



Marine Functional Foods Research Initiative (NutraMara) Project-based Award



Lead Partner: Teagasc



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Sea Change: A Marine Knowledge, Research & Innovation Strategy for Ireland

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1. Assist existing, and largely indigenous, marine sub-sectors to improve their overall competitiveness and engage in activity that adds value to their outputs by utilising knowledge and technology arising from research.
2. Build new research capacity and capability and utilise fundamental knowledge and technology to create new marine-related commercial opportunities and companies.
3. Inform public policy, governance and regulation by applying the knowledge derived from marine research and monitoring.
4. Increase the marine sector’s competitiveness and stimulate the commercialisation of the marine resource in a manner that ensures its sustainability and protects marine biodiversity and ecosystems.
5. Strengthen the economic, social and cultural base of marine dependant regional/rural communities.

The Sea Change strategy was developed as an integral part of the government’s Strategy for Science, Technology and Innovation (SSTI) and the Marine Institute as the lead implementation agency is working within SSTI policy and with government departments and agencies to deliver on the Strategy.

The Marine Institute managed Marine Research Sub-Programme, one of eight sub-programmes within the Science, Technology and Innovation (STI) Programme of the National Development Plan 2007—2013, targets funding to meet the objectives of the Sea Change strategy.

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Marine Research Sub-Programme 2007-2013

Project-based Award

NutraMara – Marine Functional Foods Research Initiative (MFFRI/07/01)

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Project Partners: National University of Ireland Galway
University College Dublin
University of Limerick
Ulster University
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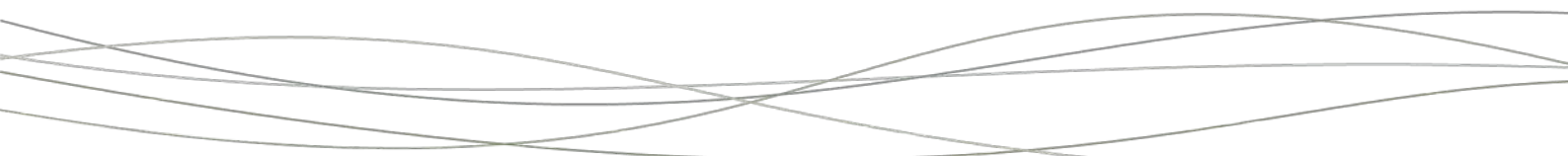
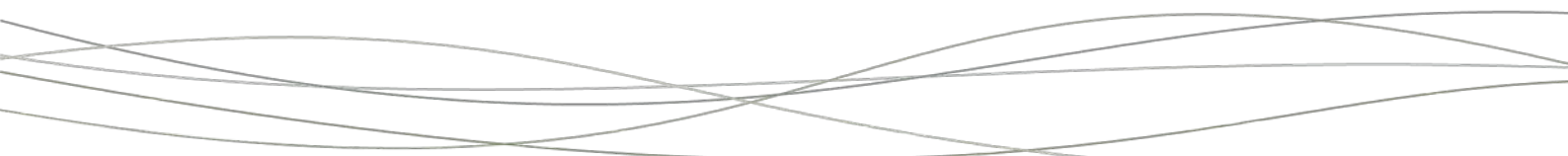


Table of Contents

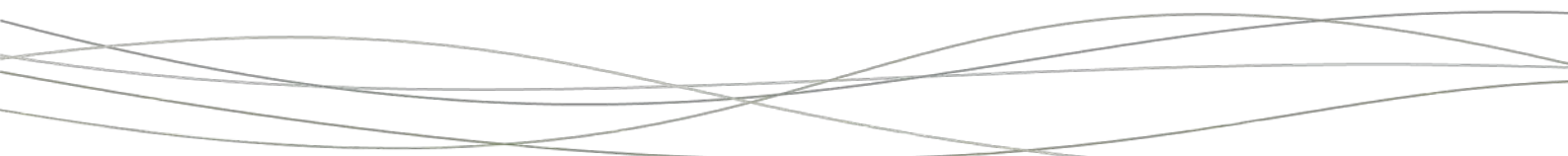
Executive Summary.....	i
1. Introduction.....	1
1.1. Background to the project.....	1
1.2. Functional foods.....	1
1.3. Summary of the project.....	2
1.3.1. Introduction.....	2
1.4. Overview of the work programme	3
1.5. Project objectives.....	4
1.6. Project deliverables.....	5
1.7. Project management.....	7
1.8. Distribution of work.....	8
1.8.1. WP1 - Marine Source Material.....	8
1.8.1.1. Research Activities in WP1	9
1.8.1.1.1. The development of a Feasibility Study	9
1.8.1.1.2. Sampling of marine bioresources	9
1.8.1.1.3. Micro- and macroalgal biomass production	9
1.8.1.1.4. Aquaculture	9
1.8.2. WP2 – Bioactive discovery and generation.....	10
1.8.2.1. Research Activities in WP2.....	10
1.8.2.1.1. The development of extraction methods.....	10
1.8.2.2. Targeted compounds.....	10
1.8.3. WP3 - Bioactive screening and profiling	10
1.8.3.1. Research Activities	11
1.8.4. WP4 - Product development	11
1.8.4.1. Research Activities	11
1.8.5. WP5- Human intervention trials.....	12
1.8.5.1. Research Activities	12



2. Feasibility study	13
2.1. Introduction to the feasibility study.....	13
2.2. Market analysis of functional foods.....	13
2.2.1. Market insights and situation analysis	13
2.3. Consumer attitudes	16
2.3.1. The NutraMara focus group study	16
2.4. Diet related health issues	18
2.5. Chronic diet related disease.....	20
2.6. The impact of marine origin compounds on health	21
2.6.1. Cardiovascular disease.....	21
2.6.2. Weight management.....	23
2.6.3. Diabetes.....	24
2.6.4. Gut health	24
2.6.5. Bone health.....	25
2.6.6. Cancer.....	26
2.6.7. Viral infections	28
2.7. International health studies.....	28
2.8. Regulatory systems for functional foods and ingredients	29
2.9. National and EU legislation.....	29
2.10. Other food related legislation/regulations relevant to marine ingredients.....	30
2.10.1. Food supplements	30
2.10.2. Foodstuffs intended for particular nutritional uses (PARNUTS)	31
2.10.3. Food additives.....	32
2.10.4. Product claims associated with marine ingredients	32
2.11. Regulation in the use of marine materials.....	34
2.12. Collection of materials from the shore.....	35
2.13. Sourcing material from seafood processing.....	36
2.14. Sourcing materials from aquaculture.....	37
2.15. Sustainability.....	37
2.16. Functional compounds from marine organisms	38
2.17. Algal sources of bioactive compounds.....	38
2.18. Macroalgal (seaweed) sources of bioactive compounds	38



2.18.1.	Proteins	39
2.18.2.	Polyunsaturated fatty acids.....	40
2.18.3.	Carbohydrates.....	40
2.18.4.	Compounds with antioxidant properties	42
2.19.	Microalgal sources of bioactive compounds.....	43
2.19.1.	Proteins	44
2.19.2.	Polyunsaturated fatty acids.....	44
2.19.3.	Carbohydrates.....	45
2.19.4.	Compounds showing antioxidant properties.....	45
2.19.5.	Vitamins and minerals.....	45
2.20.	Fish sources of bioactive compounds	46
2.20.1.	Proteins	46
2.20.2.	Polyunsaturated fatty acids.....	47
2.20.3.	Carbohydrates.....	48
2.20.4.	Compounds showing antioxidant properties	49
2.21.	Future outlook for marine origin functional ingredients.....	50
2.22.	Processing marine materials for use as functional ingredients	51
2.23.	Protein extraction	51
2.23.1.	Algal proteins.....	51
2.23.2.	Fish proteins.....	52
2.24.	Lipid Extraction.....	52
2.24.1.	Algal lipids.....	52
2.24.2.	Fish lipids.....	53
2.25.	Carbohydrate extraction.....	54
2.25.1.	Algal carbohydrates.....	54
2.25.2.	Fish carbohydrates	54
2.26.	Antioxidant compound extraction	55
2.27.	Carriers for functional ingredients.....	56
2.28.	Knowledge gaps	57
2.28.1.	Introduction	57
2.28.2.	Characterising sources and stability of supply	57
2.28.2.1.	Microalgal research	58
2.28.2.2.	Macroalgal research	58



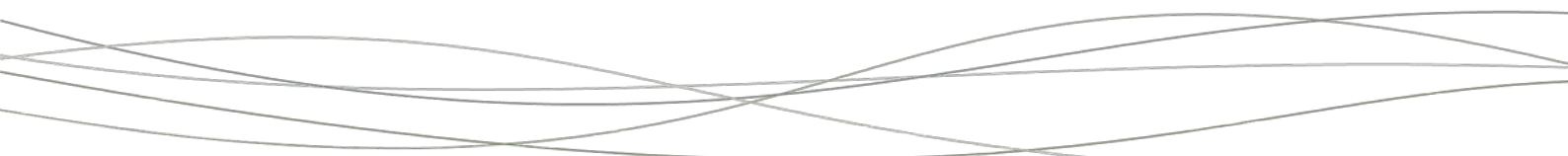
2.28.3.	Identifying and evaluating novel compounds.....	59
2.28.3.1.	Proteins, peptides and amino acids.....	59
2.28.3.2.	Polyunsaturated fatty acids.....	60
2.28.4.	Polysaccharides of fish origin.....	60
2.28.5.	Algal Polysaccharides.....	61
2.28.6.	Knowledge to demonstrate the impact of marine functional foods.....	61

3. Sourcing and Sampling.....63

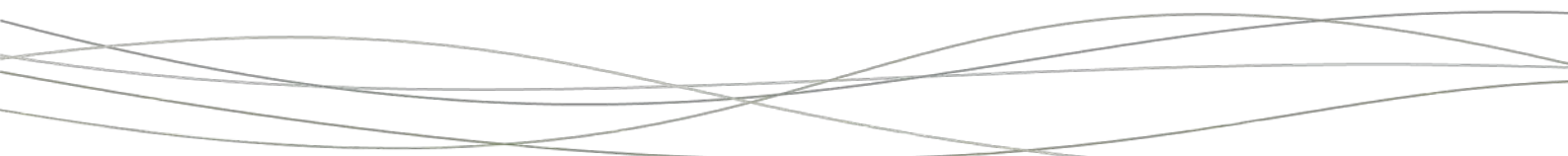
3.1.	Introduction.....	63
3.2.	Assessment of marine resources.....	64
3.2.1.	Irish fishery captures.....	64
3.2.2.	Finfish and shellfish aquaculture production.....	65
3.2.3.	Discarded fish.....	65
3.2.4.	Processing at sea.....	66
3.2.5.	On-shore processing waste.....	66
3.3.	Aquaculture waste.....	68
3.4.	Seaweed harvesting (inc. aquaculture).....	69
3.4.1.	Microalgae.....	70
3.4.2.	Species from the wild.....	71
3.4.3.	Species from algal collections.....	71
3.5.	Use of Irish marine materials as functional ingredients.....	72
3.6.	NutraMara sampling plans.....	75
3.6.1.	Introduction.....	75
3.6.2.	Sampling to test ideas about variation in concentration.....	76
3.6.3.	Sampling approaches in NutraMara.....	77
3.7.	Sample database.....	78
3.8.	Sample repository.....	81

4. Discovery and characterisation of bioactive compounds.....82

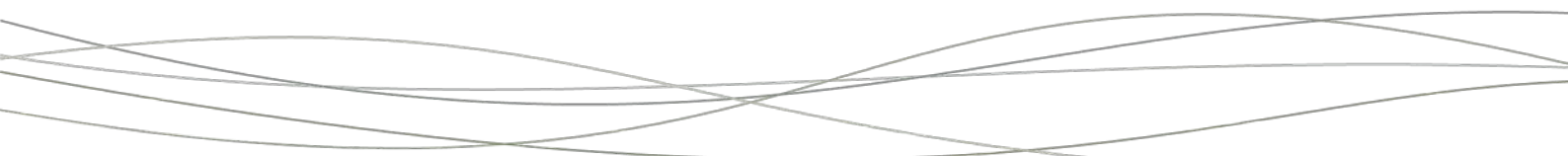
4.1.	Introduction.....	82
4.2.	Extraction of bioactive compounds.....	83
4.3.	Algal derived compounds – macroalgae.....	83
4.4.	Polyphenols.....	83



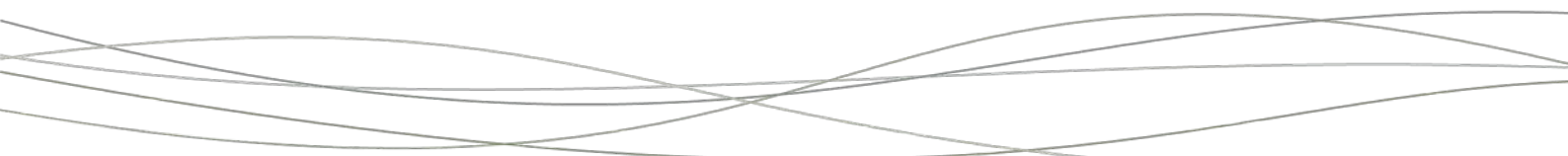
4.5. Carotenoids	84
4.6. Polysaccharides	85
4.7. Lipids.....	85
4.7.1. Extraction of total lipids from macroalgae	85
4.8. Proteins and peptides.....	86
4.8.1. Total nitrogen (TN), non-protein nitrogen (NPN) and protein nitrogen (PN) quantification	88
4.8.2. Generation of macroalgal protein hydrolysates.....	88
4.9. Algal derived compounds – microalgae.....	90
4.9.1. Introduction.....	90
4.9.2. Objectives	91
4.9.3. Overview of materials and methods.....	91
4.9.4. Cultivation and growth.....	92
4.9.5. Analysis of fatty acid composition and content.....	92
4.9.6. Pigment composition.....	92
4.9.7. Phycobiliprotein content.....	92
4.9.8. Mycosporine-like amino acid composition	93
4.10. Screening of bioactives in microalgae.....	93
4.10.1. Culture conditions.....	93
4.10.2. Results	93
4.11. Patterns of carbohydrate and fatty acid changes under nitrogen limitation and low inorganic carbon supply in two microalgae <i>P. lutheri</i> and <i>P. tricornutum</i>	94
4.11.1. Objective.....	94
4.11.2. Culture conditions.....	94
4.11.3. Results	94
4.12. Interaction of light, temperature and nitrogen for optimising the co-production of high- value compounds in <i>P. purpureum</i>	96
4.12.1. Objective.....	96
4.12.2. Culture conditions.....	96
4.12.3. Results	96
4.13. LC-PUFA-enriched oil production by <i>P. lutheri</i> : Combined effects of light, temperature and inorganic carbon availability	97
4.13.1. Objective.....	97



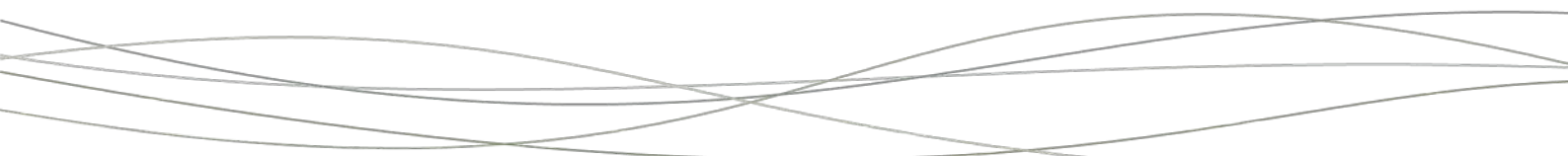
4.13.2.	Culture conditions.....	98
4.13.3.	Results	98
4.14.	Indoor microalgal large-scale production of selected microalgae for targeted bioactive production under controlled conditions.....	100
4.14.1.	Objective.....	100
4.14.2.	Cultivation system and conditions	100
4.14.3.	Results	101
4.15.	Screening of MAAs in available microalgae and investigation of the deleterious effect of UV radiation on microalgae	101
4.15.1.	Objective.....	101
4.15.2.	Microalgal species and culture conditions.....	102
4.15.3.	Results	102
4.15.4.	Conclusions.....	102
4.16.	Fish processing co-product derived compounds	103
4.17.	Peptides.....	103
4.17.1.	From salmon co-products	103
4.17.2.	From mussels.....	104
4.17.3.	Generation of protein hydrolysates from fish processing co-products	105
4.18.	Polysaccharides	106
4.18.1.	Chitin	106
4.19.	Aquaculture derived compounds.....	106
4.19.1.	Algal compounds through optimised cultivation	106
4.19.2.	Introduction	107
4.19.3.	Evaluation of natural seasonal and spatial variability in bioactive compounds in selected seaweeds	108
4.19.3.1.	Objectives.....	108
4.19.3.2.	Material and methods.....	108
4.19.4.	Results	109
4.19.5.	Evaluation of environmental control of bioactive levels and composition through experimental exposure.....	110
4.19.5.1.	Objective.....	110
4.19.5.2.	Material and methods.....	110
4.19.6.	Results	111



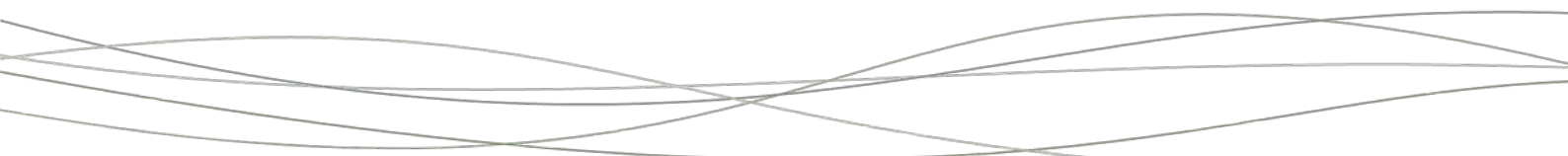
4.19.7.	Bioactive production through targeted algal cultivation.....	111
4.19.7.1.	Objective.....	111
4.19.8.	Material and methods.....	111
4.19.8.1.	Results.....	111
4.19.9.	Plasticity of lipid partitioning in Irish seaweeds.....	111
4.19.9.1.	Objective.....	111
4.19.9.2.	Material and methods.....	112
4.19.9.3.	Results.....	112
4.19.10.	Discussion.....	113
4.19.11.	Conclusions.....	114
4.19.12.	Farmed rainbow trout.....	114
4.19.12.1.	Protein extraction.....	114
4.19.12.2.	Protein hydrolysis.....	114
4.19.12.3.	MWCO - Ultrafiltration.....	114
4.19.12.4.	Protein content of rainbow trout myofibrillar protein hydrolysates, filtrates and RP-HPLC fractions.....	115
4.19.13.	Farmed mussels.....	117
4.19.14.	Biochemical composition analysis.....	118
4.19.14.1.	Results.....	118
4.19.15.	Abalone.....	121
4.19.16.	Protein extraction.....	121
4.19.17.	Enzymatic hydrolysis.....	122
4.19.18.	MWCO-Ultrafiltration.....	122
4.19.19.	Protein content of abalone myofibrillar protein hydrolysates and MWCO 3-kDa (3-kDa-UFH) and 10-kDa (10-kDa-UFH) ultrafiltration fractions.....	123
4.19.20.	Renin inhibitory bioassay of abalone thermolysin hydrolysates and MWCO 3-kDa-UFH and 10-kDa-UFH fractions.....	123
4.19.21.	Measurement of Factor Xa inhibitory activities of abalone thermolysin hydrolysate and the MWCO 10-kDa-UFH and 3-kDa-UFH fractions.....	123
4.19.22.	Water activity (aw).....	124
4.19.23.	Removal of polyethylene glycols from abalone hydrolysates, filtrates and permeates using a titanium dioxide (TiO ₂) cleanup procedure.....	124
4.19.24.	Peptide identification.....	124



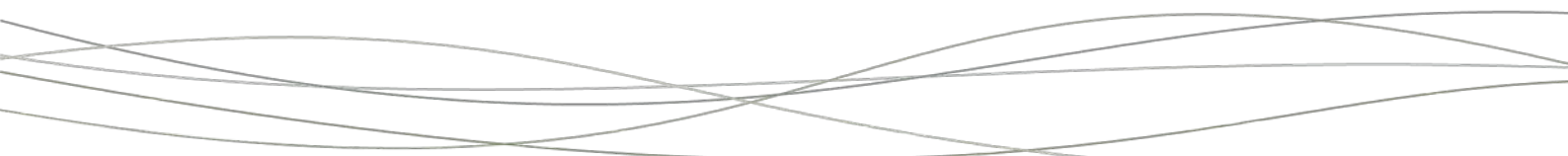
4.19.25.	Database search and confirmation of sequences.....	124
4.20.	Results.....	125
4.20.1.	Protein content of abalone myofibrillar muscle thermolysin hydrolysates, MWCO 10-kDa-UFH and 3-kDa -UFH fractions.....	125
4.20.2.	PAF-AH inhibitory activities of the 10-kDa-UFH and the 3-kDa-UFH fractions generated from abalone thermolysin hydrolysates.....	125
4.20.3.	Renin inhibitory activities of the MWCO 10-kDa-UFH and the 3-kDa-UFH fractions from abalone myofibrillar protein thermolysin hydrolysates.....	126
4.20.4.	Factor Xa inhibitory activities of the 10-kDa-UFH permeate and the 3-kDa-UFH permeate fractions from abalone myofibrillar protein thermolysin hydrolysates.....	127
4.20.5.	Water activity (a_w) of the abalone myofibrillar protein thermolysin hydrolysates, 10-kDa-UFH and 3-kDa-UFH fractions.....	127
4.20.6.	Peptides identified in abalone (<i>Haliotis discus hannai</i> Ino) myofibrillar protein thermolysin hydrolysates, 10-kDa-UFH and 3-kDa-UFH using ESI nano-spray MS and FT-ICR/Orbitrap.....	127
4.21.	The peptide database.....	130
5.	Screening and profiling bioactive compounds.....	131
5.1.	Introduction.....	131
5.2.	Cellular and metabolic studies.....	132
5.3.	Screening for anti-inflammatory potential.....	132
5.3.1.	Screening for gut anti- inflammatory potential.....	134
5.4.	Screening for anti-obesity potential.....	135
5.5.	Screening for cell viability and cytotoxicity.....	136
5.6.	Screening for pre-biotic potential.....	137
5.7.	Screening for antimicrobial potential.....	139
5.8.	Screening for anti-infective potential.....	140
5.9.	Screening for anti-diabetic potential.....	141
5.10.	Screening for anti-cancer potential.....	143
5.11.	Screening for platelet-activating factor (PAF) acetylhydrolase inhibition.....	145
5.12.	Screening for antioxidant potential.....	147
5.13.	Investigation into the molecular mode of action of marine derived bioactives.....	148
5.13.1.	Anti-adipogenic mode of action of chitosan at cell level.....	148



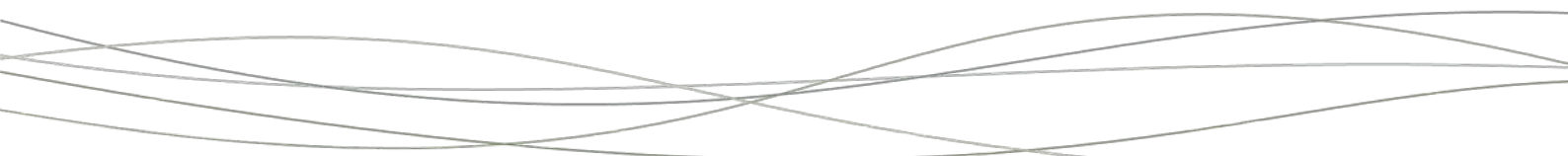
5.13.2.	Anti-inflammatory mode of action of chitosan at cell level	148
5.13.3.	Anti-inflammatory mode of action of seaweed extract derived from <i>Ascophyllum nodosum</i>	149
5.14.	An evaluation of the effects of marine bioactive compounds on fatness and anti-obesity potential in the pig model.....	151
5.14.1.	Animal selection and management.....	152
5.14.2.	Biological analysis.....	152
5.14.3.	Results	153
5.14.4.	Summary	154
5.15.	An evaluation of the potential of marine compounds to contribute to enhanced pig performance.....	154
5.16.	Comparison of the anti-inflammatory effects of seaweed extracts and novel milk hydrolysates	156
5.16.1.	Materials and methods	156
5.16.2.	Results	158
5.16.3.	Conclusion.....	160
5.17.	Human intervention trials	161
5.18.	Effect of marine polysaccharides in pig meat.....	161
5.18.1.	Source of the pork.....	161
5.18.2.	Pork processing and packaging.....	161
5.18.3.	Recruitment of participants	161
5.18.4.	Intervention.....	162
5.18.5.	Lymphocyte cell isolation	163
5.18.6.	Lymphocyte comet assay.....	163
5.18.7.	Ferric Reducing Ability of Plasma (FRAP) assay.....	163
5.18.8.	C-reactive protein	164
5.18.9.	Lipid status.....	164
5.18.10.	Dietary analysis.....	164
5.18.11.	Statistical analysis	164
5.18.12.	Results	164
6.	Scale-up and delivery of bioactives	166
6.1.	Introduction.....	166



6.2.	Carriers for functional ingredients.....	166
6.2.1.	Manufacture of dairy products fortified with seaweed-derived ingredients	166
6.3.	Moving from laboratory to pilot scale production	167
6.3.1.	Development of commercial process	167
6.3.2.	ASE Extraction of phlorotannins.....	169
6.3.3.	ASE® extraction of macro-algal lipids	170
6.3.4.	ASE extracts from Fucus serratus and Ascophyllum nodosum	170
6.4.	Large scale extractions using the Armfield Rapid extractor (5 Litre capacity)	171
6.5.	Impact of processing on functional ingredients	171
6.5.1.	The stability of seaweed extracts under storage conditions	171
6.5.2.	The effect of dietary seaweed polysaccharides on pork quality	172
6.5.3.	The effect of seaweed based fish feed on farmed salmon quality.....	173
6.6.	Bioavailability of compounds	174
6.6.1.	Bioactivity of seaweed-enriched bread.....	174
6.6.2.	Bioactivity of seaweed-enriched milk and yoghurt.....	175
6.6.3.	Bioactivity of seaweed extracts in pork	175
6.7.	Processing methods for the recovery of bioactives from seaweeds	176
6.7.1.	Introduction.....	176
6.7.2.	Process to extract agar.....	176
6.7.3.	Process to extract carrageenan.....	177
6.7.4.	Process to extract alginate	178
7.	NutraMara Technical Support.....	179
7.1.	Introduction.....	179
7.2.	Technology updates.....	179
7.3.	Service offers from partner institutions	180
8.	Impact of the NutraMara programme	181
8.1.	Introduction.....	181
8.2.	Contribution to meeting the national research agenda	181
8.3.	Capacity building.....	183
8.3.1.	Post-doctoral researchers/research officers.....	183
8.3.2.	PhD students.....	184



8.4. Training initiatives	186
8.5. Dissemination activities	187
8.5.1. Publications	187
8.5.2. Peer reviewed publications.....	187
8.5.3. Books and book chapters	194
8.5.4. Posters	196
8.5.5. Conference presentations.....	199
8.5.6. Conferences	202
8.6. Additional research activity/awards.....	203
9. Conclusions.....	206
10. References.....	209
Appendix 1: Health outcomes attributed to marine bioactives.....	238
Appendix 2 – Micro-algal strains in culture collections.....	247
Appendix 3 – NutraMara Technology Updates.....	249
Appendix 4 – Service Offers.....	268
Appendix 5 – NutraMara Conference Agendas.....	279



EXECUTIVE SUMMARY

The NutraMara – Marine Functional Foods Research Initiative was conceived by *Sea Change - A Marine Knowledge, Research and Innovation Strategy for Ireland 2007-2013*. The goal was to develop a collaborative funding mechanism that would create new research capacity and build the capabilities required to maximise the potential of Ireland’s extensive marine bioresources. By supporting a strong interdisciplinary research team, capable of exploring marine animals and plants as a sustainable source of materials for use as functional ingredients and foods, the vision for NutraMara was to position Ireland to the fore in use of marine bioresources as health beneficial ingredients.

Commencing in 2008 and supported by funds of €5.2 million from the Marine Institute and the Department of Agriculture, Food and the Marine, the research programme was led by Teagasc as the head of a multi-institutional consortium. The NutraMara consortium comprises marine bioresources and bioscience expertise, with food science and technology expertise from University College Cork; University College Dublin; the National University of Ireland Galway; the University of Limerick and Ulster University.

Research effort was directed towards exploring Ireland’s marine bioresources – including macro- and microalgae, finfish and shellfish from wild and cultured sources: and discards from processing fish as sources of novel ingredients with bioactive characteristics. This discovery activity involved the collection of over 600 samples from 39 species of algae and fish and the analysis of 5,800 extracts, which resulted in 3,000 positive “hits” for bioactivity.

The NutraMara consortium has built a strong research capacity to identify, characterise and evaluate marine-origin bioactives for use as/in functional foods. It further built the capacity to develop model foods enhanced with these marine-origin functional ingredients; providing insights to the processing challenges associated with producing functional ingredients from marine organisms.

The consortium was actively engaged in research activities designed to identify and assess bioactive compounds from available marine resources, including polyphenols, proteins/peptides, amino acids, polysaccharides, polyunsaturated fatty acids and materials with antioxidant, probiotic or prebiotic properties.

A key component of NutraMara’s activities was the development of human capital. The recruitment of M.Sc. and PhD students and their integration within a dynamic research environment that has strong links to industry, provided lasting expertise and capabilities, which are relevant to the needs of Ireland’s food and marine sectors. NutraMara research led to the awarding of eighteen PhDs and recruitment of 21 post-doctoral researchers over the eight year research programme. In excess of 80 peer reviewed publications resulted from this

research and more publications are planned. A further 100 posters and conference presentations were also delivered by NutraMara researchers and Principal Investigators.

The development and implementation of training and exchange programmes aimed at providing early stage researchers with inter-disciplinary skills that are critical to their development as researchers, enhanced the research capacity of institutions, the industry sectors and the country as a whole.

Principal Investigators involved in leading the NutraMara research programme have secured additional research grants of almost €6 million from national and international sources and are engaged in extensive research collaboration involving marine and food research expertise; an activity which did not exist prior to NutraMara.

The dissemination of knowledge and transfer of research results to industry were key activities in the research programme. The research outputs and visibility of NutraMara activity nationally resulted in 10 companies engaging in research and development activity with the consortium. Regular workshops and conferences organised by NutraMara attracted close to five hundred participants from Ireland and overseas.

Members of the NutraMara core PI group have contributed to the formulation of new national foods and marine research policy and national research agenda, both during the national prioritisation exercise and in sectoral research strategies.

This final project report describes the process by which research targets were identified, and the results of extensive screening and evaluation of compounds extracted from marine bioresources. It also highlights the development of new protocols designed to extract compounds in ways that are food friendly. Evaluating the functional properties, bioactivity and bioavailability of high potential marine compounds involved *in vitro* and *in vivo* testing. Pilot animal and human intervention studies yielded further insight to the potential and challenges in developing marine functional ingredients.

As a result of work completed within the NutraMara consortium, Ireland is well positioned to continue to contribute to the development of ingredients derived from marine organisms and in doing so support the on-going development of Ireland's food sector.

I. INTRODUCTION

I.1. Background to the project

NutraMara was formally launched as a national Marine Functional Food Research Initiative (MFFRI) in 2008. A research consortium led by Teagasc involving University College Cork, the National University of Ireland Galway, the University of Limerick, University College Dublin and Ulster University, successfully bid for what was a co-funded research programme supported by the Marine Institute and the Department of Agriculture, Food and the Marine (DAFM).

Ireland was recognised as having both the natural resources and the expertise to become a significant player in the new and expanding market for marine functional foods and food ingredients.

NutraMara's research activity was directed towards exploring the potential to use fish processing discards, the sustainable marine species, and products from aquaculture, as functional foods and ingredients. These areas were identified as research priorities at a workshop hosted by the Marine Institute and attended by food companies, food ingredient suppliers, seafood processors, biotechnology firms and researchers from industry and other institutions.

This initiative was recognised as a key activity in developing the potential of marine functional foods, as identified through the consultation process around *Sea Change - A Marine Knowledge, Research and Innovation Strategy for Ireland 2007-2013*. The aim of the collaborative funding was to create new research capacity and build the capabilities required to maximise the potential of Ireland's extensive marine bioresources, by supporting a strong interdisciplinary research team, capable of exploring marine animals and algae as a sustainable source of materials for use as functional ingredients and foods.

I.2. Functional foods

Functional foods deliver health benefits over and above meeting a basic nutritional need. The underlying concept of a functional food is that of a food, or food component(s), which can contribute beneficially to human body functions by improving the state of well-being and reducing the risk of disease. The contribution of functional foods are not confined to supporting human development, growth and body maintenance, and are recognised as helping in maintaining the quality of human life.

In 1999, a European Community (EC) Concerted Action on Functional Foods Science in Europe (FUFOSE) tightened the definition of "functional food". It declared a food as "functional" if – "it is satisfactorily demonstrated to affect beneficially one or more target

functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction in risk of disease”. Functional foods must remain foods; and they must demonstrate their effects from amounts that can normally be consumed in the diet. They are not pills or capsules, but part of a normal food pattern. Unlike dietary supplements, which are taken in pill or liquid form, functional foods also have to meet the taste requirements and preferences of consumers.

Functional foods fill the space between conventional food and dietary supplements. Some common examples of functional foods include: light or low calorie products; food with low-salt levels; cholesterol-lowering spreads and beverages; foods that are specifically low-glycemic; and products that carry an antioxidant claim such as some teas and juices. Potentially exploiting a marine sourced material is a number of spreads, breads and beverages that contain Omega-3 fats. Conventional foods consumed in their natural state (e.g. fruit, vegetables, and grains) or novel foods that incorporate functional components to provide greater health benefits, also fit within the definition of functional foods.

1.3. Summary of the project

1.3.1. Introduction

NutraMara was an all-Ireland, multidisciplinary research consortium designed to build new research capacity in an area that offers significant economic potential for Ireland’s marine foods and food ingredients sectors. The work of the consortium focused on scientific knowledge creation, establishing new capabilities and developing processes to assess and evaluate the potential of marine-origin bioactive compounds as components in functional foods and as food ingredients. NutraMara is a key national strategic research programme, which integrates Irish marine science and food science expertise and capabilities from 6 institutions throughout the island of Ireland. This partnership approach provided the capability to establish and develop a coordinated approach for the exploitation of Irish marine resources with potential food and health applications.

The NutraMara consortium built a strong research capacity to identify, characterise and evaluate marine-origin bioactive compounds for use as/in functional foods. Additionally, it is building the capacity to develop model foods enhanced with these marine-origin functional ingredients and to provide insights into the processing challenges associated with producing functional ingredients from marine organisms.

The consortium actively engaged in research activities designed to identify and assess bioactive compounds from available marine resources, including polyphenols, proteins/peptides, amino acids, polysaccharides, polyunsaturated fatty acids and materials with antioxidant, probiotic or prebiotic properties.

A key component of NutraMara’s activities was the development of human capital. The recruitment of M.Sc. and PhD students and their integration within a dynamic research

environment that has strong links to industry provided lasting expertise and capabilities, which are relevant to the needs of Ireland's food and marine sectors. The development and implementation of training and exchange programmes aimed at providing early stage researchers with inter-disciplinary skills that are critical to their development as researchers, enhances the research capacity of institutions, the industry sectors and the country as a whole. In recognising the importance of capturing and protecting research outputs, and making them available to industry, NutraMara developed and implemented Intellectual Property (IP) policies and procedures that are based upon national guidelines concerning the management of intellectual property. Allied to this, NutraMara has expanded its interaction with academic collaborators in complementary scientific fields, whilst also strengthening linkages and collaborations with industry partners.

The NutraMara consortium was acutely aware the initial research grant was provided to develop and establish new capabilities, and that continuity was required to sustain the research activities. During the final stages of the initial grant, members of the NutraMara consortium targeted national and international competitive grant support. During 2013, Principal Investigators involved in NutraMara were successful in being awarded research funds from the Department of Agriculture, Food and the Marine (DAFM), Food Institutional Research Measure (FIRM) to the value of €2.8M, allowing 5 new research projects with strong links to NutraMara. And during 2014, NutraMara PIs were again successful in winning substantial research grants from Science Foundation Ireland, DAFM and the EU. Several NutraMara PIs were also awarded funds under the Teagasc Walsh Fellowships to co-supervise research students in co-operation with Teagasc researchers to work engage in research related to the NutraMara research programme.

1.4. Overview of the work programme

The foundation of the NutraMara research programme was a Feasibility Study designed to identify sustainable marine resources with bioactive potential (*Work Package 1*) and other factors affecting the access and use of marine resources. The potential of three target resources; micro and macroalgae, by-products of primary processing and aquaculture would be refined by the feasibility study. Also included within the Feasibility Study was an examination of issues such as legislation, consumer and market analysis, all of which would influence the direction of the research. Ultimately, a national repository of sustainable marine resources was developed with due consideration to issues such as seasonal variation, geographic location and sustainability, and within this repository there is now a database holding details of materials and bioactive compounds.

Members of the consortium agreed upon a short list of marine species to allow to progress in *Work Package 2 (Bioactive discovery and generation)* whilst the feasibility study was being completed. The aim of *Work Package 2* was to develop optimised technologies to extract and

purify bioactive compounds from targeted marine resources. The research programme targeted bioactive molecules from four categories, *antioxidants and pigments*, *polysaccharides*, *peptides and amino acids* and *fatty acids*. In addition, *Work Package 2* sought to generate new compounds by mining marine based materials using chemical and enzymatic hydrolysis and fractionation techniques. High-throughput rapid screening for bioactivity was carried out to identify material with potential to be carried through to *Work Package 3 (Bioactive profiling)*. Fractions, compounds and whole materials with bioactive potential were characterised in more detail, using techniques such as high performance liquid chromatography, quadrupole time of flight mass spectrometry and high-resolution nuclear magnetic resonance.

Work Package 3 used a range of methods including model systems, cell cultures, transcriptomics, bio-informatics, computational biology and metabolomics to create a more detailed bioactive profile of candidate materials. These techniques were employed to examine the resources for bioactive potential under a number of defined biological activities i.e. anti-microbial activity, anti-thrombotic activity, anti-infective activity, anti-proliferative activity, anti-hypertensive activity, immunomodulatory/anti-inflammatory activity and prebiotic/bifidogenic activity. Whilst the majority of bioassays used in this assessment were well proven, there were some instances where specific bioassays were developed.

Work Package 4 (Product development) was designed to explore issues associated with incorporating bioactive compounds into food products. Where a particular compound demonstrated bioactivity, samples were added into a number of model foods, allowing issues such as shelf life, formulation, processing, quality and sensory properties to be examined.

Finally, on the scientific work plan, *Work Package 5 (Dietary intervention studies)* was designed to examine the health effect of selected functional ingredients when consumed in food products.

The NutraMara research programme was supported by two of the seven work packages; *Work Package 6 (Management)* supported the day to day coordination and reporting activity across the entire programme and was closely linked to the activities of *Work Package 7 (Training, Dissemination and Outreach)* which promoted researcher development opportunities, dissemination of research results and developed links between the project and industry.

1.5. Project objectives

Studies have shown that marine resources are unrivalled sources of bioactive compounds with the potential to maintain and improve health in humans and animals. Ireland's territorial waters are known for the extent of their biodiversity. Traditionally, the waters around Ireland were targeted as a source of a large variety of marine foods. Despite this attention, there was only limited activity aimed at exploiting these resources as sources of functional foods or functional ingredients. The NutraMara initiative, in utilising national research funds, planned to develop what was a neglected research area – marine functional foods, into a thriving knowledge driven

network of researchers, comprising marine sciences and food sciences expertise; transforming Ireland into an internationally recognised research hub.

The main objectives targeted by the NutraMara work programme and delivered by the consortium were to:

- Create a strong, interdisciplinary research capability, capable of exploiting marine biodiversity as a source of materials for use in functional foods.
- Support the creation of new research capacity in areas that underpin research in marine functional ingredients and foods.
- Establish new research capabilities in marine functional foods-linking indigenous and multi-national food and pharmaceutical industries with researchers at state and higher education research institutions.
- Engage in priority research activities such as polyphenols, pigments, peptides, polysaccharides, amino acids, polyunsaturated fatty acids, protein hydrolysates and materials with antioxidant, probiotic or prebiotic properties as identified by the marine functional foods workshop hosted by the Marine Institute.
- To develop model foods enhanced with marine origin functional ingredients and to develop capabilities to process marine-based materials for use by the functional ingredients sector.
- Develop and implement training programmes aimed at providing people with interdisciplinary skills critical to the industry sector.
- Secure research funds from national and international sources that will enable the Consortium to expand its research in relevant areas to the advantage of Ireland's food, food ingredients and pharmaceutical sectors.
- To commence a research programme to explore and deliver new knowledge relating to:
 - The identification, extraction and validation of bioactive compounds from marine origin material and the verification of the physiological effects of these compounds; and
 - Developing the required scientific knowledge, capabilities and processes necessary to assess and evaluate the potential of marine origin bioactive compounds for use as components in functional foods and as food ingredients.
- Ensure that commercially valuable results of the research programme are appropriately protected and made available to Irish industry for efficient and effective commercialisation.
- Promote organisational connections and linkages, both within and beyond the Programme partners, within and among campuses, industry, other research bodies and international collaborators.
- Maintain the integrity of the research programme in terms of protecting the results of the institutional research activities, and ring-fencing them from becoming obscured in non-programme related activities of the participants in terms of protection from other activities.
- Disseminate the results of the research through publications, workshops, seminars and conferences.

1.6. Project deliverables

The NutraMara consortium proposed an extensive range of project deliverables that together would become a national source of knowledge concerning the identification and processing of marine origin compounds into functional ingredients and foods. In developing scientific and technological knowledge relating to the sourcing and use of marine materials as functional ingredients, the NutraMara programme would establish both the capability and capacity

required to allow Ireland to become an internationally competitive research performer in using marine resources as functional ingredients. The work programme was designed to deliver the following outputs:

- A comprehensive report detailing common and agreed protocols developed in conjunction with the Beaufort Biodiscovery Project, addressing issues of species storage and management of referenced materials. In recognition that sources will be diverse, in terms of species, spatial and geographical distribution, seasonal variation and population differentials, the protocols will address: collection, identification, processing and storage details. This will ensure ease of access for all partners.
- A detailed review of relevant national, EU and international legislation as applied to the sourcing, refinement and application of (marine) compounds in animal and human health.
- A literature study to identify gaps in current knowledge regarding the isolation, identification and subsequent characterisation of marine components having functional food potential.
- A database comprising
 - Inventory of potentially exploitable material with signposts to known/suspected compounds.
 - Identified and quantified primary products from three sectors (fisheries, culture and processing).
 - Analytical analysis of the presence and availability of target compounds on spatial and geographical basis.
 - A report providing a full listing of the nature, availability, character, formats and quantities of various species, extracts, materials and wastes generated /available from the wild and produced by farming and downstream processing from the Irish Marine environment.
- Protocols and technologies for the quantitative and food friendly extraction of polyphenols and carotenoids from marine micro- and macroalgae.
- Detailed protocols for analytical and pilot scale purification and extraction of fatty acids from marine sources.
- A database of promising target proteins, peptides and amino acids.
- Prototype protocols for extraction of crude samples of proteins, peptides and amino acids.
- Literature review of methods for the extraction, purification and characterisation of marine origin bio-actives.
- Protocols and technologies for the quantitative and food friendly extraction and purification of β -glucans from marine micro- and macroalgae.
- Report on the effect of feeding chitin on intestinal health in porcine model systems.
- Prototypes of seaweed based fish feed.
- Cellular mechanisms of action for marine bioactives.
- Marine fractions with identifiable health promotional properties.
- Processes and protocols for the production of foods enhanced with health promoting ingredients derived from marine sources.
- Protocols for the optimal retention of marine bio-actives during processing.
- Information on the impact of marine origin bio-actives on quality, safety and shelf-life of model foods.
- Human intervention studies demonstrating the alteration of gut micro flora and immune function in humans.
- Early stage career scientists and their training and development plan needs with skills and expertise in areas which underpin marine based functional food development.
- Peer reviewed publication, newsletters, workshops and conferences, technical and financial reports.

I.7. Project management

The NutraMara programme was led by a Director, who together with support from a Programme Manager was jointly responsible for managing the entire programme. This included the provision of effective and frequent communication with the funding bodies (DAFM and the MI) through annual and interim reporting, monthly conference calls and Management Review Board meetings. The main activities comprised managing the research grant and consortium agreements, carrying out the overall scientific and administrative duties of the project including the provision of annual and 6 monthly reports, and organising scientific council meetings, management board meetings, IP meetings and external advisory board meetings in addition to financial management of the project.

The day to day management of NutraMara was the responsibility of a full-time Programme Manager based at Teagasc, Ashtown, which included coordinating and supervising work tasks, and monitoring programme milestones and deliverables. The Programme Manager worked in conjunction with the Director in directing NutraMara's outreach and dissemination activity.

A management board comprising representatives of NutraMara consortium partners and the two funding agencies - the Marine Institute and the Department of Agriculture Food and the Marine; and a scientific advisory board of international experts in areas relevant to the NutraMara research activities provided a strategic oversight function.

A schematic of the NutraMara management structure is given below in Figure I.

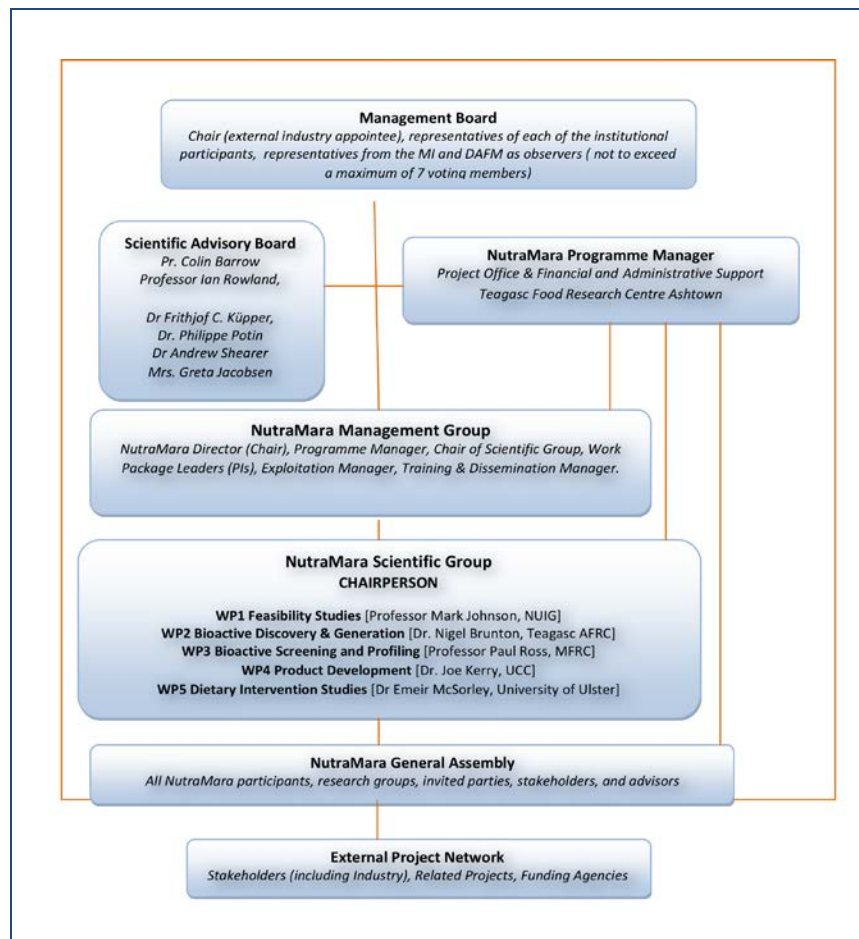


Figure 1 NutraMara management structure

1.8. Distribution of work

The NutraMara scientific work programme was developed within the consortium and built around expertise residing in Teagasc (Ashtown (A) and Moorepark (M)) Research Centres, National University of Ireland Galway, University of Limerick, University College Cork, University College Dublin and Ulster University. An overview of the role of partner institutions in each of the NutraMara work packages is given below along with insights to the nature of activity within each Work Package.

1.8.1. WPI - Marine Source Material

Work Package I	Marine source material
Lead partner	National University of Ireland Galway
Other participants	UCC, UL, UCD, UU, Teagasc (A) & (B)
Links to other WPs	WP 2, 3, 4, & 5

This Work Package was led by NUI Galway and included the sourcing, harvesting and provision of the raw materials necessary for the NutraMara research programme. Marine bioresource samples were harvested, cleaned and dried before being transferred to other NutraMara centres for extraction, characterisation and screening. In addition, research programmes in microalgae biomass production, and the production of macroalgal components through optimised cultivation and aquaculture, were completed.

1.8.1.1. Research Activities in WPI

1.8.1.1.1. The development of a Feasibility Study

This provided support for planning further research and informed the development of future, near-market or commercial projects. The Feasibility Study both summarised and critically evaluated information in the broad marine functional foods area, for example, in identifying new marine-sourced bioactives with potential for functional foods, best practices for sampling marine-origin materials, legal and regulatory issues, new product development strategies and identified research gaps with commercialisation potential.

1.8.1.1.2. Sampling of marine bioresources

In addition to the collection and identification of bioresource samples for distribution to partner institutions, facilities to store material and track data were created by the development of a repository for reference materials collected/studied and a database to track samples and extracts throughout the Programme. In conjunction with the sampling, a detailed assessment of natural variability (seasonal and spatial) in bioactive compound from marine bioresources (macroalgae, microalgae, shellfish) was undertaken.

1.8.1.1.3. Micro- and macroalgal biomass production

In an attempt to create processes to stimulate the production of bioactive compounds within macro- and microalgal species, two novel research areas involved the optimisation of microalgae cultivation to target the specific production and composition of microalgal bioactives, and the production of macroalgae compounds through optimized cultivation.

1.8.1.1.4. Aquaculture

Reflecting the potential of aquaculture in marine functional foods research as more than a source of biomass, was the development of feedstock based on seaweeds to increase the functional food value of farmed salmon.

1.8.2. WP2 – Bioactive discovery and generation

Work Package 2	Bioactive discovery and generation
○ Lead partner	○ Teagasc Ashtown
○ Other participants	○ UL, UCC
○ Links to other WPs	○ WP 3

Led by Teagasc Ashtown Food Research Centre, Work Package 2 sought to identify, extract and characterise targeted bioactive components. This was especially important in the context of drawing bioactives from marine bioresources, because in many cases, the precise chemical structures of bioactive molecules are not well defined.

1.8.2.1. Research Activities in WP2

1.8.2.1.1. The development of extraction methods

This involved the development and optimisation of technologies and methodologies for the efficient extraction, purification and chemical characterisation of bioactive compounds from marine bioresources; including seaweeds, fish and discarded processing materials.

1.8.2.2. Targeted compounds

The principal compounds are those displaying antioxidants properties, pigments polysaccharides, proteins, peptides, protein hydrolysates, amino acids and lipids, including fatty acids. Fractions and molecules displaying bioactive properties will be characterised using mass spectrometry and NMR.

1.8.3. WP3 - Bioactive screening and profiling

Work Package 3	Bioactive screening and profiling
○ Lead partner	○ Teagasc - Moorepark
○ Other participants	○ UCC, UL, UCD, UU
○ Links to other WPs	○ WP 4

Work Package 3 focused on the identification and characterisation of the bioactivities of marine origin compounds and fractions generated in Work Package 2. This work involved a

range of methodologies including *in vitro* screening assays, animal models systems, cell culture, transcriptomics, bioinformatics, computational biology and metabolomics to create a more detailed bioactive profile of candidate compounds and fractions.

These techniques are used to examine the components for bioactive potential across a range of defined biological activities. These include: anti-oxidant, anti-diabetic, anti-proliferative, anti-hypertensive, anti-inflammatory, anti-infective, anti-microbial as well as pre- and pro-biotic activities.

1.8.3.1. Research Activities

A wide range of research was undertaken in *Work Package 3* to identify extracts and compounds with prebiotic and antimicrobial activity. Marine lipids from fish and algae were screened for bioactivity and evaluated to assess their potential as anti-diabetic, anti-obesity and anti-inflammatory functional foods and ingredients. Bioactive fractions with anti-adipogenic and anti-inflammatory activities were assessed in *in vitro* and *in vivo* models. Separately, bioactive fractions with anti-cancer (cell proliferation and anti-genotoxic) activities were identified and steps taken to elucidate and understand the molecular mode of action of these bioactives. Similarly, the anti-oxidant activities of algal extracts were investigated and modes of action explored.

1.8.4. WP4 - Product development

○ Work Package 4	○ Product development
○ Lead partner	○ University College Cork
○ Other participants	○ Teagasc (A), Teagasc (M)
○ Links to other WPs	○ WP 3, 4, & 5

The focus of *Work Package 4* was the development and assessment of trial food products incorporating bioactive compounds generated in other work packages. Developing insights to the practicalities of using these compounds in the production of foods, coupled with knowledge about the consumption of foods containing marine origin compounds are required by the food sector.

1.8.4.1. Research Activities

In this UCC led work, the stability of the bioactive and bio-accessibility i.e. the degree of bioactivity available for absorption in the gut after digestion, along with the stability of extracts and purified marine-derived bioactives across a range of typical food-processing conditions was assessed. Additionally, quality and sensory analysis studies on dairy and meat products fortified

with seaweed extracts, and salmon fillets fed on a seaweed-based diet were carried out. New uses for macroalgal extracts as active packaging applications were also investigated, as were studies to assess food quality attributes such as shelf life.

1.8.5. WP5- Human intervention trials

○ Work Package 5	○ Human intervention trials
○ Lead partner	○ Ulster University
○ Other participants	○ UCC
○ Links to other WPs	○ WP 2

The health benefits of foods containing marine-derived ingredients or bioactives, which demonstrated bioactivity *in vitro* were assessed in pilot-scale human dietary intervention studies. These human dietary intervention studies involved the incorporation of test ingredients into a food matrix and the subsequent assessment of the food's effect *in vivo*.

1.8.5.1. Research Activities

The effect of consuming seaweed on markers of inflammation, cancer and anti-oxidant potential in humans and the effect of consuming pork derived from laminarin/fucoidan-fed pigs on markers of inflammation, immune function and anti-oxidant potential in humans formed the two main pilot intervention studies involving foods. A further study was undertaken to examine faecal water activity in individuals supplemented with extracts from species of laminaria.

2. FEASIBILITY STUDY

2.1. Introduction to the feasibility study

The Marine Functional Foods Research Initiative, later to become known as NutraMara, was the first research programme dedicated to exploring the potential of Ireland's marine biological resources as a source of functional ingredients. NutraMara's research challenge was to identify a range of bioactive ingredients from species inhabiting Ireland's marine territories and to assess their potential as materials that could be incorporated as functional ingredients in food products.

The feasibility study was intended to provide the knowledge required to support the research effort and inform the development of near-market or commercial projects. Whilst there were pockets of scientific expertise able to provide scientific direction to the programme, there was a need to develop a solid knowledge base as a foundation for the planned research work. At the outset, insights from related international research in the food and marine science areas were required to support and accelerate the work, and to minimise any duplication of research conducted elsewhere.

The programme faced many diverse challenges from its inception. To meet its overall research objectives, the NutraMara research team had to consider all elements of the ingredient supply chain, from harvesting or culture, to understand the various options for processing marine ingredients and the many ways of incorporating them into food products. Functional foods are designed to make positive contributions to a person's health, therefore an understanding of the role of marine origin ingredients in maintaining or improving health status, and of the range of diseases that such ingredients could affect, was required. In addition to highlighting the scientific and technical challenges, an understanding of many regulatory, legal, ethical, market and processing issues associated with utilising marine origin compounds, was required. The feasibility study was a collaborative effort involving researchers from the marine, health and food science areas. Such an approach provided the means to identify knowledge and research gaps that could be worked on by the research programme.

2.2. Market analysis of functional foods

2.2.1. *Market insights and situation analysis*

The market drivers for the growth of the functional foods category have a powerful influence more so than market inhibitors. There are many market drivers for functional foods including increased awareness among shoppers, aging population and requirements for short-term and long-term benefits. There are also another set of factors, from the perspective of consumers that are market inhibitors; these warrant consideration in developing functional food products

and include distrust, credibility and safety concerns. Table I provides a summary of these drivers.

An aging population seeks to maintain good health and prevent disease through the use of functional foods. High healthcare costs are also driving the use of functional foods. Consumers are using a more preventative approach to avoid ill health and subsequent health care costs. Convenience will also drive market success of functional foods; functional foods that are simple, convenient and easily integrated into everyday routines are product opportunities.

Table I Drivers and inhibitors of functional food development

Drivers		Inhibitors	
<i>Scientific progression</i>	Greater understanding of diet-disease link which impacts both industry and consumer	<i>Distrust</i>	Scepticism regarding health claims
<i>Aging Population</i>	Looking to off-set ill-health and facilitate healthy aging	<i>Food safety concerns</i>	Consumer aversion to artificial ingredients
<i>Self-medicating consumers and discerning consumer</i>	Looking for nutritional benefits to increase quality of life and health	<i>Time and money</i>	Functional foods development is expensive and time consuming as well as expensive for the consumer
<i>Rising health care costs</i>	Prevention better than cure. Seek out foods to improve own health status and prevent ill health and associated costs	<i>Taste</i>	Consumers think they are compromising on taste when purchasing these healthier products
<i>Time poverty & busy lifestyles</i>	Seek out convenient, healthy, quick alternatives	<i>Low awareness</i>	There is a low level of awareness of the health properties of certain ingredients
		<i>Lack of credibility</i>	Lack of belief in benefits claimed
		<i>Price sensitivity</i>	Consumer sensitivity to price due to falling incomes

In a survey of GB adults in 2008, it was found that 46 percent believed functional foods were overpriced and 35 percent expressed a lack of credibility in that they felt ‘they must do something, but not everything that is claimed’ (Mintel Group, 2008). The consumer is sceptical in general towards the claims made by functional foods and in the current economic climate maybe less willing to pay the higher price. This further cements the need to clearly communicate with the consumer the real benefits of the functional ingredient to ensure understanding, belief and ultimately purchase. The health claims legislation process that is currently underway in the EU although perceived as inhibitory by some industries, may actually help to alleviate some of the consumer scepticism.

The market value for functional foods in Europe, US and Japan is shown in Table 2. Growth has been good in these markets despite the recession, and it is predicted to continue to grow

at a compound annual rate of 5-7 percent. Although this is somewhat smaller than previous growth, it is an indication that the market is starting to somewhat mature and consumers are less willing to pay the price premium.

Table 2 International market value – functional food and drink (US\$ million)

Country/region	2007	2012	CAGR %
France	808	980	
Germany	1,983	2,525	
Italy	1,128	1,525	
Netherlands	286	346	
Spain	641	814	
Sweden	251	318	
UK	2,103	2,533	
Total Europe	8,477	10,667	4.7
Japan	16,377	21,808	5.9
USA	31,000	43,000	6.8

Source: (Heller, 2009)

The reported size and value of the market for functional foods will vary greatly and depend on the functional food definition used. The market for functional foods in Japan is highly important, which comes as no surprise. In the ten years from 1988 to 1999 more than 1700 functional foods were launched. This is the same period when FOSHU was introduced by the Japanese ministry, indicating that regulation had a beneficial effect. In the US, the functional foods market was reported to be approximately \$31 billion in 2008. A slowdown in the market has been noted which may reflect recessionary pressures on consumer spending. From 2003 to 2008 the compound growth rate was 8 percent and it is predicted that the US market will be worth approximately \$43 billion by 2013 (Heller, 2009). The European market share for functional foods is much lower than the US or Japan and is still below 1% of the EU food and drink market. Germany and the UK are the most important markets in Europe. Large differences in acceptance across the EU also exists where acceptance is higher in Northern Europe whereas the Mediterranean countries favour natural and freshness (Siro *et al.*, 2008). In terms of developing functional foods, current consumer priorities are weight loss and heart health as well as an aging population with requirement for foods designed for healthy aging. The functional foods industry should be aware that consumers have a natural dislike for too much science and are more predisposed to more natural ingredients. Given that 80 percent of functional foods fail within the first 18 months of product launches, it is important to learn from the mistakes of these failures. A report (Mellentin J, 2009) identified rules for success based on the failed products. Three of the top four rules revolve around the consumers. These three rules include:

- offer a relevant benefit and credible brand

- aim for a benefit the consumer can feel
- ingredient is not a point of difference because consumer acceptance of unfamiliar ingredients is a very slow process

It is imperative that the consumer is at the start, middle and end of the entire functional foods development process, with a clear and credible communication and marketing strategy.

2.3. Consumer attitudes

Generalisations about the typical consumer do not give information about what foods might appeal to different people, with knowledge and attitude often considered better predictors of functional food acceptance than variables such as age and gender. Some studies sought to understand the role demographics have in influencing consumer acceptance of functional foods (Verbeke, 2005); with younger consumers reported as more interested in the health benefits of foods rather than the disease reduction potential, whilst the opposite is true for some older consumers (Vassallo *et al.*, 2009).

A number of studies report cultural differences as factors in the acceptance or consumption of functional foods. French students were found to be more sceptical than North Americans with respect to information on functional foods (Kolodinsky *et al.*, 2008). French students also had a lower view of the benefits of functional foods and declared a lower intention to purchase in comparison to North Americans. Similar cultural effects were identified in different consumption levels of fortified margarines between Flemish-speaking compared to French-speaking military men (Mullie *et al.*, 2009). Saba *et al.*, (2010) found differences between Finland, Germany, the UK and Italy in the way in which health claims influence buying intentions. The message from this work being that some sort of health claim generally had a positive influence on northern European consumers, while Italians preferred foods without a health claim.

2.3.1. *The NutraMara focus group study*

An integral component of the NutraMara feasibility study was to develop insights to, and deepen the understanding of general consumer attitudes to functional foods and, in particular, to expand on the concept of marine-derived bioactive compounds in functional foods. Informed by insights from international studies, a focus group was the chosen approach, comprising seven different groups.

The composition of the groups, summarised in Table 3 below reflects key consumer segments with ranging demographics and at varying stages of life.

Table 3 Composition of focus groups

Group No	Age	Gender	Social Class	Children in home	Location	Consumer Segment
1	60-70	Female	ABCI	None	Dublin	Medically Conscious
2	60-70	Male	C1C2DE	None	Cork	Medically Conscious
3	40-55	Female	ABCI	Teens/ Young adults	Dublin	Family Nurturers
4	40-55	Female	BC1F	Teens/ Young adults	Sligo	Family Nurturers
5	30-40	Female	BC1C2	Primary/Secondary	Cork	Hectic Mums
6	30-40	Female	C2DE	Primary/Secondary	Athlone	Hectic Mums
7	18-24	Male	ABCI	Singles	Dublin	Fitness Fanatics

Key findings from the focus group study

A definition of functional foods was given at the group sessions and few participants had ever given much thought to this concept, prior to its prompting during the sessions.

Consumers were adept at identifying particular health benefits associated with consuming certain foods. Most notably, fish and seafood featured as one benefit identified – with heart health and weight management associated with the consumption of oily and white fish, respectively. Labelling emerged as a concern, though this was not felt to be a barrier to functional foods *per se*, rather the benefits of functional foods should be clearly labelled and specific ingredients detailed as clearly as possible. On balance, most consumers were perfectly comfortable with the concept of functional foods incorporating marine origin compounds.

The groups discussed seaweed, and chitin (derived from shellfish) marine-derived functional ingredients, in addition to the use of marine-derived ingredients in animal feed to naturally enhance muscle content of the meat. Attitude formation and acceptance of marine ingredients were a function of the health benefit, the source of ingredient, vocabulary used and complexity of use of the ingredient.

The concept of seaweed bread and/or yoghurts was perfectly acceptable to the vast majority of consumers as a potential marine-derived functional food. Some respondents identified seaweed as a proven source of healthy ingredients and an awareness of the use of dulse, carrageen and kelp for medicinal purposes in Ireland for generations.

The idea of feeding marine ingredients to animals to modify its inherent meat composition represented a step too far for many participants and evoked associations of Creutzfeldt–Jakob disease (CJD), although some consumers also realised that this was somehow different as illustrated in the following quote: “But the mad cow, that was cannibalism really with cows, that was dangerous, this is not though”.

The concept of extracting an ingredient from shell material was intriguing for many consumers. Whilst there was no familiarity with the term ‘chitin’, the scientific ring prompted some

respondents' interest regarding its precise health benefits. The key perceived consumer benefits related to the anti-obesity properties and identified it as important in the fight against childhood obesity, and for obesity in adults. Perceived need is important in acceptance; and clearly a societal need exists for such products in the minds of consumers. Similarly, consumers were positive regarding the potential heart-health benefits associated with chitin.

The use of chitin in preventing food spoilage as in "long life" was rejected by the mothers in the groups, where such bread was synonymous with being "high in preservatives". This finding demonstrates that acceptance of chitin is very much a function of the perceived need for the purported benefit. Consumers clearly identify a tangible need in terms of reducing obesity, but not necessarily a need to increase the shelf life of foods such as bread. Appetite suppression benefits are likely to meet a consumer need in some segments.

Countering the positive perceptions of marine functional foods is a series of potential barriers to acceptance. The greatest concern is trust or lack thereof in the acceptance and use of functional foods. Others include views that functional foods in general are likely to be over-priced, something of a food manufacturer 'gimmick' and not necessarily required when a balanced diet is consumed; potential allergic reactions to marine based ingredients (e.g. shellfish); and the source of marine origin ingredients.

Conclusions

- Marine products are universally accepted as ultimately healthy.
- Terminology around the use of functional food ingredients as 'additives' can suggest to the consumer the addition of a potentially unnatural/unhealthy substance to the carrier food, while the term 'functional' can actually suggest an uninspiring, bland foodstuff of no discernible health benefit.
- The source of marine resource ingredients will be as important to the consumer as their claimed health benefits.
- Price is not necessarily a barrier to purchase assuming, the price differential is no greater than circa 20% and consumers are convinced that they are likely to deliver upon the claimed health benefits.
- Older rural females, believed the secret to a healthy disposition lies in maintaining a balanced lifestyle, including a healthy diet.
- Few consumers are familiar with what micro algae, or indeed aquaculture actually refer to.
- The language used to communicate the likely benefits of marine and general functional foods to the consumer is absolutely vital, particularly in light of the lack of understanding as to the benefits of functional foods versus a balanced/wholesome diet.

2.4. Diet related health issues

The rate increase in global population over the past 50 years was greater than ever experienced before. From a level of 2.5 billion in 1950, the world population reached 6.5 billion in 2005. Estimates of projected increases point to a world population of more than 9

billion 2050 (United Nations, 2009). Ireland's population showed an increase of 8.1 percent between 2006 and 2011 to 5.58 million; with increases resulting from immigration and natural growth (Central Statistics Office, 2011).

Diet related health issues are increasing and affect populations in the developing and the developed world. The World Health Organisation report a consistent relationship existing between diet (an unhealthy diet) and the emergence of chronic non-infectious diseases (World Health Organisation, 2002). The risk of developing a chronic disease is not only confined to lifestyle and diet choices by adults; diet and nutrition from birth are also shown to affect health outcomes in later life (Institute of Public Health in Ireland, 2012). Coronary heart disease, cerebrovascular disease, various cancers, diabetes mellitus, dental caries, and various bone and joint diseases are shown to be related to diet (World Health Organisation, 1990). Many people living in Ireland develop chronic diseases related to poor diet, smoking, and alcohol abuse (Balanda *et al.*, 2010). Physical inactivity is also identified as a factor in the incidence of these diseases. Good health status is unevenly distributed across society; socio-economic status, levels of education, employment and housing are all linked to the prevalence of these chronic diseases (Balanda *et al.*, 2010).

A significant proportion of premature deaths in Ireland occur as a result of chronic diet related conditions. There is an increased prevalence of some of these conditions with advancing years, particularly hypertension, coronary heart disease, stroke and type-2 diabetes, and they tend to be more common amongst males and within lower socio- economic groups (Balanda *et al.*, 2010).

After cardiovascular disease, cancer is the second major cause of death in Ireland with an average of 30,000 new cases being reported each year; double the numbers reported during the 1990s. Other diet related diseases on the increase in Ireland are chronic obstructive pulmonary disease, hypertension, diabetes and coronary heart disease. Health authority estimates point to an increase of 40 percent in the number of adults with chronic diseases by 2020 (Institute of Public Health in Ireland, 2012; 2012a; 2012b).

Personal choices, psychological outlook, food availability and socio economic factors influence how much a person eats. Obesity is becoming a major public health concern in most OECD countries as the number of obese people continues to rise. If the present rate of increase continues, 2 out of 3 people in some countries will become obese by 2020 (Sassi, 2010).

Ireland is one of the OECD countries which is experiencing an increase in levels of obesity within its population. Obesity affects all levels of society and all age groups; in Ireland 61 percent of adults and 25 percent of children age 3 or under is classed as overweight or obese (Department of Health, 2013). The link between obesity and the onset of other chronic disease is well established, particularly the role of obesity in type 2 diabetes (Bray and Bellanger, 2006).

2.5. Chronic diet related disease

Dietary needs are known to change from birth to old age and unhealthy diets can contribute to ill health at all stages of life. Faced with aging populations and the negative health impact of some lifestyles, scope exists to change the way society relates to food, and to alter food preference in ways that reduce the incidence of diet related disease. There are strong indications of the existence of markets for foods that support dietary needs and help to promote good health.

The key findings of the World Health Organisation/FAO Expert Consultation on Diet, Nutrition and the Prevention of Chronic Diseases (World Health Organisation, 2002) identified the major diet related chronic diseases and gave insights to the relationship between diet, exercise and health. These major diet related diseases offer researchers involved in the development of marine functional ingredients targets for their work and include the following diseases.

- **Obesity:** This results from the imbalance between energy intake and the level of physical activity. Physical inactivity, coupled with a high energy diet where there is an excess of calories from sugar, starches or fat is the major factor in the epidemic of obesity
- **Diabetes:** Type 2 diabetes is a chronic metabolic disorder that affects the way the body metabolises glucose. It is linked to obesity and increases the risk of heart disease, kidney disease, stroke and infections.
- **Cardiovascular diseases:** These largely result from unbalanced diets and insufficient physical activity. Reducing the risk of the main forms of CVD – heart disease and stroke - can be achieved with diets that include sufficient amounts of (n-3 and n-6) polyunsaturated fats, fruits and vegetables; reducing salt intake and by taking steps to control weight.
- **Cancer:** Whilst smoking remains the main cause of cancer, dietary factors are found to contribute significantly to some types of cancer. Cancer risks, particularly cancers of oesophagus, colorectum, breast, endometrium and kidney, can be reduced by ensuring an adequate intake of fruit and vegetables and keeping a healthy weight.
- **Osteoporosis and bone fractures:** In some populations older people can suffer from osteoporosis, leading to brittle bones and increase risk of fractures. Ensuring an adequate consumption of calcium and vitamin D is known to help to reduce fracture risk.
- **Dental disease:** The erosion of teeth by dietary acids and sugars can be prevented by limiting the frequency and amount of consumption of sugars; dietary acids in beverages or other acidic foods may contribute to tooth destruction.

The human gut microflora is increasingly recognised as playing a central role in human health and disease (Tuohy *et al.*, 2003). From the standpoint of the host, these microflora can have both beneficial and detrimental outcomes, in terms of nutrition, infections, atopic disease, ulcerative colitis, xenobiotic metabolism, and cancer (Rowland and Gangolli, 1999). Research performed over the past 30 years provided new insights into the role of the human gut biota in chronic disease. Indeed, the number of diseases shown as being linked to changes in gut microbiota has grown. Much of this work focused on the possible link between gut microbiota

and chronic gastro-intestinal diseases such as irritable bowel syndrome. However, there is emerging evidence of possible links between gut microbiota and diabetes, obesity and the onset of colorectal cancer (Guinane and Cotter, 2013).

2.6. The impact of marine origin compounds on health

Marine biological resources have been exploited throughout history, with the focus largely on catching fish and collecting seaweeds for food. Some marine animals were traditionally caught to provide materials other than food. Whales and seals were once the source of oils and fats used in candle and soap making, as lubricants and as lamp oil; their bones were used in the garment industry and meat unsuitable for food, used as animal feed (Roth and Mer, 1997).

The gelling properties of some seaweeds were known in the 17th century in Japan, where agar was extracted as a thickening and gelling agent from species of red seaweed. During the 1930's extracts of brown seaweeds, containing both sodium alginate and potassium alginate, were produced commercially and sold for use in applications where their gelling and emulsifying properties were required; including for use in the manufacture of paper and textiles, drinks, paint and cosmetics (McHugh, 2003).

Research conducted over the past 30 years has brought attention to the use natural products extracted from marine animals and algae in human health. Though a large number of marine origin bioactive substances have been identified, it is only in relatively recent times that the first drugs based on compounds found in marine species were approved (Imhoff *et al.*, 2011). Largely resulting from successes as these, interest in exploiting marine species as a source of natural bioactive compounds has increased. There are now clear opportunities to use such compounds in functional foods, nutraceuticals, cosmetics and in bioprocessing applications. This section gives an overview of the potential contribution of marine derived natural products to helping to maintain human health. The scale of the NutraMara research programme was such that its work focused on the major diet related diseases as opposed to all other health challenges and life-threatening illnesses.

2.6.1. Cardiovascular disease

The Japanese population, in particular those people living on the island of Okinawa, reputedly enjoy the longest life expectancy in the world. This longevity, is strongly linked to their traditional dietary intake of fish, soya and seaweed, which is claimed to account for a low mortality rate from cardiovascular disease (CVD) and all cancers (Yamori *et al.*, 2006). Evidence from further studies in Japan and one involving people with a Japanese ancestry living in Brazil pointed to a seaweed rich diet as offering protection against CVD (Iso and Kubota, 2007) and reducing cholesterol levels (Yamori *et al.*, 2001).

Alginates extracted from brown seaweeds were shown to lower CVD risk factors and pose no toxic effect to humans (Hennequart, 2007). Clinical studies of alginate drug applications and

supplements have reported anti-obesity and anti-diabetic effects respectively (Torsdottir *et al.*, 1991).

The red seaweed *Chondrus crispus*, more generally known as Carrageen or Irish Moss, has a long standing role in historical medicine as a cure for coughs and the common cold (Morrissey *et al.*, 2001) and research using *in vitro* and animals models have suggested anti-coagulant and platelet aggregation inhibition properties of carrageen (Sen, 1994), albeit no well-designed dietary intervention study has yet been carried out to investigate these properties.

Fucoidan extracted from brown seaweeds including *Laminaria* sp. and *Ascophyllum* sp. have shown anti-coagulant properties conferring health promoting properties in the prevention of CVD (Berteau and Mulloy, 2003). Along with fucoidan, laminarin also isolated from *Laminaria* and *Ascophyllum* has excellent anti-oxidant (Xue *et al.*, 2004), anti-inflammatory (Ostergaard *et al.*, 2000) and anti-coagulant (Miao *et al.*, 1999) properties which make these two compounds excellent candidates as potential mediators in the prevention of heart disease.

Metabolic syndrome is the name for a group of risk factors that raises the risk of heart disease and other health problems, such as stroke and diabetes. Research in Korea involving 7,081 men aged 30 years and older has suggested a role for algae in the treatment and prevention of the metabolic syndrome (Shin *et al.*, 2009). A dietary intervention study carried out in Quito Ecuador, on individuals with at least one symptom of the metabolic syndrome, reported that intervention with 4-6g/d of the brown seaweed *Undaria pinnatifida* reduced blood pressure and waist circumference in comparison to the placebo group (Teas *et al.*, 2009).

Algal oils (from macro- and microalgae), similar to other marine derived oils, have both co-protective and therapeutic properties in CVD due to being good sources of the long chain polyunsaturated fatty acids (LC-PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Cohen *et al.*, 1995; Manerba *et al.*, 2010). Supplements and functional foods containing LC-PUFAs extracted from fish are in widespread use and recognised as a highly successful contribution to human nutrition. The health benefits associated with the consumption of long-chain n-3 PUFA are many, and include roles in the treatment of CVD, hypertension, diabetes, some cancers and arthritis (Barrow and Shahidi, 2008).

The freshwater blue-green microalgae *Spirulina platensis*, is a rich source of the anti-oxidants phycocyanin and carotenoids, which are reported to have benefits in lowering cholesterol in animal trials (Cheong *et al.*, 2010), (Nagaoka *et al.*, 2005), (Riss *et al.*, 2007) and in inhibiting oxidative stress and apoptosis in cardiac muscle cells (Khan *et al.*, 2006).

Astaxanthin, a natural carotenoid present in the microalgae *Haematococcus pluvialis* and other species has an antioxidant power that is 3 times that of vitamin E or beta-carotene (Kurashige *et al.*, 1990). Following the demonstration of this effect in animal trials, the anti-oxidative effect of astaxanthin together with its role in immune modulation and as an anti-inflammatory bioactive was demonstrated in a small study on healthy females (n=14) supplemented with 2g/d (*Haematococcus pluvialis*) for 8 weeks (Park *et al.*, 2010). This study provides the first human

data to suggest that astaxanthin may be a bioactive natural carotenoid important to human health.

2.6.2. *Weight management*

For overweight and obese individuals the best approach to reduce these risks is through negative energy balance, where energy expenditure is greater than energy intake. For most individuals this will involve dietary modification. Several studies have shown that an increase in dietary fibre can aid weight loss through several suggested mechanisms, including a prolonged gastric emptying rate and enhanced satiety from increased fibre-induced gastric stretch, leading to a reduction of food intake and eventually weight loss. However, it has also been suggested that different types of fibre may play a role in improving blood lipid profiles and decreasing the absorption of glucose leading to weight loss.

Alginate is a source of fibre obtained from various species of brown algae. An intervention study involving a total of 68 individuals with a BMI range from 18.5 to 32.81 kg/m², but otherwise healthy, reported that daily consumption of a strong-gelling sodium alginate drink before either breakfast or dinner for 7 days resulted in a significant ($p = 0.019$) reduction of 7% in daily energy intake compared to a fibre-rich control drink. Although the authors suggest that this reduction may be meaningful in terms of weight loss, the study was too short to measure changes in weight after treatment (Paxman *et al.*, 2008a). Further, a study by the same group demonstrated a significant ($p = 0.026$) positive correlation between percentage body fat and area under the curve cholesterolemia after a nutritionally calculated meal, indicating subjects with increasing body fat had a greater increase in cholesterol uptake, which was attenuated after alginate consumption pre-prandial (Paxman *et al.*, 2008b).

An association between alginate and decreased postprandial blood glucose levels is supported by two separate studies in healthy volunteers with BMI of 20 – 30 kg/m², one using a guar gum/alginate crispy bar ($n = 48$) (Williams *et al.*, 2004) and the other an alginate supplemented beverage ($n = 30$) (Wolf *et al.*, 2002). The alginate supplemented drink reduced glucose uptake significantly at 60 minutes postprandial, and similarly the crispy bar was shown to significantly reduce blood glucose levels at 15, 30, 45 and 120 minutes postprandial. In both cases blood glucose level were diminished in response to alginate supplementation.

Collectively these studies have investigated the short-term effects of alginate supplementation on energy intake, glucose and lipid blood profiles and various measurements of satiety in a range of normal weight to obese, but otherwise healthy individuals. Each study has suggested that alginate may serve as an aid to weight loss, however, in order to investigate this a longer term human intervention is required to determine weight loss and changes in blood profiles over the study period, and only then can major conclusions about alginate and weight management be drawn.

2.6.3. Diabetes

Alginates from seaweed have demonstrated effects that may assist weight loss. A short study by (Torsdottir *et al.*, 1991), in seven diabetic men who consumed a one-off alginate supplemented drink reported significantly reduced postprandial blood glucose levels ($p < 0.02$), serum insulin ($p < 0.02$) and plasma C-peptide ($p < 0.05$) of 31 %, 42 % and 35 % respectively, compared to a control drink (Torsdottir *et al.*, 1991). The study also demonstrated slower gastric emptying after alginate supplementation, which may have contributed to the decreased absorption rate of glucose. Other studies of seaweed supplementation on diabetic individuals used a food frequency questionnaire (FFQ) to assess efficacy on weight management, glucose response and lipid profile. A Korean cross-sectional observational study used a FFQ and measured parameters including, fasting bloods, BMI, lipid profile and blood pressure for 3405 individuals (Lee *et al.*, 2010). The findings indicated that there was a marginally significant inverse relationship between seaweed consumption and diabetes incidence in males. However, the authors pointed out the cross-sectional nature of the study limited the ability to make a causal relationship between seaweed consumption, since the methodology of the FFQ did not account for variety of species, quantity nor preparation method, or diabetes risk.

Agar is a polysaccharide gel that can be extracted from various species of red seaweed. A 12 week intervention study in individuals ($n = 76$) with diabetes or impaired glucose tolerance on an agar supplemented conventional diet and moderate exercise (three times a week) resulted in significant reductions in body weight ($p = 0.008$), BMI ($p = 0.009$) and total cholesterol ($p = 0.036$) compared to the conventional diet and moderate exercise group alone (Maeda *et al.*, 2005). A further seaweed supplemented and nutrient controlled diet with dried *Saccharina japonica* and *Undaria pinnatifida* pills containing 1:1 of each species in diabetic individuals ($n = 20$) with BMI < 35 kg/m² significantly altered the blood lipid profile and decreased fasting and postprandial blood glucose levels (Kim *et al.*, 2008). The reduction in glucose absorption, which may be a result of increased fibre in the control group (2.5 times higher than the control group), appeared to be a lasting effect with reduced fasting blood glucose levels as well as postprandial levels.

