marine drugs

Special Issue Reprint

Fatty Acids from Marine Organisms

Edited by
Giuseppina Tommonaro and Annabella Tramice

mdpi.com/journal/marinedrugs

MDPI

Fatty Acids from Marine Organisms

Fatty Acids from Marine Organisms

Editors

Giuseppina Tommonaro
Annabella Tramice

MDPI

Basel • Beijing • Wuhan • Barcelona • Belgrade • Novi Sad • Cluj • Manchester

Editors

Giuseppina Tommonaro
National Research Council
Pozzuoli
Italy

Annabella Tramice
National Research Council
Pozzuoli
Italy

Editorial Office
MDPI
St. Alban-Anlage 66
4052 Basel, Switzerland

This is a reprint of articles from the Special Issue published online in the open access journal *Marine Drugs* (ISSN 1660-3397) (available at: https://www.mdpi.com/journal/marinedrugs/special_issues/D0V374PLCE).

For citation purposes, cite each article independently as indicated on the article page online and as indicated below:

Lastname, A.A.; Lastname, B.B. Article Title. *Journal Name* **Year**, *Volume Number*, Page Range.

ISBN 978-3-0365-9088-2 (Hbk)
ISBN 978-3-0365-9089-9 (PDF)
doi.org/10.3390/books978-3-0365-9089-9

Contents

About the Editors

Giuseppina Tommonaro

Giuseppina Tommonaro is a biologist and has held the permanent position of research scientist since 2008 at the Laboratory of Natural Products, Institute of Biomolecular Chemistry (CNR), via Campi Flegrei, 34-80078 Pozzuoli, (Na), Italy. Research activities: the chemistry of natural compounds; biological activity assays (antimicrobial, cytotoxicity, AChE inhibition, and antioxidant); marine organisms; sponge- and plant-associated microorganisms; quorum sensing; nutritional capacity evaluation of tomato and new tomato hybrids. She has been involved in several international projects and is the author of more than 90 publications in international, indexed journals.

Annabella Tramice

Annabella Tramice is an organic chemist and has been a permanent researcher since 2008 at the Biomolecular Chemistry Institute of the National Research Council of Italy (CNR), via Campi Flegrei, 34-80078 Pozzuoli, (Na), Italy. She is an expert in the spectroscopic characterization of bioactive molecules from natural sources and the identification, characterization, and use of glycosidase activities from marine and thermophilic micro- and macro-organisms in biocatalytic processes. She has recently been involved in NMR metabolomic studies for the molecular profiling and characterization of pulmonary diseases and the nutraceutical improvement of Italian tomato cultivars. She has been involved in several international projects and is the author of more than 45 publications in international, indexed journals.

Preface

Marine organisms comprise a continuous resource of inestimable molecules with multiple and important activities that could lead to new drug candidates. Many marine-living organisms are soft-bodied and/or sessile, and they have developed a collection of metabolites and strategies through which they can safeguard themselves from external threats. Numerous studies on marine natural products have discovered a multitude of pharmacological activities (anti-inflammatory, antimicrobial, antitumor, and cytotoxic) as well as their involvement in the cardiovascular, immune, and nervous systems.

A modern and conscious exploitation of marine resources allows for the selection of micro- or macro-organisms as a potential source of lipids, used as biofuel, food supplements, antimicrobial bio-compounds, and animal feed, among others, in agreement with the demands of the circular economy. In addition to the major polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, a variety of fatty acids occur in marine organisms that exhibit growing interest regarding their promising biological activities.

This Special Issue is focused on the recent advances in the field of fatty acids' recovery from marine sources (algae, fungi, microorganisms, fish, and their waste tissues), and their relevance in human health and nutrition will be highlighted.

Giuseppina Tommonaro and Annabella Tramice
Editors

marine drugs

MDPI

Article

Potentially Beneficial Effects on Healthy Aging by Supplementation of the EPA-Rich Microalgae *Phaeodactylum tricornutum* or Its Supernatant—A Randomized Controlled Pilot Trial in Elderly Individuals

Lena Stiefvatter [1], Konstantin Frick [2], Katja Lehnert [3], Walter Vetter [3], Alexander Montoya-Arroyo [4], Jan Frank [4], Ulrike Schmid-Staiger [2] and Stephan C. Bischoff [1,*]

[1] Institute of Clinical Nutrition, University of Hohenheim, Fruwirthstr. 12, 70593 Stuttgart, Germany
[2] Fraunhofer Institute for Interfacial Engineering and Biotechnology, 70569 Stuttgart, Germany
[3] Institute of Food Chemistry, University of Hohenheim, 70593 Stuttgart, Germany
[4] Department of Food Biofunctionality, Institute of Nutritional Sciences, University of Hohenheim, 70593 Stuttgart, Germany
* Correspondence: bischoff.stephan@uni-hohenheim.de; Tel.: +49-71145924101

Abstract: Dietary supplements that promote healthy aging are mostly warranted in an aging society. Because of age-related risks, anti-inflammatory and anti-oxidative agents such as microalgae are potential candidates for intervention. In a randomized controlled trial, we tested *Phaeodactylum tricornutum* (PT), a microalgae rich in eicosapentaenoic acid (EPA), carotenoids, vitamins, and β-glucans, cultured in bioreactors. In this pilot trial, 19 healthy elderly received supplements for two weeks based on either the whole PT (*A*), the β-1,3-glucan-rich PT supernatant (*SupB*), the combination thereof (*A+SupB*), or a Comparator product (*Comp*). The primary outcome variable plasma interleukin-6 was reduced after treatment with *A+SupB* compared to the *Comp* group ($p = 0.04$). The mobility parameters 5 s sit-to-stand test ($p = 0.04$ in the *A* group) and by trend gait speed ($p = 0.08$ in the *A+SupB* diet) were improved compared to *Comp*. No treatment effects were observed for fatty acids, compared to *Comp* but omega-6 to -3 fatty acid ratio ($p = 0.006$) and arachidonic acid/EPA ratio ($p = 0.006$) were reduced within group *A+SupB*. Further, the *SupB* study product reduced faecal zonulin ($p = 0.03$) compared to the *Comp*. The data revealed an anti-inflammatory and potentially anti-oxidative effect of particular PT preparations, suggesting that they might be suitable for effects in healthy elderly.

Keywords: elderly; inflammageing; *Phaeodactylum tricornutum*; omega-3-fatty acids; eicosapentaenoic acid; fucoxanthin; β-glucan; chrysolaminarin

Citation: Stiefvatter, L.; Frick, K.; Lehnert, K.; Vetter, W.; Montoya-Arroyo, A.; Frank, J.; Schmid-Staiger, U.; Bischoff, S.C. Potentially Beneficial Effects on Healthy Aging by Supplementation of the EPA-Rich Microalgae *Phaeodactylum tricornutum* or Its Supernatant—A Randomized Controlled Pilot Trial in Elderly Individuals. *Mar. Drugs* **2022**, *20*, 716. https://doi.org/10.3390/md20110716

Academic Editors: Giuseppina Tommonaro and Annabella Tramice

Received: 29 September 2022
Accepted: 9 November 2022
Published: 15 November 2022

1. Introduction

The WHO declares the aging of the world population as the most important demographic problem worldwide. Therefore, healthy aging is becoming increasingly important to maintain functional abilities [1]. Aging is accompanied by immune system dysfunction, changes in intestinal epithelial barriers [2,3] chronic inflammation, and an increase in oxidative stress due to the imbalance between pro- and antioxidant species [4]. Due to these age-related changes, often named "inflammageing", microalgae have been proposed as an aid in the prevention of inflammation as their functional constituents exert multiple pharmaceutical and nutraceutical bioactivities. Microalgae contain long-chain omega-3 fatty acids (n−3 FA), especially EPA (20:5 n−3) and docosahexaenoic acid (DHA, 22:6 n−3), otherwise found in fish, but also proteins, phenols, carotenoids, vitamins and dietary fibres such as β-glucans, in particular chrysolaminarin [5–7]. The microalgae *Phaeodactylum tricornutum* (PT) is a microalgae of particular interest because it is a species highly effective in synthesizing EPA, proteins, fucoxanthin and chrysolaminarin, and therefore a candidate for promoting healthy

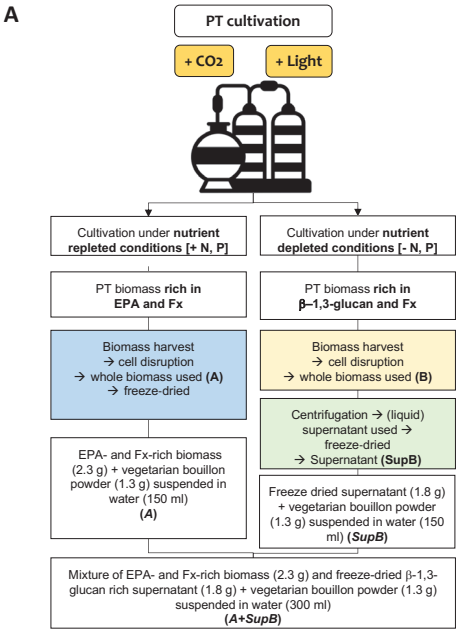
aging with preventive mechanisms. Compared to sea fish, the consumption of PT could be a sustainable nutritional alternative, as shown in previous studies from our and other groups [8,9]. By replacing sea fish consumption with microalgae such as PT the problem of overfishing of the oceans worldwide could be reduced [10]. Also, for vegans, PT could be of interest, because it offers a high content of EPA, which is available in plant food only through α-linolenic acid (ALA) known to be poorly converted into EPA in the human organism [11]. EPA plays a central role in the production of anti-inflammatory eicosanoids, cytokines and the reduction of reactive oxygen species [12]. This anti-inflammatory effect has already been demonstrated for PT [13] and could help tackle inflammageing in the elderly. Furthermore, adequate protein supply in old age is crucial due to muscle loss and the risk of sarcopenia [14]. PT contains up to 60% of protein and therefore can also serve as a valuable protein source for the elderly population. A preclinical study showed that 48% of protein could be replaced by PT without adverse effects on bioavailability [15]. Further functional compounds are phenols and carotenoids, which act as antioxidants by scavenging free radicals [16], and also the neuroprotective effects of carotenoids might be beneficial for healthy aging [17]. The most abundant carotenoid in PT is the xanthophyll fucoxanthin (Fx), a carotenoid without provitamin A activity, that is found in brown-coloured microalgae and macroalgae such as seaweeds [18,19]. Fx is converted into fucoxanthinol (FxOH) and amarouxiaxanthin A (A × A), which has already been demonstrated for PT in a clinical study [8]. Consumption of Fx may have health benefits for older people, such as neuroprotective effects [20], antioxidant and antiproliferative effects [21]. PT contains other carotenoids such as lycopene, which have antioxidant effects [22]. Also, the β-carotene with its pro-vitamin A activity is important for the visual process [23], and has antioxidant potential [24], and could provide further health benefits for the elderly. A further relevant nutrient is tocopherol, amounts of which depend on the growing conditions, and is a powerful antioxidant which could have a protective role in aging [25]. Another component of PT is the β-glucan (β-G) chrysolaminarin (Chrl), a water-soluble β-(1,3)/-(1,6)-glucan (11:1), which is produced in higher concentrations under nitrogen-limiting conditions [26]. The safe and potential gut health effect was shown in a preclinical study by the intake of the whole β-glucan- rich PT as well the EPA-rich biomass [27]. Further immunomodulatory properties and increased antioxidant status could already be shown in fish [28,29]. This regulation of antioxidant status and intestinal barrier function could benefit health, especially in older people.

