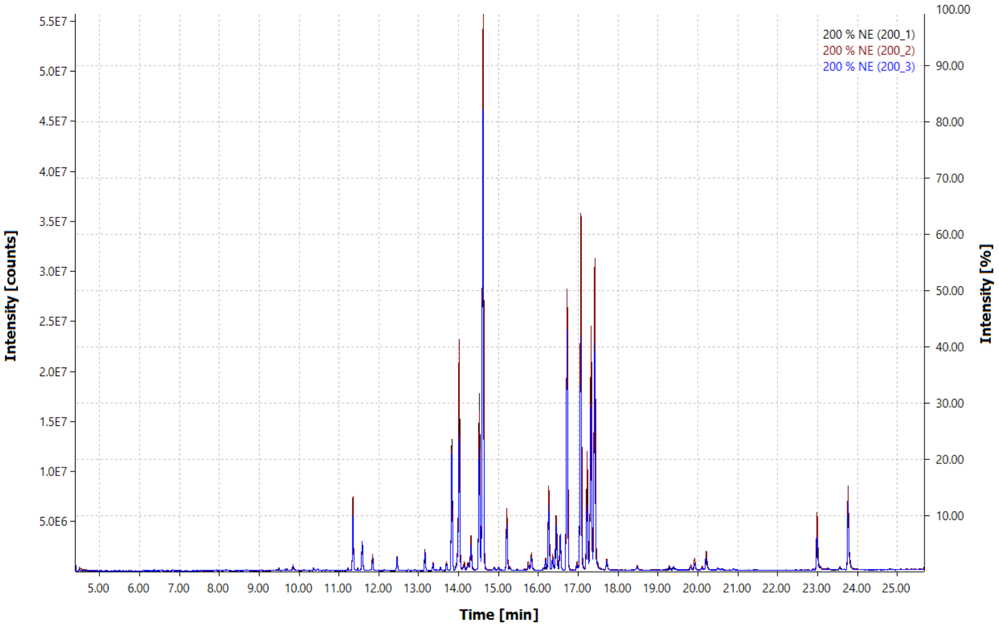


Figure S4.42: Chromatogram of fatty acid methyl esters (FAMEs) obtained from *Ulva lacinulata* cultured at 200% nutrient enrichment (NE) [100% NE = 8.05 nitrogen (N), 3.78 phosphorus (P) and 1.08 potassium (K) g.kg^{-1} *Ulva* at a ratio of 7:3:1]. 200_1, 200_2 and 200_3 are replicates.

S 4.9: FA loading data

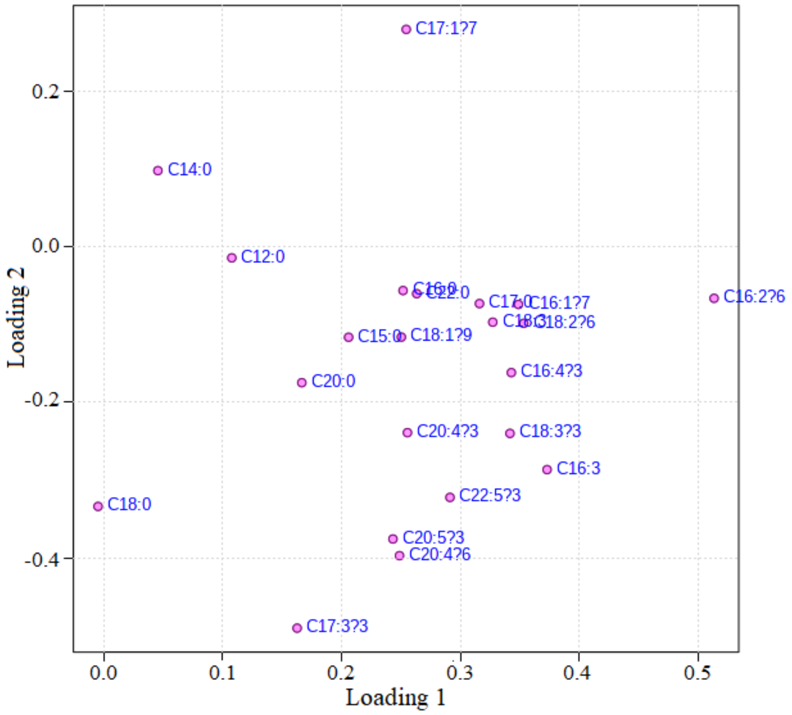


Figure S4.43: Principal component analysis (PCA) loading plot for fatty acid content of *Ulva lacinulata*.