2.6.4. Gut health

In health terms, the gut microflora establishes an efficient barrier to the invasion and colonisation of the gut by pathogenic bacteria, and produces a range of metabolites that are utilised by the host (e.g. vitamins and short chain fatty acids). Although little is known about the individual species of bacteria responsible for these beneficial activities it is generally accepted that the bifidobacteria and lactobacilli constitute important components of the beneficial gut microflora. Probiotics and prebiotics have been developed as management tools and refined to stimulate numbers and/or activities of the bifidobacteria and lactobacilli within the gut microflora. Prebiotic polysaccharides may mediate anticancer activity, reduce cholesterol, regulate glucose absorption and promote immunomodulatory effects and enhance

mineral absorption, however many of these health benefits remain to be substantiated fully (Hoyles and Vulevic, 2008).

There is evidence to show that some seaweed derived fibre can have positive effects on gut health (Vaugelade *et al.*, 2000, Deville *et al.*, 2004) and a number of studies have looked for potential prebiotic activity (Deville *et al.*, 2007). Little is known about the chemical, physicochemical and fermentation characteristics of seaweed fibre in the human gut or the individual species of bacteria that are responsible for these beneficial activities, but seaweed carbohydrates do appear to have chemical, physicochemical and fermentation characteristics that differ from higher plant carbohydrates (Deville *et al.*, 2007).

Marine sources are emerging as novel sources of prebiotic carbohydrates, which may alter gut health directly or indirectly. For example alginates, laminarin, agar, and structural components of macroalgae are highlighted as possible prebiotic candidates (O'Sullivan *et al.*, 2010) with potential for inclusion in human dietary intervention studies to investigate their efficacy. Marine compounds may offer benefits to existing probiotic research as possible delivery systems. Alginate, xanthan gum, and carrageenan gum have been shown to increase probiotic survival, however alginate based encapsulation would appear to be the option of choice; a combination of alginate with chitosan is also identified as an effective option (Islam *et al.*, 2010; Chávarri *et al.*, 2010).

2.6.5. Bone health

Osteoporosis is a degenerative disease that progresses over long periods of time and despite available therapeutic options for sufferer's (many with unwanted side effects), the key to approaching this disease is through preventative measures. Calcium and vitamin D intake have an established role to play in bone mineral density (BMD) (Tang *et al.*, 2007). An optimal bone development in infants and growing children can be strongly influenced by dietary intake. Calcium and vitamin D are the two most important micronutrients for bone health; however other dietary factors such as non-digestible prebiotic carbohydrates are emerging as possible contributors, for their ability to enhance calcium absorption and bioavailability (Cashman, 2007; Scholz-Ahrens *et al.*, 2007). Marine sources rich in minerals (in particular calcium) could be targeted for a natural product on the osteoporosis market. Seaweeds can be rich sources of several nutrients that impact positively on bone health (McArtain *et al.*, 2007). Nondigestible oligosaccharides from marine sources may present as innovative candidates for the enhancement of calcium absorption.

A marine product that has progressed to human intervention trials is active absorbable algal calcium (AAA Ca). AAA Ca is obtained from oyster shell powder heated to a high temperature (800°C), with an additional heated brown seaweed (*Cystophyllum fusiforme*) component. Fujix Corporation have carried out several interventions to investigate the influence of AAA Ca compared to AA Ca (same as AAA Ca but no heated algae component), CaCO₃ and other preparations that have been used in Japan to increase BMD and reduce the

risk of osteoporosis. AAA Ca has been shown to be more effective than AA Ca (heated oyster powder), and CaCO₃, indicating that the heated seaweed component has an influential role to play in the bioavailability of oyster shell derived minerals.

The calcified red alga *Lithothamnion calcareum* is rich in calcium carbonate. An Irish company Marigot Ltd incorporate this material in a mineralised supplement known as Aquamin®. Containing up to 34% calcium and other important nutrients including magnesium (2.4%), Aquamin® is targeted at the alleviation of the symptoms of osteoarthritis. Results from randomised, double blind, parallel placebo controlled clinical trial showed Aquamin®, and Aquamin® combined with glucosamine sulphate, each contributed to improved walking performance in subjects with osteoarthritis (Frestedt *et al.*, 2009). There are also reports that Marigot Ltd is investigating the potential role of Aquamin® alone and in the presence of short chain fructooligosaccharide on the bone mineral density of post-menopausal women (normal and osteopenic only) in a randomised double blind parallel intervention trial over a 2 year period (<http://www.controlled-trials.com/ISRCTN63118444>).

Marinova is an Australian firm that is focused on using fucoidans, sulphated polysaccharides derived from brown macroalgae for health applications. In a small scale trial (n=12), dietary supplementation with extracts from several species of brown seaweeds *Fucus vesiculosus* (85% w/w), *Macrocystis pyrifera* (10% w/w) and *Saccharina japonica* (5% w/w) plus vitamin B6, zinc and manganese, reduced osteoarthritic symptoms and showed an apparent dose dependent effect (Myers *et al.*, 2010).

Altered thyroid production has been associated with an increased risk of osteoporosis. Several studies have shown that high consumption of iodine containing seaweeds can interfere with normal thyroid function. Several studies have described hyperthyroidism in individuals consuming iodine rich kelp supplements (Shilo and Hirsch, 1986; Hartman, 1990; de Smet *et al.*, 1990; Eliason, 1998; Henzen *et al.*, 1999; Müssig *et al.*, 2006). This evidence would indicate the possible negative impact of iodine in seaweed products on thyroid health.

2.6.6. Cancer

There is a strong view that dietary seaweed consumption confers protection against cancer (Teas, 1981), a theory largely based on observations that cancer incidence is much lower among populations that consume a seaweed-rich diet, such as in Asia, in comparison to those who consume a Western style diet (Ferlay *et al.*, 2010). In support of this theory a wealth of studies have demonstrated clear anti-cancer properties of seaweed and seaweed components using *in vitro* and *in vivo* models. However, despite this, very few studies have specifically investigated the effect of seaweed consumption on cancer in humans.

A case-control study that included 362 Korean women with histologically confirmed breast cancer and controls matched according to age and menopausal status (Yang *et al.*, 2010), reported that intake of gim, a Korean style edible seaweed in the genus *Porphyra*, was inversely associated with breast cancer risk in premenopausal women (OR 0.44, 95% CI: 0.24-

0.80, $p=0.007$, 5th v. 1st quintile). Similar effects were observed in postmenopausal women, though they did not reach statistical significance possibly due to the smaller number of postmenopausal (35%) versus premenopausal women included in the study.

Previous *in vitro* and *in vivo* studies have attributed the anti-cancer effects of *Porphyra* sp. to the polysaccharide porphyran (Kwon *et al.*, 2006), protein (Hwang *et al.*, 2008), total polyphenol contents, carotenoids and chlorophyll (Okai *et al.*, 1996). The anti-cancer properties of gim may also be due to the iodine content of this seaweed which provides a major source of iodine in the Korean diet (Yang, 2010). Miyeok (*Undaria pinnatifida*; “wakame”) consumption was not significantly associated with breast cancer in Yang’s study in contrast to a previous report that miyeok suppressed mammary tumour growth in rats (Funahashi *et al.*, 1999).

Iodine-rich seaweed has long been used as a breast cancer treatment in traditional eastern Asian medicine to “soften” tumours and “reduce” nodulation (cited in Cann *et al.*, 2000 and Aceves *et al.*, 2005). In addition to Korean diets, seaweed consumption is also a major source of iodine in the Japanese diet with the iodine content of the most commonly consumed seaweeds, *Porphyra* (nori), *Undaria* (wakame) and *Laminaria* (kombu), ranging from 80 – 2500 mg/g (Cann *et al.*, 2000) and being present in several chemical forms (i.e. I-, I₂ and IO₃-) (Aceves *et al.*, 2005; Nitschke & Stengel 2014, 2016). The Japanese consume >12 mg of iodine per day (Miller, 2002), a much greater amount compared to the quantities consumed in the west e.g. 166 mg/day in the UK (Lee *et al.*, 1994) and 240 mg/day in the US (Miller, 2002).

Iodine intake is thought to be protective against breast cancer (Cann *et al.*, 2000), though to date, few epidemiological studies have investigated this association. Serra and colleagues (1988) reported that low iodine intake was associated with an increased risk of breast cancer mortality in a correlation study conducted in northeast Spain.

Clinical trials with iodine supplementation have been shown to significantly reduce the symptoms of fibrocystic breast disease in up to 70% of patients (Ghent *et al.*, 1993; Flechas, 2005). High-grade fibrocystic breast disease is considered a precursor to ductal carcinoma (Cann *et al.*, 2000).

In the study of Ghent and colleagues (1993), sodium iodide was associated with a high rate of side effects whereas molecular iodine, which is nonthyrotropic, was the most beneficial. Seaweeds and iodine supplements contain oxidized iodine, the form believed to be responsible for iodine’s tumour suppressive effects and it has been proposed that I₂ supplementation should be trialled as a breast cancer treatment (Aceves *et al.*, 2005). Other seaweeds which may offer protection against breast cancer development via their effects on oestrogen metabolism include *Alaria esculenta* (Teas *et al.*, 2009) and *Fucus vesiculosus* (Skibola, 2004).

In the Japan Collaborative Cohort Study for Evaluation of Cancer (JACC), seaweed intake was associated with lower mortality from lung cancer for men and women and for pancreatic cancer for men (Iso and Kubota, 2007). Seaweed consumption was also reported as protective

against stomach cancer (Hoshiyama and Sasaba, 1992) and *Bryopsis* (a green seaweed) was patented for use in the treatment of human lung cancer (Scheuer *et al.*, 2000).

The blue-green microalga *Spirulina* demonstrated chemopreventive effects in an oral cancer trial involving subjects with oral leukoplakia (Mathew *et al.*, 1995).

Despite the positive findings concerning the impact of extracts from various seaweeds on cancers, adverse findings are also reported. No significant associations were found between seaweed consumption and prostate cancer in the JACC study (Allen *et al.*, 2004) and the consumption of seaweeds (nori, kobo and other seaweeds) was associated with an increased risk of prostate cancer (RR 1.74, CI: 1.05-2.90, highest v. lowest tertile, $p=0.017$) (Sevserson *et al.*, 1989).

A wealth of literature highlights the anti-cancer properties of algal origin bioactives using *in vitro* and *in vivo* cancer models. However, there remains a lack of epidemiological studies that specifically investigated the effects of micro- and macroalgae and their components on cancer in humans.

2.6.7. Viral infections

The rise in resistant viral strains and the need for less aggressive anti-viral therapies indicates a role for alternative therapeutic routes such as that demonstrated by algae which has a low toxicity to the host (Cooper, 2002; Wang *et al.*, 2008; Ramjee *et al.*, 2010).

Sulphated polysaccharides extracted from various seaweeds appear to have antiviral properties. Both *in vitro* and *in vivo* animal research identified carrageenans, fuciodans and sulphated thamnogalactans, to have substantial antiviral activity against enveloped viruses such as herpes and HIV. Fucoidan, was shown to inhibit the growth of a variety of viruses (Soeda *et al.*, 1994; Aisa *et al.*, 2005; Trincherro *et al.*, 2009). The Australia marine bioactives company Marinova describe their Maritech® product as having potential anti-viral properties against the H1N1 swine flu virus. Carrageenan has also shown antiviral activity against Herpes simplex virus type 1 and type 2, HIV-1 and human rhinovirus (HRV) (Grassauer *et al.*, 2008) and has been studied as a potential vaginal microbiocide (Zeitlin and Whaley, 2002; Spieler, 2002).

The antiviral potential of a sulfated polysaccharide, galactofucan (GFS), extracted from Tasmanian *Undaria pinnatifida* was investigated in a small study (n=21) of patients with active and latent Herpetic infections (HSV-1, 2, EBV and Zoster). Ingestion of GFS was associated with lessening and disappearance of infections in those with active infections, and those with latent infections remained infection free whilst taking the supplement (Cooper *et al.* 2002).

2.7. International health studies

A summary of previous international studies, conducted to identify associations between marine bioactives on human health outcomes is included in Appendix I. The range of studies, some of which were conducted over 25 years, whilst others were considerably shorter (< 1

week) included research to identify links between marine bioactives and CVD, weight management, diabetes, bone health, cancer and viral infections.

2.8. Regulatory systems for functional foods and ingredients

The concept of functional foods originated in Japan in the 1980s and Japan remains the only nation to have legally defined functional foods. An approval system, enshrined within Japanese law was designed to help promote the manufacture of foods with the potential to address diet related health concerns, such as inadequate fibre and calcium consumption. Japans approval system for functional foods was designed to curb misleading claims. To obtain approval for a functional food product in Japan requires manufacturers to provide evidence which demonstrates: the effectiveness on the human body is clearly proven; the absence of any safety issues associated with the food; the use of nutritionally appropriate ingredients; compatibility with the product specification and the operation of quality control methods.

The European approach to functional foods differs greatly from the Japanese model, and whilst functional food is a rapidly emerging food category, it remains a “virtual category” in terms of food law, with no legal definition of a “functional food” in Irish or European law. Instead, functional foods are regulated through existing food legislation.

2.9. National and EU legislation

Manufactures and others involved in developing functional foods or ingredients may have to comply with national and European regulations before marketing the product. European regulations relevant to marine functional ingredients and foods date back to 1997 and the introduction of the Novel Food Regulation (EC No. 258/97). This regulation defines novel food as “foods and food ingredients which have not hitherto been used for human consumption to a significant degree within the Community”. This includes new or intentionally modified molecular structures, compounds isolated from microorganisms, plants, fungi or algae and foods that result from any novel processing or treatment that significantly alters their composition.

A food or ingredient with a significant history of consumption in any Member State prior to 15th May 1997, does not fall within the scope of the regulation. However, even though there may be a history of use, there remains a responsibility to comply with novel food regulations. This requires a food or ingredient to be authorised through one of two routes, a full novel food application procedure or submission under substantial equivalence as outlined – otherwise known as the “simplified procedure – notification”. Approvals will only be granted for novel foods if they do not present a risk to public health, are not nutritionally disadvantageous, and do not mislead the consumer. The submission routes for both procedures are outlined below.

The full **novel food procedure** requires the applicant to prepare and submit a dossier of scientific safety data on the product to a Member State competent authority in the country where the food will be first marketed. In Ireland, the competent authority is the Food Safety Authority of Ireland. On receipt by the competent authority, the company submits a summary of the dossier to the European Commission. The competent authority undertakes an initial assessment of the dossier and forwards this, together with the summary dossier, to the European Commission for distribution amongst member states for comment and review. Where the initial assessment is favourable and no objections are received from Member States the product may be marketed. The applicant receives a formal authorisation decision that defines the scope of the authorization, specifies how the product may be used, defines the product as a food or ingredient, its specification and any labelling requirements.

The **simplified procedure** is also known as the “substantially equivalent process”. The applicant can follow this procedure if the new product or ingredient is substantially equivalent to a similar product already on the EU market. In this case, the applicant is required to submit supporting scientific evidence directly to the European Commission or seek the opinion of a competent authority in a Member State. The onus is on the applicant to provide scientific evidence that the products are substantially equivalent with respect to composition, nutritional value, metabolism, intended use and the level of undesirable substances contained therein. Equivalence is determined on the basis of generally recognised and available scientific evidence acceptable to the Commission or by the opinion of a Member State competent authority.

2.10. Other food related legislation/regulations relevant to marine ingredients

Food is defined in Article 2 of Regulation (EC) No. 178/2002 as any substance or product, whether processed or not, that is ingested by humans. This definition of food includes beverages and water incorporated into food. Even though medicines may be ingested, they are not included in the definition and are covered by specific medicines legislation.

Ingredients obtained from the marine for consumption by humans, except when formulated in medicines, are foods. Food and food ingredients fall within categories defined by criteria such as: source, the method of pre-harvest production, history of use, intended use, or production/processing treatment. Food supplements, additives and foodstuffs intended for particular nutritional uses (PARNUTS) each fall within the intended use category of a food/ingredient and as such are relevant when considering applications for marine origin ingredients.

2.10.1. Food supplements

Legislation covering food supplements require that the supplements for sale in European markets comply with Directive 2002/46/EC, and in Ireland that they comply with Statutory

Instrument S.I. No. 506 of 2007. These regulations define food supplements as “foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities”.

Only specified vitamins and minerals, as listed in the legislation can be used in food supplements; the maximum limits of which require to be based on scientific risk assessment and data on vitamin and minerals intake from other foods, while also taking due account of what is considered an adequate vitamin and mineral intake for an average person.

Irish legislation requires food supplements are manufactured in accordance with S.I. No. 506 of 2007 and that persons placing a food supplement on the Irish market notify the Food Safety Authority of Ireland that they have done so. Food supplements are limited to vitamins and minerals listed in the annex of the relevant legislation.

2.10.2. Foodstuffs intended for particular nutritional uses (PARNUTS)

Directive 2009/39/EC and Commission Regulation (EC) No 953/2009 concerned foodstuffs for particular nutritional uses and the substances which may be added to meet a specific nutritional requirement of the consumer. Foodstuffs for particular nutritional uses are more generally referred to as PARNUTS. This category of food and the regulations surrounding it changed in July 2016 when the legislation was replaced by Regulation (EU) No 609/2013 of 12 June 2013, which establishes regulations for food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control.

The directive 2009/39/EC and associated regulations define PARNUTS as foodstuffs which, “owing to their special composition or manufacturing process, are clearly distinguishable from foodstuffs for normal consumption, which are suitable for their claimed nutritional purposes and which are marketed in such a way as to indicate such suitability.”

Products as these are intended to meet specific nutritional requirements for persons with disturbed digestive processes or metabolism; have a special physiological condition and so able to benefit from controlled consumption of certain substances; and infants or young children in good health.

The new regulations, are far more specific with regard to the category of foodstuffs, by establishing compositional and information requirements for infant formula and follow-on formula; processed cereal-based food and baby food; food for special medical purposes; and total diet replacement for weight control. Only defined substances identified in the regulation and falling within specific categories of vitamins; minerals; amino acids; carnitine and taurine; nucleotides; choline and inositol may be used in these products.

2.10.3. Food additives

The use of food additives in foods in the European Union is harmonised by Regulation (EC) No 1333/2008; only food additives meeting this regulation can be used and the EU is responsible for approval of all additives.

Regulation 1333/2008 defines a food additive as “any substance not normally consumed as a food in itself and not normally used as a characteristic ingredient of food, whether or not it has nutritive value, the intentional addition of which to food for a technological purpose in the manufacture, processing, preparation, treatment, packaging, transport or storage of such food results, or may be reasonably expected to result, in it or its by-products becoming directly or indirectly a component of such foods”. The food additives regulation identifies categories of food additives and lists the name of the additive and its associated “E” number.

The regulation (No 1333/2008) requires that additives must be safe when used; used to meet a technological need; must not mislead the consumer and must be of benefit to the consumer.

2.10.4. Product claims associated with marine ingredients

Health and nutrition claims attached to food are subject to regulation within the EU by Regulation 1924/2006 which applies to “all nutrition and health claims made in commercial communications, including, *inter alia*, generic advertising of food and promotional campaigns, such as those supported in whole or in part by public authorities.” In addition, the Regulation applies “to trademarks and other brand names which may be construed as nutrition or health claims.”

It is not uncommon to encounter a food product which is described as offering some form of health or nutritional benefit to the consumer. The inclusion or inference of such an effect is a “claim” and as such can only be attached to a food product following a rigorous scientific evaluation by the European Food Standards Authority and “shall be based on and substantiated by generally accepted scientific data”. The only allowed claims on food products are **nutrition** claims and **health** claims. A nutrition claim states or suggests that a food has beneficial nutritional properties, such as “low fat”, “no added sugar” and “high in fibre”. A health claim is any statement on labels, advertising or other marketing products that health benefits can result from consuming a given food, for instance that a food can help reinforce the body’s natural defences or enhance learning ability.

The generalised characteristics of a successful claim, include being able to substantiate a clinically relevant effect at concentrations likely to be consumed; the absence of any adverse effect, and the existence of a dose-response relationship and a complete understanding of the mechanism of action. Typically, providing these data require drawing from the results of human trials. Only nutrition claims which are listed in the annex of Regulation No 1924/2006 and amendments are permitted. Examples of permitted nutrition claims included in the Regulation and potentially relevant to marine origin compounds are listed in Table 4.

Table 4 Examples of Permitted Nutritional Claims

Permitted claim	Description of claim
Source of fibre	A claim that a food is a source of fibre, and any claim likely to have the same meaning for the consumer, may only be made where the product contains at least 3 g of fibre per 100 g or at least 1.5 g of fibre per 100 kcal.
High fibre	A claim that a food is high in fibre, and any claim likely to have the same meaning for the consumer, may only be made where the product contains at least 6 g of fibre per 100 g or at least 3 g of fibre per 100 kcal.
Source of protein	A claim that a food is a source of protein, and any claim likely to have the same meaning for the consumer, may only be made where at least 12% of the energy value of the food is provided by protein.
High protein	A claim that a food is high in protein, and any claim likely to have the same meaning for the consumer, may only be made where at least 20% of the energy value of the food is provided by protein.
Source of omega-3 fatty acids	A claim that a food is a source of omega-3 fatty acids, and any claim likely to have the same meaning for the consumer, may only be made where the product contains at least 0,3 g alpha-linolenic acid per 100g and per 100kcal, or at least 40mg of the sum of eicosapentaenoic acid and docosahexaenoic acid per 100g and per 100kcal.
High omega-3 fatty acid	A claim that a food is high in omega-3 fatty acids, and any claim likely to have the same meaning for the consumer, may only be made where the product contains at least 0,6 g alpha-linolenic acid per 100 g and per 100 kcal, or at least 80 mg of the sum of eicosapentaenoic acid and docosahexaenoic acid per 100 g and per 100 kcal.
Source of [name of vitamin/s] and/or [name of mineral/s]	A claim that a food is a source of vitamins and/or minerals, and any claim likely to have the same meaning for the consumer, may only be made where the product contains at least a significant amount as defined in the Annex to Directive 90/496/EEC or an amount provided for by derogations granted according to Article 6 of Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods.
High [name of vitamin/s] and/or [name of mineral/s]	A claim that a food is high in vitamins and/or minerals, and any claim likely to have the same meaning for the consumer, may only be made where the product contains at least twice the value of 'source of [NAME OF VITAMIN/S] and/or [NAME OF MINERAL/S]'.
Contains [name of the nutrient or other substance]	A claim that a food contains a nutrient or another substance, for which specific conditions are not laid down in this Regulation, or any claim likely to have the same meaning for the consumer, may only be made where the product complies with all the applicable provisions of this Regulation, and in particular Article 5. For vitamins and minerals the conditions of the claim 'source of' shall apply.
Increased [name of the nutrient]	A claim stating that the content in one or more nutrients, other than vitamins and minerals, has been increased, and any claim likely to have the same meaning for the consumer, may only be made where the product meets the conditions for the claim 'source of' and the increase in content is at least 30% compared to a similar product.

SOURCE: Regulation (EC) No 1924/2006 on nutrition and health claims made on foods

Examples of approved health claims in Regulation 1924/2006 where there may be a connection to a marine origin compound are given in Table 5.

Table 5 Examples of approved health claims

Claim type	Nutrient/substance,	Claim
Art.14(1)(b)	Iodine	Iodine contributes to the normal growth of children
Art.14(1)(b)	Calcium and vitamin D	Calcium and vitamin D are needed for normal growth and development of bone in children
Art.14(1)(b)	Calcium	Calcium is needed for normal growth and development of bone in children.
Art.14(1)(b)	Docosahexaenoic acid (DHA)	Docosahexaenoic acid (DHA) intake contributes to the normal visual development of infants up to 12 months of age
Art.14(1)(b)	Docosahexaenoic acid (DHA)	Docosahexaenoic acid (DHA) maternal intake contributes to the normal brain development of the foetus and breastfed infants
Art.14(1)(b)	Docosahexaenoic acid (DHA)	Docosahexaenoic acid (DHA) maternal intake contributes to the normal development of the eye of the foetus and breastfed infants
Art.14(1)(b)	Protein	Protein is needed for normal growth and development of bone in children.
Art.13(5)	Carbohydrates	Carbohydrates contribute to the recovery of normal muscle function (contraction) after highly intensive and/or long-lasting physical exercise leading to muscle fatigue and the depletion of glycogen stores in skeletal muscle
Art.13(1)	Vitamin D	Vitamin D contributes to the maintenance of normal bones
Art.13(1)	Protein	Protein contributes to the maintenance of muscle mass
Art.13(1)	Iodine	Iodine contributes to the normal production of thyroid hormones and normal thyroid function
Art.13(1)	Docosahexaenoic acid (DHA)	DHA contributes to the maintenance of normal blood triglyceride levels
Art.13(1)	Docosahexaenoic acid and Eicosapentaenoic acid (DHA/EPA)	DHA and EPA contribute to the maintenance of normal blood pressure
Art.13(1)	Chitosan	Chitosan contributes to the maintenance of normal blood cholesterol levels
Art.13(1)	Calcium	Calcium is needed for the maintenance of normal bones
Art.13(1)	Carbohydrates	Carbohydrates contribute to the maintenance of normal brain function

Source: EU Register on nutrition and health claims (<http://ec.europa.eu/nuhclaims/?event=search>)

2.1.1. Regulation in the use of marine materials

The NutraMara research objectives included targeting marine materials from known and available sources and assessing their potential as a source of functional ingredients. Initial research identified wild and cultured species of fish and algae, in addition to materials obtained from fish processing streams as prime source from which to extract these ingredients. Prospecting for marine species on the high seas was not within the scope of the research programme.

Different national and European legislation and regulations exist concerning the collection and use of these materials. Of particular relevance to the NutraMara programme was the extent to which these regulatory systems were barriers to the collection or harvesting of wild or culture species, or limited the use of marine materials as functional ingredients.

2.12. Collection of materials from the shore

The removal of any material or development activity on the shore is subject to conditions within the Irish Foreshore Act (1933). This act defines the foreshore as “the bed and shore, below the line of high water of ordinary or medium tides, of the sea and every tidal river and tidal estuary and of every channel, creek and bay of the sea or of any such river or estuary and extends outwards to 12 nautical miles (approx. 22.24 km), the seaward limit of Ireland’s territorial seas.” Under the Act, the relevant minister may issue licences to individuals or companies seeking to use the resources of the foreshore. Of relevance to NutraMara is that the majority of Ireland’s seaweed stock is on the foreshore, hence persons seeking to harvest seaweed may be required to apply for permission before collecting materials. All applications for licences should be made to the Department of Environment, Community and Local Government. Establishing an aquaculture activity on the foreshore, whether for fish or seaweed, also requires a licence. Licensing responsibility for all aquaculture activities rests with the Department of Agriculture, Food and the Marine.

A raft of other legislation is relevant to the granting of licenses to collect materials from the foreshore, in particular legislation associated with environmental conservation. The Wildlife Act 1976, the Wildlife (Amendment) Act, 2000 and the European Union (Natural Habitats) Regulations, SI 94/1997, subsequently consolidated within European Communities (Birds and Natural Habitats) Regulations 2011, establish Special Areas of Conservation (SACs). Together with areas defined as special Protection Areas (SPAs), SACs form a European network of sites known as Natura 2000, the focal point of EU policy concerning EU nature and biodiversity.

Where there are pre-existing harvesting activities within an SAC, such as harvesting seaweed, additional consent is generally not needed. However, an application to expand harvesting or to begin harvesting within an SAC (or in areas that may affect an SAC) may need further approval or be subject to an appropriate assessment.¹

In the context of NutraMara, seaweeds are the most likely material to be harvested from the foreshore. Many of the wider ecosystem roles of macroalgae, and the distribution and biomass of algal species, are yet to be fully characterised. In light of the need for an appropriate assessment if harvesting sites are located in Natura 2000 designated areas, knowledge gaps as these could influence the extent to which wild stock of seaweeds may be a major source of functional materials.

¹ An assessment to determine if a development will be damaging to a Natura 2000 site

2.13. Sourcing material from seafood processing

Different regulations apply to the use of materials obtained from fish processing. Depending how these materials are identified and treated within the processing cycle, determines which regulations apply. Materials provided from within a food processing stream, require that they are identified as a food during processing, and treated as if for direct consumption. In essence this requires full compliance on the part of the processor with food hygiene law. Two regulations are relevant. Regulation 852/2004/EC defines the requirements for food hygiene, including premises, temperature control, HACCP, equipment, transport, waste, personal hygiene and training. And since fish is a food of animal origin, specific additional hygiene regulations as defined in Regulation 853/2004/EC also apply. These rules apply in the case of wild catch and cultured species.

The use of materials from food processing waste defined as unfit for human consumption is defined in regulations concerning the use of animal by-products (ABPs). The principal legislation relating to ABPs is Regulation (EC) No. 1069/2009 and the national statutory instrument European Union (Animal By-Products) Regulations 2014 (SI No 187 of 2014). The Sea Fisheries Protection Authority (SFPA) is responsible for marine origin animal by-products in Ireland.

Different risk categories apply to ABPs, defined by the risk of the materials to animals, the public or to the environment, which also determine how materials in each risk category are to be disposed. There is a ban on material re-entering the food chain where animals may be fed material derived from the same species, for example as intra-specific fish meal. Otherwise fish processing waste is likely to be considered as category 3 (low risk) waste, provided it comes from disease-free or otherwise uncontaminated sources. It is possible for low risk waste to undergo 'technical transformation' into a product that may be consumed by humans. Examples of category 3 materials from marine sources are

- Material which has previously been fit for human consumption, including catering waste, raw fish and skins
- Parts of slaughtered animals which are fit for human consumption but which are not intended for human consumption for commercial reasons
- Animal by-products derived from the processing of products intended for human consumption, e.g. fish bones

The conversion of mussel or prawn shells into chitin or chitosan is an example of such a transformation. The mixing of materials as might occur in processing waste from wild and cultured species in the same facility places additional processing complexities on companies as a result of cross contamination (Pfeiffer 2003).

2.14. Sourcing materials from aquaculture

Aquaculture is an expanding sector with the potential to fill the gap between fish supply and demand in ways that reduce pressure on wild fisheries. The culture of seaweeds is an aquaculture activity that continues to attract interest in Ireland (Werner and Dring, 2011). The use of material obtained from aquaculture activity is subject to the same regulations relating to food processing, and the use of waste or animal by-products, as described for materials sourced from the processing of seafood.

2.15. Sustainability

The terms ‘sustainability’ and ‘sustainable development’ are increasingly visible “on the radar” of many industries, including the marine. The concept of sustainability was defined as the “social and economic advance to assure human beings a healthy and productive life, but one that did not compromise the ability of future generations to meet their own needs” (Oxford, 1987). The implication of sustainability within the food sector is that products should be:

- based on raw materials that can be produced on an on-going basis without undue environmental, social or economic harm;
- not be reliant, in the long-term, on finite energy sources; and
- food products should not adversely affect human health.

The marine sector faces numerous challenges in coping with the rapidly changing global economic and environmental conditions. Therefore, new ways of meeting the needs of the present without comprising future viability have to be embraced by the food industry. The achievement of rational use; sufficient production of marine resources for food use; avoiding needless marine processing waste; the valorisation of marine processing by-products; and the appropriate management of environmental impacts, all underpin the wellbeing, health, and longevity of human populations and the world’s environment.

The challenge of sustainability in the harvesting and exploitation of marine bioresources, equally applies to the processes used in the production of marine derived food and other products. Creating value from marine biomass typically involves some level of extraction of materials from marine organisms. Though a number of platform extraction technologies exist, many of these processes involve the use of corrosive or toxic chemicals and thus present challenges concerning their sustainability. Alternatives to the use of these processes are required.

Attention is drawn to the potential offered to the food sector to enhance its competitiveness by maximising the use of marine species described as underutilised. Europe’s ambitions for “blue growth” coupled with its strategy for smart, sustainable and inclusive growth, highlight the need to generate novel and innovative solutions to enable sustainable exploitation of marine resources. Whilst many EU regulations reflect concerns regarding sustainability, greater

effort is required to bring about the development of methodologies, which can ensure the future sustainability of commercially exploited marine resources.

2.16. Functional compounds from marine organisms

Marine organisms provide an array of proteins, fatty acids, carbohydrates and pigments that offer potential as functional ingredients. Considerable scope exists to fully explore these classes for bioactive molecules that are relevant to functional foods. Some marine origin compounds e.g. omega 3 PUFAs are already used as functional ingredients in baked products and in enriched juices and milk products (Cosgrove, 2008; Patch, 2005). This section provides insights to some of the widely diverse compounds known to exist in various species of algae and fish and considered as relevant to the aims of the food sector in expanding the range of ingredients used in functional foods.

2.17. Algal sources of bioactive compounds

Many sources highlight the potential of algae as a source of novel compounds for food and health use. The interest in macro- and microalgal sources appears to stem from their reported extensive diversity, high productivity, and because of the discovery of compounds with wide-ranging bioactives in many species. Allied to these findings, technological developments in cultivation, which offer the potential to fine tune biochemical composition to specific applications, make them an interesting research target for commercial use (Stengel *et al.*, 2011).

There remains uncertainty concerning the number of different species of algae. Britain's Natural History Museum estimates range from 27,000 to 36,000 species, with less than one third found in marine or brackish waters (Natural History Museum, 2015). There is no complete Irish checklist of algal species, and of the 53 classes of algae described, comprehensive details of just 12, mostly marine species, exist (National Biodiversity Data Centre, 2015). The Irish Biodiversity Centre (2015) reports 1,079 known species of algae from an estimated 3,000 to 5,000 likely to be in Irish waters.

Pigments are amongst the main characteristics used to distinguish between the fifteen phyla of algae. The application of molecular techniques to algae, reveals species of algae as being far more diverse and complex than originally believed; of which most are phylogenetically unrelated.

2.18. Macroalgal (seaweed) sources of bioactive compounds

The main macroalgal species are classified within three phyla; Rhodophyta (red algae) Phaeophyta (brown algae) and Chlorophyta (green algae). Though species of seaweed from each of these divisions are to be found in Irish waters, there remains a dearth of data regarding

the distribution, volume and biochemical profile of most. Irish industry identified the following nine species as priorities regarding opportunities to yield bioactive materials, *Ascophyllum nodosum*; *Laminaria digitata*; *Laminaria hyperborean*; *Palmaria palmate*; *Chondrus crispus*; *Himanthalia elongate*; *Fucus serratus*; *Lithothamnion corallioides*; and *Ulva lactuca* (per.comm Stengel D, NUI Galway). Macroalgae are generally known as sources of proteins, fatty acids, carbohydrates and compounds which demonstrate antioxidant properties. However, the structure and biological properties of algal proteins remains relatively poorly documented.

2.18.1. Proteins

Red algae generally have the highest protein content, with approximately 20-30 % of dry weight as protein, up to a maximum of 47 % in some species such as *Palmaria palmate* and *Porphyra tenera* (Fleurence, 2004). Green algae generally comprise 9-26 % protein. Although brown algae is reported as having the lowest protein content, at between 3-15 % (Fleurence, 2004), there are reports of protein content of up to 44 % in *Fucus serratus* and up to 40 % in *Undaria pinnatifida* (Marsham et al., 2007). Seasonal variation in protein concentrations is common, with the lower values often, but not exclusively, in summer and autumn (Galland-Irmouli et al., 1999, Rouxel, et al., 2001, Martínez and Rico, 2002, Hagen Rodde et al., 2004, Abdel-Fattha and Sary, 1987, Marinho-Soriano et al., 2006). Furthermore, reports have shown that different types of proteins are present in macroalgal cells at different times of the year (Galland-Irmouli et al., 1999, Yuan, 2008, Hung et al., 2009).

The most promising families of proteins from algae in terms of their bioactivities, are lectins and phycobiliproteins. Lectins are carbohydrate binding proteins typically involved in cell communication, including recognition of foreign or cancerous cells (Ziólkowska and Wlodawer, 2006, Calvete et al., 2000). Little is known regarding the biological and chemical properties of algal lectins, with some isolates showing no similarity to other known plant lectins. Investigations of algal lectins from the Asian red algae *Euclima serra* have shown evidence of activity against cancers and fish disease bacteria (Kawakubo et al., 1997, Kawakubo et al., 1999, Sugahara et al., 2001, Hori et al., 2007, Liao et al., 2003). Other lectins isolated from red algae were shown to have painkilling effects, anti-inflammatory properties, activities against HIV-1 and inhibition of human platelet aggregation and dental plaque bacteria (Hori et al., 1988, Neves et al., 2007 Viana et al., 2002, Bitencourt et al., 2008, Mori et al., 2005, Matsubara et al., 1996, Teixeira et al., 2007).

Most seaweeds are considered to contain all the essential amino acids and often a more balanced amino acid profile that found in terrestrial plants (Fleurence, 2004, Galland-Irmouli et al., 1999). Red algae are a potential source of taurine, a conditionally essential non-protein amino acid (Dawczynski et al., 2007). Taurine is associated with a large number of functions including blood pressure and blood cholesterol lowering and antioxidant properties (Lourenco and Camilo, 2002; Militante and Lombardini, 2002; Zhang et al., 2004; Houston, 2005).

Furthermore, in a recent study taurine was shown to have a significant protective effect in women with high cholesterol (Wójcik *et al.*, 2012).

Other algal sourced amino acids of note are laminine and kainoids. Laminine extracted from algae was reported to depress muscle contraction and to have a transitory hypotensive effect (Bhakuni and Rawat, 2005). The kainoids, including domoic acid, are found in many algal species and are of interest due to insecticidal, antihelminthic and neuroexcitatory properties (Parsons, 1996).

Mycosporine-like amino acids are associated with UV absorption and antioxidant functions and can be extracted from *Porphyra umbilicalis* (Oren and Gunde-Cimerman, 2007, Cardozo *et al.*, 2007). This group is relevant as a source of derivatives for natural skin protection ingredients and industrial applications where materials need to be photostable (Cardozo *et al.*, 2007).

A number of endogenous peptides with potentially useful activities have been identified from macroalgae. These include peptides with antioxidant activities and analogues of human molecules that may have therapeutic uses (Arasaki and Arasaki, 1983; Shiu and Lee, 2005; Brown, 1981; Morse, 1991; Aneiros and Garateix, 2004).

A survey of ten dipeptides extracted using hot water from *Undaria pinnatifida* showed evidence of angiotensin converting enzyme (ACE) inhibitory activity, with a smaller sub set causing reduction of blood pressure in spontaneously hypertensive rats (Suetsuna *et al.*, 2004).

Enzymatic hydrolysis of algal proteins with commercially available food-grade proteolytic enzyme preparations has the capability to release an array of peptides with potential biofunctional properties (Arihara, 2006). Macroalgal protein hydrolysates have been shown to have ACE inhibitory, antihypertensive, anti-mutagenic, calcium precipitation inhibition, antioxidant, antitumor, anticoagulant, antityrosinase (enzyme implicated in Parkinson's and similar diseases) and blood sugar or cholesterol lowering activities.

The ACE inhibitory and antihypertensive peptides identified to date have been purified from protein hydrolysates generated from three species of macroalgae (*Hizikia fusiformis*, *Porphyra yezoensis* and *Undaria pinnatifida*). The seaweed sources worked on to date are biased towards Pacific origin flora.

2.18.2. Polyunsaturated fatty acids

The fatty acid content of seaweeds can approach that of fish: *Undaria pinnatifida* may have EPA concentrations of up to 0.14 g per 100 g dry weight of thallus). The PUFA content of *U. pinnatifida* varies between locations, within the algal frond and was generally higher later in the Asian harvesting season (Khan *et al.*, 2007). More research is warranted to examine the fatty acid content of other species of seaweed as a potential source of n-3PUFAs, in particular EPA and DHA.

2.18.3. Carbohydrates

Carbohydrates are among the most abundant chemical compounds in nature and they play numerous functional and structural roles in living organisms. Complex polysaccharides from

the brown, red and green seaweeds are recognised as having a broad spectrum of properties with therapeutic potential (Patel, 2012). The carbohydrates identified in macro-algae include a number of different types of polysaccharides, e.g. sulphated galactans such as agar and carrageenans. These molecules, often termed phycocolloids, hydrocolloids or gums, are already widely used in the food industry (Renn, 1997; Bixler and Porse, 2010).

Sulfated fucans, frequently referred to simply as fucans, constitute a class of polysaccharides also known as fucoidans, fucosan, and fucanusulphate found in species of brown algae. Marine algae generally contain these polysaccharides in complex heterogeneous structures, with structural details varying between species, populations and seasons (Zhao *et al.*, 2008).

Fucoidans are known for their anticoagulant activity (Smit *et al.*, 2004), related to their sulphate content (Nishino *et al.*, 1994). This activity has been investigated as an alternative to the anticoagulant heparin, which is extracted from meat carcasses. A number of different brown algal fucoidans have been shown to have anticoagulant and antithrombotic activity, with sources including species of *Laminaria*, *Eklonia* and *Pelvetia*. Fucoidans from *Ascophyllum nodosum* and *Fucus vesiculosus* have been patented as anticoagulants.

Fucoidan attach to the cell walls of algae, this mechanism offers scope for the use of these sulphated polysaccharides in health applications. Fucoidans have been proposed as anti-inflammatory compounds, possibly altering the attachment of white blood cells, with applications in reducing reperfusion injury when circulation is restored after traumas such as heart attacks. Similarly, fucoidan may have a potential role in inhibiting graft rejection or anaphylactic shock. Fucoidan-chitosan gels can promote skin growth and contraction of wounds, making such mixtures suitable for treating dermal burns (Sezer *et al.*, 2008).

The potential antiviral activity of fucoidans was demonstrated in culture against a range of infections, such as poliovirus, herpes and HIV (Luescher-Mattli, 2003). By interfering with the attachment of viruses to cells, fucoidans can act as antivirals and have low toxicity in comparison to other antiviral drugs used in medicine. There are suggestions that consumption of brown algae may have a prophylactic effect against prion diseases (Doh-ura *et al.*, 2007). Anti-tumour and anticancer effects of fucoidans have also been found; both through stimulation of the immune response and through direct toxic effects on cancer cells (Aisa *et al.*, 2005).

The sulphated galactans have a similar activity profile to that of fucoidans, but found in red algae, typically *Chondrus crispus*. Carrageenan is the most well-known example of this group and there is evidence that they offer both anticoagulant and antiviral activity. Concerns that carrageenan, particularly in a degraded form, may produce an inflammatory reaction and ulceration of the colon, led the European Commission to recommend a limit < 5% on the amount of small molecular weight carrageenan in any food use.

Agar is another polysaccharide also extracted from red algae, mostly *Gelidium amansii* and whilst not generally considered a likely candidate source of bioactive molecules, an agar-

supplemented diet has been shown to improve short-term weight loss and lower cholesterol levels in obese subjects (Maeda *et al.*, 2005).

Alginates are polysaccharides mostly found in brown algae, with a history of use in food and related industries. They are derived from the kelps, principally *Macrocystis pyrifera*, *Laminaria* spp. and the intertidal alga *Ascophyllum nodosum*. Alginates have been shown to have effects on blood lipids, decreasing the total blood cholesterol content and causing weight loss. Sodium-calcium alginate and PUFAs have been suggested as a treatment for childhood blood lipid imbalances (dyslipidemia). As with other large polysaccharides, alginates have been shown to act as prebiotics, increasing the levels of bifidobacteria. Alginates are also used in over-the-counter medication for heartburn and acid reflux. These preparations contain weak alkaline salts (in the products for adults) and the alginate helps to form a gel that protects the oesophagus from stomach acid.

Other brown algal polysaccharides, laminaran (alternatively laminarin) may demonstrate biological activities. Laminaran extract has shown promise as an antibacterial agent in pig diets, while fucoidan has shown prebiotic properties in pig diets (Lynch *et al.*, 2010). Derivatives of laminarin may slow tumour growth and have anticoagulant activity (Adams and Thorpe 1957; Hoffman *et al.*, 1996).

Ulvan is a sulfated polysaccharide extracted from green algae. Species from the genus *Ulva* are widely eaten and have a history of medicinal uses including the use of boiled extracts against intestinal parasites and direct application of fronds to wounds (Scagel *et al.*, 1957). Experiments with ulvans have shown effects on tumour cell proliferation and hypolipidaemic activity (Kaeffer *et al.*, 1999; Sathivel *et al.*, 2008).

2.18.4. Compounds with antioxidant properties

Potential antioxidant phytochemicals from marine sources include carotenoids, phenolic compounds and organosulphur compounds. Alkaloids may also have potential as antioxidants, but little is known about marine sources of these compounds. The carotenoids, e.g., α - and β -carotene and fucoxanthin, are a widespread and diverse group of pigments, most of which have been shown to have antioxidant properties (and have other potential uses such as in food colouring). Carotenoids are found in marine algae and include pigments such as astaxanthin.

Bromophenol is a phenolic found at high levels in red seaweeds such as *Polysiphonia urceolata*. An important class of phenolic are phlorotannins (phloroglucinol, eckol). This group of compounds have been extensively studied in brown algae, where they are thought to have roles in UV damage protection and grazer deterrence (Abdala-Diaz *et al.*, 2006). Phlorotannins have potential in a number of functional food contexts, including as antioxidants (Wang *et al.*, 2010), in chelating metals and proteins out of solution (Parys *et al.*, 2007), as antimicrobials (Wang *et al.*, 2009) and antivirals (Ahn *et al.*, 2004) and as compounds with potential medical applications (Moon *et al.*, 2008). The organosulphur compounds include sulphur containing antioxidant amino acids; cysteine and methionine. Other sources of antioxidant compounds in

seaweeds include ascorbic acid, catechins (catechin, epigallocatechin, epigallocatechin gallate) and tocopherols (α -, γ -, δ -tocopherols) (Zubia *et al.*, 2009). As organic compounds containing sulphur, many of the seaweed polysaccharides have antioxidant potential (Barahona *et al.*, 2011; Ye *et al.*, 2008).

A comparison of extracts from *Fucus vesiculosus* has shown generally greater antioxidant activity in this seaweed than in a number of commercially available supplements. Other seaweed extracts, generally from species not growing in Irish waters, have shown antioxidant activity. The antioxidant content of seaweeds has been shown to vary seasonally and with shore height (Connan *et al.*, 2004). This is not surprising and is predictable in general terms on the basis of factors such as herbivory, nutrient availability and light or heat stress. Upper shore *Porphyra umbilicalis* has been found to have the highest antioxidant activity when compared to material from lower on the shore. A similar association with more stressful conditions has been observed in *Ulva lactuca*, where bromophenol content increases towards the end of summer, but declines through the rest of the year.

Several studies have indicated the potential of powdered seaweeds (López-López *et al.*, 2009a) from *Himanthalia elongata*, *Undaria pinnatifida* and *Porphyra umbilicalis* on the antioxidant capacity (determined using FRAP and TEAC (trolox equivalent antioxidant capacity) assays) of meat emulsion model systems. It was concluded that the polyphenolic compounds contained in seaweeds increased the antioxidant capacity of the meat model systems (Cofrades *et al.*, (2008); López- López *et al.*, (2009b). The inclusion of powdered (*Himanthalia elongata*) in poultry steaks did not affect the sensory properties of the meat.

The addition of phlorotannins from *Sargassum kjellmanianum* was found to act as a novel antioxidant in fish oil. Yan *et al.*, (1996) and Wang *et al.*, (2010) reported antioxidant activity of oligomeric phlorotannins isolated from *Fucus vesiculosus* in cod fish muscle.

2.19. Microalgal sources of bioactive compounds

The terms 'algae' and subsequently 'microalgae' are scientifically misleading terms which refer to a large diversity of diverse taxonomic entities with different phylogenetic backgrounds comprising both prokaryotic and eukaryotic organisms, which are as little related to each other as toadstools are to humans (and thus have little in common other than that they are mostly photosynthetic organisms living in damp environments). The estimated number of species algae is in excess of 30,000, though this is likely to be an underestimate (Falkowski and Raven, 2007). There are continuous new discoveries being made in both taxonomic entities (e.g. Stern *et al.*, 2010) and new genetic strains within different algal species, multiplying the chemical composition of 'microalgae' (e.g. Colla *et al.*, 2005; Plaza *et al.*, 2009).

Few microalgal species are currently exploited commercially, while the diversity of compounds with either nutritional or health-promoting activities, including multiple antioxidant functions, is promising (Raja *et al.*, 2008; Plaza *et al.*, 2009) Several recent studies have specifically reviewed

the potential of microalgae as food ingredients (Gouveia *et al.*, 2009; Plaza *et al.*, 2009; Chacón-Lee and Gonzales-Mariño, 2010). Microalgae also act as a food additive in animal feeds, functioning either as colourant (e.g. the freshwater genera *Haematococcus*, *Dunaliella* or *Chlorella*, (Borowitzka *et al.* 1991)) or as a nutritional enrichment of human and animal diets due to their fatty acid, vitamin, mineral and protein contents.

Traditional food uses of microalgae were based on collection from natural freshwater bloom populations in China, Chad and Mexico and the most commonly species used were the prokaryotic *Spirulina* and *Nostoc* (Pulz and Gross, 2004; Chisti, 2006). Despite their immense diversity even today only a few species are commercially grown and utilised in foods, including: the freshwater species *Haematococcus*; the halophyte *Dunaliella*; several entities of *Chlorella* (some of these falsely identified as *Chlorella*); and several *Spirulina* species (mainly *S. maxima*). It should be noted that even today most microalgal production is based on freshwater species.

2.19.1. Proteins

Early investigations of microalgae as food focused on their use as Single Cell Protein (SCP) for undernourished human populations, which was considered as a solution to world hunger in the first half of the 20th century (Jassby, 1988a). Some microalgal species contain high levels of proteins (e.g. *Spirulina*, *Anabaena*, *Scenedesmus*) (Becker, 2004) and with some exceptions, there did not appear to be any detrimental effects arising from feeding microalgal protein at high concentrations to undernourished people (extensively reviewed by Jassby, 1988a,b). The current focus of research is on the selective addition of microalgal compounds as nutraceuticals (Plaza *et al.*, 2009; Chacón-Lee and Gonzales-Mariño, 2010). Different types of the proteinaceous phycocyanin are commercially produced from *Spirulina* which has applications in foods and health foods and as dyes but also have antioxidant and pharmaceutical properties (Eriksen, 2008).

2.19.2. Polyunsaturated fatty acids

Several species from most of the major categories of microalgae (Chlorophyta, Cyanophyta, Rhodophyta, Bacillariophyceae) produce fatty acids of value to human nutrition such as EPA and DHA (Radwan, 1991; Meiser *et al.*, 2004). Fatty acid levels in microalgae, whilst specific to different groups, can be enhanced and composition modified by different culture conditions (e.g. Meiser *et al.*, 2004). Groups of species that have shown particular potential and are under investigation for potential commercial exploitation include diatoms (Otero *et al.* 1997; Guihéneuf *et al.*, 2010), cyanobacteria (Colla *et al.*, 2004), as well as red (Durmaz *et al.*, 2007) and green microalgae (Petkov and Garcia, 2007).

A breakthrough in the genetic optimisation of algae could expand the supply of omega-3 fatty acids according to published research from UC Berkeley and Aurora Algae (Kilian *et al.*, 2011). A non-transgenic strain of the unicellular alga, *Nannochloropsis*, has been developed with an altered lipid profile having a higher proportion of EPA. The authors believe this new strain will

be extremely valuable in expanding the supply of omega-3 fatty acids for the global pharmaceutical, food and dietary supplement industries.

2.19.3. Carbohydrates

Whilst most algal polysaccharides (agars, carrageenans, alginates and their derivatives, and fucoidans and laminarin) used in the food industries are derived from macroalgae, the production of sulphated exopolysaccharides from the red alga *Porphyridum cruentum* (Wang et al., 2007; Keidan et al., 2009), a Cryptophyte (*Chroomonas*) (Bermudez et al., 2004) and *Spirulina* (Nie et al., 2002) have shown potential for commercial exploitation. Besides the obvious gelling and binding characteristics of sulphated galactans more commonly extracted from macroalgae (e.g. Tuvikene et al., 2010), these compounds also have several high bioactivities and thus could present value food ingredients.

2.19.4. Compounds showing antioxidant properties

Pigments are of value to the food industry as colorants, because of the antioxidant activity of most pigments (in particular carotenoids) and their multiple functions as health promoting agents, the potential of which is only emerging (Prasanna et al., 2007, 2010). Reported health benefits from microalgal pigments include anticancer, anti-inflammatory, neuro-protective and hepatoprotective properties (Eriksen, 2008; Prasanna et al., 2010).

Microalgal pigments fall into two major categories: carotenoids are produced by all algal groups but commercially available pigments currently used in the food industry are mainly: β -carotene and astaxanthin (from *Dunaliella* and *Haematococcus*, respectively) and to a lesser extent lutein; various other xanthophylls (such as zeaxanthin); and lycopene and bixin (Gouveia et al., 2009) from other algal groups including Chlorophyta and Cyanobacteria.

Whilst most microalgal groups contain α - or more commonly, β -carotene (Falkowski and Raven, 2007), carotenoid composition is specific to different algal groups with over 600 different types reported to occur in nature (Faure et al., 1999). Total levels of carotenoids within algal cells, and the relative abundances of different types, are determined by growth conditions (e.g. light, nutrient and salinity stress) and thus vary naturally, though they can also be optimised in relevant culture conditions (Raja et al., 2007). Similarly, phycobilin production is specific to cyanobacteria (such as *Spirulina*) and the few microalgal representatives of red algae (e.g. *Porphyridum*), and its production has been optimised in certain strains (e.g. Eriksen, 2008; Gupta and Sainis, 2010).

2.19.5. Vitamins and minerals

Whilst most microalgal species examined contain a range of essential vitamins and minerals valuable to human nutrition (Becker, 2004; Pulz and Gross, 2004), some groups contain particularly high levels (e.g. certain species of *Spirulina*), but similar to other chemical constituents of interest, vitamin and mineral levels vary significantly with growth conditions (Gouveia et al., 2009). This again illustrates the potential to optimise the levels and composition of constituents that are desirable. Most research has been conducted on

freshwater species such as *Haematococcus* and *Spirulina* but the same principle can be applied to other groups. Approaches to producing algae that are rich in several compounds of interest should be investigated, but potential interactive physiological effects of different additions (with potentially antagonistic effects due to competition for chemical bindings sites), as well as possible impacts of these 'stresses' on biomass production needs to be evaluated.

2.20. Fish sources of bioactive compounds

Fish are recognised as being inherently functional foods; possessing compounds such as PUFAS, minerals and proteins that are beneficial in maintaining human health, in addition to contributing to nutritional intake. Fish, inclusive of shell fish, whether they are harvested from the wild or aquaculture farms, and fish by-products (rest raw materials or co-products) are recognised as an excellent source of nutraceuticals and bioactives (Shahidi, 2003; Alasalvar and Taylor, 2002).

2.20.1. Proteins

Proteins from fish, molluscs and crustaceans can be divided into three main groups: the water or low salt buffer soluble fraction (sarcoplasmic), the structural proteins that make up the bulk of muscle mass (myofibrillar) and the connective tissue or stroma proteins. The overall protein content of fish is between 11 and 22 %, with a greater range of variability across molluscs and crustaceans (Murray and Burt, 2001; Venugopal, 2009a).

The most familiar and most common connective tissue protein is collagen, widely used as gelling agents in the food industry (Venugopal, 2009a). Collagen is prevalent in fish bones and skin and therefore processing waste is potentially a good source for this protein (Kim *et al.*, 2008). The prevalence of non-polar amino acids may make collagen a potential source of ACE inhibitory and antioxidant peptides (Kim and Mendis, 2006).

A review of the literature shows ACE inhibitory, antioxidant, calcium binding, antihypertensive and anticoagulant activities across a range of peptides isolated from different fish and fish waste sources. Mollusc and crustacean derived protein hydrolysates also appear to be useful sources of bioactive peptides with antioxidant, appetite suppressant, antihypertensive, ACE inhibitory and HIV protease inhibitory activities reported.

Proteins and peptides with biological activity are present in fish and shellfish. For example, antimicrobial peptides may have potential as food preservation additives. Reported sources of such antibacterial peptides include skin secretions, mucus and fish milt. Protamines from fish milt contain high concentrations of arginine and have been used in medical applications to inject insulin and to reverse the effects of the anticoagulant heparin (Kamal and Motohiro, 1986; Uyttendaele and Debevere, 1994; Islam *et al.*, 1984; Gill *et al.*, 2006; Chan and Li-Chan, 2006). Patents have originated in Japan for the use of protamines as food preservatives, and as additives for the prevention of dental caries (Gill *et al.*, 2006). Equivalent antibacterial activities were demonstrated in peptides isolated from shrimp, crab and molluscs. For example, a 6.5

kDa proline-rich peptide from blood cells (haemocytes) of the shore crab *Carcinus maenas* displayed antimicrobial activity against both gram negative and gram positive bacteria (Schnapp *et al.*, 1996).

Beyond antibacterial activity, there are further activities in fish and shellfish peptides, particularly where a molecule acts as a mimic for a human protein. For example, calcitonin is a hormone involved in bone-calcium absorption (Venugopal, 2009; Kanis, 2002). Salmon calcitonin is 30 times more active than the human hormone and is used in the treatment of osteoporosis (Venugopal, 2009). This compound can have a painkilling effect and may have a role in treating pain in bone-related syndromes (Lyritis and Trovas, 2002).

2.20.2. Polyunsaturated fatty acids

Growing public awareness of the benefits and limited dietary sources of PUFAs has created a substantial interest in the production of PUFA concentrate (Sahena *et al.*, 2010a). The polyunsaturated fatty acids (PUFAs) are essential fatty acids, which must be obtained from the diet, since humans cannot synthesize these fatty acids. These are alternatively known as omega-3 and omega-6 fatty acids.

PUFAs are integral to cell membranes, influencing the physical properties of the membrane and the function of membrane-bound enzymes and receptors. Wider roles for PUFAs in the body include potential roles in blood pressure regulation, blood clotting and the development and functioning of the brain. Eicosanoids derived from PUFAs are signalling molecules involved in the regulation of inflammatory responses. The role of PUFAs in inflammatory processes is linked to eicosanoids primarily derived from 20-carbon PUFAs: arachidonic acid (*n*-6) and eicosapentaenoic acid (EPA, *n*-3). EPA and a related fatty acid docosahexaenoic acid (DHA, *n*-3) have been shown to lower the expression of genes involved in inflammatory reactions (Wall *et al.*, 2010).

A human dietary intake of 4:1 *n*-6:*n*-3 ratio is generally recommended, but western diets typically exceed 16:1 ratios. This dietary imbalance has been associated with increased prevalence of diseases characterized by inflammatory processes, including cardiovascular, psychiatric and inflammatory diseases.

Currently, PUFA concentrates are used commercially in pharmaceutical products, food additives, and in health supplements. Production of omega-3 concentrates may be carried out to offer a pure fatty acid, such as EPA or DHA or a mixture of omega-3 PUFAs (Shahidi, 2005). Typically, EPA and DHA are contained in oily fish such as salmon, lake trout, tuna and herring. Oily fish such as herring typically have the highest content of EPA + DHA (2.01 g per 100 g fish). Concentrations are lower in white fish such as cod and haddock (0.24-0.28 g per 100 g fish) and variations occur between areas, with the fat content of individual fish, over time and with different processing methods before consumption.

The greatest evidence for the health benefits of diets relatively rich in *n*-3 fatty acids are the various epidemiological studies linking fish-rich diets in Greenland and Japan to lower coronary

artery disease incidence. Other more specific studies include trials showing reduced relapse incidence in patients with Crohn's disease (an inflammatory disease of the intestine). DHA is a major fatty acid in the brain and dietary supplementation is under investigation as a potential therapy for the prevention and treatment of neurological disorders such as Alzheimer's disease. More recently, studies reported on the beneficial effects of omega-3 fatty acids on mental health, including schizophrenia and bipolar disorders. A study by Chiu *et al.*, (2012) indicated that higher omega-3 levels in membranes of erythrocytes were associated with improved cognitive function and immediate recall in older people with previous depression.

The European Food Safety Authority (EFSA) and the American Food and Drug Administration (FDA) each announced qualified health claims for dietary supplements containing EPA and DHA omega-3 fatty acids, pointing to the role of PUFAs in reducing the risk of Cardiovascular Health Disease (CHD) and beneficial in the maintenance of normal blood pressure.

Potential sources of omega-3 rich material in Ireland include the fatty, pelagic fish mackerel, herring and tuna. The fat content of these species varies, including seasonal variations (Jaobsen *et al.*, 2009). Cod and Halibut livers are also good oil sources. Catches tend to be seasonal, with the large pelagic slander in winter and spring. One of the factors that can affect the quality of fish oil is the geographic region of catching as it can influence contaminants (metals, lipophilic pollutants) building up in the food and this needs a case by case assessment.

2.20.3. Carbohydrates

In addition to many polysaccharides found in macroalgae, other major sources of potential bioactive carbohydrates are chitins obtained from the shells of crabs, lobsters, prawns, shrimp and krill. Commercial chitin is extracted from crustacean waste generated by the seafood processing industry. Crustacean shells are constituted mainly of a matrix made of chitin and protein, hardened by mineral salts. The amount of each component can vary widely among species and also in an intra-specific way as a function of season, age, gender and other environmental conditions (Beaney *et al.*, 2005). Chitin is insoluble in water, but can be treated chemically/enzymatically to produce chitosan, which, depending on molecular weight, is soluble in weakly acidic solutions.

There are suggestions that chitosan can alter bacterial cell permeability or interfere with bacterial RNA and protein synthesis (Liu *et al.*, 2004; Chung and Chen, 2008), albeit the exact mechanisms of antibacterial action are not fully established. The effectiveness of chitosan as an antibacterial agent depends on concentration and molecular weight of the chitosan molecules used.

Health benefits associated with chitosan include a lowering of blood cholesterol (Xu *et al.*, 2007), which is linked to beneficial effects on coronary heart disease and weight loss. As high molecular weight chitosan molecules are not easily digested or absorbed in the acidic environment of the small intestine, chitosan forms a gel in the intestine. This binds with dietary fats and prevents their absorption in the gut and enhances natural excretion of the fats (Gades

and Stern, 2003; Zeng *et al.*, 2008). Gades and Stern, (2003), however, point out that the fat trapping effect in their study was 'clinically negligible'.

Chitosan has been found to have anti-tumour effects in laboratory animals that are mediated through enhancing the host's immune system (Shahidi *et al.*, 1999). Immunomodulatory effects of chitin and chitosan are also observed in wound healing (Mori, 2005). At the cellular level, chitosan-mediated immune response reportedly occurs through various signalling molecules and cytokines such as tumour necrosis factor.

A number of *in vitro* studies have demonstrated that chitosan can bind fats and bile acids (recently reviewed by Egras *et al.*, 2010). It is hypothesised that once chitosan forms a complex with dietary fat, released after digestion of a meal, the absorption of the fat by the intestinal cells is prevented. Several *in vivo* studies in human and animals also demonstrate a significant reduction in body weight gain and reduction in the plasma lipid content due to feeding of chitosan. Chitosan as an effective weight loss supplement in humans was demonstrated by Schiller *et al.* (2001) and Kaats *et al.* (2006).

Chitosan is being used as a functional food in many Asian countries including Japan, Korea and China (Aranaz *et al.*, 2009). However, it is not yet enlisted in the General Standard for Food Additives although chitin and chitosan were considered by the Codex Alimentarius Commission in 2003 (Aranaz *et al.*, 2009). In the EU, chitosan is not yet approved as a food ingredient although several studies have demonstrated that chitosan is not toxic in general and has been included in the list of generally regarded as safe (GRAS) compounds (Chandy and Sharma, 1990; Thanou *et al.*, 2001).

Assays of polysaccharides from abalone have revealed relatively strong antioxidant properties in comparison to vitamin C and E controls (Zhu *et al.* 2010). A separate study of water-soluble sulphated polysaccharide extracted from abalone viscera showed evidence for tumour suppression and stimulation of the immune system in mice (Sun *et al.*, 2010).

2.20.4. Compounds showing antioxidant properties

Antioxidants can be extracted or isolated from fish and fish waste by different processing methods. Antioxidant activity was identified in protein hydrolysates of fish waste, from Alaskan pollack, tuna, eel and parasitised hake. Hydrolysates are treated with various combinations of filtration and chromatography to produce extracts of different structures and molecular weights for assay. Carotenoids have been extracted from shrimp by fermentation with enzymatic cleaving of the carotenoids from a protein matrix. Astaxanthin is the most common and stable pigment to be extracted from crustacean sources, but other carotenoids have also been extracted. A screening of crustacean-derived chitosan derivatives has identified sulfanilamide chitosan derivatives to have the highest antioxidant activity compared to chitosan sulphate or chitosan alone. Fermentation has also been used to generate antioxidant peptides from mussels.

2.2.1. Future outlook for marine origin functional ingredients

The development of functional foods is largely driven by concerns about rising levels of diet related health issues amongst the population. This is a global concern, and an issue constantly highlighted in Irish health policy. With an established food ingredients industry, and a solid background in food and marine science research, Ireland is well positioned to attempt to improve economic and health performance by directing research towards marine functional foods opportunities.

A major feature of the functional food area is the diversity of potential products. The literature contains a large number of examples of bioactivity for proteins, carbohydrates and pigments. Despite significant worldwide research into the composition of marine organisms, many species (particularly seaweed species) found in Irish waters remain to be fully characterised. Compounds extracted from marine sources have been shown to have many different activities including anti-tumour, antihypertensive and as prebiotics.

This consideration of bioactive compounds from marine organisms highlighted a number of areas of opportunity for functional food innovations relating to proteins from algal and fish sources, polyunsaturated fatty acids, carbohydrates and for compounds with antioxidant properties. These are summarised below.

- Marine algal proteins can make up a significant proportion of algal dry mass and are potential sources of bioactive proteins, peptides and amino acids or amino acid-like molecules. There is scope for defining new bioactive molecules either in extracted protein or protein derivatives.
- Given the diversity of potential products, opportunities exist for value creation by identifying and characterising functions of proteins. There is abundant evidence that fish and shellfish material can be the source of potentially valuable bioactive proteins and protein-derived compounds.
- The global omega-3 ingredient market is diverse, and indicated as continuing to expand. There are opportunities for product innovation and in defining new functionalities for particular molecular fractions extracted from fish and algal sourced omega-3.
- Marine-derived carbohydrates are already widely used in nutraceutical, functional food and pharmaceutical markets. There is good reason to expect further development of products from these marine sources. Based on successes of the traditional marine polysaccharides such as products of alginate, carrageenan, agar and chitin, areas of opportunity exist for chitosan, ulvan, laminaran and fucoidan.
- Interests in the potential of antioxidant compounds as a functional ingredient and a food preservative are increasing. Seaweeds and seafood waste are each potential sources of commercially viable antioxidants. A challenge facing the production of antioxidant compounds from marine sources is the availability of competitive production processes.
- Microalgae are already used in food supplements and incorporated as nutritional ingredients into foods and drinks.
- With only about 40 species currently grown in commercial aquaculture, the search for the ideal microalgal species for specific applications is on-going. The demand for new algal-derived bioactives for food use is behind the acknowledged rapid growth in

microalgal research. The use of pigments (phycocyanin and carotenoids) as food dyes with additional antioxidant activity, and the addition of small amounts of microalgal PUFAs into savoury foods, appear to be options with potential.

2.22. Processing marine materials for use as functional ingredients

Many factors influence the way companies compete for market space; however, the speed at which a product moves from initial concept to the shelf in a store ultimately affects overall competition. The ability to rapidly commercialise a product relies on developing early insights to the processes required to create it. Creating robust reliable products demands equally appropriate, reliable and robust production processes. Developing early insights to the manufacturing options, including any process limitations, provides knowledge that is essential in establishing the feasibility of a new product.

A range of marine organisms are identified as sources of novel ingredients, ranging from microalgae to fish, each will have specific limitations on how they are processed, from harvest to use. Biological and chemical characteristics of these organisms change in the period from capture to consumption, further changes may occur during processes used to extract functional and other compounds (Ababouch, 2005). These autolysis and other changes pose challenges in optimising processing conditions pointing to the need to develop early insights to processing methodologies and limitations.

It is most likely that species and compound specific processes are required in developing functional materials from marine organisms. In this respect, a processing challenge to be considered by NutraMara was the extent to which it may be possible to use a biorefining approach to produce several product streams, thereby improving processing capabilities.

2.23. Protein extraction

2.23.1. Algal proteins

Extraction processes are key steps in discovering algal protein sources. Such processes may be complicated due to the presence of polysaccharide (such as alginates) and polyphenolic compounds in algae (Fleurence *et al.*, 1995a; Wong and Cheung, 2001; Jordan and Vilter, 1991). These molecules can bind with proteins, hindering their extraction (Wong and Cheung, 2001). A solution to this issue is to use alkali solutions under reductive conditions (NaOH and 2-mercaptoethanol) to break polysaccharide-protein linkages (Wong and Cheung, 2001; Rice and Crowden, 1987; Fleurence *et al.*, 1995a). Alternatively, polysaccharide degrading enzymes that degrade the cell wall and intracellular polysaccharides could be employed (Fleurence *et al.*, 1995a; Fleurence *et al.*, 1995b; Fleurence, 2003; Joubert and Fleurence, 2008). A targeted or sequential use of enzymes may form part of this process. This process is likely to involve polysaccharide digesting enzymes such as cellulose, hemicellulase and β -glucanase (this

particular mixture enhanced recovery of proteins from *Ulva rotundata* (Fleurence *et al.*, 1995). Other combinations of digestive enzymes have been shown to be effective, with at least one mixture (xylanase and cellulase) being patented for use in the food industry (Fleurence *et al.*, 2001).

Membrane-based technologies such as ultrafiltration and nanofiltration may provide industrially-relevant processes to purify and enrich of peptides of specific molecular mass (Korhonen and Pihlanto, 2007; Korhonen, 2009). Other approaches include electro-membrane filtration, which involves the use of charged membranes, and enzymatic membrane reactors which integrate enzymatic hydrolysis and product separation into a single process (Korhonen and Pihlanto, 2007). These are proven in the industrial production of non-marine sourced ingredients containing bioactive peptides.

The isolation and purification process can be partly guided by the emerging structure-bioactivity profile of bioactive peptides. For example, ACE inhibitory activity is more likely with hydrophobic C-terminal amino acid residues (produced by branched or aromatic side chains), along with a number of other structural signifiers of likely activity (Murray and FitzGerald, 2007). Antibacterial peptides associate with membranes, often through a hydrophobic tryptophan containing region, but with a hydrophilic region containing positively charged residues that interfere with (generally negatively charged) bacteria (López Expósito and Recio, 2006). Antioxidant peptides are often associated with the amino acids histidine, proline, tyrosine and tryptophan (Pihlanto, 2006).

2.23.2. Fish proteins

Enzymatic hydrolysis is a common step in recovering material from fish and fish wastes, with a traditional use for the resulting material being animal feed or fertilizer (Venugopal, 2009a; Kristinsson and Rasco, 2000). The process can be modified to screen for peptides with functional properties.

While some digests involve combinations of expensive food-grade enzymes, there may be possibilities for the simpler processing of materials. For example, de-shelled mussels left in salt solution (25 % NaCl, 20 °C) for 6 months generated peptides with ACE inhibitory activity (Je *et al.*, 2005). Similarly, mussel fermentations were shown in a different study to produce peptides with metal-chelating and antioxidant properties (Rajapakse *et al.*, 2005).

2.24. Lipid Extraction

2.24.1. Algal lipids

Given the large diversity of algal species, optimising the extraction of lipids is a challenge. Algal lipids can be divided into two major types: polar lipids such as phospholipids and glycolipids, and neutral/non-polar lipids such as mono-, di- and tri-acylglycerides (Schuhmann *et al.*, 2012). Even though many extraction methods are described, algal lipid extraction remains a challenge, particularly at commercial scales where energy efficiency and cost effectiveness are important

factors in achieving overall competitiveness (Grima *et al.*, 1994). Efficient cell disruption is a prerequisite for oil extraction (Lee *et al.*, 2010).

Combinations of chloroform, methanol and water, known as the Bligh and Dyer method, can be used to extract lipids from algae. The need for more biocompatible and less toxic extraction methods for oils to be used in food related applications, resulted in alternative solvent based methods. Direct saponification is reported as such a process suitable for extracting significant yields of oils from algae (Li *et al.*, 2014).

Process developments have allowed supercritical fluid technology to be used for microalgal oil extraction for pharmaceutical and nutraceutical bioproducts. These relatively recent developments offer processors several benefits over more traditional liquid solvent based methods (Santana *et al.* 2012). The attraction of supercritical fluid extraction is it does not require the use of toxic compounds or high-temperatures, and provides a greater degree of control in the separation of products.

The continuous microwave-assisted extraction (MAE) is a further method developed for rapid oil extraction from algae. At least 77 % of recoverable oil was extracted in 30 min, compared to 47 % for control. Moreover, the MAE oil contained more unsaturated fatty acids, with more omega-3 and omega-6 essential fatty acids, indicating superior quality (Balasubramanian *et al.*, *in press*).

2.24.2. Fish lipids

Different methods are available to extract oils from fish and fish co-products, and the choice of extraction method can influence oil yield. With some methods relying on high-temperature processing or the use of toxic solvents, there are practical issues to be considered in deciding on extraction processes. Methods that require high temperatures to extract oils can degrade what is a thermally unstable compound; and those methods relying on solvents can have adverse health effects. Even allowing for such inherent disadvantages hexane extraction, vacuum distillation, urea complexation or conventional crystallisation are described as suitable for the extraction of lipids from algae (Staby *et al.*, 1993; Sahena *et al.*, 2010a).

More benign processes are used to extract omega-3 concentrates for pharmaceutical or nutritional purposes, including enzymatic methods and methods that use supercritical fluids (Rubio-Rodriguez *et al.*, 2010). Supercritical fluid extraction, which operates under mild conditions, is suited to the extraction and fractionation of edible oils containing labile PUFAs. In particular, supercritical extraction with carbon dioxide offers new opportunities for the solution of separation problems as it is non-toxic, non-flammable, inexpensive and a clean solvent (Sahena *et al.*, 2010). The pressure swing and soaking techniques of supercritical carbon dioxide extraction were found to be the most effective for extracting the omega-3 family of fatty acids from fish samples (Sahena *et al.*, 2010a; Sahena *et al.*, 2010b).

The advantage of avoiding the use of solvents and high temperatures prompted further research on the use of enzymatic technology for fish oil extraction. Liaset *et al.*, (2003)

reported that enzymatic hydrolysis of salmon frames with proteases obtained omega-3 enriched oil with good recovery (~ 77%) along with several peptides and essential amino acids. Commercially available enzymes at middle temperatures were also used to extract oil from fish processing discards – salmon heads (Linder *et al.*, 2005).

2.25. Carbohydrate extraction

2.25.1. Algal carbohydrates

Seaweeds contain a number of different types of polysaccharides, which include the sulphated galactans agar and carrageenans. These molecules, often termed phycocolloids, hydrocolloids or gums, can be used to stabilize emulsions, retain water and form gels. Sulphated fucans and galactans are negatively charged polysaccharides found in species of brown algae. A crude extract can be made using a hot water or acid soak. The Australian company Marinova, use a patented cold water process to extract fucoidan from a range of seaweeds including *Fucus vesiculosus*, *Ascophyllum nodosum* and *Laminaria digitata* which is said to cause less degradation of the polysaccharide than methods that use ethanol.

Along with agar and carrageenan, alginates are polysaccharides with a history in food and related industries. Alginates are mostly found in brown algae, with the bulk of worldwide production derived from the kelps *Macrocystis pyrifera*, *Laminaria* spp. and the intertidal *Ascophyllum nodosum*. Alginate, which may make up to 40% of the dry mass of some species, can be extracted by a multistep process that involves treating the algae with a hot alkali solution before adding salts and changing the pH to cause alginate to precipitate out of solution.

The complex nature and large sizes of seaweed polysaccharide molecules mean extraction technologies play an important role in the bioactivity, purity and composition of algal carbohydrates. Materials are often stabilised by dehydration or freeze-drying prior to extraction. A general extraction approach is to make a crude extract using aqueous (water, acid or alkali) or partially organic solvents (often ethanol). An example of an initial extraction of a sulphated polysaccharide from the red seaweed *Champia feldmannii*, involved a fairly aggressive initial treatment of enzymatic hydrolysis at pH 5.0 with papain (30 mg ml⁻¹) at 60°C.

More recently developed extraction techniques such as ultrasound (Zhu *et al.*, 2008) and microwave (Wu and Dai, 2007) assisted extraction have been used to maximise extraction yields.

2.25.2. Fish carbohydrates

The major source of bioactive carbohydrates from fish is chitin. This abundant natural compound, which is extracted from the shells of crustaceans, is made up of N-acetylated glucosamine, a glucose derivative. Chitin is insoluble in water, but can be treated chemically/enzymatically to produce chitosan, which, depending on molecular weight is soluble

in weakly acidic solutions. Native chitosan polymers with high molecular weights are associated with low solubility and high viscosity, which limits its cellular absorption and bioactivities. Therefore, low molecular weight chitosan (LMWC) and chitooligosaccharides are generally produced by hydrolysis of the chitosan polymer. A variety of methods are used in this process including chemical, enzymatic or physical hydrolysis.

The main industrial techniques used to extract chitin from different shell-waste sources rely on chemical processes for the hydrolysis of protein and removal of inorganic matter (Hayes *et al.*, 2008). Chitin isolation consists of three steps, which are demineralisation, deproteinisation and bleaching. Demineralisation can be achieved using dilute HCl while aqueous base solutions such as NaOH are used for the deproteinisation step (Hayes *et al.*, 2008). A decolourisation step is often added to remove remaining pigments resulting in a colourless product (Rinaudo, 2006).

Producing chitosan at an industrial scale involves chemical based processes. Employing high concentrations of mineral acid and alkali, they are hazardous, energy consuming and potentially damaging to the environment (Healy *et al.*, 2003). Other less hazardous extraction processes using enzymatic extracts and microbiological fermentations, are feasible alternatives. An extraction study, using a lactic acid fermentation of prawn shell, produced a lower quality of chitin compared to that normally produced by chemical extraction. This lactic acid fermentation approach may be useful as an effective pre-treatment step to chemical extraction, ultimately leading to a reduction in the use of hazardous chemicals (Beaney *et al.*, 2005).

The physicochemical characteristics of chitosan/chitooligosaccharides such as the MW and degree of acetylation (DA) are highly dependent on the type of preparation method and the rigour of the various process parameters applied (Aranaz *et al.*, 2009). It is necessary therefore to optimize an extraction protocol to generate chitosan/chitooligosaccharides with the desired level of bioactivity.

2.26. Antioxidant compound extraction

Protein hydrolysates of fish processing discards have been found to have antioxidant activity. These hydrolysates are treated with various combinations of filtration and chromatography to produce extracts of different structures and molecular weights.

Carotenoids have been extracted from shrimp by fermentation with enzymatic cleaving of the carotenoids from a protein matrix. Astaxanthin is the most common and stable pigment to be extracted from crustacean sources, but other carotenoids have also been extracted. Fermentation methods have also been used to generate antioxidant peptides from mussels. The extraction of algae for antioxidants has used various organic solvents, including acetone, methanol and ethanol.

2.27. Carriers for functional ingredients

Generalisations about the typical consumer can obscure real knowledge about the nature of foods that appeal to different groups of people. An early concern about functional foods was the ingredients might impair the taste of the product, requiring stronger health beliefs on the part of the consumer to overcome this negative factor. Verbeke, (2006) found that, although consumer surveys indicated that people with a belief in health benefits might compromise on taste, the strength of this effect has decreased in more recent surveys. Behind one trial, the thought was that the provision of health information might increase the overall acceptance of some functional beverages. However, this was not the case; health conscious consumers still preferred a palatable fruit drink with less active ingredients than a healthier drink with an impaired taste (Sabbe *et al.*, 2009). Generally functional foods have to be perceived as attractive regardless of any added functionality (Siro *et al.*, 2008). Results from studies of the role of advertising in functional foods suggested that marketing a functional food on the basis of taste, rather than a health claim, may be a suitable strategy in some cases (Kim *et al.*, 2009). Findings as these are amongst the factors to be taken into account in the design of functional foods, with special consideration given to how active ingredients are best delivered. They also highlight the importance of concurrently developing the functional ingredient and carrier.

Opportunities identified as potential carrier products for functional ingredients include:

- Incorporation of antioxidants from mussels as ingredients in seafood based sauces for use as a condiment or in a prepared consumer food.
- Drawing from Asian experiences, where seaweeds are used as a vegetable, to create salad products that incorporate active ingredients from seaweeds, or whole seaweeds.
- To introduce health beneficial compounds from seaweed into classical food or/and beverages.
- Omega-3 oils may be included in bread, crackers, cereals, cereal bars, milk and dairy products, fruit juices, salad dressing, mayonnaise, spreads, margarine, pasta, meat and lean fish products and baby food and infant formulas.
- Possibilities exist to use marine origin antioxidants in conjunction with omega-3 oils.
- The encapsulation or microencapsulation to retard lipid auto-oxidation and enzyme hydrolysis was found to improve the oil stability and control off-flavours. Scope exists to use fish oil microcapsules in functional foods such as cream to fill sandwich cookies, instant foods (soups, cocoa drinks etc.), dairy products (yoghurt, fresh cheese, butter) or pasta.
- In general, antioxidant compounds are incorporated into foods during the manufacturing process. Another approach to enhancing the oxidative stability of, for example, muscle foods (beef, pork, poultry and associated processed meat products) is to supplement pre-slaughter diets with antioxidant compounds.
- Potential exists to use the antioxidant properties of powdered seaweeds in the manufacture of meat based products, thus enhancing their functionality and delivering low sodium products with enhanced dietary fibre and mineral profiles. Powdered seaweeds can also be incorporated within pasta and pasta sauces.