Considering this background, we performed a randomized, controlled pilot trial in older individuals who received different preparations of the microalgae PT or a comparator product (*Comp*), which is considered a Placebo consisting of only vegetarian bouillon powder for two weeks to assess safety aspects, the bioavailability of selected ingredients and effects on biomarkers related to healthy aging.

2. Results

2.1. Analysis of the Phenolic Content and Oxidative Potential of the Intervention Products

The production of PT with different cultivation conditions resulted in changes in the composition of ingredients (Figure 1A,B). Under nutrient-repleted conditions, PT accumulated more EPA (Biomass A). Under nutrient-depleted conditions, Chrl-rich biomass (Biomass B) was produced. From biomass B, a supernatant was prepared (for details see 4.3) and analysed as well as biomass A and B for the total phenolic content (TPC) by measuring the gallic acid equivalents (GAE; Figure 1C). The TPC ranged from 8.31 ± 4.13 mg GAE per g dry weight in the SupB, to 14.07 ± 4.34 mg/g within biomass B and 16.82 ± 2.47 mg/g in biomass A. The whole biomass samples A and B had a higher TPC compared to SupB ($p < 0.001$ for A, $p = 0.009$ for B). To analyse the antioxidant potential, the ferric reducing ability of plasma (FRAP) assay was performed (Figure 1D). The highest FRAP value was measured for biomass B (2.65 ± 0.97 mmol FRAP per g dry weight), which was higher than for biomass A (1.97 ± 0.07 mmol/g; $p = 0.0480$) and the SupB (0.66 ± 0.27 mmol/g; $p < 0.001$). The FRAP value was also higher in the A biomass compared to the Sup ($p = 0.002$).



B

Nutrient	A	B	SupB
Protein [%]	34.9	29.9	2.4
EPA [%]	2.72	2.49	0.04
FA [%]	8.19	18.19	0.70
β-Glucan [%]	7.51	20.21	44.17
Fucoxanthin [%]	0.70	0.85	0.02

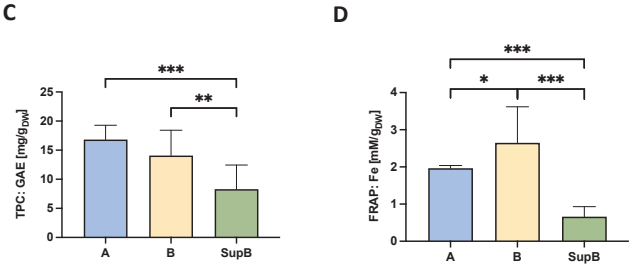


Figure 1. Different growing conditions were used for *Phaeodactylum tricornutum* (PT) production. Biomass A was grown under nutrient-repleted conditions leading to an EPA- and FX-rich biomass, whereas biomass B was grown under nutrient-depleted conditions (without nitrogen and phosphorous in the culture media) for several days before harvesting, leading to the accumulation of β-1,3-glucan. From biomass B, a supernatant was prepared (SupB). Based on biomass A and supernatant SupB, three supplements were prepared for the human trial (**panel A**). The nutrient composition of biomass A, biomass B and supernatant SupB differed (**panel B**). Total phenolics content (TPC) expressed as gallic acid equivalents (GAE) and ferric reducing antioxidant power (FRAP) were measured (**panels C**,**D**). Data are presented as mean ± SD (n = 3). Statistics: * indicates significant differences (ANOVA with Tukey post hoc test). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Further abbreviations: N, nitrogen; P, phosphor; EPA, eicosapentaenoic acid; Fx, fucoxanthin; FA, fatty acids; DW, dry weight.

2.2. Clinical Trial-Subjects' Anthropometric and Metabolic Characteristics at Baseline

Recruitment of study participants took place between June 2021 and August 2021. After the study entry and during the study, there were a total of three dropouts for personal reasons after screening (Figure 2). Thus 21 subjects were randomized into four study groups. Two additional subjects dropped out during the intervention phase due to lack of compliance, so 19 individuals could be finally analysed (12 females, 7 males). The mean (±SD) overall age of the subjects was 67.7 ± 6.5 years, with nine (37.5%) being ≥70 years old (Table 1). The participants were at the normal weight on average with a mean BMI of 24.5 ± 3.1 kg/m^2. Laboratory parameters did not show abnormal values except higher serum fat values than the reference values (cholesterol <200 mg/dl, LDL < 130 mg/dl, HOMA-Index < 1). There was no difference between study groups except the waist circumference was different within the groups ($p < 0.001$).

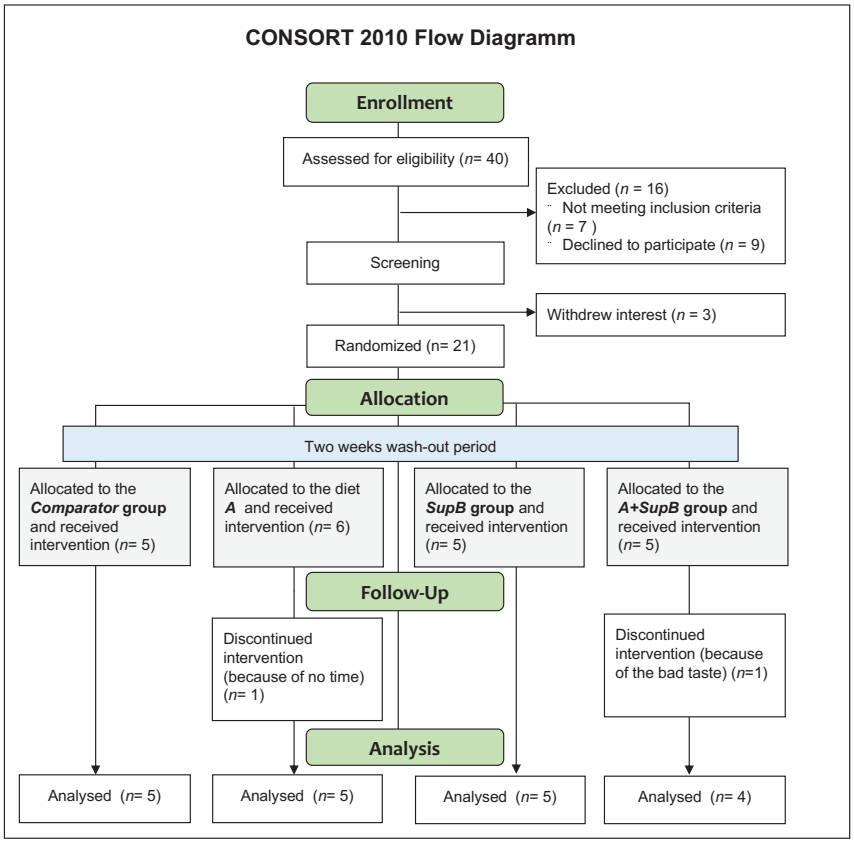


Figure 2. The study was designed as a pilot, randomized, single-blind, placebo (*Comp*)-controlled, parallel four-arm group study with a two-week wash-out and two-week intervention phase. After enrolment and screening (study start/time-point (0), a total of 21 participants were randomly assigned to four groups and underwent a two-week wash-out phase with dietary instructions. After these two weeks (time-point 2), they returned and received the study products assigned to their group. After one week of intervention (time-point 3), the third visit took place and two participants dropped out of the study. After two weeks of intervention (time-point 4), the fourth visit was conducted. The study was fully completed by 19 participants, which were analysed. Abbreviations: *Comparator*, is considered a Placebo with only vegetarian bouillon powder; *A*, diet with biomass A and vegetarian bouillon powder; *SupB*, diet with supernatant (*SupB*) of biomass B and vegetarian bouillon powder; *A+SupB*, Intervention with biomass A and supernatant of biomass B and vegetarian bouillon powder. For details of study products labelled with italic abbreviations (to separate it from biomass A and B in Figure 1) see chapter 4.3 and Table S7.

The study population was divided into four groups that received different supplements, (i) a Comparator (*Comp*) consisting of a vegetarian bouillon powder solved in 150 mL plain water (*Comp*), (ii) lyophilizate of PT biomass A mixed with bouillon powder dissolved in water (*A*), (iii) lyophilizate of supernatant of PT biomass B mixed with bouillon powder solved in water (*SupB*), (iv) a combination of (ii) and (iii) (*A+SupB*). For details of study products labelled with italic abbreviations (to separate it from biomass A and B in Figure 1) see chapter 4.3 and the nutrient composition in Table S7.

Table 1. Characteristics of the 19 study participants.

Female/Male [n]	***All*** **(*n* = 19)** **12/7**	***Comp*** **(*n* = 5)** **2/3**	***A*** **(*n* = 5)** **4/1**	***SupB*** **(*n* = 5)** **3/2**	***A+SupB*** **(*n* = 4)** **3/1**
Anthropometry					
Age [years]	67.7 ± 6.5	67.4 ± 7.9	65.4 ± 4.7	71.4 ± 5.7	67 ± 9.2
BMI [kg/m^2]	24.6 ± 3.1	26.9 ± 2.5	25.5 ± 1.8	22.3 ± 2.9	23.3 ± 3.7
Waist circumference [cm]	90.2 ± 11.6	100.5 ± 8.7	93.6 ± 4.5	81.2 ± 11.4	84.5 ± 11.2
Blood biomarkers					
Cholesterol (chol.) [mg/dl]	224.6 ± 50.0	248 ± 43.5	198 ± 44.7	219 ± 63.2	235 ± 46.5
Triglycerides [mg/dl]	99.9 ± 60.0	141 ± 102.1	83.2 ± 35.7	74 ± 26.7	101.8 ± 26.5
HDL-chol. [mg/dl]	71.1 ± 18.4	71.8 ± 24.2	64 ± 14	72.2 ± 12	77.8 ± 25.7
LDL-chol. [mg/dl]	126.4 ± 46.8	152.2 ± 24.1	120.2 ± 40.9	129.8 ± 51.2	129.7 ± 11.7
Insulin [µE/mL]	7.8 ± 4.1	10.8 ± 6.6	7.6 ± 2.8	5.9 ± 2.6	6.7 ± 1.5
HOMA-Index	1.9 ± 1.7	3.1 ± 3	1.7 ± 0.6	1.4 ± 0.6	1.5 ± 0.2

Values are expressed as mean ± standard deviation (SD). Abbreviations: diets see Figure 2; *Comp*, Comparator; BMI, body mass index; chol, cholesterol; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Statistics: Comparison of groups revealed no difference for all parameters listed in the table (p >0.05, ANOVA) except the waist circumference ($p < 0.001$).