Table S4.5: Loading features for principal component analysis (PCA) of the top 10 fatty acids from *Ulva lacinulata*.

FA	*Loading 1*	*Loading 2*
C16:3	0.3050	0.0233
C20:4ω6	0.3016	0.2548
C17:3ω3	0.3012	0.4273
C20:5ω3	0.2853	0.1520
C22:5ω3	0.2828	0.0240
C16:2ω6	0.2690	-0.4219
C18:3ω3	0.2681	-0.1171
C16:4ω3	0.2400	-0.1514
C20:4ω3	0.2318	0.0683
C18:2ω6	0.2130	-0.1386

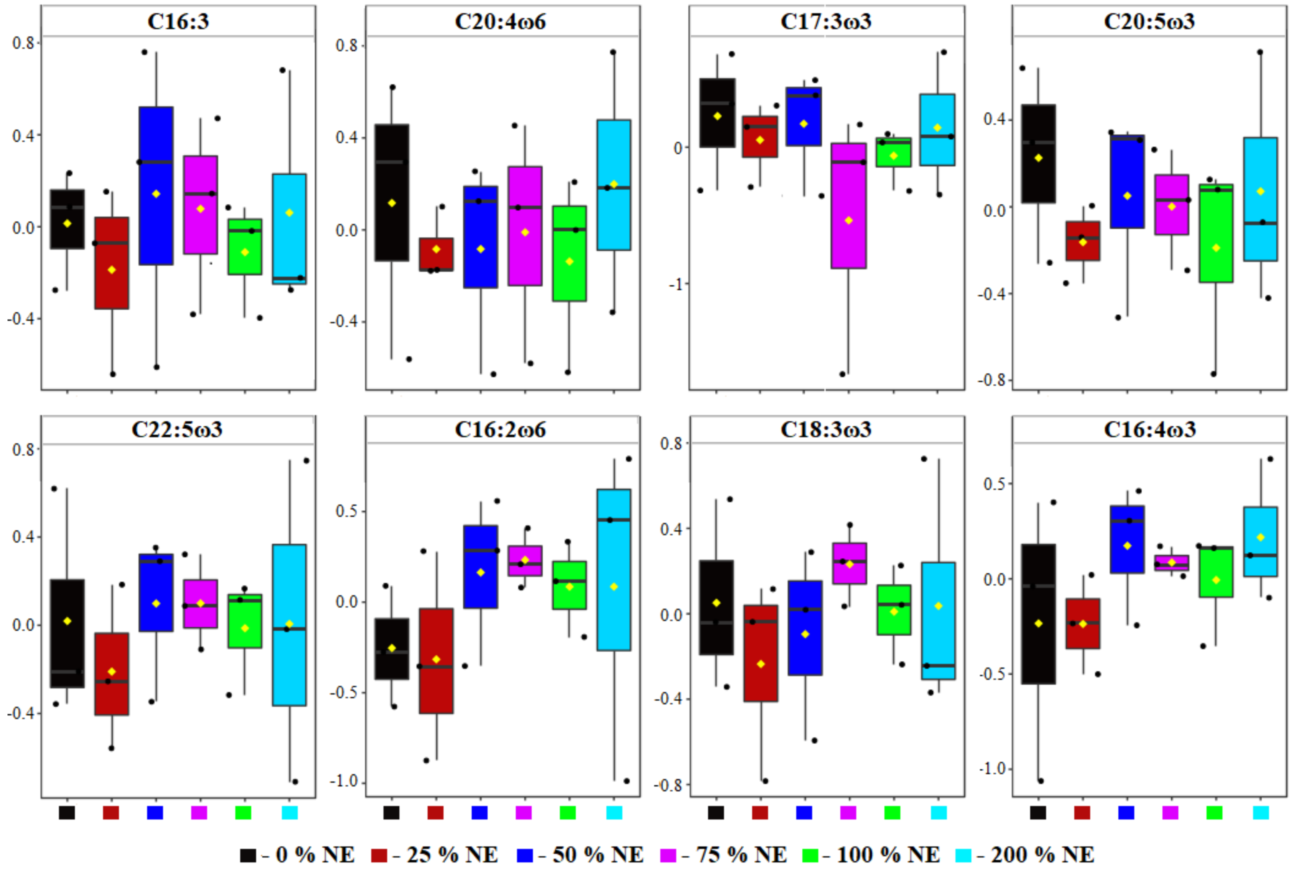


Figure S4.44: Box and whiskers plots of the 8 major contributors for PC1 using the relative abundance after log normalization of