Recent Advances in Omega-3: Health Benefits, Sources, Products and Bioavailability

Edited by

Peter D. Nichols and Matthew R. Miller

Printed Edition of the Special Issue Published in *Nutrients*

www.mdpi.com/journal/nutrients

Peter D. Nichols and Matthew R. Miller (Eds.)

Recent Advances in Omega-3: Health Benefits, Sources, Products and Bioavailability

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This book is a reprint of the special issue that appeared in the online open access journal *Nutrients* (ISSN 2072-6643) in 2014 (available at: http://www.mdpi.com/journal/nutrients/special_issues/omega-3_conference).

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Publisher
Shu-Kun Lin

Production Editor
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1. Edition 2014

MDPI • Basel • Beijing

ISBN 978-3-906980-76-8

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Preface

This volume of *Nutrients* includes twelve independent contributions that focus on – Recent advances in Omega-3: health benefits, sources, products and bioavailability. As a group of molecules, the omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA, also termed LC omega-3), are required for or essential for optimal human and animal health, yet modern western and other diets are often lacking in these key ingredients.

The papers in this volume were presented at the joint meeting of the Australasian Section of the American Oil Chemists Society and the Omega-3 Centre held November 7, 2013 in Newcastle, New South Wales, Australia. A full summary of all oral papers and posters presented at this meeting is available elsewhere [1]. The volume is provided in four sections: 1. A summary paper of the Newcastle meeting and brief summaries of the papers in this volume, 2. Health benefits and bioavailability, four papers; 3. Sources of long-chain omega-3 oils, five papers; 4. Products containing long-chain omega-3 oils, two papers. Three papers in the volume provide reviews of: 1. The Omega-3 Index (O3I), a recently developed measure of red blood cell EPA+DHA; 2. The role of LC omega-3 oils in child cognition; 3. New land plant sources of DHA as a timely solution to the issue of fish oil sustainability.

We anticipate that this selection of papers will provide readers with useful updates on this range of specific and cutting edge research on aspects of the long-chain omega-3. We very much appreciate the time and effort put forth by the authors of these manuscripts, by those who reviewed the manuscripts prior to initial publication in the journal *Nutrients*, by the sterling editorial staff at MDPI and by the Editors in Chief - Prof. Peter Howe and Jon Buckley - and in addition Kevin Krail, Executive Director Omega-3 Centre, who have all made this volume possible.

Peter D. Nichols and Matthew R. Miller
Guest Editors

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1. Overview

Recent Advances in Omega-3: Health Benefits, Sources, Products and Bioavailability

Peter D. Nichols, Alexandra McManus, Kevin Krail, Andrew J. Sinclair, Matt Miller

Abstract: The joint symposium of The Omega-3 Centre and the Australasian Section American Oil Chemists Society; Recent Advances in Omega-3: Health Benefits, Sources, Products and Bioavailability, was held November 7, 2013 in Newcastle, NSW, Australia. Over 115 attendees received new information on a range of health benefits, aquaculture as a sustainable source of supply, and current and potential new and novel sources of these essential omega-3 long-chain (LC, $\geq C_{20}$) polyunsaturated fatty acid nutrients (also termed LC omega-3). The theme of "Food *versus* Fuel" was an inspired way to present a vast array of emerging and ground breaking Omega-3 research that has application across many disciplines. Eleven papers submitted following from the Omega-3 Symposium are published in this Special Issue volume, with topics covered including: an update on the use of the Omega-3 Index (O3I), the effects of dosage and concurrent intake of vitamins/minerals on omega-3 incorporation into red blood cells, the possible use of the O3I as a measure of risk for adiposity, the need for and progress with new land plant sources of docosahexaenoic acid (DHA, 22:6ω3), the current status of farmed Australian and New Zealand fish, and also supplements, in terms of their LC omega-3 and persistent organic pollutants (POP) content, progress with cheap carbon sources in the culture of DHA-producing single cell organisms, a detailed examination of the lipids of the New Zealand Greenshell mussel, and a pilot investigation of the purification of New Zealand hoki liver oil by short path distillation. The selection of papers in this Special Issue collectively highlights a range of forward looking and also new and including positive scientific outcomes occurring in the omega-3 field.

Reprinted from *Nutrients*. Cite as: Nichols, P.D.; McManus, A.; Krail, K.; Sinclair, A.J.; Miller, M. Recent Advances in Omega-3: Health Benefits, Sources, Products and Bioavailability. *Nutrients* **2014**, *6*, 3727–3733.

1. Preface

The one day symposium; Recent Advances in Omega-3: Health Benefits, Sources, Products and Bioavailability, was co-convened by The Omega-3 Centre (O3C) and the Australasian Section of the American Oil Chemists Society (AAOCS) in Newcastle, Australia on November 7, 2013. The symposium was part of a three day Conference where over 150 scientists, researchers and industry representatives gathered for talks and discussions on a variety of lipid related topics. The Omega-3 Symposium was a full day devoted to presentations on recent advances in omega-3 research and was attended by over 115 scientists and industry representatives, with several additional presentations occurring the following day of the conference [1]. The Omega-3 Centre operates as a specialty

healthcare association and center of excellence for omega-3 fatty acids for the Australia and New Zealand region. The primary focus of the O3C is to communicate the "good science" and health benefits of long-chain ($\geq C_{20}$) omega-3 (LC omega-3) oils, and to help translate the science and nutritional health benefits of omega-3 oils to key opinion leaders, including scientists, healthcare practitioners, the media and the public at large.

2. Summary of Papers in this Special Issue

Professor Clemens von Shacky from the Preventive Cardiology Unit at the Ludwig Maximilians-University of Munich opened the Omega-3 Symposium with a keynote address on the Omega-3 Index (O3I) as a biomarker of heart health [2]. He indicated that recent large trials with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the cardiovascular field did not demonstrate a beneficial effect in terms of reductions of clinical endpoints such as total mortality, sudden cardiac arrest or other major adverse cardiac events, and that pertinent guidelines do not currently uniformly recommend EPA + DHA for cardiac patients. His paper—Omega-3 Index and cardiovascular health—therefore emphasized the importance of limiting intervention trials to those people identified with low initial omega-3 status, use of the HS-Omega-3 Index® methodology, and also ensuring that omega-3 supplements are consumed with a meal, as bioavailability data indicated that omega-3 uptake was dependent on concomitant fat intake.

Associate Professor Andrew Pipingas gave a presentation on neurocognitive benefits of omega-3 in healthy adults. In his co-authored paper—Randomized controlled trial examining the effects of fish oil and multivitamin supplementation on the incorporation of omega-3 and omega-6 fatty acids into red blood cells—healthy adult humans were randomized to receive 6 g of fish oil (1.8 g of EPA + DHA), 6 g of fish oil plus a multivitamin, 3 g of fish oil plus a multivitamin or a placebo daily for 16 weeks [3]. Treatment with 6 g of fish oil, with or without a daily multivitamin, led to higher red blood cell EPA composition at endpoint, with DHA composition unchanged. The O3I was only higher in the group receiving the combination of 6 g of fish oil and multivitamin. All treatments increased EPA incorporation in females while, in males, EPA was only significantly increased by the 6 g fish oil plus multivitamin combination. Considerable individual variability occurred in the red cell incorporation of EPA and DHA at endpoint. Gender contributed to a large proportion of this variability with females generally showing higher omega-3 composition at endpoint. It was concluded that the incorporation of LC omega-3 into red blood cells is influenced by dosage, the concurrent intake of vitamin/minerals and gender.

Professor Peter Howe presented data linking the O3I to prediction of risk for coronary heart disease [4]. As the index expresses circulating EPA + DHA as a percentage of total erythrocyte fatty acids, Professor Howe posited that this could be a novel way to measure not only the risk of heart disease, but other common health conditions such as cognitive decline and mental health disorders. The main focus of this paper was the possible use of the O3I as a measure of risk for adiposity. A review of research into adiposity, body composition and erythrocyte EPA and DHA in both men and women found that the low levels of DHA in women were predictive of adiposity risk. There was no associated risk with EPA levels in men. These findings indicate that the O3I could be a quick and cost effective clinical marker for assisting the risk of adiposity in women. Furthermore, this study supports

the trial of adequate intakes of DHA as a complimentary therapy for the management and treatment of overweight individuals and obesity.

The review paper by Dr. Welma Stonehouse from CSIRO covered the topic that LC omega-3 derived from marine sources may play an important role in cognitive performance throughout all life stages [5]. DHA, the dominant omega-3 in the brain, is a major component of neuronal cell membranes and affects various neurological pathways and processess. Despite its critical role in brain function, human's capacity to synthesize DHA *de novo* is limited and its consumption through the diet is therefore essential. However, many individuls do not or rarely consume seafood. Dr. Stonehouse critically evaluated evidence from randomised controlled trials (RCT) in healthy school-aged children, younger and older adults to determine whether consumption of LC omega-3 PUFA improves cognitive performance and to make recommendations for future research. Study design limitations in many RCTs hamper firm conclusions. The measurement of a uniform biomarker, e.g., %DHA in erythrocytes, is essential to establish baseline DHA-status, to determine targets for cognitive performance and to facilitate dosage recommendations. It was recommended that future studies be at least 16 weeks in duration, account for potential interaction effects of gender, age and apolipoprotein E genotype and include measures of speed of cognitive performance.

Several presentations at the symposium covered new sources of LC omega-3 oil, including discussing exciting developments with new land plant sources of long-chain omega-3 oils. A review paper by Dr. Soressa Kitessa and colleagues from CSIRO examined use of oils containing the shorter-chain omega-3, stearidonic acid (SDA, 18:4ω3), in a range of livestock and fish feeding trials with lamb, chicken, Atlantic salmon and barramundi [6]. Interest in the use of SDA has been enhanced by the development of SDA-containing genetically modified soyabean oil which is planned to soon enter the US market. However, neither oils from traditional oilseeds such as linseed, nor the new SDA soyabean oil have demonstrated efficient conversion to DHA in the animals' trialed or in humans. It is this knowledge that has driven the quest by a number of research groups to produce oil seeds containing LC omega-3, in particular DHA. Previous attempts to produce DHA in oilseeds only achieved low levels of DHA and also were high in omega-6 PUFA and contained a high omega-6/omega-3 ratio. Dr. Surinder Singh in his conference presentation [1] and Dr. Kitessa and colleagues [6] described a recent breakthrough that has demonstrated the ability to produce land plant-based oil particularly enriched in DHA, with low omega-6 PUFA levels, and an omega-3 to omega-6 ratio close to that occurring in marine oils/seafood [7]. Therefore, the future availability of land plant oils containing both EPA and DHA can supplement the demand for marine sources of LC omega-3 oils in a range of areas. This in turn will enhance the sustainability of global fisheries, enable the consumer to meet the recommended dietary targets for these oils, assist in aquaculture nutrition and the development of an innovative food and feed industry, and ultimately deliver improved health of consumers.

The paper by Dr. Peter Mansour and colleagues from CSIRO—Characterization of oilseed lipids from DHA-producing *Camelina sativa*: A new transformed land plant containing long-chain omega-3 oils—provided detailed lipid class and fatty acid profiles for a new land plant derived oil [8]. Triacylglycerols (TAG) were the major lipid class in hexane extracts. Chloroform-methanol (CM) extraction recovered further lipid comprising glycolipids and phospholipids and residual TAG. The main phospholipid species were phosphatidyl choline and phosphatidyl ethanolamine. The % DHA

was: 7% (of total fatty acids) in the TAG-rich hexane extract and 4% in the polar lipid-rich CM extract. The relative level of ALA in DHA-containing *Camelina* seed was higher than the control. Sterols and fatty alcohols were characterized, with iso-branched odd-chain fatty alcohols, also present.

Several members from the Centre for Chemistry and Biotechnology lead by Professor Colin Barrow at Deakin University covered aspects of single cell oil production. A paper by Thyagarajan and colleagues examined—Evaluation of bread crumbs as a potential carbon source for the growth of Thraustochytrid species for oil and omega-3 production [9]. The utilization of food waste by microorganisms to produce omega-3 fatty acids or biofuel is a low cost method with environmental benefits. It was shown that the marine microorganisms *Thraustochytrium* sp. AH-2 and *Schizochytrium* sp. SR21 were able to use breadcrumbs as an alternate carbon source for the production of lipids under static fermentation conditions. Liquid fermentation of *Thraustochytrium* sp. AH-2 with glucose produced 4.3 g/L of biomass and 44 mg/g of saturated fatty acids after seven days. Static fermentation of both species with breadcrumbs resulted in 2.5 g/L and 4.7 g/L of biomass, and 42 mg/g and 34 mg/g of saturated fatty acids, respectively. Scanning electron microscopic studies confirmed the growth of both strains on breadcrumbs. Attenuated total reflection Fourier transform infrared spectroscopy findings for *Schizochytrium* sp. SR21 were consistent with the utilization of breadcrumbs for the production of unsaturated lipids, albeit at relatively low levels. The total lipid yield for static fermentation with bread crumbs was marginally lower than for fermentation with glucose media, while the yield of unsaturated fatty acids was considerably lower, indicating that static fermentation may be more appropriate for the production of biodiesel than for the production of omega-3 rich oils in these strains.

Dr. Matthew Miller and colleagues from Plant and Food, New Zealand described the distribution of lipids in Greenshell™ Mussel (GSM) (*Perna canaliculus*) [10]. He indicated that GSM are a sustainable source of omega-3 LC-PUFA, as they require no dietary inputs, gaining all of their nutrient requirements by filter-feeding microorganisms from sea water. GSM oil is valued considerably higher than fish oils, and has been reported to have important health benefits, for example, anti-inflammatory activity. It contains several minor lipid components—such as non-methylene interrupted FA, plasmalogens and phytosterols—that are not present in most fish oil products. The lipid content of the female GSM was shown to be significantly greater than that of the male, and the major lipid class in both genders was phospholipid. Female GSM contained more LC omega-3, and stored a greater proportion of total lipid in the gonad and mantle. The higher lipid content in the female was likely related to gamete production. The mantle and digestive gland were other important sites for lipid storage and/or function/production.

Seafood continues to be one of the major sources of LC omega-3 oils. Following an update by Professor Giovanni Turchini on the use of omega-3 in aquaculture [1], Dr. Peter Nichols and co-authors provided a presentation: Readily available sources of long-chain omega-3 oils: Is farmed Australian seafood a better source of the good oil than wild-caught seafood? [11]. The two major farmed Australian finfish species, Atlantic salmon and barramundi, have higher oil and LC omega-3 content than the same or other species from the wild, and remain an excellent means to achieve substantial intake of LC omega-3 oils. Notwithstanding, LC omega-3 oil content has decreased in these two farmed species, due largely to the replacing of dietary fish oil with poultry oil in the feed. For Atlantic salmon, LC omega-3 content decreased ~30%–50% between 2002 and 2013, and the omega-

3/omega-6 ratio also decreased (>5:1 to <1:1). The development and future application of oilseeds containing LC omega-3 oils and their incorporation in aquafeeds would allow these health-benefitting oils to be maximized in farmed Australian seafood. As Australian consumers increasingly seek their LC omega-3 from supplements, a range of supplement products were also compared; all products met their label specifications, with considerable variation occurring in relative levels of EPA and DHA and the cost to consumers for consumption of 500 mg of EPA + DHA per day.

Adam Ismail from the Global Organization for EPA and DHA Omega-3 (GOED) in a plenary lecture at the Omega-3 Session presented a challenge to the fish oil industry around demand and supply [1]. He highlighted the Omega-3 ingredient marketplace as a growing US $25 billion industry, with an increase in krill and new pharmaceutical omega-3 products predicted to gain significant market penetration which will put even further demand pressure on resources.

Building on the theme of Adam Ismail, increasing the quality of products derived from smaller fishers will also help with the supply issue; work presented by the collaboration of Dr. Alex Oliveria (Kodiak, AK, USA) and Dr. Matt Miller (Nelson, New Zealand) used short path distillation as a method to improve the quality of the Alaskan pollock (*Gadus chalcogrammus*) and New Zealand's hoki (*Macruronus novaezelandiae*) oils [12]. This paper—Purification of Alaskan walleye pollock (*Gadus chalcogrammus*) and New Zealand hoki (*Macruronus novaezelandiae*) liver oil using short path distillation—demonstrated that this technology could significantly enhance oil quality parameters (free fatty acids, peroxide and *para*-anisidine values), and that purified oils obtained met the GOED standard for edible fish oils.

The paper by Susan Bengtson Nash, University of Griffiths, and colleagues addressed: The nutritional-toxicological conflict associated with fish oil *versus* Antarctic krill oil dietary supplements [13]. Fish oil supplements and complementary medicines play a role of increasing importance in meeting daily requirements of essential nutrients such as LC omega-3 and Vitamin D. A new product category, derived from Antarctic krill, has gained an increasing share of the omega-3 nutriceutical market. Antarctic krill oil is marketed as demonstrating a greater ease of absorption due to higher phospholipid content, as being sourced through sustainable fisheries and being free of toxins and pollutants. However, limited data is available on the latter. Persistent Organic Pollutants (POP) encompasses a range of toxic, man-made contaminants that accumulate in marine ecosystems and in the lipid reserves of organisms. The study provides the first quantitative comparison of the nutritional (EPA and DHA) *versus* the toxicological profiles of Antarctic krill oil products relative to other fish oil categories on the Australian market. Krill oil products adhered closely to EPA and DHA manufacturer specifications and contained intermediate levels of POP when compared to other products. Monitoring of the pollutant content of fish and krill oil products will become increasingly important with expanding regulatory specifications for chemical thresholds.

The selection of papers in this Special Issue highlights a range of new and including positive scientific outcomes occurring in the omega-3 field across the areas of health benefits, sources, products and bioavailability. Future research in the Australasia region will continue in these areas and will assist in further increasing our understanding of the key health-benefitting long-chain omega-3 oils.

Acknowledgments

The authors thank the sponsors of the Omega-3 symposium—Sanofi-Aventis Consumer Healthcare, Aker BioMarine, Nuseed, NuMega-Ingredients, Melrose Natural Health Products, together with Cargill, Bakels, Australian Oilseeds Federation and Global Specialty International, John West and Vita-blend, Newcastle Innovation, CSIRO, Malaysian Palm Oil Council, New Zealand King Salmon and Nutrients Journal—for their financial assistance towards the running costs of the meeting. We thank James Petrie and Peter Mansour for their helpful comments on the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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2. Health Benefits and Bioavailability

Omega-3 Index and Cardiovascular Health

Clemens von Schacky

Abstract: Recent large trials with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the cardiovascular field did not demonstrate a beneficial effect in terms of reductions of clinical endpoints like total mortality, sudden cardiac arrest or other major adverse cardiac events. Pertinent guidelines do not uniformly recommend EPA + DHA for cardiac patients. In contrast, in epidemiologic findings, higher blood levels of EPA + DHA were consistently associated with a lower risk for the endpoints mentioned. Because of low biological and analytical variability, a standardized analytical procedure, a large database and for other reasons, blood levels of EPA + DHA are frequently assessed in erythrocytes, using the HS-Omega-3 Index® methodology. A low Omega-3 Index fulfills the current criteria for a novel cardiovascular risk factor. Neutral results of intervention trials can be explained by issues of bioavailability and trial design that surfaced after the trials were initiated. In the future, incorporating the Omega-3 Index into trial designs by recruiting participants with a low Omega-3 Index and treating them within a pre-specified target range (e.g., 8%–11%), will make more efficient trials possible and provide clearer answers to the questions asked than previously possible.

Reprinted from *Nutrients*. Cite as: Von Schacky, C. Omega-3 Index and Cardiovascular Health. *Nutrients* **2014**, *6*, 799–814.

1. Introduction

Fish, marine oils, and their concentrates all serve as sources of the two marine omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), as do some products from algae. To demonstrate an effect of EPA + DHA on heart health, a number of randomized, controlled intervention studies with clinical endpoints like overall mortality or a combination of adverse cardiac events were conducted in populations with elevated cardiovascular risk. One early intervention study with oily fish, rich in EPA + DHA, and some early studies with fish oil or fish oil concentrate or even purified EPA at doses ranging between 0.9 and 1.8 g/day indeed demonstrated effects in terms of fewer sudden cardiac deaths, fatal or non-fatal myocardial infarctions, or a combination of adverse cardiac events [1–6]. More recent trials did not demonstrate such effects [7–12]. Recent meta-analyses found no significant benefits on total mortality, cardiovascular mortality, and other adverse cardiac or cardiovascular events [13–18]. This is in contrast to findings in epidemiologic studies, where intake of EPA + DHA had been found to correlate generally with an up to 50% lower incidence of adverse cardiac events [18,19], and in even sharper contrast to epidemiologic studies based on levels of EPA + DHA, demonstrating e.g., a 10-fold lower incidence of sudden cardiac death associated with high levels of the fatty acids, as compared to low levels [20,21]. This seemingly contradictory evidence has led the American Heart Association to recommend “omega-3 fatty acids from fish or fish oil capsules

(1 g/day) for cardiovascular disease risk reduction" for secondary prevention, whereas the European Society for Cardiology recommends "Fish at least twice a week, one of which to be oily fish", but no supplements for cardiovascular prevention [22,23]. The more recent guidelines on treating patients with stable ischemic heart disease or patients after a myocardial infarction, targeting similar patient populations, do not recommend EPA + DHA [24,25]. At least in Europe, cardiologists do not routinely use EPA + DHA to reduce cardiovascular risk.

A similar picture emerges for atrial fibrillation: In epidemiologic studies, consumption of EPA + DHA or higher levels of EPA + DHA were associated with lower risk for developing atrial fibrillation, while intervention studies found no effect [26–28]. Pertinent guidelines do not mention EPA + DHA [29]. A similar picture also emerges for severe ventricular rhythm disturbances [20,21,30,31].

Why is it that trial results are at odds with results from epidemiology? What needs to be done to better translate the epidemiologic findings into trial results? The current review will try to shed some light on this issue, with a special consideration of the Omega-3 Index.

2. The Omega-3 Index as a Cardiovascular Risk Factor

At least some nutritional surveys do not provide valid data [32]. This may explain, why the relation of EPA + DHA in the diet to clinical events has been found to be looser than the relation of levels of EPA + DHA measured in blood to clinical events (e.g., [20,33]). A detailed discussion of the pros and cons of the various fatty acid compartments in which levels of omega-3 fatty acids (whole blood, whole plasma, plasma phospholipids, and others) should be measured is outside the scope of this review and can be found elsewhere [34]. The following points argue for the use of erythrocytes: erythrocyte fatty acid composition has a low biological variability, erythrocyte fat consists almost exclusively of phospholipids, erythrocyte fatty acid composition reflects tissue fatty acid composition, pre-analytical stability, and other points [34–38]. In 2004, EPA + DHA in erythrocyte fatty acids were defined as the Omega-3 Index and suggested as a risk factor for sudden cardiac death [39]. Integral to the definition was a specific and standardized analytical procedure, conforming the quality management routinely implemented in the field of clinical chemistry [39] In fatty acid analysis, methods have a large impact on results: when one sample was sent to five different laboratories offering determination of an Omega-3 Index, results differed by a factor of 3.5 [34]. While results may be internally valid in one laboratory, a difference by a factor of 3.5 makes it impossible to compare results among laboratories. Therefore, the Omega-3 Index was renamed HS-Omega-3 Index®. In contrast, the laboratories adhering to the HS-Omega-3 Index methodology perform regular proficiency testing, as mandated in routine Clinical Chemistry labs [34]. So far, the HS-Omega-3 Index is the only analytical procedure used in several laboratories. A standardized analytical procedure is a prerequisite to generate the data base necessary to transport a laboratory parameter from research into clinical routine. Moreover, standardization of the analytical procedure is the first important criterion for establishing a new biomarker for cardiovascular risk set forth by the American Heart Association and the US Preventive Services Task Force [40,41].

As exemplified by Table 1, the HS-Omega-3 Index has been measured in many populations. Of note, a lower HS-Omega-3 Index was always associated with a poorer clinical condition (Table 1).

Table 1. Mean HS-Omega-3 Index values in various populations, Mean (±standard deviation (SD)). Please note that in every population studied, a lower value was found to be associated with a worse condition than a higher value. References are given, if not, unpublished, *n* = number of individuals measured.

Population	HS-Omega-3 Index
Western countries (high incidence of coronary heart disease)	
Germany	
Unselected Individuals (*n* = 5000)	7.15 (±2.19)%
Patients with atherosclerosis [42], (*n* = 190)	5.94 (±1.41)%
Patients with hyperlipidemia [43], (*n* = 47)	7.00 (±1.90)%
Pregnant women, week 24 (*n* = 103)	7.66 (±1.83)%
Patients with congestive heart failure (*n* = 895)	3.47 (±1.20)%
Patients with major depression [44], (*n* = 90)	3.93 (±1.50)%
Spain	
Individuals with high risk for, but without cardiovascular disease [45], (*n* = 198) (SD not reported)	7.10%
Norway	
Patients with myocardial infarction [46] (SD not reported)	
With ventricular fibrillation (*n* = 10)	4.88%
Without ventricular fibrillation (*n* = 185)	6.08%
Europe	
Unselected data from routine determinations, *n* = 10,000	6.96 (+2.15)%
USA	
Healthy in Kansas City [47], (*n* = 163)	4.90 (±2.10)%
Framingham-Offspring [48], (*n* = 3196)	5.60 (±1.70)%
Patients with stable coronary heart disease [49], (*n* = 956) (SD not reported)	4.60%
Patients with major depression [50], (*n* = 118)	2.90 (±1.50)%
Adolescents with major depression [51], (*n* = 150) (SD not reported)	3.46%

Table 1. *Cont.*

Patients with severe obstructive sleep apnea [52], (n = 52) (SD not reported)	4.00%
Saudi Arabia	
Individuals, most with diabetes (n = 69)	3.47 (±1.20) %
Asian countries (low incidence of coronary heart disease)	
Korea	
Healthy controls [53], (n = 50) (SD not reported)	11.81%
Healthy control [54], (n = 40)	10.55 (±0.48)%
Patients with myocardial infarction [53], (n = 50), (SD not reported)	9.57%
Patients with hemorrhagic brain infarction [54], (n = 40)	8.55 (±0.41)%
Patients with ischemic brain infarction [54], (n = 40)	8.19 (±0.64)%
Hemodialysis-patients without calcification on plain chest radiograph [55], (n = 11)	9.82 (±2.37)%
Hemodialysis-patients with calcification on plain chest radiograph [55], (n = 20)	9.23 (±2.34)%
Peritoneal Dialysis Patients [56], (n = 14)	12.83 (±3.30)%
Patients with a kidney transplant [57], (n = 49)	9.70 (±1.85)%
Japan	
Unselected men (n = 262), (SD not reported)	9.58%

All levels of fatty acids are determined by the balance of substance entering the body and those leaving the body. Neither a recent meal, even if rich in EPA + DHA, nor severe cardiac events altered the HS-Omega-3 Index [38,58–61]. However, while long-term intake of EPA + DHA, e.g., as assessed with food questionnaires, was the main predictor of the HS-Omega-3 Index, long-term intake explained only 12%–25% of its variability [46,62,63]. A hereditary component of 24% exists [64]. A number of other factors correlated positively (+) or negatively (−), like age (+), body mass index (−), socioeconomic status (+), smoking (−), but no other conventional cardiac risk factors [47,64–71]. More factors determining the level of the HS-Omega-3 Index, especially regarding efflux remain to be defined. Therefore, it is impossible to predict the HS-Omega-3 Index in an individual, as it is impossible to predict the increase in the HS-Omega-3 Index in an individual in response to a given dose of EPA + DHA [42,46,62,63]. In Table 2, current evidence is presented on the relation of the HS-Omega-3 Index to cardiovascular events.

This evidence is supported by measurements of EPA + DHA in other fatty acid compartments, as discussed in more detail elsewhere [72,73]. Within the framework of “Heart and Soul” and “Triumph”, it was investigated whether determination of the HS-Omega-3 Index added to the information obtained by assessing cardiovascular risk with a conventional scoring system, like the Framingham or GRACE scores for predicting fatal events. The HS-Omega-3 Index provided additional information, as demonstrated by larger areas under the curves in various c-statistics for fatal [74] and non-fatal events [53,75]. Taken together, the HS-Omega-3 Index predicts risk, appears largely independent of conventional risk factors, and adds to the information obtained by conventional risk scoring, thus fulfilling the second criterion for establishing a new biomarker for cardiovascular risk set forth by the American Heart Association and the US Preventive Services Task Force [40,41].

Table 2. Summary of epidemiologic studies relating the Omega-3 Index to cardiovascular events.

Acronym [reference]	Design	Disease	*n*	Years	Criterion	Comparison	Result
Total mortality							
Heart & Soul [49]	cohort	stable CAD	956	5.9	total mortality	HS-Omega-3 Index	HR 0.73; 95% CI, 0.56–0.94
Triumph [74]	cohort	recent MI	1144	2	total mortality	EPA in red cells tertiles	EPA < 0.25% total mortality 26%, 0.25 < EPA < 0.8% total mortality 13%, EPA > 0.80% total mortality 7%
Triumph [76]	cohort	recent MI	1424	1	total mortality	HS-Omega-3 Index < 4% *vs*. >4.0%	HR 2.0; 95% CI 1.2–3.3
Racs * [77]	cohort	recent ACS	460	2	total mortality	HS-Omega-3 Index in quartiles	not significant.
Sudden cardiac death							
[20]	case-control	SCD	82/108 cases/controls		SCD	red cell EPA + DHA in quartiles	OR 1.0–0.1 (95% CI 0.1–0.4)
Phys Health [21]	case-control	SCD	84/182 cases/controls		SCD	whole blood EPA + DHA in quartiles	OR 1.0–0.1 (95% CI 0.02–0.48) across quartiles
Cardiac morbidity							
[78]	case-control	ACS	94/94 cases/controls		ACS	whole blood EPA + DHA in quintiles	OR 1.0–0.2 (95% CI not reported), OR 0.67 (95% CI 0.46 to 0.98) per, 1 standard deviation increase EPA + DHA
[79]	case-control	ACS	768/768 cases/controls		ACS	HS-Omega-3 Index in tertiles	OR 1.0–0.31 (95% CI 0.14–0.67) across tertiles
[53]	case-control	ACS	50/50 cases/controls		ACS	HS-Omega-3 Index in tertiles	OR 1.0–0.08 (95% CI 0.02–0.38) across tertiles
no acronym [80]	case-control	ACS	24/68 cases/controls		STEMI	HS-Omega-3 Index in tertiles	OR 6.38 (95% CI 1.02–39.85)–1.0 across tertiles

Abbreviations: *n*: number of individuals studied; Coronary artery disease: CAD; HR: hazard ratio; MI: myocardial infarction; EPA: eicosapentaenoic acid; ACS: acute coronary syndrome; SCD: sudden cardiac death; DHA: docosahexaenoic acid; OR: odds ratio; STEMI: ST-elevation myocardial infarction. * No case estimate was reported in Racs. Therefore, by definition, it is unclear, whether the discriminatory power of the HS-Omega-3 Index was too small, or the study was too small to detect the discriminatory power.

Moreover, the HS-Omega-3 Index has made it possible to reclassify individuals from intermediate cardiovascular risk into the respective high risk and low risk strata [74,75], the third criterion for establishing a new biomarker for cardiovascular risk [40,41].

Increasing the HS-Omega-3 Index by increased intake of EPA + DHA in randomized controlled trials improved a number of surrogate parameters for cardiovascular risk: heart rate was reduced, heart rate variability was increased, blood pressure was reduced, platelet reactivity was reduced, triglycerides were reduced, large buoyant low-density lipoprotein (LDL)-particles were increased and small dense LDL-particles were reduced, large buoyant high-density lipoproteins (HDL)2 were increased, very low-density lipoprotein (VLDL1) + 2 was reduced, pro-inflammatory cytokines (e.g., tumor necrosis factor alpha, interleukin-1β, interleukins-6,8,10 and monocyte chemoattractant protein-1) were reduced, anti-inflammatory oxylipins were increased [43,81–94]. Importantly, in a two-year randomized double-blind angiographic intervention trial, increased erythrocyte EPA + DHA reduced progression and increased regression of coronary lesions, an intermediate parameter [95]. Taken together, increasing the HS-Omega-3 Index improved surrogate and intermediate parameters for cardiovascular events. A large intervention trial with clinical endpoints based on the HS-Omega-3 Index remains to be conducted. Therefore, the fourth criterion, proof of therapeutic consequence of determining the HS-Omega-3 Index, is only partially fulfilled [40,41].

3. Discussion of Neutral Results of Large Intervention Trials

Why is it that a low HS-Omega-3 Index can be a cardiovascular risk factor, and yet the results of the large trials testing EPA + DHA on clinical endpoints were neutral?

3.1. Bioavailability Issues

According to personal information from the respective first authors, participants of recent large intervention trials were advised to take their supplements, frequently an encapsulated EPA + DHA ethyl-ester with breakfast—in many countries a low-fat meal [7–11]. As discussed in more detail in a recent review, bioavailability of EPA + DHA depends on the chemical form in which they are bound (phospholipids > recombined triglycerides > triglycerides > free fatty acids > ethyl-esters) [96,97], on matrix effects (capsule ingestion with concomitant intake of food, fat content in food) or galenic form (*i.e.*, microencapsulation, emulsification). The chemical binding form impacts on bioavailability roughly with a factor of two, whereas matrix effects can impact bioavailability up to a factor of 13, and the galenic form up to a factor of 21 [96–99]. When the large trials mentioned here were designed, the bioavailability issues just mentioned were unknown. Thus, involuntarily, the combination used in many of the large trials—An unemulsified ethyl-ester or triglyceride with a low fat meal—guaranteed a very low bioavailability of EPA + DHA.

3.2. Issues in Trial Design

In all large intervention trials conducted so far, study participants were recruited based on clinical conditions, but irrespective of their baseline omega-3 fatty acid status [1–12]. In all populations studied so far, the HS-Omega-3 Index had a statistically normal distribution (Table 1). Thus, the

proportion of the study population with high levels was not prone to the effects of EPA + DHA, if any. In order to recruit a study population, in which an effect of EPA + DHA can be demonstrated, recruiting study participants with a low HS-Omega-3 Index is a logical choice.

In all large intervention trials conducted so far, study participants were exposed to a trial-specific, but fixed dose of EPA + DHA or placebo [1–12]. The inter-individual variability in response to a fixed dose of EPA + DHA has been found to be large, *i.e.*, vary up to a factor of 13 [42,61]. This fact alone suggests individualizing the dose given in a trial, in order to reach a predefined target range of the HS-Omega-3 Index, e.g., 8%–11%. The statistically normal distribution of the baseline HS-Omega-3 Index further complicates this problem: A large overlap of omega-3 levels in the EPA + DHA group and placebo or control group can be expected, and has been seen in at least one large trial (Mühlhäusler, B., personal communication) [100]. With levels of omega-3 fatty acids not differing between intervention and placebo or control groups, a difference in study outcome cannot be expected, even if the condition studied would be susceptible to treatment with EPA + DHA. It is worth noting that when a neutral intervention trial was analyzed in a cross-sectional way, EPA + DHA levels directly related to study outcome and less to treatment allocation [101].

Conversely, if a trial reports a positive result, it is likely to have been conducted in a study population with low baseline levels of EPA and DHA, like congestive heart failure: a positive result of a large trial was reported [6], and we found a low mean HS-Omega-3 Index in patients with congestive heart failure (unpublished data, Table 1). A similar case can be made for major depression (Table 1, references [44,50,51,84]).

In the future, recruiting study participants with a low baseline HS-Omega-3 Index and treating them within a predefined target range will allow clearer trial results to be a distinct possibility. Dose adjustments will need to be performed in the placebo group. Since a larger treatment effect can be assumed in the study size estimation, it can be expected that study sizes will be smaller and thus studies less expensive. Clearly, these thoughts are not restricted to trials with patients with cardiovascular risk, atrial fibrillation or ventricular arrhythmia, but can be extended to all areas of omega-3 fatty acid research. This will facilitate scientific progress and lead to a faster recognition of the effects of EPA + DHA.

4. Conclusions

In an inconsistent manner, EPA and DHA are either recommended or not included in guidelines of cardiac scientific societies. The use of EPA and DHA is not supported by results of recent intervention trials or their meta-analyses. However, epidemiologic data based on assessments of diet and, even more so, data based on levels of EPA + DHA measured in humans, clearly demonstrate that EPA + DHA are associated with a low risk for total mortality, sudden cardiac arrest, and fatal and non-fatal myocardial infarctions. For a number of reasons, like a standardized analytical procedure and a large data base, levels of EPA + DHA are best assessed with the HS-Omega-3 Index. According to current criteria of the American Heart Association and others, the HS-Omega-3 Index is a novel cardiovascular risk factor. Moreover, the HS-Omega-3 Index has led to a fresh look at the field of omega-3 fatty acids and has made it possible to identify issues of bioavailability and study design, explaining at least in part the neutral results of previous intervention trials. In the future, more efficient

intervention studies can be conducted based on the HS-Omega-3 Index, thus providing a clearer picture of the effects of EPA + DHA.

Conflicts of Interest

CvS operates Omegametrix, a laboratory for fatty acid analyses. Speaker honoraria were received from Reckitt-Benckiser and the Portuguese National Fisheries.

Acknowledgments

Research grants were provided by several government agencies, AkerBiomarine, Neptune, and Fresenius Kabi. The cost of travel to the AAOCS meeting was provided by Sanofi Consumer Health Care.

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Relationship between Erythrocyte Omega-3 Content and Obesity Is Gender Dependent

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Abstract: Epidemiological evidence of an inverse association between consumption of long-chain omega-3 polyunsaturated fatty acids (LC *n*-3 PUFA) and obesity has been conflicting, even though studies in animal models of obesity and limited human trials suggest that LC *n*-3 PUFA consumption may contribute to weight loss. We used baseline data from a convenience sample of 476 adults (291 women, 185 men) participating in clinical trials at our Centre to explore relationships between erythrocyte levels of LC *n*-3 PUFA (a reliable indicator of habitual intake) and measures of adiposity, viz. body mass index (BMI), waist circumference (WC) and body fat (BF) assessed by dual-energy X-ray absorptiometry. Means ± SD of assessments were BMI: 34 ± 7 and 31 ± 5 kg/m^2; WC: 105 ± 16 and 110 ± 13 cm; BF: 48 ± 5 and 35% ± 6% in women and men respectively. Erythrocyte levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were similar in men and women while docosapentaenoic acid (DPA) was higher and EPA + DHA (Omega-3 Index) slightly lower in men than in women. Both DHA and EPA + DHA correlated inversely with BMI, WC and BF in women while DPA correlated inversely with BF in men. Quartile distributions and curvilinear regression of the Omega-3 Index *versus* BMI revealed a steep rise of BMI in the lower range of the Omega-3 Index in women, but no association in men. Thus the results highlight important gender differences in relationships of specific LC *n*-3 PUFA in erythrocytes to markers of adiposity. If these reflect causal relationships between LC *n*-3 PUFA consumption and risk of obesity, gender specific targeted interventions should be considered.

Reprinted from *Nutrients*. Cite as: Howe, P.R.C.; Buckley, J.D.; Murphy, K.J.; Pettman, T.; Milte, C.; Coates, A.M. Relationship between Erythrocyte Omega-3 Content and Obesity Is Gender Dependent. *Nutrients* **2014**, *6*, 1850–1860.

1. Introduction

Polyunsaturated fatty acids (PUFA) are known to beneficially influence fat metabolism and there are numerous studies in animal models of obesity showing that consumption of PUFA, particularly the long-chain omega-3 (LC *n*-3) PUFA from marine sources, can increase fat loss and counteract adiposity [1,2]. This has been supported by a limited number of human trials of LC *n*-3 PUFA supplementation [1–3], although epidemiological evidence of an inverse association between consumption of LC *n*-3 PUFA and obesity has been conflicting [4,5].

In the Health Professionals Follow-Up Study, men with high fish consumption were less likely to be overweight than those with low fish consumption and the proportion of overweight volunteers was inversely related to LC *n*-3 PUFA intake [4]. The Nurses' Health Study, on the other hand, found that higher intakes of fish and LC*n*-3 PUFA were associated with a higher prevalence of obesity [5]. While this unexpected effect of fish intake could be accounted for by higher energy intakes, this was not the

case for LC *n*-3 PUFA intake. However, both these large studies estimated dietary intakes from semi-quantitative food frequency questionnaires which are limited in their ability to accurately assess intakes of different types of fat, particularly LC *n*-3 PUFA.

An alternative approach to assess relationships between LC *n*-3 PUFA consumption and obesity is to evaluate a surrogate biomarker of LC *n*-3 PUFA intake. Several studies have measured LC *n*-3 PUFA as a percentage of total fatty acids in plasma phospholipids with conflicting outcomes. Three early studies conducted in populations of varying ethnicity in Canada found that plasma phospholipid LC *n*-3 PUFA correlated positively with waist circumference [6–8] whereas more recent studies have reported inverse correlations with measures of adiposity [9–11].

Fatty acid levels in plasma phospholipids reflect consumption of dietary fatty acids over a relatively short period (weeks), whereas erythrocyte levels reflect intake over several months [12,13]. Hence the latter is regarded as the most reliable surrogate marker of habitual dietary intake of LC *n*-3 PUFA. Docosahexaenoic acid (DHA), in particular, is incorporated and retained predominantly inside the plasma membrane for the 4 month life of the erythrocyte [12]. Surprisingly, there is little information on relationships between erythrocyte LC *n*-3 PUFA levels and adiposity, although a recent analysis of a cohort of almost 3000 subjects from the Framingham Heart Study indicated a modest inverse relationship between erythrocyte LC *n*-3 PUFA and waist circumference [14].

As we routinely measure erythrocyte fatty acids in nutritional intervention trials, we have chosen to examine relationships between erythrocyte LC *n*-3 PUFA levels and measures of adiposity in baseline data obtained from a convenience sample of trial participants, most of whom had undergone DEXA assessments of body composition. In particular, we have sought to explore potential gender differences in such relationships.

2. Methods

2.1. Participants and Data

A secondary analysis was undertaken using de-identified pooled data obtained from volunteers who had participated in nutritional intervention trials conducted by the University of South Australia's Nutritional Physiology Research Centre between 2005 and 2009. Five trials were selected in which measures of weight and adiposity, together with analysis of erythrocyte fatty acid levels, had been undertaken at baseline. Each trial had been approved by the University's Human Research Ethics Committee.

Participants were free-living, non-smoking men and women from both metropolitan and regional locations who were predominantly overweight/obese (inclusion criterion for three of the five trials) but otherwise healthy (*i.e.*, without a diagnosed disease condition) and had limited consumption of fish or fish oil (inclusion criterion for four trials). Baseline anthropometric measurements and blood samples for determination of erythrocyte fatty acid profiles were obtained from 476 participants prior to undergoing dietary interventions. Additionally, dual-energy X-ray absorptiometry (DEXA) assessments of body composition were obtained at the same time from 376 of these participants.

2.2. Assessments

2.2.1. Anthropometric Measurements

Each participant's height and weight were recorded to calculate body mass index (BMI). Height was measured to the nearest 0·1 cm whilst barefoot using a wall-mounted stadiometer (SECA; Vogel & Halke, Hamburg, Germany). Body weight was measured to the nearest 0.1 kg with participants wearing light clothing using a TANITA Ultimate Scale 2000 (Tanita Corporation, Tokyo, Japan). Waist circumference was measured using a metric tape according ISAK international guidelines [15].

2.2.2. Body Composition

Each participant underwent a whole body DEXA scan (Lunar Prodigy, General Electric, Madison, WI, USA) to determine fat mass and lean mass, from which percentage body fat was estimated.

2.2.3. Assessment of Fatty Acid Profiles

Relative proportions of individual fatty acids in erythrocytes were assessed using a procedure adapted from previously published methods [16]. Erythrocytes were isolated within 2 h of collection by centrifugation, washed in isotonic saline and stored at −80 °C. They were subsequently thawed and the lipids were extracted with chloroform and isopropanol (2:1). The organic phase containing the lipid was evaporated to dryness under a stream of N_2 gas. The lipids were then transesterified with acetyl chloride in methanol toluene (4:1, *v*/*v*) at 100 °C for 1 h. The resultant fatty acid methyl esters were extracted with 10% potassium carbonate. Fatty acid methyl esters were separated and quantified using a Shimadzu 2010 gas chromatograph equipped with a 50 m capillary column (0·32 mm, inner diameter) coated with BPX-70 (0·25 mm film thickness; SGE Analytical Science Pty Ltd., Ringwood, VIC, Australia). The injector temperature was set at 250 °C and the detector (flame ionisation) temperature at 260 °C. The initial oven temperature was 130 °C and was programmed to rise to 220 °C at 58 °C/min. H_2 was used as the carrier gas at a velocity of 36.4 cm/s. Fatty acid methyl esters were identified based on the retention time to authentic lipid standards (GLC-463; Nu-Chek Prep, Inc., Elysian, MN, USA).

Erythrocyte contents of eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexanoic acid (DHA) were expressed as percentages of total erythrocyte fatty acids. The Omega-3 Index was calculated as the sum of the EPA and DHA contents.

2.3. Statistical Analysis

Data were analysed using SPSS for Windows (Version 21.0, 2012) and presented as means ± SD (standard deviations). Gender differences in outcome measures were determined by Student's *t*-test; statistical significance was set at $p < 0.05$. Relationships between markers of adiposity and erythrocyte fatty acid contents were assessed by correlation analysis and expressed as Pearson correlation coefficients (r). A Bonferroni correction was made for comparisons of each adiposity measure with multiple fatty acids whereby statistical significance was set at $p < 0.01$. Univariate models were used

to test for gender interactions. Quartiles of Omega-3 Index were determined for each gender and mean BMI values for each quartile were compared by ANOVA with statistical significance set at $p < 0.05$.

3. Results

3.1. Participant Characteristics

Participants were middle-aged and predominantly women. Table 1 presents anthropometric and body compositional assessments for each gender. Due to the selection of overweight/obese adults for the majority of clinical trials, average values of BMI fell within obese classifications for men and women, although there was a wide range (18–59 kg/m^2).

Table 1. Participant characteristics *.

	Males	Females
Age (year)	45.6 ± 11.6 (185)	47.5 ± 12.3 (291)
Weight (kg)	99.4 ± 17.3 (185)	91.3 ± 19.9 (291)
Height (m)	177.7 ± 7.0 (185)	163.6 ± 6.8 (291)
Body mass index (kg/m^2)	31.4 ± 5.0 (185)	34.0 ± 6.8 (291)
Waist circumference (cm)	110.0 ± 13.3 (133)	105.3 ± 16.1 (244)
Fat mass (% of total mass)	34.5 ± 6.3 (133)	48.2 ± 5.2 (243)

* Data are presented as mean ± standard deviation (number of observations provided in brackets).

3.2. Erythrocyte Fatty Acids

Table 2 shows mean values of erythrocyte fatty acid levels for each gender. Men had significantly higher erythrocyte DPA while the Omega-3 Index (EPA + DHA) was significantly higher in women.

Table 2. Erythrocyte fatty acids (% of total; mean ± SD).

	Males (185)	Females (282)
EPA	0.85 ± 0.35	0.91 ± 0.42
DPA **	2.47 ± 0.37	2.32 ± 0.36
DHA	4.25 ± 0.95	4.42 ± 1.02
Omega-3 Index *	5.10 ± 1.18	5.33 ± 1.33

Significant gender difference: * $p < 0.05$; ** $p < 0.0001$.

Table 3 summarises the linear correlation analysis of relationships between erythrocyte fatty acids and measures of adiposity. Pearson correlation coefficients (r) are presented for all participants and for men and women separately. There were strong inverse correlations in the whole dataset between DHA, DPA and the Omega-3 Index and measures of adiposity. DHA and the Omega-3 Index were associated with BMI and waist circumference, whereas DPA predicted body fat. However, the apparent influence of the Omega-3 Index can be attributed to DHA alone as EPA was weakly associated with waist circumference only.

Table 3. Correlations between erythrocyte fatty acids and adiposity measures.

	Body mass index (kg/m^2)	**Waist circumference (cm)**	**Body Fat (%)**
N (all subjects)	476	377	376
EPA	−0.016	**−0.143** *	−0.054
DPA	−0.073	−0.116	**−0.264** ***
DHA	**−0.191** ***	**−0.298** ***	−0.117
Omega-3 Index	**−0.154** **	**−0.275** ***	−0.108
N (males only)	185	133	133
EPA	0.087	−0.062	−0.147
DPA	−0.016	−0.201	**−0.228** *
DHA	−0.077	−0.144	−0.185
Omega-3 Index	−0.037	−0.133	−0.192
N (females only)	291	244	243
EPA	0.080	**−0.164** *	**−0.172** *
DPA	−0.047	−0.125	−0.143
DHA	**−0.276** **	**−0.353** ***	**−0.329** **
Omega-3 Index	**−0.236** **	**−0.322** ***	**−0.306** **

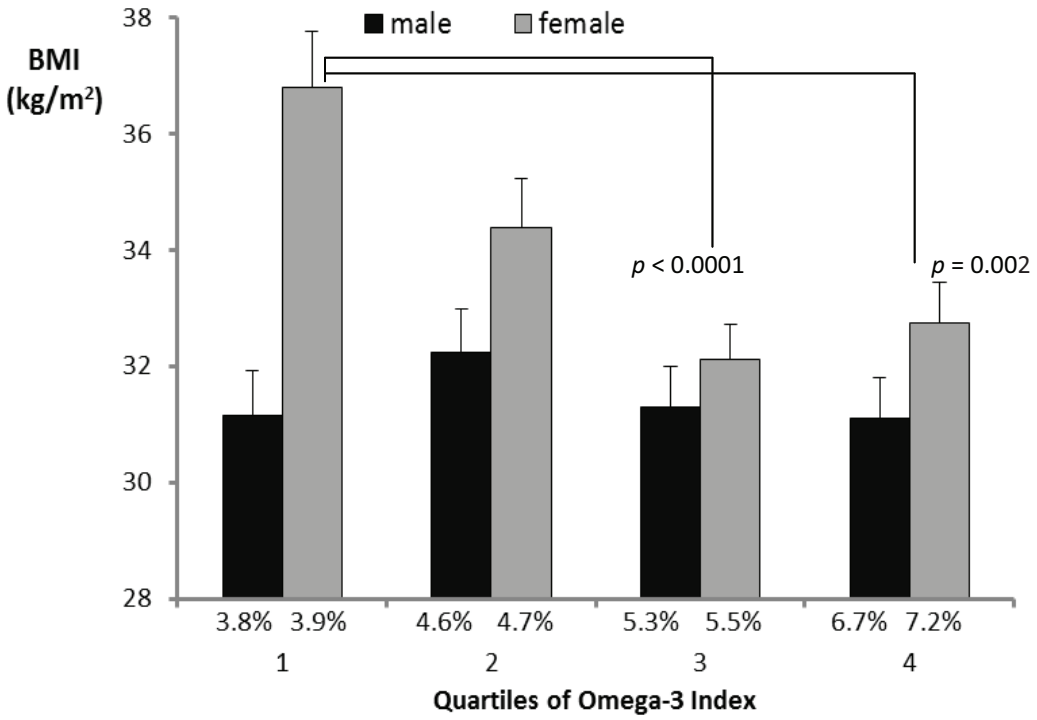
Values are Pearson *r*. Significant correlations: * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$.

Univariate analysis confirmed that there were significant gender interactions in the relationships between Omega-3 Index and BMI ($p = 0.015$) and Omega-3 Index and waist circumference ($p = 0.028$) but not between Omega-3 index and % body fat. Hence it was appropriate to split the data by gender, although gender differences in correlations with % body fat should be interpreted with caution. Erythrocyte DPA was the only significant correlate of adiposity in men; higher DPA predicted a lower percentage body fat. However, DPA was not a significant predictor in women. On the other hand, there were strong inverse correlations between all three markers of adiposity and DHA and consequently the Omega-3 Index in women.

3.3. Associations between the Omega-3 Index and BMI

BMI is the most widely used measure of obesity and the Omega-3 Index is the most widely accepted marker of habitual intake of LC *n*-3 PUFA. Hence it was of interest to further illustrate the relationship between these measures. Figure 1 shows the mean values for BMI in each quartile of the Omega-3 Index for each gender. The lack of a significant relationship in men was apparent. It was also apparent that the relationship between the Omega-3 Index and BMI in women was not linear. BMI values appeared to rise steeply in the lower quartiles of the Omega-3 Index. There were highly significant differences between mean BMI in the lowest quartile and mean BMI in the two highest quartiles of the Omega-3 Index in women.

Figure 1. Average BMI values in quartiles of the Omega-3 Index.

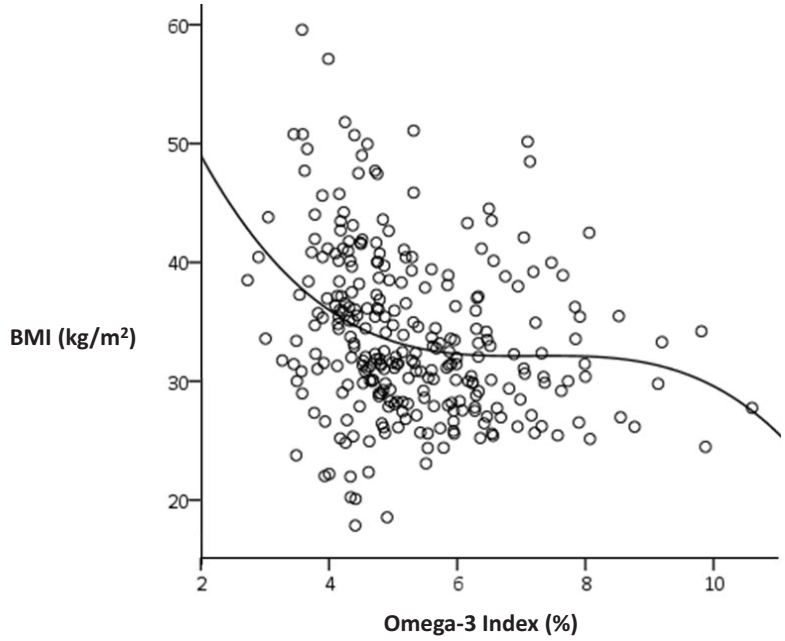


Curvilinear analysis confirmed the skewness of the relationship between Omega-3 Index and BMI in women (Figure 2). A sigmoidal curve gave the most significant fit (r^2 = 0.078, p < 0.001) and suggested a possible threshold for the Omega-3 Index around 6%, below which BMI tends to rise steeply. There was no such relationship for men (r^2 = 0.006, p = 0.74).

4. Discussion

The results of this study confirm previous indications of an inverse relationship between LC *n*-3 PUFA levels in erythrocytes and adiposity in humans [14]. Moreover, they extend previous research by revealing a primary role for DHA in this relationship. Most importantly, however, they highlight a striking gender difference, whereby the association of DHA with lower adiposity was evident in women only; men, on the other hand, tended to show an inverse association between erythrocyte DPA and adiposity. It was also apparent that erythrocyte EPA had little relationship with adiposity.

Figure 2. Sigmoidal relationship between BMI and the Omega-3 Index in women.



Recognising that erythrocyte levels reflect habitual intakes of LC *n*-3 PUFA, it is tempting to speculate that increased consumption of DHA-rich fish or fish oil may help to counteract obesity. However, the correlations derived from our cross-sectional analysis cannot imply causation. One could equally argue that being obese inclines individuals to include less fish or fish oil in their diet. Alternatively, lower intakes of fish or fish oil and a predisposition to adiposity may both be secondary to another independent factor, e.g., poor quality diet. The influence of independent factors may also account for previous anomalies in reported relationships of LC *n*-3 PUFA intake with adiposity. For example, the observation that larger waist circumferences were associated with higher plasma phospholipid LC *n*-3 PUFA levels in Canadian Inuits and Cree Indians may have been attributable to other aspects of diet in these populations, notwithstanding their habitually high intakes of LC *n*-3 PUFA [6–8].

However, preclinical research in animal models of obesity and limited data from human intervention trials suggests that LC *n*-3 PUFA consumption is causally related to adiposity. LC *n*-3 PUFA can suppress fat synthesis and increase metabolism in adipose tissue via multiple mechanisms involving altered expression of transcription factors, viz. SREBP-1 and PPARs [17]. *In-vitro* studies with lipid droplets specifically implicate DHA in these mechanisms [18]. Coincidentally, DHA was the predominant LC *n*-3 PUFA consumed in a small number of human intervention trials which reported weight loss or fat loss following supplementation [3,19,20]. Hence the highly significant inverse correlations between erythrocyte DHA and diverse measures of adiposity observed in the present study suggest that increasing DHA intake may help to reduce the incidence of adiposity (r = 0.353 indicates that erythrocyte DHA levels account for 12.5% of the variance of waist circumference in women).

The limitation of this association to women is noteworthy, particularly considering that there was no significant difference between men and women in the mean erythrocyte DHA level. Interestingly, Decsi and Kennedy [21] reported that plasma phospholipid DHA levels for almost 3000 participants in the EPIC study were approximately 10% higher in women than men, an effect that they attributed to

enhanced conversion of α-linolenic acid through to DHA in women. However, no differences were reported for DPA. The 6% higher erythrocyte DPA level for men in the present study appears to be a unique observation but would be consistent with the hypothesis of limited conversion of DPA to DHA in men relative to women. It is of interest, therefore, that DPA was a significant predictor of body fat in men, whereas DHA was the predominant predictor of all measures of adiposity in women. Garg and colleagues recently reported that DHA supplementation was effective in reducing platelet aggregation in women, whereas EPA supplementation reduced platelet aggregation in men [22]. Clearly there is a need to further characterise gender differences in LC *n*-3 PUFA and their respective functions. A useful starting point would be large epidemiological studies such as EPIC and the Framingham Heart Study, where blood samples have been routinely analysed for LC *n*-3 PUFA contents.

There is increasing recognition of the limitations of dietary intake assessment tools to estimate LC *n*-3 PUFA intake and increasing acknowledgement of the need for reliable blood biomarkers of an individual's LC *n*-3 PUFA status. Unfortunately the use of different biomarkers can lead to different interpretations. Thus the relatively simple measure of LC *n*-3 PUFA in whole plasma is at best a reflection of recent consumption, whereas assessment in a plasma phospholipid fraction reflects both consumption and incorporation of LC *n*-3 PUFA in a stable pool over a period of weeks. However, the "gold standard" biomarker for habitual LC *n*-3 PUFA consumption is their relative content in erythrocytes, reflecting, as stated earlier, their uptake and retention in the erythrocyte pool over several months [12,13]. It is unfortunate that a number of important epidemiological studies have chosen to use plasma phospholipid determinations when there is increasing recognition of the superiority of erythrocyte fatty acid determinations. Indeed the Omega-3 Index, *i.e.*, the sum of EPA and DHA in erythrocytes, has been widely promoted as both a biomarker of LC *n*-3 PUFA consumption/status and a risk factor for cardiovascular disease [23] and serves as a useful standard for comparison across populations. Hence, we quantified relationships between the Omega-3 Index and measures of adiposity in the present study, even though it was evident that erythrocyte DHA alone was a stronger predictor of adiposity than the combination of EPA + DHA.