2.3. Four-Week Food Diary and Food Frequency Questionnaire (FFQ)

Diet was assessed using an FFQ at the study start and a continuous food diary during the intervention period as described in Methods 4.10. The FFQ shows similar dietary intake between study groups (Table S1). The food diaries allowed for analysing the percentage compliance with recommended nutrient amounts for the corresponding age group, either 51–65 years or > 65 years. On average, the recommendations were exceeded for protein (124%), PUFA (151%), and vitamin A (257%) and were not fully met for energy (90%), carbohydrate (65%), fibre (73%) and vitamin D (9%) intakes.

2.4. Acceptance and Adverse Effects during the Intervention

All four study products were generally well tolerated. Adverse effects were monitored using a diary and at each study visit by the study staff (protocol). No serious adverse reactions were reported, but minimal and mild adverse effects occurred (Table 2). Most side effects occurred after taking *A+SupB*, e.g., belching, headache and increased urination. Gastrointestinal symptoms such as abdominal rumbling, flatulence and diarrhoea were described for all four treatments as well as for the *Comp*.

Table 2. Adverse effects during the two weeks intervention within the four study groups.

Side Effects	***Comp*** **(*n* = 5)** **Diary/Protocol**			***A*** **(*n* = 5)** **Diary/Protocol**			***SupB*** **(*n* = 5)** **Diary/Protocol**			***A+SupB*** **(*n* = 4)** **Diary/Protocol**		
	Min.	**Mild**	**Sev.**	**Min.**	**Mild**	**Sev.**	**Min.**	**Mild**	**Sev.**	**Min.**	**Mild**	**Sev.**
Abdominal rumbling	-	-	-	2|1$_1$	-	-	-	-	-	-	-	-
Flatulence	-	1|0$_2$	-	-	-	-	-	1|1$_3$	-	-	1|0	-
Stomach pain	-	-	-	-	-	-	-	0|1	-	-	-	-
Diarrhoea	-	1|1	-	-	-	-	-	1|0	-	-	1|0	-
Discoloration of the stool	-	-	-	-	-	-	-	-	-	1|0	-	-
Decreased frequency of bowel movements	-	-	-	-	-	-	-	-	-	1|0	-	-
Belching (at least 1×)	-	-	-	-	-	-	-	-	-	1|1	-	-
Headache	-	-	-	-	-	-	-	-	-	1|0	1|0	-
Increased blood pressure	-	1|0	-	-	-	-	-	-	-	-	-	-
Increased urge to urinate	-	-	-	-	-	-	-	-	-	1|0	-	-
Nausea	-	-	-	1|1	-	-	-	-	-	-	-	-

Adverse effects were documented in a diary by the participants and recorded upon questionnaire by the study personnel during the visits. Values from completers are expressed as absolute numbers. Minimal, transient symptoms with no impairment of the patient's daily activities; Mild, consistent symptoms with moderate impairment of the patient's daily activities; Severe, significant impairment of the patient's daily activities. Abbreviations: diets see Figure 2; *Comp*, Comparator; Min., minimal; Sev., severe; $_1$, the side effect was documented by two participants in a diary and one time during the protocol; $_2$, the side effect was documented one time in a diary but not during the protocol; $_3$, the side effect was documented one time in a diary and one time during the protocol.

2.5. Effect of Supplementation on Laboratory Parameters

Various laboratory parameters were determined at three time points at study start (0), after the wash-out phase (2) and after two weeks of intervention (4). Half of the subjects cholesterol levels were over the normal ratio. Within the *SupB* group, the cholesterol levels were lower by a trend at week four (206.2 ± 59.1 mg/dl) compared to before the intervention (209.8 ± 59.6 mg/dl; p= 0.06; Figure 3A). The LDL was higher within the *SupB* group at week 4 compared to week 2 (p= 0.04, Figure 3B). Triacylglycerols (TAG) were reduced at week 4 with 65.8 ± 17.1 mg/dl compared to week 2 (74.6 ± 22.2 mg/dl; p = 0.04, Figure 3C). Within the A group, the Homeostasis Model Assessment (HOMA) index for insulin resistance was higher at week two at 1.82 ± 0.3 compared to 1.28 ± 0.3 at week 4 (p= 0.03, Figure 3D). Further laboratory parameters are shown in Table S2. No difference was measured between groups and the change from week 4 to 2.

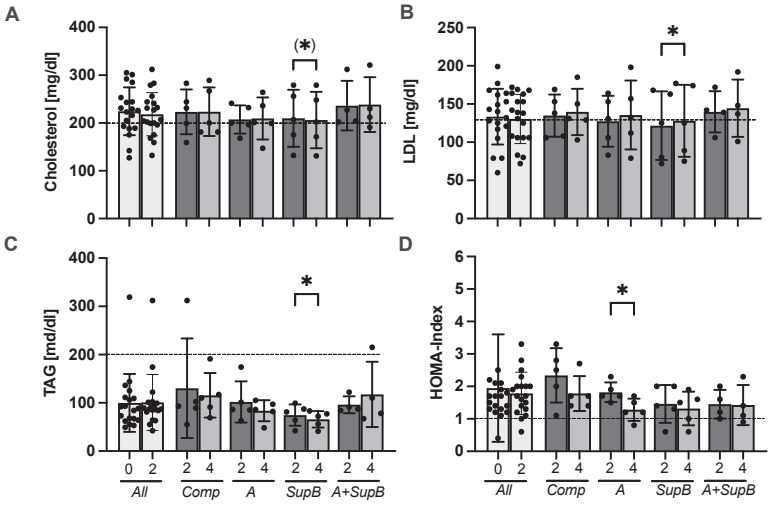


Figure 3. Effect of the study products on total serum cholesterol (**A**), LDL-cholesterol (**B**), triacylglycerols (**C**) and HOMA index (**D**). Parameters were measured at study start (0), after two-weeks wash-out (2), and after two weeks of intervention (4). Values are expressed in scatter plot with mean ± SD and individual values from 19 participants (*All* n = 19; *Comp*, *A*, *SupB* each n = 5; *A+SupB* n= 4). Below the dashed line is the normal reference range. Statistics: * indicates a difference within a group between week two (2) and week four (4) (t-test). No between-groups-differences were found at week 0, 2 and 4 (ANOVA). (*) $p < 0.1$, * $p < 0.05$. Abbreviations: diets see Figure 2; *Comp*, Comparator; LDL, Low-density lipoprotein; TAG, Triacylglycerols; HOMA index, Homeostasis Model Assessment for Insulin Resistance.

2.6. Mobility Markers and Body Composition before and after Intervention

The Western Ontario and McMaster Universities Arthritis Index (WOMAC) questionnaire was used to test the mobility of the knee and hip, including pain, stiffness, and physical functioning. There was no treatment effect measured (Table S7). The index was 9.2 ± 10.4 points at the study start and 11.1 ± 13.9 at study end. Within groups it varied at study end between 2.8 ± 3.0 points in the *A* group and 16.3± 18.9 in the *A+SupB* group. The time for the 5 s sit-to-stand test (5-STS) showed a reduction in the time from 11 ± 1.9 sec to 9.0 ± 1.2 sec within the *A* group from 0 to week 4 (p = 0.02; Figure 4A). The delta was lower by −0.7 sec within the *A* group which was significantly lower compared to the *Comp* group (p = 0.04; Figure 4B). Measurement of the gait speed test showed no changes within groups (Figure 4C), but there was a trend of a treatment effect by the delta reduction within the *A+SupB* group compared to the *Comp* group (p = 0.08, Figure 4D). The handgrip strength tended to increase

in the A group from 29.0 ± 5.2 kg at week 2 to 30.6 ± 6.3 kg at week 4 ($p = 0.09$, Figure 4E) but there was no delta change (Figure 4F). Body composition markers were not affected by the study interventions (Table S3) except the lean body mass was lowered after the *SupB* study product and by a trend of the others compared to the *Comp*.