fatty acids identified in *Ulva lacinulata*. Notches indicate 95 % confidence interval around the median of each treatment. Yellow diamonds indicate the mean content of fatty acid of each treatment.

Supplementary Information 5 (Chapter 5)

S5.1: GC-MS of solvent extracts (SE) from *Ulva lacinulata*

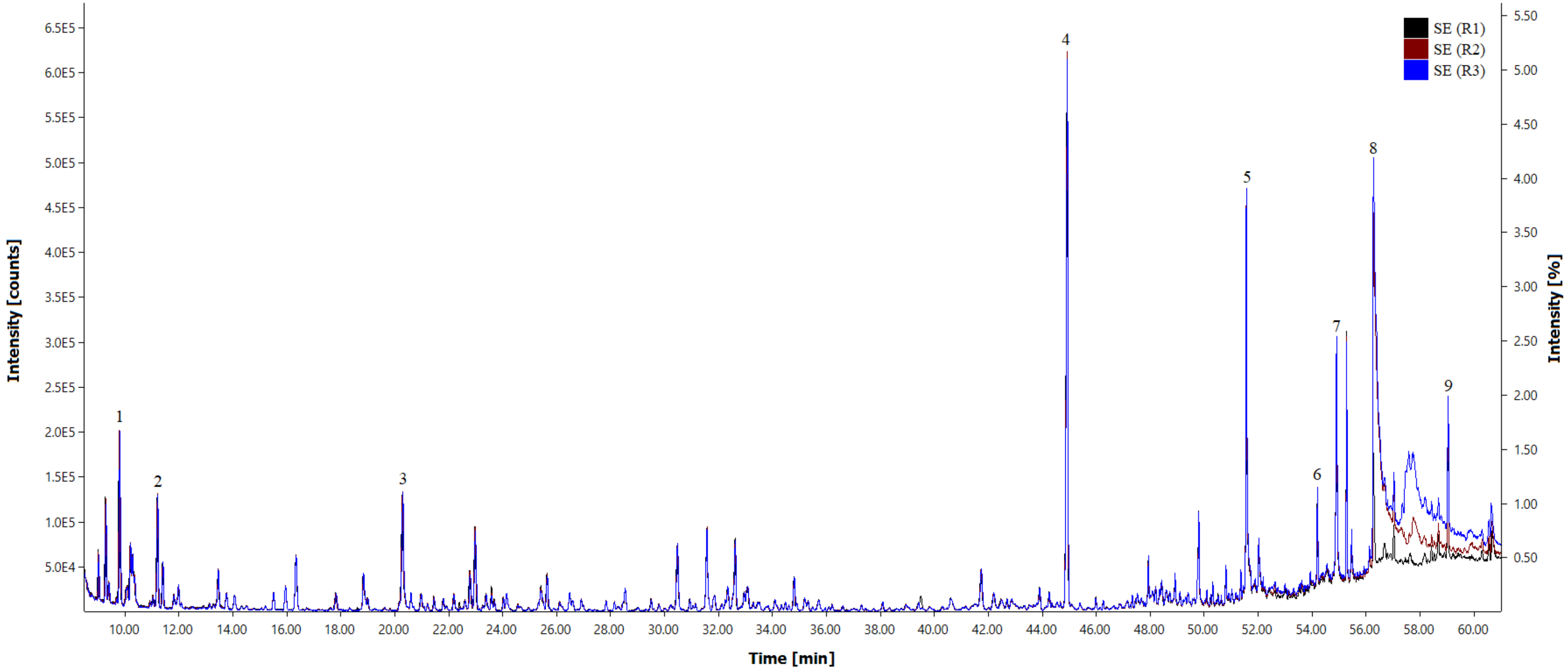


Figure S5.1: Total ion chromatograms (TIC) of volatile organic compound (VOC) detected from the solvent extraction [SE; *n*=3] samples of *Ulva lacinulata* (R1-black, R2-red, R3-blue are replicates).