Whilst there was no apparent relationship between the Omega-3 Index and measures of adiposity in men, examination of quartiles of the Omega-3 Index in women revealed a non-linear relationship with BMI (Figure 1), wherein BMI was similar in the two highest quartiles but rose sharply in the lower quartiles. This was even more evident when curvilinear relationships were tested. The best fit (shown in Figure 2) was a sigmoidal curve, indicating a plateau effect within an approximate range of 5%–9%, below which BMI appeared to increase exponentially. Bearing in mind that the Omega-3 Index predicts greater risk of cardiovascular disease below 4% and lesser risk above 8%, it appears that extremes of the Omega-3 Index may also be associated with other risk factors, including adiposity and depression [24,25].

BMI is a relatively crude measure of obesity; gender differences may reflect differences between men and women in the relative contribution of fat and lean mass to BMI. However, significant curvilinear relationships were found in women between the Omega-3 Index and both % body fat and waist circumference as well as BMI, strengthening the argument that omega-3 intake is inversely related to adiposity in women.

In conclusion, the outcomes of this cross-sectional analysis of erythrocyte LC *n*-3 PUFA content and measures of adiposity in a convenience sample of Australian adults are consistent with other

evidence suggesting an inverse relationship between LC *n*-3 PUFA intakes and obesity. In particular, DHA intake was a negative predictor of BMI, waist circumference and body fat content in women, whereas DPA was a weaker negative predictor of body fat content in men. Analysis of the Omega-3 Index indicates that women in the lower range of the Index may have increased risk of obesity. These data warrant further confirmation in larger studies where potential gender-specific effects of individual LC *n*-3 PUFA are also taken into account.

Acknowledgments

Nil.

Author Contributions

PRCH, AMC and JDB initiated and designed this secondary analysis while KJM, TP and CM were largely responsible for data collection in the contributory intervention trials. AMC undertook the data analysis and PRCH drafted the manuscript. All authors contributed to and approved the final version.

Abbreviations

BMI, body mass index; WC, waist circumference; BF, body fat; LC *n*-3 PUFA, long-chain omega-3 polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.

Conflicts of Interest

The authors declare no conflict of interest.

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Does Consumption of LC Omega-3 PUFA Enhance Cognitive Performance in Healthy School-Aged Children and throughout Adulthood? Evidence from Clinical Trials

Welma Stonehouse

Abstract: Long-chain (LC) omega-3 PUFA derived from marine sources may play an important role in cognitive performance throughout all life stages. Docosahexaenoic acid (DHA), the dominant omega-3 in the brain, is a major component of neuronal cell membranes and affects various neurological pathways and processess. Despite its critical role in brain function, human's capacity to synthesize DHA *de novo* is limited and its consumption through the diet is important. However, many individuals do not or rarely consume seafood. The aim of this review is to critically evaluate the current evidence from randomised controlled trials (RCT) in healthy school-aged children, younger and older adults to determine whether consumption of LC omega-3 PUFA improves cognitive performance and to make recommendations for future research. Current evidence suggests that consumption of LC omega-3 PUFA, particularly DHA, may enhance cognitive performance relating to learning, cognitive development, memory and speed of performing cognitive tasks. Those who habitually consume diets low in DHA, children with low literacy ability and malnourished and older adults with age-related cognitive decline and mild cognitive impairment seem to benefit most. However, study design limitations in many RCTs hamper firm conclusions. The measurement of a uniform biomarker, e.g., % DHA in red blood cells, is essential to establish baseline DHA-status, to determine targets for cognitive performance and to facilitate dosage recommendations. It is recommended that future studies be at least 16 weeks in duration, account for potential interaction effects of gender, age and apolipoprotein E genotype, include vegan/vegetarian populations, include measures of speed of cognitive performance and include brain imaging technologies as supportive information on working mechanisms of LC omega-3 PUFA.

Reprinted from *Nutrients*. Cite as: Stonehouse, W. Does Consumption of LC Omega-3 PUFA Enhance Cognitive Performance in Healthy School-Aged Children and throughout Adulthood? Evidence from Clinical Trials. *Nutrients* **2014**, *6*, 2730–2758.

1. Introduction

Optimal cognitive performance is vital throughout all stages of life. During childhood it is critical to optimize brain development; throughout adulthood it is important to maintain optimal cognitive functioning; and during old-age it is imperative to defer cognitive decline and prevent dementia. The long-chain (LC) omega-3 polyunsaturated fatty acids (PUFA) derived from marine sources, docosaehexaenoic acid (C22:6, DHA) and eicosapentaenoic acid (C20:5, EPA), may play an important role in achieving these objectives. DHA is the dominant LC omega-3 PUFA in the brain [1] and has been shown to accumulate in areas of the brain associated with learning and memory, such as the cerebral cortex and hippocampus [2,3]. DHA is incorporated into neuronal membrane glycerophospholipids at the *sn*-2 position where it regulates numerous neuronal and glial cell processes including neurogenesis,

neuroplasticity, neurite outgrowth, synaptogenesis and membrane fluidity which in turn supports membrane protein functions impacting on speed of signal transduction and neurotransmission [4–8]. In addition, DHA improves vascular tone which results in increased cerebral blood flow during cognitive tasks [9] and it regulates the transport and uptake of glucose by the endothelial cells of the blood brain barrier [7,10]. Unesterified DHA released from glycerophospholipids by phospholipase A2 are natural ligands for several nuclear receptors that regulate gene expression, and they are precursors for neuroprotectins and resolvins that counteract neuroinflammation, oxidative stress and increases neuronal survival [4,8,10]. EPA and the plant derived omega-3 fatty acid, alpha-linolenic acid (ALA), also crosses the blood brain barrier, but >99% of these fatty acids are β-oxidised and some EPA is de-esterified from glycerophospholipids [11]. Both ALA and EPA may contribute to brain function by facilitating fuel supply to the brain through ketogenesis, particularly during aging [12]. In addition, unesterified EPA may further contribute to cognitive function through the synthesis of eicosanoids that offset neuroinflammation and improve cerebral blood flow due to its antithrombotic and vasodilatory properties [7]. Thus, DHA seems to be more important for brain function, but ALA and EPA also play minor roles.

The brain retains its DHA content as indicated by a long DHA half-life of ~2.5 years in human brain [13], but studies in animal models have shown that long-term DHA depletion results in significant losses in brain DHA [2,14]. Studies in rats which involved dietary LC omega-3 PUFA depletion over successive generations or even in one-generation showed decreased brain DHA levels, particularly in the frontal cortex and hippocampus areas, with reciprocal increased levels of the omega-6 PUFA, docosapentaenoic acid (DPA, C22:5, *n*-6). With the increase in DPA the level of unsaturation was maintained, but brain function was impaired, including changes in learning, memory, auditory and olfactory responses [2,6,14]. These effects were, however, restored by repletion with dietary DHA [14]. Thus, individuals who follow omega-3 PUFA deficient diets particularly over several generations, for example families who never consume seafood, the major source of DHA, may have depleted levels of brain DHA and their cognitive function may not be optimal. Based on studies in rodents and non-human primates the brain has the capacity to nearly meet its daily needs for DHA through the conversion of ALA to DHA, mainly by the liver, when sufficient dietary ALA (>1.2 g ALA/day) is consumed [15]. The capacity of humans to synthesise DHA *de novo* is limited. While DHA is retro-converted to EPA; the extent of conversion of EPA and ALA to DHA is small [1]. The conversion of ALA to DHA is influenced by several factors; a background diet high in linoleic acid (LA, C18:2, *n*-6) reduces the conversion due to substrate competition; the conversion is more efficient in women [1]; and low dietary intakes of DHA increases conversion [15]. Vegans and vegetarians seem to have similar capacity to convert ALA to DHA than omnivores with ALA supplementation increasing EPA to a small extent, with little effect on DHA in blood lipids [16,17]. Consumption of preformed DHA from fish and seafood, supplements (marine or algae derived) or DHA enriched foods may therefore be a more efficient way of ensuring adequate supply of DHA for optimal brain development and function. Large proportions of populations consume inadequate amounts of LC omega-3 PUFA and fish and seafood. The 2008/2009 New Zealand Adult Nutrition Survey reported that ~30% of adults did not or rarely consumed seafood [18]. Only 21% of Australian children consumed fish or seafood during the two-day Australian National Children's Nutrition and Physical Activity survey [19] and Australian adults reported average consumption of 0.25 g/day of total LC

omega-3 PUFA (including EPA, DHA and docosapentaenoic acid (DPA)) [20]. Median usual intakes of total fish and fish high in LC omega-3 PUFA reported by U.S. adults was 12.2 and 1.98 g/day, respectively and median intakes of DHA from foods plus dietary supplements was only 0.04 g/day [21]. Belgian adults reported median DHA intakes ranging from 0.07 to 0.09 g/day [22]. Several studies have shown that vegan diets are devoid of DHA and vegetarian diets that include dairy food and eggs only provide about 0.02 g DHA/day (reviewed by Sanders [16]). These low intakes were accompanied by substantially lower levels of DHA in plasma, serum, red blood cells (RBC) and plasma phospholipids (PL) in vegans and vegetarians compared to omnivores [16]. Although populations following DHA deficient diets do not seem to exhibit apparent cognitive dysfunction, it is imperative to acertain whether increased consumption of DHA by individuals with low dietary intakes, but otherwise healthy will enhance cognitive performance. In summary, basic research provides strong support for the notion that LC omega-3 PUFA, particularly DHA, play an important role in brain function; but will consumption of LC omega-3 PUFA enhance cognitive performance in healthy school-aged children and throughout adulthood, particularly in populations with low dietary intakes? This review will aim to answer this question by critically examining the evidence from all the clinical trials that have been conducted on healthy school-aged children, younger adults and older adults investigating the effects of LC omega-3 PUFA on cognitive performance. Recommendations for future research will also be made.

2. Evidence from Clinical Trials in Healthy Mainstream School-Aged Children

DHA may be particularly important during periods of brain growth spurts which take place from the last trimester of pregnancy up to 2 years of age. Thereafter, the frontal lobes continue to develop throughout childhood, adolescence and into the late twenties with spurts of frontal lobe development at age 7–9 years and mid-adolescence [23,24]. Table 1 provides a summary of all nutrition intervention trials that have investigated the effects of LC omega-3 PUFA on cognitive function, learning and school achievement in healthy school-aged children. Kuratko *et al*. [25] have also reviewed studies published until November 2012 on DHA and learning and behavior in healthy children. The evidence from clinical trials focusing on healthy mainstream school-aged children is relatively new as evident from the small number of studies (n = 10) published since 2007. Most of the studies were conducted in children aged 6–12 years old. The studies varied widely with regard to duration (from 8 weeks to 12 months), dosage (from 0.1 to 1.2 g LC omega-3/day), type of LC *n*-3 PUFA (fish oil, algal oil, enriched foods) and DHA:EPA ratio of the interventions, type of outcomes measured and type of participants. LC omega-3 PUFA was generally provided in the form of fish oil or algal oil (high in DHA) and in a few studies as LC omega-3 PUFA enriched foods. Most studies were conducted in children with low baseline intakes of LC omega-3 PUFA. A landmark study by McNamara *et al*. [26] showed for the first time in humans the direct link between DHA and brain activation. Supplementation of 0.4 g/day and 1.2 g/day of DHA increased activation of the dorsolateral prefrontal cortex during a sustained attention task in boys aged 8–10 years. However, these effects were not translated into improvements in visual sustained attention performance. Eight weeks may be sufficient for DHA to increase brain activation, but longer periods may be needed to result in improved cognitive performance.

Richardson *et al*. [27] showed that DHA supplementation improved reading in children who underperformed in reading. Children with reading scores ≤20th centile gained an additional 0.8 months in reading age while children in the ≤10th centile gained 1.9 months in reading age with DHA supplementation. Parletta *et al*. [28] showed in Australian indigenous children with low literacy ability improvements with EPA + DHA on cognitive development (draw-a-person) with a larger effect in the 7–12 year old children. They were unable to show improvements in academic achievement (reading and spelling). However, these results should be interpreted in context of the myriad of factors in this indigenous population that may have affected the attainment of English literacy, e.g., language experiences, home support, socio-economic status (SES) and school attendance. Omega-3 supplementation alone may not have been sufficient to overcome these factors. The fact that improvements were seen in the draw-a-person variable, a non-verbal, culture-free test of cognitive development that does not require schooling or specific language skills, supports this argument [28]. Studies in malnourished 7–9 year old South African [29] and 8–12 year old Mexican children [30] showed improvements in learning and cognitive performance with LC omega-3 supplementation. Whereas no effects were seen over 12 months in 6–10 year old malnourished children from India [31] and Indonesia [32]. However, dosages were small and the supplement used in the Indian study was mostly ALA [31,32]. Baumgartner *et al*. [33] conducted the first LC omega-3 trial in children who were purposely recruited with iron deficiency; they showed that EPA + DHA supplementation in children with iron deficiency anemia had negative effects on working memory. They also showed treatment gender interactions where boys with iron deficiency performed better in long-term memory and retrieval with DHA while girls performed worse. These studies demonstrate the complexities of conducting omega-3 supplementation trials on cognitive performance and learning in malnourished populations where multiple other factors and nutrient deficiencies may affect the outcomes. Yet, these are the populations most likely to benefit from supplementation.

Two studies conducted in healthy mainstream school children in the UK did not show any benefit of consuming DHA on cognitive performance and learning [34,35]. The study by Kennedy *et al*. [34] was underpowered and short in duration (8 weeks). In Kirby *et al*. [35], cheek cell EPA and DHA increased in both DHA and placebo groups, although the increase was greater in the DHA group. Thus, parents of children in the placebo group may have become more aware of the benefits of omega-3 PUFA and increased their intakes.

Inconsistencies between studies could be ascribed to potential modulating effects of age and gender. Children at different phases of brain and cognitive development and boys and girls may respond differently to LC omega-3 PUFA supplementation as was shown by Parletta *et al*. [28] and Baumgartner *et al*. [33]. In a large sample of 6–16 year old American children, the relationship between dietary omega-3 PUFA and cognitive test scores was twice as strong in girls as in boys [36]. None of the other studies reviewed investigated potential interaction effects of age and gender and some studies used wide age ranges which may have resulted in greater variability or response modulating effects on outcomes.

Biomarkers of LC omega-3 PUFA intake are often not measured in studies on children because of children's fear of having a blood sample taken and consequently not wanting to volunteer for studies. Cheek cell samples are sometimes collected, which is much less invasive and has been shown to correlate well with dietary intakes, plasma and RBC levels [37]. The studies in Table 1 measured DHA

and EPA levels/concentrations in RBC, plasma, RBC or plasma phospholipids (PL) and cheek cells. The levels increased in all studies with supplementation of LC omega-3 PUFA and the magnitude of the increase often reflected the supplementation dose [26,29,31–33,35].

To date, no LC omega-3 PUFA interventions have been conducted in adolescents and the only evidence is from observational studies. A prospective study in >9000 15 year old Swedish school children showed significantly higher school grades at age 16 in adolescents consuming fish more than once a week compared to less than once a week [38]. At age 18, male adolescents who consumed fish more than once per week compared to less than once per week at age 15 had higher IQ scores which was obtained from completed intelligence tests as part of the mandatory Swedish military service conscription examination [39]. De Groot *et al*. [40] recently showed in 700 Dutch adolescents, 12–18 years, that consumption of fish providing the recommended amount of EPA + DHA of ~0.45 g/day compared to no fish intake was associated with more advanced vocabulary and higher end term grades. However, eating more fish than the recommended amount was no more beneficial [40].

In summary, it seems as if children with low literacy ability and who are malnourished with low LC omega-3 PUFA intakes may benefit most from the consumption of LC omega-3 PUFA with regard to cognitive outcomes (e.g., memory, non-verbal cognitive development, processing speed, visual-perceptive capacity, attention and executive function) and school achievement (e.g., reading and spelling). Inconsistencies between studies may have been due to different dosages, duration, other nutrient deficiencies and lack of investigating interaction effects of gender and age. Dosages may have been too low in several of the studies that did not show benefits. Nutrient deficiencies such as iron deficiency in malnourished populations may need to be corrected before supplementation with LC omega-3 fatty acids can commence to avoid potential adverse interaction between nutrient deficiencies and omega-3 supplementation.

Table 1. Nutrition intervention trials of long-chain omega-3 PUFA and cognitive function, learning and school achievement in healthy school-aged children.

Reference	Study Design	Participants	Intervention	Results	
				Cognitive Performance (LC Omega-3 *vs*. Placebo)	Biomarker
Baumgartner *et al*. 2012 [33] [a]	RCT, 8.5 months KwaZulu-Natal, South Africa	6–11 years, low-income iron deficient children (*n* = 321, analysed *n* = 288). Excluded children consuming omega-3 supplements. Low baseline omega-3 status based on low RBC DHA (~3%) and EPA (~0.16%)	Four interventions as supplements: (1) Iron + fish oil (2) Iron + placebo; (3) Fish oil + placebo; (4) Placebo + placebo. Fish oil = 0.5 g/day LC omega-3 (0.42 g DHA + 0.08 g EPA). Provided 4 days/week at school.	No effects on cognitive outcomes. LC omega-3 PUFA without iron had negative effects on working memory in children with iron deficiency anaemia and on long-term memory and retrieval in girls with iron deficiency, whereas boys with iron deficiency performed better.	RBC DHA increased by 2.21% (to ~5.9%); RBC EPA increased by 0.14% (to ~0.38%).
Dalton *et al*. 2009 [29]	RCT, 6 months Northern Cape, South Africa	7–9 years, low-income, marginally nourished indigenous children (*n* = 183, analysis on *n* = 155)	Fish flour bread spread provided at school (~0.89 g/week DHA (0.13 g/day)) *vs*. control bread spread.	Improved verbal learning ability and memory. Tendency to improve reading ($p = 0.06$). Prevented decline in spelling. Secondary analysis: effects more pronounced in children with lower baseline performance scores.	Increased EPA and DHA in plasma PC, RBC PC, RBC PE.
Kennedy *et al*. 2009 [34]	RCT, 8 weeks Newcastle-upon-Tyne area, UK	10–12 years (*n* = 90, analysis on *n* = 86). Excluded children with high intake of LC omega-3 sources	Three intervention arms: (1) Low dose algal oil: 0.4 g DHA (2) High dose algal oil: 1.0 g/day DHA (3) Placebo (vegetable oil)	No effects on comprehensive computerized cognitive test battery (including memory, working memory, attention, and reaction time) Word recognition task: Low dose: faster performance; High dose: slower performance	NR

Table 1. *Cont.*

Kirby *et al*. 2010 [35]	RCT, 16 weeks Newport, UK	8–10 years (n = 450, analysis on n = 348). Excluded children consuming omega-3 supplements.	Fish oil: (0.4 g DHA + 0.06 g EPA)/day + micronutrients *vs*. placebo (olive oil)	No effects on comprehensive cognitive performance test battery: (IQ, reading & spelling, working memory, attention, impulsivity, handwriting)	Cheek cell fatty acids: EPA and DHA increased in both DHA and placebo groups with greater increase in DHA group.
McNamara *et al*. 2010 [26]	RCT, 8 weeks Cincinnati, OH, USA	8–10 year boys (n = 38, analysis on n = 33). Low baseline omega-3 status based on low RBC DHA (3.3%).	Three intervention arms: (1) Low dose algal oil: 0.4 g/day DHA (2) High dose algal oil: 1.2 g/day DHA (3) Placebo (corn oil)	Both dosages increased activation of the dorsolateral prefrontal cortex during sustained attention task. No effect on attention or reaction time of attention.	RBC DHA increased by ~4.2% (to 7.5%) in low dose and by ~7% (to 10.3%) in high dose
Muthayya *et al*. 2009 [31][a]	RCT, 12 months Bangalore, India	6–10 years, low income, marginally nourished (n = 598, analysis on n = 550) Low baseline omega-3 status based on low RBC DHA (3.2%) and EPA (0.18%)	Four interventions provided as fortified foods: (1) High micronutrients + high omega-3 (2) High micronutrients + low omega-3 (3) Low micronutrients + high omega-3 (4) Low micronutrients + low omega-3 Low dose: 0.14 g/day ALA High dose: (0.93 g ALA + 0.10 g DHA)/day	No effects on mental processing, memory, fluid reasoning, retrieval ability or cognitive speediness.	High *vs*. low dose difference: RBC DHA increased by 1.55% (to ~5.2%); RBC EPA increased by 0.11% (to ~0.37%)

Table 1. *Cont.*

Osendarp *et al*. 2007 (NEMO Study Group) [32] [a]	RCT, 12 months Adelaide, Australia and Jakarta, Indonesia	6–10 years. Australia: well nourished, (n = 396, analysed n = 276) Indonesia: marginally nourished, (n = 384, analysed n = 367) Baseline plasma omega-3: Australia: EPA ~7.6 μg/mL, DHA ~33 μg/mL Indonesia: EPA ~1.2 μg/mL, DHA ~41 μg/mL	Four interventions provided as flavored drinks: (1) High micronutrients (2) DHA + EPA (0.09 g DHA + 0.02 g EPA)/day (3) Micronutrients + DHA + EPA (as above) (4) Placebo	No effects on general intelligence, verbal learning and memory or visual attention	Australia: Increased plasma DHA with 11.1 μg/mL, EPA 2.23 μg/mL. Indonesia: Increased plasma DHA with 7.06 μg/mL
Parletta *et al*. 2013 [28]	RCT (20 weeks) with one-way cross-over to LC omega-3 supplement (20 weeks) Northern Territory, Australia	3–13 years, indigenous children with low literacy ability (n = 408). Low intakes of omega-3 rich fish	Fish oil: 0.75 g LC omega-3 per school day (0.56 g EPA + 0.17 g DHA) plus 0.06 g/day gamma linolenic acid *vs*. placebo (palm oil)	Reading & Spelling: No effect. Non-verbal cognitive development (Draw-A Person): Improvements with strongest effects in 7–12 year olds	NR
Portillo-Reyes *et al*. 2014 [30]	RCT, 3 months low SES schools in Ciudad Juarez, Mexico	8–12 years, mild-moderately malnourished (n = 59, analysis on n = 50). Excluded children consuming omega-3 supplements. Intake of fish low	Fish oil: 0.45 g/day LC omega-3 (0.18 g EPA + 0.27 g DHA) *vs*. placebo (soybean oil)	Improved processing speed, visual-perceptive capacity, attention, executive function (large effect size improvements (Cohen d > 0.8) in >50% of children in 11/18 tests) Memory: No effect	NR
Richardson *et al*. 2012 [27]	RCT, 16 weeks Oxfordshire, UK	7–9 years, underperforming in reading (≤33rd centile) (n = 362). Excluded children with high intake of LC omega-3 sources	Algal oil: 0.6 g/day DHA *vs*. placebo (corn/soybean oil)	Reading: Baseline reading scores ≤33rd centile: No effect; ≤20th centile (n = 224): improved reading; ≤10th centile (n = 105): improved reading Working memory: No effect	NR

Abbreviations: ALA, alpha-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; SES, socio-economic status; LC, long-chain; NR, not reported; PC, phosphatidyl choline; PE, phosphatidylethanolamine; RBC, red blood cell; RCT, randomized controlled trial; [a] Multiple supplement arms, only omega-3 study arm reported.

3. Evidence from Clinical Trials in Healthy Younger Adults

The aim during younger adulthood is to maintain optimal brain function. Although brain development is established, neuroplasticity is ongoing [6]. Only seven studies to date investigated the effects of LC omega-3 PUFA on cognitive performance in younger healthy adults (Table 2). The study by Stonehouse *et al*. [41] in healthy young adults who habitually consumed diets low in DHA, has been one of the largest and longest trials to date and showed that DHA supplementation improved memory and reaction time of memory [41]. This was also the only study so far in healthy young adults that investigated whether gender and apolipoprotein E genotype (*APOE*) modulated the response to LC omega-3 PUFA supplementation. They showed that memory domains were affected differently by DHA in men and women; in women episodic memory improved whereas in men, reaction time of working memory improved compared to placebo [41]. This may be explained by men and women using different problem-solving strategies as indicated by differences in the functional organization of the brain when performing memory tasks [42,43]. *APOE* did not affect responses in the group as a whole but when stratified for gender, improvements in reaction time for working memory and attention with DHA compared to placebo were more pronounced in male *APOE4* allele carriers than in non-carriers. However, this effects needs to be further explored since the study was not statistically powered to investigate the three-way interaction of treatment*gender**APOE* [41]. Apolipoprotein E is the primary lipid transporter in brain tissue with carriers of the *APOE4* allelic variant at several fold increased risk of Alzheimer's disease (~three- and ~15-fold increase in risk in *APOE3/E4* and *APOE4/E4*, respectively, relative to the wild-type genotype) [44,45]. Structural and functional neurological changes are seen in *APOE4* carrier's decades before the appearance of any cognitive or clinical symptoms [46–48]. Surprisingly, young (20–35 years) *APOE4* carriers have been shown to perform better on cognitive tasks than non-carriers have [49,50]. This may be due to compensatory mechanisms being employed by carriers of the *APOE4* allele as suggested by increased brain activation in the frontal and temporal regions of *APOE4* carriers during memory tasks compared to non-carriers [47]. The *APOE4* carriers may compensate by taking longer to complete the cognitive tasks more accurately. Any effect of DHA supplementation in *APOE4* carriers is therefore more likely to be seen in tasks assessing reaction time as was seen in our study [41]. Considering the relatively high prevalence of *APOE4* carriers, (~24% in Caucasian populations [44,45] and 31% in the New Zealand sample [41]), it may be an important factor to take into account when investigating the cognitive benefits of LC omega-3 PUFA.

None of the other RCTs summarized in Table 2 showed any cognitive benefits with LC omega-3 PUFA [51–55]. Fontani *et al*. [56] showed improvements in sustained attention and reaction time of sustained attention. However, although the trial is described as a RCT, the authors do not report the placebo results and these results should thus be interpreted with caution. None of these trials examined gender or *APOE* interactions. If gender or *APOE* dimorphisms exist, combining groups may cancel out or dilute any potential effects. Some studies used smaller DHA dosages [51,52], had small sample sizes [51,53,54,56], included a wide age range (18–70 years) [55] and all studies were short in duration ranging from 4 to 12 weeks [51–56].

Table 2. Nutrition intervention trials of long-chain omega-3 PUFA and cognitive function in healthy younger adults.

Reference	Study Design	Participants	Intervention	Results	
				Cognitive Performance (LC Omega-3 *vs.* Placebo)	Biomarker
Antypa *et al.* 2009 [51]	RCT, 4 weeks Leiden, The Netherlands	University students, mean age ~22 years (n = 56, analysed n = 54) Excluded adults consuming fish more than once/week Baseline plasma DHA: ~1.8%, EPA: 0.48%	Fish oil: 2.3 g/day LC omega-3 (1.74 g EPA + 0.25 g DHA) *vs.* placebo (olive oil)	No effects on attention, memory or reaction time of attention	Increased plasma DHA by ~0.67% (to 2.6%) and EPA by ~2.3% (to 2.84%)
Fontani *et al.* 2005 [56]	RCT (But placebo results not reported), 35 days Siena, Italy	22–51 years (n = 33). LC omega-3 intake not considered	Fish oil: 2.8 g/day LC omega-3 (1.6 g EPA + 0.8 g DHA)	Improvements in sustained attention and reaction time of sustained attention	Poorly reported
Jackson *et al.* 2012 [52]	RCT, 12 weeks Newcastle upon Tyne, UK	18–35 years (n = 159, analysed n = 140) Low average intake of fatty fish (<2 portions/month) Baseline serum DHA: ~1.1%, EPA: ~1.1%	Three intervention arms: (1) DHA-rich fish oil: (0.45 g DHA + 0.09 g EPA)/day (2) EPA-rich fish oil: (0.2 g DHA + 0.3 g EPA)/day (3) Placebo (olive oil)	No effects on comprehensive computerized cognitive test battery (episodic memory, working memory, attention, reaction time, executive function)	DHA group: Increased serum DHA by ~0.61% (to 1.87%) and EPA by ~ 0.31% (to 1.36%) EPA group: Increased DHA by ~0.37% (to 1.49%) and EPA by ~0.62% (to 1.78%)

Table 2. *Cont.*

Jackson *et al.* 2012 [53]	RCT, 12 weeks Newcastle upon Tyne, UK Primary outcome was cerebral blood flow	18–29 years (n = 65) Excluded consumers of oily fish and omega-3 supplements	Three intervention arms: (1) Low dose DHA fish oil: (0.45 g DHA + 0.09 g EPA)/day (2) High dose DHA fish oil: (0.9 g DHA + 0.18 g EPA)/day (3) placebo (olive oil)	Increased cerebral blood flow Cognitive tasks only assessed at end of study using comprehensive computerized cognitive test battery (episodic memory, working memory, attention, reaction time, executive function). Both dosages improved reaction times on two attention tasks, but effects were lost when correcting for multiple testing	NR
Karr *et al.* 2012 [54]	Placebo controlled trial, not randomized, 4 weeks Canada	College students (mean age ~20 ± 2 years) (n = 43, analysed n = 41) Regular consumers of fish excluded	Fish oil: (0.72 g EPA + 0.48 g DHA)/day *vs.* placebo (coconut oil)	No effects on verbal learning and memory, inhibition and executive control	NR
Rogers *et al.* 2008 [55]	RCT, 12 weeks Bristol, UK Primary outcome was depressed mood	Mildly depressed adults, 18–70 years (average ± SD age 38 ± 14 years) (n = 218, analysed n = 190) Excluded consumers of >1.5 portions oily fish per week	Fish oil: 1.5 g/day LC omega-3 (0.85 g DHA + 0.63 g EPA) *vs.* placebo (olive oil)	No effects on computerised cognitive test battery (processing speed, reasoning, impulsivity, working memory)	Increased plasma EPA + DHA Mean difference between groups at 12 weeks: 3.16% (2.74%, 3.58%)

Table 2. *Cont.*

Stonehouse *et al*. 2013 [41]	RCT, 6 months Auckland, New Zealand	18–45 years (n = 228, analysed n = 176) Excluded consumers of >~0.2 g EPA + DHA/week and omega-3 supplements Baseline RBC DHA: ~5%, EPA: ~0.6%	Fish oil: (1.2 g DHA + 0.17 g EPA)/day *vs*. placebo (high oleic acid sunflower oil)	Comprehensive computerized cognitive test battery: Reaction times of episodic memory and working memory improved Gender*treatment interactions: Episodic memory improved in women and reaction time for working memory improved in men Gender*treatment**APOE* interactions: greater improvements in reaction time for working memory and attention in men. No effects on accuracy of working memory or processing speed	RBC DHA increased by 2.6% (to ~7.9%); RBC EPA increased by 0.2% (to ~0.81%)

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; *APOE*, apolipoprotein E genotype; LC, long-chain; NR, not reported; RBC, red blood cell; RCT, randomized controlled trial; Narendran *et al*. [57] not included in table because the focus was mechanistic and did not use a RCT study design.

Jackson *et al*. [9,53] investigated the effects of short-term (12 weeks) supplementation of LC omega-3 PUFA on neural tissue activation and cerebral blood flow using near-infrared spectroscopy to assess oxy-hemoglobin and deoxy-hemoglobin in the frontal cortex of adults during performance of cognitive tasks. DHA at low and high dosages [9,53], but not EPA [9], significantly increased oxyhemoglobin and total hemoglobin during several cognitive tasks indicating increased cerebral blood flow.

In summary, the trial by Stonehouse *et al*. [41] overcame the study design limitations mentioned above, namely the intervention period was adequate (6 months); a relatively large DHA dosage was used (1.2 g DHA/day) which resulted in achieving RBC DHA levels of ~8%; sufficient statistical power and gender and *APOE* interactions were investigated. They showed that DHA improved memory and reaction time of memory, demonstrating that younger adults may benefit from consumption of DHA [41].

4. Evidence from Clinical Trials in Healthy Older Adults

The main aim for cognitive function during older age is to defer cognitive decline and to prevent dementia. Age-related cognitive decline (ARCD) is decline in cognitive functioning as a consequence of the aging process that is within normal age limits [58]. Mild cognitive impairment (MCI) represents a transitional state between ARCD and dementia, but individuals with MCI are able to function normally in everyday life [59,60]. Clinical trials investigating the effects of LC omega-3 PUFA on cognitive performance in healthy older adults (without dementia) (Table 3) have been inconsistent, with some showing no effects [61–64] and others showing improvements in different measures of cognitive function, mostly memory [58–60,65–68] as well as executive function [68] and visuospatial learning [58]. The outcomes have been affected by various study design limitations such as high baseline LC omega-3 status, wide variations in cognitive impairment scores with MMSE ranging from 21 to 30, small dosages, short trial duration, insensitive outcome measures, insufficient statistical power, wide age ranges, and lack of investigating potential response modulating effects of age, gender and *APOE*. LC omega-3 PUFA were provided in the form of ethyl esters, algal oil, fish oil, enriched margarine (one study [62]) and krill oil (one study investigating effects on brain activation, not cognitive function [69]).

One of the most rigorously designed trials was unable to show any benefit of LC omega-3 PUFA on a range of cognitive outcomes [61]. The authors argued that the population may have already consumed sufficient LC omega-3 PUFA as evident from relatively high serum DHA:DPA (omega-6) ratios in both treatment groups at 24 months. Unfortunately, the authors did not assess the LC omega-3 status at baseline to confirm this. High intakes of dietary LC omega-3 PUFA of ~0.3 g/day may have also precluded any cognitive benefits with fish oil in the study by Van de Rest *et al*. [64]. In addition, wide ranges of mini-mental scale examination (MMSE) scores from 23 to 30 may have resulted in greater variability in cognitive responses that could have resulted in a null effect. The Alpha Omega trial, which had 2911 patients with stable myocardial infarction, has been the largest and longest (40 months) trial so far conducted. Neither ALA, DHA + EPA, nor a combination of ALA + DHA + EPA affected MMSE scores [62]. The study was designed for CVD as primary outcome and MMSE was used as a secondary measure of global cognitive function. MMSE may not be sensitive for

detecting small changes in cognitive function with nutrition interventions in a normal aging population. Furthermore, the effects were investigated against a background where >85% of participants were using lipid lowering and anti-hypertensive drugs which in turn may have affected cognitive function through their effects on cardio-metabolic markers, masking the effects of omega-3 PUFA. The most likely explanation for the lack of cognitive benefits in the study by Stough *et al*. [63] was the low dosage (0.25 g/day DHA from tuna oil) consumed over a short duration of 90 days and a wide age-range of 45–80 years which may have increased the variability in the outcome measures.

Yurko-Mauro *et al*. [58] showed significant improvements in several measures of memory as well as visuospatial learning in older adults with subjective memory complaints and ARCD. The improvement in the paired associate learning (PAL) test was related to a gain of 7 years in age compared to reference data. Their study was sufficient in duration (6 months), provided a large dosage (0.9 g/day DHA), had sufficient statistical power and was conducted in individuals with low habitual intakes of DHA.

Two studies were conducted in older adults with MCI [59,60]. Lee *et al*. [59] showed highly significant improvements in memory in older women with MCI. Their sample size was small (n = 35), but the effect size was large with a mean Z-score difference between fish oil and placebo of 0.8 (0.34, 1.26). The differences between this and other studies may be that participants with MCI were recruited, leaving more room for improvement in cognitive test scores, participants were from low socioeconomic background likely to consume low amounts of omega-3 rich fish, and a large dosage (1.3 g/day DHA + 0.45 g/day EPA) was consumed over a long duration (12 months). Furthermore, the study was conducted in women only which may have resulted in a more homogeneous sample. The results are consistent with that of Stonehouse *et al*. [41] who showed improvements in memory in younger women [41]. Sinn *et al*. [60], in their study on older adults with MCI, showed improvements in verbal fluency with a high DHA supplement but not with a high EPA supplement. However, this was the only significant effect out of 11 cognitive assessments and could be due to type 1 error. The lack of effects may have been due to insufficient statistical power. However, the recruitment of large numbers of participants with MCI is not an easy task.

Inconsistencies between studies could be ascribed to response modulating effects of gender, age and *APOE*, but very few studies have investigated these effects. Van de Rest *et al*. [64] identified treatment**APOE* interactions and treatment*gender interactions with *APOE4* carriers and men showing improvements in attention compared to placebo.

Improvements have been shown in cognitive performance in older adults over short duration with high dosages [66] which is most likely due to the vascular and antithrombotic effects of EPA and DHA rather than their effects on neurological changes. Nilsson *et al*. [66] showed improvements in working memory with a high dosage of 1.5 g/day EPA + 1.05 g/day DHA over a very short period of 5 weeks. They also showed significant improvements in cardio-metabolic risk markers that were inversely related to performance in working memory. Witte *et al*. [68] also showed an inverse relationship between improvements in executive function and fasting insulin.

Table 3. Nutrition intervention trials of long-chain omega-3 PUFA and cognitive function in older adults.

Reference	Study Design	Participants	Intervention	Results	
				Cognitive Performance (LC Omega-3 *vs*. Placebo)	Biomarker
Dangour *et al*. 2010 [61] (OPAL Study)	RCT, 24 months England and Wales	70–75 years, cognitively healthy, MMSE ≥ 24 (median = 29) (*n* = 867, analysis on *n* = 748) Excluded adults consuming fish oil	Ethyl ester fish oil: (0.2 g EPA + 0.5 g DHA)/day *vs*. placebo (olive oil)	No effect on global cognitive function, memory, processing speed, executive function, global delay score	Serum fatty acid levels in sub-sample (*n* = 235) at 24 months: EPA and DHA higher *vs*. placebo (EPA: 50 *vs*. 39 mg/L; DHA: 96 *vs*. 71 mg/L)
Geleijnse *et al*. 2012 [62] (Alpha Omega Trial)	RCT, 40 months Netherlands Primary outcome was CVD morbidity and mortality	60–80 years, stable MI patients, MMSE >21 (average ± SD 28 ± 1.6 points) Baseline EPA + DHA intake was low (median intake = ~118 (55–200) mg/day) (*n* = 2911)	Four interventions provided in 20 g/day margarine: (1) 0.4 g/day EPA + DHA (2) 2 g/day ALA (3) EPA + DHA + ALA (4) Placebo	No effect on global cognitive decline as measured with MMSE	Increase in plasma CE EPA and DHA in sub-sample (*n* = 600)
Johnson *et al*. 2008 [65]	RCT, 4 months Boston, MA, USA Primary outcome was eye health	60–80 years, healthy women (*n* = 57, analysed *n* = 49) Dietary intake of DHA ~136 mg/day	Four interventions provided as supplements taken with nutritional energy drink: (1) 0.8 g/day DHA (algal oil) (2) 12 mg/day lutein (3) DHA + lutein (4) Placebo	Verbal fluency (semantic/long-term memory) improved in DHA, lutein and DHA + lutein groups; DHA + lutein improved rate of learning (number of trials to learn a list) and memory in 1 of 6 recall tests (some test close to ceiling); No effects on working memory, processing speed or inhibition	Increase in serum DHA

Table 3. *Cont.*

Lee *et al.* 2013 [59]	RCT, 12 months Cheras, Kuala Lumpur, Malaysia	≥60 years, MCI, MMSE = 26.4 (25–28), middle to low-socioeconomic status (*n* = 36, analysed *n* = 35) Excluded participants consuming omega-3 supplements Baseline plasma EPA ~0.48%; DHA ~4.1%	Fish oil: (1.3 g DHA + 0.45 g EPA)/day *vs*. placebo (corn oil)	Improved memory (short-term memory, working memory, immediate visual memory, delayed recall). No effects on executive function/attention, psychomotor speed, visuospatial skills	Increase in plasma DHA and EPA
Nilsson *et al.* 2012 [66]	RCT, cross-over, 5 weeks, 5 weeks washout Lund, Sweden Aim: Relationship between cognitive and cardiometabolic outcomes	51–72 years, healthy (*n* = 44, analysed *n* = 38 No exclusion based on omega-3 intake Ordinary Swedish diet including meat and fish every week	Fish oil: (1.05 g DHA + 1.50 g EPA)/day *vs*. non-oil placebo in tablet form (dicalcium phosphate, microcrystalline cellulose, magnesium salts of fatty acids)	Treatment–consumption sequence interaction; only first period reported: Improved working memory TG and SBP improved TG, SBP, fasting glucose, TNF-α inversely related to working memory performance	NR
Sinn *et al.* 2012 [60]	RCT, 6 months Adelaide and Brisbane, Australia	>65 years, MCI, MMSE ≥ 22 (average ~27 ± 2.5) (*n* = 50) Excluded participants consuming fish >1/week and omega-3 supplements Baseline RBC EPA ~0.96%, DHA ~4.6%	Three intervention arms: (1) EPA-rich fish oil : (1.67 g EPA + 0.16 g DHA)/day) (2) DHA-rich fish oil: (1.55 g DHA + 0.40 g EPA)/day (3) Placebo (safflower oil)	DHA improved verbal fluency (test of fluid thinking/semantic memory). Only one out of 11 cognitive assessments affected	EPA group: RBC DHA increased by ~0.78% (to 5.34%); EPA by ~3.1% (to 4.06%) DHA group: RBC DHA increased by ~4.1% (to 8.65%); EPA by ~0.86% (to 1.83%)

Table 3. *Cont.*

Stough *et al.* 2012 [63]	RCT, 90 days Melbourne, Australia	45–77 years (average ~56 ± 8.7 years), healthy (n = 112, analysed n = 75) No exclusion based on intake of LC omega-3 sources Baseline plasma PL DHA: ~3.44%	Tuna oil: (0.25 g DHA + 0.06 g EPA)/day *vs*. placebo (soybean oil)	No effects on comprehensive computerized cognitive test battery (attention, secondary memory, working memory, speed of attention, speed of memory)	Plasma PL DHA increased by ~1.79% (to 5.22%)
Van de Rest *et al.* 2008 [64]	RCT, 26 weeks Wageningen, The Netherlands	≥65 years, cognitively healthy, median (25, 75 percentile) MMSE = 28 [27–29], ranged from 23 to 30, (n = 302) Excluded participants consuming high LC omega-3 sources Dietary EPA+DHA: ~0.3 g/day Baseline plasma CE EPA + DHA: 1.9% ± 1.0%	Three intervention arms: (1) Low-dose fish oil: (0.26 g EPA + 0.18 g DHA)/day (2) High dose fish oil: (1.09 g EPA + 0.85 g DHA)/day (3) Placebo (oleic acid)	No effects on comprehensive test battery (memory, executive function, attention, sensorimotor speed) Treatment–*APOE4* interactions: Attention improved in *APOE4* allele carriers Treatment–gender interactions: Attention improved in men	Increase in plasma CE EPA + DHA: Low dose: by ~0.95% (to 2.83%); High dose: by ~4.5% (to 6.4%)
Vakhapova *et al.* 2010 [67]	RCT, 15 weeks Tel-Aviv, Israel	50–90 years, non-demented participants with memory complaints, MMSE ≥ 27 (average ~28.5 ± 1.11), (n = 157, analysed n = 122) No exclusion based on intake of LC omega-3 sources	PS containing LC omega-3: 300 mg PS + 0.08 g (DHA + EPA)/day	Improved verbal immediate recall. No effect on other markers. A subset of participants with higher baseline cognitive status performed better on immediate and delayed verbal recall, learning abilities and time to copy a complex figure	NR

Table 3. *Cont.*

Witte *et al.* 2013 [68]	RCT, 26 weeks Berlin, Germany	50–75 years, MMSE < 26 (average ~29 ± 1.0, ranged from 26 to 30), (*n* = 80, analysed *n* = 65) Fish oil supplement users excluded Most participants consumed fish 1/week. Baseline omega-3 index ~8%	Fish oil: 2.2 g/day LC omega-3 (1.32 g EPA + 0.88 g DHA) *vs.* placebo (sunflower oil)	Improved executive function. No effects on memory, sensorimotor speed and attention. Sub-set who showed greatest increase in omega-3 index showed improved memory. Improved white matter microstructural integrity, grey matter volume in frontal, temporal, parietal and limbic areas. Improvements in executive function associated with peripheral BDNF and inversely with fasting insulin.	Omega-3 index (%RBC DHA + EPA) increased to ~9.6%
Yurko-Mauro *et al.* 2010 [58]	RCT, 24 weeks 19 sites in USA	≥55 years (average ~70 ± 9 years), subjective memory complaints with ARCD, MMSE >26 (*n* = 485) Excluded participants who consumed LC omega-3 supplements or >0.2 g/day DHA Baseline DHA intake: 0.14 g/day	0.9 g/day DHA from algal oil *vs.* placebo (corn + soy oil)	Improved visuospatial learning and episodic memory, immediate and delayed verbal recognition memory. No effect on working memory, executive function, MMSE	Plasma PL DHA increased with 3.2%

Abbreviations: ALA, alpha-linolenic acid; *APOE*, apolipoprotein E genotype; ARCD, age related cognitive decline; BDNF, brain-derived neurotrophic factor; CE, cholesterol esters; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LC, long-chain; MCI: mild cognitive impairment; MMSE, Mini-Mental State Examination; NR, not reported; PS, phosphatidylserine; PL, phospholipid; RBC, red blood cell; RCT, randomized controlled trial; SBP, systolic blood pressure; TG, triglycerides; TNF-α, tumor necrosis factor alpha; [a] Multiple supplement arms, only omega-3 study arm reported; Richter *et al.* [70] not included in table because it was not a RCT.

Supplementation with krill oil (0.19 g EPA + 0.09 g DHA/day) and sardine oil (0.49 g EPA + 0.25 g DHA) in 61–72 year old men for 12 weeks resulted in increased activation of the dorsolateral prefrontal cortex during a working memory task using near-infrared spectroscopy and electroencephalography compared to placebo. Krill oil also increased activity during a calculation task in the left frontal area, the dominant area for calculations [69]. Fish oil (1.32 g EPA + 0.88 g DHA/day) supplementation in 50–75 year olds over 26 weeks improved brain white matter microstructural integrity and grey matter volume in frontal, temporal, parietal and limbic brain areas [68].

In summary, many of the RCT had intrinsic design limitations which hamper drawing firm conclusions regarding the efficacy of LC omega-3 PUFA on cognitive performance in healthy older adults. However, the current evidence suggests that DHA may be of benefit for older adults with ARCD and MCI, particularly for improving memory.

5. Discussion

Trends are emerging from the current evidence suggesting that consumption of LC omega-3 PUFA, particularly DHA, by healthy school-aged children, and younger and older adults may enhance cognitive performance particularly in those who habitually consume diets low in LC omega-3 PUFA. However, the evidence is inconsistent due to various intrinsic design limitations in many of the RCTs which hamper drawing firm conclusions.

Baseline DHA status may have been an important confounding factor in the available research. Since the human brain tenaciously retains DHA [71], individuals who have been following DHA depleted diets over the long-term are most likely to show cognitive benefits with supplementation whereas individuals with already adequate DHA status may not respond. Although several studies excluded participants based on high intakes of LC omega-3 sources (supplements or seafood), the way that this was assessed and the time periods of intake considered (ranging from 2 weeks to 6 months) differed widely between studies. Some studies included biomarkers of DHA intake to verify low baseline status, but it has mostly been used to confirm compliance to LC omega-3 PUFA interventions. The studies used a wide range of biomarkers and units including RBCs, plasma, plasma PL, serum, plasma cholesterolesters (CE), plasma phosphatidylcholine (PC), RBC PC, RBC phosphatidyletanolamine (PE), cheek cells, expressed as either % of total fatty acids or in concentration units. It is therefore difficult to establish the long-term DHA status of participants and to interpret results across studies.

The use of a uniform biomarker is essential in order to establish baseline DHA status, to determine target levels for optimal cognitive performance as well as threshold levels above which no further benefits are seen. The concept of establishing an omega-3 index for mental health has been suggested by Milte *et al*. [72], based on the omega-3 index for mortality from coronary heart disease developed by Harris and Von Schacky [73]. This index expresses the levels of EPA + DHA in RBC membranes as percentage of total RBC fatty acids and an omega-3 index of ≥8% is associated with the greatest cardio-protection whereas an index of ≤4% is associated with the least protection [73]. Since DHA plays a major role in cognitive performance whereas EPA's role is probably minor, a DHA-index for cognitive performance could be established. A biomarker reflecting long-term intake of DHA may be more appropriate. Plasma DHA reflects recent intakes whereas plasma PL and RBC DHA reflects long-term DHA intakes [1] but RBC DHA has been shown to be more sensitive to long-term intakes

than plasma PL [74]. The biomarker also has to correlate well with brain tissue levels. RBC DHA was shown to be the most efficient biomarker for accumulation of DHA in the baboon neonate brain (RBC DHA, $r = 0.86$; plasma DHA, $r = 0.58$) (reviewed by [74]). However, in studies involving children a less invasive biomarker may be more appropriate such as cheek cell DHA. Cheek cell DHA levels have been shown to correlate well with dietary intakes ($r = 0.65$), plasma ($r = 0.61$) and RBC DHA ($r = 0.58$) levels [37]. In a study on piglets cheek cell DHA correlated well with brain DHA levels ($r = 0.60$), but the correlation was not as good as for plasma ($r = 0.70$) and RBC levels ($r = 0.72$) [75]. The analysis of whole blood collected by finger prick and stored on absorbent paper may also provide a non-invasive, rapid, less costly and reliable method for DHA quantification (correlation between RBC DHA and whole blood spot collected by finger prick, $r = 0.58$) [76]. There may therefore be several potential candidate biomarkers, but RBC DHA may be the preferred biomarker because of the established history of the omega-3 index for coronary heart disease [77]. Equations could to be developed to predict a uniform DHA-index level from these different biomarkers. The uniform measurement of a DHA biomarker/index in RCT could facilitate the establishment of target DHA levels at which cognitive performance is optimal which could then guide dietary intake recommendations. We know from kinetic studies that over a period of 6 months, for every 1 g/day DHA consumed, RBC DHA levels increased by 1% [78]. Arterburn *et al*. [1] showed that plasma PL DHA was highly sensitive to dietary intake of DHA up to doses of ~2 g/day after which the DHA levels approached saturation and increased only incrementally. Identification of factors that predict biomarker responses to DHA consumption would be important to estimate dietary requirements for achieving DHA targets. Flock *et al*. [79] identified increased EPA + DHA dose as the strongest predictor of the omega-3 index (% RBC EPA + DHA); lower baseline omega-3 index levels, older age, lower body weight, increased physical activity with increased dose and female sex predicted greater increases in the omega-3 index. The background diet, particularly the omega-6 PUFA content, may also be an important predictor of RBC DHA response [80] that needs to be investigated.

The duration of studies in this review have also been variable ranging from 4 weeks to 2 years. Studies in animal models showed that recovery of brain DHA levels from a state of depletion is a much slower process compared to other tissues. Rats fed an omega-3 repletion diet containing ALA and DHA after being subjected to a low omega-3 PUFA diet through two generations required 8 weeks to reach DHA levels comparable to rats fed omega-3 PUFA adequate diets whereas DHA was almost completely replete in serum and liver after 2 weeks [81]. In rhesus monkeys that were omega-3 PUFA deficient and fed a DHA rich fish oil diet, DHA in phosphatidylethanolamine of the frontal cortex increased after 2 weeks and stabilized after 12 week [82]. The half-life of DHA in the human brain is ~2.5 years [13]. Umhau *et al*. [13] commented that any potential benefit of increasing brain DHA through dietary change may therefore not be fully manifested in clinical trials of only a few weeks and if such rapid improvements occurred it may rather be due to peripheral actions which indirectly affect brain function [13]. The 5-week study by Nilsson *et al*. [66] is an example of this where improvements in working memory correlated with improvements in cardio-metabolic markers. This may also explain why several short term studies failed to show any effects of cognitive function. Several studies of 16 weeks and longer showed improvements in cognitive performance [27–30,41,58–60,68] which is the minimum time needed for RBC DHA to reach a steady state [1,78]. The brain may not be saturated

with DHA after 16 weeks of supplementation, but measurable outcomes may become apparent after 16 weeks.

The outcomes that were improved with LC omega-3 PUFA supplementation in children included verbal learning and memory [29], reading [27,29], spelling [29], non-verbal cognitive development [28] and processing speed, visual-perceptive capacity, attention and executive function [30]; in younger adults memory and reaction time of memory were improved [41]; and in older adults several studies showed improvements in memory [27–29,41,58–60,65,68], while executive function [68] and visuospatial learning [58] were also improved. Very few studies assessed the speed of performing cognitive tasks. This represents a fundamental measure of brain function and is equally informative or complementary to information on the accuracy of task performance [83]. Speed of information processing is one of the cognitive abilities in children to develop first and is fundamental to the development and expression of other cognitive abilities such as learning, memory and executive functions [23]. Bearing in mind that DHA improves neural communication through several mechanisms as discussed in the introduction, it is highly likely that DHA may affect speed of cognitive performance. Stonehouse *et al*. [41] showed improvements in reaction time of episodic memory and working memory, but not processing speed; Portillo-Reyes *et al*. [30] showed improvements in processing speed; and McNamara *et al*. [26] showed an inverse relationship between RBC DHA levels and reaction time in a sustained attention task while Muthayya *et al*. [31] could not show any effect on cognitive speediness with a ALA supplement containing a small amount of DHA (0.1 g/day). The significance of any speed change should be interpreted in the context of the function that was assessed [84]. Since memory has been the outcome most often shown to be improved by DHA supplementation, it is likely that DHA may also improve the speed at which memory tasks are performed as was shown by Stonehouse *et al*. [41]. It is suggested that future studies include this outcome in their battery of tests. The use of computerized test batteries allows for the assessment of speed of performing cognitive tasks, but also has the advantage of standardized presentation of cognitive tests, it removes the person-to-person interactions with a researcher that can bias and obfuscate data, and it allows for closely controlled collection of a large amount of data within a short period of time [83]. On the other hand, it may be tempting for researchers to assess multiple cognitive outcomes in the hope to find positive results. However, statistical significant findings from this approach are likely to result from chance alone (type 1 error) [85]. Instead, an approach where a small set of cognitive outcomes are identified and pre-specified (primary outcome) [85] based on current evidence, e.g., memory, and investigated in greater detail will be more valuable in substantiating the effects of LC omega-3 PUFA on cognitive performance than a shot-gun approach.

The increased incorporation of brain imaging technologies in future LC omega-3 PUFA interventions could provide valuable supportive *in vivo* information on the working mechanisms of LC omega-3 PUFA. Brain imaging makers can reliably reflect neurostructural, neurophysiological, neurochemical and functional cerebral changes occurring in response to the intervention. However, these imaging markers cannot be considered as a substitute of clinical endpoints in terms of cognitive or behavioral response to a task or challenge [86].

As discussed above, outcomes may have been confounded by potential response modulating effects of gender, age and *APOE*, but very few studies have investigated these interaction effects. If dimorphisms exist for any of these factors, potential effects may be diluted or cancelled out resulting

in biased conclusions. Future trials of DHA on cognitive function should take these factors into account by either recruiting homogenous samples or by planning gender-, age- or *APOE*-stratification into the study design to ensure stratified randomization and sufficient statistical power.

To date, no studies have been conducted in vegan and vegetarian populations, who have much lower dietary and blood DHA levels compared to omnivores [16], to determine the association between DHA intake and cognitive function. Sarter *et al*. [17] suggest that lifetime DHA insufficiency may put vegans at increased risk for cognitive dysfunction. It is therefore important that future research studies focus on this target population. The availability of vegetarian omega-3 supplements, e.g., algae-sourced DHA, and evidence that supplementation with these preparation result in increased plasma and RBC DHA levels in vegans and vegetarians [16,17,87] makes this possible.

6. Conclusions

Individuals with low habitual intake of LC omega-3 PUFA, children with low literacy ability and who are malnourished, and older adults with ARCD and MCI may benefit most from consuming LC omega-3 PUFA, particularly DHA. However, the evidence-base is still emerging and RCTs have been inconsistent with many study design limitations. A major challenge ahead is the design and conduct of rigorous RCT to provide the evidence-base for dietary recommendations regarding DHA. It is recommended that future studies include a uniform biomarker, e.g., % DHA in RBC, in order to establish baseline DHA-status, determine targets for improved cognitive performance and to facilitate dosage recommendations. It is also recommended that future studies be at least 16 weeks in duration, account for potential interaction effects of gender, age and apolipoprotein E genotype, include vegan/vegetarian populations, include measures of speed of cognitive performance which could be facilitated by using computerised cognitive test batteries and include brain imaging technologies as supportive information on working mechanisms of LC omega-3 PUFA.

Supplementation with DHA is unlikely to be a “magic bullet” that will create geniuses. However, because of humans’ limited capacity to synthesise DHA *de novo* and its critical role in brain function it seems prudent that healthy individuals should include DHA in their diets for optimal cognitive performance through all stages of life. While the evidence is not available yet to make specific recommendations for dietary intake of LC omega-3 PUFA and cognitive performance, we should aim to achieve country specific recommendations of LC omega-3 PUFA. Several international organisations recommend consumption of ≥500 mg/day EPA+DHA or ≥2 fatty fish meals/week [88]. The Australian–New Zealand recommended suggested dietary targets (SDT) for LC omega-3 PUFA is 610 mg/day for men and 430 mg/day for women aged 14 and older [89]. The energy adjusted SDT for 9–13 year old boys and girls are 510 and 410 mg/day and for 4–8 year old boys and girls 400 and 350 mg/day, respectively [90].

Conflicts of Interest

The author declares no conflict of interest.

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Randomized Controlled Trial Examining the Effects of Fish Oil and Multivitamin Supplementation on the Incorporation of *n*-3 and *n*-6 Fatty Acids into Red Blood Cells

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Abstract: The present randomized, placebo-controlled, double-blind, parallel-groups clinical trial examined the effects of fish oil and multivitamin supplementation on the incorporation of *n*-3 and *n*-6 fatty acids into red blood cells. Healthy adult humans (n = 160) were randomized to receive 6 g of fish oil, 6 g of fish oil plus a multivitamin, 3 g of fish oil plus a multivitamin or a placebo daily for 16 weeks. Treatment with 6 g of fish oil, with or without a daily multivitamin, led to higher eicosapentaenoic acid (EPA) composition at endpoint. Docosahexaenoic acid (DHA) composition was unchanged following treatment. The long chain LC *n*-3 PUFA index was only higher, compared to placebo, in the group receiving the combination of 6 g of fish oil and the multivitamin. Analysis by gender revealed that all treatments increased EPA incorporation in females while, in males, EPA was only significantly increased by the 6 g fish oil multivitamin combination. There was considerable individual variability in the red blood cell incorporation of EPA and DHA at endpoint. Gender contributed to a large proportion of this variability with females generally showing higher LC *n*-3 PUFA composition at endpoint. In conclusion, the incorporation of LC *n*-3 PUFA into red blood cells was influenced by dosage, the concurrent intake of vitamin/minerals and gender.