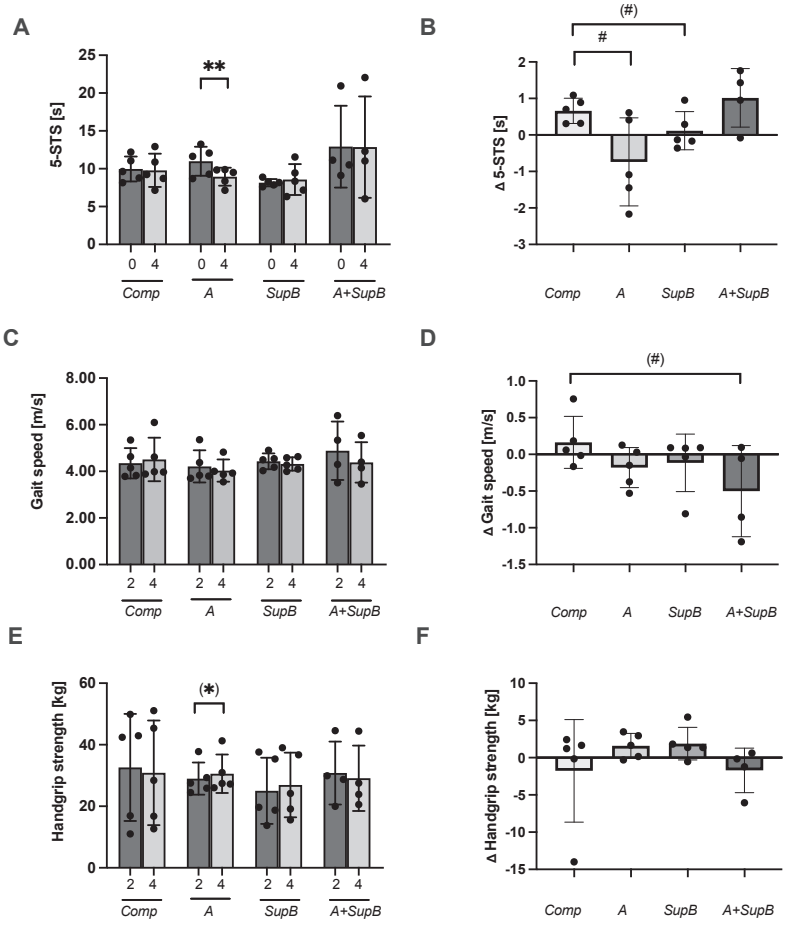


Figure 4. Effect of study products on the mobility markers gait speed (**panels A**,**B**), the 5 s sit-to-stand test (5-STS; **panels C**,**D**) and handgrip strength (**panels E**,**F**). Data at study start (0) and after (4) intervention (panel A), week 2 and 4 (**panels C**,**E**) and treatment effect expressed as differences between weeks 4 and 2 (Δ) (**panels B**,**D**,**F**) are shown. Values are expressed in scatter plots with mean ± *SD* and individual values from 19 participants (*All* $n = 19$; *Comp*, *A*, *SupB* each $n = 5$; *A+SupB* $n = 4$). Statistics: * indicate a difference within a group between study start (0) and week four (4) (t-test); $^{\#}$ indicate a difference between groups (ANOVA). (*/$^{\#}$) $p < 0.1$, $^{\#}$ $p < 0.05$, ** $p < 0.01$. Abbreviations: diets see Figure 2 *Comp*, Comparator.

2.7. Fatty acid Changes in Plasma and Erythrocyte Membrane/Red Blood Cells (RBC)

The plasma FA levels were determined at study start (0), after the wash-out phase (2) and after two weeks of intervention (4) (Figure 5 and Table S4). Comparing all plasma data at the study start (0) with after the washout period (2), when no fish was consumed, plasma levels of EPA ($p = 0.005$; Figure 5A), n−3 FA ($p = 0.02$; Figure 5B), DHA ($p = 0.008$), EPA + DHA ($p = 0.005$) decreased and others by a trend (Table S4). Further, the AA/EPA ratio increased ($p < 0.001$; Figure 5C) and n−6:n−3 ratio ($p = 0.01$; Figure 5D) after the washout period compared to the study start (Table S4).

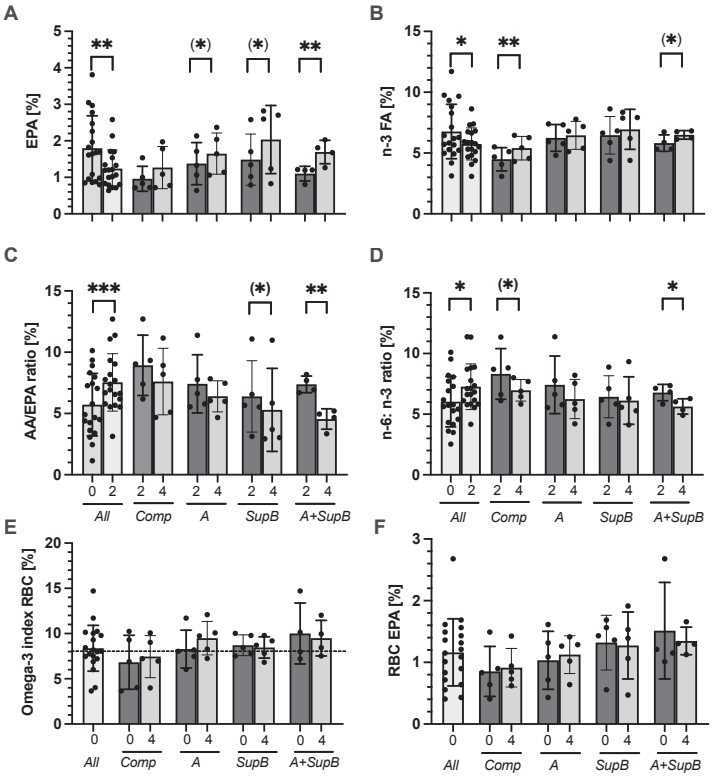


Figure 5. Effect of study products on the plasma fatty acid (FA) levels eicosapentaenoic acid (EPA; **panel A**), omega-3 fatty acids (n−3 FA; **panel B**), arachidonic acid (AA)/EPA ratio (**panel C**) and n−6 FA to n−3 FA ratio (**panel D**). Parameters were measured at study start (0), week two (2) and week four after the intervention (4). Further, red blood cell (RBC) FA were measured, namely the omega-3 index (**panel E**; marked threshold value at 8%) and the RBC EPA content (**panel F**). Values are expressed in scatter plots with mean, *SD* and individual values from 19 participants (*All n* = 19; *Comp*, *A*, *SupB* each *n* = 5; *A+SupB n* = 4). Statistics: * indicate a difference within a group between week two (2) and week four (4) (t-test). No between-groups-differences were found at week 0, 2 and 4 (ANOVA). (*) $p < 0.1$, * $p < 0.05$, ** $p < 0.01$., *** $p < 0.001$. Abbreviations: diets see Figure 2; *Comp*, Comparator.

Regarding within-group differences from week 2 to week 4, the EPA plasma concentrations tended to increase by 0.3% within the *A* group and 0.55% within the *SupB* group ($p = 0.1$; Figure 5A). Only within the *A+SupB* group, the EPA level increased significantly by 0.6% ($p = 0.006$). The DHA level did not change due to the intervention (Table S4). The EPA + DHA level did not change except for an increase within the *Comp* group (Table S4). Total n−3 FA plasma concentration increased within all interventions but only significantly within the *Comp* group by 0.9% ($p = 0.005$) and by a trend within the *A+SupB* group by 0.7% ($p = 0.05$) (Figure 5B). The AA/EPA ratio decreased from week 2 to week 4 after all supplementations but only significantly within the *A+SupB* group from 7.4 ± 0.7% to 4.5 ± 0.8% ($p = 0.006$; Figure 5C) and by a trend within the *SupB* group ($p = 0.09$). Furthermore, the n−6:n−3 ratio was lower within the *A+SupB* group at week four with 5.6 ± 0.6% compared to week two with 6.8 ± 0.1% ($p = 0.006$, Figure 5D). A reducing trend was further measured within the *Comp* group ($p < 0.01$). Minor other changes in plasma FAs were measured as shown in the Supplementary Materials in Table S4. Regarding treatment changes, which were calculated as the difference between week 4 to 2, the 18:3n−3 FA level was lowered after the *A* diet compared to the *Comp* group ($p = 0.008$). The 22:6n−3

level in the *SupB* group ($p = 0.02$) and the 20:3n−6 level within the *A* group were reduced compared to the *Comp* group ($p = 0.05$; Table S4).

The RBC FA concentration was measured at the study start (0) and after 4 weeks. The mean total Omega-3 index at the beginning was 8.4 ± 2.5% (Figure 5E), consisting of levels of EPA (1.16 ± 0.5%, Figure 5F) and DHA (7.21 ± 2.1%). Within the *A* group, the index changed from the study start from 8.29 ± 2.1% to 9.49 ± 1.9%, but not significantly, and no changes were measured comparing both time points.

2.8. Carotenoids, Vitamin E Changes in Plasma

Due to the carotenoid and tocopherol concentrations in PT, the amounts in the blood plasma were measured at four different time points and shown in Figure 6A and C at weeks 2, 3 and 4 and the delta between weeks 2 and 4. The *A* and *A+SupB* diets contained Fx in the diet and the participants took 21.4 mg or 21.6 mg daily. After one week of intake at week 3, no Fx could be measured in the blood plasma of the subjects. FxOH was detected after one week (3) (0.03 ± 0.03 µM) and two weeks (4) of *A* diet intake (0.02 ± 0.001 µM) and the level significantly increased from week 2 to 4($p = 0.007$, Figure 6A). Within *A+SupB* group, FxOH was detected after one week (3) (0.05 ± 0.05 µM) and two weeks (4) (0.08 ± 0.08) in the plasma. The change between weeks 4 to 2 (Δ) for FxOH, was higher in group *A* ($p = 0.001$; Figure 6B) and *A+SupB* group by a trend compared to the *Comp* group. A × A, to which FxOH is converted in the liver [30], could be measured in plasma at 0.003 ± 0.006 µM in only one participant after the study product *A+SupB*. Through the *A* and *A+SupB* diets, subjects consumed 0.3g of β- carotene daily. After two weeks of supplementation, plasma β-carotene increased from 0.5 to 0.7 µM. ($p = 0.02$, Figure 6C) within the *A+SupB* group. No changes between week 4 to 2 were measured (Figure 6D, Table S5). Further, carotenoids such as retinol did not change in any group, nor did the lycopene plasma levels. Furthermore, no increase in γ-tocopherol and α-tocopherol were measured (Table S5).

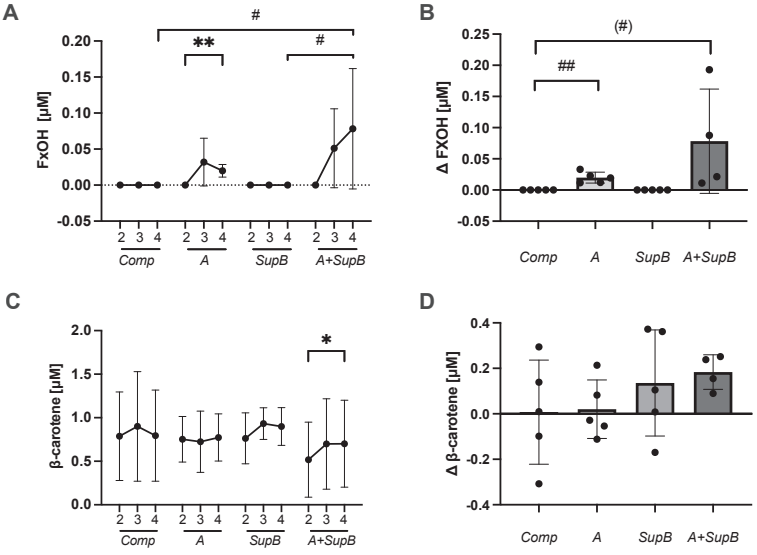


Figure 6. Plasma carotenoids changes following interventions. Fucoxanthinol (FxOH, **panels A**,**B**) and β-carotene (**panels C**,**D**) were shown before the intervention (2), after week three and one week of supplementation (3) and week 4 after two weeks of supplementation (4) (**panels A**,**C**). The treatment effect expressed as differences between weeks 4 and 2 (Δ; **panels B** and **D**) with *Comp, A, SupB,* and *A+SupB*. Values are expressed as mean and SD error bars. Statistics: *indicate a difference within a group between week two (2) and week four (4) (t-test); $^{\#}$ indicate a difference between groups (ANOVA). ($^{\#}$) $p < 0.1$, */$^{\#}$ $p < 0.05$, **/$^{\#\#}$ $p < 0.01$. Abbreviations: diets see Figure 2; *Comp*, Ccomparator.