2.28. Knowledge gaps

2.28.1. Introduction

From the outset the NutraMara research programme faced into a large research area and an almost endless list of potential research challenges. These ranged from species selection, to overcoming legal and ethical issues associated with both animal and human trials, as well as understanding key aspects of processing and commercial opportunities for marine functional foods. In commencing the NutraMara programme, early decisions on direction were influenced by knowledge embedded within the consortium and the challenge of maximising the available human resources in ways that would enable the maximum impact on Ireland's food sector.

During the early stage of the project, which explored the feasibility of drawing from available marine resources and assessing the potential of these as sources of functional ingredients, it was apparent that little research on specific Irish resources had taken place. There was a noticeable bias towards research on species that do not normally occur in Irish waters: Pacific species being far more prominent in the literature, than Northern Atlantic or other cold-water organisms. This was particularly noticeable in macroalgal research publications. Not only were Irish species poorly characterised, so too were bioactivities of compounds they produce. Of particular down-stream relevance for any commercialisation activity, is the availability of stable raw materials to process. Insights to the volume and compositional variations of organisms collected at different times of the year, and from different locations, were also quite poorly defined. The NutraMara consortium benefited from feedback from a management board and from an expert review panel. These wide contributions significantly added, when combined with insights delivered by the feasibility study, to the identification of knowledge gaps that the research programme could fill.

2.28.2. Characterising sources and stability of supply

Sources of supply for functional ingredients have previously been identified as fish, algae and discards from the fish processing sector. Insights developed from the extensive literature challenged the absence of microalgae from the work programme, particularly in light of a reported global interest in the resource and the prioritisation of microalgal research in Europe. Proposals concerning planned macroalgal research were identified as requiring a more in-depth study than was planned, with greater precision built into the sampling plans to account for geographic and temporal variations within Irish species that were of specific commercial interest and potential. There were also major gaps in understanding the profile and availability of compounds in macroalgae, and for a systematic evaluation of extraction techniques to maximise yields. Without this fundamental knowledge downstream commercialisation opportunities could be missed. An integration of the two strands of algal research (macro- and micro-) would add value to the programme.

2.28.2.1. *Microalgal research*

In Ireland, where few microalgal species are exploited, opportunities exist to explore these organisms for bioactive compounds. High returns from microalgae may be achieved by targeting the pigments, lipids and mycosporine-like amino acids (MAAs) they are known to produce. The general pathways of compound production are common to most algal groups. However, the diversity of the different, largely unrelated microalgal groups, points to these pathways being diverse. Further research is needed to fully explore factors that contribute to compound production.

Only a small number of species are being cultivated or tested for food use. Cultivation has focussed on a small number of groups, which can be easily grown for aquaculture feed production and more recently for biofuels. For food purposes, different species need to be grown in mono-specific axenic conditions. International literature and industry demands for new food ingredients from algal sources, point to the need for new knowledge regarding,

- Exploiting new species not currently grown for food purposes
- Optimising production based on biological and ecological methods
- Screening for new compounds with application in food
- Screening for new bioactivities in existing compounds
- Optimising the production of compounds of food interest (levels and composition): fatty acid profile, carotenoids (xanthophylls and carotenes), Phycobilins/MAAs.

2.28.2.2. *Macroalgal research*

Research indicates considerable knowledge gaps exist concerning the profile of seaweeds found in Irish waters, with little specific research on species of commercial relevance. An important step in the viability of an industrial application is in establishing the stability of supply raw materials. There is a need for targeted mining of macroalgae for novel bioactive food ingredients by focusing on species where a known potential exists to (naturally and artificially) optimise yield, profiles and activities based on the biological control of specific compounds.

International research in the field clearly indicates that the biochemical profile (and associated bioactivity) of algal species is highly variable. However, combining surveys of natural populations with experimental manipulations can elucidate the factors that control the compound of interest. Impacts that are known to influence bioactive profiles include habitat (or culture) conditions, seasonality and biotic interactions.

Research should thus focus on the application of new sampling and short-term culture experiments to investigate seasonal and spatial variability, and control, modify and optimise compounds (e.g. pigments, fatty acids and MAAs). Macroalgal species of particular interest are *Ascophyllum nodosum*, *Fucus* spp, *Palmaria palmata*, and *Laminaria digitata*. In addition, specific knowledge gaps exist regarding the,

- Level and mechanism of impact of different single and interactive environmental factors on compound levels (e.g. pigment, fatty acid and MAAs) in chosen macroalgal species.
- Level and mechanism of impact of different single and interacting environmental factors on compound composition (e.g. pigment, fatty acid and MAAs) in chosen macroalgal species.
- Establishing a linkage between observed variability and bioactive profiles through focussed assessment of bioactivities of well characterised materials grown under known (controlled) conditions.
- Impacts of genetic variability of different morphological types on biochemical composition of Irish target species.
- Potential to stabilise biochemical profile (and related bioactivity) by exposure to controlled conditions.

2.28.3. Identifying and evaluating novel compounds

2.28.3.1. Proteins, peptides and amino acids

Bioactive proteins, peptides and amino acids from marine sources were identified as opportunity areas in the development of novel functional foods. To fully exploit these marine resources for novel compounds requires the identification of novel bioactive nitrogenous components. Few examples of such compounds from Irish sources exist. Exploitation of marine-derived bioactive peptides as anti-diabetic agents is a relatively untapped area of research that offers significant opportunities for commercially relevant innovation. Knowledge gaps were addressed by research that would focus on,

- Directing research effort towards the identifying of macroalgal species for bioactive nitrogenous compound mining, specifically macroalgal species which grow off the coast of Ireland and which represent good candidate raw materials for further and more diverse biofunctional nitrogenous compound mining.
- Generating detailed knowledge of the parameters affecting macroalgal protein extraction efficiency, since no systematic study has been carried out to determine the parameters that affect protein extraction efficiency.
- Detailed knowledge of the parameters affecting the non-protein nitrogen compounds in macroalgae present in Irish waters. Non-protein nitrogenous components have the potential to display unique biofunctionality. However, research relating to those parameters which affect non-protein nitrogen composition and content is limited, if not non-existent, in many instances.
- Developing food-friendly bioactive peptide enrichment protocols to support the enrichment of macroalgal peptides by adapting and development of technologies and processes (e.g., membrane and electro-membrane processing) currently used to fractionate and enrich peptides from other sources.
- Exploring the stability of isolated/extracted bioactive components providing information on the interactions of marine proteinaceous compounds with other food components during processing and storage, and the effects of these interactions on their bioactivity.
- Complete sequence identification of marine-derived bioactive peptide structures, thus providing platform knowledge that will contribute to a greater understanding of the mechanism of action of macroalgal peptides.

- To date, no macroalgal-derived protein hydrolysates have been assessed for insulinotropic activity. Specific opportunities exist for the generation and subsequent screening of insulinotropic peptides from macroalgae for anti-diabetic activity.
- Target the generation and exploitation of proteins and peptides from marine to help the marine sector not only to add value, but to find solutions that address the legal restrictions, high cost and environmental problems associated with disposal of a waste material.

2.28.3.2. *Polyunsaturated fatty acids*

Opportunities are known to exist to develop PUFAs from fish, algae and discards from fish processing activity. The extent that these can be realised is dependent on developing greater insights into process and product interaction. Despite the number of health claims that have been attached to the consumption of PUFAs, and the wide recognition of biological activities of marine PUFAs, specific knowledge gaps remain. Strong evidence suggests that interactions between the gut microbiota and PUFAs, and in particular omega-3 fatty acids, may affect human health, though specific information on the influence of marine-derived lipids on gut health is currently lacking. A better understanding of potential interactions between omega-3 PUFAs and indigenous microbiota could offer strategies to optimise gastrointestinal health and improve overall health. Omega-3 fatty acids are highly unsaturated and sensitive to autooxidation. Factors affecting oxidation rates are known to include fatty acid composition, storage conditions and physical state, however, knowledge of the effect of oxidation on the bioactivities of marine lipids is limited. Specific knowledge gaps relating to marine lipids included,

- The effects of bioactive lipid fractions on gut microbiome, including developing an understanding of interactions of PUFAs with indigenous microbiota and probiotics.
- The stability of lipid fractions subjected to typical food processing, environmental and storage conditions, and the potential of natural antioxidants to prevent omega-3 oxidation.
- The bioaccessibility of marine lipids in model foods consistent with established stability and sensory profiles.

2.28.4. *Polysaccharides of fish origin*

The obesity epidemic, concerns over the increasing prevalence of inflammatory bowel disease (IBD) and the demand for animal feed additives are key drivers of research into uses of chitin and chitosan. Aqueous solutions do not dissolve either compounds, which restricts bioavailability and limit their application as functional ingredients. Alternative approaches to overcome the solubility issue, include using chemical approaches to modify chitosan to form derivatives such as chitosan hydrochloride.

Despite these barriers, the binding of chitosan molecules with dietary lipids is recognised as a possible mode of action in chitosan mediated weight loss. The diversity in physico-chemical properties of chitosan and its derivatives greatly influence the bioactivity, and hence their potential use as functional ingredients. Existing screening methods limit close examination of

the bioactivity of these compounds. Opportunities to exploit known effects of chitosan such as an enzyme immobiliser, its antimicrobial properties and role in stimulating immune function exist. However, the mechanism and modes of action of chitosan in these applications remain poorly understood. Without this knowledge, health claims for chitosan based compounds are unlikely. Research is required to resolve the following knowledge gaps,

- Factors that contribute to variations in the physico-chemical properties of chitin, chitosan and its derivatives that are associated with an unpredictable bioactivity.
- Suitable scientific methodologies to be used in screening of various types of chitin, chitosan and its derivatives and evaluation of the bioactivity.
- Adequate scientific understanding is currently lacking on the mode of action of various types of chitin, chitosan and its derivatives.

2.28.5. Algal Polysaccharides

Macroalgal polysaccharides are used in a myriad of food applications, including as emulsifiers, food stabilisers and microencapsulation ingredients. The bioactive potential of these compounds as anti-coagulants and anti-virals is well recognised, opening the way for further research. The bioactivity of macroalgal polysaccharides is not yet fully characterised in species of interest, such as *Fucus serratus*, *Laminaria digitata*, *Gracilaria gracilis*, and *Pelvetia canaliculata*. Providing new knowledge on these species could be completed by,

- Research to combine exploring polysaccharides with research on polyphenols and carotenoids from these species, in conjunction the development of food friendly extraction methods for polysaccharides.

2.28.6. Knowledge to demonstrate the impact of marine functional foods

A large scale clinical study is outside the scope of the NutraMara programme due to time-constraints, and the high costs that such an approach would incur. Small-scale targeted pilot studies can investigate the effects of marine bioactives on health. The findings from such studies will not only contribute to public knowledge of the health benefits of marine compounds, but also contribute to establishing health claims. The lack of data concerning the health benefits of consuming compounds from Irish seaweeds justifies the design of a focused study. Investigating the effects of marine bioactives from Irish algae on biomarkers of health among healthy individuals will generate useful fundamental knowledge to inform the design of larger scale human trials and identify further research needs. Pilot studies will be used to,

- Demonstrate the health effects of seaweed consumption.
- Measure the effect of seaweed consumption on weight management in humans.
- Identify mechanisms of action of seaweed constituents in type 2 diabetic individuals.
- Investigate the prebiotic activity of marine derived polysaccharides.
- Understand the influence of marine products on osteoarthritis and osteoporosis.
- Investigate the effect of seaweed consumption on cancer risk in humans.
- Assess the anti-cancer effects of Irish marine bioactives *in vitro*.
- Assess the anti-viral properties of Irish marine bioactives.

3. SOURCING AND SAMPLING

3.1. Introduction

Macroalgae (seaweeds), microalgae, fisheries and aquaculture and processing discards are all potential sources from which to obtain compounds for use as functional food ingredients. Understanding the main characteristics and scale of the Irish resource was essential when seeking to maximise the use of available resources. Several factors affect the volumes of available algae, fish and shellfish including seasonality, internationally agreed quotas, fisheries management plans and the extent of processing in Ireland. The discards and wastes associated with processing activities result from wild caught fish and from aquaculture. Compared to the variety of species of fish from the wild, aquaculture offers a narrow range, principally salmonids and mussels. Irish seaweed aquaculture production is relatively small compared to species collected from the wild. Although there may be a considerable standing crop of wild species offering potential for harvest, uncertainties exist surrounding the actual biomass and impact of harvesting on the marine environment. Microalgae occur in the wild, however, the majority of species used for food related and other applications are grown in culture and not collected from the wild.

A key aspect of assessing marine materials for their functional properties revolves around the gathering and collection of samples and preserving them for future analysis. This required a robust approach to sampling to be developed that was appropriate to the accurate determination of bioactive compounds from samples of fish, algae and processing discards. Steps also have to be taken to minimise the possibilities for samples to degrade or become contaminated. Thus it was imperative to have a process of storage (short and long term) that was efficient and preserved sample integrity over time. Faced with potentially large numbers of different species, particularly from the algal resources, an accurate taxonomic description of the species is essential. Further data concerning the habitat from which the samples were collected, including accurate location data, are also required.

Having obtained samples, there was a requirement within the programme to collect data and track information concerning the distribution and analysis of samples; thus managing the sample inventory was a core project activity. The development of standard operating procedures and plans to assure sample quality would safeguard the validity of results obtained from the analysis. Establishing procedures designed to capture the chain of custody of samples, extracts and results of analysis, supported down-stream commercialisation activities. As the research programme developed, the number of samples was expected to increase. Adding to the complexity of the data management challenge within the NutraMara programme, was that multiple locations would be involved in the analysis and characterisation of sample materials.

Reliably tracking samples and the results of analysis was essential for repeatability and for compliance with any legal or regulatory obligations: requiring steps to be taken to eliminate data entry errors or other failures that could jeopardise the validity of results.

3.2. Assessment of marine resources

3.2.1. Irish fishery captures

The composition of the Irish fish catch can be subdivided into stocks of deep sea demersal, pelagic fish and shellfish. Typical species in different groupings are demersal - cod, saithe, haddock, whiting, hake, megrim, monkfish, ling; pelagic - mackerel, horse mackerel, herring, sprat, sardines; shellfish - nephrops (Dublin Bay prawn), scallops, mussels, crabs, lobsters, squid, cuttlefish. Deep water fisheries include species like the orange roughy, but this category is in decline, with the most recent landings figure being 667 tonne. On the basis of landed weight, pelagic fish dominate the catch (Table 6). Despite making up only around 15% of the landings by weight, shellfish, particularly *Nephrops*, contribute over a third of the landed catch value. Further subdividing the catch into species, the top 10 species in terms of value are shown in Table 7

Table 6 Average annual landings of fish and shellfish 2005-2008

Fishery	Landed weight (t)	Value (€)
Demersal	30,929	59,453,467
Deep water	1,341	1,515,063
Pelagic	179,608	65,382,164
Shellfish	35,211	74,126,431

Source: Sea Fisheries Protection Authority

Table 7 Top species in Irish fisheries by value for 2008

Species	Live weight (t)	Value (€000)
Nephrops	9,391	39,819
Monkfish	7,302	28,914
Mackerel	25,738	19,123
Hake	7,584	16,664
Great Scallop	1,116	11,599
Horse Mackerel	35,640	11,439
Megrim	3,167	9,574
Blue Whiting	76,469	9,182
Edible Crab	6,331	9,149
Haddock	4,059	6,812

Source: Sea Fisheries Protection Authority

Herring has also been a significant stock in terms of landed weight in Ireland. A total of 33,167 tonnes were landed in 2006. Catches of herring are currently at a relatively low level, there is some uncertainty over stocks and the prices of some segments of the market have collapsed (Stock Book, 2008). The Irish quota for Herring in 2009 was 29,191 tonne, with an estimated landing of 19,983 tonne into Irish ports during 2008.

3.2.2. *Finfish and shellfish aquaculture production*

Aquaculture produces around 58,000 tonne of fish and shellfish per annum, just under 25 % of the equivalent figure for capture fisheries. The aquaculture of shellfish, principally oysters and mussels, has generally increased over the past 15 years. Smaller amounts of clam (*Ruditapes philippinarum*) and scallop (*Pecten maximus*) are also cultivated with mean production of 182 tonne and 72 tonne per year respectively. Mussel aquaculture is dominated by bottom grown stock, with the remaining fraction being rope grown. Finfish aquaculture is essentially static, with a peak salmon production in 2001. Other cultivated species include Arctic char, perch, turbot and ornamental fish.

3.2.3. *Discarded fish*

Waste products can be generated at any point in the value chain from harvest to consumer. Landing size regulations and species quotas have the unfortunate outcome of generating waste, in the form of fish discards at sea. Discards are notoriously difficult to estimate as the proportion of discarded catch varies seasonally, with location and with different fishing gears. An estimate of the weight of discarded deep water, demersal and pelagic fish and shellfish for Irish landings is shown in Table 8. The discard estimates used by Archer *et al.*, (2001) are consistent with more recent estimates from certain Irish fleets, although there is variability by gear, fishing area and targeted species (Borges *et al.*, 2005).

Table 8 Estimates of fish discarded at sea from Irish vessels landing into Ireland in 2007²

	Landings 2005-2008 (t)	Average discard per catch (%)	Discard (t)
Deep water	30,929	50	15,465
Demersal	1,341	12.5	168
Pelagic	179,608	12.5	22,451
Shellfish	35,211	43	15,141
Total	247,089		53,224

Developing discards as a source of functional food components, although superficially attractive, is not a reliable source. The planned reform of the EC Common Fishery Policy aims to introduce regulations that *inter alia* will prohibit discarding and require all the catch to be landed (European Commission, 2014).

² Average discard rate estimates are based on percentages provided by Archer *et al.*, 2001, with Shellfish discards based on a Nephrops fishery, Catchpole *et al.*, 2006.

3.2.4. Processing at sea

Depending on the species and market, fish may be processed at sea with the waste generally disposed of overboard – typically, viscera and heads. At-sea processing is generally greater for demersal fish than pelagics. Shellfish such as *Nephrops* are often ‘tailed’ – the head and claws are removed. A comparison of live weight with landing figures (Table 9) indicates that pelagic fish are generally not processed at sea, while demersal and some shellfish are processed at sea. The mass of fish processed at sea is close to zero for pelagic species, around 2,300 tonne for demersal species, 39 tonne for deep water species, with the shellfish figure dominated by the figure of approximately 3,000 tonne of *Nephrops*.

Table 9 Processing at sea calculations for the 10 top species landed in Ireland in 2007³

Species	Live weight (t)	Landed weight (t)	Processed at sea (t)	Processed at sea (%)
Mackerel	48,417	48,417	0	0
Nephrops	9,314	5,975	3339	36
Crab	12,518	12,434	84	1
Monkfish	3,480	2,776	704	20
Horse Mackerel	39,091	39,091	0	0
Megrim	2,034	1,937	97	5
Herring	30,821	30,821	0	0
Haddock	3,549	3,302	247	7
Lobster	242	242	0	0
Cod	1,793	1,566	227	13

Source: Sea Fisheries Protection Authority

3.2.5. On-shore processing waste

Nearly two thirds of the waste from fish and shellfish processing is generated during on-shore processing (Archer *et al.*, 2001). The nature of the waste is dependent on the species, the on-ship processing and the destination market. The latter two factors may vary within a species, for example *Nephrops* tails as scampi or whole *Nephrops* as langoustines for export. Fish fillets and other sections of meat are removed for human consumption. This leaves the viscera, fins, bones and, depending on the product, head and skin to be discarded as waste. Shells may also form part of the crustacean waste stream. Volumes of waste have been estimated from the fraction of edible product in different categories of fish (Archer *et al.*, 2000) and these fractions are shown in Table 10.

³ Note large variation in landings figures in comparison to other years for example in mackerel.

Table 10 Edible portions of fish

Species	Edible portion (%)
Demersal general	43
Cod	50
Haddock	43
Hake	50
Lemon Sole	42
Ling	48
Plaice	35
Whiting	38
Herring	53

Source: Archer et al., (2001)

Potential wastes by weight fractions are similar for shellfish and finfish. Average edible fractions are 39% for crustaceans and 20% for molluscs (Archer et al., 2001). The economically important species in Ireland are *Nephrops* (24% edible whole, 58% unshelled tails edible), crab (32% edible), and lobster (44% edible). The edible portions of some shellfish species are outlined in Table 11. The average edible portion for crustaceans and molluscs is 39% and 20% respectively.

Table 11 Edible portion of some shellfish species

Species	Edible portions (%)
Crab	32
Lobster	44
<i>Nephrops</i> Whole	24
Unshelled tails	58
Brown Shrimp	35
Prawns	40
Oyster	14
Cockle	12
Winkle	23
Scallop	14
Mussel	14
Whelk	42

Source: Archer et al., (2001)

The approach used by Archer et al., (2001) assumes that most of the catch is processed to fillets. The majority of the Irish fish catch is, however, exported whole (88% of the fish catch exported whole, CSO figures 10 year mean). This reduces the potential processing waste to an average of 11% waste by weight (Table 9). The Irish landings figures can therefore be split into whole and filleted fractions for fish to estimate waste percentages. Depending on the species in

question, between 15% and 90% of crustacean landings are exported whole (Pfeiffer, 2003). Mussels are mostly marketed whole, whereas whelks and scallops are usually shelled. If the landings figures are disaggregated to reflect species level differences before summarizing, the estimated waste volumes are shown in Table 10. The largest source of processing waste is from the pelagic sector (Table 12). The majority of waste from mollusc and crustacean fisheries is made up of shell waste.

Table 12 Landings of fish and shellfish in Ireland (2005-2008 average) and their waste production.

Fishery	Landings Weight (t)	Maximum waste (%)	Waste (t)
Deepwater	1,341	52	213
Demersal	30,929	57	5,109
Pelagic	179,608	47	27,516
Shellfish	35,211	70	16,258
TOTAL	247,089		49,096

Source: SFPA; Percentages based on Archer *et al.*, (2001)

3.3. Aquaculture waste

Aquaculture activities are concentrated in the south and west of Ireland, with nearly 60 % of licensed sites lying in the counties of Donegal, Cork and Galway (Browne *et al.*, 2008). As with caught fish, the type of processing determines the level of waste. Overall, an estimated 2,778 tonne of waste arises from processing farmed fish (Table 13).

Table 13 Percentages and volume of waste from processing of farmed fish.⁴

Product		Processed	Production	Waste %	Total	Type
			2007 (t)		waste	
Gutting for export	Salmon	70%	6,946		764	Viscera
	Trout	26%	329	11%	36	
	Other	20%	10		1	
Filleting	Salmon	10%	992		496	Skin, frames, heads
	Trout	62%	786	50%	393	
	Other	60%	29		14	
Other processing	Salmon	20%	1,985		992	Heads, skin frames, bones, meat
	Trout	12%	152	50%	76	
	Other	20%	10		5	
Total			11,238		2,778	

⁴ Production figures from Browne *et al.*, (2008), the split of product destination and waste percentages follow Pfeiffer, (2003). Trout includes both sea reared and freshwater sources.

Mussels dominate shellfish production; rope mussels are graded onshore with waste generated as shells, and rejected undersized stock. Bottom-cultured mussels, since they are graded at sea, generate little waste (Table 14).

Table 14 Wastes arising from processing of cultured mussels. ⁵

Source of waste	Rope mussel (11,200 t in 2007)			Bottom mussel (18,270 t in 2007)		
	Processing (%)	Waste (%)	Waste (t)	Processing (%)	Waste (%)	Waste (t)
Grading	50	20	1,120	10	20	365
Cooking	40	25	1,120	10	32	585
Meat extraction	10	75	840	0	75	0
Total			3,080			950

Other cultured shellfish include oysters; clams and scallops generate negligible amounts of waste. Since only scallops tend to be processed, this approximates to 28 tonne of shell waste and 19 tonne of organic waste generated in 2007.

3.4. Seaweed harvesting (inc. aquaculture)

The brown alga *Ascophyllum nodosum* and the coralline red algae known as maerl, together account for the majority of the 36,000 tonne of seaweeds harvested from Irish waters (Table 15). In general, seaweeds are not processed in a reductive manner to remove unwanted components; hence the waste fraction from processing is relatively low and estimated to be around 22 tonne per annum. Irish seaweed aquaculture activity in 2008 is confined to 4 licensed areas producing 5 tonne (Irish Seaweed Industry pers. comm.).

Table 15. Irish seaweed biomass from wild harvest and aquaculture in 2008. ⁶

Species	Production 2008 (wet t)	Primary processing Waste (t)	Secondary processing Waste (t)
<i>Alaria esculenta</i>	5	4 %	0.02
<i>Ascophyllum nodosum</i>	25,000	4 %	1000
<i>Laminaria hyperborea</i>	2,000	4 %	80
<i>Laminaria digitata</i>	500	4 %	2
<i>Fucus vesiculosus</i> and <i>F.spiralis</i>	10	4 %	0.04
<i>Fucus serratus</i>	300	100%	300

⁵ Waste percentages are taken from Pfeiffer, (2003), with production figures for 2007 from Browne *et al.*, (2008). The grading waste for bottom-cultured mussels contains only the proportion that is estimated to be graded ashore.

⁶ Data collected by NutraMara consortium from companies.

Carageen moss (<i>Chondrus crispus</i> and <i>Mastocarpus stellatus</i>)	60	4 %	2.4	2
<i>Palmaria palmata</i>	125	4 %	5	2
Maërl	8,000	4 %	320	
<i>Asparagopsis armata</i>	1	4 %	-	1
Total	36,001	4 %	1440	22

Ascophyllum nodosum followed by maërl and *Laminaria hyperborea* account for the largest amount of seaweed collected from Irish wild sources. In general 4% of the seaweed processed into a milled dried product ends up as waste (Irish Seaweed Industry pers. comm.), which yields 1,440 tonnes of waste. Several other companies then use the dried seaweed meal as input material for extractions and secondary processing (about 600 tonnes per annum). Several companies performing extractions produce together about 400 litres of sludge containing 10 % solids, i.e. 40 kg of solids a day or ca. 14.6 tonnes per annum (Irish Seaweed Industry pers. comm.).

All other species are mainly used for food or agricultural purposes and very small quantities are used for secondary processing or in the cosmetics industry. *Fucus serratus* is predominantly used for seaweed baths and once used is discarded. Used in this manner, where it is soaked in hot water is likely to remove the water-soluble fraction and denature many enzymes and other molecules from the seaweed. The amount of solid waste of seaweed generated by seaweed spas is estimated at 300 tonnes or 75 tonnes dry weight.

3.4.1. Microalgae

Microalgae are microscopic mostly unicellular organisms belonging to a large range of taxonomic entities. The major phylogenetic groups that contain microalgae are diatoms (c. 10,000 species), dinoflagellates (c. 2,000 species), Cryptophyta (c. 200 species), Haptophyta (Prymnesiophyceae; c. 500 species), Xanthophyceae (c. 600 species), Chrysophyceae (c. 1000 species) and representative groups within green algae (total number c.7,500 species), red algae (total number c. 6,000 species) and brown algae (total number c 1,500 species) (species figures adopted from Falkowski and Raven, 2007).

Although some species form blooms in natural environments, with high cell numbers particularly common in tropical eutrophic freshwater bodies, the actual biomass and the complex community structure renders commercial exploitation of natural stock as not feasible. Microalgae therefore need to be cultivated to obtain the required biomass for the desired purpose.

Despite the vast diversity of algae that exist, clearly not all are suitable for cultivation or commercial application. In selecting a species for cultivation factors such as ability to produce biomass quickly, actual growth rates, commercial processing requirements and product

stability need to be considered. Unfortunately large cells which would theoretically provide suitable biomass more quickly, commonly exhibit notoriously low productivity.

The options for microalgal cultivation in Ireland are limited; with outdoor cultivation unlikely due to largely unfavourable environmental conditions, indoor systems offer a solution. Such systems however, are complex and costly to run, (see Borowitzka, 1992; Pulz and Gross, 2004; and Milledge, 2010) possibly limiting their use for the production of high-value products. Generally there are two approaches to sourcing microalgae for commercial application; either algal samples are collected from natural environments, or species or strains previously isolated and available from culture collections worldwide are used.

3.4.2. *Species from the wild*

The isolation of microalgal species from natural populations involves the collection of water (pelagic) or biofilms (benthic) samples, followed by a series of isolation steps in the laboratory to obtain mono-specific cultures. In their natural environment the distribution of different communities and groups of species within these depends on season, location, physico-chemical factors (e.g. light, salinity, nutrients, pH), turbulence and biotic factors such as grazers and pathogens. Several isolation and separation steps need to follow the initial sample collection, with the aim to obtain an axenic (no contaminants, particularly bacterial, viral or fungal) and monospecific isolate which will grow under particular laboratory culture conditions which need to be adjusted for different species. The general challenges of this approach can be summarised as follows: poor survival of cells in sampling containers; change in species composition and loss of species before start of culture; difficulty in obtaining sterile cultures not infected by bacteria or fungi which were naturally associated with microalgal cells.

3.4.3. *Species from algal collections*

The more common and practical approach for commercial applications is the purchase of microalgal species. Different strains of these, from within a large range of available culture collections cost between €15-€100 each for small quantities (20-40ml at varying cell densities) (See Appendix 2 for details of supply). These require cultivation to obtain larger amounts of biomass, which allow the assessment (and modification) of biochemical composition and potential bioactivities.

Advantages of commercial sources of algal materials over open sourced are,

- They are monospecific and mostly axenic, and thus suitable for the production of food ingredients;
- Traceability shows that cultivation is possible at least at a small scale, and culture conditions then can be modified to optimise the production of certain compounds of interest to the food industry, and
- The diversity of species available commercially is considerable, with most culture collections supplying hundreds of species and different strains within these.

Figure 3 illustrates the diversity of species which are commercially available at just one such collection (Oban); SAG in Göttingen also provides a range of 2400 algal strains (representing 538 genera and 1424 species)

(http://www.epsag.uni-goettingen.de/cgi-bin/epsag/website/cgi/show_page.cgi?kuer_zel=about.)

Figure 3 Screenshot of options available from a sample culture collection⁷

Basic Search >>

Genus Name (545)	Strain Name (1307)	Strain No (2803)
<ALL>	<ALL>	<ALL>
Acanthamoeba	Acanthamoeba astronyxis	1001/1
Achnanthes	Acanthamoeba castellanii	1001/2
Acrochaetium	Acanthamoeba comandoni	1001/3
Actinastrum	Acanthamoeba griffini	1002/1
Agitata	Acanthamoeba hatchetti	1002/2
Agmenellum	Acanthamoeba palestiniensis	1002/5
Albomonas	Acanthamoeba polyphaga	1002/7
Alexandrium	Acanthamoeba royreba	1002/9
Allantion	Achnanthes sp.	1003/3

For more information, please view the [key to strain data](#).
 This will open in a separate window which you can minimise and maximise to view when needed while you examine the strain data.

[List of strains referenced in scientific publications](#)

Please Note the CCAP collection catalogue is constantly being added to and amended, for the most up to date information on a strain, please contact us.

Further potential sources of microalgal samples for large scale cultivation are microalgal cultures currently used in aquaculture as animal feed. Internationally, diatom species such as *Chaetoceros*, *Skeletonema*, *Thalassiosira*, *Nitzschia* and *Tetraselmis*, and others, are most commonly used for animal feed (Borowitzka, 1997; Becker, 1994; Raja et al., 2008; Brown, 2002). Whilst there may be stocks of microalgae available in Ireland for up-scaling, their potential is limited as the species have been chosen for particular purposes such as nutritional consistency, or simply local expertise, with less consideration to product range or new product potential (Borowitzka, 1997). To be used in food applications, such as functional ingredients, production facilities for these species would have to comply with food product standards. The potential of high-value products derived from microalgae grown under suitable conditions is significant and thus further research should concentrate on the application of new species, screening of new compounds and efficient, but adequate, modes of cultivation.

3.5. Use of Irish marine materials as functional ingredients

Knowledge about relevant compounds and availability of marine source material for use as functional ingredients varies greatly. For example, the characteristics of the algal polysaccharide alginate obtained in Ireland from *Ascophyllum nodosum* and a small number of other brown seaweeds are relatively well understood. In contrast, some marine origin bioactive proteins are

⁷ (http://www.ccap.ac.uk/ccap_search.php?mode=attr; accessed 17/12/2010)

still being defined; hence little is known about their availability or requirements to process them for food use.

Estimates of waste materials from fishery and aquaculture species are summarised above in Tables 11, 12 and 13. The majority of waste from the major pelagic species is processed in existing facilities into fishmeal. Any large-scale processing into functional ingredients would have to offer a price advantage to the existing route for this material.

Demersal fisheries provide a lower volume of potential waste. This source, along with aquaculture, is not as dispersed throughout coastal areas as are pelagic landings. This may favour more small-scale processing rather than collection and transport to a single processing site, which incurs additional costs and introduces the risk of spoilage for some materials such as fatty acids. Changes in export and processing patterns for stocks may also affect the availability of wastes. For example, the promotion of more Irish-based processing, particularly for demersal stocks, may increase the volume of available waste. Algal waste from existing processing, in addition to being low in volume, is unlikely to contain activities of interest. And volumes from the algal aquaculture are presently also low. Together these sources are unlikely to be of immediate interest.

The diversity of seaweed species that grows in the wild remains of interest as a source of bioactives with functional food potential. Some species such as *Ascophyllum nodosum* and *Laminaria hyperborea* are already harvested from wild standing stock. The challenge facing any wide-scale harvesting and subsequent use as a sustainable source of bioactives, is a lack of reliable data concerning the distribution of species and available biomass. Concerns have been expressed regarding the extrapolative nature of the estimates of Irish seaweed biomass (Bruton *et al.* 2009). In light of this concern, and lasting knowledge-gaps on distribution and volume, there may be insufficient biomass to support the exploitation of species other than the algal kelps and fucoids. Species of maerl (coralline red algae) grow very slowly and it is not clear that any level of harvesting is sustainable (Maerl is also protected by the EU Habitats Directive).

The diversity of European macroalgal species has not been extensively screened – there is an uneven pattern of investigation across taxa, with different target compounds and with different extractions. Further research may identify and quantify previously overlooked resources. The availability of more precise data of the potential resource and any harvesting limitations for standing stock may open opportunities for niche aquaculture of specific species. Markets may exist for purified high-value molecules based on relatively low harvests (e.g., specific pigments) that could be met through aquaculture.

The largest volume of material from Irish shellfish aquaculture comes from mussels. *Mytilus* spp.: both *Mytilus edulis*, and *Mytilus galloprovincialis* occur in Ireland with overlapping distributions that include hybrids between species (Gosling *et al.*, 2008). There are few published studies of components of *Mytilus* relevant to functional foods, so further

characterisation and extraction development may create opportunities from this widely available resource.

Although relatively low in volume at present, abalone culture is a high value capital-intensive form of aquaculture that may provide opportunities for synergistic production of functional food components. Ireland currently has a small number of farms, with two main producers. Future plans for production include increasing the capacity for individual farms up to around 80 tonne (Tower Products, 2014). Local processing of this output would create waste (viscera) of the order of 16 tonne. (waste percentage from Sun *et al.*, 2010). The species of abalone under culture in Ireland are *Haliotis discus hannai* (Ezo awabi) and *Haliotis tuberculata* (European abalone). *H. discus hannai* is generally more prevalent due to a superior production in aquaculture.

Table 16 Potential waste for processing available in Ireland ⁸

Species	Bioactive compounds	Waste available (t)	Yield (if known)	Potential Extraction (t)
Mackerel	Protein, peptides		-	-
	omega-3 (n-3) PUFAs	4,170	3 %	128
	Collagen		36-54 %	1501-2252
Horse Mackerel	Protein, peptides	5,774	-	-
	omega-3 (n-3) PUFAs		3 %	177
Blue whiting	Protein, peptides	12,388	-	-
	omega-3 (n-3) PUFAs		3 %	380
Salmon	Protein, peptides	2,252	-	-
	omega-3 (n-3) PUFAs		1.5 %	34
<i>Ascophyllum nodosum</i>	Fucoidan, alginates, fucoxanthin	1,015	30 %	305
<i>Laminaria digitata</i>	Fucoidan , laminarin, alginates, Fucoxanthin	3	30 %	1
<i>Fucus spp.</i>	Fucoidan , laminarin, alginates, fucoxanthin	300	30 %	90
Mussel	Protein,Peptides	4,030	-	-
	omega-3 (n-3) PUFAs			

⁸ Omega-3 yields for pelagic fish are based on an aggregate figure from Zuta *et al.*, (2003). The value for salmon is based on the table for long chain omega-3 content in Rubio-Rodríguez *et al.*, (2010). Figures for algae are based on alginate only (Moen *et al.*, 1999), seasonality, storage and processing are known to greatly affect the yields of polysaccharides from algae. For comparison, Laminaran and fucoidan are summarised at 14% and 5% of dry weight for *Laminaria spp.* by Reith *et al.*, (2005). Nephrops waste is based on 50 % landings being tail only with a further 50% receiving processing onshore (Archer, 2008). Crab waste is based on an estimate of 85% on-shore processing (Pfeiffer, 2003). Nephrops yields are for chitin (Healy *et al.*, 2003) and total carotenoids (Shahidi and Synowiecki, 1991). The figure for collagen recovery is only shown for mackerel, but the same figures (Arvanitoyannis and Kassaveti, 2008) can be applied to other fish.

Nephrops	Chitan, Chitosan,	3,569	20 %	713
	Astaxanthin (carotenoids)		0.009 %	0.32
Brown crab	Chitan, Chitosan,	3,659	-	-
	Astaxanthin			

Microalgae are frequently cultured as part of aquaculture operations (often as food for zoo plankton that are then fed to juvenile fish). An aquaculture-based business model that included the production of microalgae as feedstock and functional food ingredients, may create conditions to justify shared co-production of microalgae. This approach to cultivation would need to comply with regulations concerning food production for human consumption.

Changes in the ecosystem may result in the creation of additional sources of novel food components. For example, small pelagic fish are becoming relatively more abundant; a trend that is likely to continue under climate change (Perry *et al.*, 2005). Boarfish (*Capros aper*), is such an example; taken in an industrial fishery with landings of 21,584 t in 2008 (Marine Institute Stock Book, 2008), this catch is an increase on the 2005 baseline catch of c. 200 t in 2005. With no published research on novel food components from boarfish (they are too small and bony to be of direct food value) they are used to make fishmeal.

Balanced against this apparent opportunity is the possibility that the fishery may come under greater regulation, as unregulated harvests are not generally permitted to continue without assessment. Furthermore, the climatic and ecological conditions that lead to greater stocks are not well understood and the available stock could rapidly disappear even without harvesting pressure.

3.6. NutraMara sampling plans

3.6.1. Introduction

Developing functional ingredients from marine organisms is reliant on the inherent bioactivity of natural chemical compounds found within the organism. Bioactivity in natural sources is based on molecules that are likely to vary in their concentration in the target organism (Craigie *et al.*, 2008). These changes in concentration may relate to changes in environmental conditions experienced by the organism (seasonal, site or other changes) or there may be intrinsic processes such as maturation, aging or death, which cause concentrations of bioactives to vary. They may also result from contaminations during the collection or handling process, or as a result of contamination by bacteria, fungi or insects at the point of collection. A further level of complexity is that bioactivity is often described with respect to a heterogeneous extract: a term such as 'fucoidan' or 'chitosan' does not describe a single molecular structure.

When screening for bioactive compounds from natural sources, initially the exact molecule(s) and mechanisms of activities are unknown. It is also possible that effects are due to a

synergistic action among different parts of an extract. For example, Connan *et al.*, (2006) attribute differences among algae in the relationship between antioxidant assay (DPPH) response and phenolic content to possible variation in the pool of phenolic compounds or to interference from other molecules with antioxidant properties (such as carotenoids).

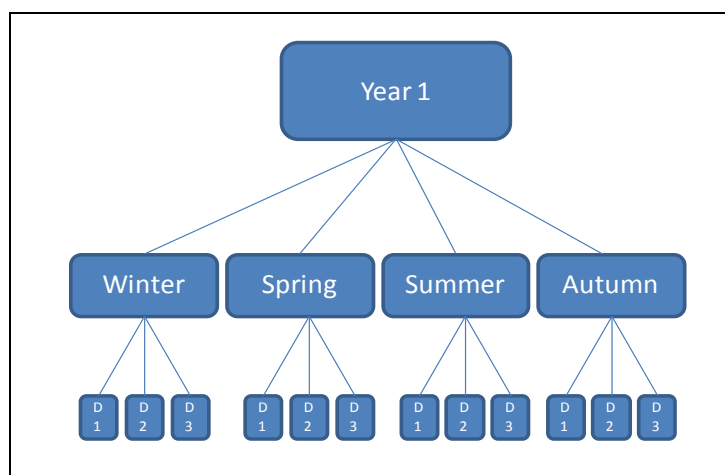
An understanding of the compositional variations often found in marine organisms is essential in attempting to make use of naturally occurring compounds. Without establishing clear causal relationships between consumption of a naturally occurring bioactive and desired health outcome, attaching the all-important health claim will be impossible. The same data is required in designing the production system that will process raw materials into a functional ingredient. Prior knowledge of any compositional variation at the input stage of the conversion process is necessary in designing robust systems with the capability of producing stable products. At the heart of the various processes and activities that contribute to the discovery of bioactives from marine organisms is the application of rigorous scientific methodologies at all stages of research.

3.6.2. Sampling to test ideas about variation in concentration

The methods for testing hypotheses about variation in mean concentration or activity of an extract are well established and use Analysis of Variance (ANOVA), the standard statistical test for comparing means. A key issue for ANOVA is the appropriate distribution of repeat measurements (replicates) to test the hypotheses of interest. In the case of seasonal variation, if different seasons are to be compared then replicated dates are required within each season. Without replicate dates, the observer cannot logically conclude that seasons are any more different than separate dates within a season - as the information on dates within a season is lacking (Underwood, 1997). The same logic applies to spatial sampling. A comparison of two sites, 10 km apart cannot discount the probability that concentrations are not even more different a short distance (metres) away from one of the sites. Studies that attempt to reveal spatial or temporal structure typically use a nested or hierarchical ANOVA (e.g., Pavia *et al.*, 2003). In these designs the replicates are partitioned in space and/or time to avoid the potential logical flaws outlined above. A typical temporally-nested design is outlined in Figure 4. Non-parametric alternatives to ANOVA are possible, but a balanced ANOVA is generally robust to departures from normality that require non-parametric designs (Underwood, 1997). Prior consideration of the statistical approach required to test a hypothesis informs decisions concerning the nature and extent of sampling activity.

The example in Figure 4 illustrates an approach used for sampling materials to test for seasonal variation. This nested design uses three replicates; generally considered as a minimum number of replicates (Pavia *et al.*, 2003 used 6 individuals at each sampling so more replicates may be generally advisable, if possible). Although a mean can be calculated from two replicates, such a design is vulnerable to any loss of samples or failure during analysis and is likely to lack statistical power - the ability to detect differences when they exist.

Figure 4 A nested design used in describing seasonal variation



Depending on the study, it may be preferable to pick random dates within each season. Ideally potentially confounding processes should occur randomly in different seasons (e.g., sampling on neaps equally distributed among months). It may be preferable to sample at the same state of tide to minimise variation with such covariates. In some cases sampling may only be possible at certain states of the tide.

A design to compare the seasonal patterns among a small number of different shores ($n = 5$) is indicative of the extent of sampling in NutraMara; this might have 360 replicates, made up as 5 shores \times 2 sites within each shore \times 4 seasons \times 3 dates within each season \times 3 replicates at each date.

3.6.3. Sampling approaches in NutraMara

Given that NutraMara is likely to generate relatively large amount of extracts, some levels of sampling may not be relevant to the overall programme. For example, age-related variation may not be relevant if harvesting or waste supply is unlikely to discriminate between individuals without extra cost. Therefore it is the question that defines the type of sampling programme required. Even addressing a limited number of factors in a nested design could create a large sample processing overhead. The adoption of a question-based approach (with reference also to any previous studies) may lead to a fully nested sampling design, a structuring of sampling to examine variability with respect to a particular variable (e.g., reproductive status) or an experimental approach. When the active elements of an extract are unknown, the variation in composition of material of the same species from different times and places can interpret variation in activity. Even in the absence of formal hypothesis testing within a nested ANOVA, sampling should include material from different dates and locations so that a representative picture of natural variability is established.

While there may be flexibility in the time and location in which some marine sources could be sampled (mostly, but not exclusively, seaweeds), other marine sources could be dependent on industrial processing. There is little point in seeking or defining high bioactivity at times or

locations outside when the bulk of the resource will be available. Within a sampling period there may be variations in bioactivity (e.g., seasonal omega-3 profile decline in fish).

When material is processed (e.g., protein hydrolysates or conversion to chitosan), a number of factors may influence yield, including sensitivity to raw materials. The question again defines what sampling is appropriate, although the dependency on industrial process requires that information on timing and logistical issues at the time of sampling should be recorded.

Requests for sample materials should be accompanied by a description of the questions that are being addressed with the samples collected. This should be used as the basis for a discussion of a sampling programme that complements the intended use of material. Dependant on the question and the effort allocation available for sample processing, an appropriate sampling scheme can be devised. This may include a nested design, a focus on a specific variable or a limited number of samples.

Where the material is collected from an intermediary (e.g., processor, fisheries co-op); information should be collected on a species by species basis. Such information should cover the availability of material, any restrictions on sampling and any known quality issues.

In the absence of a hypothesis driven approach, it is preferable to sample material from a range of sites and dates so that an estimate of background variability for different bioactives can be made from the collected material. For most algae this should include notes on the reproductive maturity of collected material.

3.7. Sample database

The NutraMara sample database was designed to record the flow of material from collection to use within the consortium. It provides users with search facilities allowing samples and actions completed on samples to be identified and tracked on an individual basis. The sample database evolved from a desk-based system, which required a centralised manual input, to a web-based system that allows remote input and provides users with search facilities.

The NutraMara database contains details of 613 samples from 39 species of algae and fish, together with results generated by submitting samples/extracts to various bioassays. The submission of 5,800 extracts to bioassays resulted in 3000 “hits”. Database users can interrogate it via queries designed to generate reports in tabular or graphic formats. Typical queries include,

- Number of samples collected in a given time frame grouped by species/location.
- List of bioactive species sorted by available weight.
- Number of bioactive hits in a given time frame grouped by species/location/bioassay type/bioassay name/institution/season.

The NutraMara database was developed using the same architecture as the Beaufort Marine Biodiscovery project database; thus offering scope to integrate the two databases into a common marine biomaterials data repository.

The following Figures 5 and 6 below are examples of searches of the database for details of samples and the results of bioassays on samples obtained from the red seaweed *Palmaria palmate*. Users of the database are also provided with a mapping facility to identify the precise (GIS coordinates) location from where all samples were collected, thus facilitating return visits to collection sites.

Figure 5 NutraMara database screenshot for *Palmaria palmate* samples

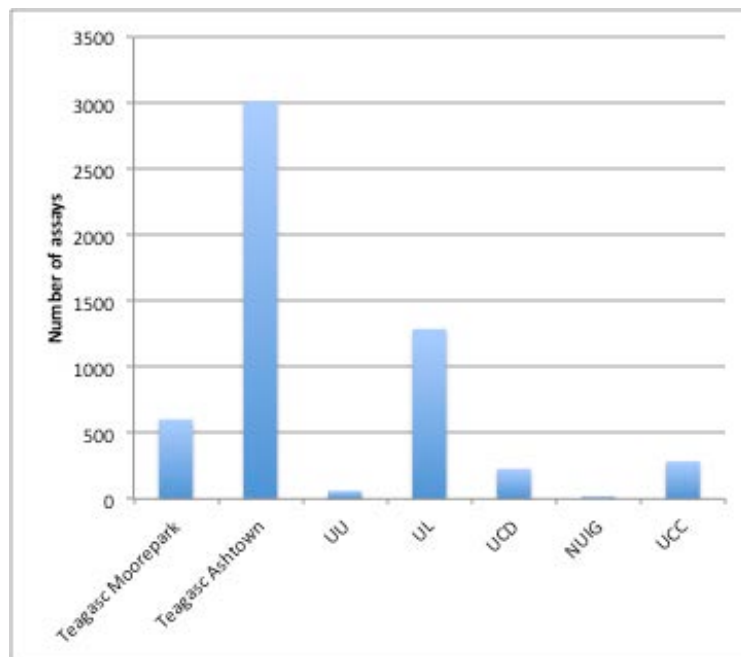
BDV Number	Species Name	Date Collected	Location	Collected By
1	71 <i>Palmaria palmata</i>	Apr 1, 2010	Doolin, Co Clare	NUIG
2	73 <i>Palmaria palmata</i>	Apr 13, 2010	Tra more beach, Mweenish Island, Co Galway	NUIG
3	74 <i>Palmaria palmata</i>	Apr 8, 2010	Ard Bay	NUIG
4	100 <i>Palmaria palmata</i>	Jul 12, 2010	Black Head, Co Clare	NUIG
5	117 <i>Palmaria palmata</i>	Oct 8, 2010	Black Head, Co Clare	NUIG
6	122 <i>Palmaria palmata</i>	Nov 4, 2010	Spiddal, Co Galway	NUIG
7	141 <i>Palmaria palmata</i>	Feb 24, 2011	Tra more beach, Mweenish Island, Co Galway	NUIG
8	383 <i>Palmaria palmata</i>	Dec 1, 2009	Spiddal, Co Galway	NUIG
9	409 <i>Palmaria palmata</i>	Mar 12, 2012	Spiddal, Co Galway	NUIG
10	607 <i>Palmaria palmata</i>	Apr 11, 2013	Spiddal, Co Galway	NUIG

Figure 6 NutraMara database screenshot for *Palmaria palmate* bioassays

BDV Number	Species	Bioassay Type	Bioassay Name	Bioassay Value	Screened By	Date Screened
1	71 <i>Palmaria palmata</i>	MTT assay	MTT assay		1 UU	Feb 8, 2011
2	71 <i>Palmaria palmata</i>	MTT assay	MTT assay		1 UU	Feb 8, 2011
3	117 <i>Palmaria palmata</i>	MTT assay	MTT assay		1 UU	Jun 14, 2010
4	117 <i>Palmaria palmata</i>	MTT assay	MTT assay		1 UU	Jun 14, 2010
5	117 <i>Palmaria palmata</i>	Cell cycle	Cell cycle		1 UU	Oct 11, 2010
6	122 <i>Palmaria palmata</i>	MTT assay	MTT assay		1 UU	Jun 14, 2010
7	122 <i>Palmaria palmata</i>	Cell cycle	Cell cycle		1 UU	Oct 11, 2010
8	122 <i>Palmaria palmata</i>	Cell cycle	Cell cycle		1 UU	Oct 11, 2010

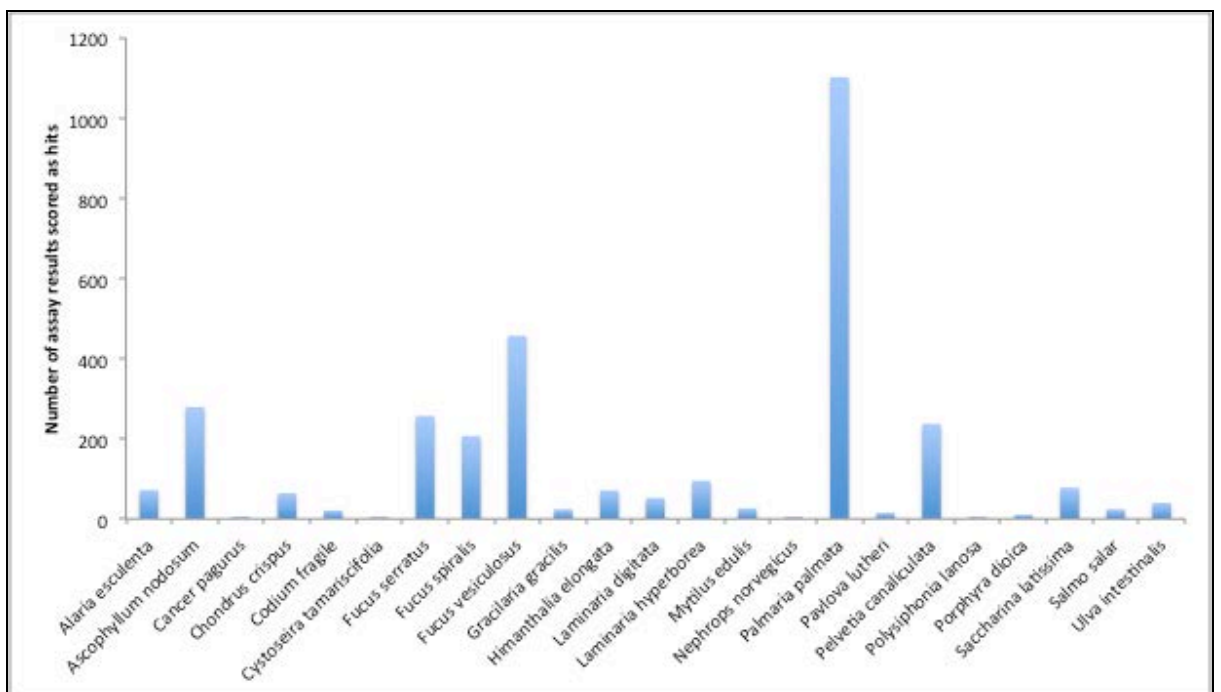
Materials were distributed upon request across the consortium, with details of 5647 assays held on the NutraMara database. The institutional breakdown of assays shows how most assays were carried out by Teagasc, but with contributions across the consortium (Figure 7).

Figure 7 Distribution of assays across the consortium



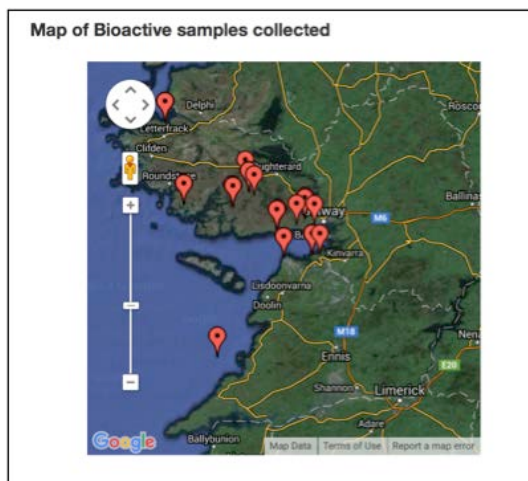
A range of species was investigated, and the number of assays of each species varied depending on the aim of the individual line of research. In summary, 23 taxa returned at least one assay result considered as a hit (positive) by the analyst. The pattern of hits was dominated by macroalgae, but this also reflects the effort put into these species (Figure 8).

Figure 8 Distribution of assay hits by taxon.



Material used by the different laboratories was generally destroyed during the process of extraction and assay (e.g., ASE accelerated solvent extraction at raised pressures and temperatures). NUI Galway retained 100g of collected materials to allow analysts the opportunity to go back and check unusual results.

Figure 9 NutraMara database screenshot showing location of bioactive “hits”



3.8. Sample repository

Sample materials were collected almost exclusively from 21 locations in counties Galway and Clare. A total of 1,500 kg of fresh biomass was collected and subsequently frozen at -80°C or freeze dried. Samples of collected and cultured species are securely retained at NUI Galway. The voucher materials from NutraMara were held in a freezer and as freeze-dried material in sealed bags. Most of the materials were maintained as individual freeze dried samples of 100g, which represents around 1 kg of fresh material. The majority of materials collected during the project were distributed to partner institutions for destructive analysis; however, some samples are available for further work on request from the Programme Director.

4. DISCOVERY AND CHARACTERISATION OF BIOACTIVE COMPOUNDS

4.1. Introduction

The NutraMara work programme identified macro-and microalgae, farmed species of fish and shellfish, and fish processing co-products as targets from which to extract compounds known to possess characteristics of functional ingredients. Bioactive compounds would be extracted from samples of macroalgae collected from various harvesting sites, cultured macro- and microalgae species, co-products obtained from Irish fish processing companies and samples of fish from Irish fish farms.

Various species of seaweeds, some of which are to be found in Irish waters, have been shown to be sources of lipids, carotenoids, polyphenols and polysaccharides. Samples of red, green and brown macroalgae, including *Chondrus crispus*, *Palmaria palmate*, *Codium fragile*, *Ulva intestinalis*, *Ulva lactuca*, *Ulva rigida*, *Pelvetia canaliculata*, *Fucus spiralis*, *Fucus vesiculosus*, *Fucus serratus*, *Alaria esculanta*, *Ascophyllum nodosum*, *Saccharina larissima*, *Laminaria digitata*, *Laminaria hyperborea* and *Himanthalia elgongata* were included amongst seaweeds identified as potential sources of bioactive compounds.

In addition to seaweeds collected from sampling sites, four macroalgal species were cultivated under controlled conditions, to provide a source of algal compounds through optimised cultivation. In selecting these species, consideration of abundance in the wild, suitability for growing in aquaculture facilities and reported as rich sources of bioactive compounds, informed the choice of *Laminaria digitata*, *Ascophyllum nodosum*, *Fucus serratus* and *Palmaria palmata* for controlled culture.

Samples of 14 microalgal species were obtained from UK, USA and German culture collections and pre-cultured in batches on receipt, and maintained in the NUI Galway Microalgal Strains Collection for further examination in the NutraMara programme.

Finfish species, included samples of whole fish and processing co-products from Atlantic salmon (*Salmo salar*), Rainbow trout (*Oncorhynchus mykiss*), Dublin Bay prawn (*Nephrops norvegicus*), Pacific abalone (*Haliotis discus hannai* Ito) and blue mussel (*Mytilus edulis*) collected from Irish aquaculture and fish processing facilities.

A specific source of farmed Atlantic salmon (*Salmo salar*) grown at the NUI Galway, Carna Laboratories, and the subject of feeding trials using combinations of macroalgae, was also included in experiments to extract and profile functional compounds.

4.2. Extraction of bioactive compounds

Standard extraction protocols for selected target compounds were used across the NutraMara consortium in support of chemical characterisation and biological studies; having been informed by desk studies. Pressurised liquid extraction using methanolic solvents was used routinely to extract antioxidant compounds and more specifically phlorotannins (Heffernan *et al.*, 2014a; Tierney *et al.*, 2012, 2013). In addition, a standard approach to protein and carbohydrate extraction was also used. An optimal alkaline soluble protein extraction occurred when using 0.12 mol/l NaOH, 0.1 g/100 ml NAC, a mass to volume ratio of 1:15 (w/v) and when stirring for 1 hour at room temperature (Harnedy and FitzGerald, 2013a). Atmospheric pressure solid liquid extraction using freeze dried powder and 0.1 M HCl at a ratio of 10: at 60 °C for 24 h at an rpm of 170 was the standard protocol when carbohydrate was the target material (Heffernan, 2015). Similar generic extraction approaches, all informed by desk studies, were developed for other bioactive compounds from algae and other marine bioresources (FitzGerald, 2014; Tierney, 2014; Heffernan, 2015).

Where necessary, purification and fractionation of extracts for biological studies and chemical characterisation followed a standard approach. This involved molecular weight cut-offs (MWCO) fractionation, followed by normal and reverse phase flash chromatography (if required) (Heffernan *et al.*, 2015; Heffernan *et al.*, 2014b; Tierney *et al.*, 2013; Tierney *et al.*, 2013a,b). This approach proved particularly useful in providing the quantities of enriched fractions required for profiling phlorotannins, which was also carried out using UPLC–MS/MS (Tierney *et al.*, 2013a; Heffernan *et al.*, 2015).

4.3. Algal derived compounds – macroalgae

The focus of the NutraMara work programme was upon the use of sustainable marine bioresources as potential sources of bioactive compounds. The NutraMara Feasibility Study identified a number of knowledge gaps concerning the availability and distribution of seaweeds and requirements for research to determine the extent to which the harvest of wild seaweed is a sustainable activity. However, despite these gaps, the work programme targeted a range of macroalgae species to explore as sources of bioactive compounds.

4.4. Polyphenols

Phlorotannins are the main class of polyphenols that occur in seaweeds. The typical recovery of these compounds was by solid liquid extraction using cold water, hot water and ethanol/water (80:20) since these solvents have previously shown to be effective for extracting antioxidants from macroalgae (Wang *et al.*, 2009; Ye *et al.*, 2008). However, experiences in NutraMara found the 80:20 ethanol:water proved to be the best extractant solvent for phlorotannins. Briefly, the extraction method involved, freeze dried macroalgal material (200-

250 grams) mixed with extraction solvent at a ratio of 1:10 w/v. The extractions were carried out at room temperature in an orbital shaker (MaxQ 6000 Shaker, Thermo Fisher Scientific, MA, USA) set at 170 rpm. Following this, the extractions were filtered three times through cotton wool and glass wool. Ethanol was removed from the extracts using a rotary evaporator (Heidolph Rotary Evaporator with WB eco bath, Germany) with the waterbath set at 40°C. All extracts were freeze-dried to remove water. Dried extracts were stored at - 80°C until further analysis.

An alternative extraction process, Accelerated Solvent Extraction (ASE®), otherwise known as pressurized liquid extraction (PLE), was also used to extract phlorotannins from species of macroalgae. Some extractions from natural products can involve lengthy and time consuming steps. An advantage of the ASE® process compared to more traditional techniques is that results are generated much more quickly. In addition, ASE offers opportunities to lower cost per sample processed by reducing solvent consumption, compared to other methods. The chosen PLE system was the Dionex PLE system (ASE 200, Dionex, Idstein, Germany). Typically, extraction involved mixing 2.5 g of freeze-dried algal mass with diatomaceous earth and 30 g of silica (Merck grade, 60 Å, Sigma Aldrich, St Louis, USA) and loading it into 33 ml sample cells. The automated extraction method used 70 % acetone in water and a pressure of 1,500 psi. The extraction time consisted of 3 cycles of 5 minutes, heat time 5 min, flush volume 50 %, purge time 60 s, static cycles 4, solvent Acetone:water 70:30 (v/v).

The recovered fractions were subsequently centrifuged at 3000 X g for 10 minutes to remove residual solids (SIGMA 2-16KL, Sigma Zentrifugen, Ostende am Hartz, Germany). Aliquots supernatants from each extract were dried under nitrogen (20 psi) using a TurboVap (Caliper LifeSciences, Runcorn, UK) and later freeze dried for 24 hrs to eliminate residual water.

4.5. Carotenoids

A solid liquid extraction process was employed to extract the carotenoids from species of macroalgae under investigation using hexane/acetone (70:30). This solvent system has previously been shown to be effective for extracting pigments from plant materials (AOAC, 1984). Within the NutraMara work programme, crude extracts were prepared by placing 10 g of a seaweed powder in a conical flask and adding the extraction solvent hexane/acetone (70:30) at a ratio of 10:1 (v/w). The mixture was then placed into a shaker (Thermo Scientific MaxQ6000) at room temperature for 24 hours. These extracts were filtered three times over a 24 h period through a Buchner funnel. The combined extracts were concentrated to remove all solvent using a rotary evaporator (BüchiRotavapour R-200 with a V710 vacuum pump) with the waterbath set at 50°C. Extracts were stored for later analysis.

4.6. Polysaccharides

Laminarins and fucoidans are the major polysaccharides in seaweeds. Within the NutraMara consortium, a traditional solid-liquid method of extraction of the seaweed polysaccharides was used. This process involved stirring powdered seaweed at 70°C for 2.5 h using distilled water and 0.1 M HCl as solvents. The extracted samples were then centrifuged at 9000 rpm for 30 min. The supernatant was separated and precipitated with ethanol overnight at 4°C. The precipitated extract was freeze dried and stored at -20°C for further analysis. The extraction yield (%) was calculated by measuring the mass of freeze dried extract over the initial mass of the sample (Kadam *et al.*, 2015; Strain *et al.*, 2015).

4.7. Lipids

4.7.1. Extraction of total lipids from macroalgae

Two methods were developed to extract total lipids from samples of macroalgae. Together, these methods generated extracts from six species of macroalgae - *Pelvetia canaliculata*, *Ulva intestinalis*, *Ascophyllum nodosum*, *Fucus spiralis*, *Fucus dichitus* and *Alaria esculenta*. A summary description of each extraction method is given below.

Accelerated Solvent Extraction of lipids from macroalgae

Extraction of lipids was carried out by Accelerated Solvent Extraction (ASE®), which is also known as pressurized liquid extraction (PLE) using the Dionex PLE system (ASE 200, Dionex, Idstein, Germany). Oil was extracted from each seaweed species in triplicate using an automated Dionex 200 accelerated solvent extraction system. 2.5 g of freeze-dried algal mass was mixed with diatomaceous earth and 30 g of silica prior to loading into 33 ml sample cells. Extraction conditions: 5 min preheat, 1,500 psi pressure, 120°C, heat time 5 min, flush volume 50 %, purge time 60 s, static cycles 4, solvent chloroform:methanol 2:1 (v/v).

Solvent extraction of lipids from macroalgae

To evaluate total lipid content, lipids were extracted from the samples with 2:1 chloroform/methanol. Specifically, 2 g of ground dry sample was weighed into a tube, 14 ml of the solvent mixture was added, the tube was closed in an atmosphere of nitrogen, and after 2 minutes in a vortex mixer the contents of the tube were filtered through Whatman No. 41 paper. The residue was re-extracted by 30 s treatment with 5 ml of solvent mixture in the vortex mixer, the resulting extract was filtered through Whatman No. 41 paper, the two filtrates were pooled and concentrated to dryness under nitrogen and the weight of the resulting residue was taken as the total lipid content of the sample.

4.8. Proteins and peptides

A desk study of published approaches to the extraction of protein from macroalgae was carried out within the NutraMara work programme and is summarised in a publication (Harnedy and FitzGerald, 2013a).

The desk study was carried out not only to detail best practices to date for extraction of the target molecules from marine sources, but also to identify gaps in knowledge with regard to extraction practices. Accordingly publications dealing with optimising the extraction of proteins, peptides, antioxidants and carbohydrates have been published by members of the NutraMara consortium including, FitzGerald *et al.*, (2012); Harnedy and FitzGerald, (2013a); Heffernan *et al.*, (2014a); Heffernan, (2015); Tierney *et al.*, (2012) and Tierney *et al.*, (2013a). This work provided the foundation for an integrated protocol for the extraction of crude samples of proteins, peptides and amino acids from red, green and brown macroalgae including *Palmaria palmata*, *Porphyra dioica*, *Chondrus crispus*, *Ulva sp./Ulva lactuca*, *Laminaria digitata*, *Fucus serratus* and *Alaria esculenta*.

The procedures used for the extraction of aqueous and alkaline soluble proteins from milled dried *Palmaria palmata* were based on methods described by Fleurence *et al.*, (1995) with some modifications. In the first instance the effect of NaOH (0.08-0.14 M) and N-acetyl-L-cysteine (NAC) concentration (0-0.5% (w/v)), mass:volume (1:10-1:30 (w/v)), agitation duration (0.5-3h) and extraction temperature (22-50°C) on the recovery of alkaline soluble proteins from milled dried *Palmaria palmata* was studied (Harnedy and FitzGerald, 2013a). The contribution of physical (osmotic shock (4°C and at room temperature for 3, 7 and 16 h) and shearing (homogenisation with an Ultra turrax at a low (15,000 rpm) and high (24,000 rpm) settings)) along with enzymatic cell disruption approaches (Celluclast® 1.5L and Shearzyme® 500L) (enzyme:substrate 1.2, 4.8 and 48.0 × 10³ units/100 g) on the extraction of aqueous and alkaline soluble proteins was also assessed (Harnedy and FitzGerald, 2013a). In most instances above the aqueous and alkaline soluble proteins were sequentially extracted. The effect of simultaneous extraction of aqueous and alkaline soluble proteins on protein recovery was also assessed. Crude aqueous and alkaline soluble proteins were separated from crude peptides and amino acids by isoelectric precipitation of the proteins. The concentration of protein in each extract was determined by the Bensadoun and Weinstein (1976) modification of the Lowry *et al.* (1951) protein quantification method.

The optimised protein extraction/semi-purification method was used to extract aqueous, alkaline and a combination of aqueous and alkaline protein extracts from *Palmaria palmata* samples *Porphyra dioica*. While the extraction protocol was optimised for extraction of protein/peptide/amino acids from the protein rich red macroalgal species *Palmaria palmata*, the method was also employed with other red macroalgal species such as, *Chondrus crispus* and *Porphyra dioica*, the green species *Ulva sp./Ulva lactuca* and the brown species *Laminaria digitata*,

Fucus serratus and *Alaria esculenta*. The protein in each fraction was quantified as described above and characterised by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, Laemmli 1970). Furthermore, a number of *Palmaria palmata* protein extracts were used as substrates for the generation of hydrolysates. Furthermore, the non-protein nitrogen (crude peptides and amino acids) fraction was used as the starting material to identify and quantify non-protein nitrogen components in macroalgae.

In addition to the integrated protocol described above, the extraction of protein from macroalgae (seaweeds) was also carried out according to known methods (Wong *et al.*, 2001; FitzGerald, 2012), with some modifications also being made. In essence, the generic extraction process was, freeze dried seaweed powder was suspended in HPLC-grade water (1:20 w/v). The suspension was sonicated for 1 hour in an ultrasonic bath and then shaken overnight in a water bath set to 35°C. The mixture was clarified by centrifugation at 4°C and 10,000 g for 40 minutes. The pellets were combined and suspended in 400 mL of HPLC-water and subjected to a second extraction procedure as described. The supernatants from both extractions were pooled and protein precipitation was carried out at 4 °C. The precipitation involved bringing the supernatant to 80% ammonium sulfate saturation, stirring for an hour and then leaving to stand for another hour. The mixture was centrifuged at the same conditions as described above. The pellet was suspended in a minimal volume of distilled water and dialyzed overnight with a 3.5 kDa membrane. The retentate, containing the protein concentrate, was freeze-dried.

The reduction and alkylation of the protein concentrate in seaweeds were carried out using a method similar to one described previously (Rai *et al.*, 2002) with some modifications. The protein concentrate was dissolved in minimal water. The dissolved algal sample, aqueous ammonium bicarbonate (IM), dithiothreitol (DTT) (100 mM), acetonitrile, iodoacetamide (200mM) and 1% formic acid were combined at a ratio of 12:1:1:12:1.2:5. The mixture was incubated at room temperature in the dark for 20 minutes prior to the addition of the 1% formic acid. The reduced samples were centrifuged in 10 kDa cut-off filter units to almost dryness. The retentate was rinsed twice with water and re-centrifuged to remove solvents. The greater than 10 kDa retentate was freeze dried. Digestion of reduced seaweed protein extract dissolved in 50 mM sodium phosphate buffer was carried out in the presence of bovine trypsin at a pH of 8.0 and 37°C. The substrate: trypsin enzyme ratio was 50:1 w/w. The pH of the mixture was altered to its optimal pH value prior to enzymatic hydrolysis. The enzymatic reaction was carried out for 8 hours at 37°C in a shaking waterbath. The digests were boiled at 99°C for 10 minutes to denature the trypsin enzyme and centrifuged with 10 kDa cut-off tubes. The less than 10 kDa digested sample was freeze-dried until further analysis by nano-UPLC-MS/MS.

4.8.1. Total nitrogen (TN), non-protein nitrogen (NPN) and protein nitrogen (PN) quantification

No statistically validated method for the extraction and quantification of the NPN and true PN content in different macroalgal species appeared to be available. In filling this knowledge gap, a method was optimised for (a) extraction of NPN and PN from macroalgae and (b) quantification of nitrogen in NPN and PN extracts within the NutraMara work plan. The use of osmotic shock (4°C for 3h) for optimum macroalgal cell disruption was selected for extraction of NPN and PN from macroalgae based on results from the optimisation study described above. Based on information in the literature, trichloroacetic acid at a final concentration of 12 % (w/v) was selected for precipitation of macroalgal proteins. The effect of homogenisation in conjunction with osmotic shock disruption, mass:volume ratio and the number of sequential extractions with TCA on the concentration of NPN and PN recovered was assessed. The Kjeldahl nitrogen quantification method was used for quantification of total nitrogen (TN), NPN and PN.

In the first instance the method was validated with a range of (sodium caseinate) standard protein solutions to identify the optimum conditions for detection of extracts with low, medium and high levels of nitrogen. This method was further validated with macroalgal extracts containing low, medium and high levels of nitrogen. The optimised extraction and quantification methods were used to determine the seasonal, geographical and cultivation variation in the TN, NPN and PN content of selected macroalgal species.

Results of seasonal and geographic variation of TN, NPN and PN in macroalgae

Results of what was the first such study of seasonal and geographic variation of TN, NPN and PN in *Palmaria palmata*, *Ulva spp*, *Fucus serratus*, *Laminaria digitata* and *Ascophyllum nodosum*, indicate significant variation of NPN, TN and PN occurs with season and geographical location. The highest NPN, TN and PN content in *Palmaria palmata* and *Ulva sp* was observed in February, while the lowest was recorded in July. Similar seasonal trends in NPN, TN and PN content were observed in samples from Spiddal Co. Galway and Finavarra Co. Clare; small differences were observed in the NPN, TN and PN content of samples harvested from the two locations. The highest NPN, TN and PN content in the brown macroalgal species was seen in samples harvested in February and April, while the lowest levels were found in samples harvested in July and October. These results provide industry direction regarding the optimum times and locations for harvesting macroalgae with high protein and/or non-protein nitrogen content.

4.8.2. Generation of macroalgal protein hydrolysates

A number of macroalgal protein hydrolysates were generated by enzymatic hydrolysis with food-grade proteolytic enzyme preparations by the procedure described by Harnedy, (2013b). All hydrolysates generated were characterised in terms of extent of hydrolysis by the TNBS method described by Spellman et al., (2005) with some modifications, molecular mass

distribution by gel permeation chromatography (GPC-HPLC) and hydrophobicity by reverse phase high performance liquid chromatography (RP-HPLC) as described by Spellman *et al.*, (2005). This work sought to determine the most appropriate protein extract and enzyme combination for the generation of *Palmaria* protein hydrolysates with high *in vitro* biological activity. Aqueous, alkaline and a combination of aqueous (aq) and alkaline (alk) *Palmaria palmata* protein extracts were hydrolysed with the food-grade proteolytic enzyme preparations Alcalase 2.4L, Flavourzyme 500L and Corolase PP (Harnedy and FitzGerald, 2013b; 2015). The aim of the second study was to assess the effect of starting protein composition and the origin and time of harvesting on the *in vitro* bioactivity of *Palmaria palmata* protein hydrolysates were also examined. Protein hydrolysates generated with Alcalase and Corolase PP, from a combination of aqueous and alkaline protein extracts from samples of *Palmaria palmata* harvested at different times of the year during 2011 were assessed.

The extraction of protein from brown macroalgal species was found to be hindered, in part, due to low levels of protein and the accessibility of proteins due to high viscosity arising from cell wall and intracellular polysaccharides. A direct hydrolysis of brown macroalgal proteins (*Fucus serratus*, *Laminaria digitata* and *Ascophyllum nodosum*) with selected food-grade proteolytic enzymes was assessed as an alternative method, to generate brown macroalgal protein hydrolysates. The effect of different approaches including the use of a range of cell disruption methods prior to incubation with proteolytic preparations, the use of specific polysaccharidases prior to or in conjunction with selected proteolytic enzymes, variations in hydrolysis conditions (e.g., pH and temperatures) and the removal of alginate prior to hydrolysis were investigated in an attempt to improve brown macroalgal protein hydrolysis.

Results from the generation of macroalgal protein hydrolysates

Results of physicochemical characterisation (extent/degree of hydrolysis and GPC analysis) studies show that the highest extent of hydrolysis of three protein extracts of *Palmaria palmata* was with Corolase PP, followed by Alcalase. However, the same protein fractions were not extensively digested when incubated with Flavourzyme. GPC analyses showed that the quantity of low molecular weight peptides <10 kDa was significantly higher in protein hydrolysates generated with Alcalase and Corolase PP, than in the samples incubated with Flavourzyme: the majority of these peptides being ≤ 2 kDa. In general, low molecular mass peptides exhibit more potent biological activity and are generally more readily absorbed across the gastrointestinal tract.

Results of physicochemical characterisation (extent of hydrolysis, GPC and RP-HPLC analysis) studies on combined aqueous and alkaline protein hydrolysates (Alcalase and Corolase PP) generated from *Palmaria palmata* samples harvested from wild and cultured sources at different times of the year during 2011 showed similar results. No differences were observed in the molecular mass profiles and extent of hydrolysis of the different protein extracts with Alcalase (8.81-10.57 mg amino group/g protein) or with Corolase PP (19.13-22.23 mg amino group/g

protein) (Harnedy *et al.*, 2014). For hydrolysates generated with each enzyme, small differences in RP-HPLC profiles were observed for hydrolysates from samples harvested at different times of the year.

4.9. Algal derived compounds – microalgae

4.9.1. Introduction

Microalgae have numerous potential applications in different fields such as human and animal nutrition, pharmaceuticals, cosmetics, CO₂ sequestration and biofuels (Spolaore *et al.*, 2006; Mata *et al.*, 2010). Interest in the development of functional foods from natural sources, including algae, is currently growing because of their beneficial health effects. Due to their taxonomic and biochemical diversity, microalgae represent a valuable alternative to existing food ingredients containing multiple bioactive molecules which could be co-extracted by applying a biorefinery approach (Lordan *et al.*, 2011; Stengel *et al.*, 2011; Mimouni *et al.*, 2012). Long-chain polyunsaturated fatty acids (LC-PUFAs), carotenoids, phycobiliproteins, polysaccharides and vitamins are the major molecules of interest, due to their capability to enhance the nutritional and functional quality of foods. Nevertheless only a few microalgal species are successfully grown commercially and included in human diets today (Pulz and Gross, 2004; Mimouni *et al.*, 2012).

Algal cultivation, induction and accumulation of bioactives is a complex problem. The metabolic plasticity of microalgae allows them to adapt quickly to changing environmental factors. In their natural environment or during outdoor cultivation, algae are subjected to different abiotic factors with daily and seasonal variations that may be stressful, such as temperature, light levels or UV radiations (Stengel *et al.*, 2011; Mimouni *et al.*, 2012). Microalgae usually grow in diluted culture media, in closed or opened systems, causing high cultivation and harvesting costs, and variable productivities due to environmental changes. Desert areas with abundant sunlight are generally considered to provide the ideal conditions for algal cultivation; successful microalgal production in Ireland thus has to take into account the regional parameters. Potential advantages could include a moderate climate with a reduced need for cooling, long day length in summer, sufficient water availability, and industrial waste streams for CO₂ and heat. In this sense, microalgal cultivation for NutraMara has focused on indoor production i.e. closed photobioreactors with controlled conditions, a system more suitable for food applications.

Microalgal production requires the development of specific culture techniques appropriate to obtain constant levels of molecules of interest to improve their nutritional value. Research conducted at NUI Galway as part of the NutraMara programme focuses on the production of microalgal biomass that is rich in bioactive molecules for food applications. The effects of various abiotic factors were investigated as an approach to enhance growth and trigger bioactive accumulation in a range of species belonging to several groups of microalgae.

Combined growth conditions and stressors (i.e. light, UV-radiation, temperature and nutrients) were tested on different marine species using controlled in-door cultivation systems (e.g. Erlenmeyer flasks, carboy vessels and low-cost flat panel photobioreactors). Bioactive compounds from the algal biomass were analysed using techniques such as HPLC-DAD/FLD (pigments, mycosporine-like amino acids), spectrophotometry (phycobiliproteins) and GC-FID/MS (fatty acids).

Several different culture strategies were developed and employed to optimise the production of specific bioactives and potential co-products in a number of microalgal species for food and health applications. Bioactive-rich microalgal biomass and extracts have been produced and provided to NutraMara partners for further bioactivity testing.

4.9.2. Objectives

The objectives were to investigate the effect of different culture conditions and stressors (i.e. light, UV-radiation, temperature and nutrients) on the growth performance and bioactive production of microalgae (small-scale experiments), and accordingly to assess indoor microalgal large-scale production of selected microalgae (large-scale experiments: low-cost flat panel photobioreactors) for targeted bioactive production under controlled conditions. And to use the results of this work to develop culture strategies for optimising the production of specific bioactives and potential co-products in a number of microalgal species for food and health applications; aiming to provide bioactive-rich microalgal biomass and extracts to partners for further bioactivity testing.

4.9.3. Overview of materials and methods

Microalgal species from various taxonomic groups were obtained from different culture collections i.e. Plymouth Culture Collection of Marine Microalgae (PLY) at the Marine Biological Association (MBA, UK); Culture Collection of Algae and Protozoa (CCAP) at the Scottish Marine Institute (SAMS Research Services Ltd., UK); Culture Collection of Algae at the University (UTEX, USA); Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, USA); Culture Collection of Algae at Goettingen University (SAG, Germany).

Table 17 displays the classification and the collection number of the main microalgal strains investigated in the microalgal biomass production work of the NutraMara programme.