Reprinted from *Nutrients*. Cite as: Pipingas, A.; Cockerell, R.; Grima, N.; Sinclair, A.; Stough, C.; Scholey, A.; Myers, S.; Croft, K.; Sali, A.; Pase, M.P. Randomized Controlled Trial Examining the Effects of Fish Oil and Multivitamin Supplementation on the Incorporation of *n*-3 and *n*-6 Fatty Acids into Red Blood Cells. *Nutrients* **2014**, *6*, 1956–1970.

1. Introduction

Two of the most commonly consumed dietary supplements in the Western world are fish oils containing long chain *n*-3 polyunsaturated fatty acids (LC *n*-3 PUFA) and multivitamins [1,2]. The high prevalence of multivitamin use can be attributed to the fact that vitamin deficiencies are common, even in affluent countries [3]. The high prevalence of fish oil use may be in response to recent health messages, made by respected medical authorities such as the American Heart Association, advocating the benefits of increasing dietary LC *n*-3 PUFA intake.

Extensive research has explored the effects of multivitamin and fish oil supplementation in isolation, however, examination into their combined effect on human health remains scarce. Data from the National Health and Nutrition Examination Survey suggests that users of complementary medicine are most likely to use more than one supplement [4] meaning that many people are using both vitamin and fish oil supplements at the same time. There is also some preliminary evidence to suggest that vitamins and fish oils may have synergistic effects. Vitamin and mineral co-factors can influence the biosynthesis of LC *n*-3 PUFA, altering levels of LC *n*-3 PUFA measured *in vivo* [5–7]. In particular, a preclinical study

demonstrated that an experimentally induced folic acid deficiency was associated with a fall in LC *n*-3 PUFA levels, suggesting that low levels of antioxidant vitamins may increase lipid peroxidation [7]. Based on their frequency of use and potentially synergistic actions, there is a clear need to understand how multivitamins and fish oils combine to affect potential health outcomes.

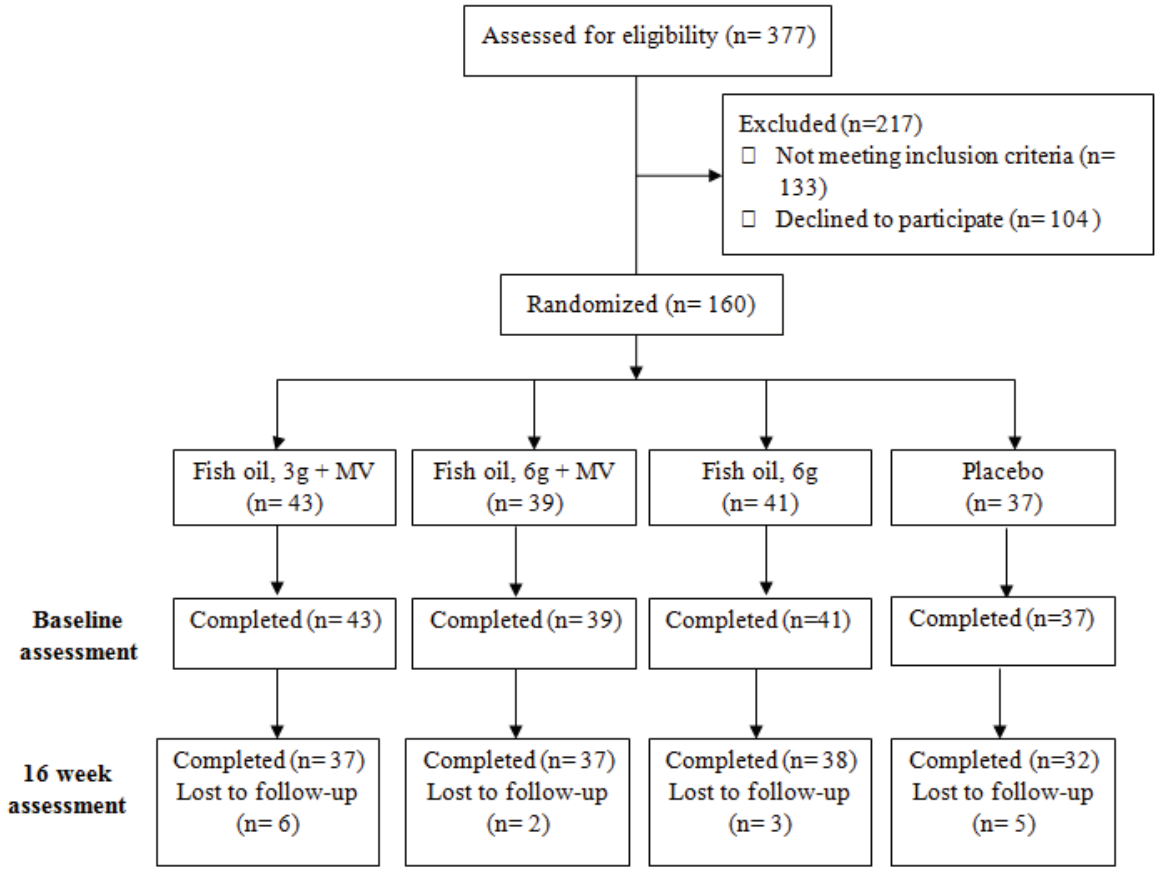
The present study investigated the effects of fish oil supplementation, with and without the addition of a multivitamin, on LC *n*-3 PUFA and LC *n*-6 PUFA incorporation measured in red blood cells. Healthy elderly participants ($n = 160$) were randomized into four groups to receive daily: (1) 6 g of fish oil; (2) 6 g of fish oil plus a multivitamin; (3) 3 g of fish oil and a multivitamin; or (4) a placebo in a double-blind, parallel groups design. The primary outcome of this trial was the effect of treatment on cognitive and cardiovascular function, which has been previously published [8]. This paper is concerned with the secondary aim of this trial which was to examine how high and low dosages of fish oil, in combination with a multivitamin, affected the incorporation of LC *n*-3 PUFA into erythrocytes. Specifically, in participants taking fish oil, we predicted increases in both EPA and DHA given that these were provided in balanced proportions in the fish oil supplements; and a dose response effect between consumption of 3 g and 6 g of fish oil. Additionally, we also examined whether combining 6 g of fish oil with a daily multivitamin increased LC *n*-3 PUFA red blood cell incorporation, over and above the effects of fish oil alone.

2. Methods

2.1. Participants

The sample consisted of 160 healthy male and female volunteers aged 50 to 70 years. Participants were recruited from the general community and were non-smoking volunteers, not currently taking any medication or vitamin/herbal supplements. Exclusion criteria were; diagnosis of dementia, diabetes, neurologic (*i.e.*, Epilepsy, Parkinson's disease, head trauma) or psychiatric disorders (*i.e.*, depression, schizophrenia), cardiovascular disease (including stroke) or past or present drug or alcohol abuse. Individuals taking anti-coagulant, anti-cholinergic, anti-depressants or acetyl-cholinesterase inhibitors were also excluded. Further exclusion criteria included those currently taking cognitive enhancing supplements regularly and current or long-term multivitamin or fish oil supplementation. The participant flow diagram is shown in Figure 1. The study randomized 160 participants and 144 completed the trial.

Figure 1. Participant flow diagram. MV: multivitamin.



2.2. Setting

The study was conducted at Swinburne University of Technology, Hawthorn, Australia.

2.3. Interventions, Randomization and Blinding

The trial was randomised, placebo-controlled and double-blind, using a parallel group design. The participants were randomly assigned to one of the following four daily treatments:

(1) Multivitamin combined with 3 g of fish oil (240 mg EPA and 240 mg DHA);
(2) Multivitamin combined with 6 g of fish oil (480 mg EPA and 480 mg DHA);
(3) Placebo multivitamin combined with 6 g fish oil (480 mg EPA and 480 mg DHA);
(4) Placebo multivitamin combined with placebo fish oil (Sunola oil).

Participants consumed their assigned treatment daily for 16 weeks. The clinical trials supplements and matching placebos were provided by Swisse Wellness Pty Ltd. (Melbourne, Australia). The active fish oil supplement was Swisse Ultiboost Wild Salmon Oil and the active multivitamin supplement was Swisse Ultivite 50+ (Mens and Womens formulations). The constituents of the multivitamins are given in the online supplement (Table S1). All participants took one multivitamin (or its corresponding placebo) daily. Participants allocated to receive 6 g of fish oil daily were required to take six active fish oil capsules daily. Participants randomized to receive 3 g of fish oil took three active fish oil capsules and three matching placebo capsules daily. Participants in the placebo group received six placebo

fish oil capsules daily. The placebo fish oil contained 1000 mg of Sunola Oil and 50 IU of vitamin E administered in a soft gelatin capsule. Sunola oil is a mono-unsaturated, high oleic (*n*-9) sunflower oil and was chosen as a control given that it is virtually *trans*-fat free and has a similar profile to olive oil. Small sachets with a few drops of fish oil were included in containers to assist with blinding by providing a fish odour when opened. The placebo multivitamin contained carrot powder with a small amount of riboflavin to produce colouration of the urine similar to the active multivitamin. The placebos were identical to the active tablets in shape, size and colour.

Participants were randomly assigned to one of the four experimental groups using a random permuted block procedure with a block size of four. The randomisation was conducted independently by the supplement supplier and the bottles labeled according to the randomization schedule. The research staff were blinded to this allocation. To ensure adequate blinding, placebo and active treatments were packaged in identical blister packs for multivitamins and sealed plastic containers for fish oil capsules. Participants were allocated the next sequential number upon enrolment in the study. Data was unblinded following the analysis of the main study aims.

2.4. Outcomes Measures

The outcome measures for this study were the incorporation of LC *n*-3 PUFA and LC *n*-6 PUFA fatty acids into red blood cells, following supplementation. Blood sampling was conducted in the morning following a 12-h fasting period. Blood was collected via venepuncture from the antecubital vein, using the BD-vacutainer system. Samples were analysed by Healthscope Functional Pathology according to standard procedures. Samples were centrifuged at 3000 rpm for 10 min before plasma was removed. Red blood cells were then washed twice by suspending in 0.9% saline, centrifuging at 3000 rpm aspirating off the supernatant. Red cells were then stored at −20 °C until assayed. Methyl ethers of fatty acids were prepared as follows: 350 µL of plasma and 1.5 mL of red cell extract were added to a 10 mL extraction tube. 3.8 mL of a methanol/chloroform mixture was added before vortexing the tube for 6 min. 0.8 mL of 0.1 M KCI solution was added and the tube was then vortexed for a further 3 min and then centrifuged at 3000 rpm for 10 min. The upper aqueous layer was discarded by aspiration. A silane treated glass wool was placed in the bottom of a glass Pasteur pipette and then filled with sodium sulphate. The organic layer was passed through sodium sulphate and the eluate was collected in 2 mL vials. The solvent was evaporated to dryness in heating block (<45 °C) with nitrogen. The dry residue was reconstituted with 130 µL of Meth-Prep II methylation agent. Vials were then closed and left to rest at room temperature overnight. 0.8 µL of the esterification mixture was then injected into a Gas Liquid Chromatography using flame ionisation detection (GLC-FID), on a Schimadzu G-2010 (Shumadzu, Kyoto, Japan), for analysis. Chromatic conditions included a detector temperature of 300 °C and injector temperature of 250 °C. Injector sampling time was 0.5 min. All fatty acid values were expressed as a percentage of red blood cell total fatty acids. The LC *n*-3 PUFA index was calculated as total DHA + total EPA + total DPA. Total *n*-3 fatty acid and total *n*-6 fatty acids were calculated as the combination of both total long and short chain *n*-3 (alpha linolenic acid + EPA + DHA + DPA) and *n*-6 (linoleic acid + gamma linolenic acid + eicosadienoic acid + eicosatrienoic acid + arachidonic acid) fatty acids respectively.

2.5. Sample Size

The sample size of 160 was determined based on the variance of the cognitive and cardiovascular study outcomes, which are reported separately [8]. Percept changes in cognitive performance were expected to be considerably smaller than changes in LC *n*-3 PUFA over the study period. Thus, the study was believed to be appropriately powered to investigate changes in LC *n*-3 PUFA due to treatment.

2.6. Procedure

Participants were required to attend testing sessions at our laboratories on three separate occasions; at baseline, following six weeks of supplementation and following 16 weeks of supplementation. Blood samples were taken both at baseline and at week 16 only.

The research was conducted in accordance with the guidelines of the Australian National Health and Medical Research Council and the Declaration of Helsinki (as revised in 2004). The study was approved by the Swinburne University Human Research Ethics Committee. Written informed consent was obtained from all subjects. This trial was registered with the Australian and New Zealand Clinical Trial Registry (ACTRN12611000094976).

2.7. Statistical Analyses

Results were analyzed using SPSS statistics (IBM, version 20, New York, NY, USA). Univariate analyses of variance (ANOVA) were used to examine whether any significant group differences existed at baseline for the basic demographic and health variables displayed in Table 1. Univariate ANCOVAs were also used to examine the effects of treatment on all outcomes variables at week 16. Significant main effects of treatment were further examined using simple planned contrasts, applying Bonferroni corrections to each contrast in order to adjust for comparisons across the treatment groups. Given that males and females may respond differently to LC *n*-3 PUFA supplementation across different clinical outcomes, we examined whether gender predicted the incorporation of LC *n*-3 PUFA and LC *n*-6 PUFA into red blood cells. For these analyses, gender was entered as a fixed factor and the respective fatty acid variable at endpoint as the dependent variable in ANOVA. All analyses were adjusted for the respective scores at baseline and all results were considered statistically significant at $p < 0.05$.

3. Results

The trial started in 2010 and was ceased in 2012 due to attainment of the desired sample size. No serious adverse events were reported.

3.1. Cohort Demographics

The descriptive demographics of the study population at baseline are given in Table 1. The mean age of the sample was 59 years. The sample was roughly gender balanced with slightly more females. On average, the sample was well educated and high functioning. Blood pressure levels were normal across the sample although Low Density Lipoprotein (LDL) cholesterol tended to be elevated across all treatment groups. ANOVA revealed that, at baseline, the treatment groups were well matched

across all continuous variables displayed in Table 1, with no significant group differences noted. ANOVA also suggested that males and females were well matched across all fatty acid variables at baseline. Mean baseline fatty acid values can be seen in Table 2, stratified according to treatment allocation. Across the whole sample, median red blood cell composition of *n*-3 fatty acid and *n*-6 fatty acid tended to be lower than those reported in a normative group of almost 160,000 people [9]. In contrast, median baseline values of saturated and monounsaturated fats were higher in the present cohort.

Table 1. Sample demographics (means and standard deviations) stratified by treatment allocation.

Variable	Fish Oil, 3 g + Multivitamin	Fish Oil, 6 g + Multivitamin	Fish Oil, 6 g	Placebo	Overall
N	43	39	41	37	160
Age, year	59.48 (5.64)	58.90 (5.60)	59.51 (5.89)	59.19 (5.96)	59.28 (5.72)
Male, %	48	48	46	46	47
Education, year	15.54 (3.10)	15.79 (3.92)	15.84 (3.94)	15.76 (3.38)	15.73 (3.57)
MMSE	28.12 (2.04)	28.25 (1.61)	28.07 (2.02)	28.14 (1.74)	28.14 (1.85)
Height, cm	170.79 (8.79)	169.63 (9.17)	173.03 (9.91)	170.23 (9.05)	170.93 (9.24)
Weight, kg	74.88 (13.78)	70.98 (12.08)	76.35 (16.28)	70.35 (11.01)	73.18 (13.57)
BMI	25.54(3.59)	24.41 (3.07)	25.31 (4.03)	24.2 (2.79)	24.88 (3.43)
LDL, mmol/L	3.31 (0.71)	3.51 (0.72)	3.37 (0.84)	3.27 (0.72)	3.36 (0.75)
HDL, mmol/L	1.52 (0.42)	1.61 (0.39)	1.56 (0.44)	1.57 (0.36)	1.56 (0.40)
SBP, mmHg	125.81 (20.97)	122.59 (16.92)	126.29 (16.92)	121.41 (21.47)	124.12 (19.10)
DBP, mmHg	77.19 (13.32)	75.46 (10.90)	77.71 (9.82)	74.62 (12.13)	76.30 (11.58)

Note: MMSE: Mini Mental State Examination, BMI: Body Mass Index, LDL: low density lipoprotein cholesterol, HDL: high density lipoprotein cholesterol, SBP: systolic blood pressure, diastolic blood pressure.

3.2. Main Effects of Treatment on n-3 Fatty Acid and n-6 Fatty Acid Blood Measures

Table 2 displays red blood cell fatty acid composition before and after treatment. Univariate ANCOVA revealed that week 16 EPA ($F(3, 136) = 12.20$, $p < 0.001$), DPA ($F(3, 136) = 3.09$, $p < 0.05$), LC *n*-3 PUFA index ($F(3, 136) = 3.98$, $p < 0.01$), AA/EPA ratio ($F(3, 136) = 53.74$, $p < 0.001$), total *n*-3 fatty acid ($F(3, 136) = 3.96$, $p < 0.05$), total *n*-6 ($F(3, 136) = 4.01$, $p < 0.01$) and the *n*-3/*n*-6 ratio ($F(3, 136) = 10.13$, $p < 0.001$) differed between treatment groups, when controlling for baseline. Week 16 DHA ($F(3, 136) = 2.01$, $p = 0.10$) did not differ according to treatment allocation, when controlling for baseline.

Table 2. Means, standard deviations and percentage change for red blood cell fatty acid status over the course of supplementation.

Variable	Fish Oil, 3 g + Multivitamin	Fish Oil, 6 g + Multivitamin	Fish Oil, 6 g	Placebo	ANCOVA *F* Value
EPA, %					12.20 ***
Baseline	0.99 (0.46)	1.01 (0.30)	1.06 (0.41)	1.00 (0.43)	
Week 16	1.41 (0.68)	1.98 (0.65) ***	1.66 (0.75) ***	1.06 (0.48)	
% change	42.42	96.04	56.60	6.00	
DHA, %					2.01
Baseline	2.66 (1.26)	2.74 (0.96)	2.92 (1.10)	2.82 (1.11)	
Week 16	2.94 (1.58)	3.64 (1.27)	3.16 (1.60)	2.86 (1.21)	
% change	10.53	32.85	8.22	1.42	
DPA, %					3.09 *
Baseline	1.69 (0.75)	1.88 (0.50)	1.94 (0.65)	1.83 (0.61)	
Week 16	1.74 (0.85)	2.31 (0.67)	1.93 (0.96)	1.87 (0.74)	
%change	2.96	22.87	−0.52	2.19	
LC *n*-3 PUFA index, %					3.98 **
Baseline	5.34 (2.31)	5.63 (1.59)	5.92 (2.04)	5.65 (2.00)	
Week 16	6.11 (2.99)	7.92 (2.46) **	6.75 (3.23)	5.79 (2.31)	
% change	14.42	40.67	14.02	2.48	
AA/EPA, ratio					53.74 ***
Baseline	10.18 (4.06)	10.18 (2.24)	9.75 (3.20)	10.58 (3.79)	
Week 16	6.11 (2.22) ***	4.48 (1.05) ***	4.64 (1.18) ***	9.72 (3.13)	
% change	−39.98	−55.99	−52.41	−8.13	
Total *n*-3 FA, %					3.96 *
Baseline	5.52 (2.32)	5.83 (1.61)	6.11 (2.06)	5.85 (2.01)	
Week 16	6.26 (3.00)	8.10 (2.47) **	6.90 (3.22)	5.99 (2.32)	
% change	13.41	38.94	12.93	2.39	
Total *n*-6 FA, %					4.01 **
Baseline	21.97 (4.82)	24.27 (3.42)	23.80 (4.50)	23.41 (4.49)	
Week 16	19.77 (5.60)*	21.63 (4.34)	19.98 (5.53) *	23.66 (4.54)	
% change	−10.01	−10.88	−16.05	1.07	
n-3/*n*-6 FA, ratio					10.13 ***
Baseline	0.24 (0.78)	0.23 (0.06)	0.25 (0.07)	0.24 (0.06)	
Week 16	0.29 (0.10)	0.37 (0.09) ***	0.33 (0.11) **	0.25 (0.08)	
% change	20.83	60.86	32.00	4.17	
n	35	37	38	31	

Note: EPA: Eicosapentaenoic Acid, DHA: Docosahexaenoic Acid, AA: Arachidonic Acid, LC *n*-3 PUFA: Long chain *n*-3 polyunsaturated fatty acid, FA: Fatty acid, ANOVA results are for univariate analysis of variance comparing blood values at end-point, by treatment allocation, whilst controlling for baseline values. Stars display results of simple planned contrasts between the placebo and treatment groups. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3. Effects of Treatment, Relative to Placebo, on n-3 Fatty Acid and n-6 Fatty Acid Blood Measures

Those treatment groups differing significantly from placebo can be seen in Table 2. The week 16 AA/EPA ratio was lower across all treatment groups relative to placebo. EPA was significantly higher at week 16 in the two 6 g fish oil groups. Despite a significant main effect, none of the treatment groups had significantly higher DPA at study endpoint, as compared to placebo. Both the LC *n*-3 PUFA index and total *n*-3 fatty acid were only higher in the group receiving the combination of the 6 g fish oil and multivitamin combination. *n*-6 Fatty acid was lower in the 3 g fish oil multivitamin group as well as in the group receiving 6 g of fish oil in isolation. The *n*-3/*n*-6 fatty acid ratio was significantly higher following 6 g of fish oil supplementation, irrespective of the multivitamin.

3.4. Effects of Fish Oil Dosage on n-3 Fatty Acid and n-6 Fatty Acid Blood Measures

Red blood cell incorporation of *n*-3 fatty acid at week 16 was expected to be higher following supplementation with 6 g as opposed to 3 g of fish oil, demonstrating a dose response. When directly comparing the 6 g fish oil multivitamin group to the 3 g fish oil multivitamin group, the higher dose fish oil group displayed significantly higher week 16 incorporation of EPA ($p < 0.01$), DPA ($p < 0.05$), LC *n*-3 PUFA index ($p < 0.05$), total *n*-3 fatty acid ($p < 0.05$) and the *n*-3/*n*-6 fatty acid ratio ($p < 0.01$). The AA/EPA ratio was also lower in the higher dose fish oil group ($p < 0.01$).

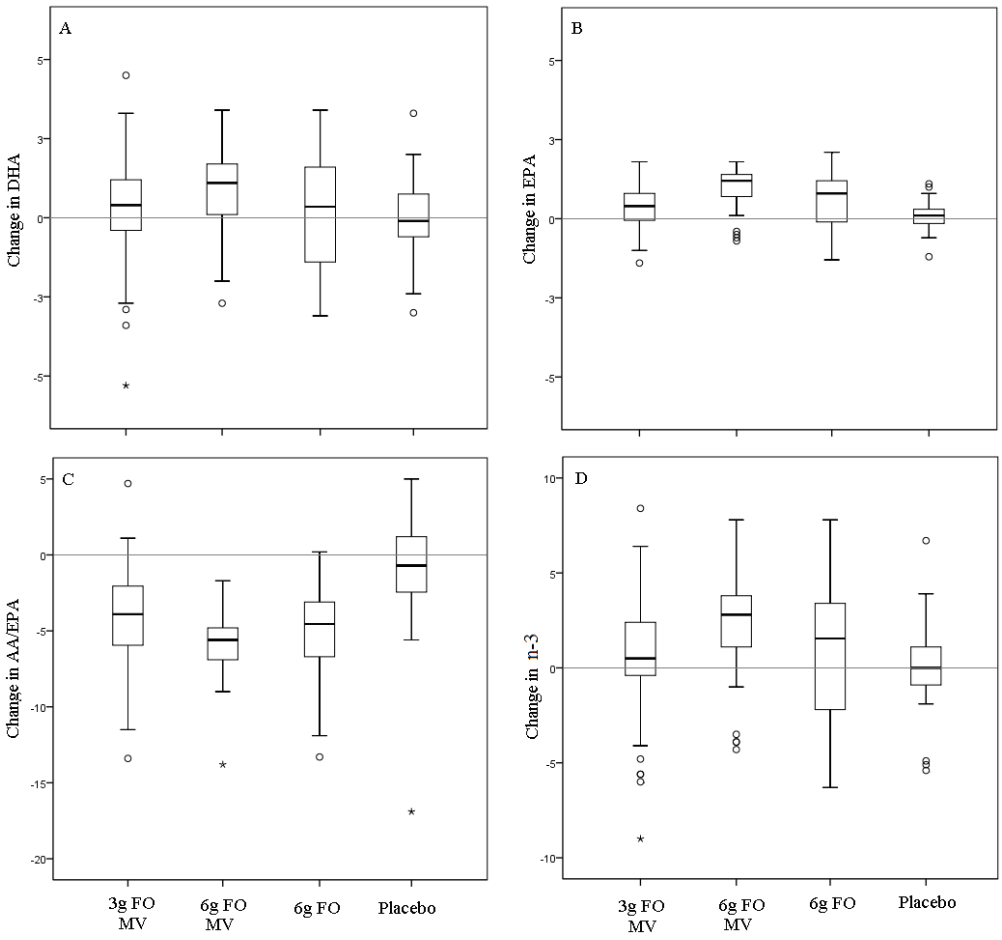
3.5. Effects of Combining Fish Oil with a Multivitamin on n-3 and n-6 Blood Measures

Adding a multivitamin to the fish oil was expected to increase week 16 *n*-3 fatty acid incorporation into red blood cells, over and above the effects of fish oil alone. When directly comparing the two 6 g fish oils groups, with and without the addition of a multivitamin, there were no significant differences between the two groups across any of the week 16 *n*-3 fatty acid or *n*-6 fatty acid variables. However, when comparing to placebo, the LC *n*-3 PUFA index and total *n*-3 fatty were only increased following the 6 g fish oil multivitamin combination (Table 2) and not the 6g fish oil alone.

3.6. Sources of Variability in Red Blood Cell n-3 Fatty Acid Incorporation

Figure 2 shows changes in total *n*-3 fatty acid, EPA, DHA and the AA/EPA ratio, over the course of the study, stratified by treatment allocation. Considerable individual variability in *n*-3 fatty acid change is evident. Interestingly, many participants allocated to the fish oil conditions decreased their amount of total *n*-3 fatty acid and DHA as measured from red blood cells over the 16 week study period. However, EPA tended to increase and the AA/EPA ratio decreased in the fish oil treatment arms suggesting that the variability in DHA and total *n*-3 fatty acid may reflect individual differences in incorporation rather than compliance to treatment. The AA/EPA ratio appears to be the best indicator of compliance to treatment as almost all participants receiving active fish oil decreased their ratio, whilst those in the control group tended to remain stable. Across all *n*-3 fatty acid measures, the coefficients of variations using week 16 treatment means and standard deviations (Table 3) tended to be lowest for the 6 g fish oil + multivitamin group and highest for the 3 g fish oil + multivitamin group. The coefficients of variation also tended to be lowest for the AA/EPA ratio.

Figure 2. Variability in red blood cell incorporation stratified by treatment allocation for measures of DHA (**A**), EPA (**B**), AA/EPA ratio (**C**) and total *n*-3 fatty acid (**D**). EP: eicosapentaenoic acid, DHA: docosahexaenoic acid, AA: Arachidonic acid, FO: fish oil, MV: multivitamin. Circles and stars represent outliers less than 2 and greater than 2 standard deviations from the mean respectively.



3.7. Gender and Variability in n-3 Fatty Acid

Recent studies suggest that males and females respond differently to LC *n*-3 PUFA supplementation across different clinical outcomes [10,11]. Males and females may therefore differ in their ability to incorporate *n*-3 fatty acids into erythrocytes. The authors investigated whether gender accounted for some of the observed variability in the incorporation of *n*-3 fatty acids into red blood cells. Gender was a significant predictor of week 16 EPA ($F(3, 135) = 4.54$, $p < 0.05$), DHA ($F(3, 135) = 4.42$, $p < 0.05$), LC *n*-3 PUFA index ($F(3, 135) = 4.53$, $p < 0.05$) and total *n*-3 fatty acids ($F(3, 135) = 5.10$, $p < 0.05$). Gender was not predictive of the week 16 AA/EPA ratio ($F(3, 135) = 0.01$, $p = 0.94$) nor DPA ($F(3, 135) = 2.58$, $p = 0.11$). Significant interactions were also found between treatment allocation and gender for EPA ($F(3, 135) = 3.40$, $p < 0.05$), DHA ($F(3, 135) = 4.99$, $p < 0.01$), DPA ($F(3, 135) = 5.10$, $p < 0.01$), LC *n*-3 PUFA index ($F(3, 135) = 5.37$, $p < 0.01$) and total *n*-3 fatty acids ($F(3, 135) = 4.86$, $p < 0.01$). Selected interactions are displayed in Figure 3 (Separate analysis of males and females across all blood measures can be seen in Tables S2 and S3 of the online supplement). It can be seen that females tended to have higher red blood cell incorporation of most *n*-3

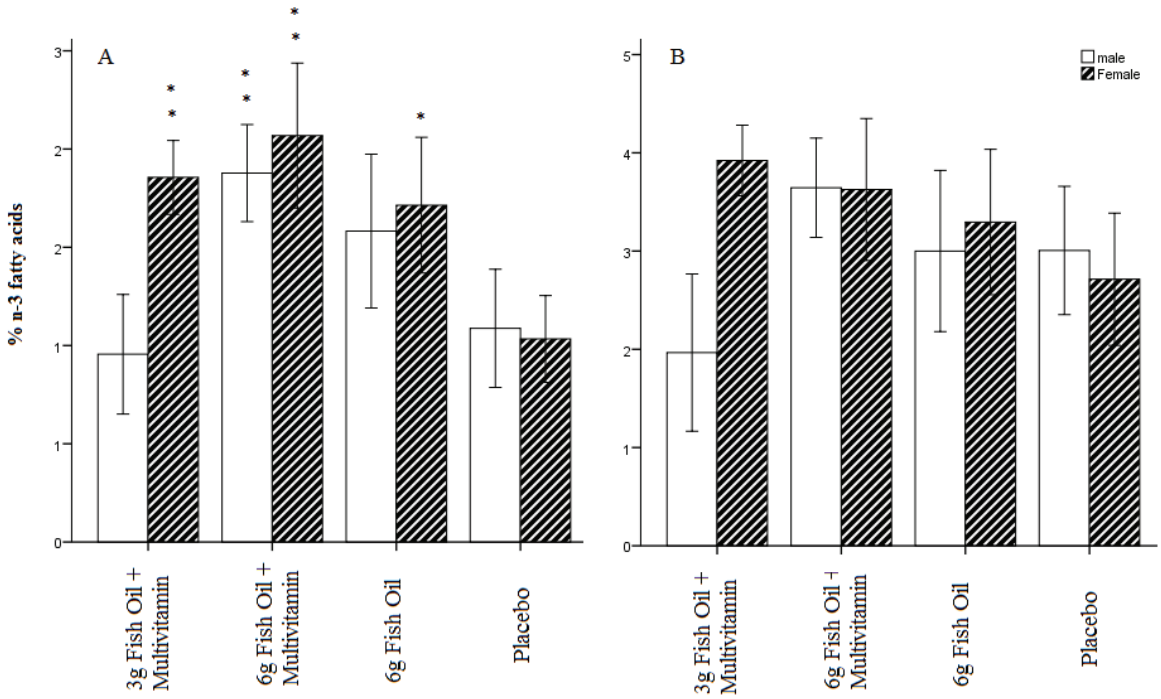
fatty acid blood measures at endpoint. The most interesting finding was that, in females, all treatment groups led to an increase in EPA relative to placebo (Figure 3A). In males, EPA only increased following treatment with the combination of 6 g fish oil and a daily multivitamin. Unlike gender, other demographic and clinical factors such as age, height, weight, physical activity, total cholesterol, high sensitivity CRP and general health status did not predict *n*-3 fatty acid incorporation into red blood cells at study endpoint.

Table 3. Coefficients of variation for each *n*-3 fatty acid blood measure at week 16, stratified by treatment allocation.

Variable	Fish Oil, 3g + Multivitamin	Fish Oil, 6g + Multivitamin	Fish Oil, 6 g	Placebo
EPA	0.48	0.33	0.45	0.45
DHA	0.54	0.35	0.51	0.42
DPA	0.49	0.29	0.50	0.40
LC *n*-3 PUFA index	0.49	0.31	0.48	0.40
AA/EPA	0.36	0.23	0.25	0.34
Total *n*-3	0.48	0.30	0.47	0.39
n-3/*n*-6	0.34	0.24	0.33	0.32

Note: EPA = Eicosapentaenoic Acid, DHA = Docosahexaenoic Acid, AA = Arachidonic Acid, LC *n*-3 PUFA = Long chain *n*-3 polyunsaturated fatty acid. Coefficients of variation calculated as week 16 standard deviation/mean for each measure respectively.

Figure 3. Incorporation of EPA (**A**) and DHA (**B**) into red blood cells at study endpoint stratified by treatment allocation and gender. Males are represented by white bars and females by shaded bars. * Group significantly different to placebo at $p < 0.05$, ** Group significantly different to placebo at $p < 0.01$.



4. Discussion

This study investigated the effects of fish oil supplementation, with and without the addition of a multivitamin, on red blood cell fatty acid composition. Daily supplementation with 6 g of fish oil for 16 weeks led to higher composition of EPA as well as a lower AA/EPA ratio. The LC *n*-3 PUFA index and total *n*-3 fatty acid only increased after 6 g of fish oil was administered in combination with a daily multivitamin. As expected, 6 g of fish oil combined with a multivitamin was more effective at increasing *n*-3 fatty acid erythrocyte composition than 3 g of fish oil combined with a multivitamin. Over the 16 week study period there was considerable individual variability in *n*-3 fatty acid change, much of which was accounted for by gender. A predicted dose response effect in *n*-3 fatty acids was seen between the 3 g and 6 g fish oil supplements.

Fish oils combined with the concomitant use of a multivitamin increased the LC *n*-3 PUFA index. The LC *n*-3 PUFA index was not increased following 6 g of fish oil alone. It thus follows that the intake of vitamins and minerals, from dietary sources, may also affect the efficacy of fish oil supplementation. These results are important because low amounts of LC *n*-3 PUFA are associated with an increased risk of death from coronary heart disease [12]. Combining fish oil supplementation with adequate vitamin/mineral intake, either through diet or supplementation, may help bolster the LC *n*-3 PUFA index thus reducing cardiovascular disease risk. Future fish oil intervention trials are advised to account for habitual intake of vitamins (*i.e.*, through food frequency questionnaires), as this may partly explain individual differences in response to fish oil treatment.

The mechanism by which vitamin/mineral intake interacts with fish oil supplementation to increase the LC *n*-3 PUFA index is not completely understood. Preliminary evidence obtained from animal studies suggests that certain vitamins and minerals, such as B vitamins and iron, influence *in vivo* composition of *n*-3 [5–7]. Although speculative, multivitamin use may boost antioxidant defence, protecting LC *n*-3 PUFA from oxidation.

The present results suggest that considerable variability exists in the individual to uptake and transfer LC *n*-3 PUFA to red blood cells. Gender was identified as one factor contributing to this variability. Females supplemented with fish oil were generally found to have higher incorporation of total *n*-3 fatty acids at the end of the study. No gender differences were found for the AA/EPA ratio suggesting that gender differences are not merely due to compliance to treatment. Instead, these results suggest that males and females differ in their ability to incorporate some specific types of LC *n*-3 PUFA, such as EPA, into red blood cells. In females, all treatments led to a significant increase in EPA over and above the placebo. In contrast, only the 6 g fish oil multivitamin treatment led to an increase in EPA composition in males. These findings are interesting in light of recent studies showing that males and females respond differently to LC *n*-3 PUFA supplementation across clinical outcomes such as platelet aggregation [10] and cognitive performance [11]. If the present findings can be replicated, they may have significant implications for health policy and guidelines because males and females may be required to consume different amounts of fatty fish or fish oil supplements in order to achieve optimal LC *n*-3 PUFA blood composition.

In certain areas of investigation, inconsistencies have been reported regarding the health benefits of fish oil supplementation. For example, randomized controlled trials have produced conflicting results as to whether fish oil supplementation can enhance cognitive performance or mitigate cognitive decline

in adults [11,13,14]. These conflicting results are surprising given that observational studies have been far more consistent in suggesting that *n*-3 fatty acid blood composition is associated with cognitive outcomes [15–18]. In light of the present findings, inconsistencies reported in fish oil intervention studies may be partly due to individual differences in the ability to incorporate LC *n*-3 PUFAs into cell membranes. These individual differences may be due to gender or vitamin/mineral intake, either through background diet or concomitant supplement use. Others have also suggested that genetic markers, such as the presence of the APOE e4 allele, may also affect response to fish oil supplementation [19]. To counteract the variability in response to fish oil supplementation, these results highlight the importance of including blood measures of LC *n*-3 PUFA status in future fish oil intervention studies.

The fish oil supplements in the current study contained a balanced ratio of EPA and DHA. Although EPA increased by as much as 96%, DHA red blood cell levels did not significantly increase following fish oil supplementation. These results are consistent with a previous report showing that EPA, as compared to DHA, was better incorporated into erythrocyte membranes following supplementation [20]. In this previous study, EPA increased by 300% while DHA only increased by 42% following 8 weeks of daily supplementation with 1296 mg EPA and 864 mg DHA. Previous studies have also shown that the uptake of DHA into erythrocyte membranes is more variable than that of EPA [20,21].

Limitations of the current study include the relatively small sample size, the relatively short follow-up period and the fact that LC *n*-3 PUFA composition was only measured at baseline and then again following 16 weeks of supplementation. Assessing red blood cell fatty acid incorporation at multiple time points, spread out over the intervention period, would provide a better indicator of *n*-3 fatty acid change across time. The multivitamin formulations used in the present study differed slightly for males and females and this may have inflated some of the observed gender differences. Furthermore, we did not monitor or examine how changes in other dietary factors may have influenced the reported results over the 16 week study period. Lastly, recent studies have shown health benefits of fish oil associated with higher dosages than that used in the present study [11,22] and it is possible that higher dosages would differentially affect *n*-3 fatty blood biomarkers.

5. Conclusions

Daily supplementation for 16 weeks with 6g of fish oil, with or without a multivitamin, led to higher EPA incorporation into erythrocytes. A dose response effect was demonstrated between 3 g and 6 g of fish oil on *n*-3 fatty acids. Treatment had no effect on DHA composition. At study endpoint, the LC *n*-3 PUFA index was only higher for those receiving a multivitamin in addition to 6 g of daily fish oil, suggesting that some vitamins/minerals aid the incorporation of LC *n*-3 PUFA into red blood cells. There was considerable individual variability in the response to supplementation with females, generally found to incorporate LC *n*-3 PUFA into red blood cells more effectively than males. Relative to placebo, all treatments increased EPA in females whereas only the 6 g fish oil multivitamin combination treatment increased EPA in males. These results suggest that some males may incorporate relatively low amounts of LC *n*-3 PUFA into red blood cells despite adhering to LC *n*-3 PUFA intake guidelines. This is an important area for future research because dietary recommendations around LC *n*-3 PUFA intake may need to be gender specific.

Author Contributions

Formulating research questions and study design: AP with input from all authors; carrying out research: NG, RC, MP, AP; data analysis: AP, MP, RC; writing manuscript: AP, MP, ASi; Revision of manuscript: all authors.

Conflicts of Interest

The study was sponsored by Swisse Wellness Pty Ltd. (formerly Swisse Vitamins Pty Ltd., Melbourne, Australia) under contract to Swinburne University of Technology and performed independently by the Centre for Human Psychopharmacology. The National Institute of Integrative Medicine, of which Professor Avni Sali is currently director, receives financial support from Swisse Wellness Pty Ltd. Andrew Pipingas and Avni Sali are currently members of the Scientific Advisory Panel for Swisse Wellness Pty Ltd. Aside from input into the supplements utilized and the broad aims of the study as well as the provision of supplements, Swisse Wellness Pty Ltd. were not involved in any other aspect of the conduct of the trial including analysis, or interpretation of the trial findings.

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3. Sources of Long-chain Omega-3

Readily Available Sources of Long-Chain Omega-3 Oils: Is Farmed Australian Seafood a Better Source of the Good Oil than Wild-Caught Seafood?

Peter D. Nichols, Brett Glencross, James R. Petrie and Surinder P. Singh

Abstract: Seafood consumption enhances intake of omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (termed LC omega-3 oils). Humans biosynthesize only small amounts of LC-omega-3, so they are considered semi-essential nutrients in our diet. Concern has been raised that farmed fish now contain lower LC omega-3 content than wild-harvested seafood due to the use of oil blending in diets fed to farmed fish. However, we observed that two major Australian farmed finfish species, Atlantic salmon (*Salmo salar*) and barramundi (*Lates calcifer*), have higher oil and LC omega-3 content than the same or other species from the wild, and remain an excellent means to achieve substantial intake of LC omega-3 oils. Notwithstanding, LC omega-3 oil content has decreased in these two farmed species, due largely to replacing dietary fish oil with poultry oil. For Atlantic salmon, LC omega-3 content decreased ~30%–50% between 2002 and 2013, and the omega-3/omega-6 ratio also decreased (>5:1 to <1:1). Australian consumers increasingly seek their LC omega-3 from supplements, therefore a range of supplement products were compared. The development and future application of oilseeds containing LC omega-3 oils and their incorporation in aquafeeds would allow these health-benefitting oils to be maximized in farmed Australian seafood. Such advances can assist with preventative health care, fisheries management, aquaculture nutrition, an innovative feed/food industry and ultimately towards improved consumer health.

Reprinted from *Nutrients*. Cite as: Nichols, P.D.; Glencross, B.; Petrie, J.R.; Singh, S.P. Readily Available Sources of Long-Chain Omega-3 Oils: Is Farmed Australian Seafood a Better Source of the Good Oil than Wild-Caught Seafood? *Nutrients* **2014**, *6*, 1063–1079.

1. Introduction

The health benefits of omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (LC-PUFA, also termed LC omega-3 oils) were first documented over three decades ago. Scientists observed that Greenland Eskimos had lower incidence of heart disease than other ethnic groups despite their high fat diet that was rich in the blubber of marine mammals [1]. The main LC omega-3 oils that have been attributed to this health benefit are eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3). For brevity, we use the term LC omega-3 here in consideration of both fatty acids, and also that of docosapentaenoic acid (DPA, 22:5ω3). Seafood has traditionally been the major source of these health-benefitting LC omega-3 oils [2]. Over the past decade the supply of farmed seafood has

steadily increased, with the contribution of aquaculture to human food supplies now similar in volume to that of the wild catch harvest.

Aquaculture is currently the main user of industrially produced fish oils, with around 90% of global fish oil production used in aquafeeds [3]. Fish oils are used in feeds for most aquaculture species to satisfy both essential fatty acid and energetic demands [4]. However, as aquaculture has expanded, the ability of existing supplies, including the increasing cost, of the wild harvest fish oil resource to meet industry needs has been largely surpassed [5]. Non-marine sources of oil, including vegetable and animal-derived, are therefore now also included in aquafeeds as alternatives to fish oil. However, both alternatives to fish oil have a lower content of LC omega-3 oil, which has the flow on effect of causing a lower concentration of LC omega-3 in farmed seafood products compared to that observed previously [6]. Generally Australian consumers and also, to our knowledge, consumers in most other countries are not fully aware of the lower LC omega-3 content now occurring in many farmed fish species. In addition to the trend of fish oil replacement largely by oil blends, other potential strategies exist, including finishing diets used prior to harvest and novel ingredients such as LC omega-3 precursors [7]. However, to ensure the long-term supply of LC omega-3 oils for aquaculture there is a clear need for new and sustainable sources of these oils from fishery independent sources.

Obtaining information on the composition of farmed seafood products is important for future developments within the aquaculture and associated feed manufacturing industries. This study was initiated to examine the fatty acid content and composition of two major Australian farmed fish species—Atlantic salmon (*Salmo salar*) and barramundi (*Lates calcifer*). Particular emphasis was given to the LC omega-3 oils, and also the comparison of the changes seen in the fatty acid profiles between samples collected over 2010–2013 and earlier data for these same species obtained during 2002 when a largely fish oil diet was in use. As many Australian consumers increasingly seek their LC omega-3 from supplements, a range of supplement products also were examined and compared.

2. Experimental Section

2.1. Sample Collection

Norwegian quality cut (NQC) samples from three fresh fillets of farmed Atlantic salmon were obtained twice per year between December 2010 and November 2013. At CSIRO, the skin was removed and each sample was independently blended for two minutes in an OSKAR 400 continuous flow homogenizer. Whole barramundi were supplied to CSIRO in November 2010 by Marine Produce Australia Pty Ltd. (Broome, WA, Australia). The right fillet of each of three fish was used and the NQC analogous cut from that fillet of the barramundi was taken. The NQC sample was then minced, with a sample analysed for moisture content by gravimetric analysis after oven drying at 105 °C for 24 h. The remainder of the sample was frozen, freeze dried, ground in a coffee grinder, and then shipped to Hobart for lipid extraction and analysis.

Fourteen commercially produced omega-3 fish oil capsules were purchased from a local Hobart pharmacy. Each product was given a laboratory code: FO1-14. Oil capsules were cut open with a scalpel and the oil transferred to a glass vial using a glass pipette.

2.2. Lipid Extraction

NQC samples (typically between 1 and 3 g wet weight for Atlantic salmon, dry weight for barramundi) were quantitatively extracted overnight using a modified Bligh and Dyer [8] single-phase methanol-chloroform-water extraction (2:1:0.8 v/v/v). The phases were separated by addition of chloroform-water (final solvent ratio, 1:1:0.9 v/v/v methanol-chloroform-water). The total solvent extract (TSE) was concentrated using rotary evaporation at 40 °C, and total lipid content was determined gravimetrically.

2.3. Fatty Acid Analysis

An aliquot of the TSE of the farmed fish samples or the fish oils dissolved in chloroform was trans-methylated to produce FA methyl esters (FAME) using methanol–chloroform–conc. hydrochloric acid (3 mL, 10:1:1, 80 °C, 2 h) [9]. FAME were extracted into hexane–chloroform (4:1, 1.8 mL). The samples were dried on a heat block (40 °C) under a stream of nitrogen gas, and an internal injection standard (C_{19} or C_{23} FAME) added.

Samples were analysed by gas chromatography (GC) using an Agilent Technologies 7890A GC (Palo Alto, CA, USA) fitted with a Supelco Equity™-1 fused silica capillary column (15 m × 0.1 mm ID, 0.1 μm film thickness (Bellefont, PA, USA) an FID, a split/splitless injector and an Agilent Technologies 7683B Series auto sampler and injector. Helium was the carrier gas. Samples were injected in splitless mode at an oven temperature of 120 °C. After injection, the oven temperature was raised to 250 °C at 10 °C min^{-1} and finally to 270 °C at 3 °C min^{-1}. Peaks were quantified with Agilent Technologies ChemStation software (Palo Alto, CA, USA). GC-mass spectrometric (GC-MS) analyses of selected samples were performed on a Finnigan Thermoquest GCQ GC-MS fitted with an on-column injector using Thermoquest Xcalibur software (Austin, TX, USA). The GC was fitted with a capillary column of similar polarity to that described above. Individual components were identified using mass spectral data and by comparing retention time data with those obtained for authentic and laboratory standards.

3. Results

3.1. Oil Content

Lipid content of the farmed barramundi samples averaged 10% (WW) in 2002 and 8.5% in 2010 (wet weight, WW). Oil content of the farmed Atlantic salmon samples ranged from 6.7% to 14.7% (WW). The 2010 and 2011 autumn Atlantic salmon samples contained lower oil content than all other samples collected from 2010 to 2013.

3.2. Fatty Acid Composition and Content

3.2.1. Farmed Fish

Major fatty acids (FA) (as % of the total fatty acids, TFA) in farmed Australian Atlantic salmon harvested in 2002 were in decreasing order of abundance: 16:0 (18%), DHA (17%), 18:1ω9 (oleic acid, OLA; 15%), EPA (10%) and 16:1ω7 (6%) (Table 1). The three LC omega-3 PUFA—EPA + DPA + DHA accounted for 30% of the TFA.

For farmed Atlantic salmon obtained in 2010–2013, the FA profile differed both from the 2002 sample, and also over the 4 year period. Major FA in the 2010–2013 samples in decreasing order of abundance were: OLA, 16:0, 18:2ω6 (linoleic acid, LOA) and 16:1ω7 (Table 1); these four FA accounted for 57% (autumn 2010) rising to 72% of TFA in late spring 2013. In 2002, these four FA had accounted for 42% of TFA in farmed salmon. The next most abundant FA in the 2010–2013 Atlantic salmon samples were: EPA, DHA and 18:0, with all three FA decreasing over this period. LOA increased from around 2.5-fold (autumn 2010) to 4-fold (spring 2013) compared to the 2002 sampling, and the relative proportion of arachidonic acid (ARA) decreased over the 2010–2013 period.

Expressed on an absolute basis (mg/100 g serve, WW), LC omega-3 content in farmed Atlantic salmon was 2010 mg/100 g in 2002, then ranged from as high as 1770 mg/100 g (spring 2010) decreasing to 980 mg/100 g in spring 2013 (Figure 1). The ratio of omega-3 PUFA/omega-6 PUFA was 7.8 in 2002 samples, and through 2010 to 2013 showed a steady decrease from 2.6 (autumn 2010) to 0.8 (spring 2013) (Figure 2).

The FA profiles of farmed and wild caught barramundi are shown in Table 2. Major FA (as % TFA) in farmed samples from 2010 in decreasing order of abundance were: OLA, 16:0, LOA and 16:1ω7; these four FA accounted for approximately 60% of TFA in farmed barramundi. The next most abundant FA in farmed barramundi in 2010 were: EPA, DHA and 18:0. The relative level values for DHA in particular are lower than recorded for the 2002 farmed samples.

Table 1. Composition of fatty acids (as percent of total FA) in farmed Atlantic salmon (n = 3)—2002 and 2010–2013.

Fatty acid	Sample Date								
	2002	2010	2010	2011	2011	2012	2012	2013	2013
	Winter	Autumn	Spring	Autumn	Summer	Autumn	Spring	Autumn	Spring
14:0	5.5	2.1	2.7	2.8	2.5	2.4	1.8	1.9	1.7
15:0	0.5	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.2
16:4	0.3	0.7	0.6	0.5	0.5	0.3	0.2	0.3	0.2
16:3	0.4	0.8	0.6	0.6	0.5	0.4	0.2	0.4	0.3
16:1ω7c	6.2	8.3	7.0	7.4	7.2	6.4	6.8	6.9	4.9
16:0	18.0	14.4	15.1	13.9	15.7	15.4	15.5	14.7	13.0
17:1ω8c + a17:0	0.4	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.3
17:0	0.5	0.2	0.3	0.2	0.2	0.2	0.2	0.2	0.2
18:3ω6	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2
18:4ω3	3.0	1.4	1.3	1.0	1.0	0.9	0.7	0.8	0.7
18:2ω6	2.8	7.2	8.2	8.4	10.0	12.8	9.6	9.8	12.4
18:3ω3	0.0	0.9	1.1	1.1	1.0	0.7	1.1	0.9	2.1
18:1ω9c	14.5	27.0	29.2	33.8	33.8	33.0	39.0	38.8	42.7
18:1ω7c	3.3	4.0	3.9	4.0	3.5	3.1	3.5	3.4	3.4
18:1ω5c	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.1
18:0	4.2	3.9	4.4	4.2	4.1	4.5	4.5	4.5	4.0
20:4ω6	0.8	0.8	0.7	0.6	0.5	0.3	0.4	0.5	0.4
20:5ω3	9.6	8.5	7.4	5.9	4.9	4.4	3.1	3.4	2.7
20:3ω6	0.2	0.4	0.3	0.3	0.4	0.4	0.4	0.4	0.4
20:4ω3	1.5	0.9	0.8	0.7	0.6	0.5	0.4	0.5	0.5
C20PUFA	0.0	0.0	0.2	0.2	0.2	0.2	0.2	0.2	0.0
20:2ω6	0.3	0.4	0.5	0.5	0.5	0.7	0.6	0.5	0.0
20:1ω9c	1.7	1.4	1.4	1.7	1.5	1.8	1.8	1.7	1.7
20:1ω7c	0.2	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2
20:0	0.2	0.2	0.2	0.2	0.1	0.2	0.2	0.1	0.2
21:5ω3	0.6	0.4	0.4	0.3	0.3	0.3	0.2	0.2	0.1
22:5ω6	0.3	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1
22:6ω3	17.0	7.8	6.7	5.0	5.1	5.4	4.8	4.9	4.0
22:4ω6	0.1	0.2	0.1	0.2	0.1	0.1	0.1	0.1	0.1
22:5ω3	3.8	3.8	2.8	2.5	2.2	1.9	1.4	1.6	1.2
22:1ω11c	0.3	0.3	0.3	0.3	0.3	0.6	0.2	0.1	0.1
22:1ω9c	0.2	0.1	0.1	0.2	0.1	0.2	0.2	0.2	0.2
24:5ω3	0.0	0.2	0.2	0.0	0.2	0.1	0.1	0.1	0.0
24:1ω11c	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0
24:1ω9c	0.5	0.2	0.2	0.0	0.2	0.2	0.2	0.1	0.2
24:0	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0
Other	2.7	2.0	1.8	1.5	1.4	1.4	1.4	1.5	1.4
Sum	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Sum SFA	29.4	21.9	23.4	22.4	23.2	23.3	22.7	21.9	19.7
Sum MUFA	29.2	43.1	44.1	49.6	48.2	46.8	53.3	52.8	54.7
Sum Omega-3 PUFA	35.5	24.0	20.9	16.5	15.4	14.3	11.9	12.4	11.3
Sum Omega-6 PUFA	4.6	9.3	10.0	10.4	11.8	14.6	11.6	11.8	13.6

SFA, Saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. Prefix a denotes anteiso branching.

Figure 1. Content (±SD) of LC omega-3 oils (EPA + DPA + DHA, mg/100 g) in farmed Tasmanian Atlantic salmon sampled in 2002 [10] and 2010–2013.

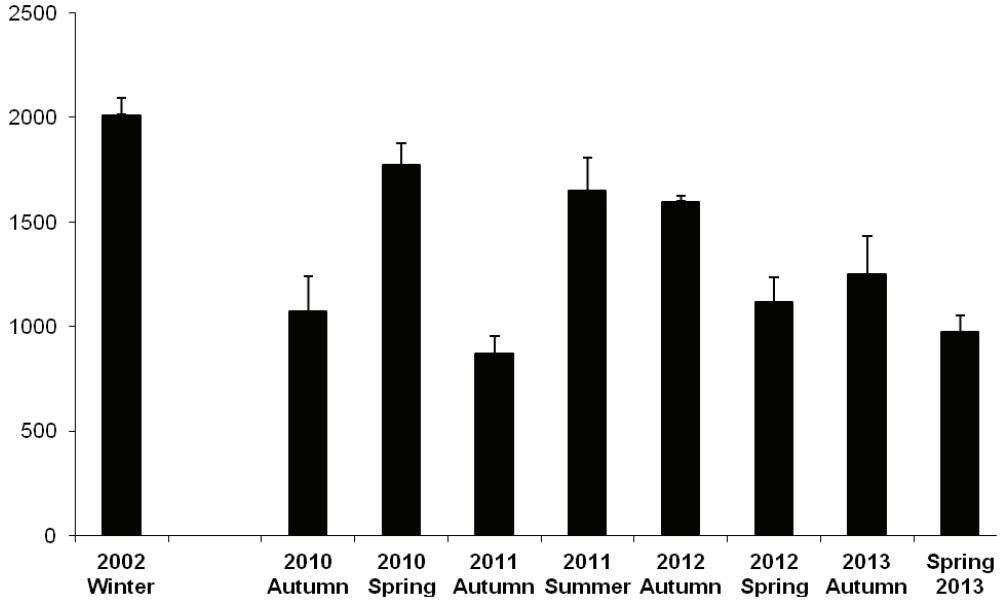
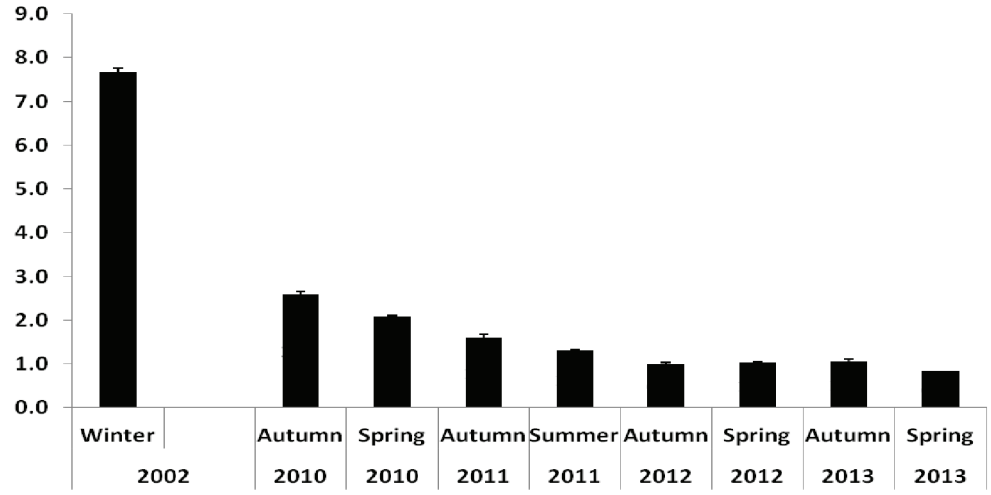


Figure 2. Ratio (±SD) of omega-3 PUFA/omega-6 PUFA in farmed Tasmanian Atlantic salmon, 2002 [10] and 2010–2013.



Major fatty acids in wild caught barramundi were 16:0, OLA, 18:0, DHA and ARA (Table 2). Less abundant components included LOA, 16:1ω7c, EPA, 18:1ω7c and DPA. Freshwater samples had higher relative levels of LOA, OLA, 16:1ω7c, 18:1ω7c, 18:3ω3 (α-linolenic acid, ALA), 14:0 and lower EPA and DHA than the saltwater specimens. The greatest difference between the wild fresh and saltwater samples for any single FA was observed for DHA (5%, freshwater; 22%, saltwater). Saltwater fish contained higher levels of LC omega-3 oils than freshwater fish. In addition to containing considerably higher relative levels of DHA, the saltwater fish contained higher relative levels of total PUFA than freshwater fish (Table 2). The relative level of ω6 PUFA was similar in freshwater barramundi and saltwater fish. In freshwater fish ARA made up 7.1% of TFA, while in the saltwater wild fish it made up 12.2% of TFA. In contrast, in farmed barramundi collected in 2002 and

2010, ARA levels were consistent at 0.6% to 0.7% and an order of magnitude lower. The ratio of ω3 PUFA/ω6 PUFA differs between salt (1.9) and freshwater (0.8) barramundi (Table 2).