2.9. Inflammatory Parameters and the XOR as an Oxidative Stress Marker

A two-week intervention with *A* and *SupB* had no negative effect on inflammatory markers such as high-sensitivity C-reactive protein (hs-CRP), interleukin (IL)-10, IL-6 and tumour necrosis factor (TNF)-α. Comparing before (2) and after the intervention (4) within the group of *A+SupB* a trend was shown to decrease the pro-inflammatory marker IL-6 level from 5.3 ± 1.6 pg/mL at week two to 3.3 ± 1.9 pg/mL at week four ($p = 0.5$; Figure 7A). The treatment effect measured as the change of weeks four to two, showed a significant difference between the *A+SupB* group (−2.0 ± 1.3 pg/mL) and the *Comp* group (+1.5 ± 2.5 pg/mL; $p = 0.042$; Figure 7B). The hs-CRP, IL-10 and TNF-α did not change according to the diet or differ between the diets. As an oxidative stress parameter, the xanthine oxidoreductase (XOR) was measured. The XOR is the final enzyme in purine catabolism and catalyses hydroxylation to xanthine and then to uric acid. The results showed a tendency to decrease levels within the *A* and *A+SupB* groups compared to the levels before the intervention (2) and after intervention (4) ($p < 0.1$). The XOR tended to increase within the *SupB* group (Figure 7C). The treatment effect showed no effect compared to the *Comp* group only between *A* and *SupB* (Figure 7D).

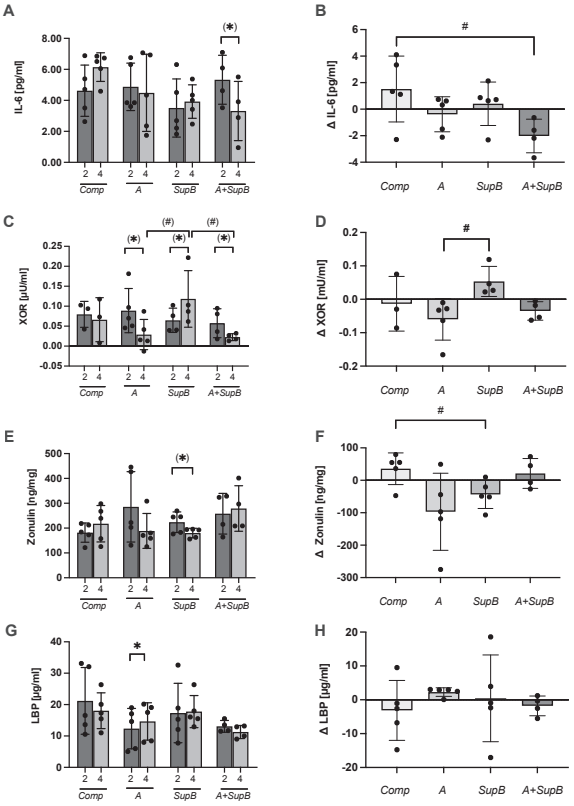


Figure 7. Effect of the study products on plasma interleukin-(IL)-6 (**panels A**,**B**), plasma xanthin oxidoreductase (XOR; **panels C**,**D**), and the gut barrier markers faecal zonulin (**panels E**,**F**) and plasma lipopolysaccharide-binding protein (LBP) in plasma (**panels G**,**H**). Data before (2) and after (4) intervention (**panels A**,**C**,**E**,**G**) and treatment effect expressed as differences between weeks 4 and 2 (Δ) (**panels B**,**D**,**F**,**H**) are shown. Values are expressed in a scatter plot with mean and *SD*-individual values from 19 participants (*Comp* , *A*, *SupB* each $n = 5$; *A+SupB* $n = 4$). Statistics: *indicates a difference within a group between week two (2) and week four (4) (t-test); $^{\#}$ indicates a difference between groups (ANOVA). (*/$^{\#}$) $p < 0.1$, */$^{\#}$ $p < 0.05$. Abbreviations: diets see Figure 2; *Comp*, Comparator.

2.10. Gut Barrier Markers and Short-Chain Fatty Acids

The gut barrier marker lipopolysaccharide-binding protein (LBP), an acute-phase protein that binds to bacterial lipopolysaccharides derived in part from translocation from the intestine and zonulin, which is an acute-phase response protein and controls intestinal permeability by reducing the stability of tight junctions (TJ), revealed small changes within the different groups. The faecal zonulin showed a trend of reduction within the *SupB* group comparing the level of 223.8 ± 41.5 ng/mg at week 2 to 180.3 ± 19.3 ng/mg at week 4 ($p = 0.088$; Figure 7E) and a treatment effect compared to the *Comp* group ($p = 0.03$; Figure 7F). The LBP was higher at week 4 with 14.6 ± 5.9 µg/mL within the *A* group compared to 12.4 ± 6.4 µg/mL at week 2 ($p = 0.045$; Figure 7G) but no treatment effect was measured compared to the *Comp* group (Figure 7H). SCFA concentrations in faeces showed no significant changes between groups or due to the interventions (Table S6).

2.11. Correlations

Spearman correlation showed a positive association between different FAs, especially EPA and the age of the participants (Figure 8A). A positive correlation was measured between the AA/EPA plasma ratio with TNF-α and IL-6 (Figure 8B,C) and the n−6: n−3 ratio with IL-6 (Figure 8D). A negative association was found between the WOMAC score and the carotenoid intake measured by the FFQ (Figure 8E). Regarding the gut barrier marker LBP, a negative association with the omega-3 Index was measured (Figure 8F).

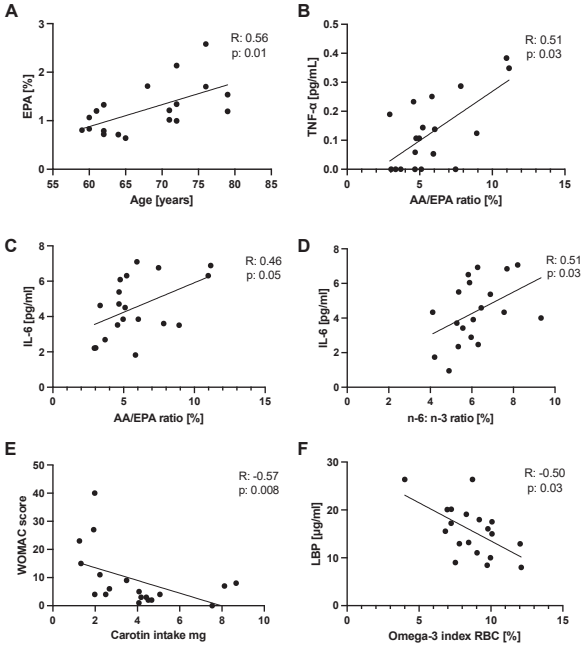


Figure 8. Positive correlations were shown by Spearman (R) correlation as the EPA plasma concentration (week 2) to the age (**panel A**), AA/EPA plasma ratio to the TNF-α (week 4) (**panel B**), Il-6 to the AA/EPA ratio (week 4) (**panel C**), and the n−6: n−3 ratio (week 4) (**panel D**). A negative association was found between the WOMAC score and the carotenoid intake measured by the FFQ (**panel E**) and the LBP level and the omega-3 index (week 4, **panel F**). Statistics: R, Spearman rho; *p*-values > 0.05. Abbreviations: EPA, eicosapentaenoic acid; AA/EPA, arachidonic- and eicosapentaenoic acid ratio; n−6: n−3, n−6 FA to n−3 FA ratio; TNF-a; tumour necrosis factor; IL-6, interleukin-6; WOMAC, Western Ontario and McMaster Osteoarthritis Index; LBP, lipopolysaccharide-binding protein; RBC, red blood cells (erythrocyte).

3. Discussion

The present study investigated the anti-aging effect of different preparations of the microalgae PT and Comparator in a randomized controlled manner. So far, PT has not been approved as a novel food; therefore, the safe intake, as well as possible health promotion effects, were investigated.

3.1. Safety and Bioavailability

Microalgae are not yet widespread on the food market and are mostly sold in capsules, tablets, or dried powder as dietary supplements. A new approach is to use the whole microalgae and convert it into novel and tasty food products with potential health-beneficial effects. The incorporation of microalgae biomass into foods has encountered some difficulties mainly due to the colour and fishy taste [31]. PT with its unique content of functional nutrients is particularly difficult to process due to its intense brown/green colouration and the oxidation of FA and carotenoids, which favours the fishy taste [32]. However, due to the combination with the vegetarian bouillon powder, the ingestion became acceptable by most study participants, although other studies rated the taste fishy and unpleasant [33]. The general intake of the β-G-rich supernatant (*SupB*) was very well accepted as it was almost tasteless when dissolved in water. Previously, it was shown that β-Gs contained in beverages and liquid test meals turned out to be the best carriers [34], as it was used in the present study.

Our study provides further evidence for safe intake by evaluating several laboratory parameters. Except for the *SupB* group, which showed an increase in LDL-cholesterol, but still within the normal reference range, no negative effects on laboratory parameters were observed. As we could not measure treatment effects compared to the *Comp*, the results still suggest health-promoting effects within the groups, such as the reduction in HOMA-index within the *A* group after the whole PT supplementation. n−3 FA could generally improve insulin sensitivity, although these results are mostly based on animal models [35]. Further n−3 FAs have been reported to lower TAG, which may lead to a reduction of the risk of cardiovascular diseases (CVD) [36]. In our study, such results could not confirm after PT supplementations (*A* or *A*+*SupB* diets), possibly because it was underpowered for such an effect. However, a pre-clinical study in Wistar rats fed a high-fat (HF) diet supplemented with 12% PT already shows a TAG and HOMA-index lowering effect compared to the HF diet without PT supplementation [37].