Table 17 Main microalgal species investigated through WP3 Task 1.3 and available at the NUI Galway Microalgal Strains Collection

Species	Phylum	Class	Family	Collection Number
<i>Phaeodactylum tricoratum</i>	Heterokontophyta	Bacillariophyceae	Phaeodactylaceae	UTEX 646
<i>Leptocylindrus danicus</i>	Heterokontophyta	Coscinodiscophyceae	Leptocylindraceae	CCMP 740
<i>Thalassiosira weissflogii</i>	Heterokontophyta	Coscinodiscophyceae	Thalassiosiraceae	PLY#541
<i>Porphyridium purpureum</i>	Rhodophyta	Porphyridiophyceae	Porphyridiaceae	PLY#539
<i>Dixoniella grisea</i>	Rhodophyta	Porphyridiophyceae	Dixoniellaceae	SAG 72.90
<i>Rhodella violacea</i>	Rhodophyta	Rhodellophyceae	Rhodellaceae	CCAP 1388/5
<i>Pavlova lutheri</i>	Haptophyta	Pavlovophyceae	Pavlovaceae	CCAP 931/6
<i>Isochrysis galbana</i>	Haptophyta	Coccolithophyceae	Isochrysidaceae	CCAP 927/1
<i>Rhodomonas salina</i>	Cryptophyta	Cryptophyceae	Pyrenomonadaceae	CCAP 978/27
<i>Nannochloropsis salina</i>	Ochrophyta	Eustigmatophyceae	Monodopsidaceae	CCAP 849/2
<i>Nannochloropsis oculata</i>	Ochrophyta	Eustigmatophyceae	Monodopsidaceae	SAG 38.85
<i>Dunaliella tertiolecta</i>	Chlorophyta	Chlorophyceae	Dunaliellaceae	PLY#83
<i>Tetraselmis suecica</i>	Chlorophyta	Chlorodendrophyceae	Chlorodendraceae	NCC 62
<i>Nodularia harveyana</i>	Cyanobacteria	Cyanophyceae	Aphanizomenonaceae	SAG 44.85

4.9.4. Cultivation and growth

All strains were pre-cultivated in batch-culture using RS-F/2 medium, a modified version of Guillard's (1975) F/2 medium where filtered seawater is substituted by Reef Salt (H2Ocean Pro+, UK). RS-F/2 medium was composed as described by Guihéneuf *et al.*, (2013).

All experiments were performed in fully controlled (temperature and light) Binder GmbH growth chambers (Tuttlingen, Germany) or temperature-controlled rooms. Microalgae were batch cultivated in Erlenmeyer flasks, Nalgene carboys or low-cost flat panel photobioreactors with growth conditions adapted according to requirements and growth parameters (i.e. cell density, optical density at 750 nm, nitrate uptake) followed and measured daily.

Total carbohydrate content was determined according to the phenol-sulfuric acid method of Dubois *et al.*, (1956).

4.9.5. Analysis of fatty acid composition and content

Fatty acid methyl esters (FAMES) were obtained by direct transmethylation on freeze dried biomass or lipid extracts as described by Guihéneuf *et al.*, (2011) and analysed by Gas-Chromatography performed on a Agilent GC-MSD 5975C Series equipped with the flame ionization detector (FID) and a fused silica capillary column (DB-Wax, 0.25 mm × 30 m × 0.25 µm).

4.9.6. Pigment composition

After extraction with 90% Acetone, pigments were analysed by High-Performance Liquid Chromatography performed on an Agilent 1200 series using the method of Wright *et al.* (1991) modified by Bidigare *et al.*, (2005).

4.9.7. Phycobiliprotein content

Phycobiliproteins (i.e. phycocyanin and phycoerythrin) were extracted in accordance with the method of Chopin *et al.*, (1995). Freeze-dried biomass being homogenised in a 0.1M phosphate

buffer (pH 6.8) and samples then centrifuged. After filtrations samples were analysed using a Cary UV50 spectrophotometer and CaryWIN software (Varian Inc., Palo Alto, CA, USA) and phycoerythrin and phycocyanin concentrations determined after Beer and Eshel (1985).

4.9.8. Mycosporine-like amino acid composition

Mycosporine-like amino acid composition and content were analysed according to an adapted version of Karsten *et al.*, (2009). Freeze-dried biomass was extracted using 100% methanol and analysed by High-Performance Liquid Chromatography performed on an Agilent 1200 series using ACE C18-AR column (particles size, 5 µm; column length, 30 mm).

4.10. Screening of bioactives in microalgae

The objective of some preliminary works conducted at NUI Galway was to investigate and pre-select valuable microalgal species obtained from culture collections for specific bioactives i.e. LC-PUFA (eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) & arachidonic acid (ARA) and pigments (Figure 10).

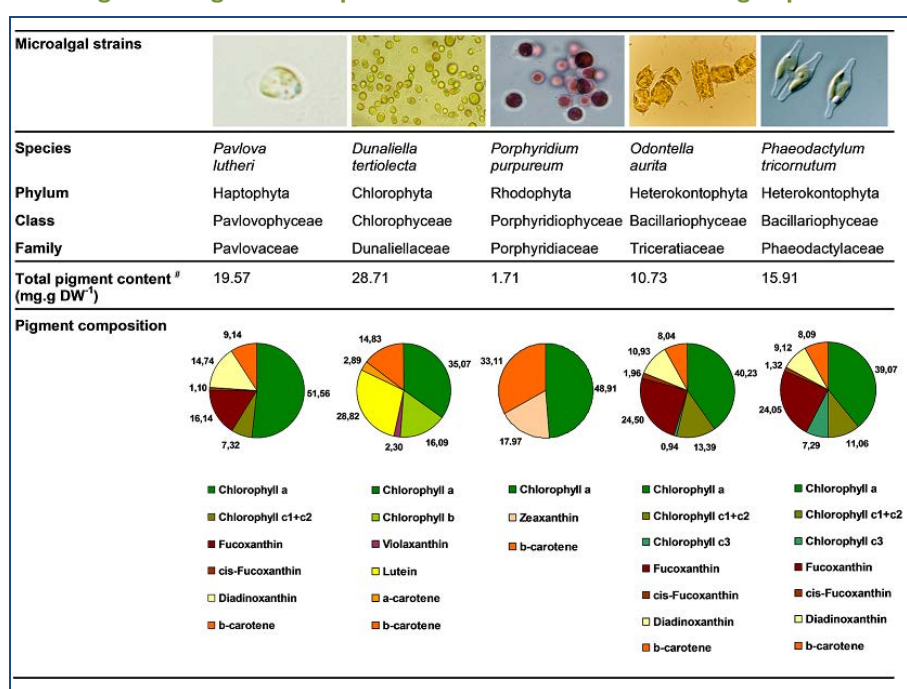
4.10.1. Culture conditions

A first screening of some bioactive compounds (i.e. PUFAs, carotenoids and phycobiliproteins) was undertaken using microalgae cultivated under intermediate growth conditions (i.e. continuous light of 100 µmol photons.m⁻².s⁻¹, 15°C and full-replete medium).

4.10.2. Results

Preliminary data allowed the pre-selection of promising species from various taxonomic groups, such as the haptophyta *P. lutheri* for EPA and DHA, the rhodophyta *P. purpureum* for EPA and phycobiliproteins, the diatom *P. tricornutum* for EPA and fucoxanthin.

Figure 10 Pigment composition and content of five microalgal species



4.11. Patterns of carbohydrate and fatty acid changes under nitrogen limitation and low inorganic carbon supply in two microalgae *P. lutheri* and *P. tricornutum*

4.11.1. Objective

The total fatty acid (TFA) content and composition of microalgae is known to be affected by environmental conditions such as light intensity, nutrient (mainly nitrogen) limitation, salinity, temperature, pH, and culture age (Mimouni *et al.*, 2012). In this context, the aim of this work was to study the impact of nitrogen limitation on the production of two major secondary metabolites, fatty acids and carbohydrates, in two marine microalgae: *P. lutheri* and *P. tricornutum*, batch-cultivated and known to contain substantial levels of LC-PUFA such as EPA and DHA.

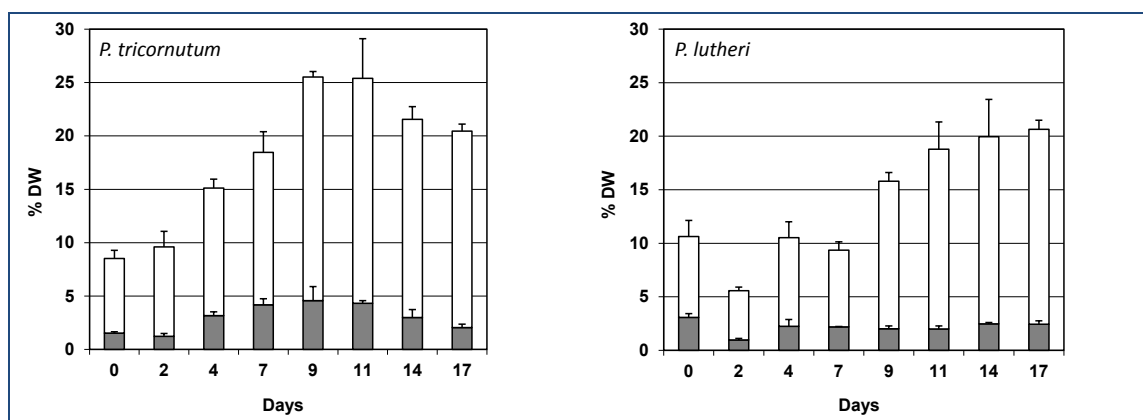
4.11.2. Culture conditions

Both species were cultivated under intermediate growth conditions (i.e. continuous light of 100 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 15 °C) using an initial nitrate concentration of 100 $\text{mg}\cdot\text{L}^{-1}$. Nitrate was fully depleted after approximately 5-6 days.

4.11.3. Results

In both species, the first response to nitrogen limitation induced by culture age was intensive production of carbohydrates (see Figures 11 and 12). However, although there was a slight lipid accumulation, these did not correspond with the findings of previous studies showing that microalgae have the capability to accumulate from 20 to 60% TFA content per dry weight after nitrogen starvation (Breuer *et al.*, 2012). One reason behind the low TFA content in this study was explained by the low inorganic carbon supply consisting of 0.03% CO_2 provided by air-bubbling.

Figure 11 TFA (grey bars) and carbohydrate (white bars) contents of *P. tricornutum* and *P. lutheri* batch-cultivated. Results are expressed as the mean \pm standard deviation ($n = 3$).

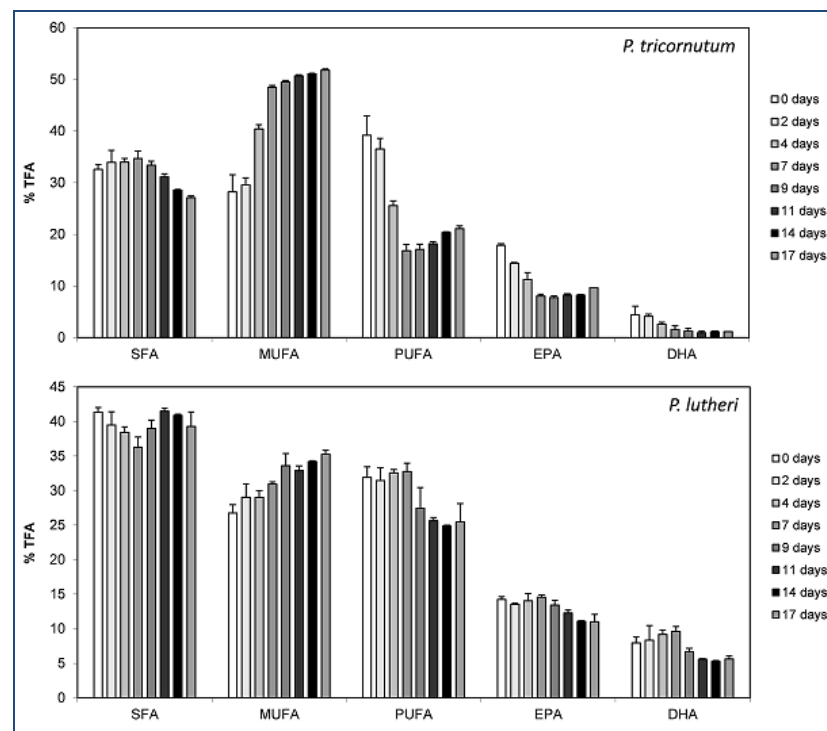


In addition, significant changes in fatty acid profiles were observed after nitrate-limitation (see Figure 12). Both species were showing a significant increase in monounsaturated fatty acids

(MUFA) which correlates with a relative decrease in the PUFA. The strongest decreases in PUFA and EPA were observed in *P. tricornutum*. The increase in MUFA content was mainly due to the large increase in the 16:1 n-7 fatty acid.

The results obtained in this study indicate that not only nitrate availability affects the growth potential and storage compound accumulations, but another important precursor, most likely inorganic carbon availability. Under nitrogen limitation, carbohydrate rather than oil is the dominant storage sink for reduced carbon. In both microalgae investigated, inorganic carbon availability and nitrogen status are therefore two key metabolic factors controlling oil biosynthesis and carbon partitioning between carbohydrates and fatty acids.

Figure 12 Fatty acid composition of *P. tricornutum* and *P. lutheri* during batch cultivation. Results are expressed as the mean \pm standard deviation (n = 3).



4.12. Interaction of light, temperature and nitrogen for optimising the co-production of high-value compounds in *P. purpureum*

4.12.1. Objective

The microalgal genus *Porphyridium* within the Rhodophyta is of increasing interest as a source of valuable chemical constituents such as phycobiliproteins (PB), sulphated exopolysaccharides and LC-PUFA. Despite the chemical richness of *Porphyridium* spp. and the potential for the co-production of multiple products, a biorefinery approach has never been fully implemented and remains a challenge. Indeed, more intensive research is needed to explore the interactive effects of multiple abiotic factors on this algal genus in order to develop multiproduct cultivation strategies that retain and enhance the production and functionality of several different cell components.

In this study, the interactive effects of light, temperature and nitrogen regime on phycobiliprotein (PB) production, and other bioactives or chemicals such as fatty acids, pigments and carbohydrates, were studied during batch-cultivation of *P. purpureum*, a red microalga, containing multiple compounds of commercial interest.

4.12.2. Culture conditions

In a first instance, three different regimes (N-replete, N-limited and N-starved conditions) were tested using nitrate (NaNO_3) as nitrogen source and intermediate growth conditions (i.e. continuous light of $100 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$, 15°C). After 10 days of cultivation, N-replete and N-starved cultures were resupplied with nutrients by dilution with full medium ($1 \text{ g L}^{-1} \text{ NaNO}_3$) in order to assess the recovery capacity of *P. purpureum* after N-starvation.

P. purpureum was then batch-cultivated for 10 days under different combinations of continuous light ($40\text{-}200 \mu\text{mol photons.m}^{-2}.\text{s}^{-1}$) and temperature ($10\text{-}30^\circ\text{C}$) using an initial nitrate concentration of 1 g.L^{-1} to avoid N-limitation.

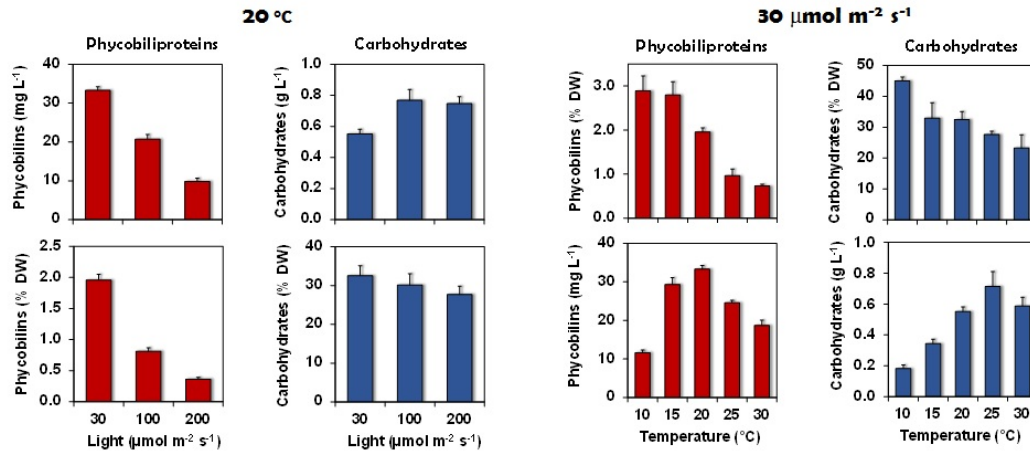
4.12.3. Results

Results indicate that nitrogen-replete modes, such as semi-continuous or continuous regime represents the most suitable culture strategy for PB, carbohydrate, total fatty acid (TFA) and eicosapentaenoic acid (EPA) production in *P. purpureum*. Nitrate-deficiency causes a strong decrease in growth performance, as well as in its PE, TFA and EPA contents which may be caused by membrane degradation; but induces carbohydrate accumulation. Nitrate-starved cells of *P. purpureum* had the ability to restore PB and TFA contents, and specifically phycoerythrin (PE) and EPA levels, after medium refreshment, suggesting an almost complete regeneration of the plastidic membranes and phycobilisomes.

Using response surface methodology (RSM), these results highlight for the first time the optimally combined light and temperature conditions necessary to promote growth and

compound production, in particular PB, in *P. purpureum* batch-cultivated in nitrogen-replete medium (Figure 13).

Figure 13 Total PB, carbohydrate and EPA productivities as a function of irradiance and temperature in *P. purpureum* cultivated under nitrate-replete medium



A simultaneous increase in light and temperature causes a strong decrease in cellular PB, TFA, EPA and pigment contents, suggesting a severe damage and possible disruption of thylakoid membranes. The highest PB content (~2.9% d.w.) was reached under combined low light (30 μmol m⁻² s⁻¹) and low temperature (10 °C). Despite this, maximal PB productivity was obtained at 20°C and under low light intensity, reaching up to 33.3 mg L⁻¹ (~2% d.w.). Under such specific growth conditions, *P. purpureum* biomass also contained substantial amounts of other valuable products (i.e., carbohydrates, EPA, Chl. a, zeaxanthin, β-carotene) which could therefore be co-extracted, with PB, by applying a biorefinery approach.

4.13. LC-PUFA-enriched oil production by *P. lutheri*: Combined effects of light, temperature and inorganic carbon availability

4.13.1. Objective

In most microalgal species, triacylglycerols (TAG) contain mostly saturated and monounsaturated fatty acids, rather than PUFA, while PUFA-enriched oil is the form most desirable for dietary intake. The ability of some species to produce LC-PUFA-enriched oil is currently of specific interest.

In this work, the role of sodium bicarbonate availability on lipid accumulation and n-3 LC-PUFA partitioning into TAG during batch cultivation of *P. lutheri* was investigated. *P. lutheri* is one of the few species reported so far where LC-PUFA are incorporated into TAG under specific conditions e.g. on the transition to the stationary phase (Tonon *et al.*, 2002). Then, the combined effect of light and temperature on omega-3 LC-PUFA partitioning into TAG was examined during bicarbonate-induced oil accumulation.

4.13.2. Culture conditions

In the first experiment, *P. lutheri* was batch-cultivated under intermediate growth conditions (i.e. continuous light of 100 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 15°C) using F/2-RSE medium supplemented with three different initial sodium bicarbonate concentrations.

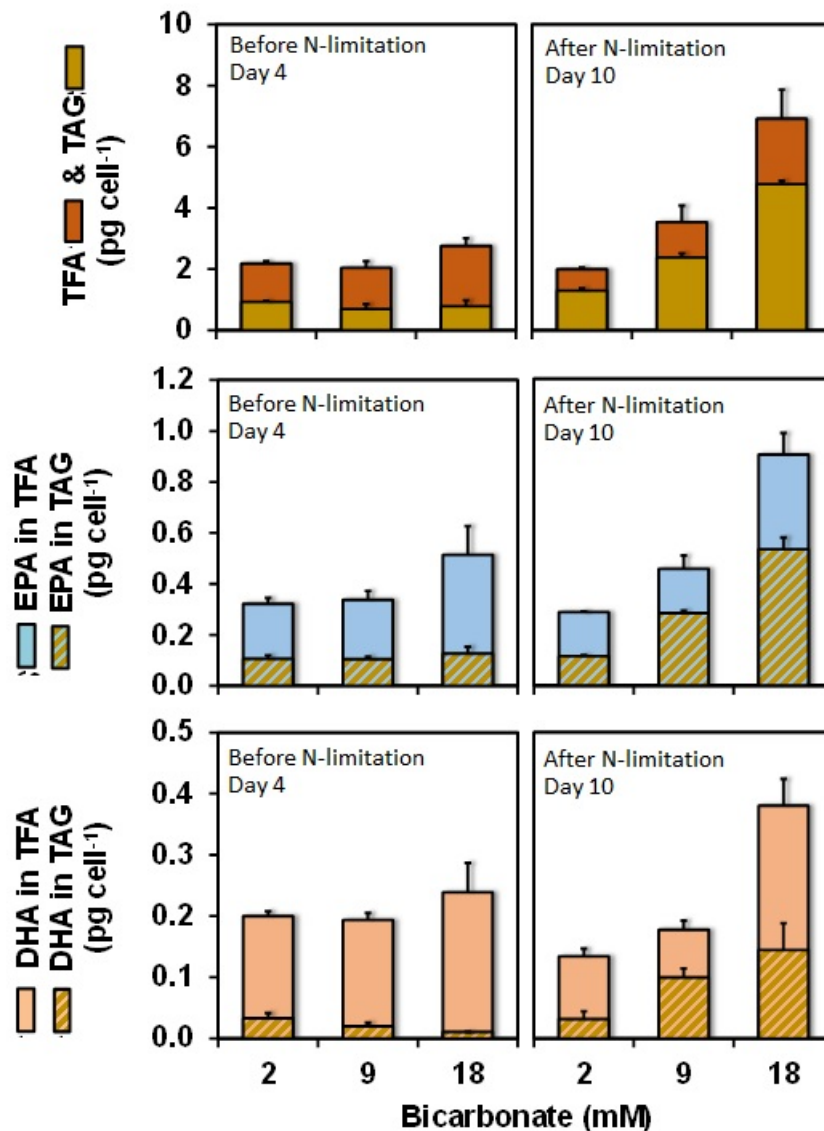
In the second part, *P. lutheri* was batch-cultivated for 18 days under different combinations of continuous light (40 and 200 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and temperature (8-28°C) using a high initial sodium bicarbonate concentration of 18 mM which previously promoted omega-3 LC-PUFA partitioning into TAG.

4.13.3. Results

Maximum growth and nitrate uptake exhibit an optimum concentration and threshold tolerance to bicarbonate addition (~9 mM) above which both parameters decreased as indicated in Figure 14. Nonetheless, the transient highest cellular lipid and TAG contents were obtained at 18 mM bicarbonate, immediately after combined alkaline pH stress and nitrate depletion (day 9), while oil body and TAG accumulation were highly repressed with low carbon supply (2 mM). Despite decreases in the proportions of EPA and DHA, maximum volumetric and cellular EPA and DHA contents were obtained at this stage due to accumulation of TAG containing EPA/DHA. TAG accounted for 74% of the total fatty acid per cell, containing 55% and 67% of the overall cellular EPA and DHA contents, respectively. These results clearly demonstrate that inorganic carbon availability and elevated pH represent two limiting factors for lipid and TAG accumulation, as well as n-3 LC-PUFA partitioning into TAG, under nutrient-depleted *P. lutheri* cultures. Therefore, accumulation of lipid and triacylglycerols containing n-3 LC-PUFA is triggered by nitrogen limitation and inorganic carbon availability in these species.

The capacity of some algal species to accumulate TAG containing LC-PUFA, particularly during environmental changes, depends on the regulation of various metabolic pathways. In this study, we demonstrated for the first time, to our knowledge, that cellular lipid content, oil body formation and TAG containing n-3 LC-PUFA accumulation induced by nitrate depletion rely mainly on inorganic carbon availability in *P. lutheri*. Therefore, this study contributes to optimised culture strategies applied to develop n-3 LC-PUFA-enriched oil production systems using autotrophic microalgae. The findings constitute an important step towards an improved understanding of the mechanisms involved in lipid metabolism regulation, and more specifically oil accumulation and LC-PUFA partitioning in microalgae.

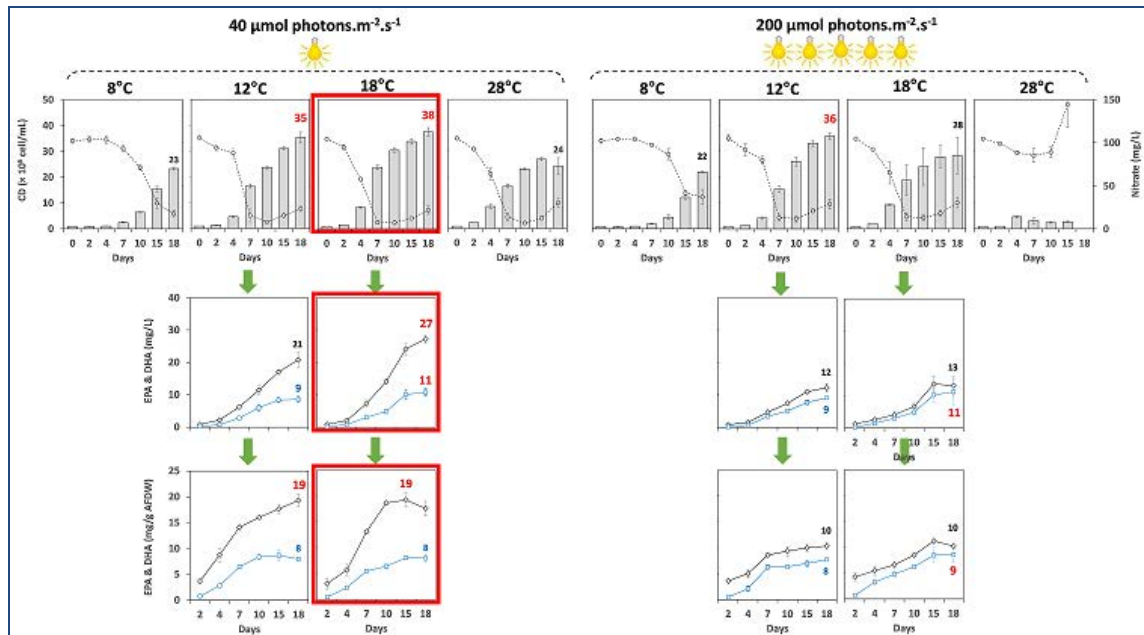
Figure 14 Bicarbonate-induced oil droplet formation (Nile red staining) and triacylglycerol (TAG) containing EPA and DHA accumulation in nitrate-depleted cells



Under both light intensity, the highest growth was observed using temperature between 12-18°C. *P. lutheri* growth was highly reduced or completely repressed using low and high temperature (8 and 28°C), highlighted by slower nitrate-uptake. Of major interest, highest lipid, EPA and DHA content and productivity were obtained under optimal growth conditions reaching N-depletion. These can be explained by TAG accumulation in all conditions able to reach N-depletion. Moreover, in addition to bicarbonate supplementation, our results (Figure 15) showed that optimised growth conditions enhanced omega-3 LC-PUFA partitioning into TAG and oil-containing omega-3 accumulation induced by N-depletion in *P. lutheri*.

In conclusion, using optimal growth conditions— light and temperature, bicarbonate supplementation represents a promising strategy to stimulate growth and trigger omega-3 enriched oil production in the haptophyte *P. lutheri*.

Figure 15 Combined effects of light and temperature on EPA and DHA content and productivity during bicarbonate-induced oil accumulation in *P. lutheri* batch-cultivated



4.14. Indoor microalgal large-scale production of selected microalgae for targeted bioactive production under controlled conditions

4.14.1. Objective

The main objectives of this task were to assess the culture strategies developed for indoor microalgal large-scale production of selected microalgae for targeted bioactive production under controlled conditions, and to produce sufficient amounts of bioactive-rich biomass necessary for extraction and further bioactivity testing within the NutraMara consortium.

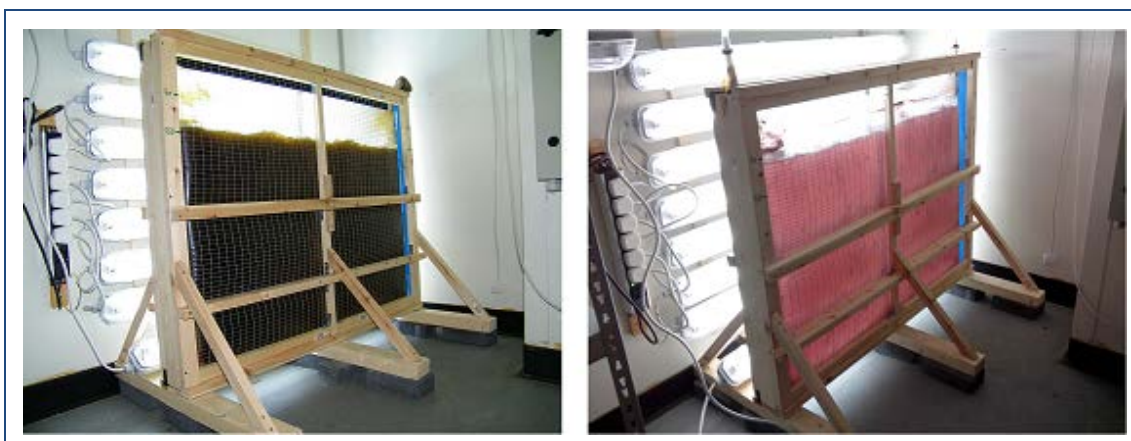
4.14.2. Cultivation system and conditions

Indoor large-scale production was performed using 80L semi-closed low-cost flat panel photobioreactors (FP-PBRs), similar to the one patented by Boussiba and Zarka, (2005) and built by Dr Freddy Guihéneuf on the NUI Galway campus. The new design of FP-PBRs consists of a plastic bag located between two iron frames; this brings a substantial cost reduction to this type of reactors (Figure 16).

Culture strategies previously developed have been tested to produce three different bioactive-rich microalgal biomass:

- Protein-rich i.e. PB-rich *P. purpureum* biomass
- Omega-3 LC-PUFA i.e. EPA/DHA-rich *P. lutheri* biomass
- Omega-3 LC-PUFA i.e. EPA-rich *P. tricorutum* biomass

Figure 16 Low cost flat-panel photobioreactors (FP-PBRs) developed to assess large-scale production of microalgae under controlled conditions. Batch-cultures of the diatom *P. tricornutum* (left) and the red microalga *P. purpureum* (right)



4.14.3. Results

Culture strategies developed during this work were successfully up-scaled, which allowed the production of substantial amounts of bioactive rich-biomass for further bioactive testing within the NutraMara consortium.

4.15. Screening of MAAs in available microalgae and investigation of the deleterious effect of UV radiation on microalgae

4.15.1. Objective

Microalgae depend on solar energy for photosynthesis and are therefore particularly susceptible to the deleterious effects of solar ultraviolet radiation (UVR). To counteract these deleterious effects, they have evolved a range of UV-protective mechanisms. Amongst them, Mycosporine-like amino acids (MAAs), a group of over 20 ultraviolet (UV) absorbing compounds are present in a diverse range of aquatic organisms where they act as sunscreens to reduce UV-induced damage. MAAs also play a role in protecting against sunlight damage by acting as antioxidant molecules scavenging toxic oxygen radicals.

Due to the role played by MAAs, they have been commercially explored as sun care products for protection of skin and other non-biological materials, e.g. as photostabilising additives in plastics, paint and varnish.

In this context, the aim of this subtask was a screening of MAAs in several targeted species (i.e. cyanobacteria, diatoms, others) and to investigate the mechanisms and environmental factors (i.e. UV-stress) inducing MAAs accumulation in selected species. Meanwhile, the deleterious effects of UV-R of microalgae on growth, photosynthesis activity, pigments, and fatty acids were investigated.

4.15.2. Microalgal species and culture conditions

Exponential growth phase cultures of eleven microalgal species from various taxonomic groups (i.e. *Pavlova lutheri*, *Tetraselmis suecica*, *Phaeodactylum tricornutum*, *Leptocylindrus danicus*, *Thalassiosira weissflogii*, *Dixoniella grisea*, *Rhodella violacea*, *Isochrysis galbana*, *Rhodomonas salina*, *Nannochloropsis oculata* and *Nodularia harveyana*) were exposed to photosynthetically active radiation (PAR) or UV-R treatment (PAR + UV-A + UV-B) for five consecutive days. Induced species-specific changes in growth and photosynthetic activity were determined during both PAR and UV-R exposure, the presence of MAAs was investigated, as well as the fatty acid and pigment content and composition.

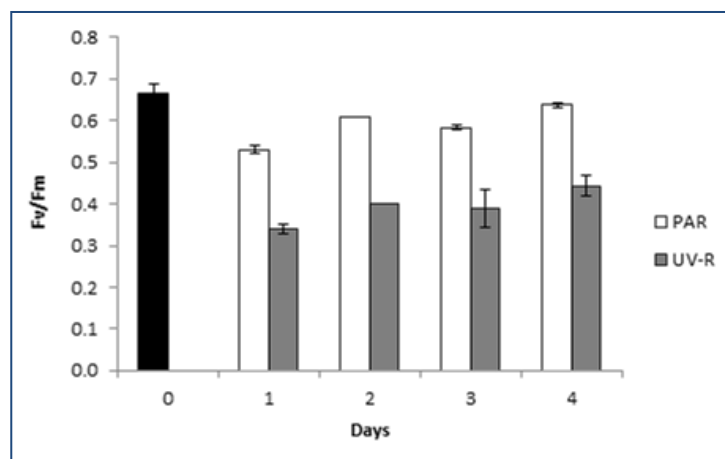
4.15.3. Results

Despite the number of species investigated, none of them showed the presence of substantial amount of MAAs even after UV-R treatment. Only the red microalgae *D. grisea*, the diatom *T. weissflogii*, and the cyanobacteria *N. harveyana* presented some traces of MAAs but insufficient for spectral characterisation.

Nonetheless, microalgae exposed to UV-R showed a significant decrease in growth, photosynthetic activity (i.e. Fv/Fm, see Figure 17 and Chlorophyll *a* levels, usually associated with an increase in carotenoids. Phycobiliproteins in red microalgae and cyanobacteria was highly degraded which suggested a strong deterioration of the thylakoid membranes. Lipid and LC-PUFA changes appeared to be species-specific.

These results contributed to a better understanding of the potential deleterious effect of UV-R on microalgae, especially during outdoor cultivation.

Figure 17 Effect of UV-R on the photosynthetic activity (Fv/Fm) of green microalgae *N. salina*. Results are expressed as the mean \pm standard deviation (n = 3)



4.15.4. Conclusions

In marine microalgae, inorganic carbon availability and nitrogen status are therefore two key metabolic factors controlling oil biosynthesis and carbon partitioning between carbohydrates and fatty acids.

Several different culture strategies were developed and employed to optimise the production of specific bioactives and potential co-products in a number of microalgal species for food and health applications.

In *P. purpureum*, maximal PB productivity is obtained at 20 °C under low light intensity using Nitrogen-repleted medium. Under such specific growth conditions, *P. purpureum* biomass also showed to be able to accumulate substantial amounts of other valuable products (i.e. carbohydrates, EPA, zeaxanthin, β -carotene) which could therefore be co-extracted, with PBs, by implementation of a biorefinery approach.

Bicarbonate addition can trigger oil containing omega-3 LC-PUFA accumulation in *P. lutheri* batch-cultivated.

In *P. lutheri*, optimised light and temperature can enhance omega-3 LC-PUFA incorporation into TAG during bicarbonate-induced oil accumulation.

In Ireland, indoor cultivation using adapted cultivation systems such as flat panel photobioreactors (FP-PBRs) under controlled conditions appears to be a pre-requisite for bioactive production from microalgae.

MAAs are not as common or are only detected as traces in most microalgal species.

UV-R strongly affects the physiology and metabolism in marine microalgae, such effect has to be considered when using outdoor cultivation in particular during summer.

4.16. Fish processing co-product derived compounds

The NutraMara feasibility study identified a number of fish processing co-products as potential sources of high quality protein and substrates for mining of bioactive peptides. Salmon trimmings (muscle and skin) were identified as a source of muscle proteins and gelatine, blue mussel meat as a source of muscle protein, and blue mussel byssus as source of collagen. Co-products from processing crab, lobster and prawns were identified as sources of the polysaccharide, chitin. Methods developed to extract bioactive compounds from these sources are described below.

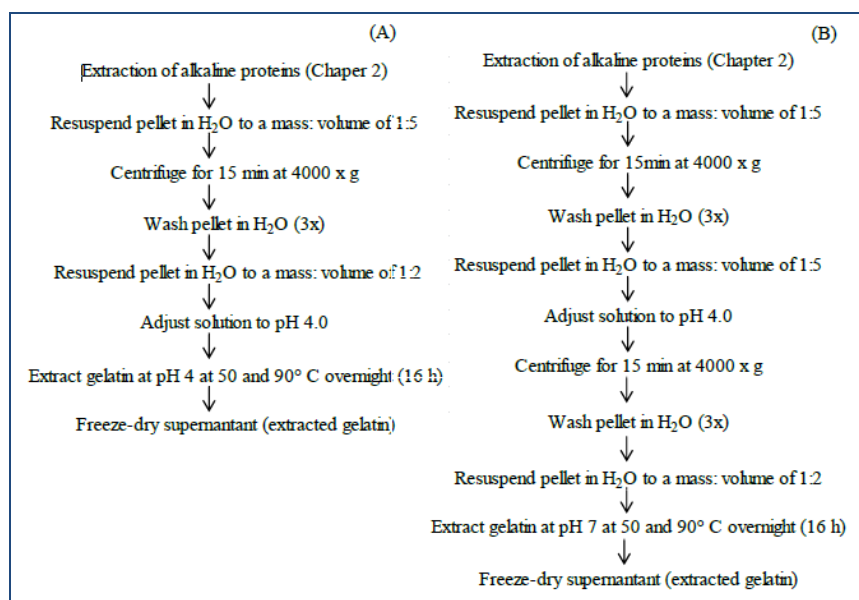
4.17. Peptides

4.17.1. From salmon co-products

Processing parameters were optimised for the extraction of soluble muscle proteins from salmon trimmings. This involved assessing the effect of weight to volume ratio (1:2.5, 1:5.0, 1:7.5 and 1:10.0), number of sequential extractions, homogenisation, extraction pH (2.5, 3.0, 3.5, 4.0, 10.0, 10.5, 11.0 and 12.0) and agitation time (5, 10, 15, 30 and 45 min) on the yield of protein recovered. Extraction pH and the use of homogenisation were identified as the critical parameters associated with the extraction of proteins from salmon muscle.

Methods to extract gelatine from salmon trimmings were also developed. A pellet (containing salmon skin, bone and membranes) obtained following removal of the soluble salmon muscle proteins by centrifugation was the starting point for extraction. The details of the two methods used for extraction of gelatine from the pellet are outlined in Figure 18 below.

Figure 18 Schematic outlining the steps involved in the extraction of gelatine from salmon trimmings (*Salmo salar*) by two different protocols



4.17.2. From mussels

The critical parameters identified in extracting salmon protein - extraction pH and the use of homogenisation were optimised (extraction pH (10.0, 10.5, 11.0 and 12.0) and homogenisation) for the extraction of protein from mussel meat.

Different food-friendly approaches were assessed to extract collagen from mussel byssus. Collagen was extracted at a 1:10 (w:v) ratio byssus:water, at pHs 7.0, 4.0, 3.0, 2.5 and 2.0, and extraction at different temperatures (50, 70, 90 and 10°C) during 1, 12, 16 and 24 h. A soluble collagen extract was obtained following centrifugation at 4,000 x g for 15 min. Different proteolytic enzymes were also assessed for their ability to liberate collagen from mussel byssus. These include pepsin, collagenase and a commercial An-PEP preparation (proline specific enzyme).

The protein content in each extract was determined using the Bradford assay (Bradford, 1976) while the protein content of the raw material and the final protein isolate was determined using a modification of the Kjeldahl procedure (IDF 1993). All protein isolates were characterised by sodium dodecyl sulphate polyacrylamide gel electrophoresis by the method described by Laemmli (1970).

The optimised protein extraction protocols were used to extract protein/gelatine/collagen from mussel meat, byssus, and salmon trimmings, for use as substrates for the generation of protein hydrolysates.

4.17.3. Generation of protein hydrolysates from fish processing co-products

Protein hydrolysates were generated from salmon and blue mussel meat protein, salmon gelatine and mussel byssus collagen isolates by using the food grade proteolytic enzymes Alcalase 2.4L, Alcalase 2.4L in combination with Flavourzyme 500L, Corolase PP and Promod 144MG for 1, 2 and 4h. This work determined the most appropriate food-grade proteolytic enzyme, marine raw material and hydrolysis duration for generation of marine protein hydrolysates with specific biological activity (Cunha Neves *et al.*, 2015; Cunha Neves *et al.*, accepted and Cunha Neves *et al.*, in preparation). Physicochemical characterisation (% degree of hydrolysis, reverse phase-high performance liquid chromatography (RP-HPLC) and gel permeation chromatography (GPC -HPLC) was performed on all hydrolysates as described by Spellman *et al.*, (2005) to determine the extent to which the proteins were hydrolysed, the molecular mass distribution and hydrophobicity of the peptides within each hydrolysate.

Results from protein extraction and hydrolysis

Extraction parameters for optimum recovery of protein from salmon trimmings include homogenization of a 1:5 trimmings:water suspension, adjusting to pH 11.0, agitation for 15 min at room temperature and centrifugation at 4000 × g (Cunha Neves, 2015 and Cunha Neves *et al.*, in preparation). The supernatant obtained following centrifugation contained the soluble muscle proteins. From these optimised conditions, 305.09 ± 7.38 mg/g wet weight of soluble muscle protein, corresponding to a protein yield of 93% (w/w) was obtained from salmon trimmings. The purity of the salmon protein isolate was determined to be 87% (w/w). Critical parameters associated with the extraction of soluble muscle proteins from salmon trimmings were extraction pH and the inclusion of a homogenisation step.

The soluble muscle protein extraction method used for salmon trimmings was adapted to extract protein from mussel meat, in which extraction pH and homogenisation conditions were optimised (Cunha Neves *et al.*, accepted). The optimum pH for extraction of mussel meat proteins was identified as pH 11.0. Furthermore, the inclusion of a homogenisation step was required for optimum cell disruption and accessibility of proteins. The yield and purity of the mussel meat protein isolate was determined to be 73% and 92% (w/w), respectively.

The most appropriate method identified to extract collagen from mussel byssus involved direct hydrolysis of the mussel byssus with a commercial proline specific enzyme (*An*-PEP). Using this food friendly approach, 138.82 ± 2.25 mg collagen g⁻¹ (d.w.) was recovered.

The optimised protein extraction protocols described above were used to extract protein/gelatine/collagen from salmon trimmings, mussel meat and byssus and used as substrates for the generation of protein hydrolysates.

Protein hydrolysates from salmon and blue mussel muscle protein, salmon gelatine and mussel byssus collagen isolates, using food grade proteolytic enzymes Alcalase 2.4L, Alcalase 2.4L in combination with Flavourzyme 500L, Corolase PP and Promod 144MG were generated over durations of 1, 2 and 4h. Results of physicochemical characterisation (degree/extent of

hydrolysis and GPC analysis) showed that in general, hydrolysis was complete after 1h. Furthermore, the highest degree/extent of hydrolysis was observed with Corolase PP and a combination of Alcalase 2.4L and Flavourzyme 500L, while the lowest was seen with Promod 144MG. In general, the majority (90-95%) of peptides present in hydrolysates generated with Corolase PP and a combination of Alcalase 2.4L and Flavourzyme 500L were <10 kDa, while 60-80% of peptides in hydrolysates generated with Promod 144MG were <10 kDa. Different RP-HPLC profiles were obtained for each of the hydrolysates generated.

4.18. Polysaccharides

4.18.1. Chitin

Chitosan was generated from prawn (*N. norvegicus*) by-product consisting of shell and protein material. Prawn shell material was heated in boiling sodium chloride (4% NaCl) for 10 min and cooled in tap water to remove excess prawn protein material. Shell was washed extensively and freeze-dried. Clean, dry shell was milled, sieved and subsequently demineralised and deproteinised using a BioFlo 110 Modular Bioreactor (New Brunswick Scientific, USA). HCl (0.25 M) was added to the prawn shell material in a 1:40 w/v ratio. The temperature of the reaction was maintained at 40°C for 6 h. Shell material was subsequently drained, washed until pH neutral (pH 7.0) using Milli-Q water, frozen, and freeze-dried to obtain demineralised shell powder. Demineralised shell powder was then deacetylated and further deproteinised using 0.25 M NaOH at a shell to solvent ratio of 1:40w/v.

Chitosan was prepared by hydrolysis of the acetamide groups of chitin, using a severe alkaline treatment. This involved chitin being further deacetylated using 3.0 M NaOH at a chitin to solvent ratio of 1:40 w/v. The reaction was maintained at 70°C for 6 h. Chitin was then washed until neutral, frozen, and freeze-dried. A final deacetylation step was carried out by subjecting chitosan to treatment with 45% NaOH at 100°C for 6 h. The final product, chitosan, was washed until neutral using Milli-Q water, frozen, freeze-dried, milled and stored for use within the consortium.

4.19. Aquaculture derived compounds

4.19.1. Algal compounds through optimised cultivation

Macroalgae (seaweeds) present a rich source of chemical compounds with bioactive properties. A major interest was to develop a better understanding of the dynamics and variability of bioactive profiles in seaweeds in the field and under controlled laboratory conditions, in order to optimally use these as a source for bioactive compounds.

Research has shown that the chemical profile of macroalgae can be variable in dependence of the abiotic conditions in their environment. A better understanding of plasticity of bioactives in

seaweeds in natural and under controlled laboratory conditions can support an optimized utilisation of macroalgae as a source of health promoting compounds.

4.19.2. Introduction

A healthy diet is becoming more and more important to the general public. This has caused a change in consumer behaviour with an increased consumption of healthy foods. The food industry has addressed this with numerous and a steadily growing product line of functional foods. With a quickly growing market there is also an increasing demand for sources of bioactive compounds for use in functional foods.

Algae contain a wide range of different compounds with multiple potential applications in the functional foods sector (Stengel *et al.*, 2011). Besides having numerous effects and physiological and ecological functions for the seaweed itself, many of these compounds have shown to have bioactive properties (Holdt and Kraan, 2011; Stengel *et al.*, 2011). Within the large inventory of seaweed compounds especially fatty acids, pigments including chlorophylls, carotenoids and phycobilins are of interest for functional foods.

It has been long realised that the composition of seaweeds is subject to significant variations in accordance to the abiotic conditions defining their environment. Seaweed species inhabit the intertidal zone, an environment which is subject to constantly changing conditions including levels of light and temperature on different temporal (diurnal, seasonal) and geographic scales (Colombo *et al.*, 2006; Mouritsen *et al.*, 2013).

These natural occurring changes in the chemical composition of seaweeds equally pose challenges but also opportunities for the utilisation of seaweeds as a resource for functional foods. Currently it is still very difficult to predict the chemical composition of seaweeds with regards to sampling time and sampling location. This can cause problems for industry that depends on a stable and chemically optimised biomass. On the other hand, the plasticity also opens opportunities to improve the chemical composition of seaweeds (Gosch *et al.*, 2015). In controlled culture conditions seaweeds can be grown under optimal conditions for the production of certain target compounds. This can improve the yield of valuable bioactive compounds from seaweed biomass.

In order to use seaweeds commercially it is important to choose species that have a valuable chemical composition rich in bioactive compounds. The target species also need to be present in sufficient abundance along the coast that allows for sustainable harvesting or be capable of being grown in aquaculture facilities. Meeting these requirements, this study focused on the investigation of the three brown seaweed species *Laminaria digitata*, *Ascophyllum nodosum*, *Fucus serratus* and one red macroalga, *Palmaria palmata*.

The single and synergistic effects of abiotic factors on the biochemical composition of seaweeds are still not fully understood. In order to address this scientific gap and to provide the base knowledge for an economical and optimized utilisation of Ireland's seaweed resource the project focused on the investigation of the chemical plasticity of seaweeds in dependence

of their abiotic growing conditions. This included the assessment of natural chemical variability and the exposure of seaweeds to controlled and modified culture conditions.

4.19.3. Evaluation of natural seasonal and spatial variability in bioactive compounds in selected seaweeds

4.19.3.1. Objectives

The objective of this work was to assess and evaluate the natural occurring variability of bioactive compounds in seaweeds. This included a comparison of seaweed species of different taxonomical background, different thallus parts of algae and of different seasonal and spatial sampling events.

4.19.3.2. Material and methods

To assess the variability of bioactive compounds and for the selection of target species a screening of 16 macroalgae species was conducted. Representatives of red (Rhodophyta), brown (Phaeophyceae) and green (Chlorophyta) algae were collected at two seasons from western Ireland and total fatty acid contents and specific profiles were determined (for details see Schmid *et al.*, (2014)).

For the evaluation of the intra-thallus variability of bioactive compounds, eight brown algae species were investigated. Species investigated belonged to the orders Fucales (*Fucus serratus* (L.) Le Jolis, (Fucaceae), *Ascophyllum nodosum* (L.) Le Jolis, (Fucaceae) and *Himanthalia elongata* (L.) Gray, (Himanthaliaceae)) and Laminariales (*Laminaria digitata* (Hudson) Lamouroux (Laminariaceae), *Laminaria hyperborea* (Gunnerus) Foslie (Laminariaceae), *Alaria esculenta* (Linnaeus) Greville (Laminariaceae), *Saccorhiza polyschides* (Lightfoot) Batters (Laminariaceae) and *Saccharina latissima* (L.) Lane (Laminariaceae)). Algal samples were dissected in morphological distinct parts and ground to fine powder for subsequent fatty acid and pigment analysis.

To support a better understanding of the variability of bioactive compounds in seaweeds in dependence of sampling location and season a seasonal sampling was conducted. Macroalgal samples of four species (*Laminaria digitata*, *Ascophyllum nodosum*, *Fucus serratus* and *Palmaria palmata*) were collected in the course of one year and chemical composition was analysed. Seaweed samples were collected at low tide every six weeks at three different sites along the coast of Galway Bay (Finavarra, Carraroe and Ballyconnely).

The quantity and composition of total fatty acids (TFA) was analysed using GC-FID (gas-chromatography coupled with a flame ionization detector). The samples analysis followed the protocol described in Schmid *et al.*, (2014). Pigment profiles (Chlorophylls and carotenoids) were analysed using HPLC (high-pressure liquid chromatography) following the protocol described in Wright *et al.*, (1991) modified after Bidigare *et al.*, (2005). Phycobilin contents were analysed after Beer and Eshel, (1985).

4.19.4. Results

Results of the seaweed screening were evaluated with particular focus on TFA contents and seaweeds as a source for EPA (see Figure 19). All investigated species showed low levels in total fatty acids compared to those achievable in microalgae (see Figure 11). Yet, PUFA levels in several macroalgae species were high with percentages of up to 50% of their TFA. Particularly *P. palmata* had an interesting fatty acid profile with high percentages (44%) of EPA (see Figure 19). The generally low ratio of omega-6 to omega-3 fatty acids in most species analysed indicate potential as food or food supplement to balance the omega-3 deficiency in western diets. The samples collected at different seasons revealed variations in TFA and EPA. Variations were species specific with similar trends partly in closely related species and seaweeds inhabiting similar shore levels. The study highlights the importance of a better understanding of seasonal dynamics in valuable fatty acids in macroalgae in order to optimally use seaweeds as a source of PUFA.

Figure 19 Total fatty acid content in % of DW at the two different sampling times in investigated brown, red and green macroalgae. Values are expressed as the mean \pm standard deviation (n = 3)

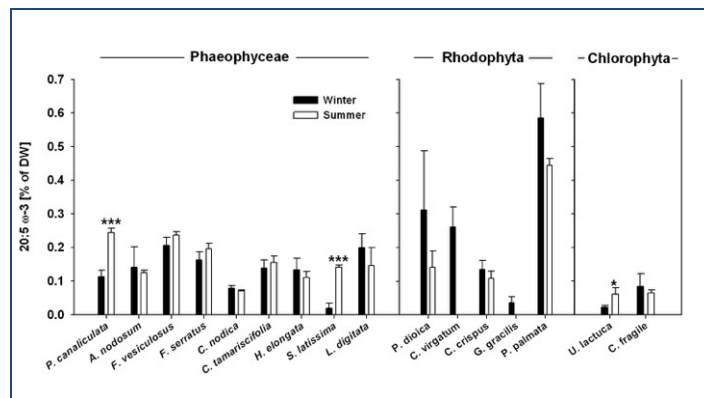
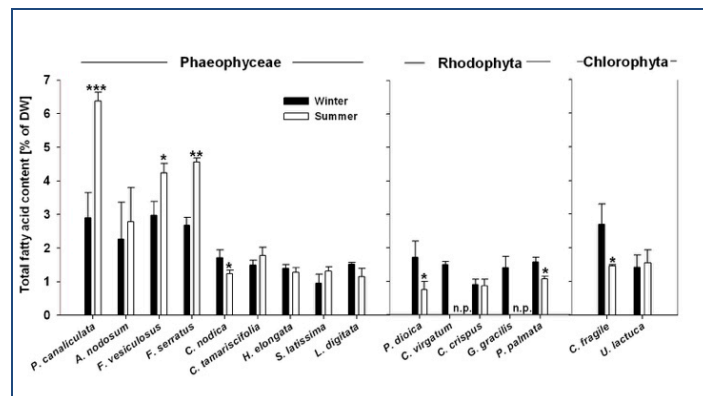


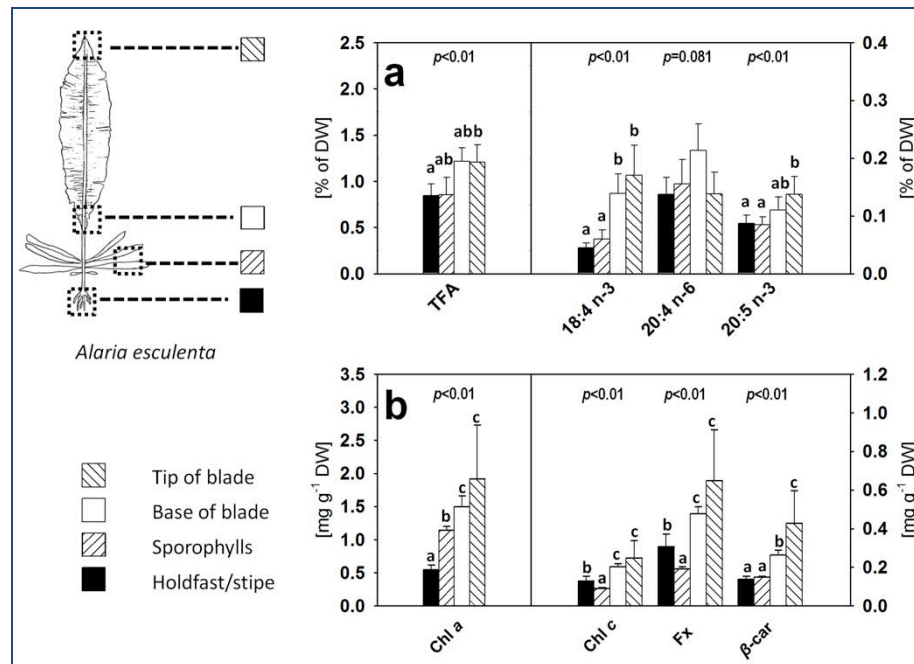
Figure 20 EPA content in investigated brown, red and green macroalgal species. EPA is displayed in percentage of DW. Values are expressed as the mean \pm standard deviation (n = 3)



The Laminariales and Fucales species investigated showed a strong biochemical differentiation comparing the different thallus parts (for example see Figure 21). In most species there was a trend of higher levels of TFA in the more distal parts, e.g. in kelps when comparing the blades to the holdfast. A similar pattern was found for the pigments in kelps, generally lower pigment

contents occurred in the apical growth region of the Fucales compared to other parts of the blade. The omega-3/omega-6 ratio was highest in blades due to an increase in the percentage of 20:5 n-3 and 18:4 n-3. When considering seaweeds as a source of PUFA and pigments, kelp blades were identified as most preferable source material of all species investigated. A detailed description of the achieved results can be found in Schmid and Stengel, (2015).

Figure 21 Fatty acids (a) and pigments (b) in *Alaria esculenta*. Different thallus parts investigated are indicated in diagram. (a) Total fatty acids (TFA) and levels of 18:4 n-3 20:4 n-6 and 20:5 n-3 in % of DW. (b) Concentrations of chlorophyll a (chl a), chlorophyll c (chl c), fucoxanthin (fx) and β -carotene (β -car) in mg g⁻¹ DW. Significant differences ($p < 0.05$) were determined using the Kruskal-Wallis test



4.19.5. Evaluation of environmental control of bioactive levels and composition through experimental exposure

4.19.5.1. Objective

The objective of this activity was to evaluate the effects of single and synergistic abiotic factors on the biochemical composition of seaweeds under controlled laboratory conditions.

4.19.5.2. Material and methods

Four seaweed species (*Laminaria digitata*, *Ascophyllum nodosum*, *Fucus serratus* and *Palmaria palmata*) were exposed to different levels of light and temperature in the laboratory. Culture conditions ranged between 10 and 20 °C and irradiances between 20 and 90 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Growth rate and photosynthetic activity ($\Delta F/F'm$) using PAM-fluorometry (Pulse amplitude modulated fluorometry) was measured during experimental exposure.

After experimental exposure the chemical composition of the seaweeds was analysed following the protocols described above and employed in the evaluation of natural seasonal and spatial variability of bioactive compounds.

4.19.6. Results

The obtained results allowed the identification of the main and combined effects of the investigated abiotic factors. The results showed similar patterns, such as lower levels of pigments under high light conditions but also species specific responses.

4.19.7. Bioactive production through targeted algal cultivation

4.19.7.1. Objective

The objective of this task was to evaluate and interpret the results from the culture experiments for the identification of optimised culture conditions.

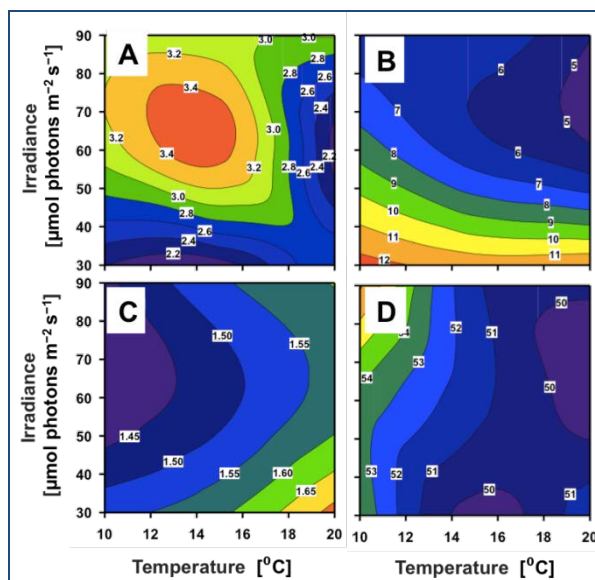
4.19.8. Material and methods

Results from the culture experiments were used to model the optimal culture conditions. A quadratic model was used for calculation of each response variable in detail described in Guihéneuf and Stengel, (2015).

4.19.8.1. Results

The response of the different seaweed species to the culture conditions was used to model optimum culture conditions for different compounds and growth rate. The results highlight how optimum condition can vary strongly between target compounds. It is also shown how the optimum conditions for certain target compounds can be distinct from optimum growth conditions (see Figure 22). For an optimal utilisation of seaweeds from aquaculture both factors need to be considered.

Figure 22 Optimum culture conditions for *P. palmata* for growth [specific growth rate % d⁻¹] (A), phycobilin [mg g⁻¹ DW] (B), TFA [% of DW] (C) and PUFA [% of TFA] (D) production.



4.19.9. Plasticity of lipid partitioning in Irish seaweeds

4.19.9.1. Objective

The results from the seasonal sampling showed distinct patterns of TFA contents comparing *P. palmata* and *F. serratus* during the seasonal sampling with high levels of TFA in summer in *F. serratus* and low levels in *P. palmata*. The objective of this task was to better understand which

underlying processes in the lipid metabolism are causing these differences. A detailed investigation of the partitioning of the fatty acids into different lipid classes at the different sampling times was conducted to investigate the different patterns.

4.19.9.2. Material and methods

Based on the data from the seasonal sampling, two sampling times with particularly high and low levels of total fatty acid were selected for samples from *F. serratus* and *P. palmata*. A detailed lipid class analysis was conducted on the selected samples and the partitioning of lipids and the profiles of the single lipid classes were compared. Lipid extraction was conducted following the protocol of Bligh and Dyer, (1959). Lipid class separation was conducted using TLC (thin layer chromatography) and fatty acid composition of individual classes was analysed using GC-FID described in Guihéneuf *et al.*, (2015).

4.19.9.3. Results

The study showed how two distinct seaweed species, the brown algae *F. serratus* (Figure 23) and the red seaweed *P. palmata* (Figure 24) had strongly differing abilities to adapt their lipid composition and fatty acid profile in response to environmental changes. Both species showed matching changes in their polar lipids, unsaturation levels of fatty acids and the linked pigment composition to maintain structural and photosynthetic function of plastidic membranes during seasonal changes (i.e. temperature and light availability). Results suggest that it is the ability to accumulate TAG that distinguishes between the two species and their tolerance to habitat related stressors. This allows *F. serratus* to prevent damage in times of excess irradiance and to divert the energy into TAG accumulation and energy storage. It appears to be a key factor in coping with environmental stresses and particularly high irradiance, and allows for a broader distribution along the shoreline and supports the exploiting of more variable habitats through a better adaptation potential of *F. serratus* in comparison to *P. palmata*.

Figure 23 Lipid class distribution in *F. serratus* in % of total lipids. Significant differences (*) were determined by Mann-Whitney-U test, $p < 0.05$

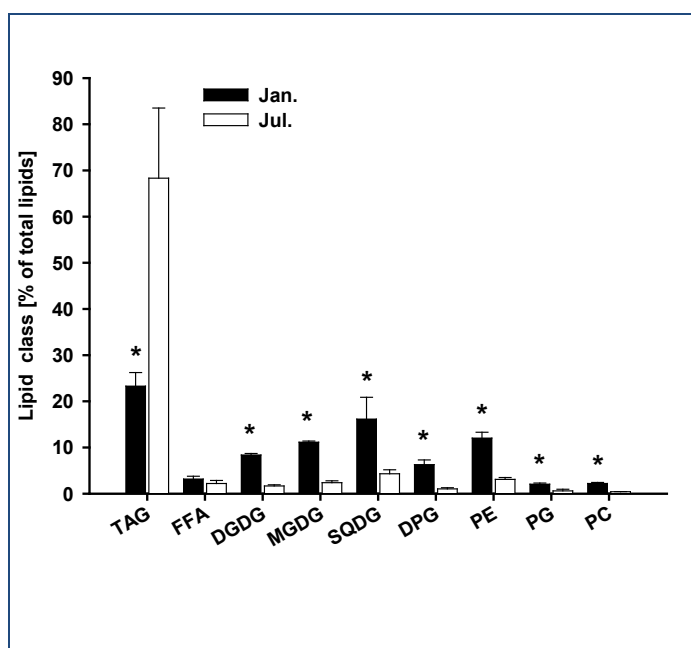
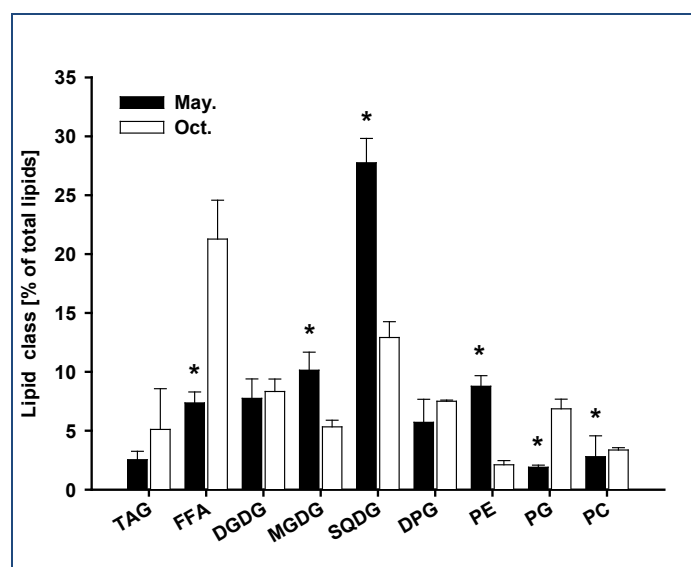


Figure 24 Lipid class distribution of *P. palmata* in % of total lipids. Significant differences (*) were determined by Mann-Whitney-U test, $p < 0.05$



4.19.10. Discussion

Results presented from this study provide a detailed overview of the plasticity of bioactives in brown and red macroalgae of commercial interest in Ireland. The results show how the chemical composition in seaweeds varied within species, and in accordance to sampling location and season, and controlled culture conditions.

The results provide knowledge to industry about the selection of target species, optimal sampling times and locations in order to apply high-value algal biomass as a source for bioactive compounds for functional foods.

4.19.11. *Conclusions*

- Results show a strong plasticity of bioactive compounds
- Chemical composition of seaweeds can vary in dependence of sampling location, season and thallus parts investigated
- Culture experiments show that the chemical composition of seaweeds can be actively altered through changes in the culture conditions
- Species specific algae responses can be linked to habitat preferences and eco-physiology of the seaweeds

4.19.12. *Farmed rainbow trout*

4.19.12.1. *Protein extraction*

Extracting proteins from rainbow trout (*Oncorhynchus mykiss*) involved blending freeze-dried samples of rainbow trout muscle and homogenising 15 g of the sample in 150 mL of 50 mM Tris-HCl buffer, pH 8.0, by using a vortex for 2 min. The homogenate was then centrifuged at 10,000g for 20 min at 4°C using a Beckman Optima™ XL-100K ultracentrifuge (Beckman Coulter Inc., UK). The supernatant constituted the fraction where all soluble proteins (sarcoplasmic proteins) were contained.

The pellet was resuspended in 150 mL of 50 mM Tris-HCl, pH 8.0, containing 6 M urea and 1 M thiourea, and homogenised in a vortex for 5 min in order to solubilise the myofibrillar proteins. The homogenate was then centrifuged at 10,000g for 10 min, collecting the supernatant which constitutes the myofibrillar extract. The residual pellet mainly contains the proteins of the connective tissue. After the extraction, myofibrillar protein samples were freeze-dried, vacuum packed and stored at -20°C until further use.

4.19.12.2. *Protein hydrolysis*

The enzyme thermolysin was used for the hydrolysis of rainbow trout proteins. The hydrolysis process involved rainbow trout (*Oncorhynchus mykiss*) myofibrillar proteins (15 g) being individually added to 500 ml of distilled water in triplicate and heated at 98°C for 15 min to inactivate endogenous myofibrillar protein enzymes. Before hydrolysis, the pH of the mixture was adjusted and subsequently maintained at 7 by the addition of 0.05 M sulphuric acid and 0.1 M sodium hydroxide. Thermolysin was dissolved in distilled water at a concentration of 3 mg/ml and added to the myofibrillar protein mixture in a substrate to enzyme ratio of 100:1 w/v. Hydrolysis was carried out in a BioFlo 110 Modular Benchtop Fermentor (New Brunswick Scientific Co., Inc. Edison, NJ) overnight at 37°C with agitation of 300 rpm. Hydrolysis was stopped by heat inactivation at 100°C for 15 min. The hydrolysates were freeze-dried, weighed, and vacuum packed and kept at -80°C until further use.

4.19.12.3. *MWCO - Ultrafiltration*

The trout myofibrillar protein hydrolysates were filtered using 10-kDa and 3-kDa molecular weight cut off (MWCO) membranes (Millipore, Tullagreen, Carrigtwohill, Co. Cork, Ireland). The 10-kDa and the 3-kDa filtrates (termed 10-kDa-UFH and 3-kDa-UFH, respectively) were freeze-dried, vacuum packed and stored at -80°C until further use.

4.19.12.4. Protein content of rainbow trout myofibrillar protein hydrolysates, filtrates and RP-HPLC fractions

The total protein content of rainbow trout myofibrillar proteins (before hydrolysis), and of the freeze-dried hydrolysates, 10-kDa-UFH and the 3-kDa-UFH filtrates was determined using the Biorad Protein Assay kit in accordance with the manufacturer's instructions (Sigma-Aldrich Chemie GmbH, Switzerland) and the method of Macart *et al.*, (1982). Bovine serum albumin was used as a standard.

Determination of the ACE-I-inhibitory activities of rainbow trout thermolysin hydrolysates, filtrates (10-kDa-UFH and 3-kDa-UFH) and RP-HPLC fractions using the method of Roy *et al.*, (2000): ACE-I-inhibitory activity is usually analysed *in vitro* and implies the determination of inhibitory activity by means of a synthetic substrate with amino di- and tri-substituted peptides such as Hippuryl-L-Histidyl-L-Leucine (HHL, HI635 Sigma), that was used at a concentration of 5 mM and dissolved in borate buffer 0.1 M containing 0.3 M NaCl, pH 8.3. The spectrophotometric method used corresponds to a modified version of the method of Roy *et al.*, (2000). Briefly, 200 μ l of HHL buffer was mixed with 20 μ l of the positive control (Captopril $\text{\textcircled{C}}$) at a concentration of 0.015 μ g/ml or the inhibitory substance and incubated at 37°C for 3 min. The reaction was initiated by addition of 20 μ l of ACE enzyme at a concentration of 0.05 units/ml and the mixture was incubated at 37°C for 30 minutes. The reaction was stopped by addition of 250 μ l of 1M HCl mixed with 1.7 ml of ethyl acetate. The mixture was centrifuged at 13,000 rpm for 15 minutes and 1.4 ml of the top layer removed using a 1 ml pipette. Solvent was evaporated from the test fractions under nitrogen and re-dissolved in 1 ml of distilled, deionized water. The absorbance of each fraction was measured at 228 nm and the percentage ACE-I-inhibition calculated using the following equation:

$$\% \text{ ACE-I inhibition} = 100 - (100 \times (C-D)/(A-B))$$

Where, A is the absorbance in the presence of ACE without the ACE inhibitor, B is the absorbance without ACE and the ACE-inhibitor, C is the absorbance with ACE and the ACE inhibitor and D is the absorbance without ACE and with the ACE-inhibitor.

Measurement of PAF Acetylhydrolase inhibitory activities of rainbow trout hydrolysates, filtrates (10-kDa-UFH and 3-kDa-UFH) and RP-HPLC fractions: PAF acetylhydrolase inhibition was assayed using the Cayman Chemical PAF Acetylhydrolase Inhibitor screening assay kit in accordance with the manufacturers' instructions (Cayman Chemical Company, Ann Arbor, MI). Briefly, 2-thio PAF was used as a substrate for PAF-AH. Upon hydrolysis of the acetyl thioester bond at the *sn*-2 position by PAF-AH, free thiols are detected using 5,5'-dithio-bis-(2-nitrobenzoic acid) using a spectrophotometer at $A_{414 \text{ nm}}$ or $A_{405 \text{ nm}}$. Samples were reconstituted in dimethylsulphoxide (DMSO) at concentrations of 1 mg/ml and assayed in triplicate. Methyl arachidonyl fluorophosphonate (MAFP) was used as a positive control at a concentration of 250 nM. MAFP has an IC_{50} value of 250 nM and is a known inhibitor of PAF-AH.

Measurement of PEP inhibitory activity in rainbow trout thermolysin hydrolyzates and filtrates (10-kDa-UFH and 3-kDa-UFH): Samples were reconstituted in HEPES buffer (50 mM, pH 7.4) to a concentration of 100 mg/mL (0.025 g in 250 μ L buffer). Bovine calf serum (Sigma Aldrich Ireland Ltd., Arklow, Ireland) was used as a source of PEP activity. Z- Gly-Pro- AMC (50 mM) (Bachem Holding AG, Bubendorf, Switzerland) was used as the fluorogenic substrate for the determination of post-proline cleaving enzyme (prolyl endopeptidase). Berberine (13.3 μ M) (Sigma Aldrich Ireland Ltd., Arklow, Ireland) was used as a positive inhibitor. HEPES buffer (50 mM) was used as a negative control.

Prolyl endopeptidase inhibitory activity (PEP inhibitory activity) was assayed as follows; 20 μ L of bovine serum albumin was added to each microtitre well in a 96 well plate (opaque black, clear-bottom; Greiner Bio-One Ltd, Stonehouse, UK). 20 μ L of each sample to be assayed for inhibitory activity or 20 μ L of berberine (as positive control) or HEPES buffer (Sigma) was subsequently added. The assay was initiated by addition of 20 μ L of Z- Gly-Pro- AMC to all wells. The plate was incubated with shaking for 1 hour at 37°C. The enzyme reaction was terminated by addition of 100 μ L of acetic acid (3 mM) to each well. All samples and controls were run in triplicate. End-point fluorescence measurements were taken at an excitation wavelength of 351 nm and an emission wavelength of 430 nm using a Tecan Safire plate reader, model IS89 (AQS Manufacturing Ltd., West Sussex, UK).

The sample background readings were subtracted from the fluorescence readings and the degree of inhibition (%) was then calculated using the following equation:

$$\% \text{ PEP inhibition} = ((\text{control} - \text{sample}) / (\text{control})) \times 100$$

RP-HPLC analysis: 3-kDa and 10-kDa ultrafiltrates were further separated using RP-HPLC analysis. Freeze-dried samples were dissolved in 5 mL to give a final concentration of 240 mg of protein powder per 1 mL of water/acetonitrile (95:5, v/v) with 0.1% trifluoroacetic acid (TFA). After filtering through PVDF 0.22 μ m membrane syringe filter the sample was injected into a Varian Pro Star Polaris HPLC system (Varian, Inc., The Netherlands). The chromatographic separation was developed using a Luna C₁₈ column (5mm particle size, 100A, 100 x 21.20 mm) and a C₁₈ security guard (15 x 21.2 mm) from Phenomenex (Phenomenex Inc., Cheshire, UK) at room temperature. Mobile phases comprised solvent A, containing 0.1% TFA in acetonitrile (v/v), and solvent B, containing 0.1% TFA in water. The separation conditions consisted of 5% of solvent A and 95% of solvent B isocratically for 5 min, followed by a linear gradient from 5 to 60% of solvent A over 30 min at a flow rate of 10 mL/min. The separation was monitored using a diode array detector at a wavelength of 214 nm, and 10 mL fractions were collected and subsequently freeze-dried and stored at -20°C.

Peptide identification by Tandem Mass Spectrometry

Fractions showing the highest levels of bioactivity were analyzed using an electrospray ionisation quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer coupled to a nano-ultra

performance liquid chromatography system (Waters Corporation, Milford, MA, USA) using positive ionisation mode.

Freeze dried samples were dissolved in water/acetonitrile (90:10, v/v) with 0.1% of formic acid and filtered through a 0.22 µm syringe filters. After filtering, 1 µl of the redissolved fractions were loaded into a nano-acquity UPLC column BEH130 C₁₈, 1.7 µm particle size, (100mm x 100mm) preceded by a trapping column Symmetry C18, 5 µm particle size, (180mm x 20mm). Mobile phases consisted of solvent A, containing 0.1% FA in water, and solvent B, containing 0.1% FA in acetonitrile. Trapping of the peptides was achieved under a loading time of 3 min at a flow rate of 5 µl/min with 97% of solvent A and 3% of solvent B and then eluted onto the analytical column at 250 nl/min. Chromatographic conditions consisted of 95% of solvent A and 5% of solvent B isocratically for 3 min, followed by a linear gradient from 95 to 50% of solvent A over 48 min.

Mass spectral data were acquired on MS^e mode with collision energy for full mass scan of 6V and a collision energy ramp of 15-35 V. In the DDA mode, a 1 s TOF MS scan from m/z 100 to m/z 1500 was performed. The Q-TOF was calibrated externally using Glu-fibrinopeptide (Glu-Fib) for the mass range m/z 100 to 1500.

Database search, confirmation of sequences, and de novo sequencing

Automated spectra processing and peak list generation was performed using the software Protein Lynx Global Server, v2.4 (Waters Corporation, Milford, MA). Database search was performed using Mascot interface 2.2 in combination with the Mascot Daemon interface 2.2.2 (Matrix Science, Inc., Massachusetts, USA), (<http://www.matrixscience.com>) against the UniProt and NCBI non redundant databases. Mascot searches were done with none enzymatic specificity and with a tolerance on the mass measurement of 100 ppm in the MS mode and 0.6 Da for MS/MS ions. Oxidation of methionine was used as variable modification.

Comparison between the sequences of proteins to determine the protein origin of peptides was done using UniProtKB/TrEMBL database. Matches of MS/MS spectra against sequences of the database were verified manually using the software mMass v3.11 (Strohalm *et al.*, 2010). The identified peptides in the 10-kDa filtrate were compared with the sequences of bioactive peptides previously identified and reported in BIOPEP database (<http://www.uwm.edu.pl/biochemia/>).

4.19.13. Farmed mussels

Blue mussel (*Mytilus edulis* L.) farming is Ireland's largest shellfish sector. Apart from their culinary value, these marine bivalves are regarded as a potential source for proteins, lipids, and carbohydrates, which may have beneficial effects on human health. Identifying the biochemical composition of mussels is relevant in assessing their potential as a source of ingredients for use in functional foods. A study was undertaken to evaluate seasonal and spatial variations in the composition of blue mussels farmed in Irish waters. In this study, mussels were collected over a one-year period from five different locations and at four different times, and their proximate

composition (glycogen, total proteins, total lipids and inorganic matter), energy content and fatty acid profiles determined. A further dimension to the study was the inclusion of samples of mussels - wild grown mussels, undersized mussels and broken specimens from aquaculture.

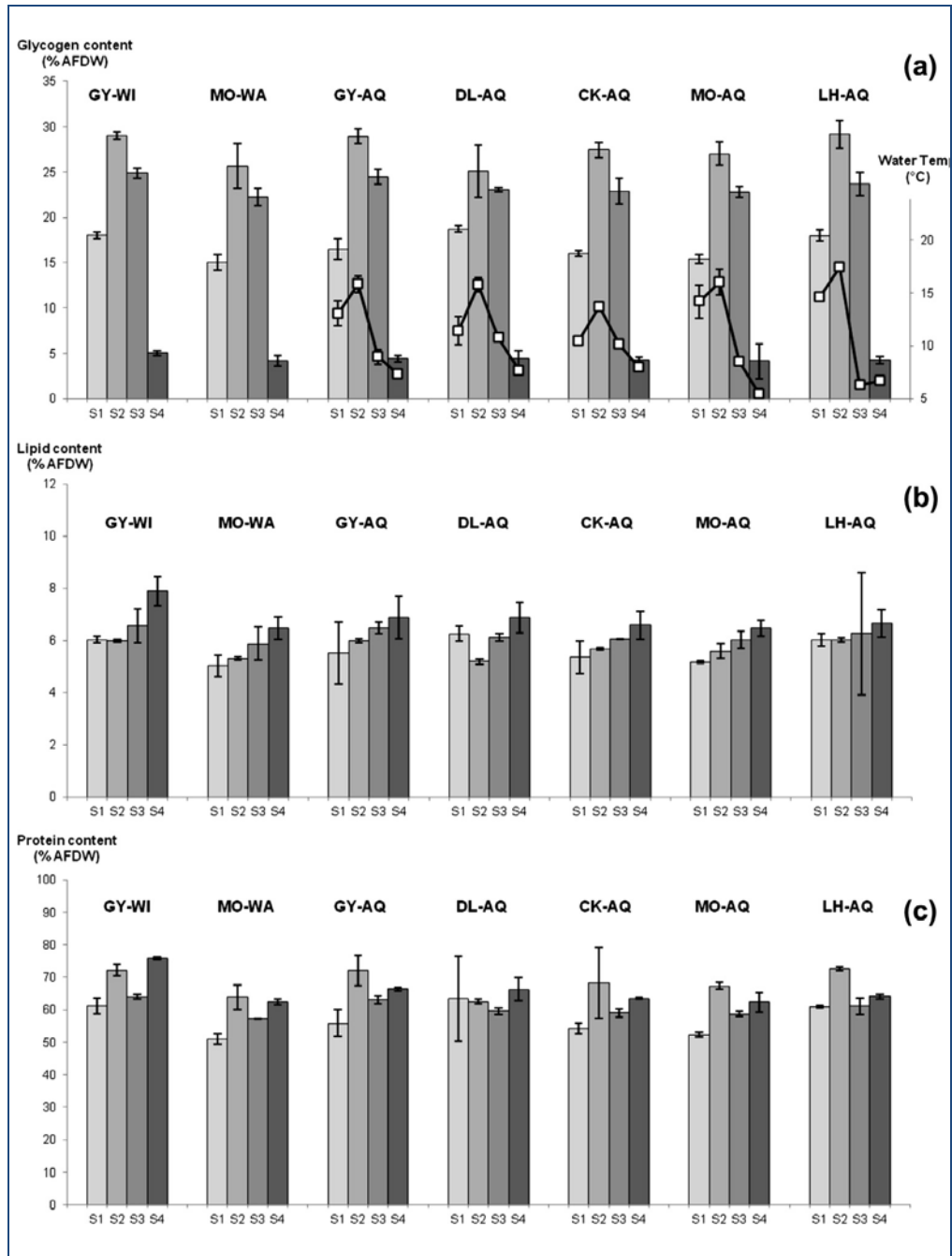
4.19.14. Biochemical composition analysis

For proximate chemical composition studies, pools of each mussel sample were freeze-dried, milled and kept in dry conditions until further analysed. Quantitative protein determination was performed by the Kjeldahl method (N_{6.25}), the lipid content was determined gravimetrically after Soxhlet extraction using petroleum ether and the amount of inorganic material was measured by incinerating the samples to ash in a muffle furnace at 550°C for 16h. The energy content was measured by combustion with a bomb calorimeter Parr-6100 (Parr Ins. Co., IL, USA) and expressed in MJ/kg. The glycogen content was determined by means of a modified phenol-sulphuric acid method (Dubois *et al.*, 1956) after enzymatic hydrolysis of the freeze-dried material with α -amylglucosidase (Murat and Serfaty, 1974). Briefly, 5 mL of the enzyme solution (0.3 mg/mL in 100 mM acetic acid) were added to 0.1 g of the freeze-dried material and the mixture was allowed to react for 3 h at 50°C. After centrifugation (6000 rpm, 15 min) and dilution of the supernatant, 0.5 mL of the samples were mixed with 0.5 mL of a 5% (w/v) phenol solution and 2.5 mL of concentrated sulphuric acid. The absorbance of the sample was measured at 490 nm using an Evolution 60S spectrophotometer (Thermo Fisher Sci., MA, USA). Glucose was used as a standard.