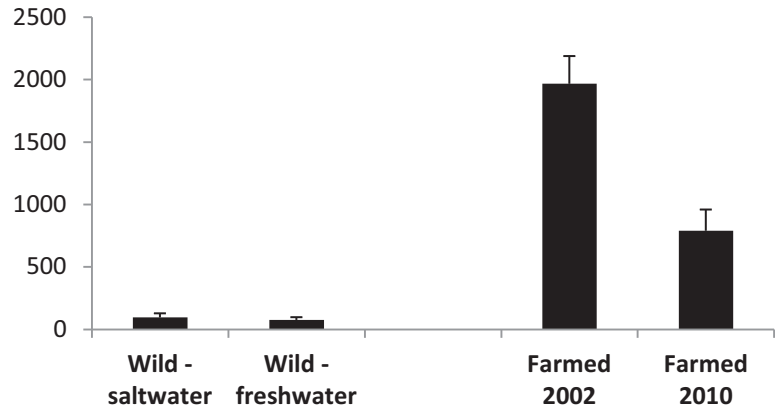
Table 2. Composition of fatty acids (as percent of total FA) in wild and farmed barramundi.

Fatty Acid	1998	1998	2002	2010
	Freshwater	Saltwater	Farmed	Farmed
14:0	2.8	0.9	5.9	3.6
15:0	0.9	0.6	0.6	0.3
16:1ω7c	7.4	2.1	6.5	7.5
16:0	27.8	22.4	19	17.7
17:1ω8c + a17:0	0.9	0.7	0.5	0.3
18:3ω6	0.4	0.3	0.3	0.3
18:4ω3	0.4	0.6	1.9	1.3
18:2ω6	4.8	1	5.5	9.8
18:3ω3	1	0.2	0	1.1
18:1ω9c	17.9	11.7	19.6	26.2
18:1ω7c	4	2.1	2.9	3.2
18:0	8.8	12.5	4.4	4.8
20:4ω6	7.1	12.2	0.6	0.7
20:5ω3	1.3	3.1	6.2	6.9
20:3ω6	0.5	0.2	0.1	0.2
20:4ω3	0.3	0	0.8	0.5
20:2ω6	0.3	0.1	0.2	0.2
20:1ω9c	0.6	0.3	3.5	1.1
20:1ω7c	0.1	0	0.3	0.2
20:0	0.3	0.2	0.2	0.3
22:5ω6	0.4	0	0.3	0.2
22:6ω3	6.1	21.6	10.2	5.4
22:4ω6	1.2	1.2	0.1	0.1
22:5ω3	1.6	2.2	2.5	2.1
22:1ω11c	0	0	1.6	0.5
22:1ω9c	0	0	0.5	0.1
24:1ω9c	0.2	0.8	0.5	0.2
Other	2.9	3	5.3	2.3
Sum SFA	42.7	39.3	31.1	27.9
Sum MUFA	31.7	17.7	37	40.7
Sum Omega-3 PUFA	11.1	28.1	21.6	17.9
Sum Omega-6 PUFA	14.5	14.9	7.2	10
Omega-3/Omega-6	0.8	1.9	3	1.55

2002 farmed barramundi [10]. 1998 and 2002 samples analysed using identical methods to 2010 fish. Wild caught barramundi data [11]. Abbreviations as used in Table 1. Prefix a denotes anteiso branching.

Absolute concentration data (mg/100 g wet weight) for the LC omega-3 oils in wild harvest barramundi are shown in Figure 3. Wild saltwater fish contained higher DHA levels. For farmed barramundi, LC omega-3 content (EPA + DPA + DHA) was markedly higher than in the wild harvest samples, in 2002, although the content decreased to 790 mg/100 g in 2010 (Figure 3).

Figure 3. Content (±SD) (mg/100 g) of LC omega-3 oils (EPA + DPA + DHA) in wild caught (1998) [11] and farmed barramundi (2002 [10], 2010).



3.2.2. Fish Oil Capsules

The capsule samples were divided into three groups of LC omega-3 containing products. The first group was those products containing elevated EPA + DHA (FO1-5, Table 3); these products contained ≥500 mg of EPA + DHA per capsule, which represents use of enrichment processes to obtain the product. One product reached this grouping largely based on its larger capsule size (Cenovis Fish Oil Plus, FO4, 1500 mg) compared with all other products. The second group of capsules (FO6-9) was the brands containing 180 mg EPA and 120 mg DHA per 1000 mg capsule. The Cenovis Fish Oil Plus (FO4) 1500 mg capsule product, when normalized to 1000 mg, contained the same DHA content as the other group 2 oils purchased, with EPA higher than for the other group 2 products. The third group (FO10-14) contained lower EPA + DHA content than group 1 and 2 oils, ranging from 52 to 160 mg per capsule.

The FA profiles of the fish oil capsule products are shown in Table 4. All fish oil capsule supplements examined generally contained EPA + DHA at levels indicated on the product labels. The five group 1 fish oils contained 41%–78% EPA+DHA. The four group 2 oils—containing 180 mg EPA + 120 mg DHA—were very similar in composition containing around 30% EPA + DHA. The group 3 oils contained 16%–25% EPA + DHA.

Other major FA present in group 2 and 3 oils included the saturated FA (SFA)—16:0, 18:0 and 20:0, the monounsaturated FA (MUFA)—OLA, 18:1ω7c and 16:1ω7c. Several group 3 oils—wild salmon oil and cod-liver oil—both contained elevated levels (~18%) of LC-MUFA—20:1ω11c (salmon oil), 20:1ω9 (cod liver oil) and 22:1ω11c (Table 4)—distinguishing these two oils readily from other group 2 and 3 oils. The group 1 PUFA-enriched oils varied in composition. The Omega Brain oil (FO2) was elevated in DHA, with the other four group 1 products (FO1, FO3, FO4, FO5) each containing EPA > DHA. Products FO3 (Healthy Care Fish Oil One a Day) and FO5 (Natures Own Omega-3 Ultra) showed similar profiles, with 32%–36% EPA and 23% DHA. The omega-3 joint product (FO1) contained the highest EPA+DHA levels (78%), with a number of other PUFA present (18%); this oil contained the highest EPA/DHA ratio (3.4), and represents a highly purified PUFA-containing oil product compared with all other products.

Table 3. Fish oil capsules—brand and composition details as supplied by manufacturers.

Brand	Description	Lab Code	Capsule Size (mg)	Number of Capsules	Cost ($)	Cost ($) per Capsule	EPA mg per Capsule	DHA mg per Capsule	EPA + DHA mg per Capsule	Number of Caps for 500 mg EPA + DHA
Group 1										
Blackmores	Omega Joint	FO1	1000	60	22.39	0.37	550	120	670	1
Blackmores	Omega Brain	FO2	1000	60	22.39	0.37	100	500	600	1
Healthy Care	Fish oil One a Day	FO3	1000	50	14.99	0.30	360	240	600	1
Cenovis	Fish oil Plus	FO4	1500	62	16.69	0.27	335	185	520	2
Natures Own	Omega-3 Ultra	FO5	1000	60	22.69	0.38	302	201	503	1
Group 2										
Cenovis	Fish oil with Omega-3	FO6	1000	180	13.69	0.08	180	120	300	2
Blackmores	Odorless Fish Oil + Vitamin D3	FO7	1000	100	12.99	0.13	180	120	300	2
Healthy Care	Fish oil	FO8	1000	400	12.99	0.03	180	120	300	2
Swisse	Odorless Fish Oil	FO9	1000	200	22.99	0.11	180	120	300	2
Group 3										
Swisse	Wild salmon oil	FO10	1000	200	22.99	0.11	80	80	160	4
Blackmores	Pregnancy	FO11	1000	120	33.99	0.28	25	125	150	4
Natures Own	Fish oil + Glucosamine	FO12	1100	90	20.99	0.23	90	60	150	4
Swisse	Wild krill oil (NKO)	FO13	333	50	43.99	0.88	50	30	80	7
Cenovis	Cod liver oil *	FO14	275	90	3.99	0.04	23	29	52	10

* EPA + DHA data not supplied on label. Fatty acid data from [11].

Table 4. Fatty acid composition (as % of total fatty acids) of fish oil capsule products.

Sample	Group 1					Group 2				Group 3				
	FO1	FO2	FO3	FO4	FO5	FO6	FO7	FO8	FO9	FO10	FO11	FO12	FO13	FO14
14:0	0.1	0.2	0.9	4.2	0.5	5.7	5.8	5.7	6.2	4.8	6.9	6.6	9.1	3.3
15:0	0.0	0.1	0.0	0.3	0.0	0.5	0.4	0.5	0.5	0.4	0.7	0.4	0.4	0.3
16:4	2.7	0.1	0.4	2.2	0.4	2.2	2.4	2.3	2.2	0.5	0.1	1.8	1.3	0.4
16:3	1.8	0.1	0.4	1.6	0.2	1.5	1.8	1.5	1.6	0.5	0.1	1.4	0.3	0.3
16:1ω7c	0.5	0.8	1.7	6.9	1.4	9.6	9.4	9.6	9.7	6.8	3.8	8.0	6.8	6.6
16:1ω5c	0.0	0.0	0.0	0.1	0.0	0.2	0.2	0.2	0.2	0.4	0.1	0.2	0.5	0.3
16:0	0.3	3.2	2.6	11.3	3.5	15.1	15.9	15.0	15.6	13.4	18.5	15.5	19.7	10.9
i17:0	0.0	0.1	0.0	0.1	0.0	0.2	0.5	0.2	0.2	0.2	0.2	0.2	0.3	0.2
17:1ω8c + a17:0	0.0	0.2	0.1	0.2	0.1	0.4	0.4	0.4	0.4	0.5	0.6	0.3	0.4	0.7
17:0	0.0	0.3	0.1	0.2	0.2	0.5	0.4	0.4	0.4	0.3	0.8	0.4	0.1	0.2
18:3ω6	0.6	0.1	0.1	0.2	0.1	0.3	0.2	0.3	0.2	0.0	0.1	0.2	0.2	0.1
18:4ω3	8.1	0.7	1.5	2.2	2.0	3.3	3.3	3.3	3.3	2.4	0.8	2.7	3.9	2.3
18:2ω6	0.2	1.1	0.9	2.5	1.6	1.4	1.1	1.6	1.4	2.5	8.3	7.8	2.1	2.2
18:3ω3	0.2	0.4	0.6	0.6	0.6	0.8	0.6	0.8	0.8	0.9	1.2	1.4	1.0	0.7
18:1ω9c	0.2	7.7	6.6	7.8	10.5	8.5	10.0	8.4	8.4	16.8	12.7	10.0	12.6	20.0
18:1ω7c	0.0	1.7	2.8	3.2	3.0	3.9	3.5	3.9	4.0	4.0	2.3	3.7	8.4	4.7
18:1ω5c	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.6	0.1	0.2	0.4	0.4
18:0	0.1	3.9	2.8	2.7	3.5	3.4	3.2	3.4	3.5	2.4	7.8	5.3	1.4	2.6
20:4ω6	1.2	2.6	0.8	1.0	0.8	0.8	0.7	0.6	0.6	0.4	1.5	0.7	0.2	0.3
20:5ω3 EPA	60.4	14.5	35.6	27.4	31.8	18.9	20.0	19.0	18.7	9.1	5.4	15.1	16.9	8.8
20:3ω6	0.2	0.3	0.4	0.3	0.4	0.3	0.3	0.3	0.2	0.1	0.1	0.2	0.0	0.1
20:4ω3	0.9	0.9	1.8	1.2	1.5	1.0	0.9	1.0	1.0	1.2	0.4	0.8	0.4	0.8
C20PUFA	0.1	0.1	0.8	0.4	0.4	0.3	0.3	0.3	0.3	0.1	0.0	0.3	0.0	0.1
20:2ω6	0.1	0.6	0.5	0.3	0.4	0.3	0.2	0.4	0.3	0.3	0.2	0.3	0.0	0.3
20:1ω11c	0.0	0.6	0.4	0.2	0.3	0.2	0.1	0.2	0.2	7.2	0.4	0.0	0.0	1.2
20:1ω9c	0.5	2.3	2.2	1.3	2.2	1.3	0.5	1.3	1.1	3.0	1.0	1.3	0.9	8.8
20:1ω7c	0.0	0.4	1.1	0.7	0.9	0.4	0.3	0.5	0.5	0.5	0.1	0.4	0.4	0.5
20:0	0.0	0.7	0.6	0.3	0.5	0.3	0.2	0.3	0.3	0.3	0.4	0.4	0.1	0.1
21:5ω3	1.5	0.6	1.4	1.1	1.6	0.7	0.8	0.7	0.8	0.4	0.2	0.6	0.5	0.4
22:5ω6	0.0	1.1	0.5	0.4	0.4	0.3	0.3	0.3	0.3	0.1	1.3	0.3	0.0	0.1
22:6ω3 DHA	17.7	46.8	23.2	13.2	23.2	11.8	10.9	11.6	11.7	6.9	18.8	9.4	8.2	10.3
22:4ω6	0.1	0.5	0.3	0.2	0.2	0.2	0.1	0.2	0.2	0.0	0.3	0.1	0.0	0.1
22:5ω3	1.5	2.9	4.5	2.9	4.3	2.3	2.1	2.3	2.2	1.8	1.2	1.8	0.4	1.2
22:1ω11c	0.0	0.6	1.6	0.9	1.0	0.7	0.1	0.7	0.6	7.6	0.5	0.5	0.0	7.5
22:1ω9c	0.0	0.4	0.4	0.2	0.3	0.2	0.1	0.1	0.2	0.7	0.1	0.1	0.5	0.4
22:0	0.0	0.4	0.2	0.2	0.2	0.1	0.3	0.1	0.1	0.1	0.2	0.2	0.0	0.1
24:6ω3	0.0	0.2	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1
24:5ω3	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.2
24:1ω9c	0.0	1.0	0.7	0.4	0.6	0.4	0.3	0.4	0.4	0.6	0.7	0.4	0.4	0.3
24:0	0.0	0.3	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.4	0.1	0.0	0.0
Sum other	1.0	1.2	0.9	1.0	1.0	1.8	2.3	1.7	1.7	2.0	1.5	0.9	1.6	2.2
Sum	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Sum EPA + DPA + DHA	79.7	64.3	63.2	43.5	59.4	33.0	33.0	32.9	32.6	17.8	25.4	26.3	25.5	20.2
Omega-3/Omega-6	16.6	9.3	11.2	4.9	9.9	6.8	7.4	6.7	7.4	4.5	2.0	1.9	6.2	5.8

Sample codes—refer to Table 3. Abbreviations as used in Table 1. Other FA (≤0.3% of total FA): 14:1ω5c, 4,8,12TMTD, i15:0, a15:0, i16:0, MBrFA:1, MBrFA, 17:1, C18PUFA, i18:0, 18:1ω7t, 18:1, 19:1, 20:1ω5c, 22:1ω7, 24:1ω11c.

4. Discussion

4.1. Farmed Fish—Oil Content and Fatty Acid Profiles

Total oil (lipid) content of farmed Atlantic salmon from 2010 to 2013 was generally similar to the oil content previously observed for Tasmanian Atlantic salmon [10]. Total lipid content in the whole body and fillet of most fish species, and barramundi are included in this regard, tends to increase with fish size [12]. In comparison, wild caught barramundi are considerably lower in lipid content (0.4% to 0.9%, WW; shoulder portion) [11]. Farmed barramundi fed a fish oil-based diet and previously analysed contained various concentrations of lipid according to study, diet and fish size and fillet section analyzed; around 10% (WW) lipid [10], 1.3% to 30% lipid [13] and 8% to 14% lipid [14].

The relative level (as % TFA) values for EPA and in particular DHA for the two farmed fish species sampled in 2010–2013 are lower than those recorded in the 2002 analyses when both species were being fed a fish oil based-diet (barramundi—EPA 6.2%, DHA 10.2%; Tasmanian Atlantic salmon—EPA 10%, DHA 17%) [10]. These 2010–2013 % composition values for EPA and DHA are also generally lower than those observed for most white muscle wild-caught fish species [11].

In addition to the reduction in EPA and DHA, the relative level of omega-6 PUFA was elevated in both farmed species sampled over the 2010–2013 period relative to 2002, due mainly to increased LOA. This leads to a dramatic shift in the ratio omega-3/omega-6 (*i.e.*, from >7:1 in 2002, to ≤1:1 currently for Atlantic salmon). A recent study [15] followed the release of an American Heart Association advisory on omega-6 fatty acids and cardiovascular risk [16] and has indicated—"advice to specifically increase omega-6 intake is unlikely to provide the intended benefits, and may actually increase the risk of CHD and death". A further change in the FA profile of the two farmed fish is a large increase in the relative level of OLA. Collectively, this profile shift is reflecting a change in diet formulation to include higher proportions of terrestrial animal and plant-based oils, with concomitant reduction in fish oil. Similar changes in salmon oil quality occur in overseas markets [17]. Also notable over the 2002–2013 period was the overall decrease in SFA which are regarded as the "least healthy" and are known precursors for endogenous cholesterol synthesis. Therefore at least one element of the current fish oil replacement strategy could be argued as improving the nutritional quality of farmed fish. It is the absolute content of the LC omega-3 that is the most critical issue in terms of the contribution that farmed fish make to the human diet. On an absolute (mg/100 g serve) basis, the LC omega-3 content values observed for farmed Atlantic salmon and barramundi sampled in 2010–2013 are considerably higher than those found in most wild caught fish species [10,11], although the values from both species are lower than previously reported a decade ago in 2002 for these two farmed species fed a fish oil-based diet [10]. Large differences were observed between the autumn and spring/summer samples in 2010 and 2011. Tasmanian waters varied in water temperature through this period, together with the presence and degree of disease and other biological factors; these may be contributing factors to the observed differences.

Wild harvest barramundi contained considerably lower absolute amounts of PUFA than the famed samples (Figure 3). However, a low oil content combined with similar high relative levels of omega-3 in saltwater fish and both ω3 and ω6 PUFA in freshwater fish are nutritionally attractive features of the wild-harvest barramundi. The overall FA profile and lower LC omega-3 content of the wild specimens

provide no major nutritional advantage in terms of these key components. Both wild specimens had an omega-3/omega-6 ratio similar to or poorer that that found in farmed barramundi.

Until about 10 years ago Australian and New Zealand fish farms were largely using feeds made with high inclusion of fishery products (fish meals and fish oils). Prior to this period, there was evidence that LC omega-3 content in farmed fish products remained high [2,3,10]. Increasing demand for fishery resources, such as fish oil, at a time where there is limited ability or capacity to increase sustainable harvest of wild fish stocks, has resulted in increased use of oils derived from non-traditional sources [7]. Oils now being routinely used include those from land plants (e.g., canola and palm oil) and rendered oils (e.g., poultry oil) [6]. The problem with the use of these oils is that they result in flesh with: (i) lower relative levels of LC omega-3 (as % of TFA); (ii) lower absolute content of the LC omega-3; and (iii) lower omega-3/omega-6 ratio. As the relative levels of these key LC omega-3 oils decrease, LOA and OLA increase. OLA and LOA are derived from the non-marine ingredients that are being increasingly substituted into aquafeeds, although most fish also have significant capacity to synthesis their own OLA from both lipid and non-lipid substrates [4].

Interest has existed in Australia and New Zealand on comparison of the LC omega-3 oil content of salmon farmed in the two locations. In Tasmania, the species farmed is Atlantic salmon (*Salmo salar*), whilst in New Zealand the predominant species is Chinook salmon (*Oncorrhynchus tshawytscha*, also termed king salmon). LC omega-3 content of the two species sampled at a similar time (spring 2012) showed Atlantic salmon containing 1117 ± 117 mg/100 g LC omega-3 with Chinook salmon at 2568 ± 153 mg/100 g. The two species generally receive similar diets [18], and the differences in LC omega-3 content (and similarly for the SFA and omega-6 PUFA) result largely from the higher oil content of the fillet of farmed Chinook salmon (~25% oil content cf 10%–15% in Atlantic salmon).

Atlantic salmon and barramundi have, when fed a FO-containing diet, provided an excellent source of beneficial omega-3 LC-PUFA for human consumption, but reduced concentrations of these nutrients, as occurs through the use of vegetable oil and/or animal fat diets, may reduce their nutritional benefit to consumers. Limited research has been performed to examine this issue. In one study [19], dietary intake of differently fed salmon (100% fish oil (FO), 50/50 FO/rapeseed oil, 100% rapeseed oil) and the influence on markers of human atherosclerosis were compared. Significant differences between the human consumer groups were observed in the serum fatty acid profiles, especially for the levels of total omega-3 PUFA and the omega-3/omega-6 ratio, which were markedly increased in the FO-fed fish consuming group in contrast to the two other groups. In addition, significant reductions of serum TAG and of vascular cell adhesion molecule-1 and interleukin-6 were observed in patients receiving the FO-fed salmon diet when compared with the two other groups. The authors concluded that Atlantic salmon fed the FO-containing diet containing very high concentrations of omega-3 LC-PUFA seemed to produce favourable biochemical changes in patients with coronary heart disease risk factors when compared with ingestion of fillets with intermediate and low levels of the marine omega-3 LC-PUFA, where FO was replaced in part or in full by rapeseed oil [19]. To our knowledge, there have been no consumer trials with fish fed diets containing LOA, ALA and/or SDA rich oils *versus* FO derived EPA + DHA, and looking at the effects on consumers.

A recent study tested whether Atlantic salmon smolt fed a diet with a higher DHA/EPA ratio and a lower content of LC omega-3 oils to that of conventional FO based diets would enhance deposition of LC omega-3 in the liver and muscle [20]. Comparisons were made between fish fed: (1) a FO diet; (2) a blend of 50% rapeseed and 50% tuna oil diet (termed model oil, MO1); (3) a blend of 50% rapeseed, 25% tuna and 25% FO diet (MO2); and (4) a blend of 50% FO and 50% poultry oil diet (FO/PO). The latter diet was representative of commercial diets in use in Australia at the time of the study, with the proportion of chicken fat increasing even further since the study was performed. The dietary DHA/EPA ratio was in the order MO1 > MO2 > FO/PO ~ FO. The LC omega-3 content was approximately 2-fold lower in the MO1, MO2 and FO/PO diets compared to the FO diet, with the relative levels (as % total FA) lowest in the MO1 diet. For the feeding trial, there were comparable contents of LC omega-3 in the muscle of the FO, MO 1 and FO/PO fed fish [20].

A major outcome for the feeding trial was the observation that a higher DHA/EPA ratio than that commonly occurring with FO-only diets used for Atlantic salmon was better suited for more efficient deposition of LC omega-3 in the flesh, in particular DHA. Evidence was therefore apparent for LC omega-3 "sparing" in the Atlantic salmon smolt fed a diet with a high DHA/EPA ratio [20]. The use of a 50% FO and 50% PO blend in aquafeeds for Atlantic salmon, as was in the range commercially practiced in Australia in 2010, resulted in comparable LC omega-3 content in the muscle [20] and liver of juvenile Atlantic salmon to a FO fed fish. It is noteworthy that such an oil blend decreases the inefficient utilization of a 100% FO diet, due to the high loss of EPA in particular, and may be considered as an appropriate current strategy, in terms of LC omega-3 sparing, for present use in aquafeeds for Atlantic salmon [20]. The reduction of FO incorporation in the aquafeeds has also enhanced the sustainability of the industry, although sufficient FO still remains in the feeds used to ensure that farmed Tasmanian Atlantic salmon remains one of the best sources of the LC omega-3 oils available to Australian consumers.

It is important to note that in spite of changes that have occurred in feeding practices, and the resulting lower content of LC omega-3 oils, the scope remains for the potential future use of new alternate sources of LC omega-3 to restore the content of these health-benefitting ingredients to those higher contents previously seen.

Further research is needed to determine the optimum relative and absolute concentrations of dietary EPA and DHA to enhance their deposition in larger-sized commercially farmed Atlantic salmon. The rationale to pursue such studies is supported by recent developments in plant genomics. As this research field has progressed, important breakthrough steps have included: the isolation and characterization of genes from the marine microalgae encoding front-end desaturases involved in DHA biosynthesis, the isolation of highly efficient desaturases and elongases, the use of genes with omega-3 substrate preference and the development and use of a land plant (tobacco) leaf-based assay using interchangeable design principles to rapidly assemble multistep recombinant pathways [21–23]. Progress with research on insertion of microalgal-derived genes leading to DHA production into a range of omega-3 C_{18} PUFA accumulating land plants has been reviewed [24–26].

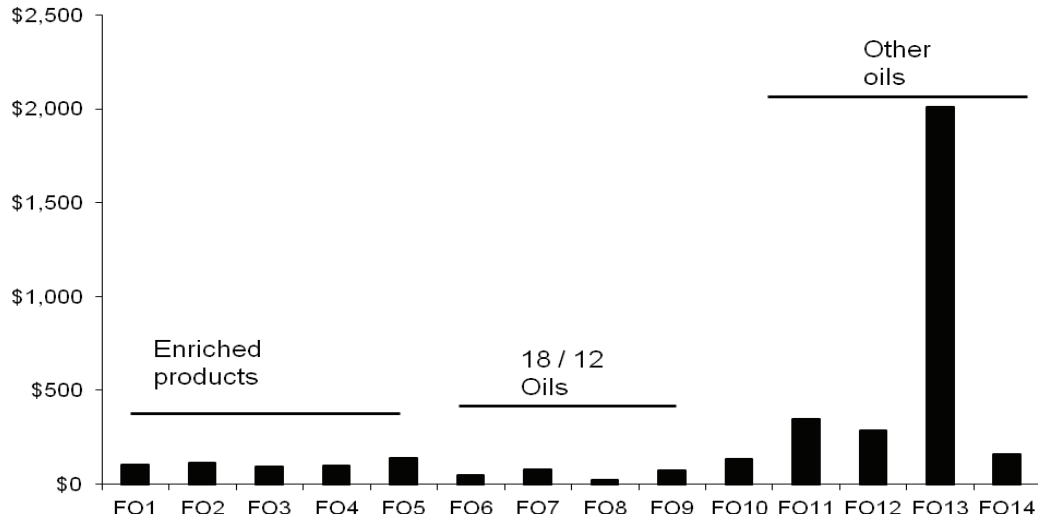
Recent developments have resulted in oilseeds containing elevated LC omega-3 oils, including with fish oil-like proportions of DHA and an elevated omega-3/omega-6 ratio (2–5) [22,23,27]. These major breakthroughs can in the future provide the feed and aquaculture industries with an opportunity to both sustainably farm this key protein source for the growing global population, and enhance

the nutritional quality of farmed seafood products in terms of the health-benefitting LC omega-3 oils. Alternate feed-grade oils containing the desired FA profiles, in particular including a high DHA/EPA ratio, are not presently available. However, a realistic examination of the future steps required suggests that they can be a commercial reality by the end of this decade [22,23].

4.2. Fish Oil Capsules as Sources of EPA + DHA

All products generally contained EPA + DHA at levels indicated on the product labels. The cost per 500 mg EPA + DHA (value generally advised for daily consumption by many nutritional and health groups [2]) varied markedly from $0.05 to $5.50 (Table 3), or from $20 to $2000 per annum (Figure 4). The lowest cost products, based on EPA + DHA levels alone, were those oils containing the conventional 180 mg EPA and 120 mg DHA per 1000 mg capsule (group 2). In the group 2 products, a four-fold cost difference occurred ($20–$79 pa, Table 3), although product specifications were identical. Consumers can have difficulties in compliance (e.g., consumption of multiple large capsules), and may prefer the range of enriched products (group 1) now available. The most expensive product based on EPA + DHA content was the krill oil, which was six-fold more expensive than the next most costly product.

Figure 4. Cost per annum of fish oil products to supply 500 mg/day EPA + DHA. Sample codes refer to brands shown in Table 3. Enriched products—denotes group 1 brands containing concentrated EPA and/or DHA. 18/12 oils denotes—group 2 standard fish oils containing 180 mg/120 mg of EPA and DHA respectively. Other oils denotes—group 3 products containing varying proportions of EPA and DHA and may contain other components.



5. Conclusions

In summary, in comparison to 2002 samples of two major farmed Australian finfish species, Atlantic salmon and barramundi sampled in 2010–2013 have been shown to contain decreased relative levels and content of LC omega-3 oils (from 2014 mg/100 g in 2002 decreasing to 975 mg/100 g in spring 2013 for Atlantic salmon; 1970 mg/100 g in 2002 decreasing to 790 mg/100 g per serve for barramundi). These changes have resulted from the use of new, lower cost and sustainable ingredients

in farmed fish feed, necessitated by the inability of existing supplies together with the increasing cost of the wild harvest fish oil resource to meet the expanding needs of the aquaculture industry. Notwithstanding, these two widely available farmed fish species still remain an excellent source of the LC omega-3 oils, and in a broader context remain one of the best of all foods available for Australian consumers. All fish oil capsule supplements examined generally contained EPA + DHA at levels indicated on the product labels and, similar to the farmed seafood, such products can also represent a viable alternative source of LC omega-3 oils for consumers.

Subject to consumer demand, one of the cost-effective strategies to increase the omega-3 content of the lipid profile of farmed fish could be to include enhanced levels of omega-3 rich vegetable oils, and/or finishing diets with higher inclusions of marine oils [6]; such a strategy may result in grades of farmed seafood product being available for purchase that contain higher content of LC omega-3 than standard products grown using a predominately non-marine oil based diet. In the longer term, other strategies to consider include: selection for enhanced delta-5 and delta-6 desaturase activities (the key enzymes converting shorter chain omega-3 to EPA and DHA) and the use of alternate long-chain omega-3 oil sources, including new oil seeds containing EPA and DHA.

Acknowledgments

The authors thank the CSIRO Food Futures Flagship Omega-3 research team for their contributions. 1998 and 2002 seafood data was produced by the CSIRO Marine Oils groups, in particular Ben Mooney, Nick Elliott and Patti Virtue, with funding support from the Fisheries Research and Development Corporation. We also thank Marine Produce Australia Pty Ltd for supplying the 2010 farmed barramundi samples, Matt Miller of NZ Plant and Food for providing NZ King salmon data, and Nick Elliott, Peter Kube and Carol Mancuso Nichols of CSIRO and two anonymous journal reviewers for valuable comments on the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Characterization of Oilseed Lipids from "DHA-Producing *Camelina sativa*": A New Transformed Land Plant Containing Long-Chain Omega-3 Oils

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Abstract: New and sustainable sources of long-chain (LC, $\geq C_{20}$) omega-3 oils containing DHA (docosahexaenoic acid, 22:6ω3) are required to meet increasing demands. The lipid content of the oilseed of a novel transgenic, DHA-producing land plant, *Camelina sativa*, containing microalgal genes able to produce LC omega-3 oils, contained 36% lipid by weight with triacylglycerols (TAG) as the major lipid class in hexane extracts (96% of total lipid). Subsequent chloroform-methanol (CM) extraction recovered further lipid (~50% polar lipid, comprising glycolipids and phospholipids) and residual TAG. The main phospholipid species were phosphatidyl choline and phosphatidyl ethanolamine. The % DHA was: 6.8% (of total fatty acids) in the TAG-rich hexane extract and 4.2% in the polar lipid-rich CM extract. The relative level of ALA (α-linolenic acid, 18:3ω3) in DHA-camelina seed was higher than the control. Major sterols in both DHA- and control camelina seeds were: sitosterol, campesterol, cholesterol, brassicasterol and isofucosterol. C_{16}–C_{22} fatty alcohols, including iso-branched and odd-chain alcohols were present, including high levels of iso-17:0, 17:0 and 19:0. Other alcohols present were: 16:0, iso-18:0, 18:0 and 18:1 and the proportions varied between the hexane and CM extracts. These iso-branched odd-chain fatty alcohols, to our knowledge, have not been previously reported. These components may be derived from wax esters, or free fatty alcohols.

Reprinted from *Nutrients*. Cite as: Mansour, M.P.; Shrestha, P.; Belide, S.; Petrie, J.R.; Nichols, P.D.; Singh, S.P. Characterization of Oilseed Lipids from "DHA-Producing *Camelina sativa*": A New Transformed Land Plant Containing Long-Chain Omega-3 Oils. *Nutrients* **2014**, *6*, 776–789.

1. Introduction

There are many beneficial health effects in humans of omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (omega-3 LC-PUFA, also termed LC omega-3 oils). The major bioactive LC omega-3 are EPA (eicosapentaenoic acid, 20:5ω3) and DHA docosahexaenoic acid, 22:6ω3). These are largely obtained through dietary consumption of seafood, primarily fish and fish oil nutraceuticals. The positive effects are across a range of degenerative and inflammatory disorders such as: heart disease, stroke, rheumatoid arthritis, asthma and some cancers, diabetes mellitus, multiple sclerosis, dementia and clinical depression [1–3]. LC omega-3 oils are also important in infant nutrition and are present in high concentrations in brain and retina and are important in the development, health and correct functioning of these organs [4,5]. They are also nutritionally important for the survival, growth and general health of aquaculture species particularly at the larval stage [6]. Highly purified or enriched LC omega-3 oils are also sought after for their bioactive function as potential pharmaceutical products [7].

Future supplies of fish oil derived LC omega-3 oils from fisheries is unlikely to be able to meet increasing demands for their inclusion in aquafeeds, foods, nutraceuticals, or for enrichment to highly

purified EPA and DHA, as pharmaceutical bioactives and other products [8,9]. Hence, there is a need to develop new and sustainable sources to supplement oils extracted from wild fish harvests.

It has been recognized that marine fish do not have the capacity to synthesize these oils, rather it is microalgae, at the base of the marine food web, that have the ability to produce these oils [10,11]. Microalgae contain the genes for the various elongase and desaturase enzymes that are responsible for the synthesis of LC omega-3 oils. Members from the brown algal line, including for example the algal class dinoflagellates in particular, produce high proportions of DHA [12–15]. These health-benefitting oils are then accumulated up the food chain into various seafoods, including shellfish and finfish.

The search for new and sustainable sources of LC omega-3 oils has led researchers to attempt to transform land plants with microalgal LC omega-3 genes. As a result, a suite of microalgal genes have successfully been incorporated into a variety of land plants including tobacco leaf [16] and *Arabidopsis* seed [17,18] as a proof of concept, showing that transformed terrestrial plants can serve as an alternative supply of these oils. A little known oilseed plant, *Camelina sativa*, a member of the Cruciferae (Brassicaceae) family is an ancient plant native to Europe and Central Asian areas. It is cultivated as an oilseed crop and used in animal feed mainly in Europe and North America and is known as camelina, gold-of-pleasure or false flax. It has several unique and desirable features which give it a competitive advantage over other commercial oilseed crops such as canola, soybean, and sunflower [19]. It grows well in semiarid, marginal and saline soils and unlike commercial oilseed crops, does not have high nutrient requirements, can tolerate insects and weeds and survive winter sowing and frost and freeze-thaw cycles after emergence during late winter and spring. *Camelina sativa* seeds can produce up to 40% oil containing high proportions of α-linolenic acid (ALA, 28%) and linoleic acid (LA, 19%) [19], which makes this plant a good candidate for transformation into a LC omega-3 oil producing land plant. A new DHA-producing *Camelina sativa* oilseed plant transformed with a suite of microalgal LC omega-3 genes sourced from several target microalgal strains has been recently reported by Petrie *et al*. [20]. The synthesis of DHA occurs by the elongation and desaturation of C_{18} PUFA through EPA and not by retro-conversion of 24:6ω3. It was also determined by ^{13}C NMR regiospecificity analysis of the TAG-containing oil that DHA is preferentially esterified at the *sn*-1,3 positions of the TAG molecule [20]. Here for the first time we present the lipid class, fatty acid, sterol and fatty alcohol composition of this new transformed DHA-producing *Camelina sativa* oilseed (hereafter termed DHA camelina) and carry out a descriptive analysis with reference to an unmodified *Camelina sativa* control seeds.

2. Experimental Section

2.1. Lipid Extraction

Details of the *Camelina sativa* seed, including: source, the full details of the gene construct inserted, which is composed of a series of elongase and desaturase genes, the metabolic pathway and methods used for the transformation to form the transgenic DHA-producing camelina oilseed event used in this study has been reported by Petrie *et al*. [20]. We used hexane as the extracting solvent since it is the industry standard and it preferentially extracts TAG-containing oil due to its solvating properties and very poor solubilization of polar lipids, particularly at room temperature. We did not use Soxhlet

extraction so as to minimize any potential degradation of DHA due to heating for prolonged periods during reflux. No antioxidants were added during extraction or analysis. Subsequent extractions of the hexane-extracted crushed seed using chloroform-methanol were used to exhaustively recover residual TAG and un-extracted polar lipids to determine the effectiveness of extraction by hexane of the TAG-containing oil from the crushed seed. Transformed and control camelina seeds (130 g and 30 g, respectively) were wetted with hexane and crushed using an electric agate mortar and pestle (Retsch Muhle, Germany), transferred to a separatory funnel and extracted four times using a total of 800 mL hexane including an overnight third extraction. For each extraction, extracts were filtered to remove fines through a GFC glass fiber filter, and then rotary evaporated at 40 °C. The extracts were pooled and constituted the TAG-rich hexane extract.

Following extraction with hexane, the remaining meal was further extracted using chloroform-methanol (CM 1:1 v/v) as above, the meal was then removed by filtration and the combined extracts rotary evaporated. The pooled CM total crude extract was then dissolved using a modified Bligh and Dyer [21] one-phase methanol-chloroform-water mix (2:1:0.8 v/v/v). The phases were separated by the addition of chloroform-water (final solvent ratio, 1:1:0.9 v/v/v methanol-chloroform-water). The purified lipid was partitioned in the lower chloroform phase, concentrated using rotary evaporation and constituted the polar lipid-rich CM extract.

2.2. Lipid Class Analysis

Lipid classes of the hexane and CM extracts were analyzed by thin-layer chromatography with flame-ionization detection (TLC-FID; Iatroscan Mark V, Iatron Laboratories, Tokyo, Japan) [12] using hexane/diethyl ether/glacial acetic acid (70:10:0.1, v/v/v) as the developing solvent system in combination with Chromarod S-III silica on quartz rods and suitable calibration curves of representative standards obtained from Nu-Chek Prep, Inc. (Elysian, MN, USA). Data was processed using SIC-480II software (SISC Version: 7.0-E). Phospholipid species were separated by applying the purified phospholipid fraction (Section 2.3) obtained from silica column chromatography and developing the rods in chloroform/methanol/glacial acetic acid/water (85:17:5:2, v/v/v) prior to FID detection.

2.3. Separation of TAG, Glycolipid and Phospholipid Fractions from the CM Extracts

Silica gel 60 (100–200 mesh) (0.3–1 g) in a short glass column or Pasteur pipette plugged with glass wool was used to purify 10 mg of the purified CM lipid extract. The residual TAG fraction in the CM extract was eluted using 20 mL of 10% diethyl ether in hexane, the glycolipids eluted with 20 mL of acetone and the phospholipids eluted in two steps, first 10 mL of methanol then 10 mL of methanol-chloroform-water (5:3:2). This second elution was shown to increase the recovery of phospholipids [22]. The yield of each fraction was determined gravimetrically and the purity checked by TLC-FID. All extracts and fractions were stored in dichloromethane at −20 °C until further analysis by GC and GC-MS.

2.4. Fatty Acid Methyl Ester Preparation

Aliquots of the hexane and CM extracts were *trans*-methylated according to the method of Christie [23] to produce FA methyl esters (FAME) using methanol–chloroform–conc. hydrochloric acid (3 mL, 10:1:1, 80 °C, 2 h). FAME were extracted into hexane–chloroform (4:1, 3 × 1.8 mL). The meal (after hexane and CM extraction) was also *trans*-methylated and the value for total lipid was determined by adding the lipid contents of the hexane and CM extracts and the FAME content of the transmethylated meal after solvent extraction**.**

2.5. Sterol and Fatty Alcohol Derivatization

Samples (approximately 10 mg) from the TAG-rich hexane extract and the polar lipid-rich CM extract were saponified separately using 4 mL 5% KOH in 80% MeOH and heated for 2 h at 80 °C in a Teflon-lined screw-capped glass test tube. After the reaction mixture was cooled, 2 mL of Milli-Q water was added and the sterols and alcohols were extracted into 2 mL of hexane: dichloromethane (4:1, v/v, 3×) by shaking and vortexing. The mixture was centrifuged and the extract in the organic phase was washed with 2 mL of Milli-Q water by shaking and centrifugation. After taking off the top sterol containing organic layer the solvent was evaporated using a stream of nitrogen gas and the sterols and alcohols silylated using 200 µL of Bis(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma-Aldrich) and heating for 2 h at 80 °C in a sealed GC vial; free hydroxyl groups were converted to their trimethylsilyl ethers.

2.6. GC and GC-MS Analysis

The sterol- and alcohol-OTMSi derivatives were dried under a stream of nitrogen gas on a heating block (40 °C) and re-dissolved in dichloromethane (DCM) immediately prior to GC/GC-MS analysis. The FAME and alcohol/sterol-OTMSi derivatives were analyzed by gas chromatography (GC) using an Agilent Technologies 6890A GC (Palo Alto, CA, USA) fitted with a Supelco Equity™-1 (Bellefont, PA, USA) fused silica capillary column (15 m × 0.1 mm i.d., 0.1 µm film thickness), an FID, a split/splitless injector and an Agilent Technologies 7683B Series auto sampler and injector. Helium was the carrier gas. Samples were injected in splitless mode at an oven temperature of 120 °C. After injection, the oven temperature was raised to 270 °C at 10 °C min^{-1} and finally to 300 °C at 5 °C min^{-1}. Eluted compounds were quantified with Agilent Technologies ChemStation software (Palo Alto, CA, USA). GC results are subject to an error of ±5% of individual component area.

GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Trace ultra Quadrupole GC-MS (model: ThermoQuest Trace DSQ, Thermo Electron Corporation). Data was processed with ThermoQuest Xcalibur software (Austin, TX, USA). The GC was fitted with an on-column injector and a capillary HP-5 Ultra Agilient J & W column (50 m × 0.32 mm i.d., 0.17 µm film thickness, Agilent Technologies (Santa Clara, CA, USA) of similar polarity to that described above. Individual components were identified using mass spectral data and by comparing retention time data with those obtained for authentic and laboratory standards. A full procedural blank analysis was performed concurrent to the sample batch.

3. Results

3.1. Total Lipid Content

The "DHA-producing camelina" seeds analyzed here contained slightly less total lipid (36% of seed wt.) than control wild-type *Camelina* (41% of seed wt.).

Of the total lipid, 31%–38% of lipid per seed weight was extracted by hexane for DHA- and control camelina, which accounted for 86% and 92% of total lipid, respectively (Table 1). The chloroform-methanol extraction post hexane extraction recovered a further 4.8% and 2.4% polar lipid-rich extract from the DHA- and control camelina, respectively and the residual lipid released by transmethylation of the remaining solvent extracted oilseed meal was 0.3% and 0.4% of seed weight, respectively.

Table 1. Lipid content (as % of seed weight) of DHA- and control *Camelina sativa* seeds after hexane extraction, post hexane chloroform-methanol (CM) extraction and subsequent transmethylation of the extracted meal.

Extract	DHA camelina	Control camelina
hexane	31.1	38.1
chloroform-methanol [1]	4.8	2.4
residual lipid [2]	0.3	0.4
Total lipid	36.2	40.9

[1] Polar lipid rich extract containing glycolipid and phospholipid with some residual TAG obtained by CM extraction after hexane extraction of the meal; [2] Residual lipid (FAME) from transmethylated meal after hexane and CM extractions.

3.2. Lipid Class Analysis

The TAG-rich hexane extract (Section 2.1) contained 96% TAG in both DHA- and control *Camelina*. The post hexane chloroform-methanol extraction recovered residual TAG amounting to 44% and 13% (of the CM extract), respectively. The chloroform-methanol extracts were rich in polar lipids (glycolipids and phospholipids) amounting to 50% and 76% (of the CM extract) for the DHA- and control camelina, respectively (Table 2). The main phospholipid was phosphatidyl choline (PC) and accounted for 70%–79% of the total phospholipids followed by phosphatidyl ethanolamine (PE, 7%–13%) with smaller relative levels of phosphatidic acid (PA, 2%–5%) and phosphatidyl serine (PS, <2%). There were several other unidentified components separated in the phospholipid fraction (Table 3).

Table 2. Lipid class composition (% of total lipid obtained for each extraction step) of Hexane and CM extracts from DHA- and control *Camelina sativa* seeds.

Lipid class	DHA camelina		Control camelina	
	Hexane	Chloroform-methanol	Hexane	Chloroform-methanol
SE/WE/HC [1]	1.0	1.4	1.0	1.4
TAG	95.6	44.2	96.0	13.1
FFA	0.9	1.3	0.8	1.4
UN [2]	0.9	1.1	0.8	1.2
ST	0.5	0.7	0.4	0.4
MAG	0.7	1.1	0.8	6.2
PL	0.3	50.3	0.3	76.3
Total	100.0	100.0	100.0	100.0

Abbreviations: sterol esters (SE), wax esters (WE), hydrocarbons (HC), triacylglycerols (TAG), free fatty acids (FFA), unknown (UN), sterols (ST), monoacylglycerols (MAG), polar lipids (PL) consisting of glycolipids and phospholipids; [1] SE, WE and HC coelute with this system; [2] May contain fatty alcohols and diacylglycerols (DAG).

Table 3. Phospholipid composition (% of total phospholipids) of CM extracts from DHA- and control camelina seeds.

Phospholipid	DHA camelina	Control camelina
PA	2.1	4.7
UN 1	5.7	2.2
UN 2	-	1.1
UN 3	-	0.6
PE	13.2	6.8
PS	1.2	1.4
PC [1] + PI	69.5	78.9
UN 4	4.8	3.6
UN 5	3.4	1.6
Total	100.0	100.0

Abbreviations: Unknown (UN), phosphatidic acid (PA), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), phosphatidyl choline (PC), phosphatidyl inositol (PI); [1] PC is the major component and PI coelutes with PC.

Table 4. Fatty acid composition (% of total fatty acids) of various lipid extracts and fractions of DHA- and control *Camelina sativa*.

	DHA camelina						Control camelina					
	Hexane	Chloroform-methanol				Meal	Hexane	Chloroform-methanol				Meal
Fatty acid	TAG [1]	Total [2]	TAG [3]	GL [3]	PL [3]	Residue [4]	TAG [1]	Total [2]	TAG [3]	GL [3]	PL [3]	Residue [4]
16:1ω7	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.2	-	-	0.3
16:0	6.2	12.8	6.8	21.3	19.4	10.4	6.7	12.8	7.8	29.6	13.7	10.3
18:4ω3	3.7	3.3	3.4	2.1	2.9	3.6	-	-	-	-	-	-
18:2ω6	7.1	3.9	8.8	7.2	3.7	8.8	22.2	28.4	29.4	20.8	29.3	27.9
18:3ω3	41.9	50.3	39.9	38.6	54.1	38.9	32.0	20.6	19.7	13.0	12.3	20.0
18:1ω9	11.1	4.7	9.6	7.2	2.8	8.1	14.0	25.4	13.3	14.7	35.7	14.3
18:1ω7	1.4	2.3	2.1	3.7	3.4	2.8	1.0	1.5	2.2	4.0	2.8	2.2
18:0	3.2	4.0	3.0	4.5	5.7	3.1	3.0	2.7	2.9	5.7	3.6	2.7
20:5ω3	0.4	0.2	0.3	-	-	0.3	-	-	-	-	-	-
20:4ω3	0.4	0.4	0.4	-	0.2	0.3	-	-	-	-	-	-
20:2ω6	0.7	0.7	0.8	0.6	0.4	0.7	1.8	0.8	2.1	1.2	-	1.8
20:3ω3	0.8	1.2	0.9	0.6	1.3	0.5	0.9	0.3	-	-	-	0.4
20:1ω9/11	11.6	6.1	10.9	5.1	1.3	8.4	12.5	3.0	11.1	4.2	1.7	9.4
20:1ω7	0.6	0.8	1.4	0.6	0.2	1.1	0.6	0.6	2.6	1.3	-	1.8
20:0	1.3	0.8	1.4	0.6	0.1	1.4	1.5	0.7	2.3	1.4	-	1.8
22:6ω3	6.8	4.2	6.1	3.0	1.6	5.4	-	-	-	-	-	-
22:5ω3	0.3	1.1	0.4	0.6	1.4	0.3	-	-	-	-	-	-
22:1ω9	1.3	1.0	1.8	0.6	0.1	1.5	2.7	0.7	3.6	0.9	-	2.9
22:0	0.3	0.2	0.3	0.6	0.1	0.7	0.3	0.2	0.7	0.8	-	0.8
24:1ω9	0.3	0.4	0.4	0.6	0.3	0.6	0.3	0.6	0.7	0.9	0.5	1.0
24:0	0.1	0.4	0.2	0.9	0.4	1.1	0.1	0.4	0.5	1.4	0.4	1.3
others [5]	0.4	1.0	1.0	1.4	0.5	1.8	0.3	1.1	0.9	0.1	-	1.1
Total	100	100	100	100	100	100	100	100	100	100	100	100

Abbreviations: triacylglycerols (TAG), glycolipids (GL), phospholipids (PL); [1] TAG-rich hexane extract; [2] Total polar lipid-rich extract containing GL and PL from chloroform-methanol (CM) extraction post hexane extraction; [3] TAG, GL and PL from CM extraction separated by silica column chromatography; [4] Residual fatty acids released by transmethylation of camelina meal post hexane and CM extraction; [5] Sum of minor fatty acids.

3.3. Fatty Acid Composition

Generally we have found that total seed fatty acid composition obtained by direct transmethylation of whole seed is very similar to that of the TAG fraction, since the bulk of the lipids present in the seed occur in the form of TAG, hence total seed fatty acid composition is not reported here. In DHA camelina, the DHA was distributed in the major lipid fractions (TAG, phospholipids and glycolipids) and the proportion ranged between 1.6% and 6.8% with an inverse relationship between the proportions of DHA and ALA. The TAG-rich hexane extract of the DHA camelina contained 6.8% DHA and 41% ALA (Table 4). The polar lipid-rich chloroform-methanol extract contained 4.2% DHA and 50% ALA. Residual TAG from the polar lipid-rich chloroform-methanol extract contained 6% DHA and 40% ALA. The glycolipid fraction isolated from the chloroform-methanol extract contained 3% DHA and 39% ALA and the phospholipid fraction contained the lowest level of DHA (1.6%) and the highest levels of ALA (54%). The DHA camelina contained higher levels of ALA and lower levels of LA (linoleic acid, 18:2ω6) compared with the control camelina in the major lipid classes (TAG, glycolipids and phospholipids). The proportions of ALA and LA for DHA- and control camelina were: ALA 39%–54% and LA 4%–9% for DHA camelina and ALA 12%–32% and LA 20%–29% for control camelina. The relative level of eurucic acid (22:1ω9) was lower in all fractions in the DHA camelina than in the control (e.g., hexane extracts–1.3% *versus* 2.7%; Table 4).

3.4. Sterol Composition

The major sterols in both DHA- and control camelina were: 24-ethylcholesterol (sitosterol, 43%–54%), 24-methylcholesterol (campesterol, 20%–26%) with lower levels of cholesterol (5%–8%), brassicasterol (2%–7%), isofucosterol (Δ5-avenasterol) (4%–6%), stigmasterol (0.5%–3%), cholest-7-en-3β-ol, (0.2%–0.5%), 24-methylcholestanol (campestanol, 0.4%–1%) and 24-dehydrocholesterol (0.5%–2%) (Table 5). These nine sterols accounted for 86%–95% of the total sterols, with the remaining components being unconfirmed sterols with partial identification obtained–including the number of carbons and double bonds.

The overall sterol profiles were similar between DHA- and control camelina for both the hexane and chloroform-methanol extracts, although there were slightly higher levels of unknown sterols in the chloroform-methanol extracts of both the DHA- and control camelina than occurred in the hexane extracts (10%–14% and 4%–7%, respectively).

Table 5. Sterol composition (% of total sterols) of DHA- and control camelina.

	DHA camelina		Control camelina	
Sterols	**Hexane**	**CM [1]**	**Hexane**	**CM [1]**
24-dehydrocholesterol	0.8	1.8	0.5	1.4
cholesterol	5.7	7.6	4.7	7.2
brassicasterol	4.4	6.5	1.9	4.2
cholest-7-en-3β-ol	0.2	0.5	0.3	0.4
campesterol	24.5	20.8	25.7	21.7
campestanol	0.4	1.1	0.4	0.9
stigmasterol	1.0	2.6	0.5	1.6
sitosterol	54.3	43.7	53.8	42.9
Δ5-avenasterol (isofucosterol)	4.2	5.2	4.7	5.5
Sum	95.5	89.6	92.6	85.9
Others				
UN1 C28 1db	0.6	1.2	0.7	1.2
UN2 C29 1db	1.2	2.0	1.2	2.4
UN3 C29 2db	0.9	1.8	1.3	2.4
UN4 C28 1db	0.3	0.9	0.6	1.1
UN5 C30 2db	1.2	1.8	1.4	1.8
UN6 C29 1db + C30 2db	0.3	2.7	2.2	5.2
Sum	4.5	10.4	7.4	14.1
total	100	100	100	100

Abbreviations: UN denotes unknown, C is number of carbon atoms and db denotes number of double bonds; [1] Polar lipid-rich extract containing glycolipids, phospholipids and residual TAG recovered by chloroform-methanol (CM) extraction post hexane extraction of crushed seed.

3.5. Fatty Alcohol Composition

A series of fatty alcohols from C_{16}–C_{22}, with accompanying iso-branched fatty alcohols, were identified in both the hexane and chloroform-methanol extracts (Table 6). Similar profiles were observed for the DHA- and control camelina, with some variation in the proportions of individual components observed. The odd-chain alcohols were present at higher levels in the chloroform-methanol extract (37%–38%) than in the hexane extract (16%–23%). Iso-17:0 (16%–38%) predominated over 17:0 (0.3%–5.7%). Another odd-chain alcohol present was 19:0 (4.5%–6.5%). Phytol, derived from chlorophyll, was the major aliphatic alcohol and accounted for 47% and 37% of the total fatty alcohols in the hexane fractions in the DHA- and control camelina, respectively. There were lower levels in the chloroform-methanol extract (9% and 12%, for DHA- and control camelina, respectively). Other alcohols detected included iso-16:0, 16:0, iso-18:0, 18:1, 18:0, with minor levels of iso-20:0, 20:1, 20:0, iso-22:0, 22:1 and 22:0 also present.

Table 6. Fatty alcohol composition (% of total sterols) of DHA- and control camelina.

	DHA camelina		Control camelina	
Fatty alcohols	**Hexane**	**CM [1]**	**Hexane**	**CM [1]**
iso-16:0	0.8	1.9	1.5	1.7
16:0	5.7	13.8	6.1	12.2
iso-17:0	16.4	37.1	23.5	38.6
17:0	3.8	0.3	5.7	1.0
iso-18:0	6.6	11.8	8.3	13.5
18:1	2.8	8.0	2.5	5.1
18:0	9.3	7.9	7.4	6.4
Phytol	47.1	9.1	37.4	11.7
19:0	4.5	5.6	5.1	6.7
iso-20:0	0.0	0.2	0.0	0.2
20:1	1.0	0.9	0.5	0.6
20:0	1.3	1.7	1.2	1.1
iso-22:0	0.0	0.2	0.0	0.2
22:1	0.0	0.5	0.0	0.3
22:0	0.8	1.0	0.7	0.5
Sum	100	100	100	100

[1] Polar lipid-rich extract containing glycolipids and phospholipids with residual TAG obtained by chloroform-methanol (CM) extraction post hexane extraction of crushed seed.

4. Discussion

The oil content from camelina seeds can range from 25% to 48% [19,24–26] and can be dependent on the location where the plant is grown and the environmental conditions during growth. Previous researchers have published the fatty acid composition of camelina oil which is similar to what we report here for the control camelina [27]. Since this was only a descriptive analysis with no replication performed, we cannot yet conclusively determine whether the insertion of the omega-3 LC-PUFA microalgal-derived genes affected the oil yield. Hence, further work including replication would need to be done to determine if there is any statistical change in the oil yield. The results for the oil extraction suggest that slow crushing using a motorized mortar and pestle with multiple extractions with hexane at room temperature is effective in recovering the majority of the TAG oil.

In addition to the oil containing moderate levels of DHA, the DHA-containing camelina also had markedly higher levels of ALA in the major lipid classes (triacylglycerols, glycolipids and phospholipids) compared with the control camelina. This finding shows that the activity of the Δ-15 desaturase gene is considerably enhanced in DHA camelina [18,20] hence there is scope to increase the DHA content further by optimizing the elongation and desaturation of ALA. Variations in the level of ALA and DHA in the transformed plants may also be influenced by the effects of cultivar variety and other factors such as the quality of soil and climatic and weather conditions. It has also been reported that, in oilseed crops, the level of PUFA in general is promoted by low temperatures (winter and spring season) during the seed filling period, while at higher temperatures (summer season) the concentration of saturated fatty acids is higher [26].

Interestingly, there were some slight differences in the fatty acid profile and proportion of DHA in the various extracts and fractions with the DHA levels being higher in the TAG-rich hexane extract and TAG from CM extraction (6%–6.8%) and lower in the polar lipid fractions (3% in glycolipids and 1.6% in phospholipids), and 16:0 being higher in the polar lipid fractions of glycolipids and phospholipids from CM extraction (19%–21%) compared with the TAG-rich hexane extract and TAG from CM extraction (6%–7%). It is not known if the low levels of residual lipid present in the meal after solvent extraction (using hexane then chloroform-methanol) and recovered by transmethylation is derived from free solvent extractable lipid or bound lipid which is liberated only by hydrolysis under the hot acid methanolic transmethylation conditions. Future research will investigate this aspect.