A reduction of cholesterol by a trend and TAG levels was measured within the *SupB* group, after supplementation with the Chrl-rich supernatant. Although our data could not confirm previous pre-clinical studies with zebrafish showing cholesterol-lowering and LDL-lowering effects following PT supernatant supplementation similar to *SupB* [38], they did suggest a possible trend. Especially in older people, in whom lipid metabolism is frequently altered [39], LDL cholesterol-lowering could be a valuable goal, since LDL cholesterol is a modifiable cardiovascular risk factor for prevention [40]. Meta-analyses have already demonstrated the β-G cholesterol-reducing and LDL-lowering effect of taking a dose >3 g of β-G per day by consuming oats and barley for at least three weeks in individuals with mild hypercholesterolemia [34]. In our study, we administered only about 0.5 g of β-G per day, which might be suboptimal. A higher supplementation of the Chrl-rich supernatant (*SupB*) could produce greater effects, which needs to be confirmed in future trials. In a pre-clinical study, the safe intake of up to 4621 mg/kg body weight of the β-G Chrl in mice has been shown [27]. For human nutrition, β-Gs are an important dietary fibre supporting preventive effects [41], and microalgae containing β-Gs like *Odontalla auritia* and *Euglena gracilis*, have already been approved by the European Food Safety Authority (EFSA). Since the recommended daily fibre levels were not reached by the participants, additional supplementation with PT Chrl-rich supernatant would be conceivable for older people and others.

In terms of FA bioavailability, the wash-out phase was effective due to the plasma FA reduction. Further, an increase of EPA was expected after the PT diets *A* and *A+SupB*, comparable to the previous study with younger participants [8]. However, no treatment effect was observed. Our study could not confirm such findings, possibly because it was underpowered. Age differences might be related to altered FA metabolism in older people, such as slower plasma clearance and lower incorporation into cell membranes [42], as well as changes related to FA release and/or β-oxidation in older people [39]. However, our present data showed that plasma n−3 FA levels were already higher in the older participants before the study than in the younger study participants [8]. This is consistent with other studies [43,44] and with our correlation analyses, which showed higher plasma EPA levels with increasing age. In addition, nutritional data also show a higher EPA+DHA intake in older women and men (65–79 years) at 232.1/277 mg/day (women/men) compared to 18–24-year-olds at 199.9/232.1 mg/day (women/men) [45]. The general recommended intake of EPA+DHA is 250–500 mg daily and might be even higher for some prevention goals [46], suggesting that no age group is meeting the recommendations. Therefore, PT could be an additional dietary source of EPA-FA. Measurement of the Omega-3 index in erythrocytes was performed to verify adequate supply. This varied between 6.8% and 10%, confirming a good supply and moderate to low risk for CVD in our study population [47]. As the Omega-3 index measured in RBC is considered a marker for long-term FA changes, no changes were expected and observed in our study after two weeks of PT supplementations. Our present study results indeed suggest a possible altered FA metabolism in older adults. Therefore, the recommended intake of EPA and DHA should be reconsidered and possibly adjusted in future for the elderly population.

Regarding the uptake of carotenoids, the present study confirms the metabolization of Fx to FxOH, as previously shown [8]. Further metabolization to A × A could only be measured in one subject. This seems to be dependent on the ingested amount of Fx, since a previously conducted study with supplementation of about 30mg Fx per day led to a significant increase of both metabolites [8]. So far, the EFSA recommends an amount of 15 mg of pure Fx per day, e.g., derived from *Undaria pinnatifida thallus* extract (wakame) [48]. Despite several possible health benefits, no health claims have been awarded so far for Fx-containing microalgae [49,50]. Future studies not only on health benefits but also regarding dose-finding and toxicity levels are needed to promote the acceptance of Fx as a valuable food supplement in future.

For tocopherol and β-carotene, PT might not be the best selection, because their content is rather low and therefore would require an intake of higher amounts of the microalgae than those chosen in our study. This might explain why we found no plasma increase of tocopherol and only some increase of β-carotene in the *A+SupB* group. This group tended to have the lowest baseline levels, suggesting that PT supplementation can increase plasma β-carotene levels as recently demonstrated [8].

In terms of gut health, minimal and mild side effects were noted after ingestion of the study products, as reported earlier [8]. Because loss of gut barrier function [2] and changes in the gut microbiome [51,52] may occur with aging, we were interested in whether PT supplementation could prevent such alterations in the study population. The gut barrier markers plasma LBP and faecal zonulin were measured to assess intestinal barrier function. The results yielded a treatment effect of zonulin, which decreases within the *SupB* group compared to the *Comp* group. Since PT contains high amounts of n−3 PUFAs, which are thought to promote the intestinal barrier [53], we expected a treatment effect as well within the *A* and *A+SupB* groups. We found no such effect, but a negative correlation between the Omega-3 index in erythrocytes and plasma LBP levels. The reduction of faecal zonulin levels within the *SupB* group could also have implications beyond the gut since zonulin is positively correlated with the concentration of pro-inflammatory cytokines (TNF-α and IL-6) and negatively correlated with muscle strength and usual physical activity [54]. In a pre-clinical study supplementing the whole EPA-rich PT biomass and β-G-rich PT biomass, we found an increase in SCFA and SCFA-producing bacteria, suggesting possible healthy

gut promoting effects [27]. However, in the present human trial, we could not confirm such findings, possibly because of smaller amounts of PT biomass administered based on a bodyweight-related dosage.

3.2. Potential Antioxidative and Anti-Inflammatory Effects

In terms of antioxidant potential in the PT biomass, the highest phenolic content was measured for both whole biomasses rich in EPA (A) and those rich in β-G Chrl (B) and half of the potential in the supernatant (Sup). The results show that for a higher phenolic content, the use of whole biomass is necessary. Previously, it was shown that PT contains fourteen phenolic compounds [55], which could be useful for human nutrition. Regarding the antioxidant potential measured by the FRAP assay, the highest value was measured for the β-G-rich PT biomass B. This biomass contained the highest Fx amount, which confirms that carotenoids and xanthophylls could have a high antioxidant potential [56]. The antioxidant effect of Fx extracts from PT has been reported previously [21]. However, the EPA-rich biomass A also exhibited antioxidant potential, as FAs may also have antioxidant activity [57].

Our human study provides the first evidence of a potential anti-oxidative effect of the EPA-rich PT biomass, as plasma XOR levels tend to be lower in group *A* and group *A+SupB*, which ingest the whole PT. In the *SupB* group, which ingested the Chrl-rich supernatant, this trend toward lowering was not observed. XOR levels are often elevated in inflammatory bowel diseases [58] and age is positively correlated with xanthine oxidase activity [59]. Therefore, the trend of reducing the XOR levels is a possible positive indication. Other studies with PT have already shown that the microalgae reduce the activity of nuclear factor kappa B (NF-κB) in mouse macrophages [13], which is activated by the production of reactive oxygen species (ROS). Moreover, it has been shown that the free radical scavenging activity is presumably related to the high Fx content of PT [30,60]. These findings are consistent with our results, as diets *A* and *A+SupB*, which have the highest Fx content, showed a trend for some XOR reduction within the groups. Not only Fx, but also n−3 FAs might have a stimulatory effect on mitochondrial function and fusion processes by reducing ROS production [61]. Higher doses and more participants are required to prove statistically significant effects.

To tackle inflammageing in elderly the current study shows potential anti-inflammatory treatment effects of the *A+SupB* group, by the reduction of the pro-inflammatory cytokine IL-6 compared to the *Comp* group. Other inflammatory parameters such as TNF-α and C-reactive protein remained unchanged. For PT, an anti-inflammatory effect has already been shown in vitro [13] and in vivo [37]. The effect could be addressed by the high Fx content, which regulates the NF-κB and NLRP3 inflammasome activation [50]. Other PT compounds such as polysaccharide and EPA could be further involved in the reduction of pro-inflammatory cytokines such as IL-6 [62,63]. A further anti-inflammatory indicator could be the reduced AA/EPA ratio and the n−6:n−3 ratio in plasma within the *A+SupB* group. Both are reliable indicators of nutritional status, and a higher n−6:n−3 ratio can lead to the development of various metabolic disorders [64]. Indeed, our correlation analyses confirm this hypothesis, as the AA/EPA ratio was positively associated with IL-6 and TNF-α levels and the n−6:n−3 ratio with IL-6 level.

Considering age-related muscle wasting, supplementation of PT could prevent such deficits and promote functional ability. If higher amounts of PT are administered, it could even be an additional source of protein. n−3 FAs are thought to have an anabolic effect [65] and indeed cause improvements in muscle strength and protein synthesis [65,66], however, other studies did not find such effects [67,68]. The current study showed some modest treatment effects after the PT supplementation in terms of improvement in mobility markers such as the 5-STSs in the *A* group and gait speed within the *A+SupB* group compared to the *Comp* group. Regarding the effects of carotenoids, higher intake is associated with better grip strength [69], and reduction of hip fractures [70], therefore PT in higher doses may be a valuable nutrient for older adults. Higher dietary intakes of α-tocopherol and lycopene are negatively associated with the WOMAC score (a higher score means more severe pain

and functional limitations) [71]. The present data suggest this association, as a negative correlation was found between the WOMAC score and carotenoid intake. In addition, supplementation with n−3 FAs, carotenoids, and vitamin E has been found to improve working memory in older adults [72], which closely matches the constituent nutrients of PT and suggests a potential benefit in the elderly.

3.3. Limitations

The study shows limitations, as some effects such as cholesterol- and TAG-lowering effects are demonstrated only in the *SupB* group, but not in the *A+SupB* group, which consumed the same amount. It has been expected that the effects would occur in both groups. The findings from this pilot trial need confirmation from larger confirmatory trials.

4. Materials and Methods

4.1. Participant Selection

Both females and males, aged between 60 to 90 years were screened with a BMI between 18.5 to 30 kg/m^2 and a weight of more than 50 kg. Participants had to be willing to adhere to certain dietary rules (no significant changes in diet, no fish or seafood and no probiotics) and the willingness to follow the prescribed diet for the duration of the study (14 days). Physical activity should not be changed throughout the study. Exclusion criteria were the intake of intestinal therapeutics, antibiotics, immunosuppressants, cholesterol-lowering drugs or similar, relevant violations of the dietary protocol, occurrence of relevant diseases—diabetes mellitus, lipid metabolic disorders, severe acute COVID disease within the last six weeks according to a case-by-case decision, and acute COVID disease. Inclusion and exclusion criteria remained unchanged throughout the study. The study was conducted according to the Declaration of Helsinki at the Center for Clinical Nutrition Stuttgart at the University of Hohenheim in 2021 and has been approved by the local Ethical Committee (Ethik-Kommission der Landesärztekammer Baden-Württemberg, F-2021-061), and was registered at ClinicalTrials.gov (NCT05120791).