4.19.14.1. Results

The spatial and seasonal variations of glycogen, total lipids, and proteins in samples of mussels collected between May 2012 and April 2013 were determined. The quantitative analyses of glycogen, revealed elevated levels in spring and summer and lower levels during autumn and winter (Figure 25a). Interestingly, this distinct seasonal cycle observed for glycogen ($P < 0.001$) correlated with the average water temperature recorded at the different sampling sites. Maximum glycogen values were recorded at the end of summer (season 2), ranging from 25.11% (DL-AQ) to 29.18% (LH-AQ). The minimum glycogen contents were observed at the end of winter (season 4), with values between 4.15% (MO-WA) and 5.05% (GYWI).

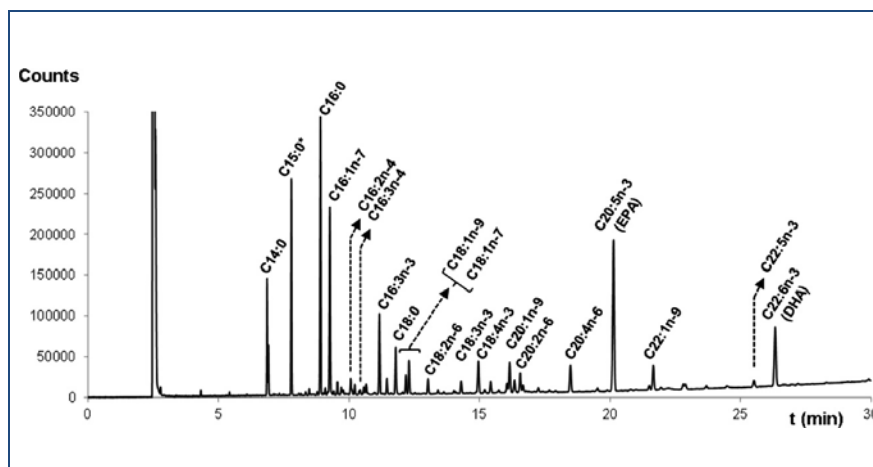
Figure 25 Seasonal and spatial variations of glycogen (a), total lipid (b), and total protein (c) content of blue mussels (*M. edulis*) collected in Ireland between May 2012 and April 2013. On the main Y-axis, percentages are represented in an Ash Free Dry Weight (AFDW) basis (bars). In addition, the average water temperature for the different aquaculture sampling sites is represented in the secondary Y-axis (curves). Water temperature data was obtained from the Irish Marine Institute. S1: May/June 2012; S2: August/September 2012; S3: November/December 2012; S4: February/March 2013



The seasonal trend observed in protein contents (Figure 25c) was not as distinct as in the case of carbohydrates or lipids. However, global seasonal differences were statistically significant ($P < 0.001$). The lowest protein level was observed in season 1 (May/June 2012), coinciding with the main spawning period of blue mussels in Ireland. Moreover, an increase in the total protein content was observed in season 2 (August/September 2012) for most of the samples. This may be related to the general accumulation of biochemical reserves during that period of time. In

all samples investigated, the protein content at the end of winter (season 4) was higher than that observed at the end of spring, i.e., season 1 (May/June 2012).

Figure 26 Fatty acid profile of a wild mussel sample collected during season 1 (May/June 2012) in Rusheen Bay, Co. Galway (GY-WI)



Seasonality stood out as an important factor influencing lipid content in Irish blue mussels ($P < 0.001$). As a general trend, a constant increase in the total lipid content was observed from May/June 2012 (season 1) to February/March 2013 (season 4), except for the case of the sample from Co. Donegal (DL-AQ). The FA profile of the wildy grown blue mussels in Co. Galway is shown in Figure 26, where a total of 28 fatty acids (FAs) were identified. The presence of PUFAs is notable not only in quantity, but also diversity. Amongst all PUFAs, a high proportion of ω -3 long-chain PUFAs, especially EPA and DHA is apparent. Within the group of saturated fatty acids (SFAs) and monounsaturated fatty acids (MUFAs), myristic (C14:0), palmitic (C16:0) and palmitoleic (C16:1n-7) acids were the most abundant.

There were distinct interactions between seasonality and sampling site, suggesting that their effects cannot be studied independently. The seasonal variations observed for glycogen and for some fatty acids appeared to be closely linked to environmental parameters, such as water temperature and food/phytoplankton availability, while the fluctuations observed in lipid or protein content depended mostly on the reproductive cycle of the mussels.

The most significant seasonal variations have been observed for glycogen, the main carbohydrate with storage reserve functions. The highest accumulation of glycogen was observed during spring and summer while a depletion was mainly observed during autumn and winter. A clear relationship between water temperature and glycogen content in mussel meat was observed for the five aquaculture samples. Smoother trends have been noticed for lipids and proteins. In the case of the energy content, the seasonal trend observed is opposite to that observed for inorganic matter.

In terms of geographical variations, the biochemical fluctuations found were significant, but not as distinct as in the case of the collection season. Thus, it appears the nutritive value of Irish blue mussels is not dependent on the collection site, but primarily on the harvesting time. Gas

Chromatography analyses emphasised the prominent presence of PUFAs in blue mussels and the correlation between EPA and DHA levels with the main phytoplankton species in the production areas.

That Irish blue mussels are a rich source of proteins is of nutritional relevance. Moreover, their fatty acid profile is characterised by elevated levels of health beneficial ω -3 PUFAs, especially EPA and DHA, plus a high ω -3/ ω -6 ratio. From a nutritional point of view, it might be concluded that the optimum season for mussel collection and marketing is the end of summer as the samples collected during this season showed the highest carbohydrate content, the highest DHA values and interesting EPA values.

Undersized and broken mussels had a similar biochemical composition as the corresponding aquaculture sample collected in the same area. Hence, in addition to cultured mussels, the exploitation of aquaculture by-products such as waste mussel meat for the extraction and investigation of high quality proteins, lipids, and carbohydrates might be of economic value.

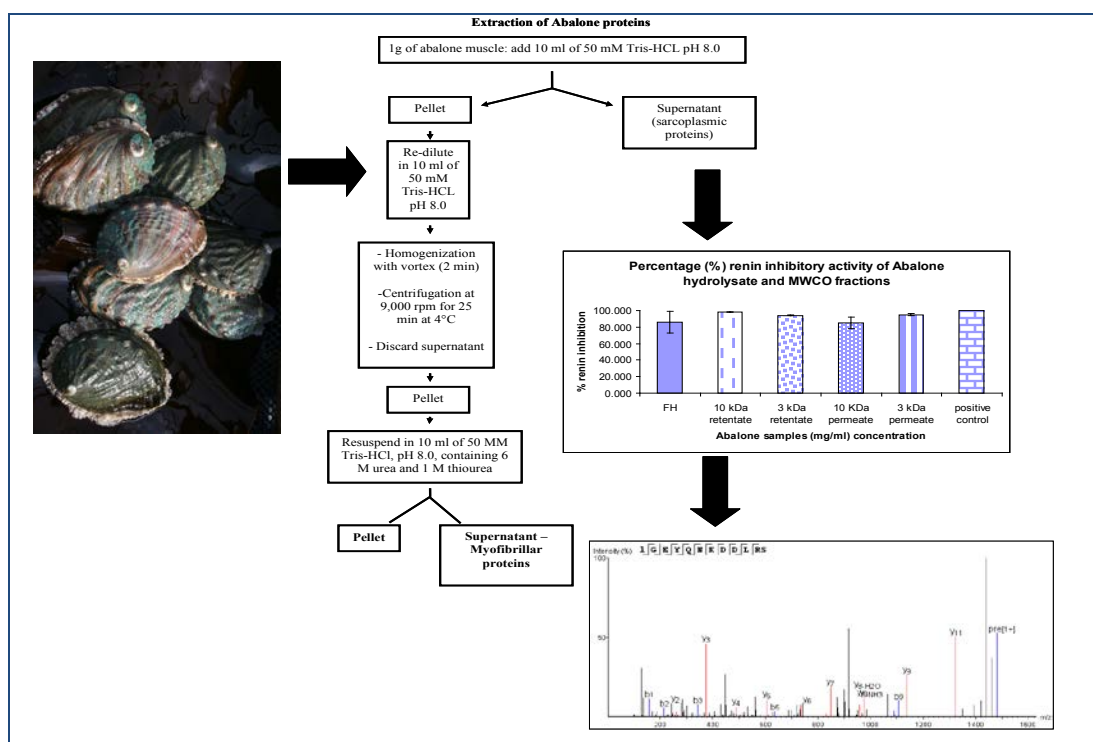
4.19.15. Abalone

Bioactive peptides are known to play an important role in metabolic modulation and regulation (Najafian, 2012). They have been isolated from a myriad of sources previously but the importance of marine invertebrates as a source of novel bioactive substances is growing (Barrow and Shahidi, 2008; Aneiros and Garateix, 2004). Recently, two peptides with antibacterial activity from the abalone *Nordotis discus discus* were reported (Park *et al.*, 2012). Against this background, and considering an opportunity to add value to Ireland's emerging Abalone aquaculture sector, NutraMara initiated work to generate thermolysin hydrolysates of abalone (Pacific abalone *Haliotis discus hannai* Ino) myofibrillar proteins and MWCO 3-kDa and 10-kDa fractions, and assess these for their abilities to inhibit renin and the serine proteases Factor Xa and PAF-AH. The Pacific abalone is cultured in Ireland.

4.19.16. Protein extraction

Whole abalone samples (including shell) were defatted by being soaked in 100 % ethanol (Sigma, Ireland) at 4°C for 5 days to decant. The samples were then removed from ethanol and the fat decanted. The foot and adductor muscle of adult abalone were removed from the abalone shell, washed with distilled, de-ionized water and subsequently freeze-dried at -80 °C for 4 days. Freeze-dried muscle was subsequently used as the substrate for protein fraction preparation using the method described below in Figure 27.

Figure 27 Schematic of steps taken in isolating bioactive peptides from abalone



4.19.17. Enzymatic hydrolysis

Thermolysin hydrolysates (X3) of the abalone muscle myofibrillar proteins were prepared using a New Brunswick (New Brunswick Scientific co., Inc., Edison, NJ) 1 L bioreactor with temperature and pH control. Abalone myofibrillar proteins (15 g) were dispersed in distilled Romil HPLC grade water in triplicate and heated at 98°C for 15 min to inactivate endogenous myofibrillar protein enzymes. Before hydrolysis with thermolysin, the pH of the mixture was adjusted and subsequently maintained at 7 by the addition of 0.1 M sodium hydroxide (NaOH). Thermolysin was dissolved in distilled water at a concentration of 3 mg/ml and added to the myofibrillar protein mixture in a substrate to enzyme ratio of 100:1 w/v. Hydrolysis was carried out overnight at 37°C with agitation of 300 rpm and stopped by heat inactivation at 100°C for 15 min. The hydrolysates were freeze-dried, weighed, vacuum packed and stored at -80°C until further use.

4.19.18. MWCO-Ultrafiltration

Abalone myofibrillar protein hydrolysates were filtered using 10-kDa and 3-kDa molecular weight cut off (MWCO) membranes (Millipore, Tullagreen, Carrigtwohill, Co. Cork, Ireland). The 10-kDa and the 3-kDa ultra-filtrates and permeates (termed 10-kDa-UFH and 3-kDa-UFH, respectively) were freeze-dried, vacuum packed and stored at -80°C until further use.

4.19.19. Protein content of abalone myofibrillar protein hydrolysates and MWCO 3-kDa (3-kDa-UFH) and 10-kDa (10-kDa-UFH) ultrafiltration fractions

The total protein content of abalone myofibrillar proteins (before hydrolysis), and of the freeze-dried hydrolysates, 10-kDa-UFH and the 3-kDa-UFH filtrates and permeates was determined using the QuantiPro BCA assay kit in accordance with the manufacturers' instructions (Sigma-Aldrich Chemie GmbH, Switzerland). Bovine serum albumin was used as the protein standard.

4.19.20. Renin inhibitory bioassay of abalone thermolysin hydrolysates and MWCO 3-kDa-UFH and 10-kDa-UFH fractions

This assay was carried out according to the manufacturers' instructions (Renin Inhibitor screening assay kit, Catalog number 10006270, Cambridge BioSciences, United Kingdom). All abalone hydrolysates and 3-kDa and 10-kDa-UFH fractions were assayed at a concentration of 1 mg/ml in triplicate. The known specific renin inhibitor, Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe (Sigma Aldrich, Dublin, Ireland), was used as a positive control. Percentage inhibition was calculated using the following equation,

$\% \text{ renin inhibition} = (100\% \text{ Initial Activity (AF)} - \text{Inhibitor (AF)}) / 100\% \text{ Initial Activity (AF)} \times 100$, where AF is the average fluorescence.

Initial activity is the assay performed without the presence of an inhibitor. Data were compared using the students' *t*-test and considered significantly different if $P < 0.05$.

Measurement of PAF Acetylhydrolase inhibitory activities of abalone thermolysin hydrolysate and the MWCO 10-kDa-UFH and 3-kDa-UFH fractions

PAF acetylhydrolase inhibition was assayed using the Cayman Chemical PAF Acetylhydrolase Inhibitor screening assay kit in accordance with the manufacturers' instructions (Cayman Chemical Company, Ann Arbor, MI). Methyl arachidonyl fluorophosphonate (MAFP) was used as a positive control at a concentration of 250 nM. MAFP has an IC₅₀ value of 250 nM and is a known inhibitor of PAF-AH. The percentage inhibition for each sample was determined using the following equation,

$\% \text{ PAF-AH inhibition} = 100\% \text{ initial activity} - \text{Inhibitor sample value} / 100\% \text{ initial activity value} \times 100$.

4.19.21. Measurement of Factor Xa inhibitory activities of abalone thermolysin hydrolysate and the MWCO 10-kDa-UFH and 3-kDa-UFH fractions

Factor Xa inhibition was assayed using the Sensolyte® Rh110 Factor Xa Assay Kit in accordance with the manufacturers' instructions (AnaSpec., Inc., Fremont, CA 94555). This kit provides a method for screening Factor Xa inhibition using a fluorogenic substrate. Upon FXa protease cleavage, this substrate generates the Rh110 (rhodamine 110) fluorophore which has a bright green fluorescence that can be detected at excitation/emission of 490nm/520nm. Abalone hydrolysate and MWCO fractions were reconstituted in dimethylsulphoxide (DMSO)

at concentrations of 1 mg/ml and assayed in triplicate. The positive control supplied in the kit was used at the recommended concentration.

4.19.22. Water activity (*aw*)

The water activities of the freeze-dried abalone full thermolysin hydrolysate, the MWCO 10-kDa-UFH and the 3-kDa-UFH powders, were assessed using an AquaLab instrument CX-2, MM/80 (Decagon, 2565 NE Hopkins, Court Pullman Wa 99163). The equipment was calibrated using a solution of 0.5 M KCl (AquaLab WP4 Dewpoint Potential Meter, Decagon Devices, Inc.) with a water activity value (*aw*) of 0.984.

4.19.23. Removal of polyethylene glycols from abalone hydrolysates, filtrates and permeates using a titanium dioxide (TiO₂) cleanup procedure

Polyethylene glycols (PEGs) were removed from total abalone myofibrillar protein thermolysin hydrolysates, 10-kDa-UFH and the 3-kDa-UFH filtrates and permeates using the Pierce™ TiO₂ Phosphopeptide Enrichment and clean-up kit (Thermo Scientific, Rockford, USA) according to the method of Zhao and O'Connor (2007). After cleanup, all protein samples were freeze-dried prior to MS analysis.

4.19.24. Peptide identification

The full abalone myofibrillar protein thermolysin hydrolysate and the MWCO 10-kDa and 3-kDa –UFH fractions were analysed by electrospray ionisation quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer coupled to a nano-ultra performance liquid chromatography system (Waters Corporation, Milford, MA, USA) using positive ionisation mode. Samples were dissolved in MilliQ purified water at a concentration of 1 mg/ml. 5 µl of the 10 and 3-kDa MWCO filtrates were loaded independently on to a nano-acquity UPLC column BEH130 C18, 10 cm, 1.7 cm) preceded by a trapping column Symmetry C18, 2cm, 5 cm). Trapping of the peptides was achieved with 4 µl/min with solvent A (0.1% HCOOH in water) and then eluted onto the analytical column at 300 nl/min. Separation of the peptides were performed in a linear gradient from 3% to 85% solvent B (0.1% HCOOH in acetonitrile) during 110 min. Mass spectral data were acquired on MSe mode for the 10 and 3-kDa filtrates. The collision energy for full mass scan was 5V and the collision energy ramp of 15-35 V was used for the MSe data acquisition mode. In the DDA mode, a 1 s TOF MS scan from *m/z* 100 to *m/z* 1600 was performed, followed by 3-8 s product ion scans from 50 to 1600 *m/z* on the most intense ions. The Q-TOF was calibrated externally with a MS/MS fragment ions of Glu-fibrinopeptide (Glu-Fib) for the mass range *m/z* 100 -1600. The Glu-Fib peptide was also used as a lock mass.

4.19.25. Database search and confirmation of sequences

Database searchers and peak list generation to identify proteins and peptides contained in the abalone myofibrillar protein, thermolysin hydrolysates and MWCO fractions was performed using the software Peaks Mass Spectrometry Software 6.0 (BioMar, Inc., USA). Searches were done with no enzymatic specificity allowing one missed cleavage and a tolerance on the mass

measurement of 1.2 Da in the MS mode and oxidation of His-Trp were used as variable modifications.

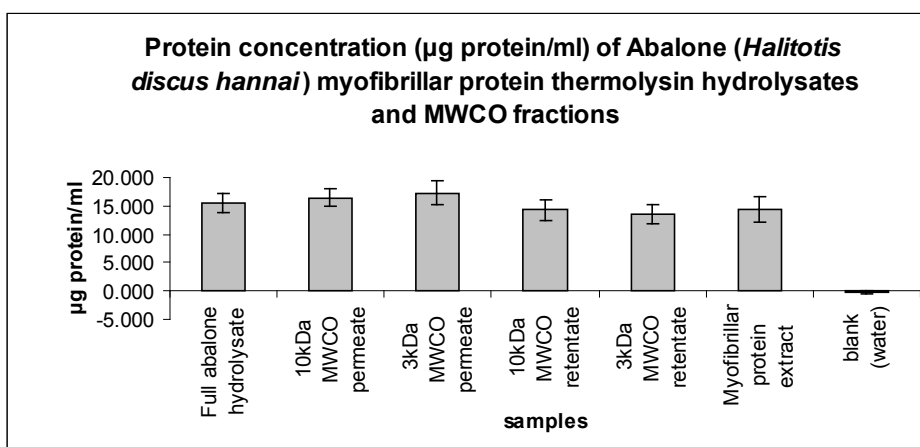
4.20. Results

The aim of this work was to screen protein and peptide fractions from muscle proteins of the abalone *Haliotis discus hannai* Ino for inhibitory activities against each of the three enzymes; renin, PAF-AH and Factor Xa *in vitro*. *Haliotis discus hannai* Ino abalones were chosen as the potential source of heart health peptides, as antimicrobial and antioxidant peptides were previously identified in this species.

4.20.1. Protein content of abalone myofibrillar muscle thermolysin hydrolysates, MWCO 10-kDa-UFH and 3-kDa -UFH fractions

The protein content of the 10-kDa-thermolysin MWCO permeate was 16.465 (\pm 1.527) g proteins / g dried sample while the protein content of the 3-kDa-UFH thermolysin permeate was 17.203 (\pm) g proteins / g dried sample. Values for the 10-kDa-UFH retentate and 3-kDa-MWCO retentate are shown in Figure 28 below.

Figure 28 Protein concentration of abalone myofibrillar protein thermolysin hydrolysates and MWCO fractions using the BCA Bioassay kit

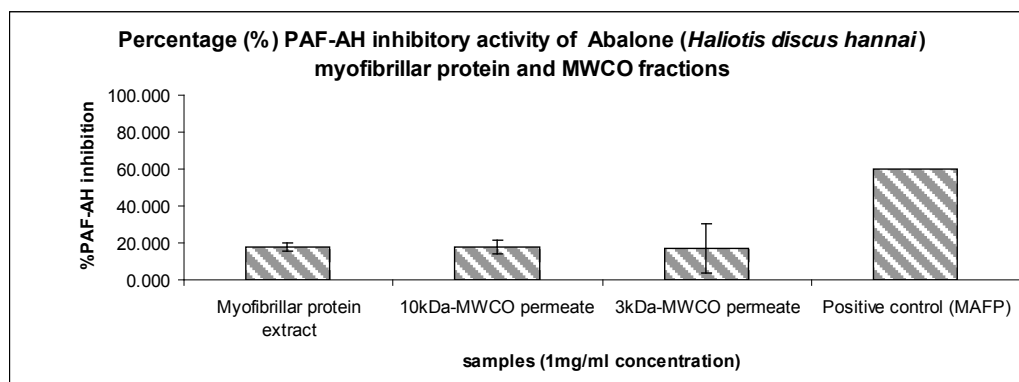


4.20.2. PAF-AH inhibitory activities of the 10-kDa-UFH and the 3-kDa-UFH fractions generated from abalone thermolysin hydrolysates

When assayed for PAF-AH inhibitory activity, the MWCO 10-kDa-UFH and the 3-kDa-UFH fractions generated from abalone myofibrillar protein thermolysin hydrolysates were reconstituted in DMSO at concentrations of 1 mg/ml and tested in triplicate. PAF-AH inhibitory activity values were less than 20 % for abalone extracts and fractions, when assayed at a concentration of 1 mg/ml. The myofibrillar protein extract from abalone inhibited PAF-AH by 17.897 % (\pm 2.26) at a concentration of 1mg/ml. The MWCO 10-kDa-UFH permeate fraction and the 3-kDa-UFH permeate fraction inhibited PAF-AH by 17.636 % (\pm 3.52) and 16.757 % (\pm 3.55) respectively compared to 60.24 % (\pm 0.22) PAF-AH inhibition by MAFF, the

positive control, which was assayed at a concentration of 250 nM. All PAF-AH inhibition values are shown in Figure 29.

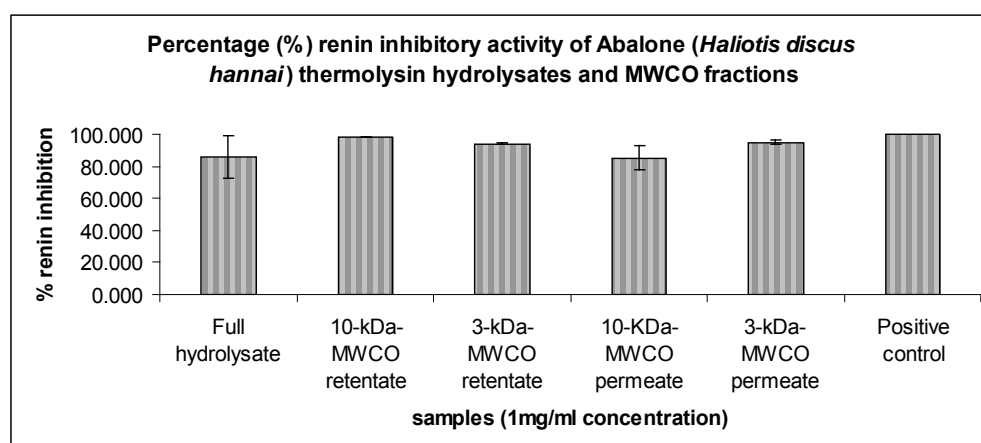
Figure 29 Percentage PAF-AH inhibitory activities of Abalone myofibrillar protein thermolysin hydrolysate and MWCO ultrafiltrates



4.20.3. Renin inhibitory activities of the MWCO 10-kDa-UFH and the 3-kDa-UFH fractions from abalone myofibrillar protein thermolysin hydrolysates

The full abalone myofibrillar protein thermolysin hydrolysate, the 10-kDa-UFH and the 3-kDa-UFH fractions were reconstituted in DMSO (1 mg/ml) and tested for their renin inhibitory activities. All fractions inhibited renin and inhibition values ranged from 86.03 % (+0.108) at a concentration of 1mg/ml for the abalone myofibrillar protein thermolysin hydrolysate to 95.428 % (+0.108) at a concentration of 1mg/ml for the 10-kDa-UFH permeate fraction. All renin inhibitory values are shown in Figure 30. These renin inhibition values compare favourably with values reported in the literature previously (FitzGerald *et al.*, 2012; Li and Aluko, 2010; Takahashi *et al.*, 2008).

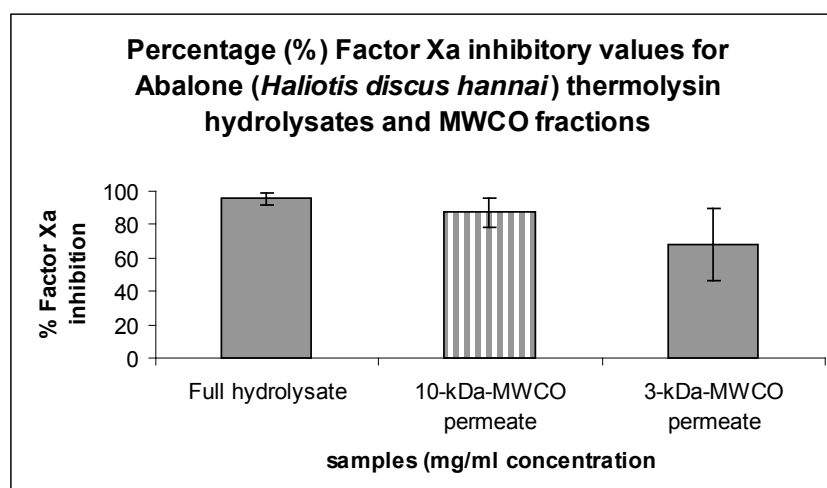
Figure 30 Percentage renin inhibitory values for Abalone myofibrillar protein thermolysin hydrolysate and MWCO ultrafiltrates



4.20.4. Factor Xa inhibitory activities of the 10-kDa-UFH permeate and the 3-kDa-UFH permeate fractions from abalone myofibrillar protein thermolysin hydrolysates

The full abalone myofibrillar protein thermolysin hydrolysate, the 10-kDa-UFH and the 3-kDa-UFH thermolysin fractions were reconstituted in DMSO (1 mg/ml) and tested for their Factor Xa inhibitory activities. All fractions inhibited Factor Xa and inhibition values ranged from 68.12 % (± 21.94) at a concentration of 1 mg/ml for the abalone myofibrillar protein thermolysin hydrolysate 3-kDa-permeate fraction to 95.69 % (± 3.52) at a concentration of 1 mg/ml for the full abalone myofibrillar protein thermolysin hydrolysate. All Factor Xa inhibitory values are shown in Figure 31 and compare favourably with values reported in the literature previously such as the commercially available Apixaban and Betrixaban (Dunwiddie *et al.*, 1989; Lim-Wilby *et al.*, 1995; Eriksson *et al.*, 2009; Jordan *et al.*, 1990) and with the internal positive control used in the Factor Xa assay kit.

Figure 31 Factor Xa inhibition values obtained with Abalone thermolysin hydrolysates and MWCO filtrates



4.20.5. Water activity (a_w) of the abalone myofibrillar protein thermolysin hydrolysates, 10-kDa-UFH and 3-kDa-UFH fractions

The water activity (a_w) values of the abalone myofibrillar protein thermolysin hydrolysate, the 10-kDa thermolysin filtrate and the 3-kDa thermolysin filtrate were $0.435 a_w (\pm 0.080)$, $0.465 a_w (\pm 0.045)$ and $0.476 a_w (\pm 0.051)$ respectively. These water activity values compare favourably with typical a_w values of meat products and are low enough to prevent growth of pathogens such as *Escherichia coli* (a_w 0.93), *Salmonella* species (a_w 0.91-0.95), *Listeria* sp. (a_w 0.93) and most moulds and yeast (a_w 0.80-0.90) (Mathlouthi, 2001).

4.20.6. Peptides identified in abalone (*Haliotis discus hannai* Ino) myofibrillar protein thermolysin hydrolysates, 10-kDa-UFH and 3-kDa-UFH using ESI nano-spray MS and FT-ICR/Orbitrap

In total, 66 proteins (data not shown) were identified in the full abalone myofibrillar protein extract hydrolysate, 191 proteins in the 10-kDa-UFH fractions (data not shown) and 114

proteins in the 3-kDa-UFH fractions. Table 18 lists the peptides identified within the 10-kDa-UFH of abalone (*Haliotis discus hannai* Ino) myofibrillar protein thermolysin hydrolysate.

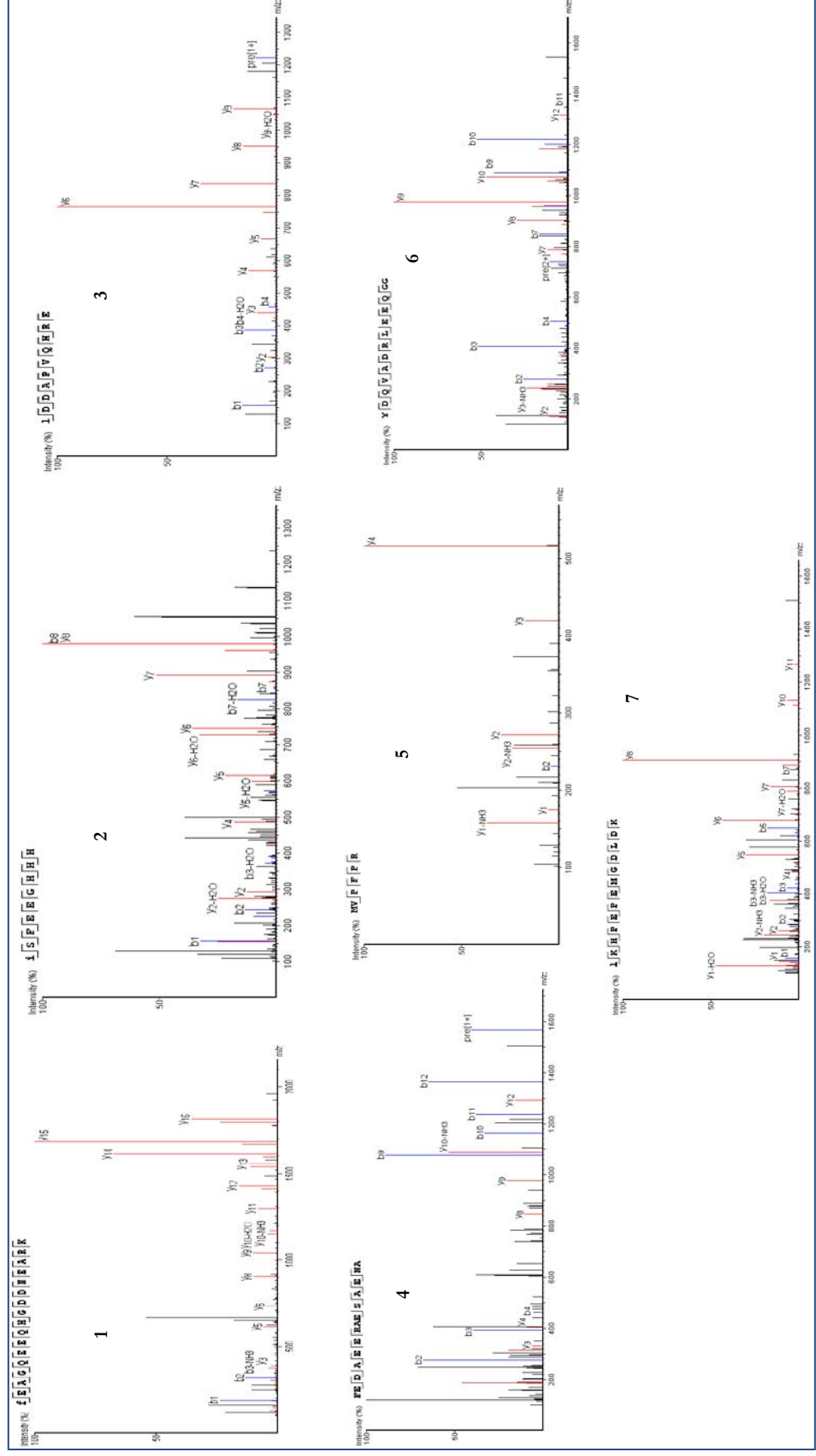
Figure 32 shows the MS/MS spectrum of pro-peptides identified in the 3-kDa-UFH filtrate fraction generated in this study. These pro-peptides were not identified in the database BIOPEP (http://www.uwm.edu.pl/biochemia/accessed_10th_November_2012) or in other peptide databases such as SwePep (<http://www.swepep.org>) and PepX.

These sequencing results show that the peptides identified in the bioactive full hydrolysate, the 10-kDa-UFH and in the 3-kDa-UFH fractions were generated from many of the main paramyosin and tropomyosin proteins found in invertebrate (abalone) tissue, such as arginine kinase, Actin A1 α -chains, and the fibrous proteins, including collagen and elastin and others (<http://pepx.switchlab.org/>). Identified pro-peptides (Figure 32) ranged from 9 amino acids (HHHGEEFSI) in length to 17 amino (KRAENDDGHQEEQGAEF) in length. Pro-peptides identified in the 10-kDa-UFH are shown in Table 18.

Table 18 Peptides identified within the 10-kDa-UFH of abalone (*Haliotis discus hannai* Ino) myofibrillar protein thermolysin hydrolysate

Peptide number	MWCO fraction	Peptide sequence	Calculated Mass	Amino acid length
1	10-kDa-UFH	AQTPKNSMEGTKVG	1447.63	14
2	10-kDa-UFH	RGHTSDYRF	1138.22	9
3	10-kDa-UFH	YRDHERSGTGS DHIV	1728.81	15
4	10-kDa-UFH	KDPYPGAMV	977.15	9
5	10-kDa-UFH	TEEYSDERQSAQDL	1670.69	14
6	10-kDa-UFH	RKGMTGTSEDAALI	1449.66	14
7	10-kDa-UFH	HHHGEEFSI	1092.14	9
8	10-kDa-UFH	EPLHDL	722.8	6
9	10-kDa-UFH	RKADFV	734.85	6
10	10-kDa-UFH	QKQDEKSMGEI	1292.43	11
11	10-kDa-UFH	GQWAPSEASEKKV	1545.67	14
12	10-kDa-UFH	APPVTRSL	840	8
13	10-kDa-UFH	SRLDDKNQY EGL	1437.53	12
14	10-kDa-UFH	AGMLPVLW	886.12	9
15	10-kDa-UFH	FTTEHI	746.83	6
16	10-kDa-UFH	TRVVKSSVDGYDM	1456.64	13
17	10-kDa-UFH	GEVIPVTHSVG	1094.23	11

Figure 32 MS/MS spectrum of pro-peptides identified in the 3-kDa-UFH filtrate fraction



Of all the identified pro-peptides, the peptide HHHGEEFSI with a molecular mass of 1092.14 was found in both the 10-kDa-MWCO and the 3-kDa-MWCO fractions and the peptides KRAENDDGHQEEQGAEF and SRLDDKNQYEGEGL were identified in both the 10-kDa and the full abalone thermolysin hydrolysates. The pro-peptides identified in this study were not identified previously in the literature or in bioactive peptide databases including BIOPEP (<http://www.uwm.edu.pl/biochemia/> accessed 10th November 2012). However, some of the pro-peptide sequences may share homologies with a number of peptides previously reported as having antioxidant and ACE-I inhibitory activities.

The a_w values reported for the dried hydrolysate product show that the hydrolysate could be used as an ingredient in soups or as a condiment as the a_w values reported do not support the growth of pathogenic and spoilage bacteria. This work demonstrates that Abalone is a potential source of heart health propeptides with Factor Xa and renin inhibitory activities. The hydrolysate and/or MWCO filtrates have potential for use as an ingredient for the manufacture of a natural condiment or sauce with heart health effects.

4.2.1. The peptide database

During the project a database containing details of marine-derived bioactive proteins, peptides and amino acids from macro- and microalgal, fish and shellfish sources with a range of biological activities was generated. The final version of the database contains 94 protein, 574 peptide and 52 amino acid entries. Of these entries, 39 peptides and 12 amino acids arose from NutraMara funded research/non-NutraMara funded research. Peptides with activities associated with *in vitro* cardioprotection (renin, platelet activating factor acetylhydrolase and acetylcholinesterase inhibition), anti-diabetic (dipeptidyl peptidase IV inhibition and glucagon-like peptide-I protection) and antioxidant and *in vivo* antihypertensive activity were derived from red macrolagal *Palmaria palmata* proteins. Two peptides with *in vitro* cardioprotective (angiotensin converting enzyme inhibition) and antioxidant activity were derived from Thai fermented shrimp paste (*Mesopodopsis orientalis/Acetes indicus/ Acetes japonicus/Acetes erythraeus*). Five peptides with metal chelating activities were mined from Alaska Pollack skin collagen hydrolysates while peptides with *in vitro* cardioprotective (angiotensin converting enzyme inhibition), anti-diabetic (dipeptidyl peptidase IV inhibition) and antioxidant were derived from Atlantic salmon (*Salmo salar*) trimming muscle proteins and gelatine. Amino acids with angiotensin converting enzyme and anti-diabetic inhibition and antioxidant were also derived from Atlantic salmon (*Salmo salar*) trimming muscle proteins and gelatine.

5. SCREENING AND PROFILING BIOACTIVE COMPOUNDS

5.1. Introduction

With many organisms having evolved to live in extreme conditions and adapt to environmental changes, the marine environment is recognised as a rich source of biodiversity. Naturally occurring bioactive compounds obtained from marine organisms have been shown to offer considerable scope for use as functional food ingredients. Marine organisms are known as a rich source of bioactive compounds such as lipids, minerals, vitamins, proteins, polysaccharides, pigments and compounds with antioxidant properties. Compounds as these have shown promise as potential ingredients in functional foods, whilst some, such as the omega-3 polyunsaturated fatty acids are associated with beneficial health effects.

The NutraMara research programme extracted a wide range of bioactive compounds from fish, algae and discards from the fish processing industry. However, the challenge to be overcome in basing food ingredients on these compounds is to demonstrate their potential by proving they have a health benefit. This has involved screening marine bioactive compounds against various bioassays, and employing different methods including model systems, cell cultures and “omic” approaches to determine the characteristics of compounds which demonstrated *in-vivo* potential to contribute positively to human health.

The NutraMara feasibility study and market feedback resulted in the identification of specific health effects as targets for marine bioactives including, prebiotic, anti-microbial, anti-infective, anti-obesity, anti-inflammatory, anti-cancer and the effect of some marine compounds on mineral absorption and glucose metabolism. In most cases the research involved using standard, proven bioassays; however, there were instances when it was necessary to develop specific bioassays.

In the context of developing ingredients for use in functional foods it is necessary to demonstrate the efficacy of the bioactive component(s). This is a critical step in the process of providing a sufficiently strong scientific basis in order to support and justify a health claim related to the intake of a functional food. Developing a scientific understanding of the mode of action of a compound identified as a potential functional ingredient is a vital step in the approval of such claims. Some of the investigations of bioactive ingredients by the NutraMara consortium included investigating the molecular mode of action of marine origin bioactive compounds. A variety of extrinsic factors can influence the efficacy of functional

compounds, including; the stability and bioavailability of bioactive compounds in food matrices; the physical and chemical form of the food compound; the effect of the total diet on the compound; food processing effects; and a range of environmental factors. The impact of different processing methods on the bioactivity of functional compounds was assessed for a small number of bioactives extracted from samples of seaweeds.

5.2. Cellular and metabolic studies

The use of cellular and metabolic approaches formed a sizable proportion of the screening activities within the NutraMara programme. Compounds derived from algae, fish and fish processing discards were all screened.

5.3. Screening for anti-inflammatory potential

A widely used *in-vitro* model for studying human gut immunity to inflammation is the human intestinal epithelial cell model (Caco-2) bioassay. This assay was optimised and used to screen NutraMara samples for anti-inflammatory properties. This bioassay requires the bioactive compound to be soluble in water. The Caco-2 cells were induced by tumour necrosis factor alpha (TNF α) to produce inflammatory cytokine (interleukin 8 – IL8) in the presence of NutraMara bioactives extracted from various seaweed species. The IL-8 secreted by the cell was measured using an ELISA kit. .

A total of 171 samples of seaweed extracts were evaluated for anti-inflammatory properties *in vitro* and 54 samples were identified to have positive hits (see Table 19 and Table 20)

Table 19 *In vitro* screening for anti-inflammatory bioactivity – whole extract

Species	Sample ID	Extract	Hit
<i>A. nodosum</i>	84	Cold water	Yes
<i>A. nodosum</i>	84	Ethanol 80%	Yes
<i>A. nodosum</i>	84	Hot water	Yes
<i>Palmaria palmata</i>	141	Cold water	No
<i>Palmaria palmata</i>	141	Ethanol 80%	No
<i>Palmaria palmata</i>	141	Hot water	No
<i>Alaria esculanta</i>	36	Cold water	No
<i>Alaria esculanta</i>	36	Ethanol 80%	No
<i>Alaria esculanta</i>	36	Hot water	Yes
<i>Codium fragile</i>	130	Cold water	No
<i>Codium fragile</i>	130	Ethanol 80%	No
<i>Codium fragile</i>	130	Hot water	No
<i>F. serratus</i>	55	Cold water	No
<i>F. serratus</i>	55	Ethanol 80%	Yes
<i>F. serratus</i>	55	Hot water	No
<i>F. spiralis</i>	123	Cold water	No
<i>F. spiralis</i>	123	Ethanol 80%	Yes
<i>F. spiralis</i>	123	Hot water	Yes

<i>F. vesiculosus</i>	51	Cold water	Yes
<i>F. vesiculosus</i>	51	Ethanol 80%	Yes
<i>F. vesiculosus</i>	51	Hot water	No
<i>Himanthalia elongata</i>	82	Cold water	No
<i>Himanthalia elongata</i>	82	Ethanol 80%	No
<i>Himanthalia elongata</i>	82	Hot water	Yes
<i>Laminaria digitata</i>	37	Cold water	No
<i>Laminaria digitata</i>	37	Ethanol 80%	No
<i>Laminaria digitata</i>	37	Hot water	No
<i>Laminaria hyperborea</i>	136	Cold water	Yes
<i>Laminaria hyperborea</i>	136	Ethanol 80%	No
<i>Laminaria hyperborea</i>	136	Hot water	Yes
<i>P. canaliculata</i>	115	Ethanol 80%	Yes
<i>P. canaliculata</i>	115	Hot water	No
<i>P. canaliculata</i>	115	Hot water	No
<i>Chondrus crispus</i>	128	Cold water	No
<i>Chondrus crispus</i>	128	Ethanol 80%	No
<i>Chondrus crispus</i>	128	Hot water	Ins
<i>Saccharina latissima</i>	113	Cold water	No
<i>Saccharina latissima</i>	113	Ethanol 80%	No
<i>Saccharina latissima</i>	113	Hot water	No
<i>Ulva intestinalis</i>	180	Cold water	No
<i>Ulva intestinalis</i>	180	Ethanol 80%	No
<i>Ulva intestinalis</i>	180	Hot water	No

Table 20 *In vitro* screening for anti-inflammatory bioactivity – molecular weight fractions

Species/Extract	Sample ID	Extraction	Fraction	Hit
<i>A. nodosum</i>	84	Cold H ₂ O	< 3.5 kDa	No
<i>A. nodosum</i>	84	Cold H ₂ O	3.5-100 kDa	Yes
<i>A. nodosum</i>	84	Cold H ₂ O	> 100 kDa	Yes
<i>A. nodosum</i>	84	EtOH/H ₂ O(80:20)	< 3.5 kDa	No
<i>A. nodosum</i>	84	EtOH/H ₂ O(80:20)	3.5-100 kDa	Yes
<i>A. nodosum</i>	84	EtOH/H ₂ O(80:20)	> 100 kDa	Yes
<i>A. nodosum</i>	84	EtOH/H ₂ O(80:20)	ethyl acetate	No
<i>F. spiralis</i>	123	Cold H ₂ O	< 3.5 kDa	No
<i>F. spiralis</i>	123	Cold H ₂ O	3.5-100 kDa	Yes
<i>F. spiralis</i>	123	Cold H ₂ O	> 100 kDa	No
<i>F. spiralis</i>	123	EtOH/H ₂ O(80:20)	< 3.5 kDa	No
<i>F. spiralis</i>	123	EtOH/H ₂ O(80:20)	3.5-100 kDa	Yes
<i>F. spiralis</i>	123	EtOH/H ₂ O(80:20)	> 100 kDa	Yes
<i>F. spiralis</i>	123	EtOH/H ₂ O(80:20)	ethyl acetate	No
<i>P. canaliculata</i>	115	Cold H ₂ O	< 3.5 kDa	No
<i>P. canaliculata</i>	115	Cold H ₂ O	3.5-100 kDa	Yes
<i>P. canaliculata</i>	115	Cold H ₂ O	> 100 kDa	Yes
<i>P. canaliculata</i>	115	EtOH/H ₂ O(80:20)	< 3.5 kDa	Yes
<i>P. canaliculata</i>	115	EtOH/H ₂ O(80:20)	3.5-100 kDa	Yes
<i>P. canaliculata</i>	115	EtOH/H ₂ O(80:20)	> 100 kDa	Yes
<i>P. canaliculata</i>	115	EtOH/H ₂ O(80:20)	ethyl acetate	No
<i>Laminaria digitata</i>	155	Cold H ₂ O	<3.5KDa	No
<i>Laminaria digitata</i>	155	Cold H ₂ O	3.5-100KDa	No
<i>Laminaria digitata</i>	155	Cold H ₂ O	>100KDa	Yes
<i>Laminaria digitata</i>	155	EtOH/H ₂ O(80:20)	<3.5KDa	No
<i>Laminaria digitata</i>	155	EtOH/H ₂ O(80:20)	3.5-100KDa	No

<i>Laminaria digitata</i>	155	EtOH/H ₂ O(80:20)	>100KDa	Yes
<i>F. serratus</i>	156	Cold H ₂ O	<3.5KDa	No
<i>F. serratus</i>	156	Cold H ₂ O	3.5-100KDa	Yes
<i>F. serratus</i>	156	Cold H ₂ O	>100KDa	Yes
<i>F. serratus</i>	156	EtOH/H ₂ O(80:20)	<3.5KDa	No
<i>F. serratus</i>	156	EtOH/H ₂ O(80:20)	3.5-100KDa	Yes
<i>F. serratus</i>	156	EtOH/H ₂ O(80:20)	>100KDa	Yes
<i>F. vesiculosus</i>	51	Cold H ₂ O	<3.5KDa	No
<i>F. vesiculosus</i>	51	Cold H ₂ O	3.5-100KDa	Yes
<i>F. vesiculosus</i>	51	Cold H ₂ O	>100KDa	Yes
<i>F. vesiculosus</i>	51	EtOH/H ₂ O(80:20)	<3.5KDa	No
<i>F. vesiculosus</i>	51	EtOH/H ₂ O(80:20)	3.5-100KDa	Yes
<i>F. vesiculosus</i>	51	EtOH/H ₂ O(80:20)	>100KDa	Yes

5.3.1. Screening for gut anti-inflammatory potential

An *in vitro* evaluation of anti-inflammatory properties identified a number extracts from seaweed with highly potent anti-inflammatory bioactivity. A conventional approach to further assess their potential would be to conduct a large-scale animal trial. As an alternative screening strategy, an *ex-vivo* evaluation technique to screen for anti-inflammatory properties of these seaweed bioactives was developed. This approach used an *ex-vivo* tissue challenge experiment using a porcine colon to evaluate the anti-inflammatory bioactivity of whole seaweed extracts and seaweed MW fractions previously found to have anti-inflammatory bioactivity in the Caco-2 cell line. A total of 15 whole seaweed extracts and 14 MW extracts were tested in the *ex-vivo* porcine colon excised from six different weaned pigs. The effect of seaweed variety and extraction methods on the *ex-vivo* anti-inflammatory properties is shown in Table 21. Of the 15 whole seaweed extracts, 10 had anti-inflammatory properties *ex-vivo*.

Table 21 Screening for anti-inflammatory bioactivity (*ex-vivo*)

Species	Sample ID	Extract	Hit
<i>F. spiralis</i>	123	Cold water	Yes
<i>F. spiralis</i>	123	Ethanol 80%	Yes
<i>F. spiralis</i>	123	Hot water	Yes
<i>F. serratus</i>	55	Ethanol 80%	Yes
<i>A. nodosum</i>	84	Cold water	Yes
<i>A. nodosum</i>	84	Ethanol 80%	Yes
<i>A. nodosum</i>	84	Hot water	Yes
<i>F. vesiculosus</i>	51	Cold water	Yes
<i>F. vesiculosus</i>	51	Ethanol 80%	Yes
<i>F. vesiculosus</i>	51	Hot water	Yes

Based on their high anti-inflammatory activity *in vitro* (Caco2 cell line) five seaweeds; *Ascophyllum nodosum*, *Fucus serratus*, *Fucus spiralis*, *Fucus vesiculosus* and *Palmaria palmata* were screened in the *ex-vivo* assay (live tissue model). An *ex-vivo* experiment was designed to

examine the effect of different extraction methods (cold water, hot water or ethanol) of five different varieties of seaweeds.

To examine their anti-inflammatory potential in porcine colon tissue when challenged with lipopolysaccharide (LPS), six inflammatory-marker genes (IL8, IL6, IL1B, TNF, IL17A and TLR4) were evaluated and the qPCR data analysed. In contrast to the low level of expression of IL-8, IL-6, and TNFA genes in the colonic tissue at 0 h, LPS treatment increased ($P < 0.05$) the expression of IL-8, IL-6, and TNFA genes to 2.38 ± 0.86 , 1.90 ± 0.66 , and 1.90 ± 0.57 fold, respectively. The pro-inflammatory response induced by the LPS was suppressed by the extracts of *Ascophyllum nodosum*. *Ascophyllum nodosum* extract reduced ($P < 0.05$) the expression of IL-8, IL-6, and TNFA genes to 0.99 ± 0.53 , 0.75 ± 0.33 , and 1.01 ± 0.17 fold, and *Fucus ssp* extracts reduced ($P < 0.05$) the expression of the corresponding genes to 0.70 ± 0.32 , 0.69 ± 0.38 , and 1.15 ± 0.25 fold, respectively. These results indicate that the extracts of *Ascophyllum Nodosum* and *Fucus ssp* seaweeds have potential to suppress the pro-inflammatory response induced by the bacterial LPS in the pig colon.

5.4. Screening for anti-obesity potential

A cell bioassay was established to screen compounds for their anti-obesity potential and optimised to screen samples generated within the NutraMara programme. This bioassay used mouse 3T3-L1 adipocyte model, a model that is widely used as an *in vitro* model of mammalian adipogenesis – a process through which excessive fat is deposited in obese individuals. This bioassay requires the bioactive compound to be soluble in water. Mouse 3T3-L1 pre-adipocytes were allowed to differentiate in presence of extracts and lipid accumulation was quantified by free glycerol/triglyceride estimation at the end of the differentiation process. A total of 171 samples of whole seaweeds extracts were evaluated for anti-obesity properties *in vitro* and 45 samples were identified to have positive hits (see Table 22 and Table 23).

Table 22 Screening for anti-obesity properties – *in vitro*

Species	Sample ID	Extract	Hit
<i>Alaria esculanta</i>	036	Hot water	Yes
<i>A. nodosum</i>	084	Cold water	Yes
<i>A. nodosum</i>	084	Ethanol 80%	Yes
<i>A. nodosum</i>	084	Hot water	Yes
<i>Chondrus crispus</i>	128	Cold water	Yes
<i>Chondrus crispus</i>	128	Hot water	Insoluble
<i>Fucus serratus</i>	055	Ethanol 80%	Yes
<i>Fucus spiralis</i>	123	Ethanol 80%	Yes
<i>Fucus spiralis</i>	123	Hot water	Yes
<i>Fucus vesiculosus</i>	051	Cold water	Yes
<i>Fucus vesiculosus</i>	051	Ethanol 80%	Yes

<i>Himanthalia elongata</i>	082	Hot water	Yes
<i>Laminaria hyperborea</i>	136	Cold water	Yes
<i>Laminaria hyperborea</i>	136	Hot water	Yes
<i>Pelvetia canaliculata</i>	115	Ethanol 80%	Yes
<i>Pelvetia canaliculata</i>	115	Hot water	Yes

Table 23 Screening for anti-obesity bioactivity *in-vitro* - molecular weight fractions

Species	Sample ID	Extraction	Fraction	Hit
<i>Fucus spiralis</i>	123	Cold H2O	< 3.5 kDa	Yes
<i>Fucus spiralis</i>	123	Cold H2O	3.5-100 kDa	Yes
<i>Fucus spiralis</i>	123	Cold H2O	> 100 kDa	Yes
<i>Fucus spiralis</i>	123	EtOH/H2O(80:20)	< 3.5 kDa	Yes
<i>Fucus spiralis</i>	123	EtOH/H2O(80:20)	3.5-100 kDa	Yes
<i>Pelvetia canaliculata</i>	115	Cold H2O	3.5-100 kDa	Yes
<i>Pelvetia canaliculata</i>	115	EtOH/H2O(80:20)	< 3.5 kDa	Yes
<i>Pelvetia canaliculata</i>	115	EtOH/H2O(80:20)	3.5-100 kDa	Yes
<i>Pelvetia canaliculata</i>	115	EtOH/H2O(80:20)	> 100 kDa	Yes
<i>Ascophyllum nodosum</i>	084	Cold H2O	3.5-100 kDa	Yes
<i>Ascophyllum nodosum</i>	084	Cold H2O	> 100 kDa	Yes
<i>Ascophyllum nodosum</i>	084	EtOH/H2O(80:20)	< 3.5 kDa	Yes
<i>Ascophyllum nodosum</i>	084	EtOH/H2O(80:20)	3.5-100 kDa	Yes
<i>Laminaria digitata</i>	155	EtOH/H2O(80:20)	3.5-100KDa	Yes
<i>Laminaria digitata</i>	155	EtOH/H2O(80:20)	>100KDa	Yes
<i>Fucus serratus</i>	156	Cold H2O	>100KDa	Yes
<i>Fucus serratus</i>	156	EtOH/H2O(80:20)	>100KDa	Yes
<i>Fucus vesiculosus</i>	051	Cold H2O	<3.5KDa	Yes
<i>Fucus vesiculosus</i>	051	Cold H2O	3.5-100KDa	Yes
<i>Fucus vesiculosus</i>	051	Cold H2O	>100KDa	Yes
<i>Fucus vesiculosus</i>	051	EtOH/H2O(80:20)	<3.5KDa	Yes
<i>Fucus vesiculosus</i>	051	EtOH/H2O(80:20)	3.5-100KDa	Yes
<i>Fucus vesiculosus</i>	051	Cold H2O	<3kDA	Yes

5.5. Screening for cell viability and cytotoxicity

Cell viability and cytotoxicity of the mouse 3T3-L1 pre-adipocyte cells (anti-adipogenic assay) and human Caco-2 cells (anti-inflammatory assay) were assessed in response to bioactives extracted from marine sources including, fish processing discards and seaweeds. In this process, cells were plated in a 96 well cell culture plate (Greiner Bio-One GmbH, Frickenhausen, Germany) at an initial plating density of 6×10^5 cells/ml. The cells were allowed to first reach full confluence and then kept at a fully confluent state for 24 hrs before treating with the bioactive. On the day of treatment, cells were washed with sterile phosphate buffer saline (Sigma-Aldrich) and incubated with serum and antibiotic free media containing the bioactive at final concentrations of 1000 $\mu\text{g/ml}$ and incubated for 24 and 48 hrs. Cell viability test was performed on the cell monolayer using the 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) (Sigma-Aldrich) test. Cell cytotoxicity was performed on 25 μl cell lysate using Tox-7 kit (Sigma-Aldrich).

5.6. Screening for pre-biotic potential

The gastrointestinal tract plays host to a complex and diverse microbial ecosystem, which can affect host health. Diet is a major determinant of gut microbiota (GM) structure and function, and as such offers an approach to modify the gut microbiota to improve health and well-being and reduce risk of disease. One such dietary strategy is prebiotics. There is accumulating evidence to suggest that prebiotics have potential to improve a number of health parameters including bone health and weight management. Although poorly understood, one mechanism whereby the GM may improve these health benefits is the production of short chain fatty acids through fermentation of polysaccharides within the colon. Macroalgae have been suggested as suitable candidates to mine for potential prebiotics owing to the richness of atypical polysaccharides within some macroalgal species. Extracts from *Laminaria digitata* were the subject of *in vitro* and *in vivo* studies. The *in vitro* study (2 sample extracts) assessed the effects of a crude extract and a depolymerised extract on the composition and activity of human faecal bacterial populations. The faecal fermentations were run in triplicate, with three healthy donors selected for each run. The composition was assessed by spread plating of culturable *bifidobacteria* and *lactobacillus* species and a more in depth analysis of all bacteria was attained by 454 pyrosequencing. The activity of the faecal populations was assessed by gas chromatography and also by colourmetric methods. Fructooligosaccharides (FOS) was used as a positive control and cellulose as a negative control. Both extracts failed to stimulate culturable bifidobacteria and lactobacillus populations in comparison to cellulose. Sequencing the 24 hour samples revealed that both crude and depolymerised extracts increased the abundance of *Lachnospiraceae*, while only the crude extract increased *Porphyromonadaceae* populations and decreased *Streptococcus* populations.

The *in vivo* studies comprised two animal studies, conducted to assess whether the extracts can have positive effects on health outcomes including bone, body composition and lipid metabolism.

The first study assessed the potential health effects of supplementing the *Laminaria digitata* extract at 5% to mice maintained on a standard chow diet. The inclusion of the seaweed extract had an impact on the GM of mice. It significantly altered 27 bacterial genera compared to non-supplemented control animals. The extract also decreased the pH of the caecum ($P < 0.001$) and increased caecal tissue mass ($P < 0.001$), both of these effects on the caecum are considered to contribute towards a prebiotic effect. Furthermore, the seaweed extract also modulated bone remodelling in adult mice in a manner which could be considered beneficial by increasing a marker for bone formation, osteocalcin ($P = 0.049$) and decreasing a marker of bone resorption, TRACP-5b ($P = 0.024$). In addition the inclusion of

the seaweed extract also resulted in reduced body weight ($P = 0.038$), energy intake ($P = 0.038$), serum cholesterol ($P = 0.025$) and serum leptin ($P = 0.049$), highlighting the potential of the extract as an anti-obesity functional food ingredient.

The second study assessed the effects of supplementing the extract at 5% to a diet induced obese mouse model. The inclusion of the extract altered the GM by altering the Firmicutes Bacteroidetes ratio in a manner which is associated with weight loss. The extract also reduced the percentage of fat tissue ($P = 0.006$), increased the respiratory exchange ratio during the day ($P = 0.034$) and night ($P = 0.014$), and also increased energy expenditure during the night period ($P = 0.008$). Serum cholesterol ($P = 0.003$), leptin ($P = 0.001$) and lipopolysaccharide binding protein ($P < 0.001$) were all reduced, indicating an anti-obesity effect. As with the former mouse study, caecal tissue mass ($P < 0.001$) was increased and caecal pH ($P < 0.001$) was decreased with the supplementation of the extract suggesting the putative beneficial effects reported could be through modulation of the GM. In addition, the two extracts tested significantly increased the production of acetate ($P < 0.001$), propionate ($P < 0.001$) and butyrate ($P < 0.001$) as well as total SCFA ($P < 0.001$) in comparison to controls. Two further studies were carried out using polysaccharide rich extract prepared from *Fucus serratus* and *Chondrus crispus* in order to evaluate prebiotic activity using an *in vitro* faecal fermentation model. During the *Fucus serratus* study, a 1.5 fold increase was observed in the production of total SCFAs, particularly the production of propionate (2.3-fold increase) and acetate (1.4-fold increase). There was also an associated significant difference ($P < 0.05$) in the ratio of propionate production, rising from 15% in the control to 24 % during the *Fucus serratus* fermentation. However, there was no significant change in levels of butyrate production. High throughput DNA sequencing analysis revealed that the *Fucus serratus* extract had no notable effect on the abundance of members of the genera Bifidobacterium and Lactobacillus. However, there were notable increases in several propionate-producing members of the microbiota such as the genus *Parabacteroides*, the family *Veillonellaceae* and the family *Erysipelotrichaceae*, which is peripherally related to the butyrate-producing superfamily *Lachnospiraceae*.

During the *Chondrus crispus* fermentation study, significant increases ($P < 0.05$) were recorded in the production of total short-chain fatty acids and in particular, the biologically important SCFAs, acetate and propionate. However, there was no significant alteration in the molar ratio of SCFA production in comparison with the control or impact on the production of butyrate. High-throughput DNA sequencing revealed that there was no notable impact on the relative abundance of the major probiotic genera, the bifidobacteria and lactobacilli. The results of this study revealed that fermentation of depolymerised polysaccharides from

Chondrus crispus have only a minimal stimulatory effect on the in vitro microbial population and would not be considered prebiotic by the current definition of the term.

The fermentation of *Fucus serratus* polysaccharides resulted in a significant alteration in the molar ratio of SCFA formation in favour of the production of propionate. Propionate is been positively associated with enhancing satiety. Increasing propionate production by the colonic microbiota through dietary intervention would be an attractive prospect in preventing overeating and maintaining good host health.

5.7. Screening for antimicrobial potential

Food related illness is a common and often preventable problem that affects approximately 30% of individuals in industrialised countries every year. Antimicrobial agents, such as preservatives and organic acids, have been used to inhibit food-borne pathogens and prolong the shelf life of processed goods but resistance to some traditional antimicrobials is spreading quickly. Many naturally occurring compounds found in edible and medicinal plants, herbs and spices have been shown to possess antimicrobial activities but the search for new antimicrobial to date has mainly focused on the terrestrial environments. Algae have proven to be a rich source of novel bioactive compounds. The long evolution of marine plants, compared with their terrestrial counterparts, has resulted in the generation of a huge diversity of genes, species etc. This diversity coupled with the ability of these plants to adapt, compete and survive in extreme environmental conditions has made marine organisms potentially an almost unlimited base for applied research. Moreover, they possess the ability to synthesise unique chemical structures, many of which have potent bioactive activity. Ten ethanol extracts, derived from Irish brown seaweeds, were examined for antimicrobial activity against a selection of food-borne pathogens. Extracts from the seaweeds *Fucus vesiculosus*, *Fucus serratus*, *Fucus spiralis*, *Ascophyllum nodosum* and *Pelvetia canaliculata* significantly inhibited ($p < 0.05$) the growth of *Listeria monocytogenes* 5788 at 24 h at a concentration of extract of 2 mg/ml. The *Fucus vesiculosus* extract was chosen for further antimicrobial evaluation. Molecular weight fractions of *Fucus vesiculosus* were tested against several strains of *L. monocytogenes*. It was found that the anti-listerial activity was concentrated in the 0-3 kDa and the 3-100 kDa molecular weight fractions, with the 3-100 kDa exhibiting the highest activity of all. Antimicrobial activity was found to be positively correlated with levels of phenolic content. Seaweed extracts exhibiting anti-listerial activity could potentially be developed into agents used to control food spoilage and food-borne illness especially, based on these results, caused by the pathogenic bacterium *L. monocytogenes*.

5.8. Screening for anti-infective potential

The over reliance on antibiotics and inappropriate use by both health care workers and the public has resulted in the emergence of antibiotic-resistant bacterial strains, which has in turn led to a reduction in effectiveness of antimicrobial therapy. As a consequence, the normal human gut microbiota can be altered in cases allowing for the enrichment of antibiotic resistant bacteria which further complicates treatment. Resistance makes the development of novel strategies to prevent and treat bacterial infections crucial. Therapies which target bacterial virulence properties (e.g. adhesion, colonization, invasion and the production of toxins) have the advantage of combating the infection without selecting for resistant bacteria and also not causing harmful effects on the host microbiota. Anti-adhesion therapy aims to reduce contact between host tissues and pathogens. This is done either by preventing the adhesion in the first place or reversing adhesion after it has occurred. Galactooligosaccharides (GOS) have been demonstrated to mimic molecular receptors and can competitively inhibit bacterial adherence to the intestine. Here, polysaccharide rich extracts prepared from the brown seaweed *Fucus serratus* and the red seaweed *Chondrus crispus* were investigated for their anti-infective potential against strains of *Staphylococcus aureus*, *Salmonella enterica*, *Listeria monocytogenes* and *Escherichia coli* using the human colonic carcinoma cell line, Caco-2 as a model system. CaCo-2 cells were grown as previously described in literature. The polysaccharide extracts were both prepared at a concentration of 5 mg/ml using DMEM (FBS 2%, NEAA 1%) and sterilized using 0.45 µm filters. The extracts were pre-incubated with the target bacteria for one hour at 37°C. Following the pre-incubation, old media was removed from the seeded 12 well plate and each well was washed with PBS (x3). One millilitre of the extract/bacterial cells mixture was added to each well and the plate was then incubated at 37°C for 2 hours. The plate was taken from the incubator and the extract/bacterial media was removed. Each well was then washed with PBS (x4) to remove all non-adherent bacteria with 500µl of Triton X being added to each well. The plate was then replaced in the 37°C incubator for 45 minutes. After 45 minutes, the contents of each experimental well was collected in individual, sterile, clean Eppendorf's and serial dilutions were carried out in MRD and plated on to BHI agar. The plates were then incubated in an inverted position at 37°C for 24 hours. It was found that neither the *Fucus serratus* or *Chondrus crispus* extract had a significant effect ($p < 0.05$) on the levels of recoverable bacteria in comparison with the control condition. This indicates that polysaccharide extract produced by dilute hot-acid extraction offers poor anti-infective potential against major food-borne pathogens such as *Staphylococcus aureus*, *Salmonella enterica*, *Listeria monocytogenes* and *Escherichia coli*.

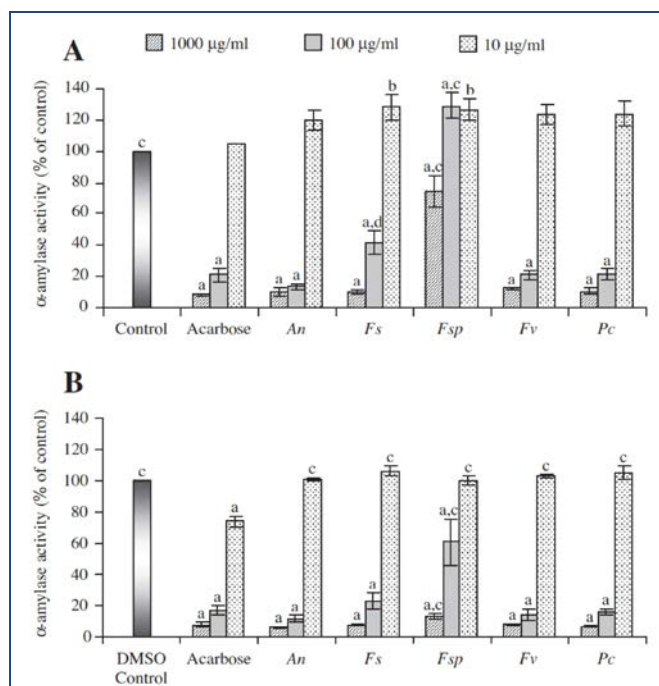
5.9. Screening for anti-diabetic potential

Hydrolysis of dietary starch is the major source of glucose in the blood, with α -amylase and α -glucosidase being the key enzymes involved in starch breakdown and intestinal absorption, respectively. It is believed that inhibiting these enzymes can significantly decrease the postprandial increase of blood glucose level after a mixed carbohydrate diet, offering a strategy in the management of hyperglycaemia linked to type II diabetes.

This study explored the potential role of seaweed extracts for diabetic care. Extracts (cold water and ethanol) of 15 species of Irish seaweeds; *Alaria esculenta*, *Ascophyllum nodosum*, *Fucus serratus*, *Fucus spiralis*, *Fucus vesiculosus*, *Himanthalia elongata*, *Laminaria digitata*, *Laminaria hyperborea*, *Pelvetia canaliculata* and *Saccharina latissima*, *Chondrus crispus*, *Gracilaria gracilis*, *Palmaria palmate*, *Codium fragile* and *Ulva intestinalis* were screened for potential α -amylase and α -glucosidase inhibitory activity, with extracts of interest subsequently tested for their effect on Caco-2 cell function. The undifferentiated Caco-2 human colon cancer cell line was chosen as it is a reliable model for cultured colonocytes, is well characterized, and is widely used for biochemical and nutritional studies.

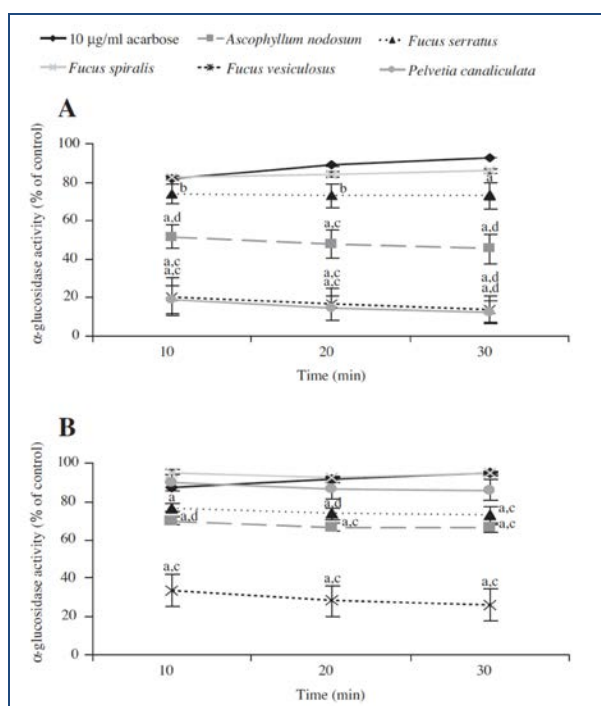
The initial screening process resulted in extracts from five brown seaweed species *Ascophyllum nodosum*, *Fucus serratus*, *Fucus spiralis*, *Fucus vesiculosus* and *Pelvetia canaliculata* being chosen for further evaluation and the pharmacological inhibitor, acarbose, included as a positive control. The extracts of interest were then examined at lower concentrations (1000–100 μ g/ml for α -amylase inhibition, 1000–100 μ g/ml for α -glucosidase inhibition) using a modified protocol, whereby the enzyme was added at the final stages of the experiment. For α -amylase inhibition, the IC_{50} values for cold water extracts of *Ascophyllum nodosum*, *Fucus serratus*, *Fucus spiralis*, *Fucus vesiculosus* and *Pelvetia canaliculata* were 53.6, 86.1, 282.7, 63.5 and 66.1 μ g/ml, respectively. Four of the cold-water extracts had a similar inhibitory effect as the positive control, with concentrations of 1000 and 100 μ g/ml significantly ($P < 0.01$) inhibiting α -amylase activity (Figure 33A). However, the cold-water extract of *Fucus spiralis* was not an effective inhibitor. As shown in Figure 33, ethanol extracts of *Ascophyllum nodosum* (IC_{50} 44.7 μ g/ml), *Fucus serratus* (IC_{50} 70.6 μ g/ml), *Fucus vesiculosus* (IC_{50} 59.1 μ g/ml) and *Pelvetia canaliculata* (IC_{50} 51.0 μ g/ml) also had the same inhibitory profile as acarbose. The IC_{50} for α -amylase inhibition of the ethanol *Fucus spiralis* extract was 109.0 μ g/ml.

Figure 33 Inhibitory effect of cold water (A) and ethanol (B) extracts on α -amylase activity. Data represent the mean (\pm SE) of at least three independent experiments ($^aP < 0.01$, $^bP < 0.05$ relative to control, $^cP < 0.01$, $^dP < 0.05$ relative to acarbose, ANOVA followed by Dunnett's Multiple Comparison test). An, *Ascophyllum nodosum*; Fs, *Fucus serratus*; Fsp, *Fucus spiralis*; Fv, *Fucus vesiculosus*; Pc, *Pelvetia canaliculata*



These investigations of α -glucosidase inhibition, identified that all extracts abolished α -glucosidase activity at 1000, 100 and 10 lg/ ml (data not shown). Even at 1 lg/ml, cold water extracts of *Ascophyllum nodosum*, *Fucus serratus*, *Fucus vesiculosus* and *Pelvetia canaliculata* significantly ($P < 0.01$) inhibited α -glucosidase activity over 30 min relative to the control (Figure 34A). With the exception of *Pelvetia canaliculata*, comparable levels of inhibition were also found with the ethanol extracts (Figure 34B). Further, the *Fucus vesiculosus* extract was identified as the strongest α -glucosidase inhibitor regardless of the extraction method used.

Figure 34 Inhibitory effect of 1 lg/ml cold water (A) and ethanol (B) extracts on α -glucosidase activity. Data represent the mean (\pm SE) of at least three independent experiments ($^aP < 0.01$, $^bP < 0.05$ relative to control, $^cP < 0.01$, $^dP < 0.05$ relative to 10 lg/ml acarbose, ANOVA followed by Dunnett's Multiple Comparison test). An, *Ascophyllum nodosum*; Fs, *Fucus serratus*; Fsp, *Fucus spiralis*; Fv, *Fucus vesiculosus*; Pc, *Pelvetia canaliculata*



The IC_{50} values for α -glucosidase inhibition of the cold water and ethanol extracts of *Fucus vesiculosus* were 0.32 and 0.49 lg/ml, respectively, making it amongst the most potent seaweed extract studied to date. Indeed, with IC_{50} values for α -glucosidase inhibition at <2 lg/ml, the physiological relevance of all the cold water and ethanol extracts is quite strong. The presence of the extracts in the blood at these concentrations is attainable, and so makes their α -glucosidase inhibitory capabilities an attractive and realistic approach to diabetes management.

The results of this study demonstrate the efficacy of brown seaweed extracts, in particular *Fucus vesiculosus* and *Pelvetia canaliculata*, to inhibit enzymes involved in intestinal carbohydrate digestion and assimilation. Due to their availability and strong inhibitory properties, these algal extracts have potential for use in functional food applications aimed at lowering glycaemic response. Additionally, the extracts are capable of inhibiting α -amylase and α -glucosidase at non-toxic levels, and cold water and ethanol extraction are desirable for food products because of the absence of solvent residues.

5.10. Screening for anti-cancer potential

The aetiology of colorectal cancer, the third most prevalent cancer in the world, is linked to several risk factors, including age, genetic factors and diet. Seaweed consumption has been

negatively correlated with colorectal cancer risk within Asian populations, and the anti-cancer properties of certain seaweed species have also been demonstrated within *in vitro* and animal models.

The anti-cancer potential of the seaweeds was investigated as a source of pharmaceutical agents in breast cancer cells and the subsequent anticancer activity of the seaweeds in colon cancer cells before and after gastrointestinal digestion was investigated in relation to its anti-cancer activity as a dietary constituent.

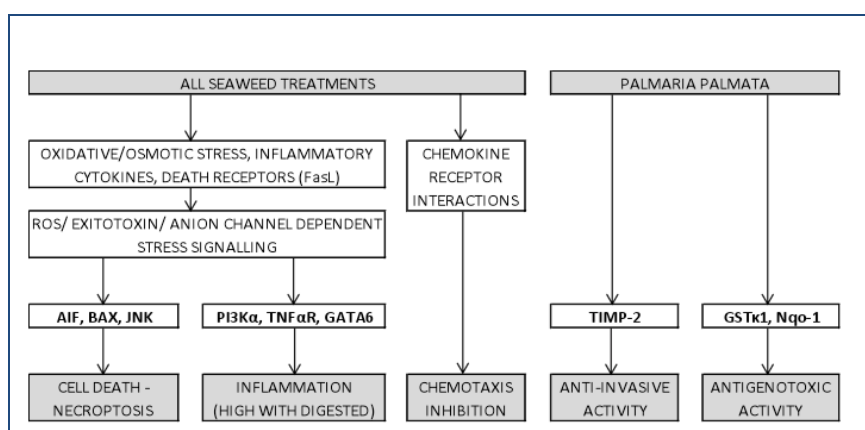
The anti-cancer activity screening was carried out using the MTT assay with a range of aqueous (hot and cold water), ethanolic and methanolic extractions from *Fucus serratus*, *Fucus vesiculosus*, *Ascophyllum nodosum*, *Laminaria digitata*, *Palmaria palmata*, *Chondrus crispus* and *Ulva intestinalis* at concentrations between 32.5-500 µg/ml. Cold water (FVE) and ethanolic (FVC) extractions of *Fucus vesiculosus* emerged as the extracts with potent anti-cancer activity in metastatic cancer cells (MDA-MB-231). The anti-cancer activity of <3 kDa phlorotannin fraction and 3-100 kDa fraction of both FVE and FVC were subsequently investigated using the real-time proliferation assay, the MTT assay and the Annexin V apoptosis assay. The <3 kDa phlorotannin fraction and 3-100 kDa fraction of FVC and FVE all harboured the capacity to significantly reduce cell proliferation of MDA-MB-231 cells in a dose dependant manner (33.3-500 µg/ml). MTT assays showed that apoptosis is induced in MDA-MB-231 cells following exposure to 125µg/ml *Fucus vesiculosus* fractions. Subsequent flow cytometry analysis confirmed that MDA-MB-231 cells undergo early apoptosis when treated with either the <3 kDa phlorotannin fraction or the 3-100 kDa fraction. Low molecular weight ethanolic fraction (<3kDa), rich in phlorotannins, was the most effective in reducing cell proliferation (IC50 67.5 µg/ml) and inducing apoptosis. Transcriptional profiling of apoptosis related genes revealed down regulation of anti-apoptotic modulators BCL2 and BCL-X and upregulation of pro-apoptotic markers BAK and BAX, indicating induction of the intrinsic apoptosis pathway in MDA-MB-231 cells following treatment with *Fucus vesiculosus* extracts. The data suggests that aqueous and ethanolic *Fucus vesiculosus* extracts have the capacity to reduce breast cancer proliferation and induce the intrinsic apoptosis machinery in MDA-MB-231 cells, with low molecular weight phlorotannin-enriched fractions harbouring the most promising anti-cancer activity.

A second study was initiated to examine the anti-cancer potential of selected Irish seaweed species, namely *Ascophyllum nodosum*, *Laminaria digitata*, *Palmaria palmata* and *Ulva intestinalis* using *in vitro* cell models of colon cancer. In order to account for the compositional changes occurring during gastrointestinal digestion, seaweed samples were subjected to *in vitro* simulation of gastric and pancreatic digestion. The chemopreventive properties of crude and

digested seaweed treatments were tested using cell models, representing the key stages of colorectal cancer initiation, promotion and invasion.

Of the seaweed species included in the study, *Ascophyllum nodosum* had the highest polyphenol-content and antioxidant power, both reduced by >60% after *in vitro* gastrointestinal digestion. Yet, digested *Ascophyllum nodosum* extracts had a strong anti-genotoxic activity, connected to up-regulation of a detoxifying enzyme GST_k-1. All crude and digested treatments had anti-proliferative effects in all cell types, inducing a necroptotic, mitochondrial cell death pathway with the up-regulation of AIF, JNK, Bax and PTEN. Digested extracts simultaneously induced the inflammatory or stress-related PI3K pathway, inhibiting the expression of Caspase-8 and p53. The invasion and migration of metastatic HT115 cells was inhibited by *Palmaria palmata* treatments both pre- and post-digestion, the up-regulation of an anti-invasive tissue inhibitor of metalloproteinases TIMP-2 was observed.

Figure 35 Proposed bioactivity of the Irish seaweed prior and post gastrointestinal digestion simulation



This work has demonstrated, for the first time, how a simulated gastrointestinal digestion significantly affects the bioactivity and composition of seaweed homogenates. The Irish seaweed species were shown to have anti-proliferative, anti-genotoxic and anti-metastatic activity in *in vitro* models of colorectal cancer, however pro-inflammatory activity of digested treatments was also observed.

5.11. Screening for platelet-activating factor (PAF) acetylhydrolase inhibition

Platelet-activating factor (PAF) is a biologically active phospholipid that is known to have a negative effect on cardiac health. In addition to the activation of platelets, other biological effects include decreases in cardiac output and increased hypotension in humans. Lipids found in species of five seaweeds - *Alaria esculenta*, *Ascophyllum nodosum*, *Fucus dichitus*,

Pelvetia canaliculata, *Fucus spiralis* and *Ulva intestinalis* were investigated to assess their potential as PAF acetylhydrolase inhibitors.