The sterol composition of the camelina DHA and control camelina samples analysed here were similar to that found in refined camelina oil [28] with the same major sterols present, indicating that the added genes did not affect sterol synthesis. Previous workers reported the major sterols as being: cholesterol (4%), brassicasterol (3%), campesterol (21%), stigmasterol (2%), sitosterols (45%), Δ5-avenosterol (9%), cycloartenol (12%) and 24-methylene cycloartenol (3%). They also noted ten minor components which were unidentified due to their very low levels. We also observed several unidentified components at low relative levels (Table 5). Schwartz *et al*. [29] reported campestanol (1.6 mg), sitostanol (2.5 mg), stigmasta-5,24-dienol (6.2 mg), gramisterol + α-amyrin (1.9 mg), Δ7-avenosterol (trace levels) and citrostadienol (1.30 mg) in camelina oil in addition to the sterols identified by Shukla *et al*. [28]. They reported the levels of the common sterols (mg/100 g oil) in camelina oil as cholesterol (35 mg), brassicasterol (27 mg), campesterol (117 mg), stigmasterol (5.6 mg), sitosterol (300 mg), Δ5-avenosterol (37 mg), cycloartenol (10 mg) and 24-methylene cycloartenol (1.0 mg). The level of cholesterol in camelina oil was higher than occurs in most vegetable oils and brassicasterol is a characteristic sterol found in the Brassicaceae family of which camelina is one.

Based on the combined analyses of a wide suite of lipid classes in DHA camelina and control camelina, it would seem then that the omega-3 LC-PUFA genes have, as expected, had little or no effect on the sterol composition. Further work will need to be carried out to determine the contributions of these sterols from the sterol ester and free sterol fractions, in each of the extracts, by separating the sterol ester and free sterol lipid classes and analysing them separately.

In relation to the presence of fatty alcohols, those with chain length C_{16}–C_{24} have previously been observed in camelina after release from wax esters following saponification [30], but to our knowledge the presence of iso-odd-chain fatty alcohols such as found here, in both DHA camelina and control camelina, have not been reported previously. Further research needs to be performed to determine which fractions these alcohols are present in, e.g., in free or in an esterifiedform such as in a wax ester.

5. Conclusions

This is the first detailed report of the lipid composition of a new transformed terrestrial oilseed capable of producing DHA-containing (6.8% of total fatty acids) TAG oil with a simple fatty acid profile and a high preference of ω3 over ω6 LC-PUFA. Fish oils also have a high preference of ω3 over ω6 LC-PUFA. However, several key distinguishing features found in the transformed camelina fatty acid profile is the high level of α-linolenic acid (ALA, 18:3ω3) which is only a minor fatty acid in fish oils and the much lower levels of EPA (eicosapentaenoic acid, 20:5ω3) making the fatty acid

profile of the oil unique. The chloroform-methanol extract is rich in polar lipids (glycolipids and phospholipid), since these are not extracted by hexane. Hence, the hexane extracted seed meal, from which most of the TAG was removed may be useful as an animal feed supplement (e.g., in aquaculture) or as a source of high value DHA-containing polar lipids (e.g., phosphatidyl choline containing DHA). The profiles of other lipid classes such as sterols and fatty alcohols were very similar to the control camelina. These results hold promise for the development and commercial production of new and sustainable terrestrial sources of LC omega-3 oils, which will supplement or in part replace LC omega-3 containing marine oils, hence alleviating pressure on wild harvest fisheries arising from increasing demand for these oils. Future research will be extended to canola and include similar detailed lipid class characterisation, examination of extraction efficiencies, oil stability and enrichment of DHA.

Acknowledgments

This research was funded by CSIRO Food Futures Flagship, Nuseed Pty Ltd. Laverton, Australia and the Australian Grains Research and Development Corporation, Canberra, Australia. The funders had no role in study design, data collection and analysis, or preparation of the manuscript. The authors were granted permission to publish the manuscript by the funders. We acknowledge Daniel Holdsworth who managed the CSIRO Marine and Atmospheric Research laboratories GC-MS facility and Diana Davies, Graeme Dunstan and two unknown reviewers for reviewing the manuscript and providing useful suggestions.

Conflicts of Interest

The authors declare no conflict of interest.

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DHA-Containing Oilseed: A Timely Solution for the Sustainability Issues Surrounding Fish Oil Sources of the Health-Benefitting Long-Chain Omega-3 Oils

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Abstract: Benefits of long-chain ($\geq C_{20}$) omega-3 oils (LC omega-3 oils) for reduction of the risk of a range of disorders are well documented. The benefits result from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); optimal intake levels of these bioactive fatty acids for maintenance of normal health and prevention of diseases have been developed and adopted by national and international health agencies and science bodies. These developments have led to increased consumer demand for LC omega-3 oils and, coupled with increasing global population, will impact on future sustainable supply of fish. Seafood supply from aquaculture has risen over the past decades and it relies on harvest of wild catch fisheries also for its fish oil needs. Alternate sources of LC omega-3 oils are being pursued, including genetically modified soybean rich in shorter-chain stearidonic acid (SDA, 18:4ω3). However, neither oils from traditional oilseeds such as linseed, nor the SDA soybean oil have shown efficient conversion to DHA. A recent breakthrough has seen the demonstration of a land plant-based oil enriched in DHA, and with omega-6 PUFA levels close to that occurring in marine sources of EPA and DHA. We review alternative sources of DHA supply with emphasis on the need for land plant oils containing EPA and DHA.

Reprinted from *Nutrients*. Cite as: Kitessa, S.M.; Abeywardena, M.; Wijesundera, C.; Nichols, P.D. DHA-Containing Oilseed: A Timely Solution for the Sustainability Issues Surrounding Fish Oil Sources of the Health-Benefitting Long-Chain Omega-3 Oils. *Nutrients* **2014**, *6*, 2035–2058.

1. Introduction

There is a vast array of reviews, systematic reviews, meta-analysis and industry reports on seafood consumption in general and the health benefits of omega-3 long-chain ($\geq C_{20}$) polyunsaturated fatty acids (omega LC-PUFA) (also termed LC omega-3 oils) in particular. Our review begins from the premise that, as far as human health claims are concerned, the health benefits of seafood and their omega-3 fatty acids are largely related to the supply of eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) [1–9]. Thus, one aim of our review is to examine the occurrence of a marked rise in demand for oils high in EPA and DHA and against this demand, the sustainability of marine sources of these LC omega-3 oils. The review summarises recommended intake targets proposed and/or set by national and international bodies. It analyses the bio-conversion of shorter-chain (C_{18}) omega-3 fatty acids to EPA and DHA in humans, monogastric animals, ruminants and aquaculture species. As the major user of global fish oil (FO) supply, the aquaculture sector is considered in a relatively greater detail with respect to both biosynthesis of EPA and DHA as well as FO supply issues. Research trends in the area of metabolic engineering of plants to produce novel omega-3 oil sources, and the biological efficacy of currently available oils are covered.

2. Consensus for, and Recommendations on, Human Nutrition Needs for Long-Chain Omega-3 Oils

Increasing numbers of clinical and epidemiological studies provide evidence supporting the case that omega-3 LC-PUFA are responsible for a multitude of health benefits [10]. The dietary intake of preformed omega-3 LC-PUFA—EPA and DHA—has been recognised as important [1] since *in vivo* conversion of the shorter-chain (C_{18}) fatty acids, namely ω-linolenic acid (ALA, 18:3ω3) to DHA in particular is relatively poor [11,12]. In 2009, the International Society for the Study of Fatty Acids and Lipids (ISSFAL) [13] provided the following position statement: "The majority of evidence from isotopic tracer studies shows that the conversion of ALA to DHA is on the order of 1% in infants, and considerably lower in adults." Hence, it is critical that there are various and sustainable sources of oils containing preformed DHA for the whole population to meet targets for adequate intake of EPA plus DHA. Table 1 summarises the dietary intake targets proposed by various national and international bodies [14–20].

Although the recommended daily intakes vary, they are a culmination of years of clinical and epidemiological research that have clearly established a strong body of evidence for the beneficial health effects of LC omega-3 oils for the improvement of cardiovascular health [21]. The recommendations provided in Table 1 are based on consumption of both EPA and DHA. As currently there are only limited and more niche supply alternatives to the marine supply of DHA, we also aim to highlight specific roles of DHA in the following section to caution against reliance on LC omega-3 sources that do not provide DHA.

Table 1. Selected suggested long-chain (LC) omega-3 (eicosapentaenoic acid + docosahexaenoic acid (EPA + DHA)) intakes (mg/day) for adults available from various agencies and bodies.

Group	EPA + DHA, mg/day
SACN/COT, UK 2004 [14]	450
National Heart Foundation (Australia), 2008 [15]	500
American Dietetic Association and Dieticians of Canada, 2007 [16]	500
FAO/WHO Expert Consultation, 2008 [17]	250–2000 *
American Heart Association, 2002 [18]:	
Coronary heart disease sufferers	1000
Those seeking to reduce triacylglycerols (blood fats)	2000–4000
National Health and Medical Research Council (Australia) [19]:	
Male adults	610
Female adults	430
European Food Safety Authority, 2010 [20]	250

* For secondary prevention of coronary heart disease.

3. The Role of DHA

LC omega-3 oils containing EPA and DHA are considered beneficial for certain aspects of cardiovascular health and pharmaceutical-grade omega-3 LC-PUFA therapies have expanded rapidly for treatment of cardiovascular-related diseases [22–24]. Whilst the majority of studies have reported

both EPA and DHA as being protective, there is a growing body of evidence that suggests differential effects depending on the nature of cardiovascular risk factor itself and/or the disease endpoint. For example, DHA has been more effective than EPA for its actions on blood pressure, heart rate and vascular health [10,25–27]. On their effects on plasma lipids, a meta-analysis of randomized placebo controlled trials of EPA or DHA monotherapy has concluded that DHA is more effective in lowering triacylglycerols (TAG) and raising HDL-cholesterol (HDL-c) than EPA [28]. Although DHA has been found to raise LDL-cholesterol (LDL-c), this was also associated with increased LDL and HDL particle sizes [29]—an outcome not observed with EPA [26]. Furthermore, only DHA was effective in reducing the number of small, dense LDL particles [27] which are known to be more atherogenic. Increased LDL and HDL particle sizes are negatively correlated with cardiovascular risk. Furthermore, DHA, but not EPA, was inversely associated with intima-media thickness—an independent predictor of cardiovascular events—in the Japanese [30], suggesting more potent anti-atherogenic properties of DHA. Similarly, DHA but not EPA supplementation reduced the vulnerability to experimentally-induced atrial fibrillation and secondary structural changes (re-modeling) of the atria [31]; these findings are in agreement with observations made in several human clinical studies that reported that lower incidence of atrial fibrillation is correlated positively with plasma DHA, but not EPA [32,33].

The human nervous system contains a significant amount of DHA, which is required for brain development and function especially in infants [12]. With the poor conversion of ALA and EPA to DHA, together with the particularly important roles for DHA in humans, inclusion of DHA in infant formula is now widespread.

With regard to mental health conditions, a range of studies have examined the effect of the LC omega-3 oils on mild cognitive impairment (MCI) [34]. Depressive symptoms may increase the risk of progressing from MCI to dementia. Consumption of LC omega-3 may alleviate both cognitive decline and depression. A recent study investigated the benefits of DHA and EPA supplementation for depressive symptoms, quality of life (QOL) and cognition in elderly people with MCI [34]. In a 6-month, double-blind, randomised controlled trial, individuals aged 65 years with MCI were allocated to receive a supplement rich in EPA, DHA or the omega-6 PUFA linoleic acid (LA, 18:2ω6). Compared with the LA group, Geriatric Depression Scale (GDS) scores improved in the EPA and DHA groups and verbal fluency (Initial Letter Fluency) improved in the DHA group. Improved GDS scores were correlated with increased DHA plus EPA. Improved self-reported physical health was associated with increased DHA. There were no treatment effects on other cognitive or QOL parameters. Increased intakes of DHA and EPA benefited mental health in older people with MCI. Increasing omega-3 LC-PUFA intakes may reduce depressive symptoms and the risk of progressing to dementia. The authors concluded that this needs to be investigated in larger, depressed sample groups with MCI.

The same research team also examined the effects of LC omega-3 oils on literacy and behaviour in children with attention-deficit/hyperactivity disorder (ADHD) [35]. The effects of an EPA-rich oil and a DHA-rich oil *versus* an omega-6 PUFA-rich safflower oil (control)—as LA—were compared in a randomized, controlled trial. The effect of supplementation on cognition, literacy, and parent-rated actions was assessed by linear mixed modelling. There were no significant differences between the supplement groups in the primary outcomes after four months. However, the erythrocyte fatty acid

profiles indicated that an increased proportion of DHA was associated with improved word reading and lower parent ratings of oppositional behaviour. These effects were more evident in a subgroup of children with learning difficulties: an increased erythrocyte DHA was associated with improved word reading, improved spelling, an improved ability to divide attention, and lower parent ratings of oppositional behaviour, hyperactivity, restlessness, and overall ADHD symptoms. The authors concluded that increases in erythrocyte omega-3 PUFA, specifically DHA, may improve literacy and conduct in children with ADHD. The greatest benefit may be observed in children who have co-morbid learning difficulties. In a recent randomized controlled intervention trial, DHA supplementation was observed to improve both memory and reaction time in healthy young adults whose habitual diets were low in DHA [36]; the response was found to be modulated by gender.

Another very recent application of DHA has been in the development of neuroprotective strategies for treatment of spinal cord and head injuries. These studies—albeit at early stages involving animal models—illustrate the significant potential of DHA, but not EPA, in the treatment of acute neurological injury [37].

Against the increasing scientific literature pointing to the importance of the LC omega-3 oils and in particular DHA, in human health, global supplies of fish oil (the current main source of EPA and DHA) obtained from wild-harvest low-value marine species, termed forage fish, will not meet future market demands [23,38]. The following sections in this review paper examine sustainability of fish oil and future sources of the LC omega-3 oils, recent findings for the use of SDA containing oils, current practices with aquafeeds, and further research needs.

4. Supply, Demand and Environmental Issues—A Need for Alternative Sources of LC Omega-3 Oils

The harvest of low trophic species such as anchovy, sardines, mackerel, menhaden, capelin and sandeel for the production of fish meal and fish oil (FO) represents the current main source of the health-benefiting LC omega-3 oils used in aqua and animal feeds, health supplements, pharmaceuticals and other products including functional foods.

Fish oil processing involves a range of steps after the initial meal and oil production [39]. Many of these steps use the same processes that are used for vegetable oils. The final use for the oil determines the level of processing, with human nutrition and pharmaceutical applications generally requiring greater processing and accompanying quality assurance and quality control procedures than for fish and animal nutrition. Several changes in the usage pattern have occurred over the past decades. FO was initially widely used in livestock feed, then, as aquaculture expanded this industry became the major user. Therapeutic uses of LC omega-3 PUFA are increasingly being recognized and recently new pharmaceutical grade LC omega-3 products (containing 85%–95% EPA and DHA) have gained increasing market share, and this industry has expanded its share of use of the fish oil resource, with less oil available for the aquaculture sector [31,40]. This changing pattern of use of FO is predicted to continue [40]. One aspect of this recent change is that the processing of the FO to achieve the higher grade (or more pure) LC omega-3 products results in considerable losses, with product yields in the 5%–10% range. For instance, in a purification process reported by Belarbi *et al.* [41], production of 1 kg EPA ester required 15 kg of FO, or 56 kg (dry basis) of the marine alga *Phaeodactylum*

tricornutum or 70 kg of another alga *Monodus subterraneus*. Further research and development for more efficient production of the pharmaceutical grade products will assist all users to maximize and better utilize this important finite resource.

Global production of FO is around 1 million tonnes per annum, with fish meal in the range of 6–7 million tonnes per annum, except during the periodic El Niño years [42]. Production has generally remained at this level for the past decade [23], and requires an annual catch of 25–30 million tonnes of feed-grade fish and unwanted fish processing waste; 4–5 kg of wet fish yields 1 kg of FO and fishmeal. Although the current harvest of low trophic (also termed forage) species for fish meal and FO has been regarded as sustainable for several decades, a recent development has been the foreseen need to reduce the harvest of small oceanic forage fish like sardines and anchovies in some areas by 20%–50% in order to protect larger predators that rely on these species for food [43,44]. Should such recommendations be implemented, this would have significant flow-on ramifications for the range of industries currently utilizing the FO resources.

An emerging source of LC omega-3 oils over the past decade has been krill oil. The current krill harvest is around 200,000 MT [40], and is actually much less than in the 1980s prior to the break-up of the USSR. The total allowable catch is set by the Committee for the Conservation of Antarctic Marine Living Resources (CCAMLR) and is three times greater than the present harvest. Of the major krill oil producers, a further development has been that Aker BioMarine has been granted certification by the Marine Stewardship Council (MSC) in 2012. MSC has validated the harvesting and traceability for Aker's Antarctic fisheries. The total krill harvest is deemed sustainable at present levels in view of a number of, although not all, bodies including various Non-Government Organizations. If there is to be a major expansion of the krill fishery, the sustainability topic will clearly need to be revisited by these and other expert groups including in particular CCAMLR.

5. Alternative Sources of LC Omega-3 Oils

Against the background provided above on the current and future status of marine-derived oils—fish oil and more recently krill oil—considerable progress has occurred with the development of new, alternate and sustainable sources of the LC omega-3 oils. Single cell organisms (SCO), such as heterotrophic dinoflagellates and thraustochytrids (both grown and harvested for DHA containing oils) and other algal groups and recently a genetically modified (GM) yeast (containing EPA) are now in commercial production; strong uptake has occurred for the DHA oils in particular areas including infant formula, health supplements and some functional foods [45]. In addition to this excellent progress with SCO production of the LC omega-3 oils, a large number of groups are conducting research and development with a suite of microalgae towards co-production of biofuels and other by-products [46]. Whilst the cost of production of microalgae for biofuel production is presently greater than for fossil fuels, future breakthroughs in culturing, harvesting and other processing are anticipated [46], which can be expected to reduce costs. It is anticipated that these uses for SCO will largely remain for the high value applications including in nutraceutical and pharmaceuticals rather than in aquafeeds.

The past decade has also seen several groups using genetic engineering to allow oilseed crops to produce LC omega-3 oils [23]. As this research field has progressed, important breakthrough steps have included: the isolation and characterization of genes from the marine microalgae encoding front-end desaturases involved in DHA biosynthesis [47], the isolation of highly efficient desaturases and elongases [48–51], the use of genes with omega-3 substrate preference [49–51] and the development and use of a land plant (tobacco) leaf-based assay using interchangeable design principles to rapidly assemble multistep recombinant pathways [52]. Progress with research on insertion of microalgal-derived genes leading to DHA production into a range of omega-3 C_{18} PUFA accumulating land plants has been reviewed [2,53–55]. Transfer of genes from microorganisms to land plants has seen accumulation in oilseeds of SDA, EPA and DHA [2] (Table 2, Figure 1). Good progress has been made in engineering the EPA genes into crop plants, with several groups reporting the production of EPA at levels similar to that observed in bulk fish oil (approximately 18%) [56,57]. The conversion of the C_{20} EPA to the particularly important C_{22} DHA, however, had been problematic with many attempts resulting in the accumulation of EPA and DPA, but until very recently little DHA [56,58–62].

For SDA and EPA, levels achieved in the engineered oilseed plants are comparable to levels from other naturally occurring land plant (SDA) and/or marine (EPA) sources [23]. Elevated levels of DHA had not been achieved prior to 2012, except for one *in planta* observation where the isolated TAG fraction from the leaf of *Nicotiana benthemiana* contained high DHA, although high levels of the omega-6 PUFA 18:2ω6 were also present [53]. In 2012, a further key breakthrough occurred, with the reporting, for the first time, of fish oil-like profiles for a DHA-containing oilseed plant *Arabidopsis thaliana* [58]. Features of the new oil were: (i) a DHA level of 15% (of the total FA); (ii) a total of 25% new omega-3 PUFA and omega-3 LC-PUFA; (iii) 30% ALA; and (iv) an omega-3/omega-6 ratio that was similar to that observed for marine oils. The latter feature is a further important and distinguishing attribute for the land plant derived LC omega-3 oils, with this report being, to our knowledge, the first time this oil trait has been observed. More recently a similar profile has been observed in a commercial oilseed plant (*Camelina sativa*) [59].

Table 2. Levels of SDA, EPA and DHA (as % of fatty acids) in new land plant oil seeds.

Oil Seed and Comparison to *Farmed salmon*	Reference	SDA%	EPA%	DHA%
CSIRO Oil Seeds (includes model plants)	[51]	10		
	[59]		5	1
	[51]	1	26	
	[53]		15	14
	[58]	5	2	15
	[59]	9	3	13
BASF Mustard	[61]		15	1.5
Monsanto-Soy	[56]	20		
Dupont-Soy	[55,57]		20	3
Farmed salmon				
fed fish oil (FO)	[63,64]		10	17
fed plant oil/chicken fat-FO	[65]		1–5	5

Abbreviations: SDA, stearidonic acid EPA; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

These findings clearly indicate the feasibility of developing oilseed crops with high concentrations of omega-3 LC-PUFA. Further research and development is needed to develop commercial oilseed crops with LC omega-3 oil enriched in DHA as has been achieved in model plants (*Arabidopsis,* tobacco) and also in *Camelina*. Future novel land-based plants can provide an economically viable source of LC omega-3 oil for aquaculture and other higher value applications. A land plant source of LC omega-3, if achieved, and assuming cultivation is permitted, will be considerably cheaper than that from microorganisms, and could be used as an additional source of these essential ingredients in feed, food and pharmaceutical products to improve human health (Figure 1).

6. ALA and SDA in Animal Feeds

The availability of the SDA-containing *Echium* oil (around 14% SDA of total FA) has enabled this potential EPA/DHA precursor, which is one step more advanced than ALA to be trialed in animal, including farmed fish, and human nutrition research. The general hypothesis driving this line of research has been that animals and farmed fish fed SDA oils would produce tissue containing greater proportion of EPA and DHA than those fed ALA oils. This is based on the assumption that the inefficiency in the biosynthesis of EPA and DHA from ALA is related to the rate-limiting step of converting ALA to SDA (Figure 2).

Selected poultry data from feeding experiments [66–74] where different oils have been used to enrich thigh muscle are summarised in Figure 3. There were only modest changes in EPA and DHA content in broiler muscle samples when the oil supplement itself did not contain the omega-3 LC-PUFA. The evidence from such a large range of experiments did not indicate marked benefit from using C_{18} oils in enriching tissues particularly with DHA (Figure 3). Similar trends were noted when we reviewed EPA and DHA enrichment of egg from omega-3 oil-supplemented laying hens and breast muscle in broilers (data not shown). Similarly, data from lamb meat studies [75–84] are summarized in Figure 4. Levels of EPA and DHA in lamb meat were lowest in studies where vegetable oils were used. As the dietary fat supplement shifted towards marine sources, the levels of EPA and DHA in lamb meat increased across experiments. The evidence so far suggests that the best way to enhance the DHA content of livestock products is to include DHA containing fat supplements in the diet. There is as yet no convincing evidence that current fat supplements containing ALA or SDA are suitable alternatives for those containing preformed EPA and especially DHA.

Figure 1. The potential future sources of omega-3 LC-PUFA are shown, with current sources (left) being seafood and microalgae, with possible future sources through genetically engineered plants also indicated at the right.

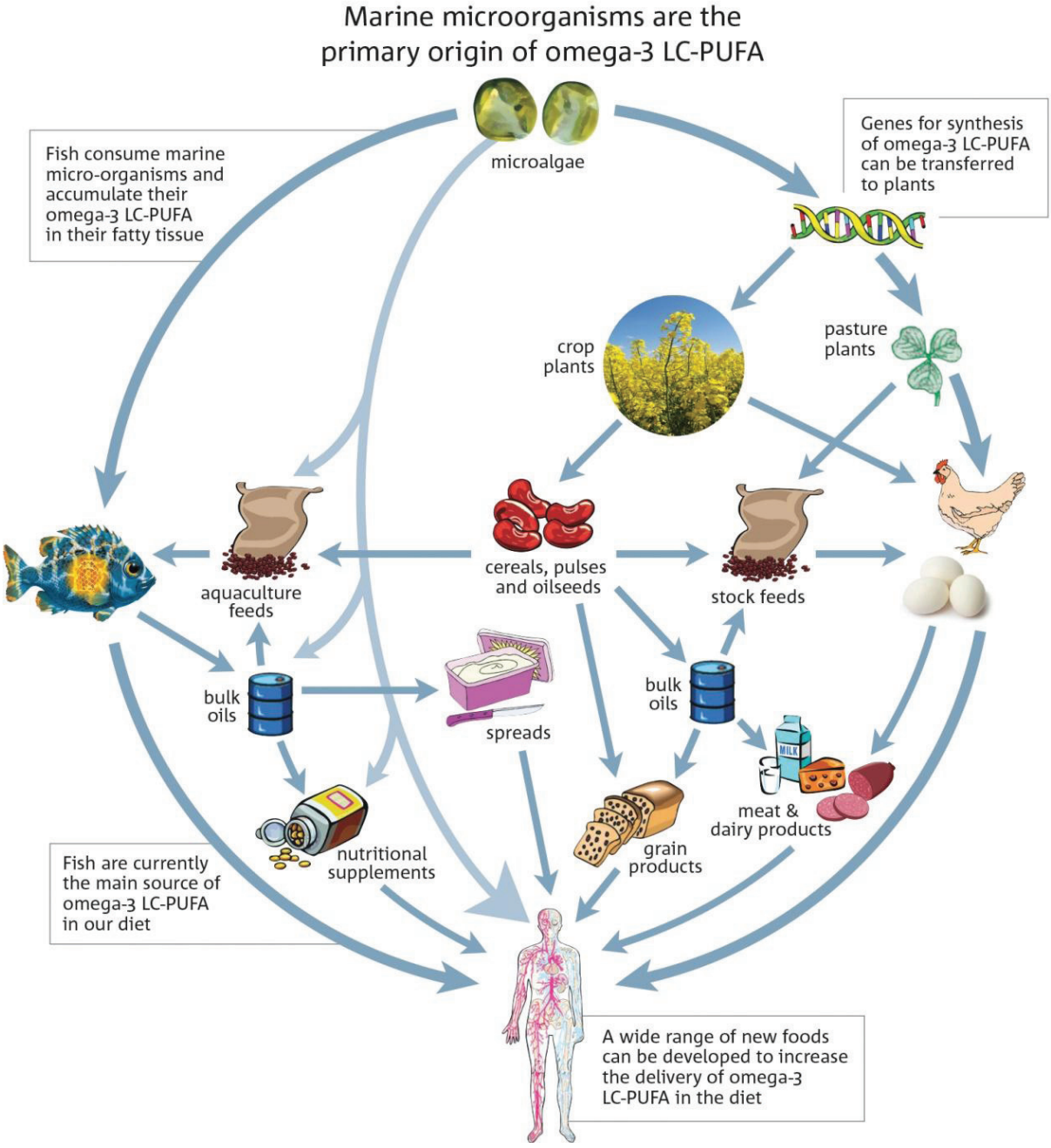


Figure 2. Schematic showing synthesis of shorter-chain fatty acids in land plants (**black horizontal arrows**), followed by addition of genes from marine microalgae (**blue vertical arrows**) resulting in new LC omega-3 containing oilseeds. elo, elongase; des, desaturase.

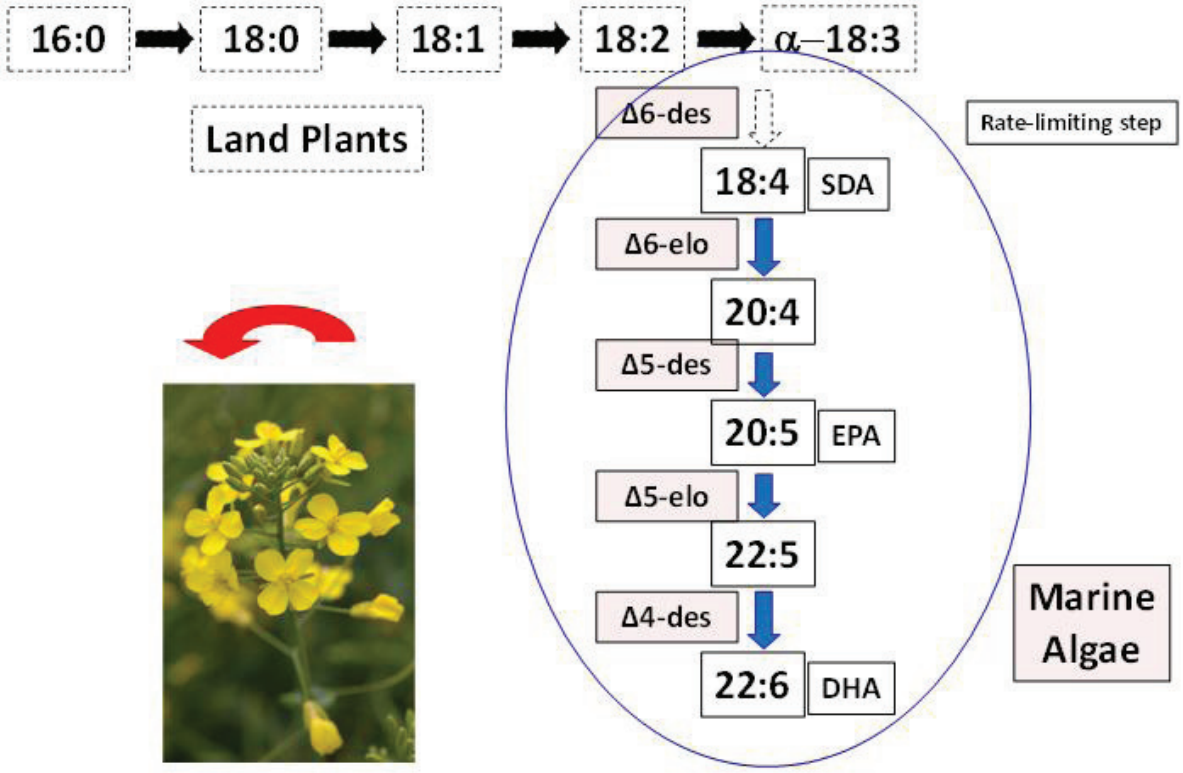


Figure 3. Levels of omega-3 PUFA (ALA) and omega-3 LC-PUFA (EPA, DPA and DHA) in thigh muscle from broilers on a diet with: (1) no oil supplement; (2) vegetable oil; (3) fish oil; (4) marine algae; or (5) SDA-containing oil [66–74].

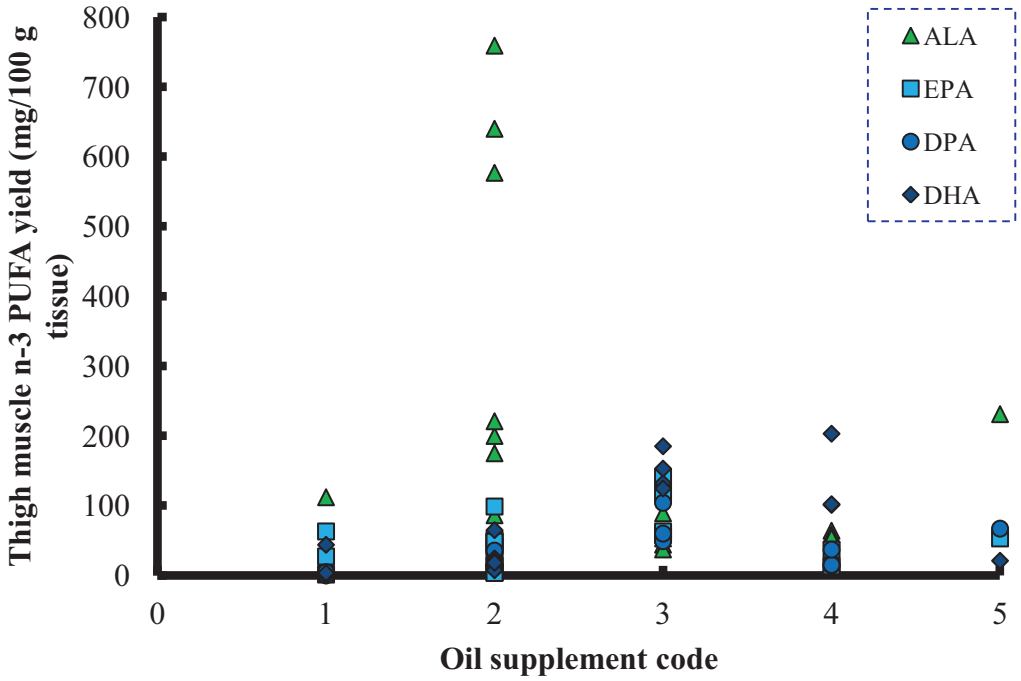
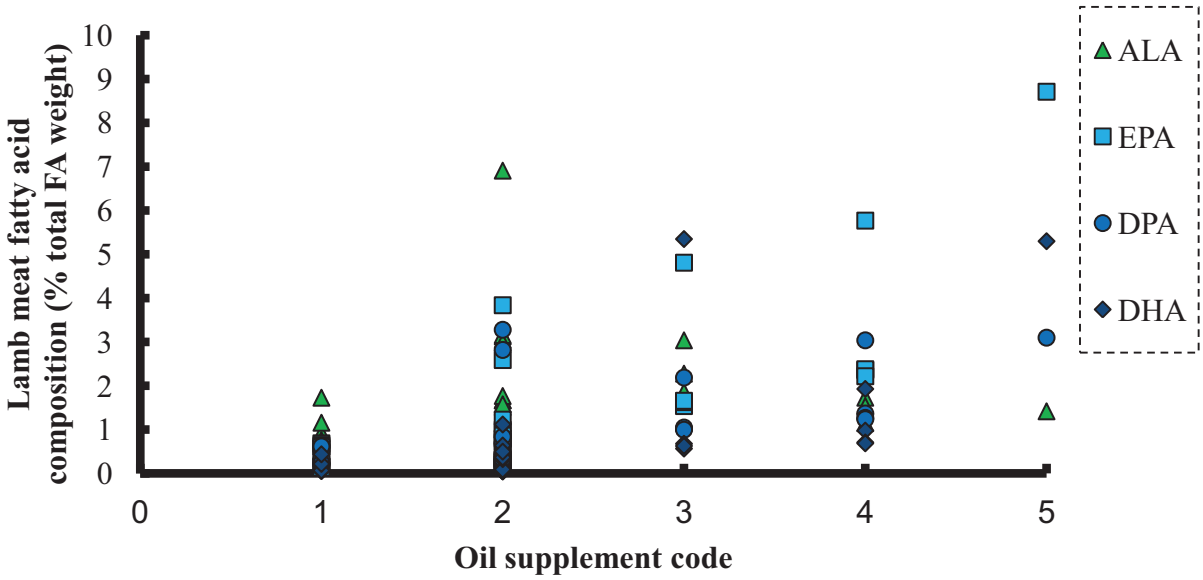


Figure 4. Levels of omega-3 PUFA (ALA) and omega-3 LC-PUFA (EPA, DPA and DHA) in trimmed lean muscle of lambs on a diet with: (1) no oil supplement; (2) linseed or linseed oil; (3) fish oil–vegetable oil mix; (4) fish oil; or (5) fish oil–marine algae mix [75–84].



7. ALA and SDA in Aquafeeds

Aquaculture continues to be the main end user of global sources of FO, yet available sources of FO generally remain static and will likely not increase significantly. Against this background of either static or decreasing resource availability, the demand for FO and its range of uses continues to increase as noted earlier. The increasing demand for FO is in line with the growing global population (due to rise by a further 34% by 2050) [85], the expanding aquaculture industry (around 10% growth per annum), the increasing recognition of the health benefits of the LC omega-3 oils, and more recently the resulting and therefore competing use of FO for production of pharmaceutical grade products containing ≥85% EPA and/or DHA.

A number of trials have been conducted for Atlantic salmon and barramundi using the SDA rich *Echium* oil *(Echium plantagineum)*. Initial work with Atlantic salmon parr (freshwater stage) showed that SDA was effective in producing EPA and DHA [86,87]. This freshwater phase is only a short period of the total life cycle for farmed Atlantic salmon. Trials for the same species during the seawater stage (bulk of the life cycle) showed SDA was not so effective [63,64,88]. Some EPA was produced and also DPA, but no DHA. The same observations occurred for barramundi feeding trials [89–91], that is limited or no omega-3 LC-PUFA, in particular DHA, was produced or accumulated in the flesh of this species. Conversion of the C_{18} SDA for barramundi to the omega-3 LC-PUFA was even lower than that observed for Atlantic salmon. Other researchers have generally observed similar findings where SDA inclusion has occurred in aquafeed trials with other fish species, including Atlantic cod, striped bass, rainbow trout and gilthead seabream [92–95].

A further feeding trial with the SDA-containing *Echium* oil for early juvenile barramundi used the plant bioactive sesamin as a potential modulator of lipid biosynthesis [96]. Relative to the control fish, growth of the SDA treatment group was hindered, although interestingly both EPA and DHA

increased. It was proposed that sesamin is a potent modulator for LC-PUFA biosynthesis in barramundi, but probably will have more effective impact at advanced ages. By modulating certain lipid metabolic pathways, the use of sesamin as a feed ingredient probably disrupted the body growth and development of organs and tissues in the early juvenile barramundi.

Atlantic salmon and barramundi have, when fed an FO-containing diet, provided an excellent source of beneficial omega-3 LC-PUFA for human consumption, but reduced concentrations of these acids, together with a markedly decreased omega-3/omega-6 ratio, as occurs through the use of vegetable oil and/or animal fat diets, may compromise their nutritional benefit to consumers. Limited research has been performed to examine this issue. In one study [97], dietary intake of differently fed salmon (100% FO, 50/50 FO/rapeseed oil, 100% rapeseed oil) and the influence on markers of human atherosclerosis were compared. Significant differences between the consumer groups were observed in the serum fatty acid profiles, especially for the levels of total omega-3 PUFA and the omega-3/omega-6 ratio, which were markedly increased in the FO-fed fish consuming group in contrast to the two other groups. The authors concluded that Atlantic salmon fed the FO-containing diet and containing very high concentrations of omega-3 LC-PUFA seemed to impose favorable biochemical changes in patients with CHD when compared with ingestion of fillets with intermediate and low levels of the marine omega-3 LC-PUFA, where FO was replaced in part or in full by rapeseed oil [93]. There have been no consumer trials with fish fed diets containing ALA / SDA rich oils *versus* FO derived EPA + DHA, and looking at the effects on consumers.

8. SDA Oils in Animal Models and Humans

It is apparent from examination of the research performed to date on SDA diets, that the benefits from use of SDA (like for ALA) are due to its conversion to EPA and DHA. Whilst SDA is more efficiently converted to EPA than ALA [98], it is important to record that the elongation of dietary SDA to DHA has been found to be absent (or negligible at best) in humans [99–101]. Furthermore, with respect to enrichment with EPA, the conversion efficiency of SDA to EPA was only 17% even after four months of feeding soybean oil preparation containing 16% SDA and 11% ALA. Similarly, three months treatment with soybean oil with even higher SDA content (28% SDA alone and 40% total omega-3) failed to change erythrocyte DHA from baseline values. These studies clearly show the inability of SDA-rich oils to influence the endogenous DHA pool in humans. The elongation and desaturation of SDA appears to terminate at the DPA level as increased levels of this fatty acid have been observed [101]. However, evidence of direct physiological benefits of DPA in humans is yet to be elucidated. The accumulation of DPA in the EPA and DHA biosynthesis pathways of many species, and its relative abundance in red meat and some marine species also point to a need to determine whether or not DPA should be included in the omega-3 content claim of foods and ingredients. Inclusion of DPA will broaden the range of foods that can reach the “good source” and “very good source” bars in the omega-3 content claims.

Similar results have also been found following animal feeding studies where dietary *Echium* oil rich in SDA (and ALA; 15% SDA, 29% ALA) failed to increase plasma or tissue DHA levels compared to supplementation with fish oil. In particular, *Echium* oil diet did not lead to any increase in EPA or DHA in cardiac muscle membranes, but resulted in a dose-related increase in DPA [102]. Fish oil

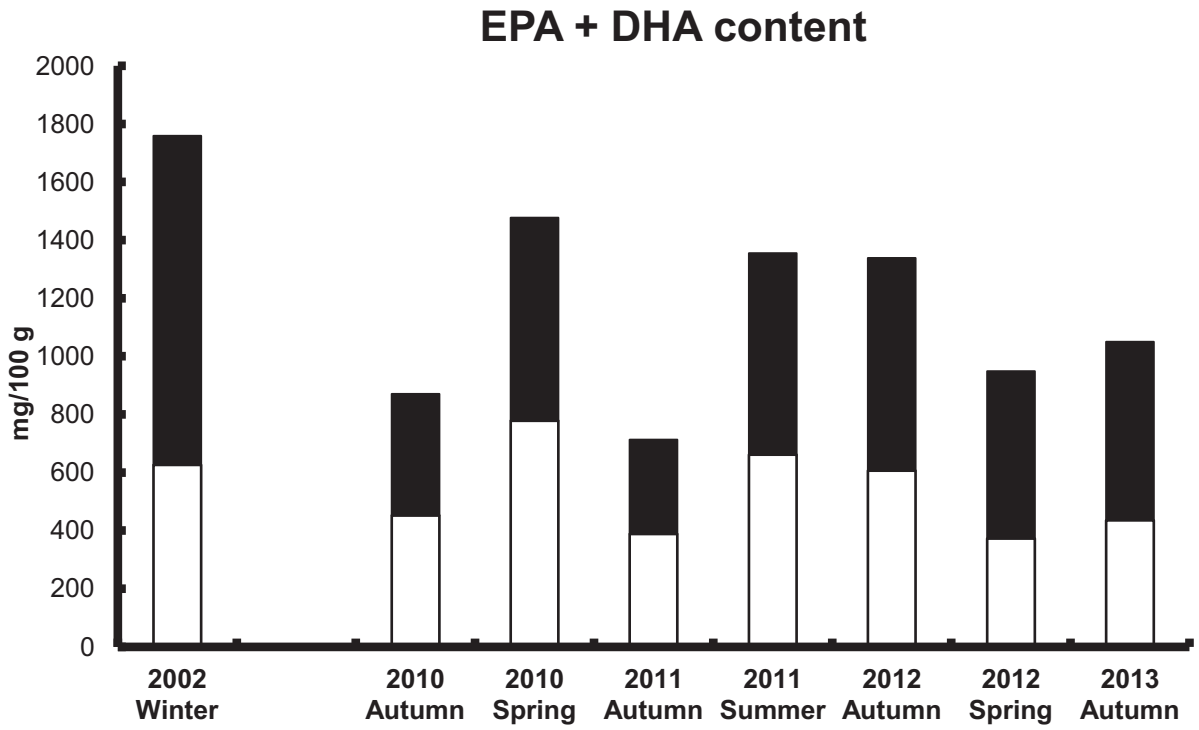
feeding on the other hand displayed considerable accumulation of DHA but not DPA. Also it is of interest to note that anti-arrhythmic action (protection against ischemia induced cardiac arrhythmia and sudden cardiac death in rats) was significantly greater following feeding with FO compared to SDA-rich *Echium* oil [65].

Albert *et al.* [103] reported over a decade ago that plasma LC omega-3 levels are inversely associated with the risk of sudden cardiac death. More recently, the omega-3 index which is the combined total proportion of erythrocyte membrane EPA and DHA has emerged as a novel biomarker that predicts cardiovascular risk [104]. It has also been reported that the omega-3 index correlates well with the EPA + DHA levels of myocardial membranes [105], thus favouring its use when assessing the risk of sudden cardiac death. Since there was no increase in DHA following feeding oils rich in SDA [99–101], the reported beneficial rise in omega-3 index has been driven solely by an increase in EPA. In view of the overall cardiovascular benefits specific to DHA, including the anti-arrhythmic actions discussed above, it can be concluded that further research is needed to determine the important question whether or not an omega-3 index that increased solely due to EPA, is of less benefit as compared to rise in omega-3 index achieved via greater incorporation of DHA into erythrocytes, and ultimately whether an increased SDA consumption would translate into improved human health outcomes [101]. Taken collectively, the observations summarized in these two sections covering the potential use of SDA-rich oils presently imply that direct supply of the pre-formed omega-3 LC-PUFA (EPA and in particular DHA) is the preferred strategy to improve the omega-3 status in diverse applications ranging from aquaculture, livestock and in humans.

9. Current Practices with Commercial Aquafeeds and Future Sources of LC Omega-3 Oils

Aquaculture can be considered as a traditional industry with fish culture occurring for many centuries. Modern aquaculture expansion began in the 1980s and has continued to rise steadily since, with the high value salmonoid fish such as Atlantic salmon being the species of choice. Fish oil, produced as a by-product of the fish meal industry, had been the main oil incorporated into fish feed until recent years. As noted earlier, the past decade has seen fish oil availability decrease and also prices increase substantially. From the use of 100% FO (of the oil component), feed manufacturers are now using up to 75% or higher of vegetable or animal-derived oils [106]. The topic of FO replacement and alternative lipid sources has been recently examined in considerable detail, with a review book now available for researchers, industry and other end users [107]. Given the availability of such a substantial resource, it is not the purpose of this section to further review this topic. This FO substitution can include mixes such as FO/rapeseed oil, FO/chicken fat, and also other combinations. Whilst fish growth and performance is generally not affected, the concentration of omega-3 LC-PUFA and the omega-3/omega-6 ratio in fillet products is markedly changed. For farmed Atlantic salmon grown in Tasmania, Australia, the concentration of EPA and DHA has reduced by ≥30%–50% or more (Figure 5), and the omega-3/omega-6 ratio also has reduced markedly. Marine fish typically show an omega-3/omega-6 ratio of between 5 and 10, and for the first time in 2013 the ratio in farmed salmon in Australia has decreased to less than 1 [108].

Figure 5. Farmed Atlantic salmon from Tasmania, Australia from 2002 (fish oil diet) [23] and 2010 to 2013 (chicken fat/fish oil diet) [108]: Content of EPA (**white bars**) and DHA (**black bars**) (mg/100 g, wet weight).



A recent study tested whether Atlantic salmon smolt fed a diet with a higher DHA: EPA ratio and a lower content of LC omega-3 oils to that of conventional FO based diets would enhance deposition of LC omega-3 in the liver and muscle [109]. Comparisons were made between fish fed: (1) a FO diet; (2) a blend of 50% rapeseed and 50% tuna oil diet (termed model oil, MO1); (3) a blend of 50% rapeseed, 25% tuna and 25% FO diet (MO2); and (4) a blend of 50% FO and 50% chicken fat diet (FO/CF). The latter diet was representative of commercial diets in use at the time of the study, with the proportion of chicken fat increasing even further since the study was performed. The dietary DHA:EPA ratio was in the order MO1 > MO2 > FO/CF ~ FO. The LC omega-3 content was approximately 2-fold lower in the MO1, MO2 and FO/CF diets compared to the FO diet, with the relative levels (as % total FA) lowest in the MO1 diet. For the feeding trial, there were comparable contents of LC omega-3 in the muscle of the FO, MO1 and FO/CF fed fish. A major outcome was that a higher DHA:EPA ratio than that commonly occurring with FO-only diets used for Atlantic salmon was better suited for more efficient deposition of LC omega-3, in particular DHA, with evidence therefore apparent for LC omega-3 "sparing" in Atlantic salmon smolts when fed a diet with a high DHA:EPA ratio [109].

The use of a 50% FO and 50% CF blend in aquafeeds for Atlantic salmon, as was in the range commercially practiced in Australia, resulted in comparable LC omega-3 content in the muscle [108] and liver of juvenile Atlantic salmon to a FO fed fish. It is noteworthy that such an oil blend decreases the inefficient utilization of a 100% FO diet, due to the high loss of EPA in particular, and can be considered as an appropriate current strategy, in terms of LC omega-3 sparing, for present use in

aquafeeds for Atlantic salmon [108]. It is important to note that in spite of changes that have occurred in feeding practices, farmed Tasmanian Atlantic salmon still remains one of the best sources of omega-3 LC-PUFA oils available to Australian consumers. However, the scope can exist with the potential future use of new oilseed-derived LC omega-3 to restore the content of these health-benefitting ingredients, and also the omega-3/omega-6 ratio to that previously seen.

Further research is needed to determine the optimum relative and absolute concentrations of dietary EPA and DHA to enhance their deposition in larger-sized commercially farmed Atlantic salmon. The rationale to pursue such studies will be reliant on research in plant genomics since oils with the desired FA profiles, in particular containing a high DHA:EPA ratio [58,59], whilst not presently available, will likely be a commercial reality by the end of this decade.

10. TAG Structure of Plant-Based LC Omega-3 Oils for Optimum Bioactivity and Food Processing

The melting characteristic of a fat/oil is an important determinant of its suitability for the manufacture of food products. For example, a certain melting range is required before a fat can be used for the manufacture of margarine or fat spreads. The positional distribution of omega-3 LC-PUFA within the TAG molecules can significantly influence the melting characteristics of LC omega-3 oils. In general, the melting point is increased when the omega-3 LC-PUFA is located at the *sn*-2 position compared with the *sn*-1(3) positions [110]. This has practical implications enabling the conversion of liquid oils to semi-solid fats for margarine manufacture or use as *trans* fat substitutes in bakery products. Furthermore, omega-3 LC-PUFA such as DHA are more resistant to oxidative deterioration when located at the *sn*-2 position compared to the *sn*-1(3) positions [111].

The effects of fatty acid positional distribution on absorption and nutrition of oils and fats are less well understood. Evidence from animal and human infant studies suggests that TAG structure affects digestibility, atherogenicity and fasting lipid levels, with fats containing palmitic and stearic acid in the *sn*-2 position being better digested and considered more harmful for cardiovascular health [112–114]. However, a few studies in human adults have indicated that fatty acid positional distribution has no effect on digestibility or fasting plasma lipids [115,116]. There have been very limited studies on the physiological effects of TAG positional distribution of omega-3 LC-PUFA such as EPA and DHA on either animals or humans. These fatty acids are predominantly located at the *sn*-2 position in fish oil TAG with the notable exception of seal blubber oil. Though it has been hypothesised, there are presently not sufficient data with humans to conclude that location of omega-3 LC-PUFA at the *sn*-2 position confers greater physiological benefit when compared to location at the *sn*-1 *or sn*-3 positions. We consider this as an area for further fruitful research including with animal model and clinical trials. The available evidence does not yet support a preference as to how the LC omega-3 containing TAG in novel oilseeds should be best assembled to maximize the nutritional benefits to human consumers. As we acquire this knowledge, the prospect of tailoring the DHA positional distribution of novel plant-based DHA oils (both during metabolic engineering and post-harvest) can be used to meet optimum health and food processing properties

11. Conclusions

The need for further clinical trials to better refine our understanding of the mode of action of the health-benefitting LC omega-3 oils will continue, including with emphasis towards the mode of action of the individual components namely EPA and DHA. Similar requirements exist for farmed species, in particular cultured fish. The latter are being increasingly fed non-marine oils, although limited research has occurred on the possible deleterious effects to the farmed species of the lower dietary proportions of LC omega-3 oils, accompanied by a lower omega-3/omega-6 ratio, and ultimately to human consumers. The issue of the finite supply of the fish oil resource is very clearly upon us, and new sustainable sources of these oils are required. SCO derived oils are in use, although remain, and likely will remain, relatively expensive and therefore better suited to niche applications. The past five years have seen expanded interest in applications with krill oil, although considerable care with exploitation of this environmentally sensitive and important resource must occur. After several decades of research for production of LC omega-3 oils from oil seeds, the prospects for such a supply are now a reality, including most recently the difficult to achieve yet nutritionally important DHA. LC omega-3 oils derived from GM oil seed crops may in the future provide the most economically viable source of these key essential ingredients for aquaculture and a range of other applications. It is estimated that the cost and availability of oils from GM plants would be similar to that of currently available commercial oilseed crops such as rapeseed and soya. Further research and development in this area has the potential for significant commercial, health, social and environmental benefits. This exciting field of research will now move into the commercial development phase, with feeding and other trials and associated approvals and consumer acceptance to occur. Other areas of research for continuing effort will include: improved processing and yields for pharmaceutical grade products, improvement and/or further development of novel delivery modes for application of LC omega-3 oils in functional foods, examination of the effects of omega-3 LC-PUFA positional distribution on the bioactivity and processing properties of various LC omega-3 food products, and also for the omega-3 index to gain increasing acceptance and use. Collectively research and development in these and other areas will ensure that enhanced intake of the LC omega-3 oils can occur for a wider range of consumers, with resultant global heath, economic and social benefits resulting.

Acknowledgements

We thank the wider CSIRO Food Futures Flagship Omega-3 research team for their contributions and Allan Green who also assisted in the preparation of Figure 1. Surinder Singh, James Petrie, Rick Phleger, Manny Noakes and Welma Stonehouse and three anonymous journal reviewers provided helpful review comments on the manuscript.

Author Contributions

All authors contributed to the review of the literature and the preparation of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Detailed Distribution of Lipids in Greenshell™ Mussel (*Perna canaliculus*)

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Abstract: Greenshell™ mussels (GSM–*Perna canaliculus*) are a source of omega-3 (*n*-3) long-chain polyunsaturated fatty acids (LC-PUFA). Farmed GSM are considered to be a sustainable source of LC-PUFA as they require no dietary inputs, gaining all of their oil by filter-feeding microorganisms from sea water. GSM oil is a high-value product, with a value as much as 1000 times that of fish oils. GSM oil has important health benefits, for example, anti-inflammatory activity. It also contains several minor lipid components that are not present in most fish oil products, and that have their own beneficial effects on human health. We have shown the lipid content of the female GSM (1.9 g/100 g ww) was significantly greater than that of the male (1.4 g/100 g ww). Compared with male GSM, female GSM contained more *n*-3 LC-PUFA, and stored a greater proportion of total lipid in the gonad and mantle. The higher lipid content in the female than the male GSM is most likely related to gamete production. This information will be useful to optimize extraction of oils from GSM, a local and sustainable source of *n*-3 LC-PUFA.

Reprinted from *Nutrients*. Cite as: Miller, M.R.; Pearce, L.; Bettjeman, B.I. Detailed Distribution of Lipids in Greenshell™ Mussel (*Perna canaliculus*). *Nutrients* **2014**, *6*, 1454–1474.

1. Introduction

The New Zealand Greenshell™ mussel (GSM), *Perna canaliculus*, is indigenous to the coastlines of New Zealand. GSM are distinguishable by the green coloration of the shell near the lip, which gives the mussel its name. Since the establishment of GSM aquaculture in New Zealand in 1969 [1], the GSM industry has seen consistent and substantial growth. In 2011, the export value of GSM was 218 million New Zealand dollars (increased from approximately 40 million NZ dollars in 1989) from a product volume of more than 38,000 t (~90,000 t at harvest) [2]. GSM are sold as food, and are also used to produce high-value nutraceuticals including oil extracts and freeze-dried mussel powders, for example, Lyprinol® and Seatone®. Studies have shown that lipid extracted from GSM has numerous health benefits, including the ability to reduce inflammation [3–13]. GSM lipid contains a high proportion of omega-3 long-chain ($C \geq 20$) polyunsaturated fatty acids (*n*-3 LC-PUFA), predominantly docosahexaenoic acid (DHA, 22:6*n*-3) and eicosapentaenoic acid (EPA, 20:5*n*-3), which are split between the triacyglycerol and polar lipid classes [14]. There are also several minor lipid components in GSM oil, including non-methylene-interrupted (NMI)-FA, plasmalogen, phytosterols, and furan fatty acids [14,15]. These minor lipids are not present in most fish oil products, and some have been shown to have beneficial effects on human health [15–18]. Because of the low concentration of oil in GSM [14,19] and the expensive extraction techniques (supercritical CO_2 with ethanol as a co-solvent, or chemical extraction) required to extract it, GSM oil is very expensive. The estimated price is NZ $3000/kg.

There has been extensive research confirming the health benefits of increased consumption of *n*-3 LC-PUFA, especially DHA and EPA [20–23]. Consequently, there is an increasing market for *n*-3 marine oil supplements. The proportion of *n*-3 LC-PUFA is higher in GSM oil [14] than in certain fish oils, such as those extracted from sardine, anchovy, and cod liver. GSM obtain *n*-3 LC-PUFA from their diet, which is rich in zooplankton and phytoplankton [24,25]. GSM are considered to be one of the most sustainable sources of *n*-3 LC-PUFA as they are farmed, rather than wild harvested, and do not require any dietary inputs for their nutrition. The lipid content and FA profile of GSM have been analyzed previously [14,19,26,27]. Those studies reported that polar lipids (PL) were the major lipid class (42.5–61.7 g/100 g) in GSM, followed by triacylglycerides (TAG) (17.8–49.2 g/100 g) with the remainder made up of sterols (5.5–6.8 g/100 g), free fatty acids (FFA) (2.9–14.9 g/100 g), and trace amounts of wax esters [14,19]. Previous studies reported that the concentration of PUFA in GSM lipid ranged from 19 to 49.1 g/100 g, with 6–12 g/100 g DHA, and 8–24 g/100 g EPA [14,28]. The lipid content of GSM, and the lipid classes and FA profile of GSM oil, are affected by many factors, including the season, location, and the types and amounts of algae consumed. To date, there have been no reports on the FA profiles of lipid extracted from different organs of the GSM.

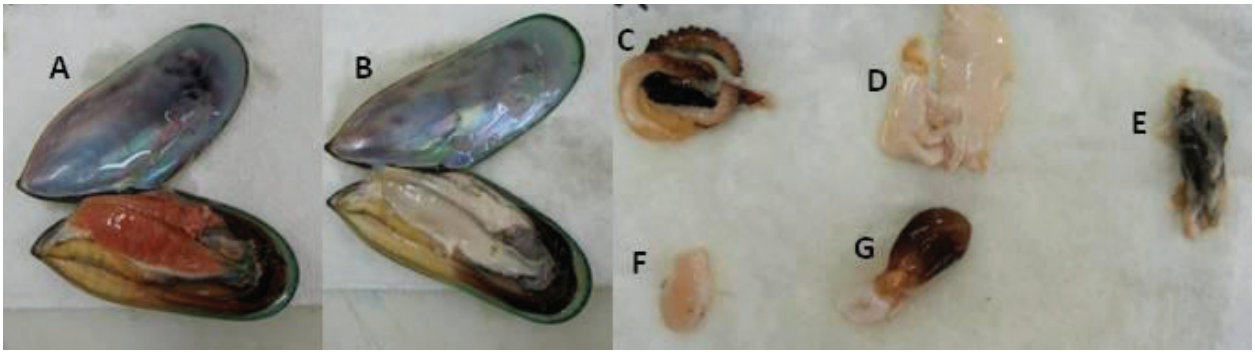
In this study, we investigated the lipid classes and fatty acid profile of GSM lipids, and analyzed the lipid content of male and female GSM. To analyze lipid localization within the GSM, we dissected the GSM body into five components: the mantle, gonad, heart and foot, posterior adductor muscle, and the digestive gland. A knowledge of the differences in the amounts and composition of lipids between genders, and of how physiological function affects GSM lipids, will be useful to optimize the lipid extraction process, which would benefit the GSM marine oil industry.