4.2. Study Design and Outcome Parameters

The study was designed as a randomized, single-blind, 1:3 Comparator controlled (considered a Placebo with only vegetarian bouillon powder) parallel group with four visits (Figure 2). An intervention period of two weeks was chosen due to the increase in FA of the previous human study [8] and to estimate a safe intake of the β-G supernatant (*SupB*). When participants fulfilled all inclusion and no exclusion criteria at the study start, visit parameters were assessed. They were requested to eat no fish and seafood for all four weeks. The first two weeks were planned as a wash-out period for the n−3 FAs and the second visit was after two weeks (2). The third visit was after three weeks (3) and the fourth visit was after four weeks (4). The study was planned as a proof-of-principle pilot study; therefore, no formal calculation of case numbers was performed.

The primary outcome parameter was the effect on inflammation markers (hs-CRP, IL-6, 10). Secondary outcome parameters were laboratory parameters, n−3 FA in plasma, erythrocyte/ RBC, the improvement of the n−6: n−3, and AA/EPA ratio, carotenoids, body weight, waist circumference, handgrip strength, BIA assessing, gut barrier marker (LBP, zonulin) and SCFAs. For muscle function 5-STS, gait speed, handgrip, and the WOMAC questionnaire were used. Diet was evaluated using a food diary and an FFQ. At the study entry, the subject got verbal and written informed consent and the inclusion and exclusion criteria were obtained. The demographic data were collected, the medical history and the collection of former and current medication. The bioelectrical impedance analysis and the 5-STS were done and the fasting venous blood collection for blood markers and FAs (plasma and RBC), as well as anthropometric measurements and hand strength were done at the institute. The nutrition diary, FFQ and the instructions for the faecal sample collection were handed out.

At the second visit (after week 2) the products were distributed, and fasting blood samples were taken to analyse blood markers and FAs (plasma). Faecal samples were collected and used for SCFA measurement and barrier permeability. Further, the gait speed and the 5-STS were completed. Participants started to take the capsules after the second visit. In the third visit after three weeks (3), fasting blood samples were obtained for blood markers and FAs (plasma) and the instructions for the faecal sample collection were handed out. The fourth visit after four weeks (4) was the same as the study entry and on the second visit and participants returned their investigational products.

4.3. Study Products, Randomization and Blinding

The EPA and Fx-rich PT biomass (biomass A) was produced under nutrient-repleted conditions in flat panel airlift reactors. The biomass was harvested and concentrated via centrifugation to 250–270 g/L (Clara 20, Alfa Laval, Glinde, Germany) as described before [8]; see also Figure 1. The cells were disrupted using a bead mill (PML 20, Bühler, Uzwil, Switzerland), and freeze-dried (VaCo 5, Zirbus). For the generation of supernatant, PT biomass B was produced in flat panel airlift reactors under nutrient-depleted conditions (without nitrogen or phosphorous source in the culture media) for several days before harvesting. Harvesting and concentrating were performed by centrifugation to 250–270 g/L (Clara 20, Alfa Laval) as described before [8]. After cell disruption via bead milling (PML 20, Bühler), the biomass was centrifuged again, and the liquid supernatant was separated from the biomass pellet to obtain the supernatant (SupB). Afterwards, the supernatant was freeze-dried (Avanti J-26 XP, Beckman Coulter, Brea, USA). The detailed nutrient composition of the study products is shown in Table S7.

For a better taste of PT and for the blinding, 1.3 g vegetarian bouillon powder (Gemüse Bouillion, Knorr, Hamburg, Germany) was added to the lyophilised biomass/supernatant. The study population was divided into four groups receiving different study products. The first study product was the *Comp*, which consisted of daily 1.3 g of vegetarian bouillon powder solved in 150 mL plain water. The second study product *A* consisted of 2.3 g of lyophilised biomass A containing 312.1 mg n−3 FA (293.5 mg EPA+DHA) per day and additionally 1.3 g of vegetarian bouillon powder suspended in water (Table 3). The amount of PT was chosen based on the national n−3 PUFA/EPA + DHA recommendation of 250 to 300 mg per day. The third study product was based on β-G recommendations of yeast β-G by the EFSA, which is 600 mg per day [73]. Because Chrl is not yet a novel food, a total concentration of 500 mg Chrl was taken, which was 1.8 g daily of lyophilised Sup B biomass and 1.3 g vegetarian bouillon powder suspended in water (*SupB*). The fourth study product *A+SupB* consisted of 2.3 g of biomass A, 1.8g of SupB and 1.3 g of vegetarian bouillon powder suspended in water (Table 3). The study groups are named as the study products *Comp*, *A*, *SupB* and *A+SupB*.

All participants were given a diary to document their intake of the study products. The participants were instructed to suspend the study products freshly in a glass of water (around 150 mL) before usage and to take these products at breakfast time. To meet the compliance criteria, participants had to achieve 100% compliance, if the intake was forgotten for one day, the intake was extended by one day. The study was single-blinded; study participants were blinded for the study products. The randomization was generated using the Randlist software (datinf GmbH, Tübingen, Germany, available at randomisation.eu).

Table 3. Daily nutrient intake of the participants through the study products.

Daily Nutrient Intake	*Comp*	*A*	*SupB*	*A+SupB*
Protein g/day	0.007	0.81	0.05	0.85
β-1,3-glucan g/day	0	0.17	0.54	0.71
FAs [mg/day]	13.02	678.3	22.9	688.2
n−3 FA	0.34	312.51	1.79	313.97
n−6 FA	5.19	63.56	5.57	63.94
SFA	3.69	90.85	7.35	94.51
MUFA	3.80	104.38	7.70	108.28
PUFA	5.53	483.07	7.85	485.38
n−6: n−3 ratio	20.00	20.43	20.47	20.90
AA/EPA ratio	0.00	0.22	0.00	0.22
EPA+DHA	0.03	293.65	1.20	294.82
EPA	0.02	288.52	1.44	289.94
DHA	0.01	5.13	0.06	5.19
Carotenoids [mg/day]				
Fucoxanthin	0.00	21.39	0.22	21.61
β-carotene	0.01	0.29	0.01	0.30
α-carotene	0.00	0.00	0.00	0.00
Lycopene	0.00	0.21	0.01	0.21
Vitamine E [mg/day]				
α-Tocopherol	0.00	0.10	0.03	0.13
β-Tocopherol	0.00	0.00	0.00	0.00
γ-Tocopherol	0.00	0.01	0.00	0.01

Abbreviations: diets see Figure 2; FA, fatty acids; n−3 FA, omega-3 fatty acids; n−6 FA, omega-6 fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; n−6: n−3, n−6 FA to n−3 FA ratio; AA/EPA, arachidonic- and eicosapentaenoic acid ratio; EPA, eicosapentaenoic acid; DHA, docosahexaeonic acid.

4.4. Blood Plasma, Serum and Faecal Measurements

Blood samples were collected in two ethylenediaminetetraacetic acids (EDTA)-coated tubes, and one serum tube. One EDTA- tube was used for blood count analysis (Sindelfingen laboratory GbR, Sindelfingen, Germany). The other one was centrifuged at 500 g for 7.5 min at 15 °C, followed by plasma separation. Plasma was stored at −80 °C until further analysis (FA, carotenoid, inflammatory markers, LBP). The RBC (underneath the centrifuged plasma) were washed with NaCl and centrifuged three times and stored at −80 °C. The serum tube was centrifuged for 15 min at 3000× g at 15 °C. 1 mL Serum was stored at −80 °C and the rest was used for quantification of gamma-glutamyl transferase (γ-GT), aspartate aminotransferase (AST), alanine transaminase (ALT), prealbumin, albumin, triacylglycerols (TAG), total cholesterol (Chol), high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), haemoglobin, haematocrit, erythrocytes, leucocytes, platelet count, haemoglobin beta-N-1-deoxy fructosyl component of haemoglobin (HbA1c), insulin, HOMA-index, plasma glucose, thyroid-stimulating hormone = thyrotropin (TSH), C-reactive protein (CRP), uric acid, 25-hydroxy vitamin D (25(OH)D) (measured at Laborärzte Sindelfingen GbR, Sindelfingen, Germany).

Stool samples were collected before the study visit (max. two days before) in two tubes and stored at −20 °C at home or transported directly to the laboratory. In our laboratory, the two tubes were stored at −80 °C until further analysis. One tube was used to measure the gut barrier marker zonulin and the other for SCFA analysis.

4.5. Antioxidant Assays

The total phenolics content (TPC) was measured as was measured using Folin–Ciocalteu method as described previously for microalgae (compounds) by Neumann et al. [21]. It is expressed as gallic acid equivalents (GAE). The unit GAE (mg/g dry weight (DW)) describes how many milligrams of gallic acid are needed to achieve the same antioxidant

effect as one gram of (biomass) sample. For conducting the TPC assay a sample solution with a defined biomass concentration was prepared. 20 mg freeze-dried biomass sample was suspended in 5 mL of dimethyl sulfoxide. 150 µL Folin–Ciocalteu reagent (diluted 1:10 in ddH_2O) and 120 µL sodium carbonate solution (75 g/L) were mixed with 30 µL sample in a 96-well plate. The samples were then incubated and protected from light for 120 min at room temperature. Afterwards, the absorbance was measured at 765 nm using a plate reader (infinite M200 PRO, Tecan Group, Männedorf, Switzerland). Each sample was analysed in triplicate. As a blank, each sample was mixed with sodium carbonate solution and ddH_2O, to obtain an individual blank for each sample. Using a calibration curve, made with gallic acid (10–50 mg/L), the total phenolic content of the defined sample solution was calculated as gallic acid equivalents (GAE in mg/L). Via the biomass concentration of the defined sample solution (see above), the total phenolic content per (biomass) dry weight (DW) was calculated (GAE in mg/g_{DW}). As part of the antioxidative effect, the ability of substances to reduce substances by absorbing electrons was quantified by the ferric reducing antioxidant power assay (FRAP) and expressed in the form of FRAP values (ferric-reducing-antioxidant-power-values). The unit FRAP (mM/g_{DW}) describes how many µM of iron (II) sulphate are needed to produce the same antioxidant effect as one gram of (biomass) sample. The FRAP assay was performed based on the method of Benzie and Strain [74] as described by Neumann et al. [21]. For conducting the assay a solution with a defined biomass concentration was prepared as already described for the TPC assay. FRAP reagent was prepared with 10 mL sodium acetate buffer (300 mM, pH 3.6), 1 mL TPTZ solution (10 mM in 40 mM HCl) and 1 mL of iron (III) chloride solution (20 mM) immediately before the experiment and stored at 37 °C until use. In a 96-well plate, 220 µL FRAP reagent was mixed with 10 µL of sample solution before incubation at 37 °C for 90 min. Absorbance was measured at 593 nm using a plate reader (infinite M200 PRO, Tecan Group). Each sample was analysed in triplicate. Individual blanks for each sample were prepared by mixing 10 µL of sample with 220 µL of ddH_2O. To establish a calibration curve iron (II) sulphate was used (100–1000 µM) and the results are presented as FRAP values (iron (II) sulphate equivalents.