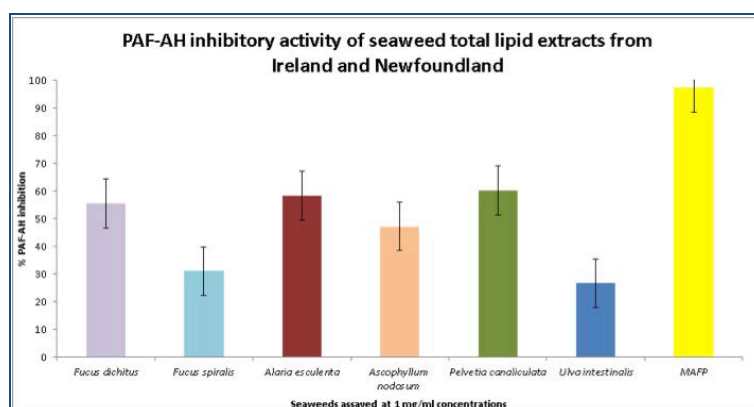
PAF acetylhydrolase inhibition was assayed using the Cayman Chemical PAF Acetylhydrolase Inhibitor screening assay kit in accordance with the manufacturers' instructions (Cayman Chemical Company, Ann Arbor, MI). Briefly, 2-thio PAF was used as a substrate for PAF-AH. Upon hydrolysis of the acetyl thioester bond at the *sn*-2 position by PAF-AH, free thiols were detected using 5,5'-dithio-bis-(2-nitrobenzoic acid) using a spectrophotometer at A_{414} nm or A_{405} nm. Samples were reconstituted in dimethylsulphoxide (DMSO) at concentrations of 1 mg/ml and assayed in triplicate. Methyl arachidonyl fluorophosphonate (MAFP) was used as a positive control at a concentration of 250 nM. MAFP has an IC_{50} value of 250 nM and is a known inhibitor of PAF-AH. The percentage inhibition for each total lipid extract was determined using according to

$$\% \text{ PAF - AH inhibition} = ((100\% \text{ initial activity value} - \text{inhibitor sample value}) / (100\% \text{ initial activity value})) \times 100$$

Pelvetia canaliculata had the highest percentage total crude lipids per dry weight (0.503 g) followed by *Ulva intestinalis* (0.40 g), *Ascophyllum nodosum* (0.372 g), *Fucus spiralis* (0.393 g), *Fucus dichitus* (0.30 g) and *Alaria esculenta* (0.20 g).

Total lipid extracts generated from *Pelvetia canaliculata* using the method reported above were found to inhibit PAF-AH by 60.08 % (+/- 24.93) when assayed at a concentration of 1 mg/ml compared to the positive control MAFP which inhibited PAF-AH by 97.37 % when assayed at a concentration of 134 nM. Total lipids extracted from *Alaria esculenta* inhibited PAF-AH by 58.29 (+/- 3.85) when assayed *in vitro* at a concentration of 1 mg/ml. Figure 36 shows the percentage PAF-AH inhibition of each of the total seaweed lipid extracts when assayed at a concentration of 1 mg/ml.

Figure 36 PAF-AH inhibitory activities of total lipid extracts from Irish and Newfoundland seaweeds



5.12. Screening for antioxidant potential

The antioxidant potential of methanolic extracts of brown seaweeds was assessed using four bioassays, Total Phenol Content, Ferric Reducing Antioxidant Power (FRAP), β -carotene bleaching and the DPPH scavenging assays. *Ascophyllum nodosum*, *Pelvetia canaliculata*, and *Fucus serratus* contained the highest phenol concentrations while *Fucus vesiculosus* and *Fucus serratus* exhibited the highest FRAP activities. *Fucus vesiculosus* and *Ascophyllum nodosum* were the most effective extracts at scavenging DPPH radicals and preventing β -carotene bleaching. The antioxidant activity of the seaweed extracts was also evaluated in Caco-2 cells. All extracts significantly ($P < 0.05$) increased glutathione (GSH) content of cells after 24 h. Caco-2 cells were also pre-treated with seaweed extract for 24 h followed by exposure to hydrogen peroxide (H_2O_2). Antioxidant enzyme activity (catalase (CAT) and superoxide dismutase (SOD)) was assessed and DNA damage was measured using the comet assay. *Pelvetia canaliculata* was the most effective at preventing H_2O_2 -mediated SOD depletion in Caco-2 cells while *Fucus serratus* exhibited the best DNA protective effects.

The ability of brown seaweed extracts from *Ascophyllum nodosum*, *Laminaria hyperborea*, *Pelvetia canaliculata*, *Fucus vesiculosus* and *Fucus serratus* to protect against *tert*-butyl hydroperoxide (*tert*-BOOH) induced stress in Caco-2 cells was also investigated. Oxidative stress was determined by measuring alteration in the enzymatic activity of catalase (CAT) and superoxide dismutases (SOD) and cellular levels of glutathione (GSH). *Laminaria hyperborea*, *Pelvetia canaliculata* and *Fucus serratus* significantly protected against *tert*-BOOH induced SOD reduction but did not protect against the reduction in CAT activity or the increased cellular levels of GSH. The ability of *Fucus serratus* and *Fucus vesiculosus* to protect against H_2O_2 and *tert*-BOOH induced DNA damage was also assessed. The DNA protective effects of the two seaweed extracts were compared to those of three metal chelators; deferoxamine mesylate (DFO), 1, 10-phenanthroline (o-phen) and 1,2-Bis (2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid tetrakis (BAPTA-AM). *Fucus serratus* and *Fucus vesiculosus* significantly protected ($P < 0.05$) against H_2O_2 (50 μ M) induced DNA damage but not *tert*-BOOH induced damage.

The antioxidant activities of extracts from *Ascophyllum nodosum* (AN), *Fucus vesiculosus* (FV) and *Fucus serratus* (FS) prepared using different solvents were assessed in Caco-2 cells. The extracts were prepared using 100% H_2O (AN₁₀₀, FV₁₀₀, FS₁₀₀), 60% ethanol (AN_{60e}, FV_{60e}, FS_{60e}), 80% ethanol (AN_{80e}, FV_{80e}, FS_{80e}) or 60% methanol (AN_{60m}, FV_{60m}, FS_{60m}) combined with an accelerated solvent extraction (ASE®) technique. The cellular antioxidant status was determined by measuring catalase (CAT) and superoxide dismutase (SOD) activity and glutathione (GSH) content. The protective effects of the extracts against H_2O_2 and *tert*-BOOH-induced DNA damage were assessed using the comet assay. AN₁₀₀ and AN_{80e}

significantly protected ($P < 0.05$) against H_2O_2 -induced DNA damage. AN_{60e} , AN_{80e} , FS_{100} , FS_{80e} and FV_{60m} protected against *tert*-BOOH -induced DNA damage. Extracts prepared from AN, particularly those prepared using 80% aqueous ethanol, appeared to have the greatest antioxidant potential, based on their ability to protect against oxidant-induced DNA damage.

5.13. Investigation into the molecular mode of action of marine derived bioactives

5.13.1. Anti-adipogenic mode of action of chitosan at cell level

Chitosan is a derivative of chitin, a natural polymer that is found in exoskeletons of crustaceans - shrimp, lobster and crab: and chitooligosaccharides result from the hydrolysis of chitosan. Four chitooligosaccharides (COS) with different Molecular weights (MW) (<1000, 1-3,000, 3-5,000 and 5-10,000 Da) were evaluated in this study to determine their effects on cell health and inhibition of adipogenesis *in vitro*. The 3T3-L1 pre-adipocyte cells were induced to differentiate in presence or absence of COS at day 8 of induction of differentiation, lipid accumulation, free glycerol release and gene expression were measured. Results indicated that the effect of COS on cell health was dependent on the MW, concentration and incubation time. Where the COS with high, MW 5-10,000 Da tended to have a high cell viability and low cytotoxicity profiles. During the adipogenesis process, COS had MW and concentration dependent inhibitory effects on lipid accumulation and free glycerol release. The highest level of inhibition of adipogenesis was observed with MW 5-10,000 Da that caused 36.1, 43.7, 58.0 and 82.4% inhibition of lipid accumulation at 600, 1200, 2400 and 4800 mg/ml, respectively. Quantitative gene expression data suggested that COS mediated inhibition of adipogenesis involved an up-regulation of interleukin 6 (*IL6*) and prostaglandin-endoperoxide synthase-2 (*PTGS2*) genes and down regulation of a panel of genes involved in lipid biosynthesis. These results suggest that COS has the potential to be a functional food against adipogenesis in humans.

5.13.2. Anti-inflammatory mode of action of chitosan at cell level

The objectives of this study were two-fold, firstly to evaluate the effect of chitooligosaccharide (COS) on expression of a specific panel of cytokine genes involved in inflammation and secondly, to delineate the signal transduction pathway underlying the COS mediated inflammatory response. Human intestinal epithelial-like (Caco-2) cells were treated with COS (5000-10,000Da) and expression of a panel of eighty-four cytokine genes was analyzed by quantitative real-time PCR. COS induced up-regulation of a total of 11 genes including CCL20 and IL8 and concurrent down-regulation of 10 genes including pro-inflammatory mediators CCL15, CCL25 and IL1B. To further establish the signal transduction pathway of COS mediated response in Caco-2 cells, two major inflammatory

signal transduction pathways (NF- κ B and AP-1) were investigated. COS had inhibitory effect (P<0.01) on TNF- α induced NF- κ B binding activity while stimulatory effect (P<0.001) on AP-1 binding activity. COS also inhibited the expression of RELA (P<0.01) and IKBKB (P<0.01) genes of NF- κ B pathway while stimulating the expression of JUN (P<0.05) gene of AP-1 pathway. In conclusion, COS elicits an acute inflammatory cytokine response in Caco-2 cells and hence it has the potential to stimulate the immune system in the gut epithelium.

5.13.3. Anti-inflammatory mode of action of seaweed extract derived from *Ascophyllum nodosum*

A number of extracts from different seaweed species demonstrated highly potent anti-inflammatory properties during *in vitro* screening. Three extracts (cold water, hot water and 80% ethanol) from one of these species, *Ascophyllum nodosum*, was the subject of an investigation of the molecular mode of action. Three different molecular weight (MW) fractions were screened in the Caco-2 inflammation assay and MW fractions with high anti-inflammatory bioactivity chosen for further evaluation in the *ex-vivo* porcine colonic tissue. Table 24 shows the differentially expressed immune genes in porcine colonic tissue treated with 80% ethanol extract of *Ascophyllum nodosum* versus LPS challenged control tissue. A number of pro-inflammatory gene markers were inhibited by 80% ethanol extract of *Ascophyllum nodosum* in colonic explant.

Table 24 List of differentially expressed genes in porcine colonic tissue *ex-vivo* as induced by the 80% ethanol extract of *Ascophyllum nodosum*

Genes	Fold up (+)/down (-)
Toll like receptor 4 (TLR4)	-56.32
Lysozyme (LYZ)	-21.14
Interleukin 17A (IL17A)	-12.45
Prostaglandin-endoperoxide synthase 2 (PTGS2)	-9.51
Nuclear factor of kappa light polypeptide gene enhancer in B-cells	-8.24
Toll like receptor 6 (TLR6)	-8.13
Interleukin 17F (IL17F)	-7.92
NF-kappa B repressing factor (NKRF)	-7.14
Interleukin 8 (IL8)	-5.79
Chemokine (C-X-C motif) ligand 10 (CXCL10)	-5.73
Interleukin 6 receptor (IL6R)	-5.70
Intercellular adhesion molecule 1 (ICAM1)	-5.69
Toll like receptor 7 (TLR7)	-4.92
Chemokine (C-X-C motif) ligand 2 (CXCL2)	-4.68
Interleukin 6 (IL6)	-4.21
TRAF family member associated NFKB activator (TANK)	-4.09
Toll like receptor 8 (TLR8)	-3.97
v-akt murine thymoma viral oncogene homolog 1 (AKT1)	-3.79
Interferon (alpha, beta and omega) receptor 1 (IFNAR1)	-3.42
Complement component 5 (C5)	-3.41
Interleukin 10 (IL10)	-3.27
Mitogen-activated protein kinase kinase kinase 8-like (MAP3K8)	-3.05
Chemokine (C-X-C motif) ligand 11 (CXCL11)	-3.03

Chemokine (C-C motif) ligand 2 (CCL2)	-3.02
Macrophage migration inhibitory factor (MIF)	-2.94
Interleukin 4 receptor (IL4R)	-2.87
MHC class I related antigen 2 (MIC2)	-2.62
Vascular cell adhesion molecule 1 (VCAM1)	-2.52
Colony stimulating factor 1 (CSF1)	-2.41
Interleukin 21 (IL21)	-2.23
Mitogen activated protein kinase 9 (MAPK9)	-2.09
Toll like receptor 1 (TLR1)	+4.92
Interleukin 23A (IL23A)	+4.15
Interferon alpha 1 (IFNA1)	+3.35
Apolipoprotein A-I (APOA1)	+2.29
S100 calcium binding protein A3 (S100A9)	+2.01

The three different MW fractions of 80% ethanol extract of *Ascophyllum nodosum* were also evaluated for their anti-inflammatory effect in gene expression *ex-vivo*. There are groups of common and unique genes up-regulated by all the three MW fractions and these are presented in Table 25 while the groups of common and unique genes down-regulated by all the three MW fractions are presented in Table 26.

Table 25 List of genes up regulated in porcine colonic tissue *ex-vivo* by different molecular weight fractions of 80% Ethanol extract of *Ascophyllum nodosum*

Genes	Molecular weight (kDa)		
	<3.5	3.5-	>100
<u>Cluster 1: Common genes (affected by all MW fractions)</u>			
Toll like receptor 1 (TLR1)	7.85	10.10	6.32
Interferon alpha 1 (IFNA1)	5.81	5.31	6.61
Apolipoprotein A-I (APOA1)	3.30	11.09	6.50
Interleukin 1 receptor associated kinase 1 (IRAK1)	3.34	8.64	4.90
Transforming growth factor beta 1 (TGFB1)	2.70	6.43	3.01
Interleukin 16 (IL16)	2.36	6.15	2.07
<u>Cluster 2: Unique genes (affected by each MW fraction)</u>			
CD 80 molecule (CD80)	5.74	-	4.62
Interferon regulatory factor 3 (IRF3)	3.06	4.42	-
Interleukin 23A (IL23A)	-	5.57	2.41
Tumour necrosis factor receptor superfamily, member 1B	-	3.91	-
V-rel avian reticuloendotheliosis viral oncogene homolog A (RELA)	-	3.91	-

Table 26 List of genes down regulated in porcine colonic tissue *ex-vivo* by different molecular weight fractions of 80% Ethanol extract of *Ascophyllum nodosum*

Genes	Molecular weight (kDa)		
	<3.5	3.5-100	>100
<u>Cluster 3: Common genes (affected by all MW fractions)</u>			
Lysozyme (LYZ)	-41.31	-48.37	-51.38
Interleukin 8 (IL8)	-14.42	-31.91	-33.53
Prostaglandin-endoperoxide synthase 2 (PTGS2)	-8.34	-3.81	-6.28
Nitric oxide synthase 2, inducible (NOS2)	-7.67	-4.95	-9.11
Toll like receptor 6 (TLR6)	-7.14	-3.25	-5.37
Chemokine (C-C motif) ligand 20 (CCL20)	-6.60	-5.35	-4.32
Chemokine (C-X-C motif) ligand 10 (CXCL10)	-5.03	-2.29	-3.79

Interleukin 6 (IL6)	-4.50	-2.05	-3.39
Chemokine (C-X-C motif) ligand 11 (CXCL11)	-4.09	-2.64	-2.84
Intercellular adhesion molecule 1 (ICAM1)	-2.61	-2.51	-4.64
Chemokine (C-X-C motif) ligand 9 (CXCL9)	-2.56	-3.08	-2.17
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	-2.05	-2.63	-2.36
<u>Cluster 4: Unique genes (affected by each MW fraction)</u>			
Chemokine (C-X-C motif) ligand 10 (CXCL2)	-4.10	-	-3.09
Interleukin 17A (IL17A)	-3.67	-	-2.12
Interleukin 5 (IL5)	-3.58	-	-2.70
Toll like receptor 8 (TLR8)	-3.48	-	-2.62
Mitogen-activated protein kinase kinase kinase 8-like (MAP3K8)	-3.24	-	-2.44
Colony stimulating factor 1 (CSF1)	-3.10	-	-2.14
Interferon (alpha, beta and omega) receptor 1 (IFNAR1)	-3.00	-	-2.26
Tumour necrosis factor alpha (TNFA)	-2.26	-	-2.21
Chemokine (C-C motif) ligand 2 (CCL2)	-2.37	-	-
Mitogen activated protein kinase 9 (MAPK9)	-	-3.35	-
Mitogen activated protein kinase 8 (MAPK8)	-	-2.36	-
TNF receptor-associated factor 4 (TRAF4)	-	-2.87	-
v-akt murine thymoma viral oncogene homolog 1 (AKT1)	-	-	-2.53
TRAF family member-associated NFKB activator (TANK)	-	-	-2.36

The evaluation of three MW fractions *ex-vivo* indicated that a) the immuno-modulatory bioactivity of the 80% ethanol extract is also shown by each of the MW fractions and b) each of the MW fractions also alter the expression of unique immune genes.

This *ex-vivo* experiment demonstrated that *Ascophyllum nodosum* ethanol extract and its MW fractions can be utilised to alter immune genes in porcine colonic tissue. These results demonstrate that extracts of *Ascophyllum nodosum* can effectively inhibit the pro-inflammatory signalling pathways in porcine gut, which provides opportunities to utilise this anti-inflammatory seaweed extract in the treatment of chronic pro-inflammatory diseases of the intestinal gut in human.

5.14. An evaluation of the effects of marine bioactive compounds on fatness and anti-obesity potential in the pig model

Chitosan, a natural polysaccharide comprising copolymers of glucosamine and N-acetylglucosamine, has been shown to have anti-obesity properties. The effect of 1000 ppm Irish prawn shell chitosan on dietary intake, body weight gain and fat deposition of animals at a starting weight of 70kg was investigated in a randomised trial. The aim was to investigate the anti-obesity effect of chitosan (1000ppm) on animals at a body weight that represented a normal healthy human being.

Two dietary treatments were designed, firstly a basal control diet, high in protein and carbohydrate, similar to the vast majority of European diets and secondly, the same diet with

an inclusion of 1000ppm of chitosan - equivalent to approximately 3g per day of chitosan. Both diets were offered in meal form. The diets were fed for sixty-nine days *ad libitum*.

5.14.1. Animal selection and management

Forty gilts (female pigs that had not farrowed) were selected from a commercial pig unit and blocked on the basis of live weight and housed in groups of 10 in partially slatted pens. They were individually fed and given *ad libitum* access to food and water. The house temperature was controlled at 21°C for the duration of the experiment. The pigs were weighed at the beginning of the experiment (day 0) and every seven days to the end of the experiment (day 69). The pens were equipped with single-space computerised feeders, which recognised when each individual animal entered the feeder. After each animal had fed and withdrawn from the trough, difference between the pre- and post-visit trough weight was collected automatically and with other data (animal identification number, date and the time of entry and exit) unique to each animal stored; allowing individual dietary intake to be calculated. Automated data collection also captured feeding behaviour (feeding time, feeding duration, amount consumed per visit) of each animal.

5.14.2. Biological analysis

Blood samples (10 ml) were taken from each animal every seven days to facilitate adipokine and hormone (leptin, IL-6 and CRP) quantification. All the animals were sacrificed on day 69 of the experiment. Samples were taken from the duodenum, jejunum, ileum and colon for nutrient transport assessment; allowing for the extraction of RNA, cDNA synthesis and qPCR analysis to establish where nutrients were being broken down in the gut and the potential effect of chitosan on this process.

Research has already shown that gut microbiota can vary in obese and non-obese individuals; hence colon samples were taken to assess the microbiota present in the control and chitosan groups.

Measurements of fat thickness 6 cm from the edge of the split back, at the level of the third and fourth last rib, were taken 48 hr after slaughter using the Hennessy grading probe. Fat samples from the abdominal region and the back of each animal allowed for gene expression work. Three samples of brain (periventricular nucleus, arcuate nucleus and the lateral hypothalamic area) were taken from each pig since each of these regions are highly correlated with satiety and hunger, and provide RNA to investigate genes associated with hunger such as PPARG and NPY.

5.14.3. Results

Dietary intake, body weight and carcass characteristics

Pigs offered the chitosan diet had lower dietary intake ($P < 0.01$), body weight gain ($P < 0.05$) ($P < 0.01$) and lower final body weight ($P < 0.05$), compared to pigs offered the basal diet. Chitosan inclusion had no effect on feed conversion ratio ($P > 0.05$). Animals offered chitosan had lower depths of back fat and total carcass fat compared to the basal group ($P < 0.05$). Lean meat percentage was higher in chitosan-supplemented pigs ($P < 0.01$) compared to the basal group.

Coefficient of apparent ileal digestibility and coefficient of total tract digestibility

Pigs offered the chitosan diet had decreased CAID of DM and GE ($P < 0.05$) compared with the control group. Pigs offered the chitosan diet had reduced CATTD of GE and N compared to the control group ($P < 0.05$).

Serum leptin

There was a time effect ($P < 0.05$) and treatment effect ($P < 0.05$) on serum leptin concentrations. Serum leptin concentrations were higher in pigs offered the chitosan diet compared to the basal treatment group ($P < 0.05$). There was no interaction between time and treatment on serum leptin concentrations ($P > 0.05$).

Nutrient transporter gene expression

The gene expression of *FABP2* was down-regulated in the ileum of animals supplemented with chitosan compared to the basal group ($P < 0.05$). The gene expression of *GLUT5* was up-regulated in the jejunum of chitosan-offered animals compared to the control group ($P < 0.05$). No supplementation effect was observed on the gene expression of nutrient transporters in the duodenum ($P > 0.10$).

Digestive enzyme gene expression

There was no effect of dietary supplementation on digestive enzyme gene expression in the duodenum, jejunum and ileum ($P > 0.10$).

Microbiology

Pigs offered chitosan had decreased gene copy number (GCN) of *Lactobacillus spp.* in both the caecum ($P < 0.05$) and colon ($P < 0.001$), and increased GCN of *Bifidobacterium* in the caecum ($P < 0.05$) compared to the control group. Animals offered chitosan had decreased GCN of *Firmicutes* in the colon compared with the control group ($P < 0.05$). There was no dietary supplementation effect on *Bacteroidetes* and *Enterobacteriaceae* population in either the caecum or colon ($P > 0.10$).

Volatile fatty acids concentration

Total VFA concentration was increased in the colon of chitosan-supplemented animals compared with the control group ($P < 0.05$). Chitosan-supplemented animals had an increased proportion of acetate in the colon compared to the control group ($P < 0.05$).

Hypothalamic regulators of appetite

Dietary chitosan up-regulated Orexin (*HCRT*) and Growth hormone receptor (*GHR*) gene expression in the arcuate nucleus (ARC) ($P < 0.05$) when compared to the control group. Dietary chitosan resulted in an up-regulation of Peroxisome proliferator activated receptor gamma (*PPARG*) gene expression in the paraventricular nucleus (PVN) ($P < 0.01$) when compared to the control animals. Dietary chitosan had a tendency to increase both Neuromedin B (*NMB*) ($P = 0.09$) and Insulin receptor (*INSR*) ($P = 0.07$) in the PVN when compared to the control group. There was no effect of diet on hypothalamic regulators of appetite in the lateral hypothalamic area (LHA) ($P > 0.05$).

Gut appetite hormones

Dietary supplementation of chitosan decreased Neuropeptide Y (*NPY*) gene expression in the jejunum when compared to the control group ($P < 0.05$). There was no effect of dietary supplementation of chitosan on the remaining genes analysed ($P > 0.05$).

5.14.4. Summary

Dietary supplementation of prawn derived chitosan resulted in reduced feed intake and body weight in a pig model. This effect may be orchestrated through multiple responses both within the intestinal tract and bloodstream including; decreased nutrient digestibility, decreased nutrient transporter expression, increased serum leptin and altered gut microflora.

5.15. An evaluation of the potential of marine compounds to contribute to enhanced pig performance

A complete randomised design experiment was conducted to investigate the effects of supplementing different molecular weights (MW) of chitooligosaccharide (COS) on pig performance, selected microbial populations and nutrient digestibility post-weaning. Three hundred and ninety six weaned piglets (24 days of age, $7.3 \text{ kg} \pm (\text{S.D}) 1.7 \text{ kg}$ live-weight) were blocked on the basis of live-weight and were assigned to one of 6 dietary treatments (twenty two replicates/treatment) for a 33-day experimental period. The dietary treatments were; (1) control diet (0 ppm COS), (2) control diet plus $< 1 \text{ kDa}$ COS, (3) control diet plus 3-5 kDa COS, (4) control diet plus 5-10 kDa COS, (5) control diet plus 10-50 kDa COS and (6) control diet plus 50-100 kDa COS. The COS was included at 250 ppm in the diets. There was no significant effect of dietary treatment on piglet performance during the starter

period (days 0-18) ($P>0.05$). However, there were quadratic responses in both daily gain ($P<0.05$) and gain to feed ratio ($P<0.05$) to the increased MW of COS inclusion during the weaner period (days 18-33) with all COS supplemented treatments improving daily gain and gain to feed ratio compared to the control. There was a quadratic response in faecal scoring to the increased MW of COS inclusion from day 0-7 ($P<0.001$), day 7-14 ($P<0.001$) and during the overall experimental period ($P<0.01$) with all the COS supplemented treatments having an improved faecal score compared to the control. During the weaner period, there was a cubic response in *lactobacilli* and *E.coli* populations as the MW of COS increased ($P<0.05$). The 5-10 kDa and 10-50 kDa COS increased *lactobacilli* populations compared to the control while *lactobacilli* populations decreased at 50-100 kDa. The 5-10 kDa, 10-50 kDa and 50-100 kDa COS decreased *E.coli* populations compared to the control. There was a cubic response in the apparent total tract digestibility of dry matter (DM) ($P<0.01$), organic matter (OM) ($P<0.01$), ash ($P<0.01$), nitrogen (N) ($P<0.01$), and gross energy (GE) ($P<0.01$) to the increased MW of COS inclusion during the weaner period. The 5-10 kDa COS had a higher apparent total tract digestibility of DM, OM, ash, N and GE in comparison to the control, while the apparent total tract nutrient digestibility of these nutrients decreased at 10-50 kDa. The current results indicate that the MW ranges of 5-10 kDa and 10-50 kDa COS decreased *E.coli* numbers while increasing nutrient digestibility of the diets.

A second experiment (complete randomised design) was conducted to investigate the effects of supplementing different molecular weights of chitooligosaccharide on performance, intestinal morphology, selected microbial populations, volatile fatty acid concentrations and the immune status of the weaned pig. Twenty-eight piglets (24 days of age, 9.1 (\pm s.d. 0.80) kg live weight) were assigned to one of four dietary treatments for 8 days and then sacrificed. The treatments were (1) control diet (0 ppm COS) (2) control diet plus 5-10 Kda COS (3) control diet plus 10-50 Kda COS and (4) control diet plus 50-100 Kda COS. The COS was included in dietary treatments at a rate of 250 mg/kg. Tissue samples were taken from the duodenum, jejunum and ileum for morphological measurements. Digesta samples were taken from the colon to measure *lactobacilli* and *E. coli* populations and digesta samples were taken from the caecum and colon for VFA analysis. Gene expression levels for specific cytokines were investigated in colonic tissue of the pig. Pigs fed the 10-50 Kda COS had a higher villous height ($P<0.05$) and villous height/crypt depth ratio ($P<0.05$) in the duodenum and the jejunum compared to the control treatment. Pigs fed the 5-10 Kda COS had a lower *lactobacilli* population ($P<0.05$) and *E. coli* population ($P<0.05$) in the colon compared to the control group. The inclusion of COS at all MW in the diet significantly reduced faecal scores compared to the control treatment ($P<0.01$). Pigs offered the 5-10 Kda COS had significantly lower levels of acetic acid and valeric acid compared to the

control group ($P < 0.05$). Supplementation of different MW of COS had no significant effect on pig performance or on the expression of the cytokines TNF- α , IL-6, IL-8, and IL-10 in the gastro-intestinal tract of the weaned pig. The current results indicate that the lower molecular weight of 5-10 Kda COS possessed strong antibacterial activity while the higher molecular weight of 10-50 Kda was optimum for enhancing intestinal structure. The COS supplementation exerted no deleterious effects on immune function or growth performance of the pigs while reducing the incidence of diarrhoea.

Reliable alternatives to in-feed antibiotics need to be identified and chitooligosaccharide may be a possible substitute. It was observed in the current experiment that the MW ranges of 5-10 kDa and 10-50 kDa COS decreased *E.coli* numbers while increasing nutrient digestibility of the diets.

5.16. Comparison of the anti-inflammatory effects of seaweed extracts and novel milk hydrolysates

The anti-inflammatory effects various seaweed species extracts generated within the NutraMara programme were compared with the anti-inflammatory effects of novel milk hydrolysates derived from milk protein substrates generated within the Food for Health Ireland Project.

5.16.1. Materials and methods

Preparation of seaweed solubility extracts

A selection of 14 species of seaweed was harvested from the west coast of Ireland. Samples were washed, stored at -18°C , subsequently freeze-dried and ground into powder using a Waring blender. Samples were stored in vacuum-packed bags at -80°C before extraction. Solubility extraction of ground seaweed samples was performed with either cold water (CWE), hot water (HWE) or ethanol (80%): water (20%) (EE) as solvent following the solid-liquid extractions procedure described by Tierney *et al.*, (2013a). Molecular weight fractions (<3.5 kDa, 3.5-100 kDa and >100 kDa) were generated for a proportion of interesting extracts. All extracts and fractions were freeze-dried and the dried extracts were stored at -80°C until used for further analysis.

Preparation of milk hydrolysates

The hydrolysis of milk substrates was carried out following the method of either Nongonierma *et al.*, (2012) for enzymatic hydrolysis, Simpson *et al.* (2004) for microbial fermentation or Kuchroo *et al.* (1982) for isoelectric precipitation of colostrum. The hydrolysates (50 L) generated using different hydrolysis techniques were spray dried, subsequently further concentrated (to ca 40 % total solids) before spray drying again.

Hydrolysates were subjected to microfiltration (MF) using a GEA Model F unit (GEA Process Engineering A/S, Skanderborg, Denmark).

The permeate stream prepared above was then subjected to ultrafiltration (UF) using the same GEA model F unit fitted with two Koch KMS HFK™-328 spiral wound membranes (96 x 965 mm, Koch Membrane Systems, Wilmington, MA, US). The 5 kDa permeate stream was finally processed on the GEA model F plant fitted with two Alpha Laval UF-ETNA spiral wound membranes (95 x 965mm, Alpha Laval AB, Lund, Sweden). The 0.14 µm and 5 kDa retentate streams described above were dehydrated in a pilot scale Anhydro Lab 3 spray drier (SPX Flow Technology A/S, Soeborg, Denmark) at an inlet temperature range of 185 – 190°C and outlet of 85–90°C. The 1 kDa retentate and permeate were further concentrated (to ca 40 % total solids) before spray drying, as outlined above.

In vitro anti-inflammatory assay

Caco-2 cells (American Type Culture Collection, ATCC, Manassas, VA, USA) 10⁵ cells/ml were seeded in a 24-well cell culture plate containing Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Corp., San Diego, CA, USA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen Corp.), 1% (v/v) non-essential amino acids, 1% sodium pyruvate and penicillin (100U)/streptomycin (100µg/ml) (All sourced from Sigma–Aldrich Corp., St. Louis, MO, USA). Plates were incubated for 8–10 days in 5% CO₂ at 37 °C. Before treatment, the growth media was removed and the cells were washed with sterile phosphate buffer saline (PBS) and incubated for 3 h in serum and antibiotic free media. To induce a pro-inflammatory response, cells were treated with 10 ng/ml TNF-α. The anti-inflammatory bioactivity of milk hydrolysates and seaweed preparations were tested through co-treatment of cells with each sample at a final concentration of 1mg/ml and TNF-α. Following 24 h incubation, the media was harvested and the concentration of IL-8 in the supernatants was measured using a human IL-8 sandwich ELISA (R&D Systems Europe, Ltd., Abingdon, UK).

Ex-vivo challenge of colonic tissues

A section of the pig colon (Large White x Landrace; n= 6 pigs) was dissected along the mesentery immediately post-slaughter. The faecal material was removed and the tissue section was washed with sterile PBS. The overlying smooth muscle layer was removed and a colonic section of approximately 1.25 cm x 1.25 cm were transferred into 1 ml DMEM in a 12-well cell culture plate containing 10 µg/ml bacterial lipopolysaccharide (LPS) (*Escherichia coli* serotype O111:B4, Sigma Aldrich, St. Louis, MO) in the presence or absence of 1 mg/ml of each sample.

RNA extraction

Total RNA was extracted from 25 mg colonic tissue samples using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich Corp.) according to the manufacturer's instructions. Total RNA was subjected to DNase I (Sigma-Aldrich Corp.) treatment, followed by further purification using a phenol-chloroform extraction method. The quality and quantity of the total RNA were assessed in a NanoDrop-ND1000 Spectrophotometer (Thermo Fisher Scientific Inc. MA, USA). The cDNA synthesis was performed with 1 µg of total RNA using a first strand cDNA synthesis kit (Qiagen Ltd. Crawley, UK) following the manufacturer's protocol.

Quantitative Real-Time PCR (qPCR)

For milk hydrolysates, qPCR was carried out to quantify the following targets; interleukins (*IL-1 α* , *IL-1 β* , *IL-4*, *IL-6*, *IL-8*, *IL-10*, *IL-17A*, *IL-21*), interferon (*IFN- γ*), tumour necrosis factor (*TNF α*), transforming growth factor (*TGF- β*) and forkhead box P3 (*FOXP3*). For seaweed extracts, qPCR was carried out for a panel of 96 genes involved in the immune signalling pathways in pig using a customized PCR array in a 7300 Real time PCR system (Applied Biosystems). Results are expressed as fold change compared to LPS stimulated cells.

5.16.2. Results

Inhibition of IL-8 production in *TNF α* induced Caco-2 cells by seaweed extracts and milk hydrolysates

All samples were screened for their ability to inhibit IL-8 production in a *TNF α* induced Caco-2 cells. All Caco-2 cells were treated with *TNF α* , which stimulated the cells to produce IL-8 at a concentration of 110 ± 6.60 pg/ml over a 24 h period (control). Depending upon the effect on IL-8 concentration, the milk hydrolysates were divided into either highly anti-inflammatory, moderately anti-inflammatory, no effect or pro-inflammatory groups. Seventy-seven seaweed extracts were evaluated and of these, 53% of samples were associated with a highly anti-inflammatory effect, 5 % with an anti-inflammatory effect, 13% with no effect and 29 % with a pro-inflammatory effect. One hundred and seven milk hydrolysates were also evaluated, out of which 22 % of samples were associated with a highly anti-inflammatory effect, 18 % with an anti-inflammatory effect, 43% with no effect and 17 % with a pro-inflammatory effect. There was a higher percentage of anti-inflammatory results among the seaweed extracts (58 %) compared to the milk extracts (40 %).

Cold water extract (CWE), ethanol (80 %) extract (EE), and hot water extracts (HWE) of *Fucus vesiculosus* and *Ascophyllum nodosum* were further analysed. Co-treatment with CWE and EE of *F. vesiculosus* resulted in a reduction of IL-8 concentration by 72% ($P < 0.05$) and 70% ($P < 0.05$) respectively, relative to control. However, co-treatment with HWE of *F. vesiculosus* had no effect on IL-8 concentration. Co-treatment with CWE, EE or HWE of *A.*

nodosum resulted in a reduction of IL-8 concentration by 53% (P<0.05), 38% (P<0.05) and 33% (P<0.05) respectively, relative to control.

Milk sodium caseinate (NaCAS) and NaCAS enzyme hydrolysates (EH) generated by low (8 %) and high (16 %) degrees of hydrolysis along with their associated 5kDa retentate (5kDaR), a 1 kDa retentate (1kDaR) and a 1 kDa permeate (1kDaP) fractions were also further investigated. Co-treatment with NaCAS, EH-8 %, or 5kDaR resulted in a reduction of IL-8 concentration by 31.1% (P<0.05), 31% (P<0.05) and 32.7% (P<0.05) respectively, relative to control. The greatest reduction in IL-8 concentration was observed following co-treatment with the 1kDaR-8 %, 1kDaP-8 %, 5kDaR-16 % and EH-16 %, which resulted in the reduction of IL-8 concentration by 68.7% (P<0.01), 66.15 (P<0.01), 59% (P<0.01) and 56.6% (P<0.01) respectively, relative to control.

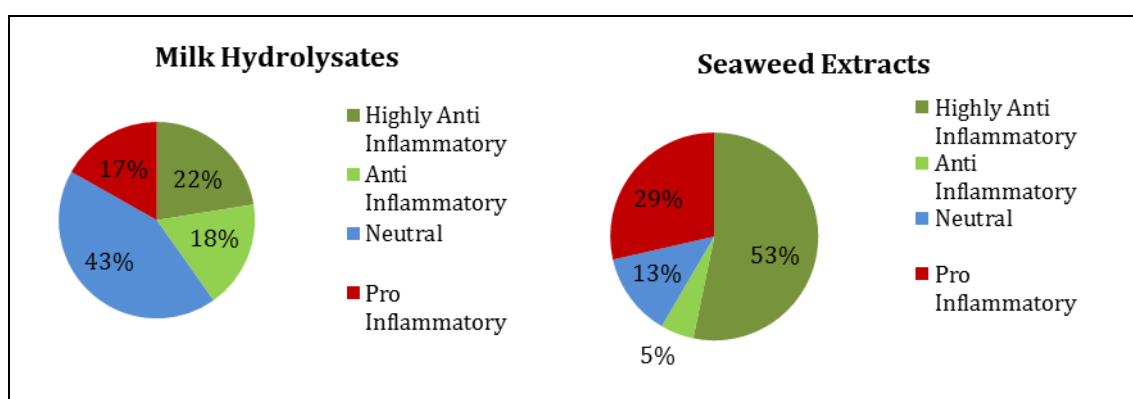
Anti-inflammatory effects of seaweed extracts and milk hydrolysates in LPS stimulated porcine colonic explants

The (CWE) of *F. vesiculosus* and (EE) of *A. nodosum* and three of its molecular weight fractions (<3.5 kDa, 3.5-100 kDa and >100 kDa), had a significant effect on the expression of LPS-induced inflammatory mediators (*PTGS-2*, *C5*, *LYZ*), cytokines (*IL17A*, *IL10*, *IL8*), chemokines (*CXCL2*, *CXCL10*), cell adhesion molecules (*ICAM1*, *VCAM1*), toll like receptors (*TLR4*, *TLR6*, *TLR7*, *TLR8*) and components of the NF- κ B (*NFKB1*, *TANK*, *NKRF*) pathway. The (CWE) of *F. vesiculosus* and (EE) of *A. nodosum* and three of its molecular weight fractions (<3.5 kDa, 3.5-100 kDa and >100 kDa), all reduced the expression (> 2 fold) of *PTGS-2*, *LYZ*, *IL8*, *CXCL2*, *CXCL10*, *ICAM1*, *TLR6* and *NFKB1*. The treatment of porcine colonic tissues with (CWE) of *F. vesiculosus* and (EE) of *A. nodosum*, its 3.5-100 kDa and >100 kDa fractions was associated with down-regulation (> 2 fold) of *TLR4*, *TLR8* and *TANK* relative to LPS challenged tissues. In contrast to the milk hydrolysates, treatment of porcine colonic tissues with (CWE) of *F. vesiculosus* and (EE) of *A. nodosum*, its <3.5 kDa and >100 kDa fractions was associated with down-regulation of *IL17A*, relative to LPS challenged tissues. Co-treatment of porcine colonic tissues with (CWE) of *F. vesiculosus* and (EE) of *A. nodosum*, and its <3.5 kDa fraction were associated with down-regulation of *C5*, while treatment with (CWE) of *F. vesiculosus* and (EE) of *A. nodosum*, and its 3.5-100 kDa fraction were associated with down-regulation of *TLR7*, relative to LPS challenged tissues. The treatment of porcine colonic tissues with (CWE) of *F. vesiculosus* and (EE) of *A. nodosum* was associated with reduced expression of *VCAM1* and *NKRF*.

Of the milk hydrolysates analysed, co-treatment of colonic tissues with EH-8%, 5kDaR-8% and 1kDaR-8% was associated with a down-regulation of *IL-1 α* and *TNF α* expression relative to LPS challenged control. Similarly, the co-treatment of tissues with 5kDaR-16% was also associated with a down-regulation of *IL-1 α* expression. Co-treatment with 5kDaR-8%,

5kDaR-16% and 1kDaR-8% hydrolysates was associated with a down-regulation of *IL-1 β* and *IL-8* expression. Co-treatment of colonic tissues with NaCAS was associated with an up-regulation of *IL-8* expression relative to LPS challenged control. Co-treatment with milk hydrolysates also had significant effects on *IL-10*, *IL-17A* and *TGF- β* expression relative to unchallenged tissues. The treatment of porcine colonic tissues with EH-8%, 5kDaR-8%, 1kDaR-8% and 1kDaP-8% were associated with a down-regulation of *IL-10* expression relative to unchallenged tissues. Co-treatment with NaCAS and 5kDaR-16% was associated with an increase in *IL-17A* expression relative to unchallenged tissues. The co-treatment with EH-8%, 5kDaR-8%, 5kDaR-16% and 1kDaR-8% hydrolysates were associated with a down-regulation of *TGF- β* expression in colonic tissues relative to unchallenged tissues. The effects of seaweed extracts and milk hydrolysates and their fractions are summarised in Figure 37.

Figure 37 The anti-inflammatory activity of seaweed extracts, milk hydrolysates and their fractions in *in vitro* TNF α stimulated Caco-2 cell culture models



5.16.3. Conclusion

This comparison of seaweed extracts and milk hydrolysates identified a number of extracts that contained anti-inflammatory potential within both the seaweed sources from NutraMara (Bahar *et al.*, 2016 a,b; Egan *et al.*, 2016) and the milk sources from Food for Health Ireland (Mukhopadhyaya *et al.*, 2014; 2015). The cold water extract of *Fucus vesiculosus* and ethanol extract of *A. nodosum* and three of its molecular weight fractions (<3.5 kDa, 3.5-100 kDa and >100 kDa), have strong anti-inflammatory bioactivity in porcine colonic tissue *ex vivo*, that is comparable to the bioactivity of milk NaCAS and NaCAS enzyme hydrolysates (EH) generated by low (8 %) and high (16 %) degrees of hydrolysis as well as their associated 5kDaR and 1kDaR retentates. Further characterisation and quantification of the bioactive molecules within the extracts from these two different sources is of significant potential interest and they are unlikely to be similar compounds but all worthy of further exploration for human and animal health.

5.17. Human intervention trials

The health benefits of marine based foods, or foods containing novel isolated marine bio-actives were identified within the NutraMara consortium in dietary intervention studies. Marine derived compounds identified as having promising bioactive potential were assessed in dietary intervention studies to validate their bioactivities *in vivo*. A human dietary intervention study was designed, which also involved the incorporation of test ingredients into different food matrices; pork meat and bread, and subsequent assessment of the foods effect *in vivo*.

5.18. Effect of marine polysaccharides in pig meat

5.18.1. Source of the pork

Pork meat was prepared from sixteen cross breed pigs (Large White × Landrace consisting of 12 males and 12 females, average live weight ~ 14.51 kg) randomly assigned to one of two treatments (n=8) and fed *ad libitum* for 21 days pre-slaughter following a completely randomised experimental design. The control group of pigs were fed with a basal diet.

The second group of pigs were fed with the basal diet plus a spray-dried seaweed extract containing laminarin and fucoidan (LAM/FUC) at an inclusion rate of 5.37 kg/tonne of feed. Inclusion rates being based on the laminarin and fucoidan content of the spray-dried LAM/FUC. The treatment group received diets containing laminarin (500 mg/kg feed) and fucoidan (420 mg/kg feed). The pigs from both groups were slaughtered and butchered at the end of the feeding period.

5.18.2. Pork processing and packaging

M. longissimus dorsi muscles were trimmed of visible fat and connective tissue and minced twice through a plate with 4 mm holes (Model P114L, Talsa, Valencia, Spain). Pork mince was packaged into 325 g raw weight portions. Minced pork was formed into pork patties. All pork products were labelled as either Control or LAM/FUC based on the feeding regime described above.

5.18.3. Recruitment of participants

Healthy participants from across Northern Ireland were recruited between July 2011 and August 2011 through email, leaflets and posters. Participants who expressed an interest completed a screening questionnaire to assess their eligibility and those who qualified were invited to participate in this double-blind, randomized placebo controlled human dietary intervention study. Participants were excluded if they regularly consumed seaweed (>5 g/week) or antioxidant supplements; those on prescribed medication, have had a history of autoimmune disease or diabetes, taking non-steroidal anti-inflammatory drugs, immune suppressant drugs, echinacea or other immune stimulating herbs, developed a cold or other

upper respiratory tract infection during the course of the study or were unwilling to comply with the study protocols and those who were pregnant or had a BMI of < 18.5 or > 30 kg/m². In addition participants who did not regularly consume pork or pork products were excluded in order to minimise the risk of non-compliance. Eligible participants provided written informed consent before taking part in the study. This research was approved by the Research Ethics Committee of Ulster University (REC/11/0080) and conducted in accordance with the Declaration of Helsinki.

5.18.4. Intervention

Participants (n=40) were randomly assigned to either the treatment group (meat from pigs fed LAM/FUC mix, n=20) or placebo group (meat from pigs fed standard feed, n=20) using an online randomization software (www.randomisation.com). LAM/FUC and placebo pork meat were labelled and coded with a participant ID according to the randomisation sequence output by an independent researcher not involved in the design or the analysis of the study to ensure that the study was double-blinded to both researcher and participants. During the study, participants were required to consume three pork burgers (125g raw weight each) and pork mince (325 g raw weight) per week for four consecutive days. Taking into account cooking, which results in approximately 32% reduction in weight (Matthews, Garrison, 1975), the provided pork comprised participants' total weekly red meat intake (476g) and did not exceed the limit of 500g cooked weight red meat per week as recommended by the World Cancer Research Fund Research (2007). Participants were advised not to consume other red meat products during the intervention study to ensure they remained within the recommended limits for weekly red meat consumption, but were advised to otherwise follow their normal dietary behaviour. Pork meat was stored at -20°C prior to delivery to the participants in a thermos cool bag with two large frozen ice packs. Participants were provided with instructions on how to prepare and cook the meat for consumption at home. Participants were supplied with a diary to record their consumption of pork to provide an indication of study compliance.

Participants were invited to the human intervention studies unit at Ulster University at baseline and post-intervention. Height and weight were measured to determine Body Mass Index (BMI) at baseline and post-intervention. Body weight (kg) was recorded without footwear or heavy clothing and was measured to the nearest 0.1 kg using portable scales (Seca; Brosch Direct Ltd, Peterborough, United Kingdom). Standing body height (m) was measured to the nearest 0.1 cm using a calibrated stadiometer (SECA, Model 220, Germany). Blood pressure was measured using an Omron 705CP electronic blood pressure monitor (Medisave, Dorset, UK) from both arms of each participant and the arm with the highest reading for each individual was subsequently used as the reference arm for the post-

intervention appointment. A trained phlebotomist obtained fasting blood samples between 7am and 9am from participants by venepuncture for the baseline and post-intervention appointment. The blood was processed within 3 hrs and serum, plasma and lymphocyte samples were aliquoted and stored at -80 °C until analysed.

5.18.5. Lymphocyte cell isolation

Lymphocytes were isolated using Leucosep tubes containing Ficoll-Paque Plus separation medium (Greiner Bio-One, Germany) according to manufacturers' instructions. Briefly, whole blood was diluted 2:1 with RPMI 1640 medium (Gibco, Life Technologies Ltd, Paisley, UK) and layered onto the separation column. After centrifugation at 1000 g for 10 minutes, brake rate zero, lymphocytes's were separated from other blood components. After removal, lymphocytes were washed in RPMI 1640 medium and harvested by centrifugation at 250 g for 10 minutes. The pellet was reconstituted in freezing medium (90 % foetal bovine serum, 10 % glycerol) and stored as aliquots at -80°C until analysis.

5.18.6. Lymphocyte comet assay

Peripheral blood lymphocytes previously isolated and stored at -80°C, were thawed and screened for single strand DNA breaks (SBs) using the single cell gel electrophoresis (Comet) assay. Endogenous and H₂O₂-induced DNA damage was assessed by pre-treating lymphocytes with either phosphate buffered saline (PBS) or 150 µM H₂O₂ for 5 minutes at 4°C before analysis of DNA SBs as described previously (Singh *et al.*, 1988). An additional modification to this described method was included to enable assessment of oxidised purine bases using the enzyme formamidopyrimidine DNA glycosilase (FPG) as described by Collins *et al* (1993). Briefly, after embedded cells are lysed, slides used to assess oxidative purine damage were washed in FPG buffer (0.02 mmol/L Tris-HCL, 0.4MNaCL, 1 mmol/L EDTA, and 0.5 mg/mL BSA, pH 7.5) for 3x5 minutes at room temperature. Following washing 40µl of FPG (16 U/ml) was applied to each gel and the slides were incubated at 37°C for 45 minutes. Slides were stained with ethidium bromide prior to analysis with Komet 5.0 software (Kinetic Imaging Ltd, Liverpool, UK). Percentage tail DNA was scored for 50 cells per gel and data for each sample given as a mean of duplicate measures for statistical analysis.

5.18.7. Ferric Reducing Ability of Plasma (FRAP) assay

The antioxidant potential of plasma samples was measured using the Ferric Reducing Ability of Plasma (FRAP) assay performed using the automated ILAB 650 Clinical Chemistry Analyser (Instrumentation Laboratory, Warrington, Cheshire, UK). Reagents for this assay were prepared as described previously (Benzie and Strain, 1996). A total of 300 µl freshly prepared FRAP reagent was used to record a reference blank at 593nm following which a total of 10 µl of the sample diluted in 30 µl distilled water was added. Absorbance was

recorded every 17.5 seconds, and the change in absorbance at 593nm after 4 minutes in relation to the change in absorbance at 593nm of a Fe²⁺ standard solution was calculated. Results are expressed as µmol Fe²⁺/L.

5.18.8. C-reactive protein

C-reactive protein (CRP) levels in serum were assessed using the ILAB 650 Clinical Chemistry Analyser using Quantex CRP plus reagent and buffer (BIOKIT, S.A., Barcelona, Spain). Each sample was measured in duplicate and results presented as mean values in mg/L.

5.18.9. Lipid status

Plasma triacylglycerols (GPO-PAP colorimetric end-point assay), HDL cholesterol and total cholesterol (CHOD-PAP colorimetric end-point assays) were measured using the ILab 650 chemistry analyser. LDL cholesterol was calculated using the Friedewald formula (LDL-cholesterol = total cholesterol – HDL-cholesterol – (TAG/2.2)).

5.18.10. Dietary analysis

Dietary intake was assessed in each participant by means of a prospective 4-d semi-quantitative food diary at baseline. Comprehensive verbal and written instructions were given to all participants on the method of recording data, and participants were encouraged not to modify their usual dietary habits. Food portion sizes were estimated by the participant by using household measures and were later quantified by using published food portion size data. The food-composition database Weighted Intake Software package (WISP, version 3; Tinuviel Software, Anglesey, UK) based on McCance and Widdowson's Composition of Foods was used to estimate dietary nutrient, including fibre intake.

5.18.11. Statistical analysis

The statistical software package, SPSS version 20.0 (Chicago, IL, USA) was used for all data analysis. The dataset were checked for normality using Shapiro-Wilk test and transformed, where appropriate, to acquire normality and the homogeneity of variance determined using Levene's test of equality of error variances. Descriptive statistics (means ± SDs) were determined for all variables and differences in baseline characteristics between the 2 groups were analysed using independent t-tests. Analysis of covariance (ANCOVA) (with baseline values as covariates) was used to assess between group differences over time (time x treatment interaction effects) controlling for age, sex, BMI and smoking status.

5.18.12. Results

The consumption of pork from LAM/FUC fed pigs by humans for 4 weeks in this human intervention study was shown to have no effect on oxidative or inflammatory markers as noted with a minimal change in serum FRAP score or CRP, respectively. The consumption of pork from the LAM/FUC fed pigs resulted in a significantly greater reduction in serum

triglycerides compared to those who consumed pork from pigs fed a standard diet (17.2% compared to a 5.5% reduction in the control group; $P=0.039$).

6. SCALE-UP AND DELIVERY OF BIOACTIVES

6.1. Introduction

Extrinsic factors are known to influence the functionality and bioavailability of bioactive compounds. Throughout the food production chain there are many environmental, chemical, biological and processing factors that influence the bioavailability of functional ingredients and other food components. And during consumption, these compounds are further influenced by their passage through the digestive system.

Irrespective of the origin of the functional ingredient, gaining insight to such influences is crucial in the development of functional foods. Whilst the challenge of creating a commercial functional food was outside the scope of the NutraMara consortium, significant effort was directed towards understanding some of these extrinsic factors and developing capabilities to address them. However, the multi-disciplinary approaches of the NutraMara programme provided the climate within which to create an awareness of the broad factors that affect the health value of the functional ingredient and of the options to incorporate these ingredients within food products.

Factors identified as affecting the functionality of ingredients, some of which were investigated within the consortium include the form of the original compound; the nature of the food matrix; interactions between the compound and other ingredients; methods employed during processing and production; storage conditions; absorption within the gut and variations, including genetic variation within the population. The interactions of foods and ingredients outside the body are complex; the range of physicochemical processes, and the metabolism of individual consumers add further to the overall complexity of developing functional foods.

Various factors associated with the production of functional ingredients and their incorporation into foods can alter the appearance and texture of the end product. In addition to being demanding regarding the safety of food products, the discerning consumer is concerned with how foods look and taste. These factors have been found to greatly contribute to the market success of foods. Trained sensory panels are widely used in assessing food products and their contributions inform food product development activities.

6.2. Carriers for functional ingredients

6.2.1. Manufacture of dairy products fortified with seaweed-derived ingredients

In addition to the inherent functionality of some food products, there are foods that are attractive for their potential to act as carriers for added functional ingredients. Dairy

products fit into this category, and have already demonstrated this capacity in products that incorporate PUFAs, probiotic bacteria and plant sterols.

To outline the effects of seaweed extracts on the quality and shelf-life of milk and yoghurt, *Ascophyllum nodosum* (100% water (AN100), 80% ethanol (AN80e)) or *Fucus vesiculosus* (60% ethanol (FV60e)) seaweed extracts were added to milk (0.25% and 0.5% (w/w)). AN80e and FV60e (0.25 and 0.5%) milk had higher ($P < 0.05$) “-a*” and “b*” values. FV60e (0.25%) and phloroglucinol (Phl) (0.5%) milk had lower ($P < 0.05$) lipid oxidation. Milk microbiology was not affected by the addition of seaweed extract. A trained sensory panel compared all the combinations of milk and extracts, finding the control and AN100 milk the most acceptable. Yoghurt is a widely consumed food product and frequently enriched with health promoting functional ingredients. Brown seaweeds (*Ascophyllum nodosum* and *Fucus vesiculosus*) contain a range of bioactive compounds (e.g. antioxidants) with numerous reported health benefits. Seaweed extracts were prepared from *Ascophyllum nodosum* (100% H₂O (AN100), 80% ethanol: 20% H₂O (AN80e)) and *Fucus vesiculosus* (60% ethanol: 40% H₂O (FV60e)) using a solid-liquid extraction technique.

This element of the research programme manufactured yoghurt containing AN100, AN80e and FV60e (0.25% and 0.5%) extracts. Yoghurt quality and shelf-life parameters, stability and bioactivity (pre and post *in vitro* digestion) of seaweed extracts in yoghurt were examined during 28 days storage at 4°C. Greenness (-a*) was lower ($P < 0.05$) in yoghurts containing AN100 (0.25 and 0.5%). Yellowness (b*) was higher ($P < 0.05$) higher in yoghurts enriched with AN80e (0.5%) and FV60e (0.25% and 0.5%). Lipid oxidation was lower ($P < 0.05$) in yoghurts containing AN80e (0.5%) and FV60e (0.5%). Proximate composition, pH, microbiology and whey separation in yoghurt were unaffected by seaweed extract addition. Yoghurt modulus was higher in yoghurt controls compared to extract-enriched yoghurts. Sensory evaluation by the taste panel identified the control and AN100 (0.25% and 0.5%) yoghurts as the most acceptable products.

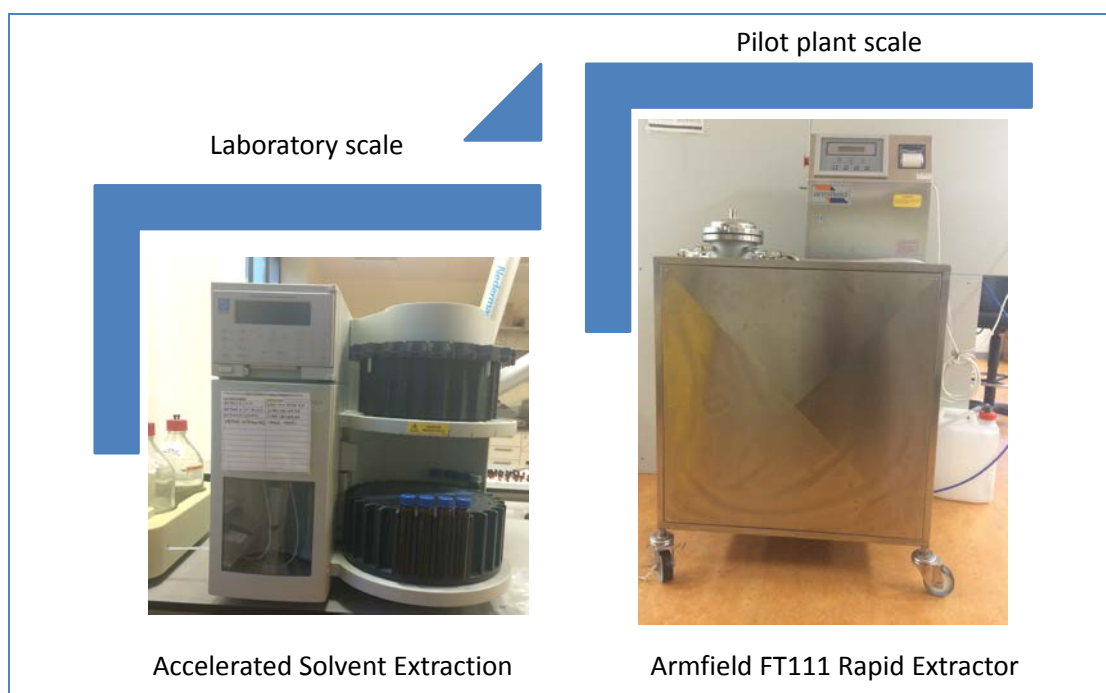
6.3. Moving from laboratory to pilot scale production

6.3.1. Development of commercial process

Research activities carried out at a laboratory scale are important early-stage tools in the technical assessment and scaling of new techniques for the development of commercially viable processes. The translation of batch type processes carried out in controlled conditions in the laboratory, to pilot scale production and further into a continuous commercial operating plant presents new challenges for those involved in the development of functional ingredients.

Typical of the processing challenges within the NutraMara programme was the assessment of new technologies and the development of protocols to enhance productivity in processing marine materials. One such novel process is Accelerated Solvent Extraction (ASE®) or pressurised liquid extraction (PLE); an automated extraction technique that uses elevated temperatures and pressures to achieve extractions in very short periods of time. Many of the organic solvents used in traditional extractions boil at relatively low temperatures and this is a limitation to methods such as Soxhlet. If sufficient pressure is exerted on the solvents during the extractions, temperatures above the boiling point can be used. Using ASE® all of the advantages of working at elevated temperatures can be realised even with solvents with relatively low boiling points. Elevated pressure also enhances the extraction process and allows the extraction to proceed at a faster pace (Pablo *et al.*, 2009). The difference between laboratory scale and pilot scale processes are clear in Figure 37 below.

Figure 37 Laboratory and pilot scale extraction system



ASE was used in the NutraMara programme to generate extracts with potential for use as health beneficial ingredients. The Armfield Rapid Extractor FT111 5L (Figure 37) was used to up-scale positive extraction results achieved using ASE. Lipid and phlorotannin containing extracts were generated using the protocols developed as part of the NutraMara programme and outlined in Table 27. Examples of the use of ASE in extracting compounds

from marine materials using the protocols developed within the research programme are described below.

Table 27 Extraction protocols developed using ASE

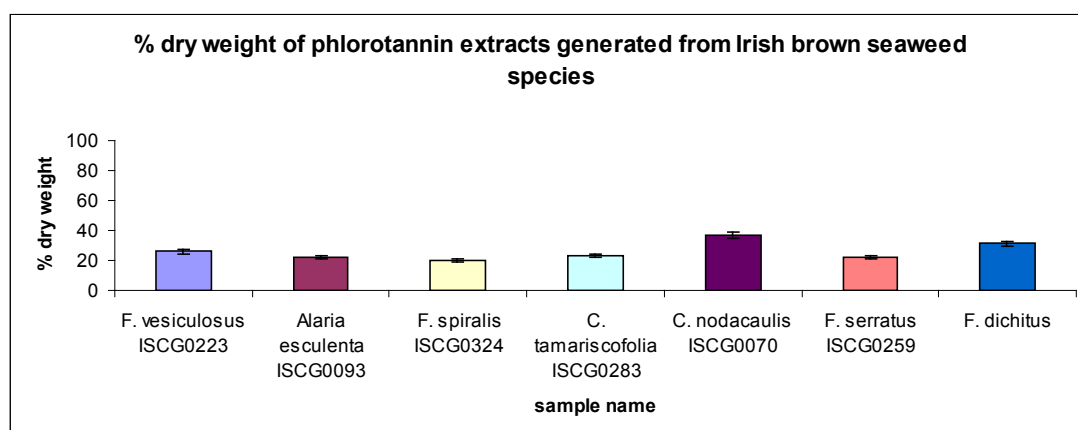
C000 ASE	A000	M8	MH	ASE® Water (100 %)
C001 ASE	A001	M9	MH	ASE® EtOH:H2O (80:20)
C002 ASE	A002	M10	MH	ASE® EtOH:H2O (60:40)
C003 ASE	A003	M11	MH	ASE® Acetone (100%)
C004 ASE	A004	M12	MH	ASE® Hexane (100 %)
C002 ASEMeOH	A005	M13	MH	ASE® MeOH:H2O (60:40%)
C002 ASEMeOH	A006	M14	MH	ASE® MeOH:H2O (70:30)
CH001 chitin/chitosan	CH001	M15	MH	chitin/chitosan extraction method
CH002 chitin/chitosan	CH002	M16	MH	chitin /chitosan extraction method
CH003 chitin/chitosan	CH003	M17	MH	chitosan extraction using enzymes
H1 - Fermentation with Viscozyme	H001	M18	MH	Fermentation of seaweed with viscozyme
P1 - protein extraction method	P001	M19	MH	Protein extraction of myofibrillar proteins from muscle tissue
P2 - protein extraction method	P002	M20	MH	Protein extraction method for seaweed proteins (i.e. <i>P. palmata</i>)
C006 ASE	A006	M21	MH	ASE®70% methanol: 30% water
Rapid extraction C001	A007	M22	MH	Rapid extraction with EtOH:H2O (80:20)
Rapid extraction C006	A008	M23	MH	Rapid extraction with MeOH:H2O (70:30)
Rapid extraction	A009	M24	MH	Rapid extraction with water (100 %)
ASE extraction of lipids	A012	M25	MH	ASE® methanol:chloroform (2:1)
C000 ASE	JA000	M26	JV	ASE® MeOH:H2O (70:30)
C001 ASE	JA001	M27	JV	ASE® EtAcO
C002 ASE	JA002	M28	JV	ASE® H2O
ASE extraction of lipids	A012	M29	JV	ASE® methanol:chloroform (2:1)

6.3.2. ASE Extraction of phlorotannins

Extraction of phlorotannins was carried out by Accelerated Solvent Extraction (ASE®) in which a sample (2.5 g) of freeze-dried algal mass was mixed with diatomaceous earth and 30 g of silica (Merck grade, 60 Å, Sigma Aldrich, St Louis, USA) and loaded into 33 ml sample cells. The automated extraction method used 70 % acetone in water and a pressure of 103.5 bar. The extraction time consisted of 3 cycles of 5 minutes, heat time 5 min, flush volume 50 %, purge time 60 s, static cycles 4, solvent Acetone:water 70:30 (v/v).

The recovered fractions were subsequently centrifuged at 3000 X g for 10 minutes to remove residual solids (SIGMA 2-16KL, Sigma Zentrifugen, Ostende am Hartz, Germany). Aliquots of supernatants from each extract were dried under nitrogen (20 psi) using a TurboVap (Caliper LifeSciences, Runcorn, UK) and subsequently freeze dried for 24 hrs to eliminate residual water. Yields of phlorotannins on a dry weight basis are shown in Figure 38. *Cytoseria nodacaulis* and *Fucus vesiculosus* gave the greatest percentage yield on a dry weight basis.

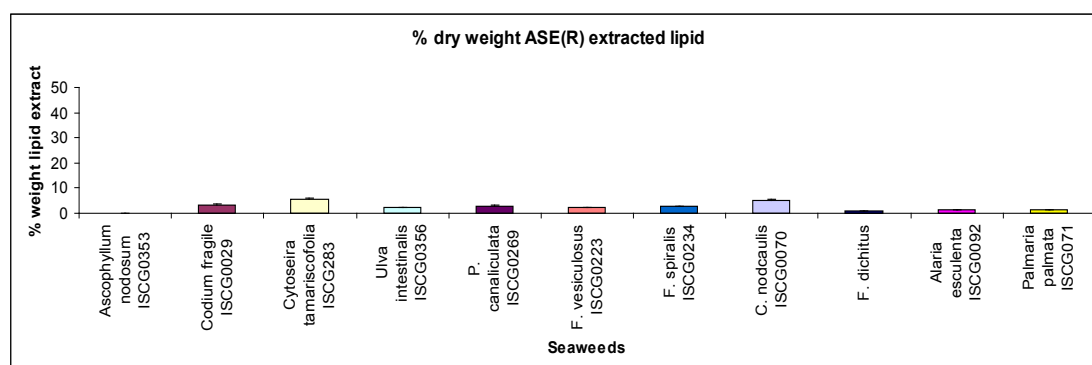
Figure 38 Percentage dry weight crude phlorotannin extracted generated from seaweed species using ASE® method II (70:30 acetone:water v:v)



6.3.3. ASE® extraction of macro-algal lipids

Accelerated Solvent Extraction (ASE®) was used to extract lipids from samples of macro-algae. Oil was extracted from each seaweed species in triplicate using an automated Dionex 200 accelerated solvent extraction system. In this process, 2.5 g of freeze-dried algal mass was mixed with diatomaceous earth and 30 g of silica before loading into 33 ml sample cells. The extraction conditions were: 5 min preheat, 103.5 bar pressure, 120°C, heat time 5 min, flush volume 50 %, purge time 60 s, static cycles 4, solvent chloroform:methanol 2:1 (v/v). Yields of lipids are shown in Figure 39 below.

Figure 39 Percentage dry weight lipid extracted/generated from seaweed species using ASE® method II (70:30 acetone:water v:v)



6.3.4. ASE extracts from *Fucus serratus* and *Ascophyllum nodosum*

Extracts from samples of each seaweed species were generated in triplicate using an automated Dionex 200 accelerated solvent extraction system. In this method, 2.5 g of freeze-dried algal mass was mixed with diatomaceous earth and 30 g of silica before loading into 33 ml sample cells. The extraction conditions were: 5 min preheat, 103.5 bar pressure,

120°C, heat time 5 min, flush volume 50 %, purge time 60 s, static cycles 4, solvent water 100%.

6.4. Large scale extractions using the Armfield Rapid extractor (5 Litre capacity)

To demonstrate the potential of using ASE methods at a larger scale, an Armfield FT111 rapid extractor was used to extract constituents from relatively large samples. Three methods were developed to up-scale the extraction of promising extracts with identified bioactivities from ASE phlorotannin seaweed derived extracts - from *Pelvetia canaliculata* and a betaine rich-extract from *Ulva intestinalis*. A phlorotannin rich extract was generated from 1 kg of *Pelvetia canaliculata* using the Armfield FT111 rapid extractor. The solvents used in this process were 70 % acetone in water and the extraction programme was: temperature 38.7°C, TPI: 5 minutes, TP0: 6 minutes, cycles: 3 TTOT: 33 minutes.

Large-scale extraction using the Armfield rapid extractor allows for the fast, efficient solid-liquid extraction of active constituents from seaweeds and microalgae. The use of high pressure and a combination of both static and dynamic extraction phases allow a rapid extraction of the bioactive materials with minimum degradation to the quality of bioactive ingredient, while the solvent is passed through the material providing a forced percolation and agitation. However, scale up of extraction protocols involving highly flammable solvents predominantly those involving batch processes as in the case of ACE remain problematic. The application of high temperature and pressure during large-scale extraction involves the excessive use of organic solvents. The disposal of large quantities of organic solvent poses environmental hazards, in addition to significant health and safety risks. Alternative, more benign methods remain to be developed.

6.5. Impact of processing on functional ingredients

In general, extraction techniques can have negative effect on functionality of bioactive ingredients. The optimisation of extraction conditions is critical to minimise the impact of the process on the bioactivity of functional ingredients. The use of optimised conditions for different variables known to influence extraction performance could significantly enhance the recovery or extraction yield of target compounds. Pilot investigations of processing conditions on the stability of marine extracts were completed within the NutraMara work programme.

6.5.1. The stability of seaweed extracts under storage conditions

Seaweed extracts (ASE®) prepared from *Ascophyllum nodosum*, *Fucus vesiculosus* and *Fucus serratus* using 100% H₂O were dissolved in 0.1 M potassium phthalate (pH 4, pH 5.5) and 0.1

M potassium dihydrogen phosphate (pH 7.0) buffers at a concentration of 2 mg/ml. Incubates were stored in vials at three storage temperatures (-20°C, 4°C, 25°C) for a duration of 12 weeks. Antioxidant activity (stability) was assessed using *in vitro* antioxidant assays (total phenol content (TPC) and DPPH free radical scavenging activity). All extracts displayed similar levels of *in vitro* antioxidant activity under the experimental conditions employed indicating stability of the extracts against pH and storage temperature. The antioxidant activity of all seaweed extracts was assessed in 25% pork muscle homogenates at concentrations of 1 and 5 mg/ml. Antioxidant activity was assessed using the 2-thiobarbituric acid (TBA) assay after 1 and 4 hours storage at 4°C. Antioxidant activity increased with seaweed extract concentration and antioxidant potency followed the order: *Fucus vesiculosus* > *Fucus serratus* > *Ascophyllum nodosum*. These results demonstrate the potential for using seaweed extracts as functional antioxidant ingredients in pork meat products.

6.5.2. The effect of dietary seaweed polysaccharides on pork quality

A seaweed extract containing laminarin (L) and fucoidan (F) (L/F) was manufactured from brown seaweed (*Laminaria digitata*) in spray-dried (L/F-SD) and wet (L/F-WWS) forms. The effect of supplementation of pig diets with L/F-SD and L/F-WWS (L, 500 mg/kg feed; F, 420 mg/kg feed) for 21 days pre-slaughter, on quality indices of fresh (*longissimus thoracis et lumborum* (LTL)) steaks was examined. Susceptibility of porcine liver, heart, kidney and lung tissue homogenates to iron-induced (1 mM FeSO₄) lipid oxidation was also investigated. Dietary supplementation with L/F did not increase plasma total antioxidant status (TAS). In LTL steaks stored in modified atmosphere packs (80% O₂: 20% CO₂) (MAP) for up to 15 days at 4°C, muscle pH, surface colour (CIE 'L*' lightness, 'a*' redness and 'b*' yellowness values) and microbiology (psychrotrophic and mesophilic counts, log CFU/g pork) were unaffected by dietary L/F. In general, levels of lipid oxidation (TBARS, mg MDA (malondialdehyde)/kg pork) followed the order: C > L/F-SD > L/F-WWS. A statistically significant reduction in lipid oxidation (P < 0.05) was observed in LTL steaks from 75% of pigs (n = 6) fed L/F-WWS compared to controls. Iron-induced lipid oxidation increased in liver, heart, kidney and lung tissue homogenates over the 24 hr storage period and dietary L/F-WWS reduced lipid oxidation to the greatest extent in liver tissue homogenates. These results demonstrate the potential for incorporating marine-derived bioactive antioxidant components into muscle foods via the animal's diet.

A spray-dried seaweed extract containing laminarin (L, 9.3%) and fucoidan (F, 7.8%) (L/F extract) from brown seaweed (*Laminaria digitata*) was added directly to minced pork (*longissimus thoracis et lumborum*) (LTL) at levels of 0.01%, 0.1% and 0.5% (w/w). Fresh and cooked minced pork patties were stored in modified atmosphere packs containing 80% O₂: 20% CO₂ and 70% N₂:30% CO₂, respectively, for up to 14 days at 4°C. The L/F extract

reduced the surface redness ('a*' values) of fresh patties as a function of concentration. The L/F extract (0.5%) exerted the greatest lipid pro-oxidant activity in fresh patties. The L/F extract (0.5%) significantly decreased ($P < 0.05$) lipid oxidation in cooked patties. The L/F extract had no effect on the microbiological status, pH, water holding capacity (WHC) or cook loss of patties. Pork patties containing 0.01% L/F were preferred by sensory evaluation panellists.

To develop further insights into the incorporation of seaweed at various levels into meat products during animal rearing, feeding trials of the polysaccharides laminarin and fucoidan were carried out. A taste panel was employed to assess the visual characteristics of cooked pork. The effect of level (450 or 900 mg laminarin (L) and fucoidan (F) /kg feed) and duration (3 or 6 wks) of feeding a seaweed (*Laminaria digitata*) extract containing L/F on the quality of pork (*longissimus thoracis et lumborum* (LTL)) stored in modified atmosphere packs and on organ lipid stability was examined. Mechanisms of L/F antioxidant activity in LTL were evaluated. Plasma total antioxidant status, LTL pH, colour, microbiology and 'eating quality' sensory analysis were unaffected by dietary L/F. 'Visual' sensory descriptors (purchasing appeal and overall visual acceptability) were enhanced ($P < 0.05$) in L/F450 - 3 LTL. Lipid oxidation was lower ($P < 0.05$) in L/F450 - 3 and L/F900 - 3 LTL and reduced in L/F900 - 6 kidney homogenates. In cooked minced pork, lipid oxidation was not reduced by dietary L/F. Saturated fatty acids were lower ($P < 0.05$) in L/F900 - 6 LTL. Results indicated L/F in pig diets for 3 weeks enhanced pork quality.

6.5.3. The effect of seaweed based fish feed on farmed salmon quality

The availability of sustainable feedstock is amongst the various challenges in moving to sustainable aquaculture. Typically, fish feed relies on the use of manufactured fishmeal, some processes and materials used in its production can have negative environmental impacts and be demanding of energy inputs. Developing fish feeds that incorporate seaweeds offers a potential alternative feedstock.

The effect of using two seaweeds *Ulva rigida* (UR) and *Palmaria palmata* (PP) in farmed Atlantic salmon (*Salmo salar*) diets was investigated during the NutraMara research programme. The effect of supplementation of salmon diets with *Ulva rigida* (0, 5, 10 and 15% UR) or synthetic astaxanthin (positive control, PC) for 19 weeks pre-slaughter on quality indices of fresh (raw) salmon fillets was examined. Susceptibility of salmon fillets/homogenates to oxidative stress conditions (cooking/iron-ascorbate induced oxidation) was also measured. In salmon fillets stored in modified atmosphere packs (60% N₂:40% CO₂) (MAP) for up to 15 days at 4°C, *Ulva rigida* increased surface 'a*' greenness and 'b*' yellowness values in a dose-dependent manner resulting in a final yellow/orange flesh colour. Proximate composition, pH and lipid oxidation (fresh, cooked and fillet

homogenates) were unaffected by dietary addition of *Ulva rigida*. On day 12, 5% UR psychrotrophic total viable cell counts were significantly lower than the controls. Salmon fed 5% UR did not influence 'eating quality' sensory descriptors (texture, odour, oxidation flavour and overall acceptability) in cooked salmon fillets (180°C for 12 min) compared to 0% UR. Results indicated *Ulva rigida* may prove to be a functional ingredient in salmon feed to enhance salmon fillet quality.

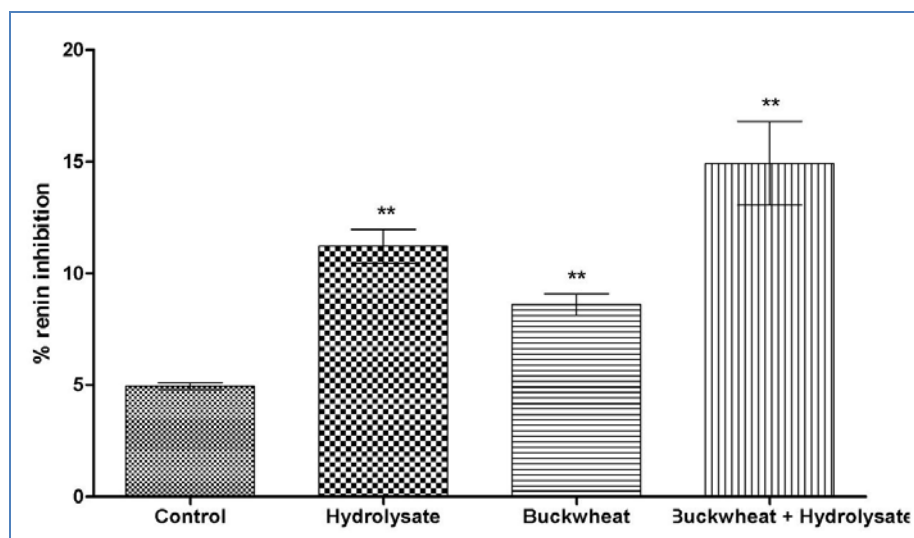
The effect of salmon diet supplementation with *Palmaria palmata* (0, 5, 10 and 15%) or synthetic astaxanthin (positive control, PC) for 16 weeks pre-slaughter on quality indices of fresh salmon fillets was examined. Susceptibility of salmon fillets/homogenates to oxidative stress conditions was also measured. In salmon fillets stored in modified atmosphere packs (60% N₂ : 40% CO₂) for up to 15 days at 4°C, *Palmaria palmata* increased surface 'a*' greenness and 'b*' yellowness values in a dose-dependent manner resulting in a final yellow/orange flesh colour. In general, the dietary addition of *Palmaria palmata* had no effect on pH, lipid oxidation (fresh, cooked and fillet homogenates) and microbiological status. 'Eating quality' sensory descriptors (texture, odour and oxidation flavour) in cooked salmon fillets were not influenced by dietary *Palmaria palmata*. Salmon fed 5% PP increased overall acceptability compared to PC and 0% PP. Dietary *Palmaria palmata* was ineffective at providing red coloration in salmon fillets however pigment deposition enhanced fillets with a yellow/orange colour. Carotenoids from *Palmaria palmata* may prove to be a natural pigment alternative to canthaxanthin in salmon feeds.

6.6. Bioavailability of compounds

6.6.1. Bioactivity of seaweed-enriched bread

The feasibility of introducing bioactive hydrolysates and peptides isolated from *Palmaria palmata* into a baked product was explored along with the potential bioavailability of seaweed derived hydrolysed proteins and peptides. Bread formulations were tested with the incorporation of 4% of the *Palmaria palmata* hydrolysate in a blend of 70% wheat: 30% buckwheat. Breads containing 4% *Palmaria palmata* hydrolysate demonstrated significantly higher renin inhibitory activity than that of the control bread ($P < 0.01$). The *Palmaria palmata* papain protein hydrolysate bread inhibited renin by 11.21% (± 0.77) when tested at a concentration of 1mg/ml compared to the positive control, which was higher than the buckwheat bread formulation that had a renin inhibitory value of 9.546% (± 0.48). Combining the buckwheat and seaweed protein hydrolysate in bread formulation increased the renin inhibitory activity to the bread to 14.92% (± 1.88) as shown below in Figure 40.

Figure 40 Renin inhibitory assay of the control, control + hydrolysate, buckwheat and buckwheat + hydrolysate breads. Values are mean \pm S.E.M. (n=3). *P<0.05, **P<0.01 and *P<0.001 compared with control**



6.6.2. Bioactivity of seaweed-enriched milk and yoghurt

Ascophyllum nodosum (100% water (AN100)), 80% ethanol (AN80e) or *Fucus vesiculosus* (60% ethanol (FV60e) seaweed extracts were added to milk and yoghurt (0.25% and 0.5% (w/w)). The antioxidant activity of the seaweed extracts was measured on the basis of the scavenging activity of the stable 1, 1- diphenyl 2-picrylhydrazyl (DPPH) and found to be stable in milk.

Milk and digestates exhibited DPPH and FICA activities, and did not affect cellular antioxidant activity or protect against DNA damage. The antioxidant activity (DPPH) of seaweed extracts in yoghurt was stable during storage. *In vitro* antioxidant activity was higher ($P < 0.05$) in FV60e (0.5%) yoghurt pre- (DPPH and FICA assays) and post *in vitro* digestion (DPPH assay). Ferrous ion chelating activity (FICA assay) was higher ($P < 0.05$) in all yoghurts post-digestion. Yoghurt and yoghurt digestates did not alter the antioxidant status (catalase (CAT), superoxide dismutases (SOD) and glutathione (GSH) assays) or protect against H₂O₂-induced DNA damage in Caco-2 cells.