S5.2: GC-MS of steam distillation–liquid/liquid (SD-LLE) extract from *Ulva lacinulata*

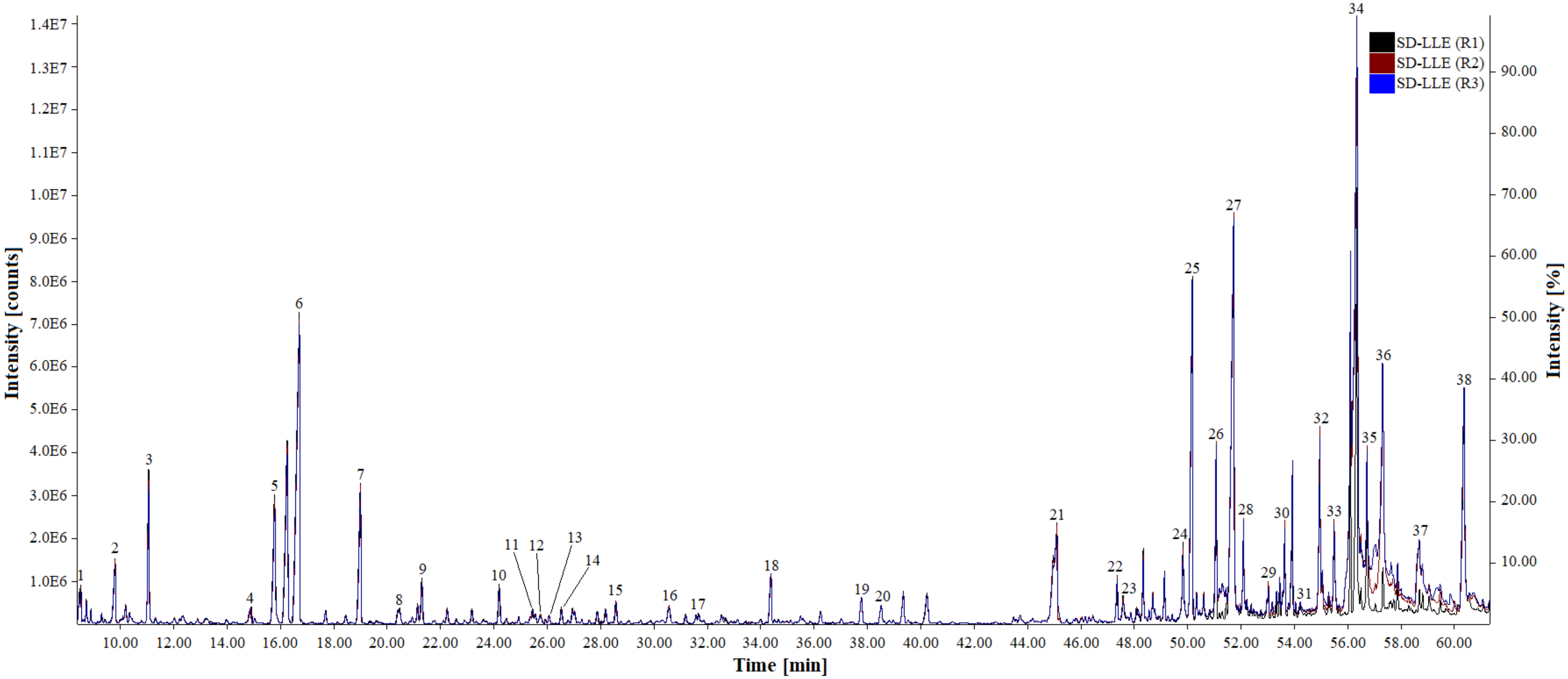


Figure S5.2: Total ion chromatograms (TIC) of volatile organic compound (VOC) detected from the steam distillation–liquid/liquid extraction [SD-LLE; *n*=3] samples of *Ulva lacinulata* (R1-black, R2-red, R3-blue are replicates).