2. Experimental Section

2.1. Sampling

Whole live GSM were collected 12 November 2012 from the Marlborough Sounds (South Island, New Zealand) and stored in a recirculating sea water system until experiments began on 16 November 2012. After transport and dry storage, mussels were rehydrated for 24 h in 100 L bins supplied with 10 L/min filtered seawater on a flow-through basis with auxiliary aeration. The mussels were weighed, shucked, and then the mussel meat was weighed. 10 Female and 10 male mussels were selected for lipid extraction based on gonad color; gonads of males are creamy white while those of females are various shades of orange (Figure 1). The data for one mussel was excluded from the analysis because that individual was an outlier in terms of size and oil content. Six female and six male mussels were used for analyses of the various components of the GSM body. The shell and meat were weighed and then the meat was dissected into the mantle, gonad, posterior adductor muscle, heart/foot, and the digestive gland (Figure 1). The gonad was not able to be completely separated, and some gonad tissue remained attached to the digestive gland. Other un-dissectible smaller organs including the gill and labial palp were omitted from this analysis. All samples were stored at −80 °C prior to freeze drying before lipid extraction.

Figure 1. (**A**) Female Greenshell™ mussel (*Perna canaliculus*) with orange gonad; (**B**) Male Greenshell™ mussel with creamy white gonad. Dissected organs of a male Greenshell™ mussel: (**C**) mantle; (**D**) gonad; (**E**) digestive gland and digestive gland; (**F**) posterior adductor muscle; (**G**) heart and foot.



The mussels were assigned a condition index as follows:

$$\text{condition index (CI)} = \text{dry meat weight}/(\text{whole weight-shell weight}) \times 100 \text{ [29]} \quad (1)$$

2.2. Lipid Extraction, Fractionation, and Fatty Acid Analysis

GSM oils were extracted using a modified Bligh and Dyer protocol [30]. A single phase extraction with $CHCl_3$:MeOH:H_2O (1:1:0.9, v/v/v) yielded the total lipid extract (TLE). Lipid classes were analyzed with an Iatroscan MK V thin-layer chromatography-flame ionization detector (TLC-FID) (Iatron Laboratories, Tokyo, Japan). Samples were spotted onto silica gel SIII Chromarods (5-µm particle size) and developed in a glass tank lined with pre-soaked filter paper. The solvent system used for lipid separation was hexane: diethyl ether: acetic acid (60:17:0.1, v/v/v). After development for 25 min, the Chromarods were oven-dried and analyzed immediately to minimize adsorption of atmospheric contaminants. Lipid classes were quantified using Azur v5.0 software (DATALYS, St Martin D'Heres, France). The FID was calibrated for each compound class using the following compounds: phosphatidylcholine; cholesterol; cholesteryl ester; oleic acid; hydrocarbon (squalene); wax ester (derived from fish oil); and triacylglycerol (TAG, derived from fish oil).

An aliquot of the TLE from each sample type was trans-methylated in methanol: chloroform: hydrochloric acid (10:1:1, v/v/v) for 1 h at 100 °C. After addition of water the mixture was extracted three times with hexane: chloroform (4:1, v/v) to obtain fatty acid methyl esters (FAME). Samples were completed to 1 mL with an internal injection standard (23:0 or 19:0 FAME) and analyzed by gas chromatography mass spectrometry (GC-MS). The analytical system consisted of a Shimadzu 2010 QP GC-MS equipped a Restek GTx silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Samples (1 µL) were injected via a splitless injector at 220 °C. The column temperature program was as follows: 60 °C at 0 min; 40 °C min^{-1} to 100 °C; then 10 °C min^{-1} to 170 °C; then 5 °C min^{-1} to 185 °C; 2 min hold; then 3 °C min^{-1} to 197 °C; then 0.5 °C min^{-1} to 199 °C; 1 min hold; then 5 °C min to 230 °C; 3 min hold; then 5 °C min^{-1} to 250 °C; 5 min hold. Helium was the carrier gas. GC results were typically repeatable to within ±5% of the area of each individual component in replicate analyses.

Different classes of lipids were separated using a solid phase extraction (SPE) cartridge containing 500 mg silica (Grace Pure, Grace Davison Discovery Sciences, North Shore City, New Zealand). The SPE column was pre-treated with chloroform, and then a TLE sample from whole mussel, mantle, gonad, or digestive gland samples was loaded onto the column. Lipids were eluted with 10 mL chloroform followed by 10 mL methanol [31]. The oil fractions were concentrated by rotary evaporation and added to vials for analysis. Success of elution was confirmed via thin-layer chromatography (TLC).

2.3. Sterol Analysis

Sterols were isolated by saponifying 200 µL TLE in 5% w/v KOH in methanol/water (8:2 v/v) at 60 °C for 3 h. The mixture was cooled to room temperature and then 1 mL water was added. The sterols were then extracted twice using 1 mL hexane/chloroform (4:1). The extracted total non-saponifiable neutral (TSN) lipid fraction was transferred to labeled vials for further analysis. The TSN lipid fraction was blown down under a nitrogen stream and then heated at 60 °C with 50 µL *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 40 min. BSFTA was evaporated under nitrogen and the sample was made up to 2 mL with chloroform before GC-MS analysis. Samples were injected into the GC at an oven temperature of 100 °C, which was increased to 300 °C at a rate of 10 °C/min and then held for 30 min. The mobile phase carrier gas was He. The silylated sterols were identified by their retention times and characteristic peaks in the mass spectrum [32].

2.4. Statistical Analysis

Mean values and standard error are reported. Normality and homogeneity of variance were confirmed and mean values were compared by one-way analysis of variance (ANOVA). Multiple comparisons were conducted using the Tukey-Kramer HSD (honestly significant difference) test. Differences were considered significant at $p \leq 0.05$. Statistical analyses were performed using GenStat 14th Edition software.

3. Results

3.1. Lipid Content of Whole GSM

The lipid content of whole female GSM (1.9 g/100 g ww; 9.3 g/100 g dw) was significantly ($p < 0.01$) higher than that of male GSM (1.4 g/100 g ww; 7.4 g/100 g dw). The average total meat mass per individual was 18.3 g ± 3.3 g for females and 19.87 g ± 2.2 g for males. The Condition Index (CI) was 11.25 ± 2.0 for females and 9.57 ± 1.6 for males. There was no statistically significant difference between the two genders in the CI, size, or weight.

3.2. Lipid Classes of GSM

The major lipid class in both genders of GSM was PL. There was a significantly ($p < 0.03$) higher concentration of PL in males (82.3 g/100 g TLE) than in females (77.8 g/100 g TLE) (Table 1). The concentration of TAG in females (19.4 g/100 g TLE) was significantly higher ($p < 0.03$) than that in males (13.2 g/100 g TLE). The minor lipid classes in males and females were sterols (2.8–2.9 g/100 g TLE) and trace amounts (0.1 g/100 g TLE) of free fatty acids (FFA).

Table 1. Absolute FA (wet weight) and proportion of lipid classes (g/100 g TLE) in female and male Greenshell™ mussel (GSM) oil.

Fatty acids (mg/g mussel ww)	Female	Male	F	*p*
14:0	10.6 ± 2.1	7.3 ± 1.2	18.2	<0.001
16:0	47.3 ± 6.9	42.2 ± 6.1		
17:0	2.4 ± 0.4	2.6 ± 0.4		
18:0	11.7 ± 1.8	12.1 ± 2.0		
Other SFA	5.6 ± 1.1	5.4 ± 0.7		
Total SFA	77.7 ± 12.0	69.5 ± 9.6		
16:1*n*-7	18.3 ± 3.1	12.0 ± 2.0	26.5	<0.001
18:1*n*-9	3.0 ± 0.6	2.8 ± 0.7		
18:1*n*-7	7.6 ± 1.5	5.9 ± 1.0	7.7	<0.05
20:1*n*-9	9.8 ± 1.5	10.0 ± 2.1		
20:1*n*-7	4.3 ± 1.5	3.0 ± 0.6	5.9	<0.05
Other MUFA	1.7 ± 0.5	0.9 ± 0.3	15.5	<0.001
Total MUFA	44.7 ± 7.4	34.6 ± 5.1	11.4	<0.01
18:4*n*-3	5.7 ± 1.0	3.2 ± 0.7	37.8	<0.001
18:2*n*-6	3.7 ± 0.7	3.1 ± 0.6		
18:3*n*-3	2.7 ± 0.3	2.0 ± 0.5	14.1	<0.01
20:4*n*-6	4.6 ± 0.7	4.5 ± 1.0		
20:5*n*-3	60.9 ± 11.4	42.5 ± 6.3	18.0	<0.001
20:2	5.3 ± 0.7	5.8 ± 1.8		
20:2NMI	3.2 ± 0.5	2.7 ± 0.5		
22:6*n*-3	59.2 ± 10.2	52.9 ± 8.1		
22:5*n*-3	4.1 ± 0.7	3.2 ± 0.5	10.05	<0.01

Table 1. *Cont.*

22:2NMI	5.0 ± 0.8	4.7 ± 1.1		
Other PUFA	7.7 ± 1.5	7.6 ± 2.1		
Total PUFA	162.1 ± 25.4	132.2 ± 19.3	7.9	<0.01
Total *n*-3	132.8 ± 22.5	104.3 ± 14.5	10.2	<0.01
Total *n*-6	12.0 ± 1.7	11.1 ± 2.2		
Total other	9.7 ± 6.4	10.1 ± 7.8		
Lipid Classes (g/100 g TLE)				
TAG	19.4 ± 4.9	13.8 ± 3.2	5.8	0.03
ST	2.8 ± 1.3	2.9 ± 0.5		
FFA	tr	tr		
PL	77.8 ± 4.5	82.3 ± 3.2	6.0	0.03
Lipid content				
g lipid /100 g wet weight	1.9 ± 0.2	1.4 ± 0.3	14.7	0.001
g lipid/100 g dry weight	9.2 ± 1.1	7.4 ± 1.5	8.7	0.01

Values are mean ± standard error (n = 9). ww; wet weight; Other SFA: Sum of 15:0, 17:0, 20:0, 22:0 and 24:0; Other MUFA: Sum of 16:1*n*-5, 18:1*n*-7trans, 18:1*n*-5, 22:*n*-11 and 22:1*n*-9; Other PUFA: Sum of 16:2*n*-6, 18:3*n*-6, 20:4*n*-6, 20:2*n*-6, 22:5*n*-6, 22:4*n*-6 and 22:2*n*-6; Other includes fatty aldehydes and 4,8,12 trimethyl tetradecanoic acid (4,8,12-TMTD); NMI, non-methylene interrupted; TAG; triacylglycerols; PL: polar lipid; ST: sterols, FFA; free fatty acids; tr: trace.

3.3. FA Profiles of GSM

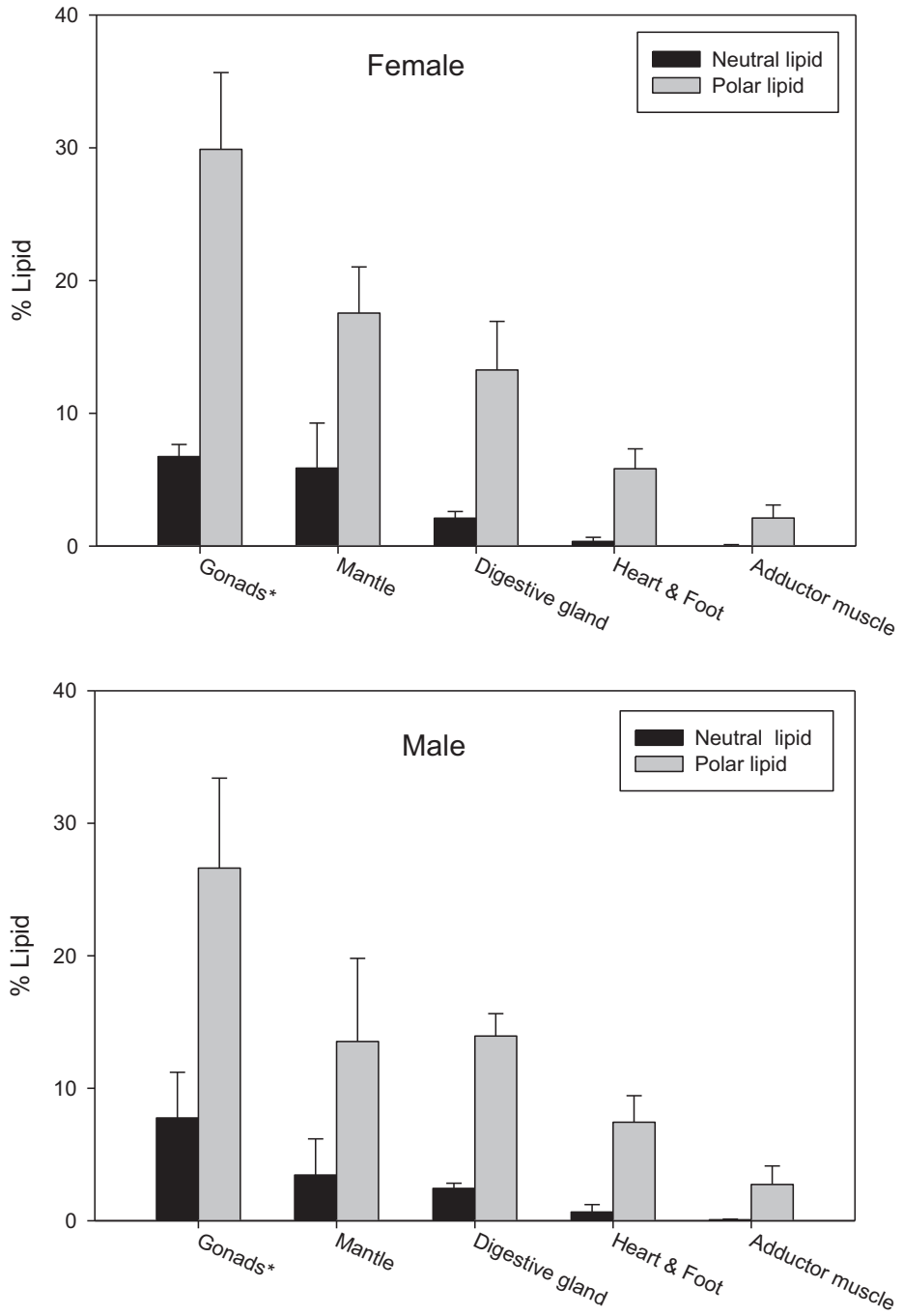
The major FA class (in % FA) was PUFA (53.8–55.9 g/100 g TLE), the main component of which was *n*-3 LC-PUFA (42.4–45.6 g/100 g TLE). The *n*-3/*n*-6 ratio was 6.6 in male GSM and 8.3 in female GSM. The major fatty acids (>10 g/100 g TLE) in both male and female GSM were EPA, DHA, and palmitic acid (16:0 PA). There were small amounts of NMI FA, including 20:2 NMI FA (2.8–3.4 g/100 g TLE) and 22:2 NMI FA (1.0–1.9 g/100 g TLE). The absolute FA content of the male and female (in mg/g ww) are shown in Table 1. Compared with male GSM, female GSM showed significantly ($p < 0.01$) higher concentrations of alpha-linolenic acid (ALA, 18:3*n*-3), stearidonic acid (SDA 18:4*n*-3), EPA, docosapentaenoic acid (DPA(*n*-3), 22:5*n*-3), total *n*-3s, and total PUFA.

3.4. Lipid Content and Lipid Classes in Different Organs

We used SPE to separate the non-polar and polar lipid fraction of TLE from the whole male and female GSM and from the various organs (Figure 2). The success of chromatographic separation was confirmed by TLC-FID. The most lipid-rich organs were the gonad (male, 3.1 g lipid/100 g ww; female, 4.0 g lipid/100 g ww), digestive gland (male, 3.1 g lipid/100 g ww; female, 3.7 g lipid/100 g ww) and mantle (1.5 g lipid/100 g ww; female, 2.0 g lipid/100 g ww). There were lower concentrations of lipid in the heart/foot (male, 1.3 g lipid/100 g ww; female, 1.0 g lipid/100 g ww) and adductor muscle (male, 1.2 g lipid/100 g ww; female, 0.8 g lipid/100 g ww) (Table 2, Supplementary Table S1). In all organs, PL was the major lipid class in the TLE. There were higher concentrations of TAG in the

digestive gland (male, 28.8 g/100 g TLE; female, 33.5 g/100 g TLE) and gonad (male, 38.5 g/100 g TLE; female, 33.7 g/100 g TLE) than in the other organs. The lowest concentrations of TAG were in the heart/ foot and adductor muscle samples (<8 g/100 g TLE). The main lipid class in the TLE from those samples was PL (>80 g/100 g TLE).

Figure 2. Lipid distribution in female and male Greenshell™ mussels (GSM) organs. * Gonad also contained gill and labial palp. Values are mean ± standard error (n = 9).



3.5. FA Profiles of Lipids from Different Organs

In both male and female GSM, there were no differences in the proportions of the major FA (those detected at more than 10 g/100 g TLE; *i.e.*, EPA, DHA, and PA) among the five different organs. For the minor FA, the proportions of SDA and ALA in the TLE from the digestive gland were significantly ($p < 0.01$) greater in the female GSM than the male GSM.

3.6. Sterols

Saponification of the TLE produced a total non-saponifiable neutral (TSN) fraction, which contained mainly sterols (Table 3). GC-MS analysis was performed after silylation of the TSN fraction. In total, 18 sterols were identified in GSM, with cholesterol being the predominant sterol. There was a significantly ($p < 0.01$) higher proportion cholesterol (30.2% of total sterols) in male GSM than in female GSM (28.5% of total sterols). The second most abundant sterol was brassicasterol; the proportion of brassicasterol was significantly ($p < 0.01$) higher in female (23.4% of total sterols) than in male GSM (21.05% of total sterols). Other major sterols were 24-nordehydrocholesterol, occelasterol, trans-22-dehydrocholesterol, and 24-methlenecholesterol.

4. Discussion

4.1. Male vs. Female GSM

In this study the lipid content of female GSM was significantly ($p < 0.01$) higher than that of male GSM even though it was previously reported that female mussels contain lower levels of total lipids [33]. Also, there was a significantly ($p < 0.01$) higher concentration of the TAG lipid class in the female than in the male. Because female GSM contained larger amounts of lipid than did males, the total amount of FA was higher in females than in males. In particular, the amounts of FA in the *n*-3 LC-PUFA class (ALA, SDA, EPA, and DPA) were all significantly ($p < 0.01$) higher in female than in male GSM. Differences in the lipid profile between male and female GSM have not been published previously, although a recent report showed similar results for the mussel species *Mytilus galloprovincialis* [34]. In another study, the FA composition of male and female GSM from two locations (Marlborough Sounds and Stewart Island) was profiled over three seasons (winter, spring, summer) but the types of lipids, that is, the lipid classes, were not reported [26]. Further, it was reported that GSM contained higher levels of lipids in summer and autumn than in winter and spring, but that the highest *n*-3 content was in winter [28]. In our study, we analyzed GSM collected in late spring (November) of 2012. Our results showed differences in lipid content, lipid classes, and total FA content between male and female GSM. In future research, it would be interesting to analyze changes in lipid content, lipid class composition, and the FA profile of male and female GSM on a finer time scale.

Table 2. Concentrations of fatty acids in oil extracted from mantle, gonad, and digestive gland of Greenshell™ mussels.

Fatty acids (g/100 g TLE)	Mantle		Gonad		Digestive gland		*f*	*p*
	Female	Male	Female	Male	Female	Male		
14:0	2.8 ± 0.2	3.4 ± 2.2	3.5 ± 0.3	2.9 ± 0.4	3.4 ± 0.7	3.0 ± 0.4		
16:0	15.5 ± 0.4	17.1 ± 4.3	16.5 ± 1.1	16.8 ± 1.5	17.1 ± 3.8	15.6 ± 1.3		
18:0	4.3 ± 0.4	4.5 ± 0.4	4.2 ± 0.3	5.2 ± 1.5	5.1 ± 1.6	4.8 ± 0.4		
Other SFA	1.8 ± 0.2 ^{ab}	1.8 ± 0.1 ^{ab}	1.7 ± 0.2 ^{a}	1.7 ± 0.1 ^{a}	1.7 ± 0.2 ^{ab}	2.4 ± 0.9 ^{b}	2.9	0.03
Total SFA	25.3 ± 0.4	27.7 ± 6.6	26.6 ± 1.4	27.5 ± 2.6	28.1 ± 6.3	26.5 ± 1.3		
16:1*n*-7	5.2 ± 0.6 ^{ab}	5.3 ± 1.8 ^{ab}	6.3 ± 0.4 ^{ab}	4.7 ± 1.6 ^{a}	6.8 ± 0.6 ^{b}	6.2 ± 0.8 ^{ab}	3.2	0.02
18:1*n*-9	1.3 ± 0.5 ^{ab}	0.9 ± 0.2 ^{a}	1.1 ± 0.3 ^{ab}	1.0 ± 0.2 ^{ab}	1.7 ± 0.6 ^{b}	1.4 ± 0.3 ^{ab}	3.0	0.03
18:1*n*-7	2.2 ± 0.3 ^{ab}	2.0 ± 0.2 ^{a}	2.7 ± 0.2 ^{c}	2.6 ± 0.4 ^{ab}	2.5 ± 0.2 ^{bc}	2.4 ± 0.2 ^{abc}	6.2	<0.001
20:1*n*-9	4.2 ± 0.4	4.2 ± 0.7	3.5 ± 0.3	3.5 ± 0.6	3.8 ± 0.5	3.7 ± 0.3		
20:1*n*-7	1.3 ± 0.1	1.0 ± 0.4	1.3 ± 0.1	1.2 ± 0.3	1.5 ± 0.4	1.2 ± 0.2		
Other MUFA	0.5 ± 0.2 ^{ab}	1.9 ± 1.3 ^{b}	0.5 ± 0.1 ^{ab}	0.3 ± 0.1 ^{a}	0.9 ± 0.8 ^{ab}	1.6 ± 1.6 ^{ab}	3.1	0.02
Total MUFA	14.6 ± 0.9 ^{ab}	15.3 ± 1.0 ^{bc}	15.3 ± 0.8 ^{bc}	13.3 ± 1.2 ^{a}	17.1 ± 1.3 ^{c}	16.4 ± 1.2 ^{bc}	9.4	<0.001
18:4*n*-3	1.2 ± 0.4 ^{ab}	1.0 ± 0.3 ^{a}	2.0 ± 0.4 ^{bc}	1.4 ± 0.6 ^{abc}	2.1 ± 0.6 ^{c}	2.1 ± 0.4 ^{c}	6.4	<0.001
18:2*n*-6	1.0 ± 0.2 ^{a}	1.0 ± 0.1 ^{a}	1.3 ± 0.3 ^{ab}	1.1 ± 0.2 ^{a}	1.5 ± 0.3 ^{b}	1.3 ± 0.2 ^{ab}	4.5	0.01
18:3*n*-3	0.6 ± 0.2 ^{a}	0.7 ± 0.2 ^{ab}	1.0 ± 0.2 ^{ab}	0.8 ± 0.3 ^{ab}	1.0 ± 0.2 ^{b}	1.0 ± 0.1 ^{b}	4.4	0.01
20:4*n*-6	1.9 ± 0.2 ^{b}	1.9 ± 0.5 ^{b}	1.3 ± 0.1 ^{a}	1.2 ± 0.1 ^{a}	1.3 ± 0.2 ^{a}	1.3 ± 0.2 ^{a}	9.3	<0.001
20:5*n*-3	18.9 ± 1.5	17.9 ± 1.5	22.0 ± 1.5	19.5 ± 3.7	18.1 ± 4.0	17.6 ± 3.0		
20:2NMI	2.9 ± 0.7	3.0 ± 0.9	1.6 ± 0.2	1.2 ± 0.2	1.9 ± 0.3	1.7 ± 0.2		
20:4*n*-6	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	1.3 ± 0.2	1.3 ± 0.3		
20:2*n*-6	1.5 ± 0.3 ^{c}	1.4 ± 0.3 ^{bc}	0.8 ± 0.1 ^{a}	0.7 ± 0.1 ^{a}	1.0 ± 0.1 ^{ab}	0.9 ± 0.1 ^{a}	14.8	<0.001
22:6*n*-3	22.0 ± 1.4	20.5 ± 4.0	19.2 ± 1.3	21.9 ± 2.4	17.9 ± 3.8	19.1 ± 1.5		
22:5*n*-3	1.5 ± 0.3	1.3 ± 0.4	1.2 ± 0.1	2.0 ± 2.1	1.4 ± 0.3	1.1 ± 0.1		
22:2NMI	2.2 ± 0.4 ^{b}	1.9 ± 1.0 ^{ab}	1.2 ± 0.1 ^{a}	1.2 ± 0.2 ^{a}	1.7 ± 0.3 ^{ab}	1.6 ± 0.3 ^{ab}	4.4	0.01

Table 2. *Cont.*

Fatty acids (g/100 g TLE)	Mantle		Gonad		Digestive gland		*f*	*p*
	Female	Male	Female	Male	Female	Male		
Other PUFA	1.8 ± 0.3 ^{ab}	1.8 ± 0.5^{a}	2.9 ± 2.0 ^{ab}	2.6 ± 1.3 ^{ab}	3.3 ± 1.4 ^{ab}	4.4 ± 2.2 ^{b}	2.7	0.04
Total PUFA	56.0 ± 2.3	52.8 ± 7.1	54.0 ± 1.8	54.5 ± 2.5	51.6 ± 7.1	52.5 ± 2.5		
Total *n*-3	44.2 ± 1.6	41.5 ± 4.5	45.4 ± 1.9	45.7 ± 4.1	40.7 ± 8.1	41.2 ± 4.0		
Total *n*-6	5.5 ± 0.5	5.2 ± 1.0	4.8 ± 0.8	4.9 ± 0.9	6.0 ± 1.1	6.7 ± 1.8		
Total other	3.9 ± 1.8	3.8 ± 1.4	2.9 ± 2.1	4.4 ± 1.5	2.8 ± 1.1	4.2 ± 1.7		
Lipids class (g/100 g)								
TAG	22.9 ± 8.0 ^{ab}	18.4 ± 6.0 ^{a}	33.7 ± 4.3 ^{ab}	38.5 ± 17.6 ^{b}	33.5 ± 10.8 ^{ab}	28.8 ± 8.1 ^{ab}	3.3	0.02
ST	3.5 ± 0.9	3.2 ± 0.4	1.2 ± 0.6	6.1 ± 11.0	1.0 ± 1.1	1.7 ± 0.6		
PL	73.5 ± 7.4 ^{b}	77.3 ± 6.4 ^{b}	64.5 ± 4.0 ^{ab}	54.6 ± 12.7 ^{a}	65.1 ± 10.6 ^{ab}	68.7 ± 7.6 ^{ab}	5.1	0.002
Lipid content								
g lipid /100 g wet weight	2.0 ± 0.4 ^{a}	1.5 ± 0.7 ^{a}	4.0 ± 0.6 ^{b}	3.1 ± 0.6 ^{b}	3.7 ± 0.3 ^{b}	3.1 ± 0.3 ^{b}	19.8	<0.001
g lipid/100 g dry weight	11.8 ± 1.8 ^{b}	7.9 ± 3.6 ^{a}	13.3 ± 1.4 ^{b}	11.7 ± 2.0 ^{b}	13.2 ± 1.0 ^{b}	11.9 ± 1.1 ^{b}	5.6	<0.001

TLE: Total lipid extract. Values are mean ± standard error (n = 6). Other SFA: Sum of 15:0, 17:0, 20:0, 22:0, and 24:0. Other MUFA: Sum of 16:1*n*-5, 18:1*n*-7trans, 18:1*n*-5, 22:1*n*-11, and 22:1*n*-9. Other PUFA: Sum of 16:2*n*-6, 18:3*n*-6, 20:4*n*-6, 20:2*n*-6, 22:5*n*-6, 22:4*n*-6, and 22:2*n*-6. Other includes fatty aldehydes and 4,8,12 trimethyl tetradecanoic acid (4,8,12-TMTD). TAG: triacylglycerols, PL: polar lipid; ST: sterols.

Table 3. Sterol content of female and male Greenshell™ mussel (GSM) oil extract.

Sterols g/100 g total sterols	Sterol name	Female	Male	f	*p*
24-nordehydrocholesterol	24-nordecholesta-5,22*E*-dien-3β-ol	6.3 ± 0.9	6.7 ± 0.4		
24-nordehydrocholestanol	24-nor-5α-cholest-22*E*-en-3β-ol	0.1 ± 0.0	0.2 ± 0.0		
Occelasterol	27-nor-24-methylcholest-5,22*E*-dien-3β-ol	4.3 ± 0.3	4.5 ± 0.3		
Trans-22-dehydrocholesterol	Cholesta-5,22*E*-dien-3β-ol	12.4 ± 0.7	13.1 ± 0.8		
Trans-22-dehydrocholestanol	5α-cholesta-22*E*-en-3β-ol	0.3 ± 0.1	0.2 ± 0.1		
Cholesterol	Cholest-5-en-3β-ol	28.5 ± 1.5	30.2 ± 0.8	11.1	0.004
Cholestanol	5α-cholestan-3β-ol	0.3 ± 0.2	0.5 ± 0.4		
Brassicasterol	24-methylcholesta-5,22*E*-diene-3β-ol	23.4 ± 0.9	21.0 ± 1.4	21.3	<0.001
Brassicastanol	24-methyl-5α-cholest-22*E*-en-3β-ol	0.3 ± 0.3	0.3 ± 0.2		
Ergosterol	24-methylcholesta-5,7,22*E*-triene-3β-ol	0.6 ± 0.4	0.0 ± 0.1	16.6	<0.001
24-Methylenecholesterol	24-methylcholesta-5,24(28)-en-3β-ol	16.2 ± 1.2	16.6 ± 1.6		
24-Methylcholesterol	4-methyl-5α-cholestan-3β-ol	1.9 ± 0.4	1.6 ± 0.7		
Stigmastanol/Porifasterol	24-ethyl-5α-cholesta-5,22*E*-diene-3β-ol	0.6 ± 0.2	0.7 ± 0.2		
Sitosterol	24-ethyl-5α-cholestan-3β-ol	1.8 ± 0.4	2.1 ± 0.3		
Isofucosterol	24-ethylcholesta-5,24(28)*Z*-dien-3β-ol	1.7 ± 0.4	1.7 ± 0.4		
Unknown sterols		1.3 ± 0.1	0.3 ± 0.1	40.8	<0.001

Values are mean ± standard error (n = 9); Unknown sterols could not be identified from MS data.

There is significant biological investment by the GSM into the production/storage of lipids during gonad development during winter, before spawning [28]. In general, the amount of lipid increases in GSM before spawning, and decreases to its lowest level immediately after spawning as a result of lipid loss during reproduction. GSM can also spawn in autumn (March–April) or shed eggs throughout their life cycle [28]. These reproductive events could explain differences in FA and lipid accumulation throughout the seasons. The higher lipid content in the female GSM is likely associated with oogenesis, during which lipid globules and small quantities of glycogen accumulate in the eggs [35]. There is a large variation in the timing and duration of gonad development in GSM and other mollusks, and the timing of gametogenesis varies according to the season and location [35]. A better understanding of the reproductive cycle, and how it affects the amount of lipid in GSM, may allow GSM oil producers to optimize the harvest time to obtain higher lipid yields.

Little is known about FA biosynthesis in GSM. In general, most bivalve species gain the majority of FA from their diet, although biochemical modifications of some FA occur in some species. The idea of "you are what you eat" has led to the study of signature lipids that can help to shed light on the prey-predator relationships in ecosystems. When analyzed using high-powered statistical models, signature FA profiles can reveal prey-predator relationships in a food web. GSM are filter feeders that consume a variety of phytoplankton and zooplankton [24,25]. Recently, studies on feeding blue mussels (*Mytilus edulis*) organic waste from an aquaculture facility showed that when they were fed on a non-traditional (fish waste) diet, they did not feed or grow, but instead showed significant decreases in growth and total lipid content [36,37]. Phytoplankton and zooplankton are high in *n*-3 LC-PUFA, which results in the FA profile of GSM oil being rich in EPA and DHA. The types of FA present in the food sources of GSM will depend on factors such as season, location, and temperature. The similarity in the relative proportions of FA (g/100 g TLE) in males and females (and even among organs) suggested that there was little adaption of FA from their shared diet by either gender. However, the

larger amount of lipid in the female GSM suggests that it has a greater capacity than the male GSM to store, and possibly biosynthesize, FA, especially *n*-3 LC-PUFA.

The biosynthetic pathway for *n*-3 LC-PUFA is unknown in GSM. Vertebrates have a poor capacity to produce DHA from precursors via the Sprecher pathway [38]. However, there is some evidence that invertebrates, such as the nematode *Caenorhabditis elegans*, have different pathways to produce *n*-3 LC-PUFA [39]. The FA results confirmed that the major biosynthetic precursors in the *n*-3 LC-PUFA pathway are present in the FA profile of GSM. These biosynthetic precursors include ALA, SDA, EPA, and DPA (*n*-3), with DHA being the final product. All of these FA except for DHA were present in significantly ($p < 0.01$) higher proportions of total FA in the female than in the male GSM. The higher concentrations of ALA, SDA, EPA, and DPA (*n*-3) may indicate that the female GSM has a greater *n*-3 LC-PUFA biosynthetic capacity during gametogenesis. Furthermore, in the digestive gland, the proportions of ALA and SDA in the FA profile were significantly ($p < 0.01$) higher in female than in male GSM. This may indicate a higher rate of FA biosynthesis in the digestive gland before lipids are transported for storage in the gonad. However, these FA results could also indicate that female GSM have an enhanced capacity to filter, process, and store *n*-3 LC-PUFA. To date, there have been no published reports of feeding trials to clarify aspects of lipid metabolism and/or storage in GSM. Therefore, it remains a matter of speculation whether the higher concentrations of FA in female than in male GSM are a result of enhanced biosynthesis or greater storage capacity.

Minor bioactive FA present in the GSM profiles included NMI PUFA, 4,8,12 trimethyl tetradecanoic acid (4,8,12-TMTD), and fatty aldehydes, all of which have been reported previously [14,19]. NMI FA are present in various mollusks at concentrations of up to 20% of wet weight [16]. Mollusks can synthesize NMI, which are believed to have structural and functional roles in biological membranes [16], via biosynthetic pathways that are still not fully understood. Our results showed that there were differences in the amount of NMI between male and female GSM, and that there was significantly less NMI in the gonad than in the other organs. Fatty aldehydes and 4,8,12-TMTD were present in GSM, but at very low concentrations (<1 g/100 g TLE), and were not included in the FA profiles. The amounts of both 4,8,12-TMTD and fatty aldehydes were substantially lower than those detected in our previous analyses of GSM that were collected from a similar location but at a slightly earlier time of the year (August and September 2009) [14]. Both NMI and 4,8,12-TMTD are possible indicators of red algae and/or some zooplanktonic pteropods, which could have been directly or indirectly consumed [19]. However, in other mollusks such as the green abalone, *Haliotis fulgens*, there is some evidence that 20:2 and 22:2 NMI may be metabolic products of desaturation of LC-MUFA [40]. Therefore, their presence may indicate an endogenous biosynthetic capacity of GSM.

4.2. Differences among Organs

Although the gonad contained the highest concentrations of lipid, the digestive gland also contained high lipid concentrations, which may be indicative of lipid-rich stomach contents. The lipid extracted from the digestive gland was darker and stickier than that extracted from the other organs. The dark color may represent non-enzymatic browning during extraction; this reaction occurs between oxidized lipids and primary amine groups, and has been observed in PL emulsions [41]. The digestive gland will likely contain the highest concentrations of endogenous lipases and low pH which may lead to the

oxidization of lipid because of the more extreme conditions. Further, the dark color could be due to pigment residues derived from algae from the GSM diet

Generally, different lipid classes perform different functions in biological systems. Neutral lipids (*i.e.*, TAG) are used as energy storage, while PL are mainly components of structural and functional parts of the cell. The lipid content of bivalves is directly linked to the gametogenic cycle, as TAGs and PLs play important roles as structural components and energy reserves in the gonads and gametes and during embryonic development [35]. Previous studies have shown the accumulation of neutral lipid reserves in eggs for a number of bivalve species, although studies looking at lipid production in GSM are not as detailed with regards to the effects of the gametogenic cycle [26,28]. It has been suggested that the digestive gland plays an important role in storing metabolic reserves, such as lipids, for use in gametogenesis and during periods of stress [35,42]. As previously mentioned, the higher proportion of *n*-3 LC-PUFA biosynthetic precursors (ALA and SDA) in the GSM digestive gland indicates that this organ is the most likely site of FA biosynthesis, and that these FA may have structural and functional roles in gamete development. The GSM analyzed in this study were collected in single sampling during a period of gametic growth; therefore, we cannot comment on changes in lipid composition during the year.

The mantle was the largest of the organs analyzed in this study, and accounts for approximately 20% of the wet weight of the mussel (Figure 2). The mantle is an important store of lipids in GSM; our results showed that this organ contained 21.5% (male mussels) and 28.0% (female mussels) of the total lipid. The main lipid class in the mantle was PL (73.5 g/100 g TLE in females; 77.3 g/100 g TLE in males) because of the large amount of structural tissue in this organ. The mantle consists of vascular connective tissue and plays a role in directing particles to the gills and deflecting other materials [35]. Our results suggested that the mantle of GSM also functions in lipid storage. The proportion of neutral lipids, which are generally used for storage, was higher in the mantle (22.9 g/100 g TLE in females; 18.4 g/100 g TLE in males) than in organs such as the adductor muscle (<3.0 g/100 g of TLE), which have different functions.

The three major organs of the GSM that store and use lipids are the gonad, digestive gland, and mantle. Our results showed that the adductor muscle and the heart and foot contained lower concentrations of lipids (3.9 g/100 g of TLE for adductor muscle and 5.4 g/100 g of TLE for heat and foot). The lipid extracted from the adductor muscle and the heart and foot showed similar FA profiles to that of the mantle. In the TLE from both the adductor muscle and the heart and foot samples, the major lipid class was PL (83.1–91.78 g/100 g of TLE) with minor amounts of TAG (1.9–7.5 g/100 g of TLE) and sterols (4.7–8.1 g/100 g of TLE). Profiles are included in the Supplementary Files. In general, the differences in lipid classes among different organs and between genders were minimal, suggesting that the diet is the main source of these FA and has the greatest influence on FA composition.

4.3. Sterols

The sterols in marine invertebrates are generally derived from sterols in the diet. However, some unconventional sterols are more common in primitive invertebrates, while cholesterol is found in greater proportions in more complex organisms [43]. The sterol profile of GSM reported here (Table 3) is similar to that of the only other published GSM sterol profile [19]. In a previous study,

GSM were sampled from a similar region (Marlborough Sounds) at a similar time of year (October, compared with our sampling time in early November). In that study, there were only minor differences in the sterol profile among samples collected from four different sites [19]. This finding suggested that there was little difference in the diets of GSM among sites.

Phytosterols have wide bioactivity in humans. In particular, they are considered to be effective in lowering cholesterol, and consequently may have a preventive role against vascular disease [18,44]. Further beneficial properties of phytosterols include cancer prevention [18,45]. Phytosterols and cholesterol are likely metabolized by internal and external microorganisms into other bioactive substances. Phytosterols cannot be synthesized by humans and, therefore, can only be obtained through the diet. However, GSM may have some capacity to biosynthesize cholesterol into different phytosterols depending on their stage of the life cycle, sexual maturity, and gender [46]. As they are lipophilic substances with positive health benefits, phytosterols have been added to margarines and spreads to create "functional" foods. The amount of phytosterols derived from land sources is increasing in the marine environment as a result of land base ingredients increased used in aquafeeds. Changes from marine to terrestrial sources of protein and oil for use in aquafeeds has resulted in increased amounts and different types of phytosterols in farmed Atlantic salmon [47]. Here, we report that GSM oil contained approximately 3% phytosterols, of which about one-third was cholesterol. Larger amounts of sterols (5.5%–6.9%) have been reported previously for GSM [19]. Phytosterols such as isofucosterol and occelasterol are not common in our diets as they are not typical in terrestrial food sources, but may have novel beneficial functionalities. Isofucosterol and occelasterol are most likely derived from marine algae. In future research, it would be interesting to investigate whether novel phytosterols, along with the high content of ω3 LC-PUFA in GSM, can provide increased protection against coronary heart disease in humans.

4.4. Implications of Results for the GSM Industry

The major implications of this work are for the GSM oil industry, which is growing in both value and volume. A detailed understanding of the location of *n*-3 LC-PUFA in GSM may allow novel processing techniques to be established. The firmer parts of the mussel (posterior adductor muscle, heart and foot) contained very low concentrations of oil and *n*-3 LC-PUFA, and removal of these organs would reduce the amount of material processed using expensive freeze-drying and super-critical oil extraction procedures. This could potentially improve yields and benefit producers. Further, development of all-female lines of mussels would give mussel oil producers a greater source of oil and *n*-3 LC-PUFA. We estimate that oil yields could increase by as much as 35%, *n*-3 yields by 27%, and EPA yields by 43% if all-female mussels were extracted at the appropriate time of year. Highest yields could be obtained if female GSM were harvested in the peak reproductive state, since the gonad is the major storage centre for lipid. Higher investment in reproduction by the female GSM gonad may be to supply ample nutrition to offspring/eggs. The FA data suggested that female GSM have a higher biosynthetic capacity than that of males, and that they also store more lipid than do males. Compared with male GSM, female GSM contained larger amounts of the *n*-3 LC-PUFA biosynthetic precursors SDA and ALA. If both genders obtain the same FA from the shared diet, the increase in these biosynthetic precursors provides further evidence of an enhanced biosynthetic capacity in female GSM.

5. Conclusions

The lipid profiles of GSM were evaluated on the basis of gender and anatomy. The lipid content of the female GSM (1.9 g/100 g ww) was significantly greater than that of the male (1.4 g/100 g ww). The major lipid class in both genders was PL. Compared with male GSM, female GSM contained more *n*-3 LC-PUFA, and stored a greater proportion of total lipid in the gonad and mantle. The higher lipid content in the female than the male GSM is most likely related to gamete production. The mantle and digestive gland were other important sites for lipid storage and/or function/production. Novel bioactives, such as NMI-FA, plasmalogens, and phytosterols were identified in GSM oil.

Acknowledgments

We thank Helen Cleaver for technical assistance. This research was supported by MBIE (NZ Ministry of Business, Innovation and Employment) under project C02X0806, "Engineered Marine Molecules" with additional funding from MBIE programme "Export Marine Products".

Author Contributions

Matthew R. Miller made substantial contributions to conception and design, interpretation of data and development of manuscript. Luke Pearce and Bodhi Bettjeman made substantial contributions to acquisition of data, analysis and interpretation of data and development of manuscript.

Abbreviations

ANOVA, 1-way analysis of variance
ALA, alpha-linolenic acid
BSTFA, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide
CI, Condition index
DHA, docosahexaenoic acid
DPA, docosapentaenoic acid
EPA, eicosapentaenoic acid
FA, fatty acid(s)
FFA, free fatty acid(s)
FALD, dimethylacetals of aliphatic aldehydes
FAME, fatty acid(s) methyl ester
GC-MS, gas chromatography mass spectrometry
GSM, Greenshell™ mussel
HSD, honestly significant difference
LC, long chain ($C \geq 20$)
MUFA, monounsaturated fatty acid(s)
NMI, non-methylene interrupted
TSN, non-saponifiable neutral
n-3, Omega 3
n-6, Omega 6

PUFA, polyunsaturated fatty acid(s)
SDA, stearidonic acid
SFA, saturated fatty acid(s)
TAG, triacylglycerol
TLE, total lipid extract
TMTD, trimethyl tetradecanoic acid
tr, trace amounts

Supplementary Information

Table S1. Fatty acid concentration (mg/g) of Greenshell™ mussels. Polar and neutral lipid fractions were separated by solid phase extraction (SPE).

Fatty acid	Female	Male	Female	Male	F	*p*
(mg/g ww)	Polar lipid	Polar lipid	Neutral lipid	Neutral lipid		
14:0	5.3 ± 0.9 ^{c}	3.5 ± 1.1 ^{b}	1.9 ± 0.9 ^{a}	0.9 ± 0.5 ^{a}	50.8	<0.001
16:0	23.2 ± 2.8 ^{b}	19.8 ± 4.3 ^{b}	5.9 ± 2.7 ^{a}	3.0^{a} ± 1.6 ^{a}	110.8	<0.001
18:0	5.6 ± 0.8 ^{b}	5.8 ± 1.3 ^{b}	1.0 ± 0.5 ^{a}	0.7 ± 0.3 ^{a}	113.0	<0.001
Other SFA	2.6 ± 0.4 ^{b}	2.5 ± 0.7 ^{b}	0.5 ± 0.3 ^{a}	0.3 ± 0.2^{a}	84.8	<0.001
Total SFA	37.9 ± 4.5 ^{b}	32.8 ± 7.4 ^{b}	9.5 ± 4.4 ^{a}	5.2 ± 2.7 ^{a}	105.7	<0.001
16:1*n*-7	9.2 ± 1.6 ^{b}	5.7 ± 1.8 ^{b}	3.3 ± 1.7 ^{a}	1.6 ± 1.0 ^{a}	45.5	<0.001
18:1*n*-9	1.5 ± 0.4 ^{b}	1.3 ± 0.3 ^{b}	0.6 ± 0.3 ^{a}	0.4 ± 0.2 ^{a}	26.6	<0.001
18:1*n*-7	3.7 ± 0.5 ^{b}	2.9 ± 0.9^{b}	1.1 ± 0.6 ^{a}	0.6 ± 0.3 ^{a}	59.5	<0.001
20:1*n*-9	4.7 ± 0.7 ^{b}	4.6 ± 0.8 ^{b}	0.7 ± 0.3	0.6^{a} ± 0.2^{a}	223.3	<0.001
20:1*n*-7	2.0 ± 0.6 ^{c}	1.4 ± 0.4 ^{b}	0.5 ± 0.2 ^{a}	0.3 ± 0.1 ^{a}	28.5	<0.001
Other MUFA	0.8 ± 0.2 ^{c}	0.4 ± 0.2 ^{b}	0.3 ± 0.1 ^{ab}	0.1 ± 0.1 ^{a}	27.6	<0.001
Total MUFA	21.7 ± 2.8 ^{c}	16.3 ± 4.0 ^{b}	6.5 ± 2.9 ^{a}	3.6 ± 1.8 ^{a}	80.8	<0.001
18:4*n*-3	2.8 ± 0.4 ^{d}	1.5 ± 0.5 ^{c}	1.1 ± 0.6 ^{b}	0.5 ± 0.3^{a}	42.2	<0.001
18:2*n*-6	1.8 ± 0.3 ^{b}	1.5 ± 0.4 ^{b}	0.5 ± 0.3 ^{a}	0.3 ± 0.2 ^{a}	48.2	<0.001
18:3*n*-3	1.3 ± 0.3 ^{c}	0.9 ± 0.2 ^{b}	0.4 ± 0.3 ^{a}	0.2 ± 0.1 ^{a}	45.9	<0.001
20:4*n*-6	2.2 ± 0.3 ^{b}	2.1 ± 0.4 ^{b}	0.2 ± 0.1 ^{a}	0.2 ± 0.1 ^{a}	201.5	<0.001
20:5*n*-3	30.5 ± 5.2 ^{c}	20.5 ± 5.7 ^{b}	7.5 ± 3.2 ^{a}	4.1 ± 1.9 ^{a}	80.0	<0.001
20:2*n*-6	2.6 ± 0.5 ^{b}	2.6 ± 0.4 ^{b}	0.3 ± 0.1 ^{a}	0.2 ± 0.0 ^{a}	172.2	<0.001
20:2NMI	1.5 ± 0.1 ^{b}	1.3 ± 0.3 ^{b}	0.4 ± 0.1 ^{a}	0.2 ± 0.1 ^{a}	146.7	<0.001
22:5*n*-6	0.3 ± 0.1 ^{b}	0.3 ± 0.1 ^{b}	0.1 ± 0.1 ^{a}	0.1 ± 0.0 ^{a}	6.8	<0.001
22:6*n*-3	28.7 ± 3.7 ^{b}	24.8 ± 4.9 ^{b}	6.3 ± 3.3 ^{a}	3.9 ± 1.7 ^{a}	121.9	<0.001
22:5*n*-3	2.0 ± 0.2 ^{c}	1.5 ± 0.4 ^{b}	0.3 ± 0.2 ^{a}	0.2 ± 0.1 ^{a}	139.4	<0.001
22:2NMI	2.4 ± 0.4 ^{b}	2.2 ± 0.4 ^{b}	0.2 ± 0.1 ^{a}	0.2 ± 0.0 ^{a}	177.7	<0.001
Other PUFA	4.3 ± 1.0 ^{b}	4.1 ± 1.4 ^{b}	1.3 ± 1.8^{a}	0.8 ± 0.6 ^{a}	20.7	<0.001
Total PUFA	79.5 ± 9.6 ^{c}	62.7 ± 14.0 ^{b}	17.8 ± 7.9 ^{a}	10.4 ± 4.5 ^{a}	112.7	<0.001
Total *n*-3	65.5 ± 8.6 ^{c}	49.5 ± 11.6 ^{b}	15.6 ± 6.9 ^{a}	9.0 ± 3.9 ^{a}	107.5	<0.001
Total *n*-6	5.8 ± 1.1 ^{b}	5.5 ± 1.7 ^{b}	1.1 ± 0.6 ^{a}	0.8 ± 0.3 ^{a}	62.73	<0.001
Total other	4.3 ± 2.9 ^{b}	3.9 ± 2.5 ^{b}	0.2 ± 0.1 ^{a}	0.2 ± 0.1 ^{a}	11.2	<0.001

Values are mean ± standard error (n=9). ww; wet weight; Other SFA: Sum of 15:0, 17:0, 20:0, 22:0 and 24:0; Other MUFA: Sum of 16:1*n*-5, 18:1*n*-7trans, 18:1*n*-5, 22:*n*-11, and 22:1*n*-9; Other PUFA: Sum of 16:2*n*-6, 18:3*n*-6, 20:4*n*-6, 20:2*n*-6, 22:5*n*-6, 22:4*n*-6, and 22:2*n*-6; Other includes fatty aldehydes and 4,8,12 trimethyl tetradecanoic acid (4,8,12-TMTD).

Table S2. Fatty acid concentrations in oil extracted from Greenshell™ mussel (GSM) adductor muscle and heart and foot.

Fatty acids (g/100 g TLE)	Adductor Muscle		Heart & foot	
	Female	Male	Female	Male
14:0	1.9 ± 0.6	2.4 ± 0.5	2.6 ± 0.5	2.3 ± 0.7
16:0	18.6 ± 6.1	26.3 ± 10.1	19.0 ± 1.8	16.1 ± 4.1
17:0	1.4 ± 0.5	1.5 ± 0.4	1.5 ± 0.2	1.3 ± 0.2
18:0	5.6 ± 0.9	6.8 ± 2.7	5.2 ± 0.3	5.0 ± 0.4
Other SFA	1.7 ± 0.5	1.3 ± 0.5	2.6 ± 0.2	2.4 ± 0.5
Total SFA	29.2 ± 7.9	38.1 ± 13.4	30.8 ± 2.4	27.1 ± 5.3
16:1*n*-7	2.0 ± 1.2	2.2 ± 0.6	4.4 ± 0.7	4.0 ± 1.2
18:1*n*-9	0.7 ± 0.2	0.7 ± 0.3	0.8 ± 0.1	0.8 ± 0.2
18:1*n*-7	2.6 ± 0.8	2.1 ± 0.7	1.9 ± 0.2	1.9 ± 0.3
20:1*n*-9	4.3 ± 0.7	3.5 ± 0.9	4.7 ± 0.6	4.8 ± 0.3
20:1*n*-7	0.9 ± 0.1	0.6 ± 0.3	1.1 ± 0.1	1.2 ± 0.2
Other MUFA	0.4 ± 0.4	0.6 ± 1.0	0.4 ± 0.0	0.4 ± 0.1
Total MUFA	10.8 ± 1.1	9.8 ± 2.3	13.2 ± 0.7	13.2 ± 1.1
18:4*n*-3	0.8 ± 0.5	0.3 ± 0.4	0.7 ± 0.3	0.8 ± 0.2
18:2*n*-6	1.5 ± 0.5	1.1 ± 0.6	1.5 ± 0.2	1.5 ± 0.1
18:3*n*-3	0.8 ± 0.3	0.6 ± 0.4	0.8 ± 0.1	0.8 ± 0.1
20:4*n*-6	1.9 ± 0.3	1.2 ± 0.6	3.1 ± 0.4	3.2 ± 0.2
20:5*n*-3	13.5 ± 1.8	12.0 ± 1.0	11.3 ± 1.5	12.9 ± 3
20:2NMI	1.0 ± 0.3	0.5 ± 0.4	1.8 ± 0.3	1.7 ± 0.2
20:4*n*-6	0.4 ± 0.2	0.2 ± 0.2	0.5 ± 0.1	0.6 ± 3.2
20:2*n*-6	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
22:6*n*-3	25.3 ± 6.4	23.7 ± 10.8	19.6 ± 3.0	21.7 ± 0.2
22:5*n*-3	3.2 ± 0.9	2.4 ± 1.8	1.7 ± 0.4	1.9 ± 0.1
22:3	0.5 ± 0.1	0.3 ± 0.3	0.8 ± 0.2	0.9 ± 0.4
22:2NMI	2.1 ± 0.8	1.7 ± 1.5	2.3 ± 0.4	2.4 ± 0.2
Other PUFA	1.5 ± 0.5	1.0 ± 0.5	1.8 ± 0.2	2.2 ± 0.2
Total PUFA	55.9 ± 9.6	47.6 ± 13.0	50.5 ± 3.8	54.5 ± 6.6
Total *n*-3	43.6 ± 8.0	39.1 ± 11.5	34.1 ± 3.5	38.1 ± 5.8
Total *n*-6	4.9 ± 1.3	3.2 ± 1.3	6.5 ± 1.2	6.4 ± 0.3
Total other	4.0 ± 1.2	4.4 ± 2.8	5.2 ± 1.5	4.9 ± 1.9
Lipids				
TAG	1.9 ± 2.2	2.7 ± 1.7	4.9 ± 3.3	7.5 ± 5.8
ST	7.1 ± 1.6	4.7 ± 2.7	8.1 ± 1.8	6.9 ± 0.7
PL	87.8 ± 6.0	91.3 ± 2.5	86.8 ± 1.8	83.1 ± 7
Lipid content				
g lipid /100 g wet weight	0.8 ± 0.4	1.2 ± 0.6	1.1 ± 0.1	1.3 ± 0.2
g lipid/100 g dry weight	3.2 ± 1.7	4.6 ± 2.7	5.0 ± 0.7	5.8 ± 1

TLE: Total lipid extract; Values are mean ± standard error (n = 6); Other SFA: Sum of 15:0, 17:0, 20:0, 22:0, and 24:0; Other MUFA: Sum of 16:1*n*-5, 18:1*n*-7trans, 18:1*n*-5, 22:*n*-11, and 22:1*n*-9; Other PUFA: Sum of 16:2*n*-6, 18:3*n*-6, 20:4*n*-6, 20:2*n*-6, 22:5*n*-6, 22:4*n*-6, and 22:2*n*-6; Other includes fatty aldehydes and 4,8,12 trimethyl tetradecanoic acid (4,8,12-TMTD); TAG: triacylglycerols, PL: polar lipid; ST: sterols.

Conflicts of Interest

The authors declare no conflict of interest.

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Evaluation of Bread Crumbs as a Potential Carbon Source for the Growth of Thraustochytrid Species for Oil and Omega-3 Production

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Abstract: The utilization of food waste by microorganisms to produce omega-3 fatty acids or biofuel is a potentially low cost method with positive environmental benefits. In the present study, the marine microorganisms *Thraustochytrium* sp. AH-2 and *Schizochytrium* sp. SR21 were used to evaluate the potential of breadcrumbs as an alternate carbon source for the production of lipids under static fermentation conditions. For the *Thraustochytrium* sp. AH-2, submerged liquid fermentation with 3% glucose produced 4.3 g/L of biomass and 44.16 mg/g of saturated fatty acids after seven days. Static fermentation with 0.5% and 1% breadcrumbs resulted in 2.5 and 4.7 g/L of biomass, and 42.4 and 33.6 mg/g of saturated fatty acids, respectively. Scanning electron microscopic (SEM) studies confirmed the growth of both strains on breadcrumbs. Attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopy for both strains were consistent with the utilization of breadcrumbs for the production of unsaturated lipids, albeit at relatively low levels. The total lipid yield for static fermentation with bread crumbs was marginally lower than that of fermentation with glucose media, while the yield of unsaturated fatty acids was considerably lower, indicating that static fermentation may be more appropriate for the production of biodiesel than for the production of omega-3 rich oils in these strains.

Reprinted from *Nutrients*. Cite as: Thyagarajan, T.; Puri, M.; Vongsvivut, J.; Barrow, C.J. Evaluation of Bread Crumbs as a Potential Carbon Source for the Growth of Thraustochytrid Species for Oil and Omega-3 Production. *Nutrients* **2014**, *6*, 2104–2114.

1. Introduction

Thraustochytrids are marine protists that belong to Labyrinthulomycetes and were first reported by Sparrow in 1936. These microorganisms are epibiotic in nature and represent a diverse group of organisms living in marine and estuarine habitats throughout the world and exhibiting a saprotrophic mode of nutrition [1]. Due to their ability to produce a large amount of oil, including polyunsaturated fatty acids (PUFAs), research has focused on lowering their cost of production using low cost carbon and nitrogen sources, particularly for large-scale industrial fermentation [2]. Biomass produced through heterotrophic fermentation of thraustochytrids is a potentially sustainable approach to the production of PUFAs for food, feed and supplement applications and oil for biofuel applications [3].

Thraustochytrids can act as microbial cell factories for the production of omega-3 PUFAs, squalene, and other secondary metabolites such as carotenoids and sterols, along with enzymes and extracellular polysaccharides [2]. For commercial utility in the production of these materials, thraustochytrid growth should be low cost, particularly to compete in the food market as a replacement for fish oils. Because heterotrophic organisms require a carbon source for bioconversion into oil, they are more expensive to grow in terms of consumables than autotrophic organisms. Heterotrophic fermentation has some

advantages over autotrophic fermentation, particularly the ability to use standard industrial fermentation equipment at scale and to get much higher cell density than can be achieved with autotrophic organisms, which often need complex and expensive equipment to enable light to reach the cells while preventing contamination [4,5]. The cost of glucose as a carbon source in the growth medium to produce biomass can account for up to 30% of the overall production cost and so commercial biofuel production using heterotrophic fermentation requires the use of low cost carbon sources such as glycerol or food waste [6,7]. Food wastes generated worldwide are about 1.3 billion tons [8]. Management of food wastes through landfill dumping is common and is environmentally problematic. Food in landfills can rot and release methane gas, which is a major contributor to carbon emissions worldwide [9].