6.6.3. Bioactivity of seaweed extracts in pork

The antioxidative potential of laminarin (L), fucoidan (F) and an L/F seaweed extract was measured using the DPPH free radical scavenging assay in 25% pork (*longissimus thoracis et lumborum* (LTL)) homogenates (TBARS) (3 and 6 mg/mL) and in horse heart oxymyoglobin (OxyMb) (0.1 and 1 mg/mL). The DPPH activity of fresh and cooked minced pork LTL containing L (100 mg/g; L100), F100 and L/F100,300, and bioaccessibility post *in vitro* digestion (L/F300), was assessed. Theoretical cellular uptake of antioxidant compounds was measured in a transwell Caco-2 cell model. Laminarin displayed no activity and fucoidan reduced lipid oxidation but catalysed OxyMb oxidation. Fucoidan activity was lowered by

cooking while the L/F extract displayed moderate thermal stability. A decrease in DPPH antioxidant activity of 44.15% and 36.63%, after 4 and 20 h respectively, indicated theoretical uptake of L/F antioxidant compounds. Results highlight the potential use of seaweed extracts as functional ingredients in pork.

6.7. Processing methods for the recovery of bioactives from seaweeds

6.7.1. Introduction

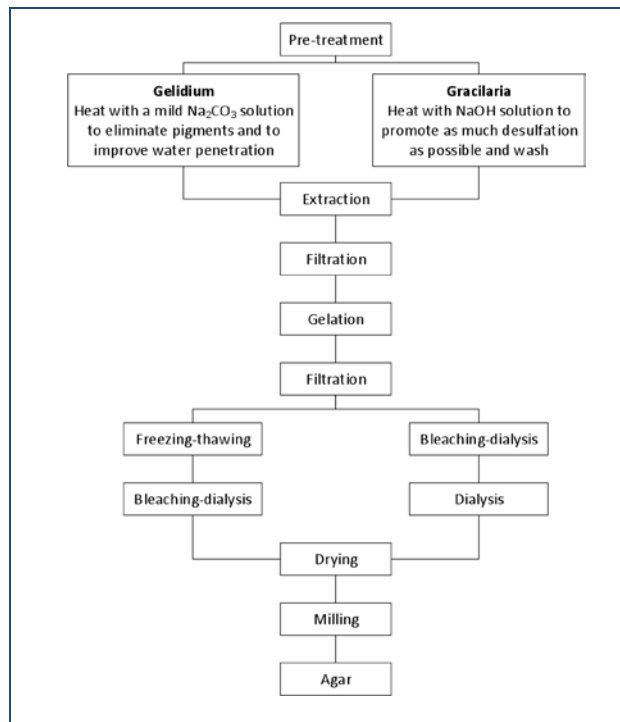
Irish seaweeds were the source of the majority of bioactives investigated in the NutraMara work programme. Bioactive compounds recovered from this source included polysaccharides, polyphenols, lipids, carotenoids, and proteins. The research involved only the use of seaweeds collected from wild sources, and processing and extraction methods varied according to the species under investigation and compounds of interest.

This section provides details of some of the commercial scale extraction methods used in the production of commercially available compounds. Process flow diagrams illustrate the number of processing steps and the complexity of typical extraction methods. Prior to extraction, seaweeds typically undergo a pre-treatment designed to remove any sand, salt or other debris. The economic feasibility of extraction methods is linked to factors such as desired throughput, yield, purity and end use of the extract.

6.7.2. Process to extract agar

Agar is a polysaccharide widely used as a gelling agent and a medium for culture of bacteria. Two genera account for the majority of world-wide agar production – *Gelidium* spp. and *Gracilaria* spp. Figure 41 illustrates the typical extraction process.

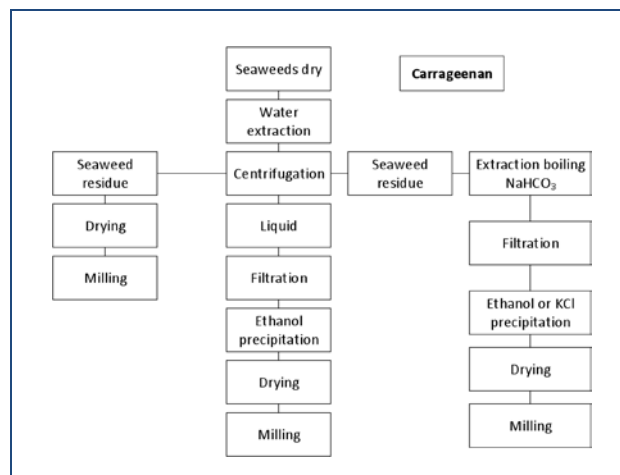
Figure 41 Agar extraction process



6.7.3. Process to extract carrageenan

This seaweed derived polysaccharide is widely used by the food industry in processed food products such as meats, cheese, confectionary, iced creams, etc. as a thickening and emulsifying agent. *Chondrus crispus* was once the main source of carrageenan, however, most of the world's supply now comes from other red seaweed species including *Kappaphycus alvarezii*, *Eucheuma denticulatum*, *Gigartina skottsbergii*, *Sarcothalia crispata*. Figure 42 shows a typical process flow chart for carrageenan production.

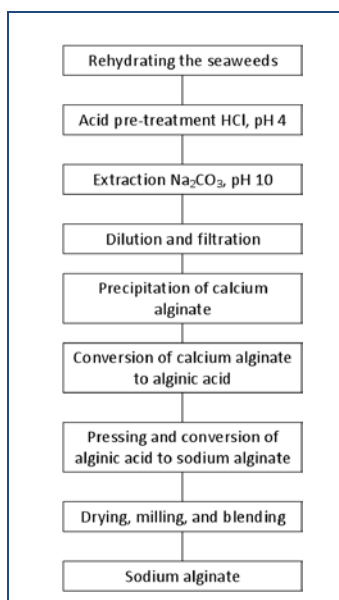
Figure 42 Carrageenan extraction process



6.7.4. Process to extract alginate

Most species of brown seaweeds contain alginates. This polysaccharide is used as an emulsifier or a thickening agent in various food and industrial products. In Ireland, the wild harvest of *Ascophyllum nodosum* is used as a source of alginates. Figure 43 shows a typical process for alginate production.

Figure 43 Alginate extraction process



7. NUTRAMARA TECHNICAL SUPPORT

7.1. Introduction

Results from the 5-year research programme support national policy and commercial goals to maximise the sustainable use of marine biomaterials in the production of functional foods and functional ingredients. The NutraMara research programme has to date generated 80 peer-reviewed publications across a range of marine functional foods research related areas. These publications provide open access to knowledge generated within the NutraMara programme. Additionally, partners in the NutraMara consortium have developed considerable scientific and technological know-how, which can be accessed directly from individual research institutions. Much of this knowledge is proprietary and is only accessible via a technology transfer agreement.

A technology portfolio developed as part of the NutraMara outreach and technology transfer activity provides “technology-updates” and describes the expertise and services available from members of the NutraMara consortium.

7.2. Technology updates

NutraMara published 19 technology updates to coincide with the 2015 NutraMara Conference. These wide-ranging insights are relevant to interested parties in industry, academia, government departments, state agencies and funding agencies. They provide a summary of results and describe opportunities from the research programme. The title of each update is given in Table 28 below and the full update is included in the Appendix 3.

Table 28 Technology updates available from NutraMara

Update	Institution
Seaweed Inclusion in Fish Food	NUIG
Extraction, Purification and Characterisation of Biofunctional Peptides from Marine Processing Co-Products	UL
Marine Sourced Peptides for Glycemic Management	UL
Mining, Extraction and Purification of Proteins and Peptides from Macroalgae	UL
Extraction of Nitrogenous Compounds from Macroalgae	UL
Irish Seaweed Polysaccharides for Gut Health	Teagasc
Chitosan Generation and Characterisation from Shell	Teagasc
Novel Proteins and Peptides from Seaweeds	Teagasc
Seaweed Derived Glycine Betaine and DMSP	Teagasc
The Anti-inflammatory Effect of Algal Lipid Extracts	Teagasc
The α -amylase and α -glucosidase Inhibitory Effects of Irish Seaweed Extracts	Teagasc
The Potential of Yoghurt as a Functional Food Matrix for an Omega-3 PUFA-rich Algae Extract	Teagasc

Pork Meat Enhanced with Seaweed Polysaccharides	UCC
Seaweed Polysaccharides as Functional Ingredients in Pork Meat: Mechanistic Studies	UCC
Brown Seaweed Extracts as Potential Functional Ingredients in Dairy Products	UCC
Bioavailability and Bioactivity of Phenolic Enriched Extracts from Brown Seaweed	UU
Anticancer Properties of Marine Bio-Actives	UU
The Incorporation of Seaweed into Commonly Consumed Foods – Bread	UU
The Incorporation of Seaweed into Commonly Consumed Foods – Pork	UU

7.3. Service offers from partner institutions

Service offers from NutraMara partner institutions describe specific areas where a research group is available to undertake work related to the use of marine bioresources in foods and as functional and other ingredients. In many cases, the available offers are relevant to non-food applications. Typically the services include testing and analysis of marine materials, the characterisation of compounds and the assessment of marine bioresources. Full details of the offers are available in the Appendix 4 to this report. Table 29 provides summary details of the service offer.

Table 29 NutraMara service offers relevant to marine bioresources

Description	Institution	Contact
Algal Biochemical/Bioactive analysis	NUIG	Dagmar Stengel
Polysaccharide extraction and characterisation	NUIG	Zoe Popper
Seaweed biomass resource assessment	NUIG	Dagmar Stengel
Biofunctional characterisation of peptides	UL	Dick FitzGerald
Extraction, quantification and characterisation of peptides	UL	Dick FitzGerald
Generation and enrichment of peptides	UL	Dick FitzGerald
Identification of peptides	UL	Dick FitzGerald
Physicochemical and techno-functional characterisation of peptides	UL	Dick FitzGerald
Food packaging assessment and testing	UCC	Joe Kerry
Chemical and shelf-life testing of muscle foods	UCC	Joe Kerry
Muscle foods processing	UCC	Joe Kerry

8. IMPACT OF THE NUTRAMARA PROGRAMME

8.1. Introduction

It is now widely recognised that continued growth and competitiveness of the Irish agri-food and fisheries industry will play a pivotal role in Ireland's economic recovery. Food Harvest 2020 and more recently, FoodWise2025 each identified key industry, consumer and global trends and opportunities for dynamic growth in the food sector. In highlighting opportunities for Ireland's food and related sectors to respond to global challenges and the emergence of new markets, national policy – including science and technology policy recognised the need for a national response. Targeted research and development, partnerships between industry and the scientific community and the development of an innovative, value added value products are common themes stressed by national policy.

NutraMara's focused workplan was designed to underpin and contribute to these issues. In common with the call from within Food Research Ireland, NutraMara created new knowledge, which supports the goals for “smart, green, growth”. Central to the NutraMara mission was its support for science-based innovation in the marine and food sectors. Accompanying the research activity within the NutraMara programme was a robust, dynamic outreach activity that was focused on linking industry with commercially relevant scientific outputs.

NutraMara was positioned to interact with ongoing related food and marine research activity; offering scope to develop research synergies by creating new linkages. Such an outlook was responsible for bringing new expertise into research proposals, building new capacity and was behind the expansion of research activity into new priority areas. The consortium was well placed to address marine foods related research areas in sustainable foods production and processing, and foods for health, as defined by the recently published national Strategic Research and Innovation Agenda.

8.2. Contribution to meeting the national research agenda

From its inception, and having an origin within *Sea Change - Ireland's Marine Knowledge, Research and Innovation Strategy*: NutraMara was relevant to national and European policy and was of strategic importance to Ireland, particularly the plan to maximise the economic potential of sustainably exploiting our marine resources. On a broader front, NutraMara was contributing to Ireland's efforts to support the achievement of Europe's “grand challenges of ensuring food security; the sustainable management of natural resources; the creation of jobs and maintaining competitiveness; and investing in knowledge, innovation and skills.

Experiences and outputs from NutraMara are reflected in successive national food research plans. Contributions from NutraMara highlighted opportunity areas described within Ireland's first food research strategy – Food Research Ireland. Food Harvest 2020, stressed the importance of further developing marine functional foods research and building Ireland's marine biotechnology related research capability.

Principal Investigators and others associated with NutraMara were involved in Ireland's research prioritisation exercise; drawing attention to the capabilities of NutraMara to support food production and processing and creating an awareness of Ireland's new capacity to engage in related research. New marine functional foods research initiated within NutraMara complemented Ireland's demonstrated ability in dairy based functional ingredients. Indeed scientists with international reputations in dairy functional foods research developed new expertise to engage in marine related research through an involvement in NutraMara. Together, these areas – dairy and marine functional foods and ingredients were identified as the foundation of an entirely new priority research area; Foods for Health by the Research Prioritisation Exercise.

Just as was the case in the development of *Food Research Ireland*, experiences from NutraMara contributed to the development of Ireland's most recent food research strategy - *Sustainable Healthy Agri-Food Research Plan (SHARP)*, A Strategic Research and Innovation Agenda for the “Sustainable Food Production and Processing” and Food for Health priority areas of the National Research Prioritisation Exercise.

FoodWise 2025 recognises NutraMara as one of a number of complementary centres of research excellence which have roles in supporting the development of Ireland's food sector, particularly in respect of contribution to the Foods for Health opportunity area. Ireland's emerging reputation in marine biotechnology research, largely achieved by the efforts of NutraMara and its sister programme the Beaufort Biodiscovery Project, is identified by FoodWise2025 as an opportunity to drive home Ireland's “*strategic advantage in the marine biotechnology field*”.

NutraMara also has a role in the development of a SMART NAUTRIENTS research programme referred to in FoodWise2025 that will seek to explore the potential of marine species of fish, shellfish and seaweed as possible high value sources of pharmaceutical, cosmetic products. The importance and market potential of this emerging area has been recognised by the EU and domestically under the National Research Prioritisation Exercise and the integrated marine plan “*Harnessing our Ocean Wealth*”.

The Marine Institute is currently developing a strategic action plan and roadmap for Marine Biotechnology. This initiative, informed by contributions from industry and the research community, includes major contributions from a cohort of Principal Investigators from

within the NutraMara consortia possessing specific expertise in marine biological resources research.

8.3. Capacity building

One of the major objectives and a basic principle of the NutraMara research programme was to create new research capacity. Within the research programme and across all partner institutions there is an ongoing development of core scientific leadership and a focus on developing young researchers. Creating this additional research capacity has enabled individual researchers and institutions active within the NutraMara programme, to be responsive to new research opportunities in Ireland and in European programmes. Ireland's marine foods and ingredients research capabilities revolve around the NutraMara core group of PIs comprising food and marine scientists. New expertise at the level of senior researcher officer was recruited into the NutraMara programme; some of whom brought international marine research expertise, whilst others brought food related experience. Since the start of NutraMara in 2008, 21 post-doctoral researchers have worked in support of the programme. A further 18 PhD students have been trained as a result of working in areas of research that underpins and contributes to the development of marine functional foods. Researchers funded to work within the NutraMara programme are listed below.

8.3.1. Post-doctoral researchers/research officers

NutraMara presented unique opportunities to attract experienced post-doctoral researchers, from food, marine, chemical and biological related areas to work within a focused work programme. Irish and international scientists were recruited to NutraMara; bringing specific expertise which supported overall research objectives and providing further support to the early stage researchers as they embarked on their post-graduate studies. These researchers significantly enhanced both the research capacity and capabilities of NutraMara. The researchers recruited into the NutraMara programme are listed below in Table 30.

Table 30 Post-doctoral and research officers associated with NutraMara

Name	Host institution	Duties	PI
Dr Anna Soler Vila	NUI Galway	Sampling, sample distribution and database management	Prof Mark Johnson
Dr Freddy Guiheneuf	NUI Galway	Production and optimisation of compounds of food value from microalgal species	Dr Dagmar Stengel
Dr Stefan Kraan	NUI Galway	Sampling, sample distribution and database management	Prof Michael Guiry
Dr Ulrike Grienke	NUI Galway	Natural products chemistry and structural elucidation of bioactive compounds	Prof Deniz Tasdemir

Dr Ayoa Fernandez	NUI Galway	Natural products chemistry and structural elucidation of bioactive compounds	Prof Deniz Tasdemir
Dr Thomas Smyth	Teagasc	Large scale extractions, fractionation/enrichment of extracts, purification and structural elucidation of bioactive compounds	Dr Nigel Brunton
Dr Fiona Manning	Teagasc	Programme manager	Mr Declan Troy
Dr Juan Valverde	Teagasc	Extraction, purification and characterisation of compounds from marine species	Dr Nigel Brunton
Dr Carlos Alvarez	Teagasc	Extraction, purification and characterisation of proteins from boarfish	Dr Brijesh Tiwari
Dr Owen Kenny	Teagasc	Extraction, purification and characterisation of compounds from marine species	Dr Brijesh Tiwari
Dr Prabhesh Kumar	Teagasc	Extraction, purification and characterisation of compounds from marine species	Dr Brijesh Tiwari
Dr Pádraigín Harnedy	UL	Extraction, purification and characterisation of nitrogenous components from macroalgae	Prof Dick FitzGerald
Dr Adriana CunhaNeves	UL	Extraction, purification and characterisation of biofunctional peptides from marine processing co-products	Prof Dick FitzGerald
Dr Sinead Lordan	Teagasc	Assessment of the pre-biotic potential and anti-diabetic potential of marine extracts	Prof Paul Ross
*Dr Snehel Gite	Teagasc	Assessment of the mental health benefit of marine extracts	Dr Catherine Stanton
*Dr Supriya Yadav	Teagasc	Assessment of the prebiotic potential of marine extracts	Dr Catherine Stanton
Dr Philip Allsopp	UU	Bioactive screening and profiling of marine bioactives using breast cancer cell models	Dr Emeir McSorley
Dr Emma Brown	UU	Bioactive screening and profiling of marine bioactives using colon cancer cell models, Human Intervention Studies	Dr Emeir McSorley
Mr James Burnside	UU	Creating and updating website	Dr Emeir McSorley
Dr Olaf Sonnetel	UU	Bioactive screening and profiling of marine bioactives using breast cancer cell models	Dr Emeir McSorley
Dr Michael O'Grady	UCC	Model foods and carriers for marine bioactives	Dr Joe Kerry
Dr Bojlul Bahar	UCD	Bioactive screening and profiling of marine bioactives	Prof John O'Doherty
Dr Paul McAlpine	UCD	Bioactive screening and profiling of marine bioactives	Prof John O'Doherty
Dr Mary McDonnell	UCD	Bioactive screening and profiling of marine bioactives	Prof John O'Doherty

**Staff not directly funded by NutraMara research programme, but funded from other sources.*

8.3.2. PhD students

A core objective of the NutraMara programme was to create additional research capacity by recruiting high-potential graduates to engage in research leading to the award of a PhD. Nineteen graduates, including some from overseas, were attracted by the challenge of working within a new, rapidly developing research area, and the chance to work within a multi-disciplinary project. Table 31 lists the diverse topics these PhD students worked on and the year in which the award was made.

Table 31 PhD students associated with NutraMara

Name	Host	Thesis title	Start	Award
Ms Ann Walsh	UCD	The use of marine derived bioactives in pig diets	2008	2012
Mr Anthony O'Sullivan	UCC	Cellular and <i>in vitro</i> models to assess antioxidant activities of seaweed extracts and the potential use of the extracts as ingredients	2008	2013
Mr Alex Wan	NUI Galway	Irish macroalgae as feed supplements in farmed Atlantic salmon (<i>Salmo salar</i>): Safety, viability, quality and economic feasibility	2009	2016
Mr Benoit Queguineur	NUI Galway	Phlorotannins, current and future implications for the seaweed industry	2009	2013
Ms Natalie Heffernan	Teagasc	Extraction, characterisation and seasonal variation of bioactive compounds	2009	2015
*Ms Michelle Tierney	Teagasc	Investigation of macroalgal polyphenols and peptides with potential antioxidant and antihypertensive activities	2009	2014
Ms Natasha Moroney	UCC	Macroalgae and commercial macroalgal polysaccharides as potential functional ingredients in muscle foods	2009	2015
*Mr Ciaran Fitzgerald	Teagasc	Development of a bioactive bread enriched with seaweed peptide fractions with potential heart health effects	2010	2014
Ms Sonja Nitecki	UU	Anticancer activity of raw and digested Irish seaweed extracts on colorectal cancer models <i>in vitro</i>	2010	2014
Mr Matthias Schmid	NUI Galway	Biochemical plasticity in seaweeds: assessment and optimisation of high value compounds	2011	2015
Mr Kenneth Collins	Teagasc	Potential of seaweed derived bioactives for human health	2011	2017 (Expected)
Mr Ruairi Robertson	Teagasc	Algae-derived PUFA as novel functional ingredients	2012	2016
Ms Tara Flaherty	UL	Extraction of nitrogenous compounds from macroalgae	2012	Discontinued (2016)
Ms Adriana CunhaNeves	UL	Extraction, purification and characterisation of biofunctional peptides from marine processing co-products	2012	2015
Ms Aine Egan	UCD	An evaluation of the effect of marine compounds as anti-obesity and anti-inflammatory agents using <i>in vitro</i> , <i>ex vivo</i> and <i>in vivo</i> pig models	2012	2017 (Expected)
*Ms Dara Kirke	NUI Galway	Optimisation and standardisation of phlorotannin profiles of commercially valuable seaweeds with food applications (Walsh Fellowship)	2013	Ongoing
*Ms Sinéad Mackey	Teagasc	Marine Bioactives	2013	2016
Ms Claire McGreagh	UU	Marine Bioactives	2009	Withdrew (2009)
Mr Conall Strain	UU	Marine Bioactives	2013	2016

***PhD students not directly funded by NutraMara research programme, but funded from other sources.**

8.4. Training initiatives

The NutraMara research programme developed a training initiative to encourage early stage researchers to be exposed to international conferences and acquire new skills as a result of visiting other laboratories. Awards totalling nearly €15,000 were made to 12 researchers during the period 2011 to 2013. Details of the training activities undertaken and the level of grant support are given below in Table 32.

Table 32 Training activity supported from within NutraMara

Beneficiary	From	Purpose	Location	Duration	Grant	Date
Kenneth Collins	Teagasc	To perform laboratory work	Ashtown	53 days	€1,050	2011
Maria Hayes	Teagasc	To attend EuroFoodChem Conference	Gdansk, Poland		€250	2011
Ciaran Fitzgerald	Teagasc	To attend EuroFoodChem Conference	Gdansk, Poland		€300	2011
Maria Hayes	Teagasc	To attend EuroFoodChem Conference	Copenhagen, Denmark		€500	2011
Pádraigín Harnedy	UL	To attend Kiel Food Science Symposium	Kiel, Germany	3 days	€700	2012
Emma Brown	UU	To attend a meeting of the British Phycology Society	Belfast	3 days	€211	2012
Alex Wan	NUI Galway	To attend Aqua 2012	Prague, Czech Republic	4 days	€2,000	2012
Anna Soler Vila	NUI Galway	To attend Aqua 2012	Prague, Czech Republic	4 days	€2,000	2012
Ruairi Robertson	Teagasc	To perform research on algae-derived polyphenols	CSRO Werribee, VIC, Australia	34 days	€2,000	2013
Kenneth Collins	Teagasc	To perform research on algae-derived polysaccharides	Ulster University	2 days	€250	2013
Natalie Heffernan	Teagasc	To attend Trends in natural product research: a young scientist meeting of PSE & OPhG	Austria	4 days		2013
Thomas Smyth	Teagasc	To attend Trends in natural product research: a young scientist meeting of PSE & OPhG	Austria	4 days	€1,520	2013
Matthias Schmid	NUI Galway	To attend 10th International Phycology Congress	Orlando Florida, USA	7 days	€1,440	2013
Conall Strain	UU	Sample extractions and in-vitro batch culture analysis and pyrosequencing	Ashtown and Moorepark	Intermittent	€1,965	2013

8.5. Dissemination activities

8.5.1. Publications

Dissemination is an important component of NutraMara’s activities. The overall aim of the NutraMara consortium is to generate knowledge, capabilities and processes to identify, characterise and evaluate marine-derived bioactives as components of functional foods and as food ingredients. This knowledge and capability has the potential to have a significant impact not only within the scientific community but also to benefit the wider community.

NutraMara’s dissemination strategy aimed to raise awareness of NutraMara, its activities and outputs. The dissemination activities were designed to inform different audiences in a targeted way, to promote NutraMara’s expertise, results and outputs and to actively engage with the wider community.

The NutraMara research programme continues to add to an already extensive list of publications. The many outputs greatly contribute to raising the international profile of the NutraMara programme and the many scientists involved in it. In addition to the peer reviewed publications – many of which are in “high impact factor” journals, NutraMara researchers have produced large numbers of posters and delivered presentations at national and international events. Details of the publications to date, including those recently accepted and others described as “in-press” are given below.

8.5.2. Peer reviewed publications

The high number of co-authors on papers produced within the NutraMara programme reflects the extent of inter- and intra-institutional research collaboration that is facilitated by NutraMara. These publications are listed below in Table 33.

Table 33 NutraMara peer reviewed publications

Article Title	Journal Title	Year	Authors
The effects of lactose inclusion and seaweed extract derived from <i>Laminaria</i> spp. on performance, nutrient digestibility and microbial populations in newly weaned piglets.	Animal Feed Science and Technology	2010	O’Doherty, J.V., Dillon, S., Figat, S., Callan, J.J. and Sweeney, T. (2010).
The contribution of chemistry to improvement of food quality.	European Food Research and Technology	2010	Valverde, J., Hayes, M.,
A review of antihypertensive and antioxidant activities in macroalgae.	Botanica Marina	2010	Tierney, M.S., Croft, A.K. and Hayes, M. (2010).
The effects of supplementing the diet of the sow with seaweed extracts and fish oil on aspects of gastrointestinal health and performance of the weaned piglet.	British Journal of Nutrition	2011	Leonard SG, Sweeney T, Pierce KM, Bahar B, Lynch BP and O’Doherty JV (2010).

The effects of laminarin derived from <i>Laminaria</i> spp. on measurements of gut health: select microbiota, intestinal fermentation, mucin gene expression and cytokine gene expression in the pig.	British Journal of Nutrition	2011	Smith, A.G., O'Doherty, J.V., Reilly, P., Ryan, M.T., Bahar, B. and Sweeney, T. (2011).
Marine bioactive as functional food ingredients: Potential to reduce the incidence of chronic diseases.	Marine Drugs	2011	Lordan, S., R.P. Ross and Stanton, C. (2011).
In vitro and cellular antioxidant activities of seaweed extracts prepared from five brown seaweeds harvested in spring from the west coast of Ireland.	Food Chemistry	2011	O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Queguineur, B., Hanniffy, D., Troy, D.J., Kerry, J.P., and O'Brien, N.M. (2011).
Heart health peptides from macroalgae and their potential use in functional foods.	Journal of Agricultural Food Chemistry	2011	Fitzgerald, C., Gallagher, E., Tasdemir, D. and Hayes, M. (2011).
Bioactive proteins, peptides and amino acids from macroalgae.	Journal of Phycology	2011	Harnedy, P.A., and FitzGerald, R.J. (2011).
Angiotensin-I-converting enzyme and prolyl endopeptidase inhibitory peptides from natural sources with a focus on marine processing by-products.	Food Chemistry	2011	Wilson, J., Hayes, M. and Carney, B. (2011).
A potential role of interleukin 6 (IL6) in the chitooligosaccharide mediated inhibition of adipogenesis.	British Journal of Nutrition	2011	Bahar, B., O'Doherty, J.V. and Sweeney, T.
Isolation of heart healthy peptides derived from red algae and incorporation into bread.	Polish Journal of Food and Nutrition	2011	Fitzgerald C, Gallagher, E, Hayes M.
The effects of supplementing varying molecular weights of chitooligosaccharide on performance, faecal scoring, selected microbial populations and nutrient digestibility in the weaned piglet.	Animal	2012	Walsh, A.M., Sweeney, T., Bahar, B., Flynn, B. and O'Doherty, J.V. (2012).
Isolation and characterization of bioactive propeptides with in vitro renin inhibitory activities from the macroalga <i>Palmaria palmata</i> .	Journal of Agricultural and Food Chemistry	2012	Fitzgerald, C., Mora-Soler, L., Gallagher, E., Prieto, J., Mora-Soler, A. and Hayes, M. (2012).
Influence of pressurised liquid extraction and solid-liquid extraction methods on the phenolic content and antioxidant activities of Irish macroalgae.	International Journal of Food Science & Technology	2012	Tierney, M.S., Smyth, T.J., Hayes, M., Soler-Vila, A., Croft, A.K. and Brunton, N. (2012).
Extracts of brown seaweeds can attenuate the bacterial	Journal of Animal Science	2012	Bahar, B., O'Doherty, J.V., Hayes, M. and Sweeney, T. (2012).

lipopolysaccharide (LPS) induced pro-inflammatory response in the porcine colon ex-vivo.				
Chitooligosaccharide elicits acute inflammatory cytokine response through AP-1 pathway in human intestinal epithelial-like (Caco-2) cells.	Molecular Immunology.	2012	Bahar, B., O'Doherty, J.V., Maher, S., McMorrow, J. and Sweeney, T. (2012).	
Bioactive peptides from marine processing waste and shellfish: A review.	Journal of Functional Foods	2012	Harnedy, P.A., and FitzGerald, R.J. (2012).	
Assessment of the antioxidant activities of the brown macroalga <i>Fucus spiralis</i> Linnaeus harvested from the west coast of Ireland.	Journal of Biological Sciences	2012	Tierney, M.S., Soler, A., Croft, A.K. and Hayes, M. (2012).	
Addition of seaweed (<i>Laminaria digitata</i>) extracts containing laminarin and fucoidan to porcine diets: Influence on the quality and shelf-life of fresh pork.	Meat Science	2012	Moroney, N.C., O'Grady, M.N., O'Doherty, J.V. and Kerry, J.P. (2012).	
Assessment of the ability of seaweed extracts to protect against hydrogen peroxide and tert-butyl hydroperoxide induced cellular damage in Caco-2 cells.	Food Chemistry	2012	O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Queguineur, B., Hanniffy, D., Troy, D.J., Kerry, J.P. and O'Brien, N.M. (2012).	
The α -amylase and α -glucosidase inhibitory effects of Irish seaweed extracts.	Food Chemistry	2013	Lordan, S., Smyth, T.J., Soler-Vila, A., Stanton, C. and Ross P.R. (2013).	
The effects of supplementing varying molecular weights of chito oligosaccharide on performance, selected microbial populations and nutrient digestibility in the weaned pig.	Animal	2013	Walsh, M., Sweeney, T., Bahar, B., Flynn, B. and O'Doherty J.V. (2013).	
The effect of solvents on the antioxidant activity in Caco-2 cells of Irish brown seaweed extracts prepared using accelerated solvent extraction (ASE®).	Journal of Functional Foods	2013	O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Hayes, M., Kerry, J.P., and O'Brien, N.M. (2013).	
The effect of solvents on the antioxidant activity in Caco-2 cells of Irish brown seaweed extracts prepared using accelerated solvent extraction (ASE®).	Journal of Functional Foods	2013	O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Hayes, M., Kerry, J.P., and O'Brien, N.M. (2013).	
Prawn chitosan containing bread: Assessment of bioactive and sensory qualities.	Journal of Chitin and Chitosan Science	2013	Lafarga, T., Hayes, M., Walsh, D., Valverde, J. and Gallagher, E., (2013).	
Multi-functional roles of chitosan as a	PLoS One	2013	Walsh, A.M., Sweeney, T., Bahar, B.	

potential protective agent against obesity.			and O'Doherty J.V. (2013).
LC-PUFA-enriched oil production by microalgae: accumulation of lipid and triacylglycerols containing n-3LC-PUFA is triggered by nitrogen limitation and inorganic carbon availability in the marine haptophyte <i>Pavlova lutheri</i> .	Marine Drugs	2013	Guihéneuf, F. and Stengel, D. (2013).
Influence of pressurised liquid extraction and solid-liquid extraction methods on the phenolic content and antioxidant activities of Irish macroalgae.	International Journal of Food Science & Technology	2013	Tierney, M.S., Smyth, T.J., Hayes, M., Soler-Vila, A., Croft, A.K., Brunton N. (2013).
In vitro assessment of the cardioprotective, anti-diabetic and antioxidant potential of <i>Palmaria palmata</i> protein hydrolysates.	Journal of Applied Phycology	2013	Harnedy, P.A., and FitzGerald, R.J. (2013).
Extraction of protein from the macroalga <i>Palmaria palmata</i> .	Lebensmittel-Wissenschaft und-Technologie	2013	Harnedy, P.A. and FitzGerald, R.J. (2013).
Enrichment of polyphenol contents and antioxidant activities of Irish brown macroalgae using food-friendly techniques based on polarity and molecular size.	Food Chemistry	2013	Tierney, M.S., Smyth T.J., Rai, D.K., Soler-Vila A., Croft A.K. and Brunton N. (2013).
Development of a seaweed derived platelet activating factor acetylhydrolase (PAF-AH) inhibitory hydrolysate, synthesis of inhibitory peptides and assessment of their toxicity using the Zebrafish larvae assay.	Peptides	2013	Fitzgerald, C., Gallagher, E., O'Connor, P., Prieto, J., Mora-Soler, L., Grealy, M. and Hayes, M. (2013).
Chitosan containing bread made using marine shellfishery by-products: functional, bioactive and quality assessment of the end product.	Journal of Agricultural and Food Chemistry	2013	Lafarga, T., Gallagher, E., Walsh, D., Valverde, J., and Hayes, M., (2013).
Chito-oligosaccharide inhibits the demethylation of a 'CpG' island within the leptin (LEP) promoter during adipogenesis of 3T3-L1 cells.	PLoS One	2013	Bahar, B., O'Doherty, J.V., O'Doherty, A.M. and Sweeney, T. (2013).
Cardioprotective peptides from marine sources.	Current Protein & Peptide Science	2013	Harnedy, P.A. and FitzGerald, R.J. (2013).
Antioxidant activity of the brown macroalgae <i>Fucus spiralis</i> harvested from the West Coast of Ireland.	Current Research Journal of Biological Sciences	2013	Tierney, M.S., Soler-vila, A., Croft, A.K. and Hayes, M. (2013).
Effect of a brown seaweed (<i>Laminaria</i>	Meat Science	2013	Moroney, N.C., O'Grady, M.N.,

<i>digitata</i>) extract containing laminarin and fucoidan on the quality and shelf-life of fresh and cooked minced pork patties.				O'Doherty, J.V. and Kerry, J.P. (2013).
UPLC-MS profiling of low molecular weight phlorotannin polymers in <i>Ascophyllum nodosum</i> , <i>Pelvetia canaliculata</i> and <i>Fucus spiralis</i> .	Metabolomics		2014	Tierney, M.S., Soler-Vila, A., Rai, D.K., Croft, A.K., Brunton, N. and Smyth, T.J. (2014).
The effect of time and origin of harvest on the in vitro biological activity of <i>Palmaria palmata</i> protein hydrolysates.	Food Research International		2014	Harnedy, P.A, Soler-Vila, A., Edwards, M.D., FitzGerald, R.J. (2014).
Seaweed and human health.	Nutrition Reviews		2014	Brown, E.M., Allsopp, P.J., Magee, P.J., Gill, C.I., Nitecki, S., Strain, C.R. and McSorley, E.M. (2014).
Potential of a renin inhibitory peptide from the red seaweed <i>Palmaria palmata</i> as a functional food ingredient following confirmation and characterization of a hypotensive effect in spontaneously hypertensive rats.	Journal of Agricultural and Food Chemistry		2014	Fitzgerald, C., Aluko, R.E., Hossain, M., Rai, D.K. and Hayes, M. (2014).
Increasing the health benefits of bread: assessment of the physical and sensory qualities of bread formulated using a renin inhibitory <i>Palmaria palmata</i> protein hydrolysate.	Lebensmittel-Wissenschaft und Technologie		2014	Fitzgerald, C., Gallagher, E., Doran, L., Auty, M., Prieto, J. and Hayes, M. (2014).
In vitro assessment of the multi-functional bioactive potential of Alaska pollock skin collagen following simulated gastrointestinal digestion.	Journal of the Science of Food and Agriculture		2014	Guo, L., Harnedy, P.A., Li, B., Zhang, Z., Hou, H., Zhao, X. and FitzGerald, R.J. (2014).
Food protein-derived chelating peptides: biofunctional ingredients for dietary mineral bioavailability enhancement.	Trends in Food Science & Technology		2014	Guo, L., Harnedy, P.A., Li, B., Hou, H., Zhang, Z., Zhao, X. and FitzGerald, R.J. (2014).
Fatty acid contents and profiles of 16 macroalgae collected from the Irish coast at two seasons.	Journal of Applied Phycology		2014	Schmid, M., Guihéneuf, F., Stengel, D.B. (2014).
Bioactive peptides and their potential use for the prevention of diseases associated with Alzheimer's disease and mental health disorders: Food for thought?	Annals of Psychiatry and Mental Health		2014	Hayes, M. (2014).
Bioactive compounds from marine mussels and their effects on human health.	Food Chemistry		2014	Grienke, U., Silke, J., and Tasdemir, D. (2014).

Antioxidant activity and phenolic content of pressurised liquid and solid-liquid extracts from four Irish origin macroalgae.	International Journal of Food Science & Technology	2014	Heffernan, N., Smyth, T.J., FitzGerald, R.J., Soler-Vila, A. and Brunton, N.P. (2014).
An examination of the potential of seaweed extracts as functional ingredients in milk.	International Journal of Dairy Technology	2014	O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Waldron, D.S., Smyth, T.J., O'Brien, N.M., and Kerry, J.P. (2014).
Towards the biorefinery concept: interaction of light, temperature and nitrogen for optimizing the co-production of high-value compounds in <i>Porphyridium purpureum</i> .	Algal Research	2015	Guiheneuf, F. and Stengel, D.B. (2015).
Seaweed polysaccharides (laminarin and fucoidan) as functional ingredients in pork meat: an evaluation of anti-oxidative potential, thermal stability and bioaccessibility.	Marine Drugs	2015	Moroney, N.C., O'Grady, M.N., Lordan, S., Stanton, C., and Kerry, J.P. (2015).
Seasonal and geographical variations in the biochemical composition of the blue mussel (<i>Mytilus edulis L.</i>) from Ireland.	Food Chemistry	2015	Fernández, A., Grienke, U., Soler-Vila, A., Guihénéuf, F., Stengel, D.B. and Tasdemir, D. (2015).
Purification and identification of dipeptidyl peptidase (DPP) IV inhibitory peptides from the macroalga <i>Palmaria palmate</i> .	Food Chemistry	2015	Harnedy, P.A., O'Keeffe, M.B. and FitzGerald, R.J. (2015).
Profiling of the molecular weight and structural isomer abundance of macroalgae derived phlorotannins.	Marine Drugs	2015	Heffernan, N., Brunton, N.P., FitzGerald, R.J. and Smyth, T.J. (2015).
Phenolic content and antioxidant activity of fractions obtained from selected Irish macroalgae species (<i>Laminaria digitata</i> , <i>Fucus serratus</i> , <i>Gracilaria gracilis</i> and <i>Codium fragile</i>).	Journal of Applied Phycology	2015	Heffernan, N., Smyth, T.J., Soler-Vila, A., FitzGerald, R.J. and Brunton, N.P. (2015).
Optimization of ultrasound assisted extraction of bioactive components from brown seaweed <i>Ascophyllum nodosum</i> using response surface methodology.	Ultrasonics sonochemistry	2015	Kadam, S.U., Tiwari, B.K., Smyth, T.J. and O'Donnell, C.P. (2015).
Intra-thallus differentiation of fatty acid and pigment profiles in some temperate Fucales and Laminariales.	Journal of Phycology	2015	Schmid, M. and Stengel, D.B. (2015).
Influence of level and duration of feeding polysaccharide (laminarin and fucoidan) extracts from brown seaweed (<i>Laminaria digitata</i>) on	Meat Science	2015	Moroney, N.C., O'Grady, M.N., Robertson, R.C., Stanton, C., O'Doherty, J.V. and Kerry, J.P. (2015).

quality indices of fresh pork.			
Influence of green seaweed (<i>Ulva rigida</i>) supplementation on the quality and shelf-life of Atlantic salmon fillets.	Journal of Aquatic Food Product Technology	2015	Moroney, N.C., Wan, A.H.L., Soler-Vila, A., O'Grady, M.N., FitzGerald, R.D., Johnson, M.P. and Kerry, J.P. (2015)
Inclusion of <i>Palmaria palmata</i> (red seaweed) in Atlantic salmon diets: effects on the quality, shelf-life parameters and sensory properties of fresh and cooked salmon fillets.	Journal of the Science of Food and Agriculture	2015	Moroney, N.C., Wan, A.H.L., Soler-Vila, A., FitzGerald, R.D., Johnson, M.P. and Kerry, J.P. (2015).
Hydrodynamic characterisation of chitosan and its interaction with two polyanions: DNA and xanthan.	Carbohydrate Polymers	2015	Almutairi, F.M., Erten, T., Adams, G.G., Hayes, M., McLoughlin, P., Kok, M.S., Mackie, A.R., Rowe, A.J. and Harding S.E. (2014).
Fractionation and identification of Alaska pollock skin collagen-derived mineral chelating peptides.	Food Chemistry	2015	Guo, L., Harnedy, P.A., O'Keefe, M.B, Zhang, Z., Li, B., Hou, H. and FitzGerald, R.J. (2015).
Extraction, structure and biofunctional activities of laminarin from brown algae.	International Journal of Food Science & Technology	2015	Kadam, S.U., Tiwari, B.K. and O'Donnell, C.P. (2015).
Extraction of antioxidant and ACE inhibitory peptides from Thai traditional fermented shrimp pastes.	Food Chemistry	2015	Kleekayai, T., Harnedy, P.A., O'Keefe, M.B, Poyarkov, A.A., CunhaNeves, A., Suntornsuk, W. and FitzGerald, R.J. (2015)
Effects of polysaccharide rich extracts obtained from the brown seaweed <i>Laminaria digitata</i> on human microbiota in an in vitro model of the distal colon.	Proceedings of the Nutrition Society	2015	Strain, C.R., Collins, K.C., Smyth, T.J., Soler-Vila, A., Rea, M.C., Stanton, C., Ross, R.P., Allsopp, P.J., Naughton, V., and McSorley, E.M. (2015)
Effect of ultrasound pretreatment on the extraction kinetics of bioactives from brown seaweed (<i>Ascophyllum nodosum</i>).	Separation Science and Technology	2015	Kadam, S.U., Tiwari, B.K., O'Connell, S., O'Donnell, C.P. (2015).
Effect of ultrasound pre-treatment on the drying kinetics of brown seaweed <i>Ascophyllum nodosum</i> .	Ultrasonics Sonochemistry	2015	Kadam, S.U., Tiwari, B.K. and O'Donnell, C.P. (2015).
Cardioprotective potential of Irish macroalgae: Generation of glycine betaine and DMSP containing extracts by accelerated solvent extraction.	Planta Medica	2015	Valverde, J., Hayes, M., McLoughlin, P., Rai, D. K. and Soler-Vila, A. (2015).
The anti-inflammatory effect of algae-derived lipid extracts on human THP-1 macrophages.	Accepted by Marine Drugs	2015	Robertson R., Stanton C. Guihéneuf F., Ross P., Bahar B., Schmid M., Stengel D.B. and Fitzgerald G.
Cardioprotective cryptides derived from fish and other food sources: Generation, application and future	Journal of Agriculture and Food Chemistry	2015	Mora, L. and Hayes, M. (2015)

markets.			
Angiotensin converting enzyme and dipeptidyl peptidase-IV inhibitory, and antioxidant activities of a mussel meat protein extract and its hydrolysates.	Journal of Aquatic Food Product Technology	2015	CunhaNeves, A., Harnedy P.A., and FitzGerald, R.J. (2015).
Prospects and challenges for industrial production of seaweed bioactives	Journal of Phycology	2015	Hafting, J.T., Craigie, J.S., Stengel D.B., Loureiro, R.R., Buschmann, A.H., Yarish, C., Edwards, M.D. and Critchley, A.T.
Prawn shell chitosan has anti-obesogenic properties, influencing both nutrient digestibility and microbial populations in a pig model	PLoS One. 2015; 10(12): e0144127	2015	Á. M Egan, T. Sweeney, M. Hayes, J. V. O' Doherty.
The Omega-3 Polyunsaturated Fatty acid Docosahexaenoic acid (DHA) reverses Corticosterone-induced Changes in Cortical neurons.	Int J Neuropsychopharmacol	2015	Pusceddu, M.M., Nolan, Y. M., Green, H. F., Robertson, R. C., Stanton, C. Kelly, P., Cryan, J. F., Dinan, T. G
The effect of consuming <i>Palmaria palmata</i> -enriched bread on inflammatory markers, antioxidant status, lipid profile and thyroid function in a randomised placebo-controlled intervention trial in healthy adults	Eur J Nutr	2015	Allsopp P, Crowe W, Bahar B, Harnedy PA, Brown ES, Taylor SS, Smyth TJ, Soler-Vila A, Magee PJ, Gill CI, Strain CR, Hegan V, Devaney M, Wallace JM, Cherry P, FitzGerald RJ, Strain JJ, O'Doherty JV, McSorley EM.
Cardioprotective cryptides derived from fish and other food sources: Generation, application and future markets	Journal of Agricultural and Food Chemistry	2015	Mora L, Hayes M,
Red and green macroalgae for fish and animal feed and human functional food development	Food Research International	2015	Vaquero M, Hayes M
Prawn shell chitosan exhibits anti-obesity effects through alterations to hypothalamic and intestinal gene expression in vivo.	PLoS One. 2016	2016	Á. M Egan, T. Sweeney, S. Vigors, J. V. O' Doherty. PLoS One (Accepted).
The anti-inflammatory potential of seaweed extracts in in vitro and ex vivo models of the gastrointestinal tract.	Innovative Food Science and emerging technologies	2016	Á. M Egan, B. Bahar, T. Sweeney, T. Smyth, J. V. O' Doherty (Submitted)

8.5.3. Books and book chapters

Members of the NutraMara consortium contributed chapters to books or were responsible for editing major books relating to the use of marine bioresources. These are listed below in Table 34

Table 34 Books and Book chapters by NutraMara researchers

Title	Publication	Date	Authors
Antihypertensive peptides from marine origin.	In Hernández-Ledesma, B. and Herrero, M. (Eds.): Bioactive Compounds from Marine Foods: Plant and Animal Sources, Wiley-Blackwell.	2013	Norris, R., Harnedy, P.A. & FitzGerald, R.J.
Bioactive proteins and peptides from macroalgae, fish, shellfish and marine processing waste.	In: Kim, S.K. (Ed.): Marine Proteins and Peptides: Biological Activities and Applications, Wiley-Blackwell	2013	Harnedy, P.A. & FitzGerald, R.J.
Algae-derived polyunsaturated fatty acids: Implications for human health.	In: Polyunsaturated Fatty Acids: Sources, Antioxidant Properties and Health Benefits	2013	Robertson, R., Guihéneuf, F., Schmid, M., Stengel, D.B., Fitzgerald, G., Ross, P. and Stanton C. (2013).
Bioactive peptides from marine processing by-products.	In: Bioactive Compounds from Marine Foods: Plant and Animal Sources, First edition, Edited by Blanca Hernández-Ledesma and Miguel Herrero, (Wiley & Sons, 2014), 57-72	2013	Hayes, M., and Flower, D. (2013).
Biological activities of proteins and marine-derived peptides from by-products and seaweeds.	In: Marine Proteins and Peptides: Biological Activities and Applications, First edition, Se-Kwon S Kim (editor), John Wiley & Sons, 139-166	2013	Hayes, M., (2013).
Algae-derived polyunsaturated fatty acids: Implications for human health.	In: Polyunsaturated fatty acids: Sources, antioxidant properties and health benefits. Ed: Catalá A. Nova Sciences Publishers, Inc., Hauppauge, NY 11788 USA.	2013	Robertson R., Guihéneuf F., Schmid M., Stengel D.B., Fitzgerald G., Ross P., and Stanton C., 2013.
Nutraceuticals derived from marine sources: Extraction and application of bioactive carbohydrates, proteins and peptides as functional ingredients.	In: Nutraceuticals and Functional Foods: Natural Remedy. Nova Publishers.	2014	Hayes, M., and Valverde, J. (2014).
Natural Products from Marine Algae: Methods and Protocols.	Humana Press, Springer	2015	Stengel, D.B. and Connan, S. (editors) (2015).
Marine processing proteinaceous by-products: sources, characterization, purification and	In:Dhillon, G.S. (Ed): Waste-derived proteins: Transformation from environmental burden into	2015	Neves, A.C. Harnedy, P.A., and FitzGerald, R.J.

applications.	value-added products. Elsevier (accepted)		
Extraction and enrichment of protein from red and green macroalgae.	In Stengel, D.B. and Connan, S (Eds): Natural Products from Marine Algae, Methods and Protocols, Springer.	2015	Harnedy, P.A., and FitzGerald, R. J. (2015).
Marine algae a source of biomass for biotechnological applications.	In: Natural Products from Marine Algae: Methods and Protocols, Springer.	2015	Stengel, D.B. and Connan, S. (2015).
Lipids and fatty acids in algae: extraction, fractionation into lipid classes, and analysis by gas chromatography coupled with flame ionisation detector (GC-FID).	In: Natural Products from Marine Algae: Methods and Protocols.	2015	Guiheneuf, F., Schmid, M. and Stengel, D.B. (2015).
The Gut-Brain Axis: Dietary, Probiotic, and Prebiotic Interventions on the Microbiota	The Gut-Brain Axis: Dietary, Probiotic, and Prebiotic Interventions on the Microbiota: Academic Press	2016	Niall Hyland, Catherine Stanton (Eds)

8.5.4. Posters

Byrne A., Schmid M. and Stengel D.B. Omega 3s under the seas: Increasing bioactive compounds in seaweeds. Environ - Irish Environmental Researchers' Colloquium Galway February 2013

Guihéneuf, F. and Stengel, D.B. Culture Strategies to Enhance the Potential of Microalgae as a Source of Bioactives for "Healthy" Foods. Natural Product Biotechnology 2014, 18-20 November 2014, Inverness, Scotland

Kirke, D., Smyth, T., Rai, D. and Stengel, D.B. Optimisation of phlorotannin-enriched food products from commercially valuable Irish seaweeds. Natural Product Biotechnology 2014. 18-20 November 2014, Inverness, Scotland

Kirke, D., Smyth, T., Rai, D. and Stengel, D.B. Optimisation of phlorotannin profiles for the enrichment of seaweed-based food products. 43rd Annual Food Research Conference, UCD, Ireland (poster)

Schmid M., Guihéneuf F. and Stengel D.B. Optimised utilisation of Irish seaweeds as a source for bioactive compounds with potential applications in Functional Foods, NutraMara conference, Dublin, June 2015

Schmid M. and Stengel, D.B. Optimised utilization of Irish seaweeds as a source for bioactive compounds, Natural Product Biotechnology, Inverness Scotland, November 18th-22nd 2014

Schmid M., Guihéneuf F. and Stengel, D.B. Irish seaweeds as a source of pigments and fatty acids as bioactive compounds with potential applications in functional foods, NutraMara conference, Dublin, April 2012

Stack, J., Le Gouic, A., Guihéneuf, F., Tobin, P., Stengel, D.B. and FitzGerald, R.J. Antioxidant activity of protein hydrolysates from *Porphyridium purpureum* and *Phaeodactylum tricornutum*. NutraMara Conference, Dublin, Ireland, 29-30th June 2015.

Guihéneuf, F. and Stengel, D.B. Challenges and strategies for optimised production of bioactive compounds from microalgae for food applications. NutraMara Conference, Dublin, Ireland, 29-30th June 2015.

Guihéneuf, F. and Stengel, D.B. Culture Strategies to Enhance the Potential of Microalgae as a Source of Bioactives for “Healthy” Foods. Natural Product Biotechnology 2014, 18-20 November 2014, Inverness, Scotland.

Guihéneuf, F. and Stengel, D.B. LC-PUFA-Enriched Oil Production by the Marine Haptophyte *Pavlova lutheri*: The Importance of Inorganic Carbon Availability. Royal Society of Chemistry Conferences: Sustainable Chemicals from Microalgae: Encompassing Biocrude through to Fine Chemicals. The Chemistry Center, 19 November 2013, London, UK.

Guihéneuf, F. and Stengel, D.B. Marine microalgae as potential sources of nutritionally important PUFA. NutraMara Conference, Dublin, Ireland, 25th and 26th April 2012.

Schmid, M., Guihéneuf, F. and Stengel, D.B. Irish seaweeds as a source of pigments and fatty acids as bioactive compounds with potential applications in functional foods. NutraMara Conference, Dublin, Ireland, 25th and 26th April 2012.

Guihéneuf, F. and Stengel, D.B. Marine microalgae as potential alternative sources of bioactive compounds for food applications. 9th European Workshop “Biotechnology of Microalgae”, Nuthetal, Germany, 4th and 5th June 2012.

Guihéneuf, F. and Stengel, D.B. Marine microalgae as potential sources of nutritionally important PUFA. Marine Biodiversity/discovery 'event', Ryan Institute, NUI Galway, Ireland, 7th June 2012.

Guihéneuf, F. and Stengel, D.B. Marine microalgae as potential alternative sources of bioactive compounds for food applications. Ryan Institute Launch and Symposium, NUI Galway, Ireland, 10th July 2012.

Harnedy P.A., O’Keeffe M.B. & FitzGerald, R. Isolation and characterisation of dipeptidyl peptidase (DPP) IV inhibitory peptides from *Palmaria palmate*, NutraMara Conference Dublin June 2015

Mullan, C., Harnedy, P.A., Mc Laughlin, C., Parthasarathy, V., Allsopp, P.J., McSorley, E.M., FitzGerald, R.J. & O’Harte, F.P.M. Synthetic peptides isolated from the macroalga *Palmaria palmata* demonstrate dipeptidyl-peptidase-4 inhibitory actions on GLP-I degradation using a HPLC assay system. NutraMara Conference Dublin June 2015

CunhaNeves, A., Harnedy P.A. and FitzGerald, R.J. Extraction, purification and characterisation of biofunctional peptides from marine waste/co-products. The world is your oyster: How You Eat It Is Up to You, NutraMara Student Conference, Dublin, Oct 2013.

Flaherty, T., Harnedy P.A. & FitzGerald, R.J. Assessment of the biological activity of peptide and amino acid extracts from the macroalgae *Palmaria palmata* NutraMara Student Conference, Dublin, Oct 2013

CunhaNeves, A., Harnedy P.A. & FitzGerald, R.J. Adaption and optimisation of a protein extraction protocol for salmon processing by-products NutraMara Conference, Dublin, April 2012

Flaherty, T., Harnedy P.A. & FitzGerald, R.J. Seasonal and Geographical Variation of Total Nitrogen, Protein Nitrogen and Non-Protein Nitrogen Content of Macroalgae Native to the Irish Coastline NutraMara Conference, Dublin, April 2012

O’Sullivan, A.M., O’Grady, M.N., O’Callaghan, Y.C., Smyth, T.J., O’Brien, N.M. and Kerry, J.P. (2015). Seaweed extracts as potential functional ingredients in yoghurt. *NutraMara Conference (Harnessing Marine Bioresources for Innovations in the Food Industry)*, Royal Dublin Society (RDS) Dublin. 29 – 30 June 2015 (p. 75).

Moroney, N.C., O'Grady, M.N., Wan, A., Soler-Vila, A., Johnson, M., Fitzgerald, R. and Kerry, J.P. (2012). Inclusion of seaweed (*Palmaria palmata*) in salmon diets: Effects on fresh salmon fillet quality. *41st Annual UCC Food Research Conference*, University College Cork. 26 – 27 April 2012.

Moroney, N.C., O'Grady, M.N., Wan, A., Soler-Vila, A., Johnson, M., Fitzgerald, R. and Kerry, J.P. (2012). Supplementation of salmon diets with red macroalgae (*Palmaria palmata*): Effects on fresh salmon fillet quality. *Nutramara Conference (Bioactive Components from Marine Sources and By-Product Utilisation and Valorisation)*, Dublin. 25 – 26 April 2012 (p. 50-51).

Moroney, N.C., O'Grady, M.N., O'Doherty, J.V. and Kerry, J.P. (2011). Addition of seaweed polysaccharides from brown seaweed (*Laminaria digitata*) to porcine diets: Influence on the oxidative stability of organ tissues and fresh pork quality. *62nd Annual EAAP (European Association for Animal Production) Meeting*, Stavanger, Norway. 29 August – 2 September 2011 (p. 358).

Moroney, N.C., O'Grady, M.N., O'Doherty, J.V. and Kerry, J.P. (2010). Assessment of the effect of dietary laminarin and fucoidan supplementation on the quality of fresh pork. *Proceedings of the EFFoST (European Federation of Food Science and Technology (EFFoST) Conference*, Dublin. 10 – 12 November 2010.

O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Hayes, M., Kerry, J.P. and O'Brien, N.M. (2012). Influence of the extraction solvent on the DNA-protective compounds extracted from brown seaweed. *Nutramara Conference (Bioactive Components from Marine Sources and By-Product Utilisation and Valorisation)*, Dublin. 25 – 26 April 2012 (p. 40).

O'Sullivan, A.M., O'Callaghan, Y.C., O'Grady, M.N., Kerry, J.P. and O'Brien, N.M. (2009). Determination of the antioxidant potential of seaweed extracts using different methods. *Proceedings of the Nutrition Society*, 68 (OCE3), E110, Belfast, N. Ireland. 17 – 19 June 2009.

Robertson, R. C., Guihéneuf, F., Bahar, B., Schmid, M., Stengel, D.B., Fitzgerald, G., Ross, P. and Stanton C. – The anti-inflammatory effect of algae-derived lipid fractions in THP-1 macrophages. *NutraMara Postgraduate Dissemination Open Day* (October 2013)

Robertson, R. C., Fitzgerald, G., Ross, P. and Stanton C. - An investigation into the modulation of the gut microbiota following *in vitro* fermentation of a seaweed polyphenol extract. *8th World Congress on Polyphenol Applications*, Lisbon, Portugal, June 2014

Robertson, R. C., Fitzgerald, G., Ross, P. and Stanton C. An investigation into the modulation of the gut microbiota following *in vitro* fermentation of a seaweed polyphenol extract. *UCC and Imperial College London Joint Postgraduate Symposium in Food and Health*, September 2014

Robertson, R. C., Fitzgerald, G., Ross, P. and Stanton C. An investigation into the modulation of the gut microbiota following *in vitro* fermentation of a seaweed polyphenol extract. *Alimentary Pharmabiotic Centre Scientific Advisory Board Day*, Cork, October 2014

Robertson, R. C., Guihéneuf, F., Bahar, B., Schmid, M., Stengel, D.B., Fitzgerald, G., Ross, P. and Stanton C. The anti-inflammatory effect of algae-derived lipid fractions in THP-1 macrophages. *New York Academy of Sciences Journey Through Science Day*, New York, December 2014

Nitecki, SS, Allsopp, PJ, Brown, EM, Soler-Vila, A, Magee, PJ, McSorley, EM, Gill, CIR, Strain, JJ. Anticancer activity of raw and digested Irish seaweed extracts on colorectal cancer cell lines *in vitro*. *NutraMara international conference 2012*, Dublin.

Nitecki, SS, Magee, PJ, McSorley, EM, Strain, JJ, Gill, CIR. Anticancer activity of raw and digested *Irish Palmaria palmata* seaweed extracts on colorectal cancer cell lines in vitro. The Institution of Biomedical Sciences (IBMS) Centenary Symposium 2012, Belfast.

Nitecki, SS, Magee, PJ, McSorley, EM, Strain, JJ, Gill, CIR. Anticancer activity of raw and digested Irish *Palmaria palmata* seaweed extracts on colorectal cancer cell lines in vitro. European Nutraceutical Association international conference 2012, Munich.

Allsopp, PJ, Brown, EM, Soler-Vila, A, Magee, PJ, Nitecki, SS, , Gill, CIR, Strain, JJ McSorley EM. “An investigation into the effect of consuming bread containing *Palmaria palmata* on markers of inflammatory status in healthy adults” IUNS 20TH International Congress of Nutrition September 15th 2013

Strain, CS, Allsopp, PJ, Brown, EM, Soler-Vila, A, Magee, PJ, Nitecki, SS, Gill, CIR, Strain, JJ McSorley EM. Prebiotic potential of polysaccharide rich extracts obtained from *Laminaria digitata*. Society of general microbiology

Strain, CS, Allsopp, PJ, Brown, EM, Magee, PJ, Gill, CIR, McSorley EM. Prebiotic potential of polysaccharide rich extracts obtained from *Laminaria digitata*. The World is Your Oyster: How you Eat It Is Up To You NutraMara student workshop

C.R. Strain, K.C. Collins, T.J. Smyth, A. Soler-Vila, M.C. Rea, C. Stanton, R.P. Ross, P.J. Allsopp, V. Naughton and E.M. McSorley Effects of polysaccharide rich extracts obtained from the brown seaweed *Laminaria digitata* on human microbiota in an in vitro model of the distal colon. Poster presentation at Nutrition Society Summer meeting, ‘Carbohydrates in health: friends or foes?’ University of Glasgow

8.5.5. Conference presentations

Allsopp, P.J., Brown, E.M., Soler-Vila, A., Magee, P.J., Nitecki, S.S, Gill, C.I.R., Strain, J.J. and McSorley, E.M. Mining seaweeds for bioactive ingredients. BIM seaweed workshop, December, 2012

Allsopp, P.J., Brown, E.M., Soler-Vila, A., Magee, P.J., Nitecki, S.S, Gill, C.I.R., Strain, J.J. and McSorley, E.M. Insights into Research Careers in Marine and Food Science. NutraMara student workshop, October 2013

Allsopp, P.J., Brown, E.M., Soler-Vila, A., Magee, P.J., Nitecki, S.S, Gill, C.I.R., Strain, J.J. and McSorley, E.M. An investigation into the effect of consuming bread containing *Palmaria palmata* on markers of inflammatory status in healthy adults. Infection and Immunity TRG meeting. Feb 2013

Allsopp, P.J., Brown, E.M., Soler-Vila, A., Magee, P.J., Nitecki, S.S, Gill, C.I.R., Strain, J.J. and McSorley, E.M. Marine Bioactives in human health – NutraMara human intervention studies Sea to Shelf workshop – University of Ulster Coleraine, July 2013

Allsopp, P.J., Brown, E.M., Soler-Vila, A., Magee, P.J., Nitecki, S.S, Gill, C.I.R., Strain, J.J. and McSorley, E.M. “Potential anticancer activity of Irish brown seaweed extracts in models of colon cancer”. British Phycology Society Meeting 7-10 July 2013

Brown, E.M., Allsopp, P.J., Soler-Vila, A., Magee, P.J., Nitecki, SS, , Gill, C.I.R., Strain, J.J. and McSorley E.M. “Marine Bioactives in human health – NutraMara In vitro studies”. Sea to Shelf workshop – University of Ulster Coleraine, July 2013

CunhaNeves, A., Harnedy P.A. and FitzGerald, R.J. Anti-diabetic and anti-oxidant potential of marine by-products derived peptides. Department of Life Sciences 2013 Annual Research Day, UL, May 2013.

CunhaNeves, A., Harnedy P.A. and FitzGerald, R.J. Extraction, purification and characterisation of biofunctional peptides from marine processing co-products, Department of Life Sciences, UL, 2014 Annual Research Day, Limerick, May 2014.

CunhaNeves, A., Harnedy P.A. and FitzGerald, R.J. Extraction, purification and characterisation of biofunctional peptides from marine processing co-products. Young Life Scientists Ireland, Dublin, March 2014.

CunhaNeves, A., Harnedy P.A. and FitzGerald, R.J. Extraction, purification and characterisation of biofunctional peptides from marine waste/co-products. The world is your oyster: How You Eat It Is Up to You, NutraMara Student Conference, Dublin, Oct 2013.

CunhaNeves, A., Harnedy P.A. and FitzGerald, R.J. Mussel byssus Collagen hydrolysates Angiotensin converting enzyme Dipeptidyl peptidase IV Oxygen radical antioxidant capacity, 43rd Annual Food Research Conference, Dublin, December 2014.

Fitzgerald, C., Gallagher, E. and Hayes, M. Isolation of heart healthy peptides derived from *Palmaria palmata* and incorporation into bread. EuroFoodChem XVII, July 2011 6th-8th. Gdansk, Poland.

Fitzgerald, C. and Hayes, M. Characterisation of bioactive peptides from *Palmaria palmata* and the INFOGEST method. INFOGEST, COST Action I005 meeting Madrid, Spain, 2014.

Fitzgerald, C., Mora, L., Gallagher, E., Prieto, J., Soler-Vila, A. and Hayes, M. Isolation and characterisation of bioactive peptides with in vitro renin inhibitory activities from the macroalga *Palmaria palmata*, 10th Nordic Nutrition Conference, Reykjavik, Iceland June, 2012.

FitzGerald, R.J. and Harnedy P.A. Biologically active peptides from marine protein sources. NutraMara Conference Dublin, June 2015

FitzGerald, R.J. and Harnedy P.A. Proteins and peptides from marine sources: their potential as functional food ingredients. NutraMara Conference, Dublin, April 2012.

Flaherty, T., Harnedy P.A. and FitzGerald, R.J. Assessment of the biological activity of peptide and amino acid fractions from the macroalgae *Palmaria palmata*. Department of Life Sciences 2013 Annual Research Day, UL, May 2013.

Flaherty, T., Harnedy P.A. and FitzGerald, R.J. Extraction of nitrogenous compounds from Irish macroalgae. The world is your oyster: How You Eat It Is Up to You, NutraMara Student Conference, Dublin, Oct 2013.

Flaherty, T., Harnedy P.A. and FitzGerald, R.J. Seasonal and Geographical Variation of Total Nitrogen, Protein Nitrogen and Non-Protein Nitrogen Content of Macroalgae Native to the Irish Coastline. Department of Life Sciences 2012 Annual Research Day, UL, May 2012.

Guihéneuf, F. and Stengel, D.B. LC-PUFA-enriched oil production by the marine haptophyte *Pavlova lutheri*: combined effects of light, temperature, and inorganic carbon availability. 5th Congress of the International Society for Applied Phycology, Sydney, Australia, June 2014.

Guihéneuf, F. and Stengel, D.B. The potential of microalgae as a source of food ingredients. NutraMara Conference, Dublin, Ireland, April 2012.

Guihéneuf, F. and Stengel, D.B. The potential of Microalgae for "Healthy" Foods. The 23rd Irish Environmental Research' Colloquium, ENVIRON 2013, Galway, Ireland, January 2013.

Harnedy, P.A. and FitzGerald, R.J. Bioactive proteins, peptides and amino acids from marine sources. NutraMara Training Day, Dublin, May 2009.

Harnedy, P.A. and FitzGerald, R.J. Bioactivity of *Palmaria palmata* hydrolysates in vitro. 2nd Kiel Food Symposium, Max Rubner-Institut, Kiel, Germany. May 2012.

Harnedy, P.A. and FitzGerald, R.J. Macroalgae: a reservoir of biofunctional peptides. NutraMara Industry Day, Dublin, Oct 2009.

Harnedy, P.A. and FitzGerald, R.J. Macroalgal protein a potential source of biofunctional peptides: Extraction optimisation. 1st Kiel Food Symposium, Max Rubner-Institut, Kiel, Germany. May 2010.

Hayes, M. Collagen and chitin generation from Irish Marine processing waste streams. NutraMara conference 2012, 24th and 26th of April 2012, Castleknock Hotel and Country Club, Dublin, Ireland.

Hayes, M. Las algas como Fuentes de ingredientes funcionales para alimentacion. CETMAR 2nd workshop, 24th June, Vigo, Galicia, Spain 2010.

Hayes, M. Marine bioactive peptides and proteins including betaines: potential industry applications and challenges. International Marine Ingredients Conference 2013 September 22nd-24th, Oslo, Norway.

Hayes, M. Marine bioresources for use as techno-functional and health beneficial ingredients. NutraMara conference 2015, 29th and 30th of June, 2015. Royal Dublin Society, Dublin.

Hayes, M. Proteins from Macroalgae: Isolation and characterisation of bioactive peptides from red and green macroalgal species, Natural Product Biotechnology, Aberdeen, Scotland. 18th November 2014

Hayes, M. Transport of food derived peptides across the blood brain barrier and development of an in vitro model. 106th AOCS annual meeting, 3-6th May 2015, Orlando, Florida, USA.

Hayes, M., Valverde, J. and Soler-Vila, A. Edible seaweeds as a source of bioactive ingredients for functional foods: Screening through pressurised liquid extraction and H-NMR. EuroFoodChem XVII, Istanbul, Turkey, May 10-13th 2013.

Hayes, M. Waste not want not: Bioactive peptides from marine resources including macroalgae and by-products. 105th AOCS annual meeting and expo, May 4-7th 2014, San Antonio, Texas, USA.

Kirke, D., D. K. Rai, Smyth, T. and Stengel, D.B. Environmental impact on phlorotannin profiles of commercially valuable Irish brown seaweeds. 6th European Phycological Congress (EPC6). London, 2015.

Kirke, D., D. K. Rai, Smyth, T. and Stengel, D.B. The influence of environmental factors on phlorotannin profiles of commercially valuable Irish brown seaweeds. Environ 25: Sustainability and Opportunities for Change. Sligo, 2015

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Schmid M. and Stengel D.B. Enhancement of PUFAs, phycobilins and carotenoids in two Irish macroalgal species (*Palmaria palmata*, *Fucus serratus*) through optimised cultivation. International Phycological Congress, Orlando Florida, USA, August 2013.

Schmid M., Guihéneuf F. and Stengel D. B. Plasticity of fatty acid profiles and contents in seaweeds. 6th European Phycological Congress, London, August 2015.

Schmid M., Guihéneuf F. and Stengel D. B. You are where and when you eat: seasonal and spatial variability in edible seaweed bioactives. The 23rd Irish Environmental Researchers' Colloquium, ENVIRON 2013, NUI Galway, Ireland, January 2013.

Stanton, C. Marine ingredients for enhanced gut and brain function. NutraMara Conference, Dublin, June 2015.

Stengel D. B. 'Variability in bioactivity of algal compounds – implications for commercial applications', NutraMara Conference, Dublin, April 2012.

Stengel D.B. Bioactives from marine algae – overview of some research activities at NUI Galway. 18-20 November Inverness, Scotland, oral presentation at Natural Product Biotechnology Inverness, UK, November 2014.

Stengel D.B. Variability in algal bioactives – the good, the bad and the unknown International Society of Applied Phycology, Sydney, invited symposium presentation, June 2014.

Stengel, D. B. Applications of marine algae in food and health – recent developments and remaining challenges. NutraMara Conference. Dublin June, 2015.

Stengel, D. B. Bridging the gap between algal ecology and biotechnology – more than just learning a new language. 6th European Phycological Congress (EPC6) London, 2015.

Stengel, D.B. Ireland has vast & valuable seaweed resources – fact or fiction? INFOMAR Seminar 2013 Achievements, Opportunities, Innovation. University of Limerick/Marine Institute. October 2013. Invited talk.

Stengel, D.B. The Algal Biorefinery Concept. The European Marine Biotechnology ERA-NET Stakeholders' Meeting, Portugal. Invited keynote address, Lisbon, October 2014.

Strain, C.S., Allsopp, P.J., Brown, E.M., Magee, P.J., Gill, C.I.R., McSorley E.M. Prebiotic potential of polysaccharide rich extracts obtained from *Laminaria digitata*. NutraMara student workshop, The World is Your Oyster: How you Eat It Is Up To You. October 2013.

Troy, D.J. Marine Functional Foods – Research Needs Biomarine International Conference, Halifax Nova Scotia September, 2013.

8.5.6. Conferences

The NutraMara programme organised various conferences and workshops aimed at securing insights from industry; to highlight research programme outputs; to promote opportunities for industry collaboration; and to provide opportunities to early stage researchers to discuss their work. The two international conferences organised by the NutraMara consortium provided an opportunity to build new national and international linkages, raise the international profile of Ireland's marine functional foods research capacity and showcase research outputs. Together, the 2012 and the 2015 conferences attracted over 350 participants from industry, national research funding agencies, government departments and research performers from Ireland and overseas. Dedicated conferences targeting specific groups were also organised; including an early stage research conference and industry

conference. Table 35 below lists the conferences and the numbers of participants. Appendix 5 contains the programmes from these conferences and indicates the diversity of topics presented and discussed.

Table 35 NutraMara Conferences and Workshops

Conference	Target audience	Date	Number of attendees
Research opportunities for industry	Industry	2010	60
NutraMara Conference	Industry/research	2012	102
NutraMara Student Conference	Early researchers	2013	40
NutraMara Conference	Industry/research	2015	250

8.6. Additional research activity/awards

NutraMara has created a solid research platform and generated new research capacity that is being used to develop functional food ingredients from marine bio-resources. The strength of the NutraMara research programme is reflected in the successes of Principal Investigators working within the research consortium in winning significant further research funds. These awards build on the expertise of the PI, and the network of multi-disciplinary research activity of NutraMara. A characteristic of the additional research awards is the inter-institution collaborations involving PIs working within the NutraMara programme. Not only have NutraMara PIs been successful in securing funds from national agencies, they have also secured research funds from international sources and in doing so, built new collaborative linkages (Table 36). Principal Investigators involved in the NutraMara consortium secured additional funds of close to €6.2 million for research on the use of marine organisms in food and health related applications.

Table 36 Research awards to NutraMara Scientists

Project title	Host	PI	Partners	Grant	Source
SMART FOOD-Science based 'Intelligent/Functional and Medical Foods for Optimum Brain Health, Targeting Depression and Cognition	Teagasc	Catherine Stanton	NUI Galway, UCC	€595,846	DAFM
Marine Compounds to enhance productivity and health in pigs	UCD	John O'Doherty	n/a	€99,387	DAFM
Seaweed extracts to reduce Campylobacter in chickens	UCD	Torres Sweeney	n/a	€95,195	DAFM
Seaweeds as a source of non-digestible complex polysaccharide components for the development of novel prebiotic ingredients for the functional food industry	UCC	Paul Ross	NUI Galway, UU	€601,078	DAFM
The anti-inflammatory and microbial modulating effects of marine derived laminarin and omega-3 fatty acids on inflammatory bowel disease in an	UCD	Torres Sweeney	UCC	€493,064	DAFM

experimental porcine model					
Marine sourced Peptides for Glycaemic Management	UL	Dick FitzGerald	UU	€581,117	DAFM
Iodine in commercially valuable Irish seaweeds: variability, pathways, and implications for industrial applications	NUI Galway	Dagmar Stengel	n/a	€244,843	SFI
Optimisation and standardisation of phlorotannin profiles of commercially valuable seaweeds with food applications	NUI Galway	Dagmar Stengel	Teagasc	€78,000	Teagasc
Comparative extraction technologies applied to selected micro- and macroalgae for optimised compound yield	NUI Galway	Dagmar Stengel	n/a	€24,000	IRC
Technology Development Award	UL	Dick FitzGerald	n/a	€115,682	SFI
The Macroalgal Fibre Initiative: 'natural molecules naturally'.	UCD	John O'Doherty	UCD	€1,222,140	SFI
Delivering Processed Meat Products with Health Benefits – NutriMeat	UCD	Joe Kerry	Teagasc, UCC	€141,400	DAFM
Fulbright Scholarship	Harvard University	Ruairi Robertson	Teagasc, UCC		Fulbright
Isolation of fatty acids from seaweeds	Teagasc	Michelle Tierney	Memorial University Canada	Ireland-Newfoundland partnership	
Natural compounds to enhance productivity, quality and health in intensive farming systems	UCD	John O'Doherty	UCD	€533,125	EU
Novel Extraction Processes for multiple high-value compounds from selected Algal source materials	NUIG	Dagmar Stengel	UCC, Ghent, Unilever, & UiTromso	€759,976	EU

The high visibility of the NutraMara nationally, and the significant research outputs, together have attracted “new blood” into the marine functional foods research area. As a result, a number of NutraMara Principal Investigators were invited to join other research consortia in making submissions in response to national and international research calls. Table 37 lists the projects where NutraMara PIs are involved in funded projects and where new PIs were successful in winning marine foods related research awards that are linked to the work of NutraMara. Research grants in excess of €3 million contribute to expanding marine functional foods related research as well as generating new research capacity, much of which leverages upon the work done within the NutraMara consortium, including access to marine materials collected by the consortium.

Table 37 Projects with links to NutraMara research activity

Project title	Host	PI	Partners	Grant	Source
The use of marine derived antibacterial agents to combat the prevalence of Salmonella in pork products	UCC	Alan Dobson	Teagasc	€490,000	DAFM
Antioxidant and anti-inflammatory ingredients for health enhancement in the older population	UL	Dick FitzGerald	UCC	€495,472	DAFM
Shelf-life Extension ingredient and processing technologies Applied to Fish	Teagasc	Declan Bolton	UCD	€583,100	DAFM
Adding value to ready to eat crustacean products by improving their quality, safety and shelf life enhanced conventional and novel processing methods	UCD	James Lyng	Teagasc	€599,983	DAFM
Arsenic in Marine Macroalgae and Implications for Commercial Uses	MI	Evin McGovern	NUIG	€277,437	DAFM
Mining marine materials for novel functional ingredients that modulate the immune response for benefit in inflammation and allergy	DCU	Christine Loscher	UL	€557,526	DAFM
Anticancer activity of seaweed extracts	UU	Philip Allsopp	IT Sligo	€6,000	UU

9. CONCLUSIONS

The NutraMara programme spanned the period 2008 to 2015 and involved six research partners collaborating on a jointly agreed research agenda to explore marine organisms as a source of functional ingredients. Allied to this goal, was a series of initiatives designed to provide feedback on research findings to industry and other audiences; attract additional research funds; and provide early stage researchers with opportunities for professional development. From the outset, NutraMara sought to develop synergies with other research projects possessing a marine bioresources or functional food research component. The funds provided by the Marine Institute and the Department of Agriculture, Food and the Marine led to the creation of a unique research capability, where expertise from the marine sciences and food sciences work on a common research agenda. This agenda anticipated and initiated areas of research subsequently identified as priorities in national strategies and development plans. NutraMara contributed to the international profile of Ireland as a focal point for functional foods research with the capabilities to engage in high-quality research. Findings from NutraMara have contributed to the world body of knowledge regarding the potential of developing novel functional ingredients from marine bioresources. The vision of providing dedicated funds to build research competencies in marine functional food has been amply rewarded as evidenced by;

- Ireland's capacity to engage in leading edge functional foods research being further strengthened and reflected in the large number of peer reviewed publications, PhDs awarded and other publications produced by the project.
- NutraMara has created research critical mass, attracting food and marine scientists to collaborate, training 39 new researchers (PhD and PDoc) to work in an area of increasing global importance; and in doing so establishing a solid foundation of expertise on which to build further capabilities for marine functional foods research.
- As the first such integrated research programme in the marine-food space, it placed Ireland to the fore in marine functional foods research and raised the international visibility of Irish researchers leading to international research collaboration.
- Research collaborations established between researchers in the NutraMara initiative facilitated a role for Irish researchers as partners in EU projects that target the use of marine bioresources.
- Highlighted the strategic importance of understanding marine bioactives and functional compounds from marine bioresources and of the need to employ environmentally, socially and economically sustainable methods in further developing them into value-added products.
- Established Ireland as a focal point for marine functional ingredients research, resulting in invitations from companies, research institutions and regional governments in Europe, North America and the Asia-Pacific region inviting NutraMara staff to participate in a variety of discussions overseas and to receive fact-finding missions to Ireland.

- Enabled linkages and synergies between the Beaufort Biodiscovery project and the industry oriented Food for Health Ireland initiative, leading to shared services in managing data/materials, access to equipment and the provision of marine origin materials for evaluation on foot of formal agreements between projects.
- Attracted a wide range of research expertise and competencies from areas typically unconnected with the marine foods/marine research to work on related projects, creating an awareness of new methods and providing access to specialised equipment and facilities.
- Broadened the awareness of the potential of marine bioresources in national policy and strategy development, through the provision of knowledge and personnel to participate in working groups, steering groups etc. on Food Research Ireland, the National Research Prioritisation Exercise, Harnessing Our Ocean Wealth, SHARP, FoodHarvest 2020, Foodwise2025 and others.
- Contributed to marine bioactive compounds, functional foods and marine biotechnology research being prioritised by the Research Prioritisation Exercise, and identified as key opportunity areas requiring further research activity by SHARP and FoodWise 2025.
- Stimulated links between industry and research providers and created an awareness in industry, much of it unconnected with the marine sector, of the potential of marine bioresources as a source of novel compounds for food use as ingredients and in functional foods.
- The knowledge transfer activity of NutraMara resulted in the provision of know-how to companies seeking to create value from marine bioresources; leading to contracts for in-company consultancy between various firms and members of the NutraMara consortium.
- Provided specialised training to the next generation of research scientists by supporting students engaged on the NutraMara project to work in overseas laboratories to access specialised equipment, receive mentoring and attend conferences.
- Resulted in the creation of a network of leading experts in food and marine bioresources, which has demonstrated a lasting and successful collaboration in securing new research funding for marine functional foods and marine biotechnology oriented projects. This informal network supports the exchange of early stage researchers and has provided opportunities for PIs to work within new research areas.
- The initial involvement of Ulster University (UU) in the NutraMara initiative led to further and extensive cross-border collaboration between UU and Universities in the rest of the country, and with Irish industry, where the nutritional sciences expertise of UU is integrated within other marine foods/nutrition related projects.
- Returned significant value from the original investment; producing 80 peer reviewed publications at an average cost of €65,000 per publication and generating additional research funds of €9.2 million, almost double the original grant award to the NutraMara consortium.
- Results from the NutraMara project highlight the importance of understanding the biology of marine species when seeking to utilise them as sources of food ingredients. Knowledge of the natural variations that exist in the same species is essential when seeking to optimise processing conditions. The results also point to the importance of quantifying the extent of Ireland's marine bioresources, if the harvesting and use of marine species is to be labelled as "sustainable".