S5.3: GC-MS of headspace extracts (HS–SPME) from *Ulva lacinulata*

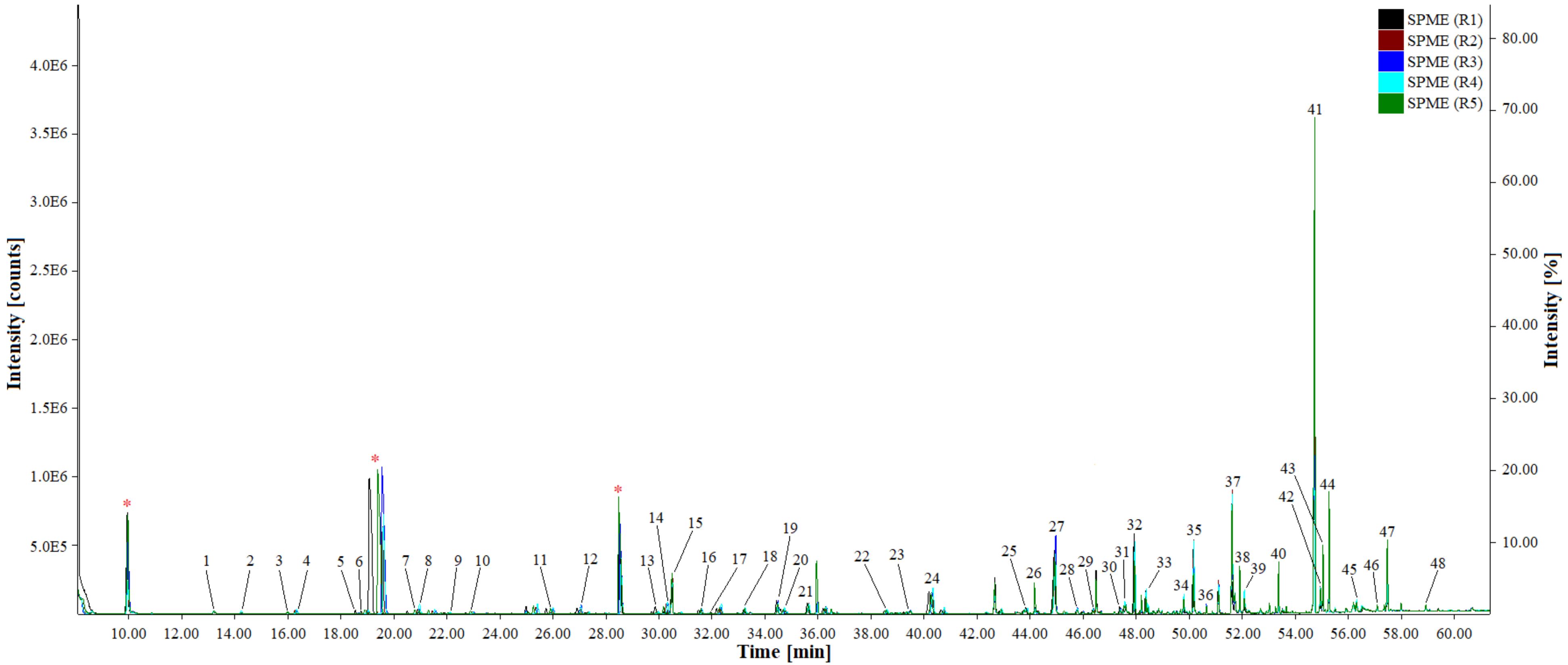


Figure S5.3: Total ion chromatograms (TIC) of volatile organic compound (VOC) detected from headspace solid–phase microextraction [HS-SPME; *n*=5] samples of *Ulva lacinulata* (R1-black, R2-red, R3-blue, R4-cyan and R5-green are replicates; *n*=5; * are signal peaks from the Divinylbenzene/Carboxen/Polydimethylsiloxane fiber).