Research on using microbial fermentation to convert food waste into value added products is limited [10]. *Schizochytrium mangrovei* and *Chlorella pyrenoidosa* have been grown using food waste obtained by fungal hydrolysis and were reported to produce biomass potentially useful as a feed supplement or for biodiesel production [6]. *Schizochytrium mangrovei* KF6 was also reported to utilize processed bread crust to produce docosahexaenoic acid (DHA) from shake flask fermentation at 200 rpm under fluorescent light for eight days [11]. Polyunsaturated fatty acids were produced by *Mortierella alpina* utilizing rice bran as the carbon source in a solid-state column reactor under static conditions. Static conditions, where there is no agitation during the growth phase, are often used in solid substrate fermentation, as opposed to submerged liquid fermentation which requires higher energy inputs for continuous shaking of the flask at constant speed [12]. Other inexpensive carbon sources derived from food wastes that were studied includes okara powder [13], residues from beer and potato processing [14], sweet sorghum juice [15], coconut water [16], marine aquaculture waste water [17] and crude glycerol [18].

The objective of the present study was to investigate the ability of some thraustochytrid strains to utilize bakery waste, specifically breadcrumbs (BC), as an alternate carbon source in the fermentation media to produce lipids under static conditions. Scanning electron microscopy (SEM) was used to investigate the growth pattern of these microorganisms during static fermentation, and attenuated total reflection Fourier transform infrared (ATR-FTIR) spectroscopic analysis was performed to observe the production of unsaturated fatty acids during fermentation. ATR-FTIR spectroscopy and SEM were shown to be useful for monitoring static fermentation.

2. Experimental Section

All chemicals including fatty acid methyl ester standards used in this study were procured from Sigma-Aldrich (Sydney, Australia) and Merck Chemicals (Victoria, Australia) and were of analytical grade.

2.1. Preparation of Seed Culture

Thraustochytrid strains used in the study were *Thraustochytrium* sp. AH-2 (ATCC® PRA-296™, Manassas, VA, USA) and *Schizochytrium* sp. SR21 (ATCC® MYA-1381™, Manassas, VA, USA) and these strains were procured from the American Type Culture Collection (ATCC, Manassas, VA, USA), and grown in liquid media containing 1 g yeast extract, 15 g peptone, 20 g glucose (1 g yeast

extract, 1 g peptone, 5 g glucose for *Schizochytrium* sp. SR21) in 1 L of artificial sea water (ASW) at 70% strength, according to ATCC product information sheets. In brief, the cultures were grown at 20 °C with shaking speed of 120 rpm for 96 h. Seed culture of 5% (v/v) culture was used in subsequent submerged liquid glucose fermentation and static fermentation with breadcrumbs as an alternate carbon source.

2.2. Submerged Liquid Fermentation with Glucose Medium

Cultures were grown in 250 mL flasks with 50 mL of medium altered and adopted from Li *et al.* [19]. Flasks were kept at 20 °C with shaking speed of 120 rpm for 7 days with medium at a starting pH of 6.0 (prior to autoclaving) and the pH was not controlled over the fermentation period. Flasks were collected for the dry weight determination and fatty acid estimation, each for 24 h.

2.3. Preparation of Bakery Waste Bread Crumbs (BC) for Static Fermentation

Breadcrumbs (crude powder; non-uniform size) were purchased from a local bakery (Geelong, Australia) for evaluating their utilization under static fermentation. BC powder was used for carrying fermentation experiments. Static fermentation was carried with the same media as submerged fermentation but with BC (at 0.5% and 1%) substituted for glucose in the media. Flasks were kept under static conditions in an incubator at 20 °C for 7 days, with pH of medium adjusted at 6.0 prior to autoclaving but not adjusted during the fermentation phase. Flasks were inoculated with 5% (v/v) seed culture (Section 2.1) under aseptic conditions. Samples were collected for the cell dry weight and fatty acid estimation after 24 h. Freeze-dried BC were analyzed using an EuroEA elemental analyzer (Euro Vector, Milan, Italy) to determine the percentages of carbon and nitrogen content.

2.4. Scanning Electron Microscopy (SEM)

A small flake of freeze-dried cells was mounted onto carbon tape on an aluminum stub and air dried, after which 60 nm of gold was deposited on its surface using a sputter coater. The cells were examined under a scanning electron microscope (SEM Supra 55 VP, Zeiss, Berlin, Germany) at accelerating voltage 3–5 KV using secondary electron detector.

2.5. Fatty Acid Extraction and Gas Chromatography (GC) Analysis

Fatty acid extraction was performed as previously described with some modifications [20]. In brief, 10 mg of freeze-dried cells were used for lipid extraction. Fatty acids were extracted with a mixture containing a 2:1 ratio of chloroform to methanol and repeated 3 times. For trans-esterification, 1 mL toluene was added followed by addition of 200 µL of internal standard, methyl nonadecanoate (C19:0) and 200 µL of butylated hydroxytoluene (BHT). Acidic methanol (2 mL) was also added to the tube and kept for overnight incubation at 50 °C. Fatty acid methyl esters (FAMEs) were extracted into hexane. The hexane layer was removed and dried over sodium sulphate. FAMEs were concentrated using nitrogen gas prior to GC analysis [21]. The samples were analyzed using a GC-FID system (Agilent Technologies, 6890N, Santa Clara, CA, USA). The GC instrument was equipped with a capillary column (Suplecowax 10, 30 × 0.25 mm, 0.25 µm thickness). Helium was used as the carrier

gas at a flow rate of 1.5 mL·min^{-1}. The injector was maintained at 250 °C and a sample volume of 1 µL was injected. Fatty acids were identified by comparison to external standards (Sigma-Aldrich, Sydney, Australia). Peaks were quantified with Chemstation chromatography software (Agilent Technologies, Santa Clara, CA, USA) and corrected using theoretical relative FID response factors [22]. Samples are analyzed in duplicate and compared to external standards.

2.6. Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) Spectroscopic Analysis

ATR-FTIR measurements of the freeze-dried samples were conducted using an Alpha FTIR spectrometer (Bruker Optik GmbH, Ettlingen, Germany) equipped with a deuterated triglycine sulfate (DTGS) detector and a single-reflection diamond ATR sampling module (Platinum ATR QuickSnap™, Ettlingen, Germany). The ATR-FTIR spectra were acquired at 4-cm^{-1} spectral resolution with 64 co-added scans within the 4000–400 cm^{-1} spectral region. Blackman-Harris 3-Term apodization, power-spectrum phase correction, and zero-filling factor of 2 were set as default acquisition parameters using OPUS 6.0 software suite (Bruker Optik GmbH, Ettlingen, Germany). Background spectra of a clean ATR surface were acquired prior to each sample measurement using the same acquisition parameters.

2.7. Statistical Analysis

Data were statistically compared using one-way analysis of variance (Anova), and the significant difference was identified using Tukey's and Scheffe tests. The analysis was carried out using SPSS software (IBM® SPSS® Statistics 20, Sydney, Australia).

3. Results and Discussion

Thraustochytrium sp. AH-2 was previously isolated from coastal and mangrove habitats of Goa and further studied for its extracellular alkaline lipase production [23]. In the present study, control fermentation profiles were obtained using submerged liquid fermentation with 3% glucose as the carbon source. Under glucose conditions, biomass of 4.3 g/L and total lipid yield of 941 mg/L were achieved (Table 1). The biomass and lipid yield for our species is similar to that reported for *T. aureum* ATCC 34304 [24], although optimization of controlled fermentation conditions would probably enable higher biomass and lipid production for strain AH-2. Oleic acid (C18:1n9) was the major fatty acid at 63.19 mg/g, followed by palmitic acid (C16:0) 32.33 mg/g, DHA (C22:6n3) 23.74 mg/g and stearic acid (C18:0) 11.82 mg/g. C14:0, C15:0. C16:1n7, C17:0, C17:1n7 were present in lower amounts. DHA (C22:6n3) was the major PUFA, followed by docosapentaenoic acid (DPA) (C22:5n6) at 4.32 mg/g and eicosapentaenoic acid (EPA) (C20:5n3) at 3.03 mg/g.

Table 1. Fermentation profiles for *Thraustochytrium* sp. AH-2.

Fermentation type	Biomass (mg/L)	Lipids (mg/L)	Saturated fatty acids [1] (mg/g)	Mono-unsaturated fatty acids [2] (mg/g)
Submerged liquid fermentation (3% glucose)	4300	941.32	44.16	63.19
Static fermentation (0.5% bread crumbs)	2530	260.0	42.4	29.00
Static fermentation (1% bread crumbs)	4760	390.0	33.6	22.6

[1] Palmitic acid (C16:0); stearic acid (C18:0); [2] Oleic acid (C18:1n9).

3.1. Fermentation Growth Using Bread Crumbs as the Carbon Source

Elemental analysis of the freeze-dried BC revealed approximately 40.95% carbon and 3.23% nitrogen. The fatty acid analysis of unfermented BC is presented in Table 2 and shows oleic acid as the major fatty acid in the profile, followed by palmitic acid (C16:0), stearic acid (C18:0), linoleic acid (18:2n6) and other fatty acids were also present. The polyunsaturated fatty acids EPA, DPA and DHA were not detected in the fatty acid profile of BC.

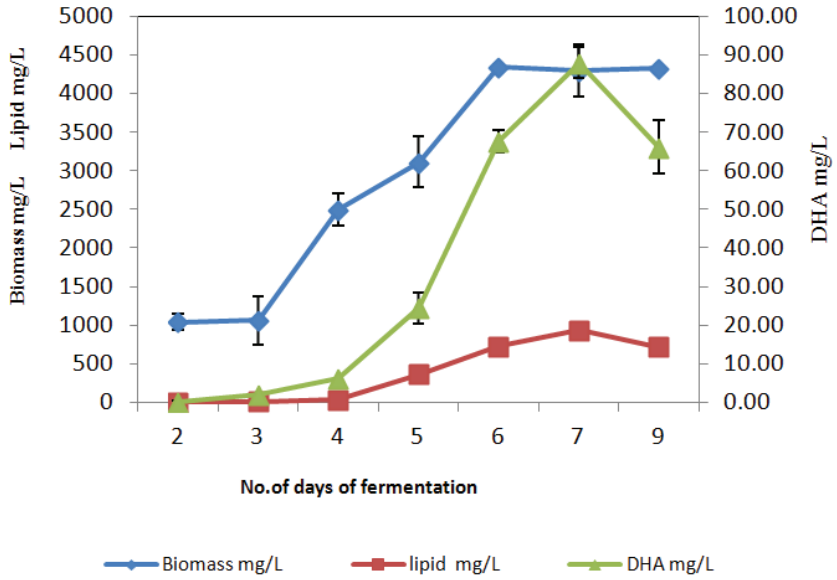
Thraustochytrium sp. AH-2 was grown using BC as the carbon source and the results compared with those obtained using glucose. BC is known to contain primarily complex carbohydrate in the form of starch [25]. BC was used at levels of 0.5% and 1%. Higher levels of 3% and 5% BC gave no improvement in cell growth and made lipid extraction difficult and were not pursued further. Fermentation with 0.5% BC gave 2.5 g/L of biomass and 260 mg/L of total lipid yield. Fermentation with 1% BC gave 4.7 g/L of biomass and 390 mg/L of total lipid yield (Figure 1). Compared to liquid fermentation with glucose, the total lipid yield was relatively low for static fermentation.

Table 2. Fatty acid profile [1] (mg/g) of unfermented bread crumbs, submerged liquid fermentation and static fermentation with bread crumbs, for *Thraustochytrium* sp. AH-2.

C16:0	C18:0	C18:1n9	C18:2n6	C18:3n3	C20:5n3	C22:5n3	C22:6n3	others
Unfermented bread crumbs fatty acid profile								
2.20	1.50	3.10	1.00	0.00	0.00	0.00	0.00	1.50
Submerged liquid fermentation fatty acid profile [2]								
32.33 [a]	11.82 [a]	63.19 [a]	2.76 [a]	0.00 [a]	3.03	4.32	23.74	33.35
Static fermentation fatty acid profile [3]								
25.9 [b,4]	16.5 [b]	29.0 [b]	12.9 [b]	1.2 [b]	0.00	0.57	2.40	14.40
20.4 [b,5]	13.2 [b]	22.6 [b]	11.2 [b]	1.2 [b]	0.00	0.00	1.30	11.50

[1] Palmitic acid (C16:0); stearic acid (C18:0); oleic acid (C18:1n9); linoleic acid (C18:2n6); linolenic (C18:3n3); EPA (C20:5n3); DPA (C22:5n3); DHA (C22:6n3); [2] Submerged liquid fermentation media with 3% glucose incubated for 7 days at 20 °C with shaking speed of 120 rpm; [3] Static fermentation with same medium composition as submerged liquid fermentation, with breadcrumbs as 5 and 10 g substituted for glucose in 1litre of 70% ASW at pH 6; [4] Static fermentation with 0.5% BC, incubated at 20 °C for 7 days; [5] Static fermentation with 1% BC, incubated at 20 °C for 5 days; [a,b] indicates statistically significant difference for submerged liquid fermentation ([a]) and static fermentation ([b]) ($p < 0.05$).

Figure 1. Fermentation profile of *Thraustochytrium* sp. AH-2 under submerged liquid fermentation with 3% glucose as the carbon source.



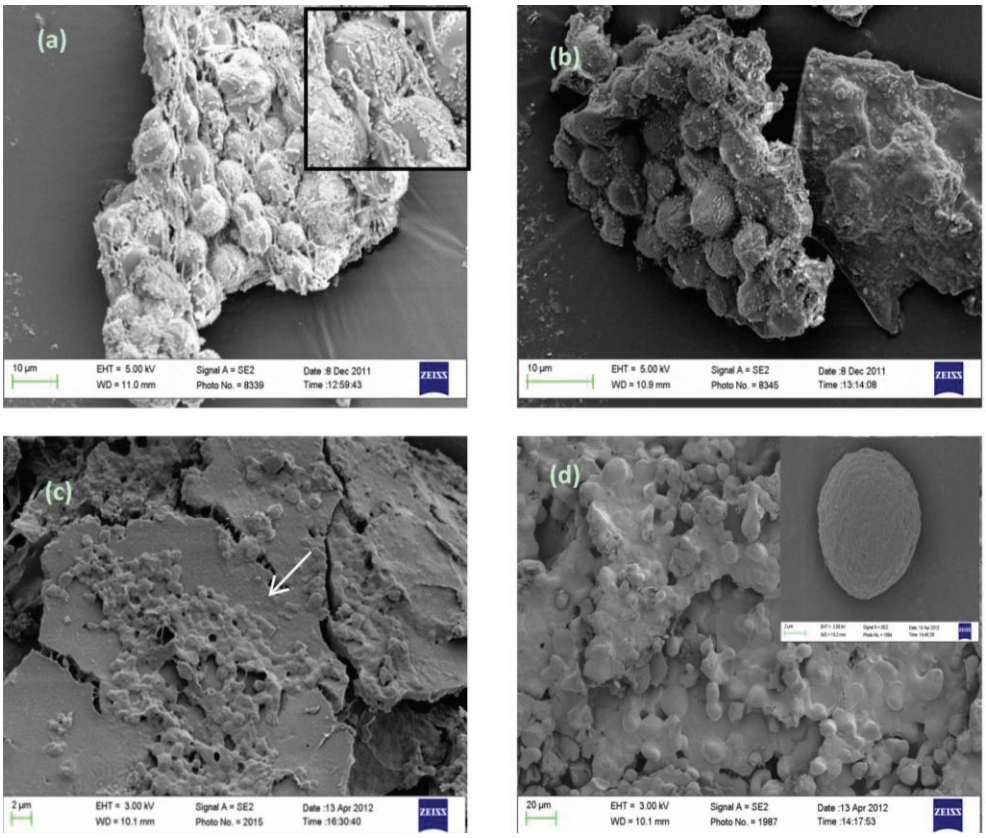
Under static fermentation, unsaturated fatty acids were poorly synthesized, although the presence of C18:3n3 (linolenic acid), EPA and DHA were clearly observed. Previous study on *Schizochytrium mangrovei* KF6 under heterotrophic conditions with processed bread crust also reported low levels of unsaturated fatty acids [11]. In general, monosaccharide sugars in the media result in the production of higher levels of PUFAs compared to di- and poly-saccharides [26]. As BC contains mainly starch, the observed poor synthesis of unsaturated fatty acids is not unexpected. In this study, the maximum fatty acid profile with 1% BC was observed on day 5, after which fatty acid content decreased, probably due to lipid consumption by the organism. Lipids and fatty acids accumulated in oleaginous microorganisms can act as energy sources for growth that are utilized when there is a lack of available carbon in the media [27].

The total amount of saturated fatty acids, which was primarily C16:0 and C18:0, were 42.4 mg/g with 0.5% BC and 33.6 mg/g with 1% BC, whilst submerged liquid fermentation with glucose as the carbon source gave an only slightly higher level of saturated fatty acids at 44.1 mg/g. Since PUFA production is low and unsaturated production is relatively high, fermentation with BC provides a fatty acid profile more consistent with that of biofuel, than did submerged liquid fermentation. Static fermentation may be a useful method for converting BC to oil, since parameters are readily standardized at industrial scale.

3.2. Scanning Electron Microscopy (SEM) Observation of Cell Growth

The fermentation growth for *Thraustochytrium* sp. AH-2 and *Schizochytrium* sp. SR21 were compared for BC and glucose as the carbon source using SEM. The morphology of freeze-dried unfermented BC was observed using SEM as a control material. The SEM images of cells grown using submerged liquid fermentation with glucose show spherical cells that are clumped together (Figure 2a). When grown in the presence of BC, cell clusters are attached to the BCs, confirming that cells do grow on this complex carbon source (Figure 2b,c).

Figure 2. SEM images of freeze-dried cells for (**a**) freeze-dried cells of *Schizochytrium* sp. SR21 grown under submerged liquid fermentation; (**b**) *Schizochytrium* sp. SR21 cells grown with 1% BC as alternate carbon source; (**c**) *Thraustochytrium* sp. AH-2 cells grown with 1% BC as alternate carbon source; (**d**) *Thraustochytrium* sp. AH-2 grown under submerged liquid fermentation.

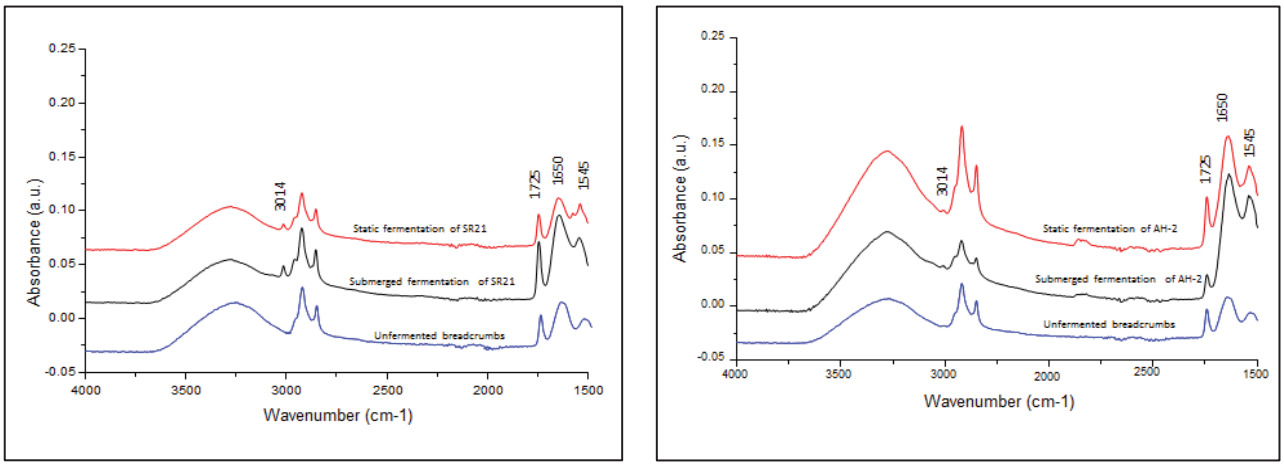


3.3. ATR-FTIR Spectroscopy Analysis of Cell Lipid Content

ATR-FTIR spectroscopic measurement of *Schizochytrium* sp. SR21 was performed to confirm the production of unsaturated fatty acids when BC was used as an alternative carbon source, in comparison to the submerged liquid fermentation with glucose of the same strain. Figure 3 shows the comparison of the ATR-FTIR spectral features of the raw unfermented BC, the static fermented 1% BC and glucose fermented cells. In particular, the olefinic C=CH stretching vibration found at ~3014 cm^{-1} is commonly known as a representative band for unsaturated fatty acids [28,29]. This band is clearly observed in the freeze-dried cells that were grown in 1% concentration of BC under static fermentation and with glucose, suggesting that observable amounts of unsaturated fatty acids were produced in the cells grown by both fermentation methods. The triplet bands found in the range of 3000–2800 cm^{-1}, on the other hand, are attributed to C-H stretches of lipids and proteins [29]. At the low wavenumber region, the strong bands centered at 1650 and 1545 cm^{-1}, known as amide I and II bands, respectively, occurred due to the protein moieties in the BC and the cells. The sharp band at 1725 cm^{-1}, on the other hand, represents ν(C=O) stretches of ester functional groups from lipids and fatty acids, and is therefore indicative of total lipids produced by the cells [28–30]. According to the intensities of this band, fermentation with glucose led to a substantially higher amount of total lipids

produced in the microorganisms. However, the ratios of unsaturated fatty acids per total lipids (*i.e.*, I_{3014}/I_{1725}) were found to be comparable between both fermentation approaches, suggesting that similar yields of unsaturated fatty acid can be achieved using BC as a carbon source under static fermentation of *Schizochytrium* sp. SR21. Therefore, growth of these strains on BC is potentially useful both for the utilization of food waste and the production of lipid.

Figure 3. ATR-FTIR spectra of: (**a**) *Thraustochytrium* sp. AH-2; and (**b**) *Schizochytrium* sp. SR21. Black line—submerged fermentation; Blue line—unfermented breadcrumbs; Red line—static fermentation with breadcrumbs.



(**a**) (**b**)

4. Conclusions

Thraustochytrium sp. AH-2 and *Schizochytrium* sp. SR21 were tested for their ability to utilize BC during static fermentation as a low-cost and environmental friendly carbon source for producing oil for either biofuel (saturated fatty acid rich) or food (PUFA rich). The fatty acid profiles from *Thraustochytrium* sp. AH-2 indicated low levels of PUFA and higher levels of saturated oil. ATR-FTIR spectroscopy of *Schizochytrium* sp. SR21 was also consistent with higher levels of saturated fatty acids. Fermentation on BC containing complex carbohydrate appears to be more appropriate to the production of biofuel from these organisms than for the production of high levels of PUFA for food applications, due to the suppression of PUFA when BC is used as the carbon source.

Acknowledgments

We thank the Centre for Chemistry and Biotechnology for funding towards this project, including some scholarship costs for T. Thyagarajan.

Author Contributions

T. Thyagarajan performed the research and contributed to writing the manuscript. J. Vongsvivut performed and interpreted the ATR-FTIR. M. Puri contributed to research and supervision. C. Barrow supervised the project and contributed to writing the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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4. Products containing Long-chain Omega-3

Purification of Alaskan Walleye Pollock (*Gadus chalcogrammus*) and New Zealand Hoki (*Macruronus novaezelandiae*) Liver Oil Using Short Path Distillation

Alex C. M. Oliveira and Matthew R. Miller

Abstract: The beneficial health effects of a diet rich in *n*-3 long chain polyunsaturated fatty acids (*n*-3 LC-PUFA) have been extensively researched in recent years. Marine oils are an important dietary source of *n*-3 LC-PUFA, being especially rich in two of the most important fatty acids of this class, EPA (eicosapentaenoic acid; 20:5*n*-3) and DHA (docosahexaenoic acid; 22:6*n*-3). Oils rich in *n*-3 LC-PUFA are prone to oxidation that leads to loss of product quality. Alaskan pollock (*Gadus chalcogrammus* Pallas, 1814) and New Zealand's hoki (*Macruronus novaezelandiae* Hector, 1871) are the highest volume fisheries of their respective countries. Both produce large quantities of fishery byproducts, in particular crude or unrefined *n*-3 LC-PUFA containing oils. Presently these oils are used as ingredients for animal feed, and only limited quantities are used as human nutritional products. The aim of this research was to investigate the applicability of short path distillation for the purification of pollock and hoki oil to produce purified human-grade fish oil to meet quality specifications. Pollock and hoki oils were subjected to short path distillation and a significant decrease in free fatty acids and lipid oxidation (peroxide and *para*-anisidine values) products was observed. Purified oils met the Global Organization for EPA and DHA Omega-3 (GOED) standard for edible fish oils.

Reprinted from *Nutrients*. Cite as: Oliveira, A.C.M.; Miller, M.R. Purification of Alaskan Walleye Pollock (*Gadus chalcogrammus*) and New Zealand Hoki (*Macruronus novaezelandiae*) Liver Oil Using Short Path Distillation. *Nutrients* **2014**, *6*, 2059–2076.

1. Introduction

The beneficial health effects of *n*-3 long chain polyunsaturated fatty acids (*n*-3 LC-PUFA) are well established. There is evidence that *n*-3 LC-PUFA play a role in the treatment and possible prevention of cardiovascular diseases, hypertension, diabetes, arthritis, and other inflammatory and autoimmune diseases [1–4]. Marine oils are an important dietary source of *n*-3 LC PUFA, being especially rich in two of the most important fatty acids of this class, namely EPA (eicosapentaenoic acid; 20:5*n*-3) and DHA (docosahexaenoic acid; 22:6*n*-3). Due to its nutritional value, there is growing interest in refining fish oil from different marine sources for human consumption. Refined edible fish oil can be consumed in the form of a pharmaceutical (e.g., Omacor™, Lovaza™), or nutraceutical (e.g., fish oil capsules), or it can be added as an ingredient to boost levels of *n*-3 LC-PUFA in various food items such as baked goods, orange juice and yogurt [5].

Aquaculture is presently the major user of *n*-3 LC-PUFA oils. Therefore, the nutraceutical and pharmaceutical industries are in competition with aquaculture industries for access to *n*-3 LC-PUFA, and the increased demand for this marine oil has lead to an increase in the value of this commodity [6]. Currently, most of the fish oil used in aquaculture feeds is produced from non-food forage fish species from Peru and Northern Chile; Mexican and Central American Pacific coasts; the US Gulf and Atlantic coasts; Norway; Iceland; and other regions [7]. Most of these industrial fisheries are harvested at sustainable levels and increased oil production from these sources is unlikely [8]. A possible method for increasing the human intake of *n*-3 LC-PUFA is improving the quality and processing yields of oils rendered from abundant although presently underutilized marine byproduct streams.

Alaskan walleye pollock and New Zealand's hoki are major world fisheries with large economic importance to their respective countries. Alaska walleye pollock, previous scientific name *Theragra chalcogramma* and recently reassigned to *Gadus chalcogrammus* [9], is the largest volume fishery in Alaska and estimated at over 1,000,000 tons per year [10]. The Bering Sea and Aleutian Islands, and the Gulf of Alaska pollock fisheries are considered sustainable and were certified by the Marine Stewardship Council (MSC) in 2005 and 2010. The combination of a high production volume and the year-round availability of pollock byproducts make these raw materials ideal for the production of human-grade fish oils [10]. Hoki (*Macruronus novaezelandiae*, family Merlucciidae) is the major commercial fishery in New Zealand. Hoki is a hake species and has MSC certification as being a well-managed species. The quota varies depending on stocking information and was set at 150,000 tons for the 2013–2014 fishing season. Hoki are found in the waters surrounding New Zealand, in particular the Chatham Rise, Cook Straight, around the Sub Antarctic islands as well as southeastern Australia. Both of these species store large amounts of oil in their liver making them a good source of *n*-3 LC-PUFA. Pollock liver oil has been reported to contain *n*-3 LC-PUFA concentrations of 23 g/100 g, with 5 g/100 g DHA and 15 g/100 g EPA [11]. Hoki liver oil contains similar concentrations of *n*-3 LC-PUFA (23 g/100 g), with a different ratio of DHA (12 g/100 g) to EPA (6 g/100 g) [12].

Hoki and pollock oils are rich in *n*-3 LC-PUFA and susceptible to lipid degradation processes which cause loss of the valuable LC-PUFA, and development of rancid odours and flavours [13]. Lipid oxidation is a degradative free radical reaction which can be triggered by many mechanisms such as singlet oxygen or peroxide radicals, from sources such as light and/or oxygen and catalysts such as iron [13]. Some important triggers of lipid oxidation in fish byproducts are iron from hemoglobin, as well as temperature and the presence of oxygen during processing [13]. Lipid oxidation products do not only impart unpleasant taste and smell to fish oils but they also exert cytotoxic and genotoxic effects [14,15]. Ingestion of these compounds may cause low density lipoprotein cytotoxicity [16], atherogenesis and atherosclerosis [17], and liver enlargement indicating nutrition-induced toxicity [18]. For these reasons, it is critical to monitor the quality and oxidative stability of edible fish oils. Oxidation products in crude marine oils, such as pollock and hoki liver oil, can be high, reducing the oil value and quality. Oxidation is measured by a series of tests. The Peroxide Value (PV) measures the primary products of lipid oxidation (lipid peroxides) while *para*-Anisidine Value (*p*-AV) is a method used to measure secondary products of oxidation (aldehydes and ketones). Acid Value (AV), often used as an indication of quality of the oil, quantifies free fatty acids (FFA) that have been cleaved from their parent molecules (e.g., triglycerides or phospholipids) as a result of hydrolytic breakdown.

Cleavage of a FFA from a parent molecule, commonly known as lipid hydrolysis, results from increased enzymatic activity in the fish tissues post-mortem; the lipases triggering the degradation process are either innate to the organism or of bacterial origin. As there is no compulsory or regulatory qualitative parameters for lipid oxidation for marine oils to date, many marine oil processers use the Global Organization for EPA and DHA Omega-3 (GOED) voluntary monograph [19]. The 2012 GOED monograph states that for oils for human consumption, the following values are required: AV < 3 mg KOH/g; PV < 5 mEq/kg; *p*-AV < 20; and a resulting TOTOX of <26 (result of calculation, (2 × PV + *p*-AV) is also a requirement.) The GOED monograph also contains specifications for the maximum level of dioxins, PCBs and heavy metals with relevance to the process technology used in this study. In addition, the European Community has implemented hygienic, raw material quality and process requirements for fish oil intended for human consumption (Regulation (EC) 853/2004). The Codex Alimentarius Committee on Fats and Oils has recently started to assemble the Standard for Fish Oils and currently this document is at Step 2 [20].

In Alaska and New Zealand, large quantities of fishery byproducts are already utilized for the production of fishmeal and fish oil. However, most fish oil produced in Alaska and New Zealand is crude, only serving as an ingredient for animal/aquaculture feed. Food-grade fish oils can be produced from crude fish oils by including further processing steps that add value to marine byproducts for the respective fishing industries. Traditionally, fish oil purification is composed of four consecutive steps: degumming, neutralization, bleaching and deodorization. Degumming removes soluble and insoluble impurities such as proteins, phospholipids, waxes and trace metals [21]. Degumming is accomplished by washing the oil with an aqueous solution of an organic acid such as citric or phosphoric acid under mild heat [22]. Neutralization, often referred to as alkali refining, is used to remove FFA and this is accomplished by treating the degummed fish oil with sodium hydroxide (aqueous solution) under mild heat [21]. Bleaching the neutralized fish oil further purifies it by removing pigments, traces of soap, sulfur- and carbonyl-containing compounds, pigment breakdown products and trace metals [21]. Bleaching is accomplished by treating the oil with an adsorbent such as activated earth (bleaching clay), activated carbon, or chitosan [23]. Deodorization is the final purification step and consists of removing aldehydes and ketones that are responsible for the peculiar fish oil odor, which in most cases is not appealing to consumers. Aldehydes and ketones are formed during lipid oxidation, and this degradation of fatty acids may occur during raw material handling and storage, and/or during the rendering process. Since the late 1980's, an additional step has been added in which the fish oil is also subjected to another step of molecular distillation to remove persistent organic pollutants (POP) [21]. In summary, the general objective of purification is to remove impurities that have negative health effects and detrimental sensorial and qualitative impacts on marine oils such as odor and taste.

Molecular distillation offers advantages for separation, purification and/or concentration of natural products, usually consisting of complex and thermally sensitive molecules such as fat-soluble vitamins and PUFA, because it minimizes losses caused by thermal degradation [24,25]. In this context, short-path distillation (SPD), provides an alternative to the traditional fish oil purification process by removing unwanted free fatty acids, deodorizing (removing aldehydes and ketones), and removing environmental contaminants under low pressure conditions [26–28]. One of the main advantages of using this technology, as compared with traditional fish oil purification steps, is that SPD does not require chemical treatments during processing, thus reducing processing effluents and decreasing the

number of steps needed to refine fish oils. It is noteworthy to mention that it is expected that a majority of odorants, that is, low-molecular weight volatiles, in fish oils will be distilled off during short-path distillation due to the low-pressure used in the SPD system. However, SPD has its limitations and will not remove most pigments or heavy metals and therefore, cannot replace all physical refining steps used in traditional fish oil refining when either depigmentation or removal of heavy metals are required. The aim of this work was to investigate the applicability of short-path distillation to refine crude pollock and hoki oils to produce purified human-grade fish oil that meet GOED quality specifications.

2. Experimental Section

2.1. Materials

Pollock oil was produced in November of 2008 onboard the *F/T American Triumph* (American Seafoods Group) during the Bering Sea Pollock season. The pollock oil, produced at sea, was rendered from a mixture of fresh byproducts using a sequence of three inline horizontal contherm heat exchangers operated at 85–90 °C, and product cook time was less than 2 min. The cooked material was then separated into oil, water, and a protein sludge using a three-phase centrifuge operated at about 85 °C. Ascorbyl palmitate was used as an antioxidant. Ascorbyl palmitate (Sigma Aldrich, St. Louis, MI, USA) was mixed into the centrifuged crude Pollock oil, immediately after rendering, at a ratio of 250 mg/kg [29]. The oil was stored in 25 kg containers fitted with screw-top caps and frozen at −20 °C. Pollock oil was received frozen at the Kodiak Seafood and Marine Science Center (Kodiak, Alaska) and kept at −30 °C until used.

Hoki oil was produced by Sealord Group Ltd. (Nelson, New Zealand) at their rendering plant on 6 August 2006. Barox™ (Kemin, Des Moines, IA, USA) was used as an antioxidant and was added at 750 ppm immediately after rendering. The oil was kept at −40 °C until shipment to Kodiak, Alaska, in March of 2011.

2.2. Purification of Fish Oil Using Short-Path Distillation

The SPD process was conducted using a combination of processing variables in a sequence similar to previously reported [30–32]. The SPD apparatus (Figure 1) consisted of a Pope 2″ Wiped-film Still (Pope Scientific Inc., Saukville, WI, USA) connected to a Diffstak® Mk2 diffusion pump model 63/150 (BOC Edwards, Crawley, West Sussex, UK) and also to a high-vacuum pump model RV3 (BOC Edwards). A Penta-Drive DC Meter Speed Control (Pope Scientific Inc.) for controlled rotation of the carbon blades in the evaporator was set at 450 or 500 revolutions per minute and digitally displayed by a RPM meter (Minarik, Aneheim, CA, USA). The surface area of the wiped film is 0.033 m^2, the evaporator was contained in a heated jacket (Pope Scientific Inc., Saukville, WI, USA), and temperature of the evaporator was digitally controlled with a Digital Indicating Controller model UT35A (Yokogawa Electronic Corporation, Sugarland, TX, USA) and system pressure was monitored by a Digital Pressure Monitor (Kurt J. Lesker Company, Philadelphia, PA, USA). Distillation was conducted in a two-step procedure. The SPD cold-trap was cooled with dry-ice in acetone.

Pollock oils were purified using the SPD system during the summer of 2009 and hoki oils were purified in the spring of 2012. A portion of 1500 mL of either crude fish oil was added to the graduated feed flask and the heat tape enclosing the flask was set to 60 °C. The first distillation (first degassing pass) parameters were as follows: internal condenser temperature 55 °C; evaporator temperature 150 °C; feeding rate 360–480 mL/h; roller speed 450 rpm (hoki) or 500 rpm (pollock) and vacuum 0.05–0.06 mbar. The degassed oil was used immediately or stored in a sealed vessel under nitrogen at 5 °C for a maximum of 24 h. The main fish oil distillation (second pass) parameters were as follows: condenser temperature 55 °C; evaporator temperatures were 190 °C, 200 °C or 210 °C; feeding rate 360–480 mL/h; roller speed 450 rpm (hoki) or 500 rpm (pollock) and vacuum 0.01–0.02 mbar. The refining yields were determined gravimetrically, with about 300 g of oil trialed for each temperature parameter. The purification process was repeated three times for each type of oil and yielded nine purified oil samples (three independent oil replicates for each evaporator temperature tested).

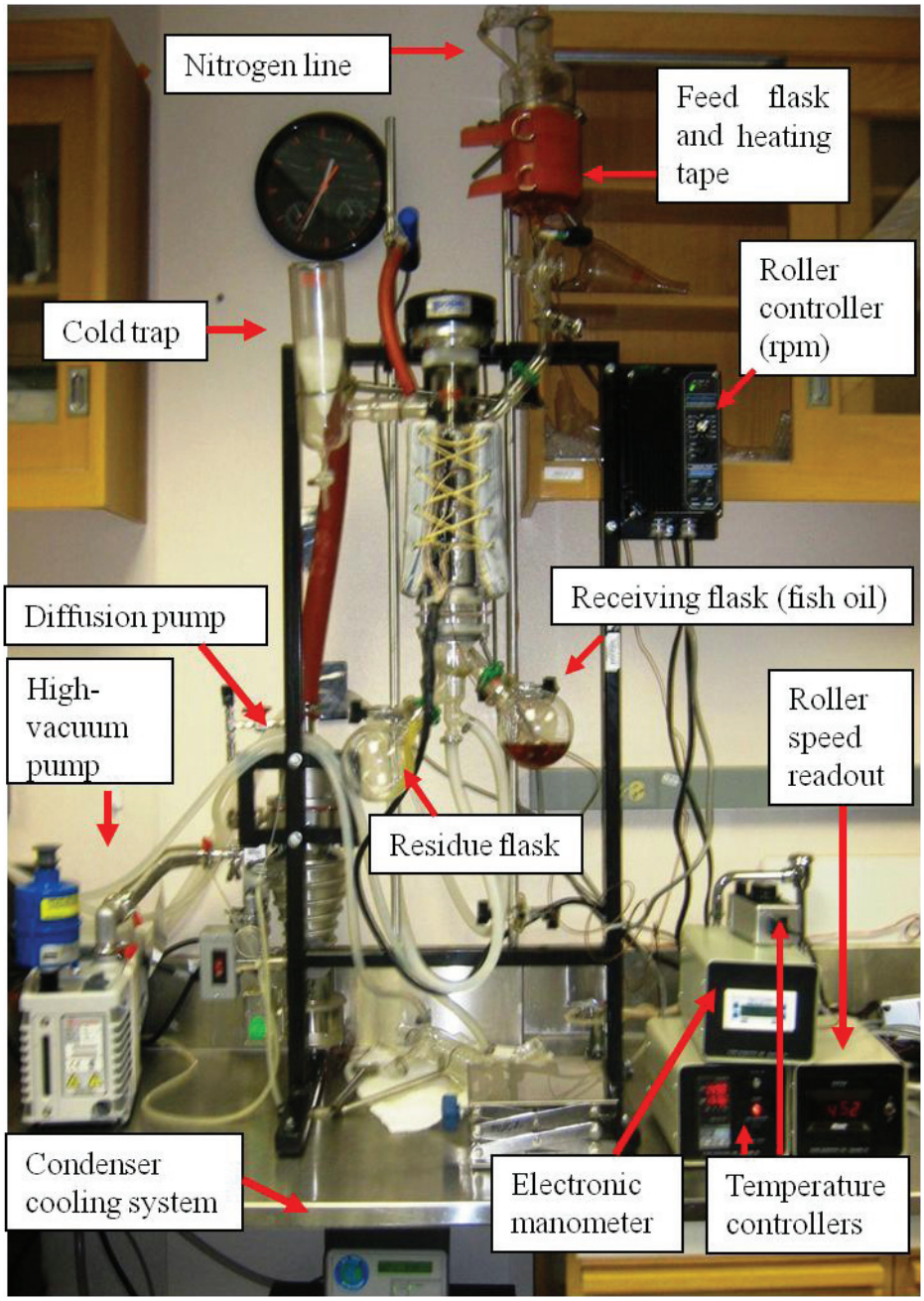
2.3. Proximate and Oxidation Analysis

Rancidity and oxidation of fish oils were assessed in accordance to AOCS official methods including Acid Value (AOCS Official Method Cd 3d-63, [33]), Peroxide Value (AOCS Official Method Cd 8-53, [34]) and *para*-Anisidine Value (AOCS Official Method Cd 18-90, [35]). Reported TOTOX values were calculated via the equation TOTOX = (2 × PV) + *p*-AV [13]. Water content was measured by the Karl-Fisher method using an automated tritrator; values were generally <0.02%.

2.4. FAME Analysis

Fatty acid methyl esters (FAME) were prepared using KOH and methanol [36]. FAME were transferred into 1.5 mL snap-cap amber GC vials (Agilent Technologies, Wilmington, DE, USA) and immediately analyzed. An internal standard, tricosanoic methyl ester (23:0), was used for quantification. Fatty acid profiles were determined with a GC model 6850 coupled to a flame ionization detector (Agilent Technologies, Wilmington, DE, USA) fitted with a DB-23 (60 m × 0.25 mm id., 0.25 μm film) capillary column (Agilent Technologies, Wilmington, DE, USA). An autosampler performed the GC injections and injection volume was 1 μL. The chromatographic conditions were as previously described [37]. Unsaponifiable matter was calculated from the initial recorded weight of the oil (~20 mg) used for methylation, compared to the total lipid converted to methyl esters (mg FAMES). FAME were quantified in mg/g oil by using an internal standard and also a five-point calibration curve for all fatty acids included in the Supleco 37 mix (Sigma Aldrich, St. Louis, MI, USA) [38].

Figure 1. Short-path distillation (SPD) system.



2.5. Statistical Analysis

Mean values were reported plus or minus standard error of the mean. In cases where data was reported in percentage composition, values were transformed using arcsin function to yield normalized data prior to statistical analysis. Normality and homogeneity of variance were confirmed and a comparison between means was achieved by one-way analysis of variance (ANOVA). Multiple comparisons were achieved by Tukey-Kramer HSD (honestly significant difference). Significance was accepted as probabilities of 0.05 or less. Statistical analysis was performed using SPSS® statistics 17.0 and GenStat version 14 software.

3. Results

3.1. Peroxide Value (PV)

Figure 2a depicts the PV of crude hoki and pollock oils together with values determined after oils were subjected to molecular distillation with set evaporator temperatures of 190, 200 or 210 °C. The crude pollock oil, with an initial PV of 6.32 ± 0.45 mEq/kg, was purified by molecular distillation with set evaporator temperatures of 190 °C (PV of 0.13 ± 0.06 mEq/kg), 200 °C (PV of 0.10 ± 0.00 mEq/kg) and 210 °C (PV of 0.10 ± 0.00 mEq/kg) (Figure 2a). PV of the three refined pollock oils did not statistically differ from each other and were all significantly ($p < 0.001$, $f = 561.1$) lower than the crude pollock oil. The crude hoki oils had significantly ($p < 0.001$, $f = 113.6$) higher PV, 10.33 ± 1.15 mEq/kg, than the values measured for any of the distilled hoki oils (190 °C 2.32 ± 0.28 mEq/kg; 200 °C 2.38 ± 0.44 mEq/kg; 210 °C 2.28 ± 0.33 mEq/kg). Significant differences were not observed in the PV of hoki oils distilled at the different evaporator temperatures tested.

Figure 2. Quality indices of crude pollock ($n = 3$) and hoki ($n = 3$) oils, purified by short path distillation with variable evaporator temperatures (190 °C, 200 °C and 210 °C). (**a**) Peroxide Values; (**b**) *para*-Anisidine Values; (**c**) TOTOX Values; and (**d**) free fatty acids (FFA) Values.

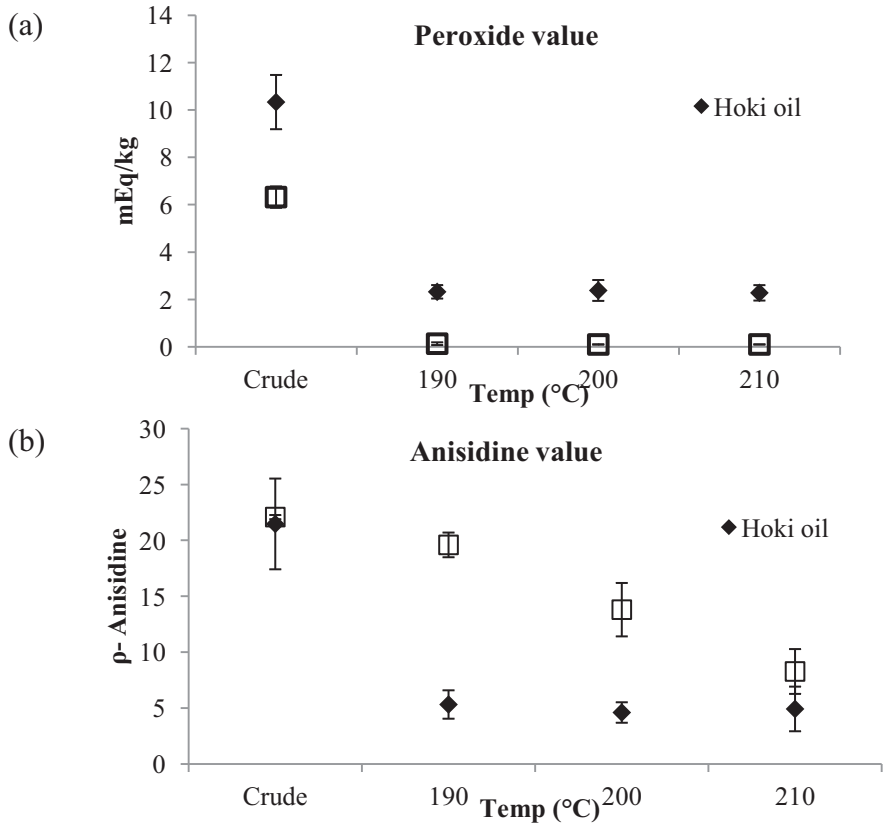
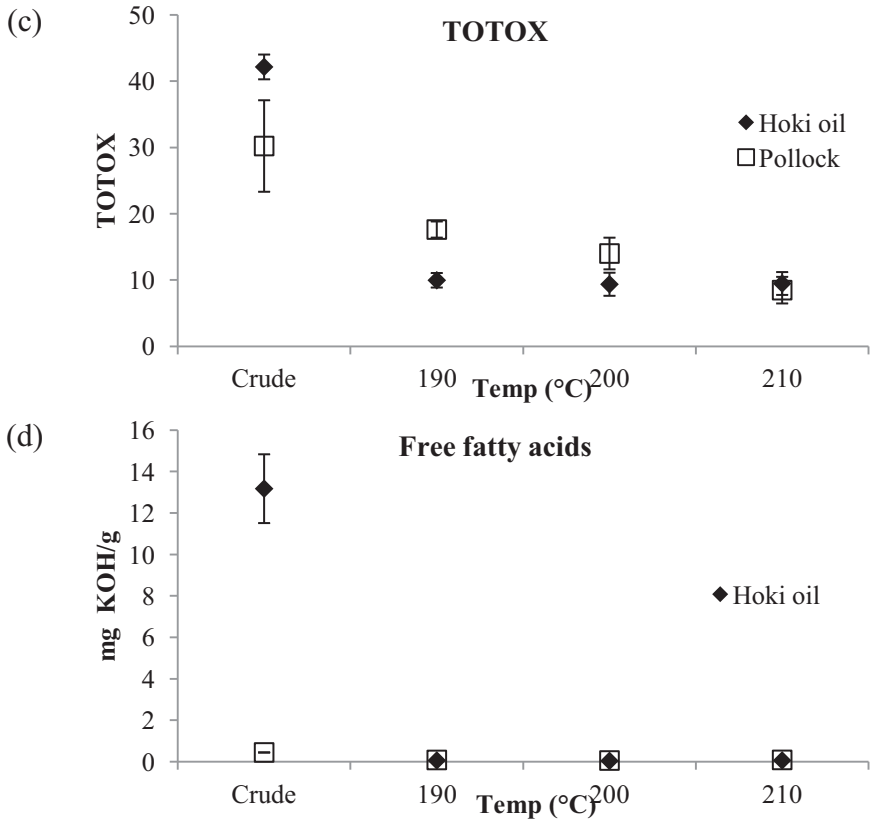


Figure 2. *Cont.*



3.2. Para-Anisidine Value (p-AV)

Figure 2b depicts the *p*-AV of crude hoki and pollock oils together with values determined after oils were subjected to molecular distillation with set evaporator temperatures of 190, 200 or 210 °C. The *p*-AV of crude pollock oils (20.09 ± 0.19) were significantly higher ($p < 0.001$, $f = 41.7$) than values determined for the higher temperature distilled oils (200 °C 13.81 ± 2.39 and 210 °C 8.28 ± 2.01). Crude hoki oils had significantly ($p < 0.001$, $f = 37.8$) higher *p*-AV (21.48 ± 4.06) than the values measured for hoki oils distilled using all evaporator temperatures: 190 °C (5.33 ± 1.27), 200 °C (4.61 ± 0.92) or 210 °C (4.93 ± 2.00). The *p*-AV of hoki oils subjected to molecular distillation at the tested evaporator temperatures were not significantly different.

3.3. TOTOX Value

Figure 2c depicts the TOTOX values of crude hoki and pollock oils together with values determined after oils were subjected to molecular distillation with set evaporator temperatures of 190, 200 or 210 °C. The TOTOX values of crude pollock oils (30.22 ± 6.90) were significantly ($p < 0.001$, $f = 17.63$) higher than those of their respective distilled counterparts at any of the tested SPD evaporator temperatures. A significant reduction in the TOTOX values ($p < 0.001$, $f = 17.63$) of pollock oils distilled at 210 °C (8.48 ± 1.22) was observed when these were compared to the TOTOX values of pollock oils distilled at either 190 °C (19.87 ± 2.39) or 200 °C (14.01 ± 2.00); however, the TOTOX values of oils distilled at the two lower temperatures did not significantly differ from one another. Crude hoki oils had significantly ($p < 0.001$, $f = 296.9$) higher TOTOX values (42.15 ± 1.87),

than those of their respective distilled counterparts at any of the tested SPD evaporator temperatures. Regardless of the evaporator temperature tested, the TOTOX values of distilled hoki oils were not significantly different (190 °C 9.98 ± 1.10; 200 °C 9.37 ± 1.74; 210 °C 9.49 ± 1.72).

3.4. Acid Value (AV)

Figure 2d depicts the AV of crude hoki and pollock oils together with values determined after oils were subjected to molecular distillation with set evaporator temperatures of 190, 200 or 210 °C. The AV values of crude pollock oils (0.45 ± 0.01 mg KOH/g) were significantly higher ($p < 0.001$, $f = 1634.6$) than values determined for distilled pollock oils, regardless of tested SPD evaporator temperatures: 190 °C (0.09 ± 0.01 mg KOH/g), 200 °C (0.06 ± 0.01 mg KOH/g) or 210 °C (0.09 ± 0.01 mg KOH/g). The AV of crude hoki oils averaged 13.18 ± 1.65 mg KOH/g of oil and were significantly ($p < 0.001$, $f = 187.4$) higher than the values obtained for distilled hoki oils (190 °C 0.06 ± 0.00 mg KOH/g, 200 °C; 0.04 ± 0.03mg KOH/g; 210 °C 0.06 ± 0.00 mg KOH/g). A significant difference was not observed in the AV values of distilled hoki oils.

3.5. Fatty Acid Profiles

The FA profiles of the crude pollock oil and three distilled fractions are shown in Table 1. In general the amount of fatty acids in the distilled fractions was significantly higher due to the reduction in unsaponifiable matter, in particular in the 210 °C fraction. The crude pollock oil had significantly higher ($p < 0.001$, $f = 45.63$) unsaponfiable matter (24.01 ± 0.94) than the oils from the three different treatments (190 °C 15.95 ± 1.76; 200 °C 17.16 ± 0.27; 210 °C 10.54 ± 0.27). The two most abundant (>10.0 g/100 g) fatty acids observed in the pollock crude oils were long chain monounsaturated fatty acids (LC-MUFA): (20:1*n*-11, 12.2 g/100 g and 22:1*n*-11, 12.0 g/100 g). The n-3 LC PUFA were also present: DHA (4.0 g/100 g) and EPA (7.8 g/100 g). The most abundant FA class was MUFA (46.5 g/100 g) with the remainder being composed of saturated (SFA 13.8 g/100 g) and PUFA (15.6 g/100 g). The *n*-3 PUFA made up a majority (91%) of the PUFA fraction.

Table 1. Fatty acid (g/100 g oil) and unsaponifiable matter content of crude pollock oils (±SD) and pollock oils (±SD) purified by short-path distillation (SPD) with variable evaporator temperatures (190 °C, 200 °C, 210 °C).

Fatty acids [1] (g/100 g)	Crude oil (±SEM)	SPD 190 °C (±SEM)	SPD 200 °C (±SEM)	SPD 210 °C (±SEM)
14:0	3.85 ± 0.09a	4.00 ± 0.13a,b	3.96 ± 0.06a,b	4.35 ± 0.02b
16:0	6.61 ± 0.12a	7.10 ± 0.20a	7.00 ± 0.07a	7.66 ± 0.01b
16:1*n*-7	7.70 ± 0.14a	8.25 ± 0.23a,b	8.13 ± 0.08a	8.88 ± 0.00b
18:1*n*-9t	1.59 ± 0.02a	1.73 ± 0.04b	1.71 ± 0.01b	1.85 ± 0.00c
18:1*n*-9	4.15 ± 0.06a	4.55 ± 0.11b	4.47 ± 0.02b	4.84 ± 0.01c
18:1*n*-7	2.50 ± 0.03a	2.75 ± 0.06b	2.70 ± 0.01b	2.93 ± 0.00c
20:1*n*-11	12.17 ± 0.14a	13.65 ± 0.25b	13.45 ± 0.02b	14.49 ± 0.04c
20:1*n*-9	3.45 ± 0.03a	3.83 ± 0.08b	3.80 ± 0.02b	4.04 ± 0.00c
22:1*n*-11	12.01 ± 0.07a	13.67 ± 0.19b	13.48 ± 0.05b	14.46 ± 0.14c

Table 1. *Cont.*

Fatty acids [1] (g/100 g)	Crude oil (±SEM)	SPD 190 °C (±SEM)	SPD 200 °C (±SEM)	SPD 210 °C (±SEM)
18:3*n*-3	0.25 ± 0.01a	0.28 ± 0.01a,b	0.27 ± 0.00a,b	0.29 ± 0.00b
18:4*n*-3	1.22 ± 0.02a	1.34 ± 0.03b	1.32 ± 0.01b	1.42 ± 0.00c
20:4*n*-3	0.29 ± 0.00a	0.34 ± 0.01b	0.33 ± 0.00b	0.36 ± 0.00c
20:5*n*-3	7.83 ± 0.07a	8.77 ± 0.17b	8.62 ± 0.01b	9.26 ± 0.01c
22:2*n*-6	0.42 ± 0.01a	0.45 ± 0.01a,b	0.46 ± 0.00a,b	0.48 ± 0.00c
22:5*n*-3	0.63 ± 0.00a	0.71 ± 0.01b	0.70 ± 0.00b	0.75 ± 0.01c
22:6*n*-3	4.00 ± 0.02a	4.55 ± 0.07b	4.48 ± 0.01b	4.79 ± 0.04c
Other SFA	3.42 ± 0.09a	3.76 ± 0.11a,b	3.70 ± 0.05a,b	4.02 ± 0.00c
Sum SFA	13.88 ± 0.29a	14.85 ± 0.44a,b	14.66 ± 0.18a	16.02 ± 0.01b
Other MUFA	2.94 ± 0.03a	3.28 ± 0.06b	3.24 ± 0.01b	3.49 ± 0.02c
Sum MUFA	46.50 ± 0.50a	51.71 ± 0.99b	50.97 ± 0.09b	54.97 ± 0.20c
Sum PUFA	15.61 ± 0.15a	17.49 ± 0.35b	17.21 ± 0.02b	18.47 ± 0.06c
Sum *n*-3	14.22 ± 0.12a	15.98 ± 0.31b	15.71 ± 0.02b	16.87 ± 0.06c
Sum Unsaponifiable matter	24.01 ± 0.94a	15.95 ± 1.76b	17.16 ± 0.27b	10.54 ± 0.27c

Note: [1] Values are means ± SEM, *n*-3. Means in a row with different letters differ significantly as determined by Tukey-Kramer HSD, $p < 0.01$. SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids.

In hoki oil, there were only minor differences observed between FA profiles of crude and distilled hoki oils (Table 2). The three most abundant (>10.0 g/100 g) fatty acids observed in the hoki oils were oleic acid (OA, 18:1*n*-9, 23.0 g/100 g), palmitic acid (PA, 16:0, 17.0 g/100 g) and DHA (22:6*n*-3, 12.0 g/100 g). Other important fatty acids were 20:1*n*-9 (7.0 g/100 g) and EPA (20:5*n*-3, 6.5 g/100 g). The most abundant FA class was MUFA (44.0 g/100 g) with the remainder being composed of SFA (24.8 g/100 g) and (PUFA 24.0 g/100 g). The *n*-3 PUFA made up a majority (85%) of the PUFA fraction. There was no statistical difference in the unsaponifiable matter in the crude and purified hoki oils.

Table 2. Fatty acid (g/100 g oil) and unsaponifiable matter content of crude hoki oils (±SD) and hoki oils (±SD) purified by short-path distillation (SPD) with variable evaporator temperatures (190 °C, 200 °C, 210 °C).