- NutraMara has provided Ireland's food ingredients, marine foods and marine bioresources companies with new knowledge about the use of compounds extracted from marine species, with a source of expertise and research leadership to draw upon and equipped early stage researchers with industry relevant experience.
- As a unique research initiative NutraMara has become strategically important in the development of Ireland's marine bioresources sector and an essential knowledge provided to end-user of marine materials. Research outputs and impacts demonstrate its potential to address the challenges and opportunities described in Harnessing Our Ocean Wealth, FoodWise 2025 and the SHARP research plan.
- The collaboration between leading Irish scientists working on the NutraMara initiative and other Irish scientists engaged in related research created a foundation on which to build a major research initiative. Such an initiative could make wide-ranging contributions to increase the competitiveness of Ireland's marine bioresources sector. These include the creation of new revenue streams from marine bioresources; building science and technology platforms to underpin efforts to increase the value of marine bioresources; using research outputs to enhance the marketability of marine origin products; and provide robust, scientifically validated knowledge of the quality attributes of marine bioresources.

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APPENDIX I: HEALTH OUTCOMES ATTRIBUTED TO MARINE BIOACTIVES

Summary of studies investigating the associations between marine bioactives and health outcomes in humans

Author, year	Study, country	Subject status (n)	Sex (age range)	Length of study	Marine bioactive	Groups	Markers examined	Main outcome	Potential limitations
Cardiovascular disease and related risk factors									
Teas <i>et al.</i> , 2009	RCT, Ecuador	Individuals with at least one symptom of the metabolic syndrome (27)	Male (47.7 \pm 9.9yr) female (45.6 \pm 12.2yr)	2 months	<i>Undaria pinnatifida</i>	Group 1 - 1m placebo + 1m 4 g/d seaweed Group 2 - 4g/d seaweed + 1m 6g/d seaweed	Blood pressure, weight, waist circumference, inflammatory markers, lipids	Reduction in symptoms of the metabolic syndrome	Small sample size. Carried out in healthy individuals.
Ionov VA & Basova, 1978	Intervention study, Russia	Ischemic heart disease; atherogenic dyslipidemia	-	-	<i>Spirulina platensis</i>	-	-	Corrected lipid and hemostatic disturbances	
Park, 2010	RCT, Korea	Healthy female college students (14)	Female (20.2-22.8 yrs)	8 weeks	Astaxanthin	0mg, 2mg or 8mg/d astaxanthin per day	Immune function and oxidative status	Asaxanthin decreases a DNA biomarker and acute phase protein and enhances immune response in healthy female individuals.	Small sample size. Carried out in healthy individuals.
Neff <i>et al.</i> , 2010	RCT, America	Overweight or obese adults (36)	Male and females (16-18 yrs)	4, 5 mo	Algal DHA	(i) 2g/d of algal DHA (ii) placebo	Plasma lipids and lipoprotein concentrations	DHA supplementation resulted in potentially beneficial changes in some markers of cardiometabolic risk, where as other markers were unchanged.	Small sample size. Limitations with the use of multiple statistical comparisons.
Geppert <i>et al.</i> , 2005	RCT, Germany	Healthy vegetarians (104)	Females & males (18-43 yrs)	8 weeks	Docosahexaenoic acid (microalgae Ulkenia sp.)	(i) daily consumption of a microalgae oil from Ulkenia sp. (0.94 DHA/d) (ii) olive oil (placebo)	RBC & phospholipid DHA and EPA	Increased the omega-3 index from 4.8 to 8.4 wt%	
Geppert <i>et al.</i> , 2006	RCT, Germany	Normolipidaemic vegetarians (114)	Females & males (18-43 yrs)	8 weeks	Docosahexaenoic acid (microalgae)	(i) daily consumption of a	Fatty acids, TG, total and HDL	Improvements with CHD risk factors (plasma TG,	

Conquer <i>et al.</i> , 1996	RCT, Canada	Healthy vegetarians (24)	Females & male (29.6 ± 1.7 yrs)	6 weeks	Algae source of Docosahexaenoic acid	microalgae oil from Ulkenia sp. (0.94 DHA/d) (ii) olive oil (placebo)	cholesterol, platelet function, full blood cell counts	TG:HDL cholesterol ratio, however LDL cholesterol worsened. Therefore the overall effect of this treatment is unclear.	Small sample size
Weight management									
Paxman <i>et al.</i> , 2008	Intervention, UK	Healthy (68) BMI 18.5 - 32.81 kg/m ²	Men and women (19 - 65 years)	4 weeks	Sodium alginate from brown algae	Placebo-controlled, single blind crossover study. 1 x 1.5 g sodium alginate drink or control slimfast p/day for 7 days followed by 2 week washout then crossover.	Energy intake (7 day food diary) analysed using NetWISP	↓ in daily energy consumption for sodium alginate group of 135 kcal (174 kcal adjusted for control calorie contribution) compared to control group. No side-effects.	Short study length, only one parameter measured.
Paxman <i>et al.</i> , 2008	Intervention, UK	Healthy (14) BMI 23.9 ± 3.4 kg/m ²	Men (26.9 ± 8.6 years (SD))	1 day measurement	Sodium alginate from brown algae	Randomized, single blind, placebo-controlled crossover study. 1 x 1.5 g sodium alginate drink or control drink on day of study 3 hours after set breakfast before consuming test lunch. 7 days washout then crossover.	Blood cholesterol, triacylglycerols and glucose levels pre and postprandial	Sig correlation of body fat with ↑ uptake of cholesterol. Higher BMI = increased uptake but lower BMI ≠ increased uptake. Alginate ↓ cholesterol uptake in subjects with higher BMI. Similar NS effect observed with glucose levels. No change in triacylglycerols.	Small sample size
Williams <i>et al.</i> , 2004	Intervention, USA	Healthy (48) BMI 19.3 - 29.8 kg/m ²	Men (39) and women (9) (19 - 75 years)	1 day measurement	Alginate	Randomized, double blind, placebo-controlled crossover study. Subjects participated in 2 separate 3-hour meal tolerance	Blood glucose levels pre and postprandial (glucose tolerance)	Alginate/guar gum crispy bar ↓ incremental blood glucose levels compared to control. Incremental peak blood glucose concentration sig ↓ by 30 % after alginate/guar gum treatment compared to	Only one parameter measured

Wolf et al, 2002	Intervention, USA	Normal and overweight but otherwise healthy (30)	Women (19) and men (11) (36 ± 2 years (SE))	1 day measurement	Alginate	tests after consuming test or control bar from overnight fasting. 7 days washout then crossover.	Randomized, double blind, placebo-controlled crossover study. Subjects participated in 2 separate 3-hour meal tolerance tests after consuming test or control drink from overnight fasting. 7 days washout then crossover.	Serum glucose and insulin levels pre and postprandial (glucose tolerance)	NS trend towards reduced average incremental change in peak serum glucose levels for alginate supplemented drink. Sig ↓ in serum glucose. Mean peak serum insulin levels over the 3 hour study were higher for alginate compared to control but no change in incremental serum insulin for alginate compared to control.	control.	
Peikman et al, 2007	Intervention, USA	Overweight and obese but otherwise healthy (29)	Women (20 - 40 years)	5 weeks	Alginate	Placebo-controlled, double blind crossover study. 3 formulations of test drink: 1.0g/drink, 2.8g/drink or control drink with no alginate. 2 x test beverage p/day for 7 days. 1 week washout then crossover.	Energy intake on day 1 and day 7 recorded by monitoring feeding of breakfast, lunch, dinner and snacks in the feeding laboratory.	↓ in energy consumption of ~ 20, 22 and 75 kcal for breakfast, lunch and evening meal after pre-breakfast and 2.5 hours post-lunch consumption of both active drinks compared to control group. Mild adverse effects reported.	Short study length, only one parameter measured.		
Odunsi et al, 2009	Intervention, USA	Overweight and obese but otherwise healthy (48) BMI 25 - 45 kg/m ²	Men and women (18 - 65 years)	10 days	Alginate from brown seaweed Laminaria digitata	Randomized, placebo controlled, blind parallel study. Participants consumed 3 x alginate or control capsules for 7 days before main meal, then 6 x capsules the following 3 days	Gastric volume, gastric emptying, satiation, appetite assessment, levels of gut hormones	No differences in effect observed for the alginate treatment group and control group on gastric volume and emptying, gut hormone levels, satiation and total calorie intake.	Lack of blood analysis to determine absorption of nutrients after consumption of meal major drawback. Also parallel nature of the study less desirable than a crossover study.		

Diabetes

Lee et al, 2010	Observation, Korea	Healthy (3405)	Men (1389) and women (2016) (20 - 65 years)	-	Porphyria yezoensis and Undaria pinnatifida	Cross sectional nationally representative survey from non-institutionalized individuals in Korea.	Food frequency questionnaire, diabetic status (fasting blood glucose levels), BMI, waist circumference, blood pressure, blood lipids	Overall prediabetes and diabetes groups had higher BMI, waist circumference, blood pressure, total cholesterol and triglyceride levels than non-diabetic group, but lower HDL cholesterol levels. Trend test showed marginal relationship between algae consumption and diabetes risk in men.	Difficult to determine causal relationship between algae consumption and diabetes using such method. Error associated with FFQ. Quantities and type of algae consumed were not measured, nor was the processing method.
Torsdottir et al, 1991	Intervention, Sweden	Type 2 diabetes (7)	Men (39 - 58 years)	1 day measurement	Alginate	Randomized, placebo controlled, crossover study. Participants consumed drink with 5 g of sodium alginate or control drink after fasting on day of measurement	Blood glucose, serum insulin and plasma C-peptide levels and gastric emptying	Sodium alginate ↓ postprandial blood glucose, serum insulin and plasma C-peptide levels by 31, 42 and 35 % respectively. Alginate also sig ↓ gastric emptying rates compared to control.	Short study therefore only a brief indication and cannot predict long term effects.
Maeda et al, 2005	Intervention, Japan	Impaired glucose tolerance and type 2 diabetes (76)	Men (28) and women (48) (58.6 ± 6.4 years (SD))	12 weeks + 4 weeks run-in	Agar	Randomized, controlled, parallel study. Participants maintained habitual diet for 4 weeks, then were assigned to either conventional diet and exercise or conventional diet + agar (180 g/day) supplementation before evening meal and exercise.	Body weight, BMI, glycaemic control, blood pressure, insulin resistance, total body fat, fat distribution and lipid profile	Body weight and BMI decreased in both groups but the ↓ in the agar group was greater (4.4 and 1.5 % reduction in body weight and BMI respectively). Fasting plasma glucose levels were ↓ after 12 weeks in both groups as was blood pressure. Total cholesterol was only significantly ↓ in the agar group. Significant ↓ in total body fat, visceral fat and subcutaneous fat in the agar treated group.	Lack of placebo in control group may affect the results.
Kim et al, 2008	Intervention, Korea	Type 2 diabetes	Men (9) and women	4 weeks	Undaria pinnatifida	Randomized,	Blood glucose, total	↓ in fasting and	Measured markers

			(20)	(11) (40 - 70 years)		and Saccharina japonica	placebo controlled, parallel study. Subjects were supplemented with either placebo or powdered seaweed pills, consumed 3 x per/d (total daily consumption = 48g) and maintained normal diet	cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, hemoglobin A1c	postprandial blood glucose in seaweed supplementation group compared to baseline but not in control group. Triglycerides were ↓ and HDL cholesterol was ↑ in seaweed supplementation group compared to baseline and ↓ LDL cholesterol and triglycerides compared to control group.	of diabetes but did not measure changes in body weight or BMI after 4 week supplementation.
Bone Health										
Fajita et al. 1997	Parallel Intervention	Osteoporotic or osteopenic (137)	51-83 predominantly female < 10% male	36 Months	900mg calcium as AAA Ca (AAACa);	(i) The control group (No specific treatment for osteoporosis) (ii) Daily oral administration of 900mg calcium as AAA	Bone Mineral density	A significantly increase BMD of 4.5% relative to baseline compared to a 3.5 % decrease observed in control treatment	Unblinded study; not placebo controlled; A lack of clarity of control conditions; A lack of continuity in group numbers; no declaration of exact number of drop out;	
Fajita et al. 1996	Randomised double blind parallel placebo controlled trial	Hospitalised females (58)	Postmenopausal females (65-96)	24 months	900mg calcium as AAA Ca (AAACa);	Treatment 1: heated oyster shell-seaweed calcium (AAA Ca) (900mg/day 6x 150mg capsules; Treatment 2: calcium carbonate 900mg Calcium/day - 6x 150mg capsules); or Treatment 3: placebo 0 mg Calcium/day - 6x 0mg capsules.	Bone Mineral density; Parathyroid hormone; alkaline phosphatase	Significantly elevated BMD compared to placebo and a significantly reduced PTH and AP relative to placebo	The study could benefit from increased sample size; this study was carried out on an extremely elderly population	
Fajita et al. 2000	Randomised double blind parallel placebo controlled trial	Osteoporotic or osteopenic females (34)	Female (26-91)	4 months	900mg calcium as AAA Ca (AAACa);	Treatment 1: heated oyster shell-seaweed calcium (AAA Ca) (900mg/day 6x	Radial Trabacular bone density and Cortical bone density	Significantly elevated Radial Trabacular bone density (P=0.0435) and Cortical bone density (P=0.0219) compared to	This small sample size severely limits any clear conclusions to be drawn from this	

Frestedt et al . 2008	Randomised double blind parallel placebo controlled trial	moderate to severe knee osteoarthritis	Male & female (68-61)	12 weeks	Aquamin	150mg capsules; Treatment 2: heated oyster shell calcium (AA Ca) (900mg/day 6x 150mg capsules) Treatment 3: calcium carbonate calcium carbonate 900mg Calcium/day - 6x 150mg capsules); or Treatment 4: placebo 0 mg Calcium/day – 6x 0mg capsules.	(a) Glucosamine sulfate (1500 mg/d); (b) Aquamin (2400 mg/d); (c) Combined treatment composed of Glucosamine sulfate (1500 mg/d) plus Aquamin (2400 mg/d) and (d) Placebo	6 minute walking distance (6 MWD), range of motion (ROM), and pain and joint mobility measured by the Western Ontario and McMaster Universities (WOMAC)	WOMAC score was significantly improved by 14.9 (P = 0.006 ANOVA) for Aquamin and 10.8 (P = 0.007 ANOVA) for Glucosamine. Aquamin and Glucosamine groups walked 101 feet (+7%) and 56 feet (+3.5%) extra respectively. Aquamin showed significant improvement over time for stiffness score (20.6, p = 0.002)	placebo and a significantly reduced PTH and AP relative to placebo	study.	
Frestedt et al . 2009	Randomised double blind parallel placebo controlled trial	moderate to severe knee osteoarthritis	Male & female (65-75)	12 weeks	Aquamin		(i) Aquamin (2400 mg/d); (ii) Placebo	6 minute walking distance (6 MWD), range of motion (ROM), and pain and joint mobility measured by the Western Ontario and McMaster Universities (WOMAC)	Significant improvement in passive and active extension ROM (p = 0.028) and 6 MWD (p = 0.03) but only following a 50% reduction in NSAID use	Unfortunately this study was a small sample size, which was made worse by dropouts during NSAID withdrawal due to pain, with only 6 in the Aquamin treatment group at the 50% NSAID withdrawal		
Myers et al . 2010	open label combined phase I and II pilot scale study	moderate to severe knee osteoarthritis (12)	Male & female (18-65)	12 weeks	Maritech® seaweed extract plus vitamin B6, zinc and manganese.		(i) 100mg/day; (ii) 1000mg/d	Comprehensive arthritis test (COAT) score which is comprised of four	Clear dose response effect seen between the two treatments (P≤ 0.0005) on the average COAT score	This is a small pilot study to investigate the safety and potential of the		

Shibata, 2004.	pilot study, USA	Pre-menopausal women with abnormal cycles and dysmenorrhea. Otherwise in general good health. (3)	women, 21-43 years	Subjects treated over 7 consecutive menstrual cycles.	<i>Fucus vesiculosus</i>	patients were put on 2mg isoflavones/kg body weight and after a 3 wk wash out period subjects were crossed over to alternative supplement schedule	OHE:16alpha-hydroxyestrone	include modulation of colonic bacteria. Inverse correlation between seaweed dose and serum E2. Seaweed plus soy increased urinary 2-OHE and ratio of 2-OHE:16alpha-hydroxyestrone.	composition typical of the population. Estimate of seaweed dose occurred post hoc.
Iso and Kubota, 2007	cohort, Japan	residents in 45 areas of Japan (109,778 at baseline)	women, <65 - >80 years	13-15 years	seaweed (algae)	<3/wk, 3-4/wk, >5/wk	length of menstrual cycle (all subjects), plasma 17beta-oestradiol and progesterone (subject 1)	Following treatment menstrual cycle length increased in all subjects by 5.5 - 14 days. In subject 1 dose-dependent decrease in plasma 17beta-oestradiol and increase in progesterone.	Small sample size, no control group, lack of data on other hormones associated with menstrual cycle.
Allen et al., 2004	prospective, Japan	residents of Nagasaki and Hiroshima (18,115)	men and women, 40-79 years	17-33 years	seaweed	<2/wk, 2-4/wk, almost daily	mortality from all causes, cancer, CVD, ischemic heart disease	Seaweed intake associated with lower mortality from lung cancer (men & women) and pancreatic cancer (men).	method of assessing seaweed intake could be more rigorous
Severson et al., 1989	prospective, Japan	Japanese ancestry living in Hawaii (7999)	males, <65 - >80 years at recruitment	18-21 years	nori, kobo and other seaweeds	≤1/wk, 2-4/wk, >5/wk	prostate cancer incidence	no association between consumption of seaweed and prostate cancer risk	Questionnaire covered limited number of foods and portion size not included. Low number of men with dietary information at 2 time points.
Hoshiyama and Sasaba, 1992	case-control, Japan	single stomach cancer (216), multiple stomach cancers (35), controls (residents of Saitama, Prefecture) (483)	males, <54 - >65 years		seaweed	≤1/wk, 2-4/wk, >5/wk	questionnaire on dietary habits including frequency of consumption of 12 food items and 12 food groups; lifestyle	seaweed consumption protective against stomach cancer (single and multiple); dose response relationship.	Authors noted could be "chance finding" given that seaweed not consumed in large amounts in western diet.

Mathew et al., 1995	intervention, India	pan tobacco smokers with oral leukoplakia (87)		12 months	Spirulina fusiformis (SF)	(i) Spirulina fusiformis 1g/day or (ii) placebo for 12 months	questionnaire lesion regression	SF supplementation induced complete regression of lesions in 45% of subjects v. 7% in placebo group. Response was more pronounced in homogenous lesions. No toxicity observed.	small sample size
Anti viral									
Cooper, et al. 2002	intervention/observational, Tasmania	HSV infected (active & latent) otherwise healthy (15)	Men and women (10 - 72 years)	1-24 months	Tasmanian Undaria pinnatifida (GFS)	4 x 560mg GFS p/day for initial 10 days followed by maintenance dose for 6 patients	Herpetic viral infection symptoms	Reported lessening or disappearance of symptoms; No further outbreak when on maintenance dose; No side-effects	Small sample size; not placebo controlled; unblinded
Abbreviations: NS, non- significant; Sig, significant; RCt, randomised control trial									

APPENDIX 2 – MICRO-ALGAL STRAINS IN CULTURE COLLECTIONS

International Culture Collections

Algobank, France:	http://www.unicaen.fr/algobank/
American Type Culture Collection:	http://www.atcc.org
Antarctic Protist Culture Collection at Woods Hole, MA:	http://www.whoi.edu/science/B/protists/
Canadian Center for the Culture of Microorganisms (includes marine algae in NEPCC and Freshwater Algal Collection at UBC):	http://www3.botany.ubc.ca/cccm/index.html
Cawthron Micro-Algae Collection:	http://www.cawthron.org.nz/seafood-safety-biotechnology/micro-algae-culture-collection.html
Chlamydomonas Genetics Center:	http://www.chlamy.org
Coimbra Culture Collection of Algae, Portugal (ACOI):	acoi.ci.uc.pt/
CSIRO Microalgae Research Center, Australia:	http://www.marine.csiro.au/microalgae/
Culture Collection at University of Marburg, Germany (based on the collection of Professor H.A.von Stosch):	http://staff-www.unimarburg.de/~cellbio/welcomeframe.html
Culture Collection of Algae and Protozoa:	http://www.ife.ac.uk/ccap
Culture Collection of Algae at the University of Cologne, Germany (CCAC):	http://www.ccac.uni-koeln.de/
Culture Collection of Algal Laboratory, Trebon, Czech Republic:	http://www.butbn.cas.cz/ccala/ccala.htm
Culture Collection of Algae of Charles University Prague:	http://botany.natur.cuni.cz/algo/caup.html
Culture Collection of Baltic Algae (Gdansk, Poland):	www.ocean.univ.gda.pl/~ccba/ien.php
Culture Collection of Cyrophilic Algae, Germany:	http://cccryo.fraunhofer.de/web/infos/welcome/
Culture Collection of Microorganisms from Extreme Environments:	http://cultures.uoregon.edu/default.htm
Dunaliella Culture Collection:	http://www.dunaliella.org/dccbc/
Hawaii Culture Collection (see HR BioPetroleum):	www.hrbp.com/Technology/CultureCollection.html
IAM Culture Collection in Japan (Transferred to National Institute of Environmental Studies (NIES), Japan):	http://www.nies.go.jp/biology/mcc/home.htm
Loras College Diatom Culture Collection, USA (Transferred to UTEX):	http://www.bgsu.edu/departments/biology/facilities/algae/html/DiatomCulture.html
Marine Biotechnology Institute Culture Collection in Japan (Transferred to National Institute of Technology & Evaluation):	www.nbrc.nite.go.jp/e/070328-e.html

National Institute of Environmental Studies, Japan (NIES):	http://www.nies.go.jp/biology/mcc/home.htm
Pasteur Culture Collection of Cyanobacteria:	http://www.pasteur.fr/recherche/banques/PCC/
Provasoli-Guillard Culture Collection of Marine Phytoplankton:	http://ccmp.bigelow.org
Roscoff Culture Collection, France (RCC):	http://www.sb-roscoff.fr/Phyto/RCC/index.php
Sammlung von Algenkulturen Goettingen Germany (SAG):	http://www.epsag.uni-goettingen.de
Scandinavian Culture Collection of Algae and Protozoa:	http://www.sccap.dk
University of Texas Culture Collection of Algae:	http://www.utex.org/

APPENDIX 3 – NUTRAMARA TECHNOLOGY UPDATES

UPDATES

Seaweed Inclusion in Fish Food

KEY EXTERNAL STAKEHOLDERS

Consumers, feed producers, fish farming operations

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

Seaweeds can act as a source of protein, fatty acids, and carbohydrate in fish feeds. This offers alternative means of formulating diets and has potential implications for fish health, growth and sustainability of feed production.



MAIN RESULTS

A number of seaweed species were trialled and there were generally no negative effects of inclusion rates up to 15% of dry feed weight.

Pigment from seaweed in feeds is taken up by salmon, resulting in a range of flesh colours.

OPPORTUNITY/BENEFIT

Depending on the amount of seaweed added and the materials cost, seaweed inclusion into salmon diets can potentially save up to 14% on feed costs. The pigments and eventual flesh colour differ from the existing market norms. This may be an opportunity to develop a market niche for salmon of a novel flesh colouration.

COLLABORATING INSTITUTIONS

National University of Ireland, Galway
University College Cork

Project Reference: NutraMara

Funding Source: DAFM/MI

Date: November, 2014

Project Dates: April 2008 – June 2015

Extraction, Purification and Characterisation of Biofunctional Peptides from Marine Processing Co-Products

KEY EXTERNAL STAKEHOLDERS

Marine processors, government authorities/legislators/funding agencies, food ingredients industry, consumer food sector, academic and food research institutes.

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

This project highlighted the potential of marine processing by-products such as salmon trimmings and skin, blue mussel meat and byssus as candidate raw materials for the generation of bioactive peptides.



MAIN RESULTS

- Sustainable protein-rich marine by-products have been identified that can be used as substrates for the generation of bioactive peptides
- Industrially relevant protocol for the extraction of protein from salmon trimmings and mussel meat, as well as gelatin and collagen from both salmon trimmings and mussel byssus, respectively, have been adapted and optimised
- A number of candidate protein hydrolysates with high *in vitro* multifunctional activity were generated
- These protein hydrolysates have applications as functional food ingredients for the management of metabolic conditions such as hypertension and Type 2 diabetes.

OPPORTUNITY/BENEFIT

The outcomes from this project provide a significant opportunity for the marine industry to add value to existing marine processes through the upgrading of low value marine co-products to high value functional food ingredients. Furthermore, it will provide industry with a means for dealing with the high disposal costs and legal restrictions associated with materials that are otherwise considered as waste.

COLLABORATING INSTITUTIONS

- Ulster University
- University College Cork
- University College Dublin
- NUI, Galway
- Teagasc

Project Reference: MFFRI/07/01

Funding Source: MI/DAFM

Date: 2007

Project Dates: Jan 2012 – July 2015

Extraction, Purification and Characterisation of Biofunctional Peptides from Marine Processing Co-Products

KEY EXTERNAL STAKEHOLDERS

Marine processors, government authorities/legislators/funding agencies, food ingredients industry, consumer food sector, academic and food research institutes.

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

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COLLABORATING INSTITUTIONS

- Ulster University
- University College Cork
- University College Dublin
- NUI, Galway
- Teagasc

Project Reference: MFFRI/07/01

Funding Source: MI/DAFM

Date: 2007

Project Dates: Jan 2012 – July 2015

Mining, Extraction and Purification of Proteins and Peptides from Macroalgae

KEY EXTERNAL STAKEHOLDERS

Seaweed processors, seaweed aquaculture sector, food ingredients industry, consumer food sector, government authorities/legislators/funding agencies academic and food research institutes.

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

This project highlighted the potential of macroalgae as rich protein sources for the mining of peptides which beneficially modulate biomarkers associated with type 2 diabetes and cardiovascular disease.



MAIN RESULTS

- Macroalgae, in particular the red species *Palmaria palmata*, have been identified as a protein rich source for the mining of bioactive peptides
- An integrated industrially relevant protocol for the extraction of crude samples of proteins, peptides and amino acids from macroalgae was developed
- A number of candidate macroalgal-derived protein hydrolysates with high *in vitro* multifunctional anti-diabetic, cardioprotective and antioxidant activity were generated
- Several novel peptides with high *in vitro* anti-diabetic, antioxidant and multifunctional bioactivity have been identified

OPPORTUNITY/BENEFIT

The outcomes from this project provide the seaweed industry with scientific knowledge for the extraction and hydrolysis of proteins from macroalgae. It also identifies macroalgae as a source of high value bioactive peptides that have potential applications as functional food ingredients for the management of type 2 diabetes and cardiovascular disease.

COLLABORATING INSTITUTIONS

- Ulster University
- University College Cork
- University College Dublin
- NUI, Galway
- Teagasc

Project Reference: MFFRI/07/01

Funding Source: MI/DAFM

Date: 2007

Project Dates: Jan 2009 – Dec 2013

Extraction of Nitrogenous Compounds from Macroalgae

KEY EXTERNAL STAKEHOLDERS

Seaweed processors, seaweed aquaculture sector, food ingredients industry, consumer food sector, government authorities/legislators/funding agencies academic and food research institutes.

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

This project highlighted the extent of seasonal and spatial variation in the content of total nitrogen, protein nitrogen and non-protein nitrogen in macroalgae. Furthermore, it highlighted that macroalgae are a rich source of biofunctional nitrogenous components.



MAIN RESULTS

- A protocol for the extraction and quantification of total nitrogen (TN), non-protein nitrogen (NPN) and protein nitrogen (PN) content in macroalgae was developed
- Significant seasonal and geographical variation in the TN, PN and NPN content was observed in 1 red (*Palmaria palmata*), 1 green (*Ulva* spp) and 3 brown seaweed (*Fucus serratus*, *Laminaria digitata* and *Ascophyllum nodosum*) over a two year period
- Amino acid profiling of NPN fractions derived from *Palmaria palmata* and *Porphyra dioica* has identified high quantities of aspartic and glutamic acid, alanine and taurine in addition to mycosporine-like amino acids
- NPN fractions were identified as exhibiting high antioxidant activity

OPPORTUNITY/BENEFIT

The outcomes from this project provide the seaweed industry with a scientifically validated method for quantification of the TN, PN and NPN content in

macroalgae. This will allow industry to add value to their produce through selection of optimum harvesting times and locations. Specific macrolagal NPN components have potential applications as functional food ingredients.

COLLABORATING INSTITUTIONS

- Ulster University
- University College Cork
- University College Dublin
- NUI, Galway
- Teagasc

Project Reference: MFFRI/07/01

Funding Source: MI/DAFM

Date: 2007

Project Dates: Sept 2009 – Aug 2013

Irish Seaweed Polysaccharides for Gut Health

KEY EXTERNAL STAKEHOLDERS

Consumers, society, government authorities/legislators and the food industries

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

Seaweed is a natural source of bioactive components such as polyphenols, vitamins, antioxidants, polysaccharides and dietary fibres. This project is aimed at understanding the role of seaweed derived polysaccharides in gut health and targeting prebiotic potential of Irish seaweeds for development of functional food products.



MAIN RESULTS

- Four Irish seaweeds viz. *Laminaria*, *Fucus*, *Ulva* and *Palmaria* were collected from the west coast of Ireland and were used for extraction and separation of polysaccharides.
- A hot HCl extraction method was used for the extraction of polysaccharides. Following neutralisation the extracts were electro-dialyzed for desalination and polysaccharides were precipitated using ethanol. The extracts were then freeze dried.
- In vitro digestion using digestive enzymes was undertaken and the digests were used for testing through faecal fermenters.
- Diafiltration was used to separate the polysaccharides and oligosaccharides depending on their molecular weight cut offs.

OPPORTUNITY/BENEFIT

Considering the nutritional profile of Irish seaweeds and functional food product development is the target of this project. Cost effective methods for extraction and purification of the polysaccharides and desalination are being developed. These methods will help to develop cost effective products for the food industry. Prebiotic potential studies through clinical trials would support health claims pertaining to gut health for these products.

COLLABORATING INSTITUTIONS

- Teagasc Food Research Centre, Moorepark
- National University of Ireland, Galway
- Teagasc Food Research Centre, Ashtown

Project Reference: MDBY 6588

Funding Source: DAFM

Date: May, 2015

Project Dates: July 2014 – Nov 2017

Chitosan Generation and Characterisation from Shell

KEY EXTERNAL STAKEHOLDERS

Marine processors, ingredient producers

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

Use of by-products from marine processing and reduction in disposal at landfill costs

Novel ingredient for use in a myriad of applications as a functional food (anti-obesity/anti-cholesterol), horticulture, plant protection.



MAIN RESULTS

- Chitosan generation and characterisation from shell material (prawn and crab)
- NMR analysis and molecular weight determination

OPPORTUNITY/BENEFIT

By-product disposal is expensive and no longer permitted under the revised CFP. We have developed methodologies to generate a high-value grade chitosan from prawn and crab shell material and methods to characterise the resultant product which has a myriad of applications in functional foods, foods, packaging and horticulture.

COLLABORATING INSTITUTIONS

- National University of Ireland, Galway
- University College Dublin

Project Reference: NutraMara – The Marine Functional Foods Research Initiative

Funding Source: DAFM and Marine Institute and Teagasc

Date: May 2015

Project Dates: October 2009 – December 2012

Novel Proteins and Peptides from Seaweeds

KEY EXTERNAL STAKEHOLDERS

Protein ingredient manufacturers, marine processors

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

- Novel protein sources for use in the sports nutrition markets, Halal and Kosher as well as vegetarian markets.
- Increases essential amino acid profile of products
- Imparts a health benefit



MAIN RESULTS

- Bioactive peptides isolated from red seaweed were found to reduce blood pressure when tested in the lab and in spontaneously hypertensive rats (animal models)
- A novel hydrolysis and purification methodology was employed and applied to red seaweed
- Optimal conditions for developing bread products with this hydrolysate were determined and blood pressure regulation activity was maintained

OPPORTUNITY/BENEFIT

Protein extracts developed as part of this project were examined for their essential amino acid content, ability to inhibit enzymes important in blood pressure control and suitability for use in cereal products such as bread. Extracts could have benefits in the manufacture of food products for the prevention of heart health associated problems such as blood pressure.

COLLABORATING INSTITUTIONS

- National University of Ireland, Galway
- University College London, UK

Project Reference: NutraMara – The Marine Functional Foods Research Initiative, Teagasc Walsh Fellowship Programme and INFOGEST (EU COST Action FA1005)

Funding Source: DAFM and Marine Institute and Teagasc

Date: May 2015

Project Dates: October 2009 – October 2014

Seaweed Derived Glycine Betaine and DMSP

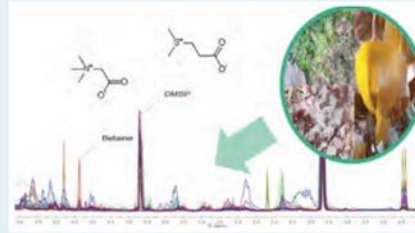
KEY EXTERNAL STAKEHOLDERS

Ingredient companies, marine processors, biochemical companies, food companies

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

Seaweeds are an abundant resource present around the Irish coastline. We have assessed a number of Irish seaweeds, which were harvested by our research partners in NutraMara – NUI Galway. Researchers at Teagasc determined the glycine betaine and DMSP levels in these seaweeds using NMR and MS methodologies.

Glycine betaine obtained a health claim under article 13 of EFSA in 2011 in relation to maintenance of normal homocysteine levels and therefore can be used for this purpose as a functional food ingredient/capsule ingredient.



MAIN RESULTS

- Two green seaweeds, harvested from around the Irish coast contained glycine betaine and DMSP.
- A novel, cost-efficient, environmentally friendly methodology was employed to generate fractions containing these zwitterionic compounds.
- NMR method developed to assess the level of glycine betaine and DMSP in the extracts.

OPPORTUNITY/BENEFIT

These extracts could be used in supplements or in functional foods to control homocysteine levels in the blood.

COLLABORATING INSTITUTIONS

- National University of Ireland, Galway
- Teagasc

Project Reference: NutraMara – The Marine Functional Foods Research Initiative

Funding Source: DAFM and Marine Institute and Teagasc

Date: May 2015

Project Dates: October 2009 – December 2012

The Anti-inflammatory Effect of Algal Lipid Extracts

KEY EXTERNAL STAKEHOLDERS

Consumers, society, government authorities/legislators and food industries.

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

Inflammation is a biological process that contributes strongly to a number of chronic diseases such as cardiovascular disease. Seaweeds and microalgae are potent sources of bioactive ingredients such as omega-3 polyunsaturated fatty acids (n-3 PUFA) and pigments which, as dietary ingredients have shown strong potential to prevent inflammation. This project identified the anti-inflammatory effect of algae extracts from the Irish coast.



MAIN RESULTS:

- Lipid extracts of four algal species contained a broad range of fatty acids including n-3 PUFA (34–62g/100g total fatty acids) and a broad range of pigments including chlorophyll a and β -carotene.
- *Palmaria palmata* extract significantly inhibited the production of the inflammatory cytokine IL-6 and IL-8 and *Pavlova lutheri* extract significantly inhibited the production of the inflammatory cytokine IL-6 in lipopolysaccharide stimulated human THP-1 macrophages.
- Moreover, all four extracts (*Pavlova lutheri*, *Palmaria palmata*, *Porphyra dioica* and *Chondrus crispus*) downregulated the expression of a number of inflammatory genes in the macrophages.
- Out of the four species tested, *P. lutheri* posed the greatest potential as a functional anti-inflammatory ingredient.
- This study suggests that algal lipid extracts may inhibit the production of inflammatory cytokines and the expression of inflammatory genes and thereby alleviate the symptoms of inflammatory disease.

OPPORTUNITY/BENEFIT:

The algae extracts studied exhibited anti-inflammatory effects and are natural sources of bioactive components. The present study identifies the potential of these extracts for use as functional food ingredients aimed at inhibiting chronic disease-associated inflammation.

COLLABORATING INSTITUTIONS:

- Teagasc Food Research Centre, Moorepark
- National University of Ireland, Galway
- University College Dublin

Project Reference: MFFRI/07/01

Funding Source: Marine Institute and DAFM

Date: November, 2015

Project Dates: 2007–2013

The α -amylase and α -glucosidase Inhibitory Effects of Irish Seaweed Extracts

KEY EXTERNAL STAKEHOLDERS

Diabetic patients, consumers, society, government authorities/legislators and the food industries

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

Antidiabetic effects can be achieved through inhibiting the carbohydrate hydrolysing enzymes involved in digestion and absorption using α -amylase and α -glucosidase inhibitors. Seaweeds and their bioactive principles such as antioxidant and polyphenols also plays role in antidiabetic effects. This project led to identification of seaweed extracts useful for diabetic care.



MAIN RESULTS:

- The cold water and ethanol extracts of *Ascophyllum nodosum* had the strongest α -amylase inhibitory effect with IC50 values of 53.6 and 44.7 μ g/ml respectively.
- Moreover, the extracts of *Fucus vesiculosus* Linnaeus were found to be potent inhibitors of α -glucosidase with IC50 values of 0.32 and 0.49 μ g/ml.
- Out of 15 seaweeds, brown seaweed extracts (in particular *F. vesiculosus* and *P. canaliculata*) showed stronger efficacy to inhibit enzymes involved in intestinal carbohydrate digestion and assimilation.
- This study revealed that brown seaweed extracts may limit the release of simple sugars from the gut and thereby alleviate postprandial hyperglycaemia.

OPPORTUNITY/BENEFIT:

Due to antioxidant and phenolic content, seaweed extracts showed antidiabetic effects. The present study reported the potential of algal extracts for use in functional food applications aimed at lowering glycaemic response. These extracts are natural sources of α -amylase and α -glucosidase inhibitors, and represent alternatives to drugs for diabetic care.

COLLABORATING INSTITUTIONS:

- Teagasc Food Research Centre, Moorepark
- University of Limerick
- Teagasc Food Research Centre, Ashtown

Project Reference: MFFRI/07/01

Funding Source: Marine Institute and DAFM

Date: November, 2015

Project Dates: 2007–2013

The Potential of Yoghurt as a Functional Food Matrix for an Omega-3 PUFA-rich Algae Extract

KEY EXTERNAL STAKEHOLDERS

Consumers, society, government authorities/legislators and food industries.

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

Omega-3 polyunsaturated fatty acids (n-3 PUFA) are bioactive ingredients that have anti-inflammatory, cardioprotective and cognition-enhancing properties. Seaweeds and microalgae are potent sources of n-3 PUFA and provide a novel vegetarian source of these ingredients over fish. This project identified the potential of yoghurt as a carrier-food for omega-3 rich algae extracts.



MAIN RESULTS:

- A lipid extract from the microalgae *Pavlova lutheri* was obtained and analysed for its fatty acid content (51g n-3 PUFA/100g total fatty acids)
- Addition of the extract (at 0.25% or 0.5%) to the yoghurt did not significantly affect pH, rheology, whey separation, starter culture survival or macronutrient composition over 28 days.
- Colour of the yogurt was significantly affected by addition of the extract at 0.25% and 0.5%
- Addition of the extract to the yoghurt at both concentrations was associated with altered sensory properties.
- n-3 PUFA concentrations were significantly increased following addition of the extract to the yoghurts at 0.25% and 0.5%.

OPPORTUNITY/BENEFIT:

Addition of a *P. lutheri* lipid extract to yoghurt did not impact the techno-functional properties of the yoghurt, while colour and sensory properties were significantly altered. The present study suggests yoghurt as a potentially suitable food-carrier for algal extracts and as a novel vegetarian source of n-3 PUFA.

COLLABORATING INSTITUTIONS:

- Teagasc Food Research Centre, Moorepark
- National University of Ireland, Galway
- University College Cork

Project Reference: MFFRI/07/01

Funding Source: Marine Institute and DAFM

Date: November, 2015

Project Dates: 2007–2013

Pork Meat Enhanced with Seaweed Polysaccharides

KEY EXTERNAL STAKEHOLDERS

Pork producers, pork meat processing companies, seaweed and functional ingredient suppliers.

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

Research into the development of healthier functional meats and meat products is advantageous to the meat industry and consumer as the link between diet and disease prevention continues to grow. Brown seaweeds contain a variety of compound classes (e.g. proteins, polysaccharides, lipids) with numerous functional properties and applications. Bioactive polysaccharides (laminarin and fucoidan) in brown seaweeds possess antitumor, antiviral, antibacterial and antioxidant activities. Collaborating researchers in University College Dublin previously



demonstrated beneficial effects of dietary laminarin and fucoidan on growth performance and gut health in pigs. This project examined potential for the manufacture of functional pork meat containing health – promoting polysaccharides (laminarin and fucoidan) extracted from brown seaweed (*Laminaria digitata*).

MAIN RESULTS

- Seaweed polysaccharides (laminarin and fucoidan) were incorporated into pork via the animals' diet and by direct addition into minced pork meat.
- Dietary supplementation of laminarin and fucoidan (450 or 900 mg/kg feed for 3 weeks) significantly reduced lipid oxidation in fresh pork stored in MAP.
- Quality, shelf life and sensory properties of pork were not negatively influenced by dietary laminarin and fucoidan.
- Direct addition of laminarin and fucoidan (0.5%) decreased lipid oxidation in cooked minced pork possibly due to Maillard reaction products.

OPPORTUNITY/BENEFIT

- Dual benefit of supplementing pig diets with seaweed polysaccharides (pig health and pork quality).
- Enhancing the healthiness of pork meat.

COLLABORATING INSTITUTIONS

University College Dublin.

Project Reference: MFFRI/07/01

Funding Source: MI / DAFM

Date: June 2015

Project Dates: Jan 2008 – July 2015

Seaweed Polysaccharides as Functional Ingredients in Pork Meat: Mechanistic Studies

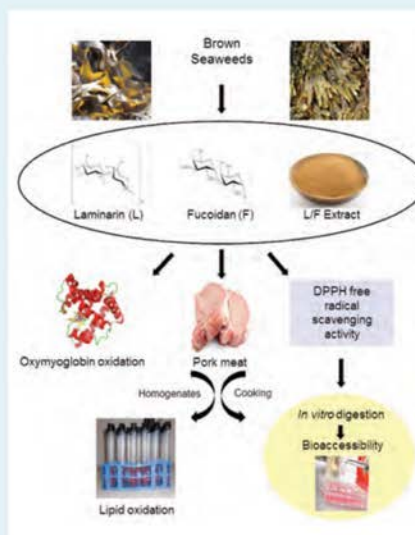
KEY EXTERNAL STAKEHOLDERS

Pork meat processing companies, seaweed and functional ingredients suppliers.

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

Previous research indicated that functional ingredients, such as laminarin and fucoidan, have beneficial effects in pigs, both pre-slaughter (animal health) and post-slaughter (meat quality). Addition of a seaweed extract, containing laminarin and fucoidan, to pig diets reduced lipid oxidation in fresh pork. However, direct addition of the same extract promoted lipid oxidation and decreased the colour stability of fresh minced pork. Elucidation of the reaction mechanisms of laminarin and fucoidan in fresh and cooked pork meat is necessary to underpin their use in the development of functional pork meat products.

Bioaccessibility is defined as the fraction of a compound transferred from the food matrix during digestion and thus made available for intestinal absorption and cellular uptake. Cooked pork containing laminarin and fucoidan was subjected to an *in vitro* digestion procedure and theoretical cellular uptake of antioxidant compounds was examined in a transwell Caco-2 cell model.



MAIN RESULTS

- Laminarin displayed no activity and fucoidan reduced lipid oxidation but catalysed oxymyoglobin oxidation (decreased redness).
- Fucoidan activity (DPPH radical scavenging) decreased following cooking while the seaweed extract displayed moderate thermal stability.
- Theoretical uptake of laminarin and fucoidan antioxidant compounds by Caco-2 cells was observed.

OPPORTUNITY/BENEFIT

Understanding the mechanisms by which seaweed polysaccharides function in fresh and cooked pork meat.

COLLABORATING INSTITUTIONS

Teagasc Food Research Centre, Moorepark.

Project Reference: MFFRI/07/01

Funding Source: MI / DAFM

Date: June 2015

Project Dates: Jan 2008 – July 2015

Brown Seaweed Extracts as Potential Functional Ingredients in Dairy Products

KEY EXTERNAL STAKEHOLDERS

Dairy products companies, seaweed and functional ingredients suppliers, and consumers.

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

Dairy products are frequently enriched with health promoting functional ingredients such as probiotics, omega-3 fatty acids and vitamins etc. Brown seaweeds contain a range of bioactive compounds and reported bioactivities include anti-inflammatory, antibacterial and antioxidant activities. Collaborating Nutramara researchers in University College Cork demonstrated *in vitro* antioxidant activity and protective effects of brown seaweed extracts against DNA damage in cell culture. Therefore seaweed represents a potentially exploitable source of functional ingredients for use in the manufacture of functional dairy products. This project examined potential for the manufacture of functional dairy products (milk and yoghurt) containing extracts prepared from brown seaweeds (*Ascophyllum nodosum* and *Fucus vesiculosus*) using food friendly solvent systems (water and aqueous ethanol).



MAIN RESULTS:

- *Ascophyllum nodosum* (100% H₂O (AN₁₀₀) and 80% ethanol : 20% H₂O (AN_{80e})) and *Fucus vesiculosus* (60% ethanol : 40% H₂O (FV_{60e})) extracts were incorporated into milk and yoghurt (0.25% and 0.5%).
- Extracts (AN_{80e} and FV_{60e}) influenced the colour of milk and yoghurt.
- Milk and yoghurt containing AN₁₀₀ extracts were most accepted by sensory panellists.
- Extract-enriched dairy products did not display antioxidant activity in cell culture – further research is necessary.

OPPORTUNITY/BENEFIT:

Dairy products enriched with health-promoting seaweed extracts.

COLLABORATING INSTITUTIONS:

Teagasc Food Research Centre, Ashtown.

Project Reference: MFFRI/07/01

Funding Source: MI / DAFM

Date: June 2015

Project Dates: Jan 2008 – July 2015

Bioavailability and Bioactivity of Phenolic Enriched Extracts from Brown Seaweed

KEY EXTERNAL STAKEHOLDERS

(Bio-) pharmaceutical companies, nutritional companies, seaweed producers, health agencies.

BACKGROUND

The EU project SWAFAX investigated the bioavailability and bioactivity of compounds from UK seaweeds for application in food and health & wellness products. Although polyphenols from land plants are widely used as functional food ingredients and food supplements, seaweed sources have been little studied or exploited.



MAIN RESULTS

- Developed new methodology to extract polyphenolic enriched fractions from seaweed.
- Identified variation in inter-individual bioavailability of polyphenolic compounds after dietary intervention trial.
- Consumption of the seaweed polyphenolic supplement decreased DNA damage and improved antioxidant status in higher risk subpopulations.

OPPORTUNITY/BENEFIT

This research represents the first comprehensive study in human subjects of the bioavailability of seaweed polyphenolics and strengthens, the very limited data from human studies on natural seaweed bio-actives and their importance to human health, specifically in chronic diseases involving oxidative stress.

COLLABORATING INSTITUTIONS

- CyberColloids Ltd
- University of Reading

Funding Source: EU SP7

Date: May 2015

Anticancer Properties of Marine Bio-Actives

KEY EXTERNAL STAKEHOLDERS

Pharmaceutical companies, food companies, nutraceutical industry, seaweed producers, health agencies.

PRACTICAL IMPLICATIONS

The brown macroalgae species, *Fucus vesiculosus* (Bladderwrack), is of interest due to its vast abundance and potential sustainability as a primary source for pharmacological and nutritional compounds. This project explored the potential anti-cancer properties of Irish seaweeds and identified *F. vesiculosus* as a leading candidate for sourcing anticancer compound candidates.



MAIN RESULTS

- Crude aqueous and ethanolic extracts of *F. vesiculosus* have the capacity to significantly reduce breast cancer proliferation in a triple negative breast cancer cell model (MDA-MB-231)
- Refining of both extracts showed that the low molecular weight fractions of each was predominantly responsible for the MDA-MB-231 cell death
- Transcriptional gene expression profiling revealed that seaweed extracts mediated cell death through the intrinsic apoptosis pathway

OPPORTUNITY/BENEFIT

This project has identified that aqueous and ethanolic extracts of *Fucus vesiculosus* have potential for sourcing anticancer components. Furthermore, subsequent fractionation of both aqueous and ethanolic extracts revealed that compounds in low molecular weight fractions are of particular interest as a source for novel anticancer

bioactive compounds from *F. vesiculosus*. There is considerable potential for further fractionation of the low molecular weight fractions to identify novel bioactive compounds using in vitro models and subsequent progression to animal models to substantiate noted anticancer activity.

COLLABORATING INSTITUTIONS

- NUI Galway
- Teagasc Food Research Centre, Ashtown

Funding Source: NutraMara

Date: May, 2015

Project Dates: Dec 2013 – June 2015

The Incorporation of Seaweed into Commonly Consumed Foods – *Palmaria palmata*

KEY EXTERNAL STAKEHOLDERS

Seaweed harvesting companies, bread industry, national food research institutes, consumers.

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

The opportunity to provide added value to the commonly consumed red seaweed, *Palmaria palmata* (commonly known as dulse or dillisk). The addition of *Palmaria palmata* could provide a novel product with enhanced taste and with enhanced but acceptable levels of iodine which could help address the existing global deficiency of iodine in the human diet.



MAIN RESULTS:

- *Palmaria palmata* enriched bread had a significant impact on thyroid stimulating hormone which would provide indications this seaweed may be a useful additive for addressing the existing iodine deficiency issue within Europe.
- Supplementation of *Palmaria palmata* enriched bread significantly increased C-reactive protein indicating that it stimulates the innate immune system.
- Supplementation of *Palmaria palmata* enriched bread significantly increased triglycerides indicating that it alters lipid metabolism.

OPPORTUNITY/BENEFIT:

The additional of *Palmaria palmata* to bread was shown to enhance consumer acceptability and the addition of this seaweed to suitable foods may be useful for enhancing the iodine concentration resulting which would help to address the existing iodine deficiency. There is a need to optimise the dose of *Palmaria palmata* dose to avoid the apparent immunostimulatory and hyperlipidemic effects.

COLLABORATING INSTITUTIONS:

- UCD
- Limerick
- National University of Ireland, Galway

Funding Source: DAFM

Date: November, 2015

Project Dates: Dec 2011 – June 2012

The Incorporation of Seaweed into Commonly Consumed Foods – Pork

KEY EXTERNAL STAKEHOLDERS

Seaweed harvesting companies, pork industry, national food research institutes, consumers.

PRACTICAL IMPLICATIONS FOR STAKEHOLDERS

The opportunity to provide added value to existing pork products through incorporation of brown seaweed fibre components (Laminarin/fucoidan) into pig feed.



MAIN RESULTS:

- Incorporation of (Laminarin/fucoidan mix) into pig feed was previously shown to impact favourably on markers of pig health.
- A human intervention study in which participants were fed meat from Laminarin/fucoidan fed pigs was shown to have no impact on the antioxidant, lipid or inflammatory status.
- Furthermore, consumption of the pig meat had no impact on liver/kidney function and was shown to have no genotoxicity.

OPPORTUNITY/BENEFIT:

Consumption of meat from pigs supplemented with a laminarin/fucoidan mix was well tolerated and had no genotoxic effect after a 4-week intervention in healthy volunteers. There were no significant changes in lipid, antioxidant or inflammatory status post intervention. This study demonstrated the safety of consuming a novel pork product. In summary, the incorporation of laminarin/fucoidan mix into feed has favourable impact on pig health and subsequent consumption of the pig meat has no negative impact on consumer health.

COLLABORATING INSTITUTIONS:

- University College Dublin (UCD)
- University College Cork (UCC)
- National University of Ireland, Galway

Funding Source: DAFM

Date: November, 2015

Project Dates: Dec 2011 – June 2012

APPENDIX 4 – SERVICE OFFERS

SERVICE

Algal Biochemical/Bioactive Analysis and Profiling

The Algal Biosciences Group at NUI Galway has extensive expertise and facilities available to identify and quantify secondary metabolites of commercial potential in cyanobacteria, microalgae, and seaweeds or algae-derived extracts which are of interest to companies in the functional foods, agri/horticultural and cosmetics sectors.

BACKGROUND

Marine algae are potentially an important resource for biotechnological multi-product development. Micro – and macroalgae contain a diverse array of metabolites with multiple bioactivities including antioxidant, antimicrobial, anti-inflammatory and anticancer activities which have many and multiple applications in food and health, including functional foods, but also agri-horticultural and home and personal care sectors, as well as cosmetics applications.

Bioactive levels and composition in algae are species-dependent and may vary with growth conditions, season and harvesting locations, which allows their selection, manipulation and induction for specific applications.

Professionals involved with algal cultivation and products, therefore require expertise and biochemical analysis of the algal-biomass or issued-extracts.

BENEFITS TO CLIENTS

Biochemical profiling provides insights into algal composition which is crucial for new product development and can be used to determine biomass quality. Potential clients' needs are assessed and advice is given on the appropriate analyses required.

SERVICE DETAILS

- We carry out a full range of biochemical analyses on algal biomass and bioactive extracts including determination of contents and composition.
- Analyses available for algal products consist of lipids and fatty acids e.g. eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, pigments i.e. chlorophylls and carotenoids, phenolic compounds e.g. phlorotannins, UV-absorbing mycosporine-like amino acids, phycobiliproteins e.g. phycoerythrin and phycocyanin, and iodine.



FACILITIES

- Our facility consists of a dedicated algal biomass processing and extraction laboratory.
- The analytical facility comprises a Cary 50 Scan UV-Visible spectrophotometer, an Agilent Gas Chromatography 7890A GC/5975C MSD series, and a Agilent High Performance Liquid Chromatography 1200 Series equipped with various detectors for specific applications.

OF INTEREST TO

- Marine functional food companies
- Seafood and aquaculture companies
- Ingredient manufacturers

SERVICE CONTRACTS

Contracts are agreed with clients and work is carried out on a confidential basis. Cost is dependent on the method and number of analyses performed, e.g. sample number.

Polysaccharide Extraction and Characterisation

The Algal Biosciences Group at NUI Galway has the expertise and necessary facilities to optimally extract and characterise algal polysaccharides of commercial interest. Post-extraction we can also alter polysaccharide properties and generate oligosaccharides which may have application in a variety of scenarios including but not limited to; as bioactive ingredients, and as emulsifying, stabilising and gelling agents.

BACKGROUND

Algal polysaccharides differ in structure and composition depending on the source alga, as well as with developmental stage, part of the alga and season. We can identify appropriate sources, extract polysaccharides, and carry out compositional analysis. Polysaccharides are impacted by extraction technique and we can identify the most suitable source and appropriate extraction technique to provide polysaccharides for a given application. For example, some extraction techniques may generate oligosaccharides/lower molecular weight products which might make the product less suitable for viscosifying and gelling properties within the food industries but have novel application for example associated with their potential bioactivities.

BENEFITS TO CLIENTS

Identifying the most suitable sources of polysaccharides and chemically characterising them allows clients to obtain suitable, quality products for their applications. It is also possible to characterise the sugar residue composition of polysaccharides and oligosaccharides for example, verifying the nature of a potential bioactive which is necessary for product development and/or marketing. Clients needs are assessed on an individual basis enabling the most suitable analyses to be selected and performed.

SERVICE DETAILS

- We can give advice on suitable sources for given polysaccharides and/or properties
- We can give advice on suitable extraction procedures and carry out extractions (depending on scale)
- Analyses of polysaccharide or oligosaccharide molecular weight, constitutive sugar residues, and in some cases in situ localisation of the polysaccharide can be performed.



FACILITIES

- Thin-layer chromatography
- Size-exclusion chromatography
- Immunocytochemistry
- Histology

OF INTEREST TO

- Marine functional food companies
- Ingredient manufacturers

SERVICE CONTRACTS

Contracts are agreed with clients and work is carried out on a confidential basis. Cost is dependent on the method of testing used and sample numbers.

Seaweed Biomass Resource Assessment

The Algal BioSciences Group at NUI Galway provides expertise in seaweed biomass assessment in the form of advice and practical support to underpin the sustainable utilisation and management of coastal resources, including brown, red, green intertidal and subtidal seaweed species, and their suitability for specific commercial applications.

BACKGROUND

Given the increasing demand for the seaweed biomass for a range of traditional and novel applications in the food, agri – and cosmetics sectors, there is increasing awareness of the quality and traceability of seaweed biomass and their sustainable harvesting, as well as appropriate resource management.

BENEFITS TO CLIENTS

Support is provided

- in selecting suitable species
- in choosing harvesting locations and methods
- in identifying high quality biomass
- in assessing biomass, growth and productivity
- in assessing and monitoring harvesting impact

SERVICE DETAILS

- Onshore seaweed biomass assessment
- Expertise, management and answers to environmental problems related to algae (i.e. invasive species, green tides)
- *In-situ* and lab-based growth and productivity assessment
- Advise on harvesting methodology and ecological monitoring

EXPERTISE

- 25 years' experience in seaweed biology
- Knowledge on Irish seaweed resources in a global context
- Extensive experience in seaweed ecology, growth and productivity assessment methods



OF INTEREST TO

- Seafood, seaweed processors and aquaculture companies
- Environmental Protection Agency
- Non-governmental organisations

SERVICE CONTRACTS

Contracts are agreed with clients and work is carried out on a confidential basis. Cost per hour is dependent on expertise provided and workload.

Biofunctional Characterisation of Peptides from Marine Sources

The Proteins and Peptides Research Group in the Department of Life Sciences at the University of Limerick has expertise, scientifically validated protocols and facilities for biofunctional characterisation of marine-derived protein hydrolysates/peptides.

BACKGROUND

The laboratory at UL has developed an array of scientifically validated in vitro bioassays for assessment of the effect of protein hydrolysates/peptides on biomarkers associated with metabolic conditions such as type II diabetes and cardiovascular disease. These include dipeptidyl peptidase (DPP) IV, α -glucosidase and α -amylase inhibitory activity assays (anti-diabetic), angiotensin converting enzyme (ACE) and renin inhibitory activity assays (cardioprotective) and an array of antioxidant activity assays including: oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), 2,2-diphenylpicrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), superoxide radical scavenging and xanthine oxidase inhibition assays.

BENEFITS TO CLIENTS

These analyses provide industry with scientifically robust in vitro biofunctional data on the effect of marine protein hydrolysates/peptides on biomarkers related to type II diabetes and cardiovascular disease.

SERVICE DETAILS

- In vitro anti-diabetic assays: dipeptidyl peptidase (DPP) IV, α -glucosidase and α -amylase inhibitory activity assays
- In vitro cardioprotective assays: angiotensin converting enzyme (ACE) and renin inhibitory activity assays
- In vitro antioxidant activity assays: oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), 2,2-diphenylpicrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), superoxide radical scavenging and xanthine oxidase inhibition assays



FACILITIES

Two plate readers with absorbance, fluorescence and chemiluminescence detection options

OF INTEREST TO

- Marine functional food companies
- Seafood companies
- Ingredient manufacturers

SERVICE CONTRACTS

Contracts are agreed with clients and work is carried out on a confidential basis. Cost is dependent on the method of testing used and sample numbers.

Extraction, Quantification and Characterisation of Proteins from Marine Sources

The Proteins and Peptides Research Group in the Department of Life Sciences at the University of Limerick has extensive knowledge, expertise and facilities for extraction, quantification and characterisation of protein isolates or specific protein components from marine sources.

BACKGROUND

Alternative protein sources are needed to meet the rapid growing global demand for high quality protein. Marine organisms contain significant quantities of protein and are promising candidate raw materials to help meet the global demand. The Proteins and Peptides Research Group has expertise and state-of-the-art facilities for the extraction, quantification and characterisation of protein isolates and specific protein components, such as collagen, from marine sources including macroalgae, microalgae, fish and shellfish, meat and by-products, and fermented marine products.

BENEFITS TO CLIENTS

Through numerous research projects, the laboratory has developed a range of industrially relevant protocols for the extraction of protein fractions or specific protein components including collagen from marine sources. This allows industry to perform preliminary laboratory-scale trials to identify the optimum conditions for extraction of protein from marine sources in addition to determining yield and purity. A significant opportunity also exists for the marine industry to add value to existing marine processes through the upgrading of low value marine protein rich co-products to high value protein isolates. This also helps in dealing with the high disposal costs and legal restrictions associated with materials that are otherwise considered as waste.

SERVICE DETAILS

- We can adapt existing extraction protocols to identify the optimum conditions for extraction of protein from specific marine sources
- Protein content, purity and extraction yield can be determined using standard protein quantification methods
- Full characterisation of extracted protein can be carried out



FACILITIES

- Protein extraction facilities
- Equipment for the spectrophotometric determination of protein and Kjeldahl nitrogen quantification
- Fast protein liquid chromatography (ion-exchange, hydrophobic, reverse phase and gel permeation columns), analytical and semi-preparative high performance liquid chromatography and analytical gel permeation chromatography systems.

OF INTEREST TO

- Marine functional food companies
- Seafood companies
- Ingredient manufacturers

SERVICE CONTRACTS

Contracts are agreed with clients and work is carried out on a confidential basis. Cost is dependent on the method of testing used and sample numbers.

Generation and Enrichment of Peptides from Marine Sources

The Proteins and Peptides Research Group in the Department of Life Sciences at the University of Limerick has extensive knowledge, expertise and facilities for targeted enzymatic hydrolysis of marine proteins and subsequent enrichment of peptides.

BACKGROUND

Numerous marine sources contain significant quantities of high quality protein (10–47 % (w/w)) which represent good candidate raw materials for the generation of bio – and technofunctional peptides. Enzymatic hydrolysis of protein with proteolytic preparations is routinely used by the food industry for the generation of protein hydrolysates as it is relatively inexpensive, highly controllable and performed under mild conditions. Selected enrichment processes such as membrane processing technologies can be employed to improve the bio – and technofunctional properties of marine peptides

BENEFITS TO CLIENTS

The laboratory at UL has significant expertise in the targeted generation of marine protein hydrolysates. This experience and associated equipment can be leveraged by industry to perform laboratory-scale trials to determine the optimum hydrolysis conditions/proteolytic enzyme preparation(s) for production of marine protein hydrolysates with selected bio – and/or technofunctional properties. Enriched peptide fractions with enhanced activity can be produced in the laboratory using food-friendly membrane processing technologies. This involves ultrafiltration (UF) through a range of membranes having different molecular mass cut-off values, e.g. through 10, 5 and 3 kDa UF membranes.

SERVICE DETAILS

- We can provide expertise, advice and facilities for the targeted generation of marine protein hydrolysates using food-grade proteolytic enzymes
- Ultrafiltration apparatus equipped with membranes having different molecular mass cut-off values, e.g. 10, 5 and 3 kDa can be employed to fractionate/enrich specific peptides within promising hydrolysates



FACILITIES

- Facilities for enzymatic generation of marine protein hydrolysates
- Membrane processing facilities for enrichment of peptides
- Protein hydrolysate drying facilities (two freeze-driers and a small scale spray-drier)

OF INTEREST TO

- Marine functional food companies
- Seafood companies
- Ingredient manufacturers

SERVICE CONTRACTS

Contracts are agreed with clients and work is carried out on a confidential basis. Cost is dependent on the method of testing used and sample numbers.

Identification of Peptides from Marine Sources

The Proteins and Peptides Research Group in the Department of Life Sciences at the University of Limerick has expertise and state-of-the-art facilities available for marine-derived peptide identification.

BACKGROUND

The laboratory at UL houses a fully commissioned liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) system, specifically for the identification of peptides. Capabilities exist for identification of long and short peptides from a range of food protein sources. To date, this facility has been used to identify peptides from *Palmaria palmata*, salmon meat and gelatine and Alaska pollack gelatine hydrolysates and shrimp fermentates.

BENEFITS TO CLIENTS

- This facility provides information on the primary sequence of peptides within a hydrolysate/fraction. This data can be used to guide peptide selection for confirmatory studies in an attempt to identify peptides responsible for an observed biological activity.
- Peptide sequence identification is a key essential requirement for European Food Safety Authority submissions.

SERVICE DETAILS

- Optimisation of peptide separation prior to MS analysis
- Identification of marine protein-derived peptide sequence

FACILITIES

- Ultra performance liquid chromatography (UPLC), high performance liquid chromatography (HPLC) and fast protein liquid chromatography (FPLC) systems for optimisation of marine peptide separation
- UPLC coupled to electrospray injection quadrupole (ESI) time-of-flight MS



OF INTEREST TO

- Marine functional food companies
- Seafood companies
- Ingredient manufacturers

SERVICE CONTRACTS

Contracts are agreed with clients and work is carried out on a confidential basis. Cost is dependent on the method of testing used and sample numbers.

Physicochemical and Technofunctional Characterisation of Peptides from Marine Sources

The Proteins and Peptides Research Group in the Department of Life Sciences at the University of Limerick has expertise and state-of-the-art facilities for physicochemical and technofunctional characterisation of marine-derived peptides.

BACKGROUND

Full physicochemical characterisation of candidate protein hydrolysates (e.g. degree/extent of hydrolysis, molecular mass distribution and peptide profiles) is an essential requirement for European Food Safety Authority health claim dossier submissions. Technofunctional characterisation of marine protein hydrolysates provides useful information on the behaviour of marine protein hydrolysates in formulated food systems. These include properties such as solubility, clarity, heat stability, emulsification, viscosity and foaming, all of which are important aspects in the utilisation of hydrolysates as food ingredients.

BENEFITS TO CLIENTS

Data obtained following extensive physicochemical characterisation of candidate protein hydrolysates provides information on the characteristics of the hydrolysates such as the degree/extent to which the protein is hydrolysed, the molecular mass distribution of the peptides and peptide profiles. This is key information required for submission of health claim portfolios to the European Food Safety Authority. Results of technofunctional assessment give an indication of how a protein hydrolysate/prototype ingredient may behave in formulated food systems.

SERVICE DETAILS

- We can provide expertise, advice and facilities for the physicochemical characterisation of marine protein hydrolysates
- Technofunctional assessment capabilities such as solubility, clarity, heat stability, emulsification, viscosity and foaming analyses are available



FACILITIES

- Equipment for determining degree/extent of hydrolysis
- High performance liquid chromatography, ultra-performance liquid chromatography and gel permeation chromatography equipment for determining peptide profiles and molecular mass distribution of peptides within hydrolysates
- Specific equipment for assessment of technofunctional properties

OF INTEREST TO

- Marine functional food companies
- Seafood companies
- Ingredient manufacturers

SERVICE CONTRACTS

Contracts are agreed with clients and work is carried out on a confidential basis. Cost is dependent on the method of testing used and sample numbers.

Food Packaging Assessment and Testing Capabilities

The Food Packaging Group in UCC have nearly 15 years of experience in food packaging science and technology, with particular expertise in package optimisation, testing, product development, reformulation, shelf life analysis/extension and trouble shooting. The state-of-the-art facilities include packaging and shelf life testing laboratories, environmental rooms, process/packaging facilities (with packaging equipment for bulk gas flushing, modified atmosphere packaging, vacuum packaging, overwrapping, canning etc.) and sensory laboratories.

PACKAGING OPTIMISATION AND TESTING

Optimising and testing food packaging materials is based on a range of physical, chemical and microscopic techniques available at UCC.

Physical:

- Packaging material identification and optimisation comprising assessment of film strength, colour and appearance. Physical strength measurements include; tensile strength puncture test, elongation, yield point, torque on or off strengths etc.
- Pack integrity testing for evidence of burst leak and seepage problems.
- Taptone testing is a non-destructive assessment of potential leaking cans.
- Heat seal and integrity testing.

Chemical/microscopic:

- Determination of the gas permeability of packaging films and plastic bottles.
- Measure and analysis of gas composition in head-spaces of food packs non-destructively and continuously, thereby, optimising packaging and gas utilisation.
- Assessment and identification of packaging materials using FTNIR-FTMIR (and IR microscopy).
- Analysis of pack colour/light barrier using advanced spectroscopic methodologies.
- Evaluation of chemical migration from pack materials to food.

Shelf life analysis and extension:

- Shelf life analysis and extension using a combination of scientific expertise in microbiology, chemistry and sensory science and can assist companies with troubleshooting i.e. problems based on chemical composition, ingredient interactions, microbial contamination and sensory quality.
- Solutions for extended shelf life.
- Microbiological assessment of foods.



New packaging technologies:

- Development and optimisation of in-pack sensors suitable for detecting levels of gases in gas-sensitive products (indicators of product quality/safety).
- Environmental packaging applications.
- Development of new smart packaging technologies for application to food products/beverages for maintenance of sensory and quality attributes and improvement in safety and shelf life.

Consumer driven development:

- Optimisation of packing, design and formation based on consumer identified attributes.
- Determination of compositional and nutritional profiles of packaged foods for compliance with food labelling requirements.

OF INTEREST TO

The expertise/facilities at UCC are of interest to all seafood processors and companies manufacturing packaging materials and components. Companies can avail of consultancy, services, contract research and collaborations.

HOW TO PROCEED:

Chemical and Shelf-life Testing of Muscle Foods

Researchers in UCC have extensive experience and expertise in the chemical analysis and shelf-life evaluation of marine-based muscle foods. The availability of state-of-the-art facilities, equipment and technical know-how enables UCC researchers to provide a detailed and complex evaluation of product quality, stability, safety and shelf-life parameters such as colour, lipid stability, microbiology and sensory analysis. The impact of quality enhancement compounds, techniques and processes can also be measured.

BACKGROUND

The shelf-life of muscle foods is of critical importance with respect to quality, safety, and sensory acceptability. The chemical composition of marine muscle foods, such as fish, renders it susceptible to a range of quality deterioration processes with a negative impact on factors such as colour, lipid stability, microbiology and sensory properties. A range of strategies (e.g. antimicrobial and antioxidant compounds, modified atmosphere packaging (MAP)) are available for the control of quality deterioration in marine-based products. Measurement of the impact, efficacy and potency of such compounds and storage conditions is key to developing marine muscle food products with adequate quality and shelf-life properties.



OF INTEREST TO

- Seafood companies
- Marine functional food companies
- Ingredient manufacturers and suppliers

FACILITIES, EQUIPMENT AND TESTING

- Muscle food processing and packaging facilities
- Fish smoking facilities
- Shelf-life/testing laboratory
- Sensory analysis unit
- Processing equipment including mincers, bowl-chopper, multi-needle injector etc.
- Modified atmosphere packaging (MAP) and vacuum packaging technologies
- Gas sensor and package integrity testing
- Compositional analysis (Protein, moisture, fat, ash)
- Microbiological analysis of muscle foods
- Texture profile analysis (TPA) testing
- Analytical instrumentation (Minolta colorimeter, HPLC, GC-MS, FT-IR spectrometer and microscope)
- A vast array of chemical analysis techniques including fatty acid analysis, measurement of lipid stability (TBA test) and *in vitro* antioxidant assays (TPC, DPPH and FRAP)

Muscle Foods Processing Facilities at UCC

The Muscle Foods Research Group in UCC has over 50 years of experience in muscle foods research, training and education at all levels within this field. The state-of-the-art processing facilities have been developed over many years to be capable of dealing with both fresh and fully processed muscle-based food products and facilities include; the muscle foods processing hall, a small to industrial-scale cooking facility, novel technologies processing room, a series of walk-in chills and freezing units and simulated retail display area all of which, is supported by the muscle food processing, analytical, packaging and sensory laboratories.

BACKGROUND

These facilities at UCC have provided a significant number of services to the seafood industry over the past 50 years, typically on a case by case basis. The services that can be availed of are;

- Mincing, flaking, bowl chopping technologies followed by a host of available forming technologies, including nugget, sausage and stuffing lines.
- Complete curing technologies line.
- Full pre-dusting, battering and breading line with flash frying facility.
- Small to industrial scale cooking facilities, including large and small electric kettles.
- Wood chip, log, friction and liquid-smoking facilities.
- A wide range of mixing and formulation equipment for niche and novel products manufacture.
- Dehumidifying and ripening chambers for fermented-styled products.
- Extrusion equipment for novel processed food manufacture.
- State-of-the-art retorting facility for can, glass and pouched product processing.
- Super-chilling and blast-freezing facilities and supported by walk-in chills and freezers.
- Simulated retail-display area for assessment of retail-ready products with respect to storage stability, shelf-life etc.
- Muscle food packaging area (MAP, vacuum, overwrapping, canning etc.).
- Muscle analytical facilities for measurement of product attributes; colour, texture, microbiological status, sensory etc.

OF INTEREST TO

The expertise/facilities at UCC are of interest to all seafood processing companies.



APPENDIX 5 – NUTRAMARA CONFERENCE AGENDAS

NutraMara Conference 2015: Harnessing marine bioresources for innovations in the food industry

Day 1: Monday 29th June 2015

Introduction and keynote addresses

Growing Ireland's Capability in Marine Functional Foods – The Nutramara Programme

Mr Declan Troy, Director of NutraMara, Teagasc

Innovation in the European marine bioresources sector

Dr Torger Børresen, Technical University of Denmark

Marine ingredients and opportunities for processing underutilised fish species: an Irish industry perspective

Mr Jason Whooley, Bio-Marine Ingredients Ireland Ltd

Marine Based Functional Food Ingredients

Applications of marine algae in food and health – recent developments and remaining challenges

Dr Dagmar Stengel, National University of Ireland, Galway

Novel ingredients from Irish seaweeds: the CyberColloids approach

Dr Sarah Hotchkiss, CyberColloids Ltd, UK

The novel role of seaweed in traditional food products

Dr Helena Abreu, AlgaPlus, Portugal

Seasonal variation in phlorotannin profiles of Irish brown seaweeds; novel knowledge for industry

Ms Dara Kirke, National University of Ireland, Galway

The challenges and pitfalls of marine bioactive characterisation

Dr Thomas Smyth, Institute of Technology, Sligo

Adding Value to Marine Processing By-Products

Development of an integrated bio-refinery for processing chitin-rich biowaste into speciality chemicals

Prof Dr Volker Sieber, Fraunhofer Institute, Germany

Marine bioresources for use as techno-functional and health beneficial ingredients

Dr Maria Hayes, Teagasc Food Research Centre, Ashtown

Biologically active peptides from marine protein sources

Prof Dick FitzGerald, University of Limerick

Protein isolation from underutilised marine raw materials

Prof Ingrid Undeland, Chalmers University of Technology, Sweden

Novel bioactive ingredients and products derived from marine algae

Ms Rósa Jónsdóttir, Matís Ltd. - Icelandic Food and Biotech R&D

Day 2: Tuesday 30th June 2015

Novel Applications of Marine Derived Bioactives and Biomaterials

Marine bacteria as a source of novel bioactive agents for use in strategies against foodborne pathogens and in food processing applications

Prof Alan Dobson, University College Cork

Commercial exploitation of marine derived bioactives for food and non-food applications

Dr Trevor Francis, CTO Byotrol plc., UK

Marine polysaccharides – potential functional foods against obesity and inflammation

Prof Torres Sweeney/Prof John O'Doherty, University College Dublin

Seafood processing research: trends and practical applications

Prof Ioanis Boziaris, University of Thessaly, Greece

Health Promoting Properties of Marine Derived Ingredients

Marine bioresources – nutritional benefits and challenges

Dr Emeir McSorley, Ulster University

Macroalgae extracts and their role as a source of functional ingredients

Prof. Yvonne Yuan, Ryerson University, Canada

Marine ingredients for improved brain and gut functioning

Dr Catherine Stanton, Teagasc Food Research Centre, Moorepark

Anticancer-activity of aqueous and ethanolic extracts of *Fucus vesiculosus*

Dr Olaf Sunnotel, Ulster University, Northern Ireland

Microalgal omega-3 supplementation during early life impacts learning, anxiety, social behaviour and brain fatty acid composition in C57BL/6 mice

Mr Ruairi Robertson, Teagasc Food Research Centre, Moorepark

Sustainability of Marine Bioresources

Marine bioresources – food security and sustainability

Dr John Forster, Forster Consulting Inc., Washington, USA

Extraction of seaweed biostimulants for agricultural and horticultural applications: an industry

perspective

Dr Franck Hennequart, Oilean Glas Teo, Ireland

Development of algae based products for high value markets

Dr John C. Dodd, Algaecytes Ltd., UK

Chitosan alters dietary intake, microbial populations and nutrient digestibility

Ms Áine Egan, University College Dublin

Provision of solutions to a global market using natural bioactives to stimulate defence, immunity and microbiota

Mr John T. O'Sullivan, BioAtlantis Ltd., Tralee, Co. Kerry

Panel Discussion

Dr Dermot Hurst Chairman

Conference closure

Simon Coveney TD Minister for Agriculture, Food and the Marine

NutraMara Conference 2012

Day 1 - Wednesday 25th April, 2012

Introduction and keynote addresses

Overview of NutraMara Programme

Mr Declan Troy, Teagasc and Director of NutraMara

Welcome of the President of Ireland, Dr Michael D. Higgins

Dr Noel Cawley

Presidential Address

Dr Michael D. Higgins

Setting the Marine Foods Research Agenda

Dr Pamela Byrne, Department of Agriculture, Food and Marine, Ireland

Marine resource sustainability

Chair: Mr Richie Flynn, IFA Aquaculture Section

Sources of Marine Materials – The NutraMara Feasibility Study

Dr Richard FitzGerald, NUI Galway, Ireland

Natural Products: The Fundamentals of Marine Functional Foods

Prof Deniz Tasdemir, Chair of Marine Biodiscovery, NUI Galway, Ireland

Variability in Bioactivity of Algal Compounds – Implications for Commercial Applications

Dr Dagmar Stengel, NUI Galway, Ireland

Marine by-product utilisation and valorisation

Chair: Mr Declan Troy Teagasc and Director of NutraMara,

Novel Enzymes from Marine Waste Products

Prof Colin Barrow, Deakin University, Australia

What's Up? – A French Perspective to Marine Waste Utilisation and Valorisation

Dr Jean-Pascal Berge, IFREMER, France

By-products and Pronova Biopharma – Opportunities for Irish and EU Industries

Dr Alexis Garras, Director, Business Development, Norway

Functional Surimi Seafood Developed from By-products

Dr H. G. Kristinsson, Matis, Iceland

Collagen and Chitin Generation from Irish Marine Processing Waste Streams

Dr Maria Hayes, Teagasc

An Approach to the Valorisation of Fishing By-products in Galicia

Dr Uxía Vázquez Ferreiro, CETMAR, Vigo (Pontevedra), Spain

Valorisation of Shell fish Wastes in Newfoundland by Canadian Industries

Ms. Heather Manuel, MI, St. John's Newfoundland, Canada

Day 2: Tuesday 26th April, 2012

Marine algae and functional ingredients

Chair: Dr Dagmar Stengel, NUI Galway

Marine Algae – A Source of Food Ingredients

Chair: Dr Maria Tuohy, NUI Galway

AlgaePARC: Algae Production and Research Centre – Potential Benefits for Industry

Dr Maria Cuaresma Franco, Wageningen University, The Netherlands

The Potential of Microalgae as a Source of Food Ingredients

Dr Freddy Guiheneuf, NUI Galway

Microalgae: Identification and Selection for Biotechnological Applications

Dr Jean-Paul Cadoret, Research Director, IFREMER, France

Integrating Aquaculture, A Source of Marine Molecules

Dr Anna Soler, NUI Galway

“Omic” Approaches to Identify Novel Bioactive Molecules with Utility in Functional Foods

Prof Fergal O’Gara, University College Cork

Marine Derived Ingredients as Potential Functional Foods

Chair: Prof Colin Barrow, Deakin University, Australia

SmPill(tm): Delivering Complex Innovation

Dr Ivan Coulter, Sigmoid Pharma, Dublin

Proteins and Peptides from Marine Sources: Their Potential as Functional Food Ingredients

Prof Dick FitzGerald & Dr Padráigín Harnedy, University of Limerick

Strategies for the Chemical Characterisation of Marine Natural Products

Dr Thomas Smyth, Teagasc

Marine Polysaccharides – Potential Functional Foods Against Obesity and Inflammation

Prof Torres Sweeney, University College, Dublin

Anti-Cancer Properties of Irish Seaweeds on Colon and Breast Carcinogenesis in Vitro

Dr Chris Gill, University of Ulster at Colerain

Health Benefits from the Incorporation of Irish Seaweed in the Diet

Dr Emeir McSorley, University of Ulster at Colerain

Food Ingredients from the Marine – An Industrial Perspective

Mr. Ross Campbell, Cybercolloids, Co. Cork

Prebiotic Potential of Marine Derived Ingredients

Prof Paul Ross, Teagasc

Pork Meat Enhanced with Seaweed Derived Ingredients

Dr Michael O'Grady, University College Cork

Conference close

Food Research Ireland – Gaps in Research and Opportunities for the Marine Sector in Ireland

Dr Dermot Hurst, Marine Institute, Galway

Concluding remarks

Mr Declan Troy, Teagasc and Director of NutraMara



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