Fatty Acid [1] (g/100 g)	Crude oil (±SEM)	SPD 190 °C (±SEM)	SPD 200 °C (±SEM)	SPD 210 °C (±SEM)
14:0	3.56 ± 0.01a	3.52 ± 0.10a	3.51 ± 0.05a	3.54 ± 0.07a
16:0	17.04 ± 0.08a	16.84 ± 0.49a	16.92 ± 0.29a	17.04 ± 0.51a
18:0	3.20 ± 0.03a	3.16 ± 0.06a	3.19 ± 0.06a	3.21 ± 0.09a
16:1*n*-7	4.76 ± 0.02b	4.74 ± 0.06a,b	4.72 ± 0.04a	4.84 ± 0.02a,b
18:1*n*-9t	0.47 ± 0.01a	0.48 ± 0.02a	0.48 ± 0.01a	0.49 ± 0.01
18:1*n*-9c	22.91 ± 0.17a,b	22.81 ± 0.29a	22.82 ± 0.03a	23.32 ± 0.08b
18:1*n*-7	3.14 ± 0.02a	3.07 ± 0.09a	3.11 ± 0.06a	3.21 ± 0.01a
20:1*n*-9	7.03 ± 0.07a	6.98 ± 0.08a	7.00 ± 0.04a	7.12 ± 0.07a
20:1*n*-7	0.32 ± 0.00a	0.31 ± 0.01a	0.31 ± 0.01a	0.33 ± 0.00a
22:1*n*-11	3.20 ± 0.05a	3.19 ± 0.04a	3.18 ± 0.02a	3.23 ± 0.06a

Table 2. *Cont.*

Fatty Acid [1] (g/100 g)	Crude oil (±SEM)	SPD 190 °C (±SEM)	SPD 200 °C (±SEM)	SPD 210 °C (±SEM)
24:1*n*-9	1.14 ± 0.01a	1.13 ± 0.02a	1.13 ± 0.02a	1.13 ± 0.02a
18:2*n*-6	2.61 ± 0.01a	2.62 ± 0.04a	2.61 ± 0.02a	2.68 ± 0.02a
18:3*n*-3	0.79 ± 0.01a	0.78 ± 0.01a	0.76 ± 0.01a	0.77 ± 0.01a
18:4*n*-3	0.93 ± 0.02a	0.94 ± 0.01a	0.93 ± 0.01a	0.96 ± 0.02a
20:5*n*-3	6.43 ± 0.06a	6.48 ± 0.13a	6.46 ± 0.08a	6.68 ± 0.15a
22:5*n*-6	2.04 ± 0.02a	2.10 ± 0.17a	2.03 ± 0.05a	2.06 ± 0.06a
22:6*n*-3	11.88 ± 0.12a	11.99 ± 0.25a	11.94 ± 0.15a	12.32 ± 0.36a
Total SFA	24.79 ± 0.06a	24.87 ± 0.07a	24.68 ± 0.12a	24.81 ± 0.17a
Total MUFA	43.97 ± 0.02a	43.76 ± 0.05a	43.76 ± 0.02a	44.69 ± 0.07a
Total *n*-3 PUFA	20.04 ± 0.06a	20.28 ± 0.20a	20.09 ± 0.04a	20.79 ± 0.11a
Total PUFA	23.50 ± 0.03a	23.77 ± 0.04a	23.66 ± 0.09a	24.46 ± 0.12a
Sum Unsaponifiable matter	7.74 ± 0.24a	7.60 ± 0.29a	7.90 ± 0.29a	6.04±0.97a

Note: [1] Values are means ± SEM, *n*-3. Means in a row with different letters differ significantly as determined by Tukey–Kramer HSD, $p < 0.01$. SFA, Saturated fatty acids; MUFA, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acids.

4. Discussion

4.1. Removal of Lipid Oxidation Products by SPD

The SPD system effectively removed the markers for lipid oxidation in both pollock and hoki oils. The primary products of oxidation, measured as PV, were significantly reduced in the distilled oils from both fish species examined and met the GOED monograph of quality marine oil. The final PV results in distilled pollock oils were lower than in distilled hoki oil, regardless of distillation temperature. Neither oil type showed a graded reduction in the PV with increasing temperature of the evaporator in the SPD system. Crude hoki oil had higher (10.3 ± 1.2 mEq/kg) starting PV values than those recorded for crude pollock oil (6.3 ± 0.5 mEq/kg). The higher PV level may reflect the differences in storage time (hoki oil 5.5 years, pollock oil 10 months) or differences in crude oil processing conditions. Previous work on better quality commercial oils showed PV values reduced from 1.8 mEq/kg to 0.7 mEq/kg [26] and all distilled oils studied had values in this range, moreover pollock and hoki oils had much lower PV than the maximum recommended in the GOED voluntary monograph (<5 mEq/kg) [19]. There was an 80% reduction in lipid peroxides between crude and distilled hoki oils (2.38–2.36 mEq/kg), while this reduction was 97% for the pollock oil (<0.14 mEq/kg). Further work is needed to understand the efficiency of the SPD system in removing peroxides from fish oils, however, our findings indicate the initial content of peroxides in the crude oil appeared to have a significant impact in the values found in distilled oils. Further adjustments to distillation process variables need to be investigated, which can counterbalance variability in the initial levels of peroxides in crude fish oil, to yield consistent rate of reduction of peroxides from crude to distilled oils. For instance, a slower flow of the oil through the evaporator may yield lower peroxide values in the distilled product because peroxides will decompose into secondary oxidation products that may be distilled off depending on molecular weight. On the other hand, prolonged exposure to high temperatures

even under extremely low pressures may promote removal of other constituents of the oil that have antioxidant properties, such as fat soluble vitamins, which may be desirable in the final product.

A previous study on marine oil purification on a larger SPD (0.06 m^2, double the size of our wiped surface area) showed reductions in PV, *p*-AV and TOTOX over a series of experiments that was greater than reductions observed for traditional activated carbon treatments [26]; this study demonstrated that reduction in PV is related to flow rate and evaporator temperature [26]. A commercial scale SPD (3m^2 wiped surface area, 350 Lh^{-1} flow) study showed PV reduction, but did not show as pronounced reduction in oxidation parameters (14%–32% reduction in PV and 0%–21% reduction in *p*-AV) as we have demonstrated (76%–98% reduction in PV and 12%–78% reduction in *p*-AV) [28]. Our slow flow rate (0.36–0.48 Lh^{-1}), high evaporator temperatures (190–210 °C), and small surface area (0.033 m^2) may have resulted in greater reductions in PV. It is difficult to make comparisons between studies using different SPD; however, differences based on flow rate per m^2 of evaporator surface area will give good indication of residence time of oil. In our study we had an estimated residence time of 10.9–14.5 $Lh^{-1}\cdot m^{-2}$, while the larger scale study was ten-fold greater at 116.7 $Lh^{-1}\cdot m^{-2}$ [28]. Traditional fish oil refining also includes a bleaching step to remove colored compounds and oxidation products. If reduction in oil color is desirable, then fish oils should be subjected to beaching prior to SPD and this likely further reduces the PV and *p*-AV values of finished product.

The SPD was an effective process to also remove secondary lipid oxidation products (carbonyl-containing compounds) that were measured using the *p*-AV method for both oils studied. In the pollock oils the results demonstrated an evaporator temperature effect, with pollock oils distilled at 210 °C showing the greatest reduction in *p*-AV when compared with values for oils distilled at either 190 or 200 °C. All distilled oils had *p*-AV below the GOED voluntary monograph recommended level of <20; however, pollock oil distilled at 190 °C had an average *p*-AV (19.6 ± 1.1) that was very close to this limit. These results suggest that within the temperature range studied, an evaporator temperature of 210 °C is preferred for distilling crude pollock oils that contained ascorbyl palmitate when using the SPD system; while for crude hoki oils any of the tested temperatures would be suitable for removing secondary products of oxidation. One potential confounding variable is ascorbyl palmitate that was added to crude pollock oil (250 ppm) for its established antioxidant properties [27], which was absent in the crude hoki oil. The hoki oil had Barox™ added post rendering as an antioxidant at a maximum of 750 ppm. A weakness in our study with this regard is the lack of data regarding quantity of ascorbyl palmitate or Barox removed from pollock and hoki oils as a result of distillation at the three tested evaporator temperatures. Further investigations are necessary to determine the influence of these additives in fish oils as it pertains to distillation efficiency of secondary products of oxidation using the SPD system.

The TOTOX values for distilled hoki and pollock oils, regardless of the evaporator temperatures tested, fell well under that required by the GOED monograph (TOTOX < 26). As TOTOX values are a function of PV and *p*-AV, not surprisingly the results for the AV were the major contributor for the significant difference observed between TOTOX values of pollock oils distilled at the three tested evaporator temperatures.

4.2. Removal of Lipid Hydrolysis Products by SPD

The SPD process was very efficient at removing free fatty acids (FFA) from both hoki and pollock oils. This method removed high amounts (13.1 mg KOH/g) of FFA from the hoki oil. The SPD process removed even minor quantities (0.5 mg KOH/g) of FFA that were found in crude pollock oil to very low levels of 0.06–0.09 mg KOH/g recorded for distilled oils. The FFA of distilled pollock oils were comparable to the levels determined for distilled hoki oils (0.04–0.06 mg KOH/g). The distilled oils FFA concentrations were far beneath the suggested maximum levels of the GOED voluntary monograph (<3 mg KOH/g) [19]. Overall, the SPD system operated at the evaporator temperatures studied yielded virtually complete removal of FFA from crude oils, regardless of their initial FFA content.

It has previously been shown that FA ethyl esters added to fish oil assisted in the removal of persistent organic pollutants using SPD [26]. It was proposed that the addition of the FA ethyl esters led to the formation of a "working fluid" that enhanced the efficacy of the process [26]. The working fluid model can include any volatile compounds in the fish oils, including FA ethyl esters, FFA, cholesterol, mono-, di- and triacyl glycol, natural vitamins and antioxidants, and added antioxidants and carriers used in the commercial formulations (e.g., propylenglycol). The fluidity of this mixture will also depend on the internal condenser temperature. It is possible that the higher content of the FFA in the crude hoki oils assisted, via a similar mechanism, in the removal of the secondary oxidation products as determined by *p*-AV. This "working fluid" model may help explain the enhanced reduction in *p*-AV of distilled hoki oils as compared with the more gradual, and temperature-dependent, effect observed for the distilled pollock oil samples (Figure 2b). Even though data in this study does not support conclusive remarks about the applicability of the working fluid model to explain the research findings, it suggests further research in this particular topic should be conducted.

4.3. Effect of SPD on Oil Fatty Acid Composition

A major consideration for the use of SPD in fish oil processing is whether it affects the concentrations of EPA and DHA in the oil. Overall there were no appreciable changes to FA profiles in either of the oils for any of the temperatures tested. There were no differences in the concentrations of EPA and DHA in any oils post SPD treatment of the hoki oil. In the Pollock oil there was a small although significant increase in both DHA and EPA in the 210 °C fraction. The preservation of PUFA at all tested temperature conditions confirmed that SPD is an effective method to purify fish oils, which have been previous shown in other studies [26,28]. Further, as expected, SPD reduced the amount of unsaponifiable matter in the oil which has also been previously demonstrated [26]. The advantages of the SPD system include the short residence time of the oil in the evaporator combined with very low operating pressures and limited reaction time available for undesirable lipid degradation processes to take place, which are known to occur during distillation of oils when using traditional oil processing [5].

4.4. Uses of SPD in Marine Oil Processing

Liver oils, historically obtained from Atlantic cod, have been consumed in Scandinavian countries as far back as the middle ages and are an important source of *n*-3 LC-PUFA [21]. More recently

pollock oils and oils from other gadoid species (which includes Hoki) and Hake have overtaken cod as the traditional source of fish oil [21]. This is the first reported use of SPD to purify hoki and pollock oils. SPD provides a rapid and gentle way to increase the quality and value of these oils. In a preliminary study (data not shown) it was determined that no significant difference (Tukey's Honest Significant Difference Test; $p < 0.05$) existed in various quality parameters examined between pollock oil purified at 210 °C using roller speeds of 500 or 450 rpm. The 50 rpm decrease in the roller speed, from the maximum allowable setting of 500 rpm, was selected for purification of hoki oils because this speed poses less mechanical stress to the internal movable parts of the evaporator that operate the wiped film blades.

Preventing fish oil lipids from undergoing undesirable oxidative chemical changes during rendering and purification steps is a key element to obtaining a final product that has prolonged shelf life and adequate sensorial and nutritive properties. It also ensures consumer's safety. It has been previously reported that SPD can strip oil of natural and added antioxidants [29,32]. A 50% reduction in tocopherol concentration was demonstrated by the application of a series of different SPD conditions in rapeseed oil [32]. In another study, reductions of up to 90% of the original concentrations of antioxidants were observed in vegetable oils subjected to short-path distillation [29]. It was suggested that through the application of antioxidants at different steps of oil processing, such as pre- and post-SPD, a reduction in the oxidation status of the oil can be achieved; however, the antioxidant systems used were not disclosed due to commercial sensitivities [29]. Liver oils such as hoki, pollock and cod are known to be a good source of fat soluble vitamins such as A, D and E. This may be advantageous if hoki or pollock oil are to be consumed on a daily basis to achieve intake of EPA and DHA at the levels recommended for certain disorders. For example, the American Heart Association's (AHA) recommendation of consumption of 2–4 g of EPA+DHA per day would require 10 to 20 g of hoki and pollock oils. In these doses the amount of minor components of the oil such as lipid soluble vitamins may be approaching the upper limits (UL) of recommend dietary intake. Pollock oil lipid soluble vitamins have been reported with vitamin A (retinol) 103 g/g and vitamin E (measured as -tocopherol) 172 g/g [11]. Unrefined hoki oil has higher levels of vitamin A 1400–1900 g/g, vitamin E (measured as -tocopherol) 600–1100 g/g and ~100 g/g of vitamin D [39,40]. The recommended dietary intake (RDI) and UL, which is maximum daily intake unlikely to cause adverse health effects, for an adult male and female are different for each vitamin. The RDI from the nutrient reference values of Australia and New Zealand of vitamin A is 900 µg/day for men and 700 µg/day for women with a shared UL of 3,000 µg/day [41]. Two grams of hoki oil would reach the UL of vitamin A consumption, and similarly for vitamin D. However, for vitamin E the UL and RDI are substantially higher than the content in hoki oil (300 mg/g UL, 4 mg/g RDI). In this study we did not measure the effect of SPD on the vitamin content of the hoki and pollock oils, but it is expected that there could be a loss of lipid-soluble vitamins using this process. Previous work has seen vitamin loss in spratt (*Sprattus sprattus*) oil up to 82% for vitamin A, 64% vitamin D and 42% vitamin K [26]. The loss of lipid soluble in vitamins by SPD pollock and hoki oils would be important to establish for commercial use.

This work was carried out using a laboratory-scale bench top SPD. This equipment has several glass-on-glass connections which do not always give a good seal and require extensive leak checks and verification that stable pressure values have been achieved before distillation is carried out. All distilling pressures were <0.02 mbar and the vacuum varied slightly between replicate distillation runs,

for instance the range of pressures recorded for nine pollock oil distillations was 0.018–0.011 mbar. This slight change in vacuum between runs due to the apparatus has been previously reported [29]; however, industrial scale stainless steel SPD equipment provides finer control of the vacuum between replicate distillations. Albeit not included in this study, determination of the concentration of antioxidants pre- and post-SPD purification of oils, together with other parameters such as sensory properties, polar lipids and oxidative stability trials, should be considered for future research and development endeavors on these oils.

5. Conclusions

The Alaskan fish oil industry is growing, with several companies refining marine oils and selling higher value products. However, there are still many companies that sell fish oil for non-edible purposes, such as for use in aquaculture. The New Zealand hoki industry does have the capability to make edible fish oils; however, the bulk of the oil is still sold as crude or unrefined due to processing costs. SPD provides a gentle and efficient way to improve the quality of these oils that could be readily added into the rendering processes in these countries. Moreover, SPD has the potential to provide major benefits for industry as it involves reduced and simplified processing for the purification of marine oils. Unfortunately, as is often the case for the new technologies, SPD involves high operating costs which have prevented the broad uptake of this technology by industry to date.

Although recognition of the importance of oil quality and sustainable processing is growing, potential cost saving and/or oil yield increases remain the prime parameters for the implementation of a new process. However, the potential for SPD to provide improved oil quality has been clearly demonstrated in this study.

Acknowledgments

The pollock oil research was funded by the Pollock Conservation Cooperative Research Center project title “Purification of pollock oil using short path distillation”. This research was also supported by MBIE (NZ Ministry of Business, Innovation and Employment) under project C02X0806, “Engineered Marine Molecules” with additional funding from the MBIE programme “Export Marine Products” and the Kodiak Seafood and Marine Sciences Center (University of Alaska Fairbanks). We thank American Seafoods Company (Seattle, UA, USA) for donation of Pollock oil and Sealord Group Ltd. (Nelson, New Zealand) for the donation of the hoki oil.

Author Contributions

A.O. and M.M. both equally contributed to conception and design, interpretation of data and development of manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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A Nutritional-Toxicological Assessment of Antarctic Krill Oil *versus* Fish Oil Dietary Supplements

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Abstract: Fish oil dietary supplements and complementary medicines are pitched to play a role of increasing strategic importance in meeting daily requirements of essential nutrients, such as long-chain ($\geq C_{20}$, LC) omega-3 polyunsaturated fatty acids and vitamin D. Recently a new product category, derived from Antarctic krill, has been launched on the omega-3 nutriceutical market. Antarctic krill oil is marketed as demonstrating a greater ease of absorption due to higher phospholipid content, as being sourced through sustainable fisheries and being free of toxins and pollutants; however, limited data is available on the latter component. Persistent Organic Pollutants (POP) encompass a range of toxic, man-made contaminants that accumulate preferentially in marine ecosystems and in the lipid reserves of organisms. Extraction and concentration of fish oils therefore represents an inherent nutritional-toxicological conflict. This study aimed to provide the first quantitative comparison of the nutritional (EPA and DHA) *versus* the toxicological profiles of Antarctic krill oil products, relative to various fish oil categories available on the Australian market. Krill oil products were found to adhere closely to EPA and DHA manufacturer specifications and overall were ranked as containing intermediate levels of POP contaminants when compared to the other products analysed. Monitoring of the pollutant content of fish and krill oil products will become increasingly important with expanding regulatory specifications for chemical thresholds.

Reprinted from *Nutrients*. Cite as: Nash, S.M.B.; Schlabach, M.; Nichols, P.D. A Nutritional-Toxicological Assessment of Antarctic Krill Oil *versus* Fish Oil Dietary Supplements. *Nutrients* **2014**, *6*, 3382–3402.

1. Introduction

Fish are a nutrient-dense food source. The role of marine-derived, long-chain (LC) ($>C_{20}$) omega-3 (ω3) polyunsaturated fatty acids (LC-PUFA), in the promotion of health is well established. Since early observations that Greenland Eskimos who subsisted on large amounts of fish suffered low levels of cardiovascular disease related mortality, epidemiological and experimental evidence has confidently shown the protective role of sufficient ω3 LC-PUFA intake against cardiovascular disease and certain types of cancer, e.g., [1,2]. In particular docosahexaenoic acid (DHA, 22:6ω3) and eicosapentaenoic acid (EPA, 20:5ω3), each with distinct roles in disease prevention, have been credited for their contribution to a healthy diet. In addition to serving as energy stores, ω3 LC-PUFA form integral structural components of cellular membranes [3]. For example, ω3 LC-PUFA are highly concentrated in the cellular membranes of the retina and brain and accumulate there rapidly in the third trimester of foetal development. Gestational ω3 LC-PUFA restrictive studies have shown significant deleterious impact to off-spring visual acuity and cognitive function [4]. Finally, symptomatic alleviation with ω3 LC-PUFA intake has been reported for a broad range of health conditions. Anti-inflammatory properties of ω3 LC-PUFA provide a molecular basis for symptomatic alleviation of inflammatory disease such as rheumatoid arthritis, lupus and asthma [5–7]. More recently improvements in

psychiatric disorders such as depression and schizophrenia with ω3 LC-PUFA administration have been observed [8].

Fish oil is also a rich source of lipid-soluble micronutrients such as vitamin D, which plays a fundamental role in bone health [9]. Consequently, The National Heart Foundation of Australia, in accordance with a host of international agencies, recommends consumption of fish at least twice a week. Paradoxically, modern diets in developed nations are characterised by severe ω3 LC-PUFA deficiency, reflecting low seafood intake. This was exemplified in a recent study which found 78% of the Australian population did not meet their daily recommended intake of ω3 LC-PUFA [10]. In fact, it must be considered that meeting health targets for seafood intake is not economically nor ecologically attainable for large fractions of the global population [11].

Effectively tackling dietary deficiency of seafood micronutrients would carry significant bearing on both the social and economic burden of disease. Increasing the dietary status of vitamin D alone in Western Europe has been estimated to alleviate the economic burden of disease by $293 billion per year [12]. In the absence of sufficient high quality, affordable seafood sources, dietary supplements and complementary medicines are pitched to play a role of increasing strategic importance.

A new product category has been launched on the omega-3 nutriceutical market and is currently gaining significant market share. A nutriceutical oil derived from Antarctic krill (*Euphausia superba*), a Euphausiid crustacean forming the basis of the Antarctic food web, has been marketed since 2002 and has recently become broadly available in Australia [13]. Marketing of krill oil centres on three characteristic properties of the oil. Krill oil contains the essential nutrient, choline and an antioxidant, astaxanthin. In addition, it is posed that Antarctic krill oil derived ω3 LC-PUFA is more bioavailable compared to fish oils. A higher fraction of ω3 LC-PUFA is associated with phospholipids in krill oil, compared to triacylglycerols in fish oils. This property has been theorised to improve absorption and bioavailability of ω3 LC-PUFA [14], based upon independent liposome carrier research [15]. Secondly, one major krill oil manufacturer has achieved Marine Stewardship Council certification of sustainability and, as a whole, the industry is often viewed as being sustainable due to the fact that the worldwide harvest constitutes only a minor fraction of established fishing quotas, e.g., [14,16]. It should be noted, however, that uncertainty surrounds the distribution and density of circumpolar krill stocks and therefore the robustness of fishery quotas remains debated [17]. Finally, krill oil is cited as being naturally free of toxins and pollutants [6]. Persistent Organic Pollutants (POPs) are toxic contaminants that bioaccumulate, and have been introduced to the environment since the mid-1900s. Their extreme persistence and effective environmental dispersal mechanisms have resulted in ubiquitous contamination of all environmental matrices. POPs are considered a substantial risk to human health [18] and are subject to the Stockholm Convention, a legally binding treaty signed by over 100 nations, and ratified by Australia in 2004 [19].

Within an ecosystem, lower trophic level species such as zooplankton, are often found to accumulate lower levels of POP contaminant burdens due to shorter life-spans. However, this cannot be assumed when comparing species from different ecosystems. Polar species are characterised by large body size and long life spans. Antarctic krill live to 5–7 years which is comparable to, or longer than, source species commonly used in fish oil production [13]. Similarly, Antarctic krill have demonstrated highly adaptable feeding, and in addition to their herbivorous feeding, have been

observed to rely on cannibalism and detritivory to endure food deprivation [20]. This dietary flexibility also confounds their trophic placement and thus the POP bioaccumulation patterns of the species.

Historical or "legacy" POPs are chlorinated compounds. Their common molecular structures predict similar environmental behaviour. In the physical environment they are semi-volatile. Volatilized fractions will undergo progressive movement towards colder and colder climates experiencing "cold-trapping" at the poles of the earth [21]. In the particulate phase they will adhere strongly to organic matter representing an effective mechanism for transfer from the terrestrial to the aquatic environment and assimilation into food-webs. Consequently, the vast majority of human exposure to POPs occurs via seafood consumption [22,23]. This clear nutritional-toxicological conflict associated with seafood intake has urged the Codex Alimentarious Commission for Contaminants in Food to convene an expert consultancy on the risks and benefits of fish consumption [18]. In the case of fish oil dietary supplements, the scenario is even more acute. Legacy POPs are extremely lipophilic and accumulate in the fat reserves of animals. When the lipid fractions of seafood are selectively isolated and concentrated for administration as dietary supplements or complementary medicines, the seafood micronutrient:POP burden conflict is exacerbated. Indeed, repeated incidences of fish oil product recalls due to exceedance of POP safety guideline have occurred and are only likely to rise as the market expands and authorities pursue greater regulatory overview [24,25].

Recently we conducted the most comprehensive analytical survey of POPs in any Antarctic environmental matrix to date [26]. Our study centred on Antarctic krill, as POP vectors to the remainder of the Antarctic food-web, and extended across almost a quarter of the Antarctic continent. Our findings highlighted that Antarctic krill POP profiles were distinct from those typical of northern hemisphere species, but that they were not insubstantial. Indeed, for some compounds such as hexachlorobenzene (HCB), levels were comparable to or greater than those of similar trophic level species in other global regions. This work has prompted the following strategic examination of commercial krill oil products. Here we will assess krill oil POP burdens, as well as product nutritional lipid class and fatty acid profiles. These will be compared to those of other categories of commercial fish oil dietary supplements available on the Australian market.

2. Experimental Section

2.1. Product Selection

Four categories of seafood-oil dietary supplements were selected for analysis, namely, (i) krill oil; (ii) enriched (in terms of EPA + DHA) fish oil; (iii) nutriceutical formulations containing fish oil; and (iv) standard or budget grade 18:12 (EPA + DHA) fish oil (Table 1). Products representative of the two major krill oil manufacturers were selected under the krill oil category. For the other remaining categories, three representative and readily available brands were selected. Efforts were made to combine capsules from two separate batches of each product for each POP and FA analysis. This was achieved for all products except Blackmores Omega Liquid Fish Oil for which only a single batch number could be sourced. Full details of selected products and batch numbers are listed in Table 1.

2.2. Sample Analysis

2.2.1. Lipid Extraction and Class Determination

Pre-weighed (*c.a.* 0.03 g) oil samples were used for lipid analyses. Individual capsule or liquid oil samples were cut open and dissolved in $CHCl_3$. A known aliquot of total lipid (achieving a final concentration of approximately 10 mg lipid/mL $CHCl_3$) was transferred into separate vials and made up to 1.5 mL of $CHCl_3$.

2.2.2. Fatty Acid (FA) Determination

An aliquot of the total lipid extract was trans-methylated by addition of MeOH/HCl/$CHCl_3$ (3 mL 10:1:1, v/v/v, 80 °C/2h) to produce fatty acid methyl esters (FAME). After cooling the mixture and addition of 1 mL of water, FAME were extracted (3×) with 4:1 hexane/dichloromethane. A C19 FAME internal injection standard was added prior to analysis by gas chromatography (GC) using a GC (Agilent Technologies 7890A) equipped with a Supelco Equity™-1 fused silica capillary column (15 m × 0.1 mm internal diameter, 0.1 μm film thickness) [27]. GC-mass spectrometry (GC-MS) confirmed FAME identifications and was performed on a Finnigan Thermoquest GCQ GC-mass spectrometer fitted with a column of similar polarity to that described above, an on-column injector and using Thermoquest Xcalibur software (Austin, TX, USA). Helium was used as carrier gas and other operating conditions were as previously described [27]. The relative levels of individual FA were expressed as percent of total FA area. A catalogue of quantified FA is presented in Table 2. FA present at less than 0.5% of total FA in all products are grouped as Other FA; this FA group comprised 1.6%–4.2% of the total FA across the products analysed.

Table 1. Selected fish and krill oil nutriceutical products compared in this study. Capsules or subsamples from two separate batches of each product were pooled for each fatty acids (FA) analysis, except for Blackmores Omega Liquid Fish Oil for which only a single batch number could be sourced.

Krill Oil	Enriched Fish Oil	Formulations Containing Fish Oil	Standard 18:20 Grade Fish Oil
	BIO organics Super Liquid Fish Oil EPA 1.6 g; DHA 810 mg 5 mL serve (Batch 11448A and 11815A)	Blackmores Omega Joint "Mercury, PCB and dioxin tested" EPA 550 mg; DHA 120 mg 1000 mg capsule (Batch 252505 and 252076)	Nature's Own Odourless Fish Oil 1000 mg EPA 180 mg; DHA 120 mg 1000 mg capsule (Batch 650566 and 652769)
Swisse Wild Krill Oil (NKO) EPA 47 mg; DHA 28 mg 333 mg capsule (Batch 022537 and 022538)	Blackmores Omega Liquid Fish Oil EPA 1.7g; DHA 1.1g 5 mL serve (Batch 10709101)	Nature's Way Kids Smart EPA 28 mg; DHA 133 mg 1000 mg capsule (Batch B8514-1 and Batch C4401)	Blackmores Odourless Fish Oil 1000 mg EPA 180 mg; DHA 120 mg 1000 mg capsule (Batch 252461 and 253417)
Norkrill (Aker BioMarine) EPA 60 mg; DHA 28mg 500 mg capsule (Batch 390048 and 443003)	Bioceuticals OmegaSure Liquid Fish Oil EPA 1050 mg; DHA 750 mg 5 mL serve (Batch 26764 B L80 and 26764 C L80)	Blackmores Pregnancy and Breastfeeding Gold EPA 25 mg; DHA 125 mg 1000 mg serve (Batch 252025 and 250973)	Cenovis Fish oil 1000 mg EPA 180mg; DHA 120 mg 1000 mg capsule (Batch 650588 and 649319)

2.2.3. Quality Control

For lipid class and FA profiling, commercial (Nuchek) and laboratory standards (e.g., tuna oil) of known composition were routinely analysed to both confirm component identifications and ensure data quality.

2.2.4. Chemical Analysis

Oil samples were analysed for chlorobenzenes (hexa- and penta-chlorobenzene); chlorinated pesticides; hexachlorocyclohexanes (α-, β-, γ- HCH); the dichlorodiphenyltrichloroethane (DDT) group (o,p'-DDE, p,p'-DDE, o,p'-DDD, p,p'-DDD, o,p'-DDT, p,p'-DDT); toxaphene (Tox-26, 32, 40 + 41, 42a, 44, 50, 62); polychlorinated cyclodienes (endosulfan-I , endosulfan-II, endosulfan-sulphate, heptachlor-exo-epoxide, heptachlor-endo-epoxide, trans-chlordane, cis-chlordane, oxychlordane, chlordene, heptachlor, trans-nonachlor, cis-nonachlor, dieldrin, aldrin, isodrin, endrin) and the individual compounds mirex and trifluralin. In addition, samples were analysed for the polychlorinated biphenyl (PCB) congeners, 18, 28, 31, 33, 37, 47, 52, 66, 74, 77, 81, 99, 101, 105, 114, 118, 122, 123, 126, 128, 138, 141, 149, 153, 156, 157, 167, 169, 170, 180, 183, 187, 189, 194, 206 and 209 (IUPAC numbers) and polychlorinated dibenzo-p-dioxin/furan (PCDD/F) congeners; 2,3,7,8-TCDD, 1,2,3,7, 8-PeCDD, 1,2,3,4,7,8-HxCDD, 1,2,3,6,7,8-HxCDD, 1,2,3,7,8,9-HxCDD, 1,2,3,4,6,7,8-HpCDD, OCDD, 2,3,7,8-TCDF, 1,2,3,7,8/1,2,3,4,8-PeCDF, 2,3,4,7,8-PeCDF, 1,2,3,4,7,8/1,2,3,4,7,9-HxCDF, 1,2,3,6,7,8-HxCDF, 1,2,3,7,8,9-HxCDF, 2,3,4,6,7,8-HxCDF, 1,2,3,4,6,7,8-HpCDF, 1,2,3,4,7,8, 9-HpCDF and OCDF.

2.2.5. Sample Preparation and Clean-up

The extraction and clean-up methods for POP have previously been described in full [26]. In brief, dioxin, furan and non-ortho PCB sample extraction and clean-up was performed on a semi-automated 3 column system (first column, Na_2SO_4, activated silica and potassium silicate; second column, single use Fluid Management Systems (FMS) silica column; third column, single use FMS activated carbon column). The sample portion containing PCDD/Fs and non-ortho PCBs was eluted from column 3 with toluene, reduced and exchanged to hexane before undergoing further clean-up by sulphuric acid coated silica column followed by potassium hydroxide coated silica column.

Samples for PCB and chlorinated pesticide analysis were extracted on a cold-column and cleaned by gel permeation chromatography, alumina and silica gel columns.

2.2.6. Quantification

The isomer identification and quantification was carried out with HRGC/HRMS using a Hewlett-Packard 5890II (1990–2003) or 6890N (2003–2006) gas chromatograph coupled to an AutoSpec mass spectrometer (Micromass Waters, Manchester, UK). Resolution of mass spectrometer was >10,000 with electron ionization mass spectrometry in the selected ion monitoring mode (GC/EI-HRMS-SIM). Two SIM values were monitored for each isomer group. The added ^{13}C-labelled isomers were used as internal standard for each group. Additionally, the recovery rates of the added internal standard compounds were determined.

2.2.7. Quality Assurance

The following quantification conditions were fulfilled for all data presented: (i) the retention time of the native compound was within three seconds of the corresponding ^{13}C-labelled isomer; (ii) the isotope ratio of the two monitored masses was within +20% of the theoretical value; (iii) the signal/noise was >3/1 for quantification; (iv) the recovery of the added ^{13}C-labelled internal standards was within 40% to 120% and thereby in agreement with EU and US guidelines and official methods; (v) prior to each new series of samples the blank values of the complete clean-up and quantification procedures were determined. Clean-up of samples only commenced when a sufficiently low blank value was obtained. At least once a year the laboratory participates in an international laboratory inter-calibration exercise.

2.3. Metrics

2.3.1. Tolerable Daily Intake (TDI)

Various regulatory bodies and food authorities have assessed the levels of chemicals that are safe for human consumption, based upon observed affect levels in animal models. The tolerable daily intake (TDI) refers to a threshold of a chemical which does not appear to carry an appreciable risk. In the current study we have used a variety of sources for our reference TDIs, namely Health Canada and the US EPA, The World Health Organisation and the International Panel on Chemical Safety (IPCS) as well as peer-reviewed literature.

2.3.2. Toxicity Equivalency Factors (TEQs)

Toxicity equivalency factors express the toxicity of similar acting, planar, dioxin, furan and certain PCBs relative to the most potent congener 2,3,7,8-TCDD which is assigned a value of 1.0. The TEQ values applied in the current study refer to Van den Berg *et al.*'s 2005 re-evaluation of TEQ values [28].

3. Results and Discussion

3.1. Lipid and Fatty Acid Profiles

The majority of categories and brands of seafood oil supplements matched or exceeded manufacturer EPA and DHA specifications, with the exception of three brands which fell slightly below (~10%–30%) the manufacturer specifications (Tables 1–3). These related to EPA levels in one enriched fish oil, namely Blackmores Omega liquid fish oil (1700 mg specified *vs.* 1500 mg observed) and DHA levels in Nature's Way Kidsmart (133 mg specified *vs.* 95 mg observed) and Blackmores Pregnancy and Breastfeeding Gold (125 mg specified *vs.* 85 mg observed). It is noted, that for pure oil capsules it is possible to compensate for EPA and DHA batch variability through marginal capsule volume adjustments. This is however, less readily achievable for formulations, such as the latter two products, and uncontrollable for liquid formulations. A listing of all FA present at >0.5% of the total FA in each product analysed is shown in Table 2.

Table 2. A catalogue of fatty acids (FA) quantified, together with the composition (as percent of total FA) of all products. Abbreviations used for oil products are: Swisse Krill Oil (SW); Norkrill (Nor); Bio-organics (Bio-O); Blackmores Omega (B-Ω); Bioceuticals (BCT); Blackmores Joint (B-Joint); Nature's Way Kidsmart (NWK); Blackmores Pregnancy (B-P); Nature's Own 1000 mg (NO-1000); Blackmore's 1000 mg (B-1000); Cenovis 1000 mg (C-1000). Capsules or subsamples from two separate batches of each product were pooled for each FA analysis, except for Blackmores Omega Liquid Fish Oil for which only a single batch number could be sourced. Other: denotes FA present at <0.5% in all products.

Product	SW	Nor	Bio-O	B-Ω	BCT	B-Joint	NWK	B-P	NO-1000	B-1000	C-1000
FA											
14:0	7.3	8	1.9	0.37	4.3	0.12	3.3	5.06	6.3	2.4	6.1
15:0	0.33	0.33	0.15	0.02	0.3	0	0.64	0.46	0.58	0.2	0.44
16:4	0.7	0.77	1.4	0.1	2.6	0.98	0.31	0.24	2.3	1.4	2.3
16:3	0.22	0.23	1	0.05	2.2	0.53	0.27	0.2	1.6	0.9	1.6
16:1ω7c	6.4	5.6	3.3	0.55	5.7	1.07	5.1	4.3	10	4.1	11
16:1ω 5c	0.48	0.54	0.07	0	0.12	0.01	0.14	0.11	0.21	0.07	0.21
16:0	19	23	5	3.2	11	0.18	19	20	17	5.6	15
Br17:1	0.1	0.06	0.16	0.03	0.28	0.12	0.57	0.52	0.35	0.19	0.31
17:1ω 8c + a17:0	0.27	0.25	0.18	0.1	0.3	0.06	0.7	0.6	0.41	0.18	0.41
17:0	0.09	0.1	0.17	0.19	0.28	0.01	0.86	0.83	0.53	0.18	0.47
18:4ω3	2.9	3.4	4.3	1.5	3.6	3.9	1.1	0.89	2.9	3.5	2.7
18:2ω6	2.3	2	1.1	0.75	1.2	0.9	3.4	9.03	1.5	1.1	1.4
18:3ω3	1	1.2	0.68	0.43	0.46	0.59	0.73	1.4	0.81	0.65	0.69
18:1ω9c	11	9.8	5.6	6.5	6.5	1.37	11	13	9	3.9	8.6
18:1ω7c	8.2	7.2	2.3	2.6	2.6	0.94	2.4	2.6	4	2	4.2
18:0	1.01	1.1	1.7	3.6	2.9	0.11	14	7.8	3.6	1.2	3.4
20:4ω6	0.19	0	3.4	0.29	0.5	0.15	1.3	1.3	0.63	0.23	0.41
20:5ω3	20	21	37	35	24	62	6.9	5.7	18	47	19
20:4ω3	0.49	0.52	1.3	1.7	1.1	2	0.48	0.43	0.89	1.6	0.9
20:1ω9c	0.69	0.7	1	3.05	1.4	0.56	1.3	1	1.09	0.73	0.97
20:1ω7c	0.38	0.4	0.26	0.72	0.5	0.07	0.17	0.15	0.45	0.2	0.47

Table 2. *Cont.*

Product	SW	Nor	Bio-O	B-Ω	BCT	B-Joint	NWK	B-P	NO-1000	B-1000	C-1000
FA											
21:5ω3	0.48	0.63	1.3	1.6	1.1	2.4	0.22	0.19	0.68	1.8	0.71
22:5ω6	0.01	0	0.25	0.46	0.17	0.29	0.71	1.1	0.27	0.27	0.22
22:6ω3	12	9.8	20	25	18	16	20	18	11	14	12
22:5ω3	0.46	0.57	2.7	4.8	3.4	4.4	1.3	1.2	2.3	3.7	2.6
22:1ω11c	0	0	0.6	2.3	1.1	0.02	0.67	0.49	0.55	0.2	0.59
22:1ω9c	0.48	0.79	0.15	0.49	0.27	0.05	0.17	0.14	0.2	0.1	0.19
24:1ω9c	0.17	0.19	0.24	0.53	0.53	0	0.37	0.39	0.46	0.15	0.45
Other	3.09	2.39	2.71	4.16	3.61	1.59	3.45	3.39	2.8	1.87	3.19
Total	100	100	100	100	100	100	100	100	100	100	100

Other fatty acids: 14:1ω5c, i15:0, a15:0, 15:1ω6c, 16:2, i16:0, 16:1ω9c, 16:1ω7t, 16:1ω13t, i17:0, 17:1, 18:3ω6, 18:1ω7t, 18:1ω5c, 18:1, 19:1 (2 isomers), 20:3ω6, 20:2ω6, 20:1ω11c, 20:1ω5c, 20:0, 21:0, 22:4ω6, 22:1ω7c, 22:0, 23:0, 24:6ω3, 24:5ω3, 24:1ω11c, 24:1ω7c, 24:0.

Table 3. Observed *versus* manufacturer labelled EPA and DHA values of selected fish and krill oil nutriceutical categories and products (labelled and values are rounded to two significant figures). Capsules or subsamples from two separate batches of each product were pooled for each FA analysis, except for Blackmores Omega Liquid Fish Oil for which only a single batch number could be sourced.

Product	Labelled EPA (mg) Per Capsule/Serve	Observed EPA (mg) Per Capsule/Serve	Labelled DHA (mg) Per Capsule/Serve	Observed DHA (mg) Per Capsule/Serve	EPA + DHA (mg) per Max Recommended Daily Serve	Cost (AUD) Per (Labelled) 500 mg DHA + EPA
Krill Oil						
Swisse (NKO)	50	55	30	33	240	3.8
Norkrill (Aker Biomarine)	60	86	28	40	180	4.0
Enriched Fish Oils						
BioOrganics super liquid	1600	1600	810	860	2400	0.21
Blackmores Omega Liquid	1700	1500	1100	1100	2800	0.24
Bioceuticals omegasure Liquid	1050	1300	750	790	1800	0.28
Formulations						
Blackmores Omega Joint	550	590	120	150	2700	0.40
Nature's way kidsmart	28	33	130	95	320	0.90
Blackmores Pregnancy	25	27	130	86	300	2.7
18:12 Standard Grade						
Nature's Own Odourless 1000	180	180	120	110	2700	0.15
Blackmores Odourless 1000	180	180	120	110	3600	0.10
Cenovis 1000	175	180	70	120	250	0.25

3.2. Persistent Organic Pollutants

None of the categories or products analysed in the current study, at their highest recommended dosage, came close to fulfilling tolerable daily intake (TDI) levels for any single analyte (Table 4). Despite the fact that environmental exposure to POPs does not occur to a single residue at a time, but rather to complex and interacting mixtures, this finding is reassuring. As a means of qualitatively comparing and contrasting the eleven products analysed in this study, and providing an overview of chemical summaries obtained, we devised a simple scoring system (Table 5). The five products with the greatest contaminant burden for five key compound groups, plus TEQ values, were ranked from 1–5 with the sample containing the highest concentrations receiving a score of 5. Bioceuticals Omegasure liquid fish oil and Blackmores 1000 mg both carried a cumulative score of 16 reflecting their placement among the top five products for five and four compound/index groups respectively. Blackmores Pregnancy and Breastfeeding Gold formula and Nature's Own 1000 mg each received a score of 12. Blackmore's Pregnancy and Breastfeeding Gold formula incorporates tuna oil, sourced from northern hemisphere oceans, thereby likely contributing to the higher contaminant burdens found in this formulation, despite its lower oil content. Blackmore's Joint formula was the only product which did not feature among the top five products for any analyte or index group.

Table 4. Chemical burdens per maximum recommended daily dose (lipid); where relevant, corresponding TEQ (2005) and percent (%) of Tolerable Daily Intake (TDI). Values are presented to two significant figures. Blank squares indicate that values fell below the method level of detection (LOD), which are given as an average concentration for all 11 products (pg/g lipid), whilst grey squares indicate that corresponding congeners were not analysed for that product. Abbreviations used for oil products are: Swisse Krill Oil (SW); Norkrill (Nor); Bio-organics (Bio-O); Blackmores Omega (B-Ω); Bioceuticals (BCT); Blackmores Joint (B-Joint); Nature's Way Kidsmart (NWK); Blackmores Pregnancy (B-P); Nature's Own 1000 mg (NO-1000); Blackmore's 1000mg (B-1000); Cenovis 1000 mg (C-1000). Capsules or subsamples from two separate batches of each product were pooled for each POP analysis, except for Blackmores Omega Liquid Fish Oil for which only a single batch number could be sourced.

Compound (LOD, pg/g Lipid)	TDI [a] (pg)/Day Per 60 kg Adult	SW (pg/1 g)	Nor (pg/ 1 g)	Bio-O (pg/4.75 g)	B-Ω (pg/4.75 g)	BCT (pg/4.75 g)	B-Joint (pg/4 g)	NWK (pg/1 g)	B-P (pg/0.86 g)	NO-1000 (pg/9 g)	B-1000 (pg/12 g)	C-1000 (pg/9 g)
HCH												
a-HCH (27)				180					260			
b-HCH (32)		72		370					270		690	260
g-HCH (29)		65	33	170					1700			
∑HCH	18,000,000 [c]	(0.00076)	(0.00018)	(0.004)					(0.012)		(0.0038)	(0.0014)
DDT												
o,p'-DDE (46)					480	810.0		1800.0		49,000		
p,p'-DDE (59)					1000	16,000	1200	5000	15,000	120,000	35,000	
o,p'-DDD (37)						840		890	7100	1300		
p,p'-DDD (40)		180		10,000		6700		4900	31,000	20,000	25,000	11,000
o,p'-DDT (43)		260		1300		1100		270	26,000	2000	3600	1000
p,p'-DDT (69)					1400	4400		5100	80,000	140,000	9000	2100
∑DDT	30,000,000 [c]	(0.0015)		(0.038)	(0.0095)	(0.1)	(0.004)	(0.06)	(0.53)	(1.1)	(0.24)	(0.047)

Table 4. *Cont.*

Compound	TDI [a] (pg)/Day Per 60 kg Adult	SW (pg/1 g)	Nor (pg/1 g)	Bio-O (pg/4.75 g)	B-Ω (pg/4.75 g)	BCT (pg/4.75 g)	B-Joint (pg/4 g)	NWK (pg/1 g)	B-P (pg/1.72 g)	NO-1000 (pg/9 g)	B-1000 (pg/12 g)	C-1000 (pg/9 g)
Chlordanes												
trans-Chlordane (60)						4000						
cis-Chlordane (160)						2300						
oxy-Chlordane (1500)					30,000							
cis-Nonachlor (28)						1700		100				
∑Chlordane	30,000,000 [c]				(0.1)	(0.027)		(0.000028)				
Endosulfan-I (34)	360,000,000 [c]			390.0 (0.00011)								
Toxaphene												
Tox-26 (61)						2400						
Tox-40 + Tox-41 (48)						2300		260	770			3500
Tox-44 (160)						7800		1100				13,000
Tox-50 (23)						6200		330	1100			5200
∑Toxaphene	12,000,000 [d]					(0.16)		(0.014)	(0.016)			(0.18)
Chlorobenzenes												
PeCB (3.1)	60,000,000 [b]	930 (0.0016)	340 (0.00057)	59 (0.0001)		300 (0.0005)	180 (0.0003)	91 (0.00015)	190 (0.00032)			160 (0.00027)
HCB (2.8)	9,600,000 [e]	9900 (0.1)	4400 (0.046)	68 (0.00071)		780 (0.0081)	160 (0.0017)	27 (0.00028)	500 (0.0052)			140 (0.0015)

Table 4. *Cont.*

Compound	TDI[a] (pg)/Day Per 60 kg Adult	SW (pg/1 g)	Nor (pg/1 g)	Bio-O (pg/4.75 g)	B-Ω (pg/4.75 g)	BCT (pg/4.75 g)	B-Joint (pg/4 g)	NWK (pg/1 g)	B-P (pg/0.86 g)	NO-1000 (pg/9 g)	B-1000 (pg/12 g)	C-1000 (pg/9 g)
PCB		Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)
18 (14)		38	14			150	86		52		140	
28 (10)		48	20		72	370	83		130	590		
31 (10)		45	21		50	230	91		68		190	
33 (10)			13			64	56				150	
47 (4.8)		48	75		210	420	46		240	260	310	
52 (5.4)		130				130	96	13	360			140
66 (4.6)		57	14			850			600	790	600	260
74 (4.0)		30				550			210	280	250	100
77 (0.11)				10 (0.0011)		18 (0.0018)		0.59 (0.000059)	9.4 (0.00094)	54 (0.0054)	110 (0.011)	
81 (0.10)		0.51 (0.00015)			0.48 (0.00014)	0.38 (0.00011)				1.1 (0.00033)		
99 (5.7)		64	12	93		2300		38	820	920	130	510
101 (6.3)		170	42	190		3700	62	69	900	1900	2800	1000
105 (7.6)		34 (0.0010)		130 (0.0039)		1200 (0.036)		19 (0.00057)	210 (0.0063)	1100 (0.033)	1600 (0.048)	720 (0.022)
114 (5.8)						120 (0.0036)					130 (0.065)	

Table 4. *Cont.*

Compound	TDI [a] (pg)/Day Per 60 kg Adult	SW (pg/1 g)	Nor (pg/1 g)	Bio-O (pg/4.75 g)	B-Ω (pg/4.75 g)	BCT (pg/4.75 g)	B-Joint (pg/4 g)	NWK (pg/1 g)	B-P (pg/0.86 g)	NO-1000 (pg/9 g)	B-1000 (pg/12 g)	C-1000 (pg/9 g)
PCB		Conc. (TEQ)	Conc. (TEQ)	Conc.(TEQ)	Conc.(TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)
118 (5.7)		75 (0.0023)	0.017 (0.000001)	340 (0.01)		3500 (0.11)		0.067 (0.000002)	690 (0.021)	2600 (0.078)	4000 (0.12)	1600 (0.048)
122 (6.3)												140
123 (6.3)						55 (0.0017)		13 (0.00039)	110 (0.0033)			230 (0.0069)
126 (0.43)		1.8 (0.18)	0.94 (0.094)	5.5 (0.55)	1.8 (0.18)	4.6 (0.46)				24 (2.4)	50 (5.0)	
128 (8.1)		21		120		1100		69	310	1200	1500	690
138 (6.9)		110	15	790	60	7100	53	380	1800	6700	10,000	4300
141 (4.8)		18		110		850		50	220	1000	1400	640
149 (4.3)		100	19	320		2700	52	120	75	3100	4400	1700
153 (4.1)		140	25	1200	76	9200	75	50	2600	9900	14,000	5500
156 (6.9)						450 (0.014)		30 (0.0009)	150 (0.0045)	530 (0.016)	810 (0.024)	460 (0.014)
157 (5.7)						100 (0.003)				110 (0.0033)	200 (0.006)	91 (0.0027)
167 (5.9)				51 (0.00051)		260 (0.0026)		19 (0.00019)	107 (0.053)	370 (0.037)	550 (0.055)	250 (0.025)
169 (0.28)		1.4 (0.042)	0.86 (0.026)	2.1 (0.063)	0.83 (0.025)					3.6 (0.11)	8.8 (0.26)	
170 (7.7)		18		320	71	1400		150		2100	3500	1700
180 (7.3)				770	130	4000	62	590	2300	6600	9800	4700
183 (5.7)				100	47	630	40	56	190	880	1100	560
187 (6.0)		43		320		1800		190	440	2600	3700	1600
189 (7.7)											250 (0.0075)	
194 (5.3)				110	59	57		83	180	910	1200	640
206 (6.8)								36		200	290	160
209 (2.4)						120		23	106			92
∑PCB (%TDI)	7,800,000 [b]	1200 (0.015)	270 (0.0035)	5000 (0.064)	1000 (0.013)	4600 (0.59)	800 (0.01)	2500 (0.032)	14,000 (0.17)	45,000 (0.58)	65,000 (0.83)	29,000 (0.37)

Table 4. *Cont.*

Compound	TDI [a] (pg)/Day per 60 kg Adult	SW (pg/1 g)	Nor (pg/1 g)	Bio-O (pg/4.75 g)	B-Ω (pg/4.75 g)	BCT (pg/4.75 g)	B-Joint (pg/4 g)	NWK (pg/1 g)	B-P (pg/0.86 g)	NO-1000 (pg/9 g)	B-1000 (pg/12 g)	C-1000 (pg/9 g)
Dioxin/Furans		Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)	Conc. (TEQ)
OCDD (0.41)				30 (0.009)	7.4 (0.0022)	73 (0.022)						
2378-TCDF (0.49)		0.54 (0.054)	0.15 (0.015)							0.72 (0.072)		
23478-PeCDF (2.3)			0.19 (0.095)									
123478/123479-HxCDF (0.19)			0.049 (0.0049)									
∑TEQ	120 TEQ/Day	0.23%	0.18%	0.53%	0.17%	0.55%		0.0018%	0.071%	1.5%	4.7%	0.10%

[a] Values are based on current scientific information and may change; [b] Health Canada, 2007 [29]; [c] US EPA [30]; [d] Man Chan *et al*. (2000) [31]; [e] IPCS (1997) [32].

Table 5. Ranking (1–5) of products according to analyte or TEQ category where a score of 5 denotes the highest concentration/index value.

	∑HCH	∑DDT	∑Chl	HCB	∑PCB	TEQ	Score
SW	1			5		1	7
Nor				4			4
Bio-O	4					2	6
B-Ω			5				5
BCT		2	4	3	4	3	16
B-Joint				1			1
NWK		1	3				4
B-P	5	4		2	1		12
NO-1000		5			3	4	12
B-1000	3	3			5	5	16
C-1000	2				2		4

Hexachlorocyclohexane (HCH) congeners did not feature prominently in any product profiles, possibly reflecting the slightly lower lipophilicity of this compound group. The DDT group included the highest concentration of any single compound, with 13 ng/g lipid p,p'-DDE detected in Nature's Own 1000 mg standard fish oil product, equalling a maximum daily dose 120 ng of p,p'-DDE. Notably, only one krill oil formulation (Swisse) showed detectable levels of ∑DDT. p,p'-DDE has repeatedly been found to be one of the dominant congeners accumulating in Antarctic krill and their predators [26,33–38]. Previously, the authors have reported a comprehensive overview of baseline contamination in Antarctic krill [26], with HCB and p,p'-DDE dominating the described profiles. Further, team studies on dependent populations of humpback whales (*Megaptera novaeangliae*), found that the profiles of these predators closely mirrored the profiles of their principal prey, Antarctic krill. In the case of the krill oil products analysed in the current study, however, only trace (440 pg/g lipid or daily dose) levels were quantified in the Swisse krill oil brand which may indicate purification through the manufacturing process.

Detectable levels of chlordanes were observed in only three products, namely Bio-Organics Super Liquid fish Oil (30 ng per maximum daily dose), Bioceuticals Omegasure fish oil (4.6 ng per maximum daily dose) and Nature's Way Kidsmart (0.1 ng per daily dose). Similarly, endosulfan-I was only detected at trace levels (390 pg/g lipid) in BioOrganics Super Liquid fish oil.

Toxaphene structures were not quantified in five of the eleven products due to loss of the analytes during clean-up. However, notable quantities were detected in Cenovis 1000 mg (19 ng/daily dose; 0.15% TDI) and Bioceuticals Omegasure fish oil (16 ng/daily dose; 0.14% TDI). Only trace levels of toxaphene were quantified in Nature's Way Kidsmart and Blackmores Pregnancy and Breastfeeding formulation. These congeners were undetectable in Blackmores Joint formula and BioOrganics Super Liquid fish oil.

Chlorobenzenes (penta- and hexa-) were quantified in eight of eleven products at levels ranging from 27–9900 pg/maximum daily dose. Antarctic krill products carried the highest levels of chlorobenzene contamination for both penta- and hexa- congeners. The higher levels of particularly HCB, in Antarctic krill oil is not surprising as this has repeatedly been shown to be the compound dominating POP profiles of the Antarctic sea-ice ecosystem food-web [26,33,34]. The finding that

levels were greater than any other product categories, sourced from other global regions, however, was unexpected as HCB has been postulated to be approaching global equilibrium [39]. This finding does not support equilibrium conditions and may be reflective of cold trapping or remobilisation processes of the compound in Polar Regions, combined with steady removal from temperate or tropical source regions.

Polychlorinated biphenyls (PCBs) were detected in all products at cumulative levels ranging from 0.01% TDI (Blackmores Joint formula) to 0.94% TDI (Blackmores 1000 mg). Krill oil products were at the lower end of the spectrum (0.034% and 0.015% TDI for Swisse and Norkrill krill oil respectively), as is expected, given the manufacturing applications of these compounds and the lower historical usage in the southern hemisphere.

Dioxins and furans encompass a class of compounds which are not intentionally produced, but originate primarily through the manufacture of other chlorinated chemicals or combustion processes. Whilst the highest detected levels of any single dioxin or furan congener was 73.0 pg/g lipid of octachlorodibenzodioxin (OCDD) found in Bioceuticals Omegasure fish oil, only the krill oil products contained multiple detectable congeners. This is surprising given the low vapour pressure of dioxins and furans which predict long range atmospheric transport in association with particles. This in turn lowers their potential for effective transport to the Antarctic. Toxicity equivalencies (TEQ) are available for dioxins, furans and a sub-set of planar PCBs, and are calculated based upon their common mode of action. The single highest TEQ for any product analysed was obtained for Blackmores 1000 mg standard fish oil product which yielded a TEQ of 5.6 TEQ or 4.7% of the 120 TEQ TDI. Swisse Krill oil, however, also featured among the top five highest ranking TEQ products. Dioxins, furans and planar PCBs are among the POP compounds most effeciently removed by common fish oil cleaning processes [40]. This finding therefore raises two possibilities. Either some of the fish oil products analysed are subject to one or more chemical purification steps during manufacture, reducing their original TEQ values to the ones observed here, with krill oil apparently not being subject to the same procedures. Alternatively processing and handling itself may have introduced contaminants to the krill oil product that were not present in the raw oil. The dioxin/furan profiles of krill oil here do not match the profiles of whole Antarctic krill previously analysed [25], providing support for the latter.

4. Conclusions

This study compared a range of readily available fish and krill oil dietary supplements for both their favourable long-chain omega-3 composition and content, as well as their persistent organic pollutant profiles. All products and categories adhered closely to manufacturer specifications and none exceeded chemical guideline thresholds. When krill oil was compared across categories to other fish oil products and formulations, it was the most expensive oil per 500 mg DHA + EPA and adhered to manufacturer EPA and DHA specifications. The two krill oil products were ranked as intermediate in terms of their levels of POP contaminants when compared overall to the remaining omega-3 nutriceutical products selected for this study, with distinct chemical profiles reflecting their geographical region of origin. This study is the first to provide quantitative evaluation of toxicological profiles of Antarctic krill products, an emerging nutriceutical category. It hereby

balances consumer information with regard to marketing of krill oil on the basis of product chemical purity. Ongoing monitoring of the pollutant content of fish and krill oil products will become increasingly important as food authorities seek regulatory overview of this rapidly expanding industry.

Acknowledgments

This study was in part funded by ARC Discovery Grant DP666891. The authors thank David McLagan and NILU laboratory staff for undertaking chemical analyses and Seanan Wild for undertaking lipid analyses. Danny Holdsworth managed the CSIRO GC and GC-MS facility. We also thank the two anonymous reviewers for their helpful comments on the manuscript.

Author Contributions

Susan Bengton Nash led and managed the study; Martin Schlabach oversaw chemical analyses at NILU whilst Peter Nichols oversaw lipid and fatty acid analyses at CSIRO. All authors contributed to the production of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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ISBN 978-3-906980-76-8
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