4.6. Quantification of Plasma and Erythrocyte Fatty Acids, Carotenoids and Tocopherols

The same protocol was used for plasma and erythrocyte fatty acids quantification by gas chromatography with mass spectrometry (GC/MS) as described before [8]. The transesterification was performed as previously reported with slight modification [75]. In short, 2 µL 10,11-dichloro-undecanoic acid (DC-11:0) as internal standard and 2 mL methanol (Carl Roth, Karlsruhe, Germany) with 1% sulphuric acid for transesterification were added to 100 µL sample. During incubation at 80 °C for 1 h, the samples were sonicated three times for 5 min. Thereafter, samples were cooled down on ice, mixed with 0.5 mL demineralised water and 0.35 saturated NaCl solution and extracted with 2 mL n-hexane. Prior to measurement, 5 µL tetradecanoic acid- ethyl ester (14:0) was added as the second internal standard. Fatty acid methyl esters (FAMEs) were analysed by GC/MS on a 5890 series II/5972A system (Hewlett-Packard, Waldbronn, Germany). A commercial standard (Supelco, Taufkirchen, Germany) was used for identification based on mass spectra and retention times in full scan mode, quantification was carried out in selected ion monitoring mode [76]. Five saturated fatty acids (14:0, 16:0, 18:0, 20:0, 22:0) were measured by GC/MS. Unsaturated fatty acids were measured in form of the monounsaturated fatty acids (MUFAs) 14:1 n−5 (myristoleic acid), 16:1, 16:1 n−7 (palmitoleic acid), 17:1, 18:1 n−9 (oleic acid), 18:1 (isomer of oleic acid), 20:1 n−9 (gondoic acid) and the polyunsaturated fatty acids (PUFAs) 18:2 n−6 (LA), 20:2 n−6 (eicosadienoic acid), 18:3 n−6 (γ-linolenic acid), 18:3 n−3 (ALA), 20:3 n−6 (dihomogammalinolenic acid), 20:4 n−6 (AA), 20:5 n−3 (EPA), 22:5 n−3 (DPA), 22:6 n−3 (DHA). The n−6:n−3 ratio in plasma levels was calculated from the total area of the n−6 FA (18:2 n−6, 20:2 n−6, 18:3 n−6, 20:3 n−6, 20:4 n−6) divided by the total area of the n−3 FA (18:3 n−3, 20:5 n−3, 22:5 n−3, 22:6 n−3). For the AA/EPA ratio, percentual contributions of both fatty acids were determined and divided through each

other. According to its definition, the Omega-3 index was determined by the percentual share of EPA and DHA in erythrocyte membranes relative to the sum of 26 fatty acids like in the method of Omegametrix (HS-Omega-3-Index®) [77].

Carotenoids and tocopherols were measured in plasma as described in detail previously. For FX, FXOH and A × A analysis, 100 µL of human plasma were mixed with 200 µL of ethanol/butanol (50/50 (*v*/*v*)) containing 5 mg butylated hydroxytoluene (BHT). After vigorous mixing and centrifugation (17,000× *g* and 4 °C, 10 min, Heraeus Fresco 17, Thermo Fischer Scientific, Waltham MA, USA) clear supernatants were injected into a Shimadzu HPLC system (Mc Kinley Scientific, New York, USA) equipped with an autosampler (15 °C), an UV detector (450 nm) and a C18 reversed-phase column (2.6 µm F5 100Å 150 × 4.6 mm, Kinetex, Phenomenex Ltd., Aschaffenburg, Germany) at 40 °C. A mixture of methanol/water (85/15 (*v*/*v*)) at 1.0 mL/min for 15 min was used as mobile phase [8].

For determination α/γ-tocopherol, lutein/zeaxanthin, lycopene, β-cryptoxanthin, α/β-carotene, and retinol, 40 µL of plasma were mixed with 200 µL of ethanol/butanol (50/50 (*v*/*v*)) containing 12 µL beta-apo-8′-carotenal-methyloxime/100 mL as internal standard. After vigorous mixing and centrifugation (17,000× *g* and 4 °C. 10 min; Heraeus Fresco 17, Thermo Fischer Scientific, Waltham, MA, USA), clear supernatants were injected into a Shimadzu HPLC system (Mc Kinley Scientific, New York, NY, USA) equipped with an autosampler (5 °C), and a ReproSil 80 ODS-2 column (3 µm, 250 × 4.6 mm) (Dr. A. Maisch GmbH, Ammerbuch-Entringen, Germany) at 40 °C. A mixture of acetonitrile/1.4-dioxane/methanol (82/15/3; *v*/*v*) containing 100 mmol/L ammonium acetate and 0.1% trimethylamine, at 1.5 mL/min for 20 min was used as mobile phase. Carotenoids were detected using an UV detector (450 nm). Retinol and tocopherols were detected using a fluorescence detector (Ex/Em at 325/ 470 nm for retinol and Ex/Em at 296/325nm for α/γ-tocopherol). carotenoids and tocopherols were quantified using authentic commercial standards and respective standard curves [78]. Quantification of FX [8], other carotenoids and tocopherols [79] in study products were performed as described before.

4.7. Inflammatory Markers and Anti-Oxidative Stress Parameter

The inflammatory markers were performed using the Human IL-10 ELISA Kit (RAB0244), the Human IL-6 ELISA Kit (RAB0306; Millipore Sigma Aldrich, Saint Louis, USA) and Human TNF-α Immunoassay (HSTA00E; R&D Systems, Inc., Minneapolis, USA) according to the manufacturer's protocol. For IL-10 and IL-6 plasma was used and TNF-α serum was used. For an oxidative stress marker, the xanthin oxidase fluorometric assay kit was performed following the manufacturer's protocol (Item No. 10010895; Cayman).

4.8. Analysis of the Intestinal Permeability Marker Plasma LBP, Faecal Zonulin, and Faecal SCFA

The measurement of the faecal samples for zonulin measurement were diluted to the working concentration in sample buffer using stool sample tubes (K6998SAS; Immundiagnostik AG, Bensheim, Germany) and for LBP analysis, 10 µL of blood plasma was used and processed. Zonulin and LBP were measured using the enzyme-linked immunosorbent assay kit (K5600, KR6813, Immundiagnostik AG, Bensheim, Germany) following the manufacturer's protocols. For SCFA analysis the same method was used as described before [8] by gas chromatography (Clarus 690, Perkin-Elmer, Waltham, MA, USA).

4.9. BIA, Muscle Function Test (5 STS, WOMAC, Gait Speed, Hand Grip Strength)

The BIA was done as a multi-frequency BIA according to Kyle et al. [80] (Data Input, Pöcking, Germany). The individual ASM and ASMI were calculated with the dietic pocket guide (www.dieteticpocketguide.com (accessed on 21 September 2022)) with the individual weight, gender, age, body height, BIA resistance (50 kH) and reactance (50 kH).

For muscle function, the 5-STS was conducted as previously described by Jones et al. [81]. Using the WOMAC questionnaire, the activity levels of the study participants were assessed at weeks zero and eight. WOMAC was developed for patients with osteoarthritis and registers signs of physical disability and relevant changes in health status because of

treatment intervention [82]. This questionnaire consists of three scales: pain, stiffness, and function. The higher the WOMAC value, the higher the pain, stiffness, and functional limitations. The maximum score which can be achieved is 96 (maximum score for pain is 20, 8 for stiffness and 68 for functional limitations). Gait speed in elderly was assessed in meters per second measured as a four-meter usual walking speed test two times. The hand grip strength was measured on each side two times (Hydraulic hand dynamometer, Jamar).

4.10. Diet Evaluation

Food diary of four weeks was evaluated with EPISpro 2016 (Software, Willstätt-Legelshurst, Germany). The dietary pattern was documented by the validated German Food Frequency Questionnaire (FFQ) [83].

4.11. Statistical Analyses

All parameters were tested for normal distribution using the Kolmogorov–Smirnov test. Normally distributed data, one-way ANOVA was used to compare statistically significant differences ($p < 0.05$) between microalga diet groups and the *Comp*. Variances were tested with the Brown–Forsythe test. Tukey's multiple comparison post hoc test was used for equal variances, and Dunnett's T3 multiple comparisons test was used for unequal variances. Within one group a t-test was performed between weeks two and four or study entry and week four. The study product treatment effect was measured as differences between the values of weeks 4 and 2 (Delta, Δ). This change was calculated for each subject. The four groups were compared with each other with a one-way ANOVA and a t-test respectively to the *Comp* group. For the mortality and body composition data, the value after intervention (4) minus the study start value (0) was calculated. Correlation analyses were performed with two-tailed Spearman-rank correlation. All statistical analyses were performed using GraphPad Prism version 9.4.1 (GraphPad Software, San Diego, CA, USA) and IMB SPSS statistics 25 (IBM Corp., Armonk, NY, USA).

5. Conclusions

The study demonstrates anti-inflammatory effects and potential antioxidant effects, as well as possible preservation of functional ability after ingestion of the whole microalgae and the β-G rich supernatant in the elderly. In addition, the present results provide the first evidence of the safe use of the β-G-rich microalgae supernatant over a two-week period and reconfirm the safe ingestion of whole PT in humans. Supplementation with PT could be a source of functional compounds that contribute to healthy aging from a nutritional perspective and, could be a candidate for anti-aging effects as a supplement. Higher doses of PT with higher amounts of functional compounds are warranted for future interventions.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/md20110716/s1, Table S1: Food Frequency Questionnaire at the study start; Table S2: Laboratory parameter and inflammatory markers; Table S3: Mobility markers and body composition at baseline and the study change; Table S4: Plasma fatty acid blood composition at different time-points; Table S5: Carotenoid plasma levels at the study start, after two-weeks and four weeks of intervention; Table S6: The concentration of short-chain fatty acids (SCFA) per dry mass in faecal samples at week 2 and week 4 after the intervention within the four study groups; Table S7: Nutrient composition of *Comp*, *A*, *SupB* and *A+SupB* diets used in the study.

Author Contributions: Conceptualization, L.S. and S.C.B.; methodology, L.S., K.L. and A.M.-A.; formal analysis, L.S.; investigation, L.S., K.L. and A.M.-A.; resources, K.F., U.S.-S., W.V. and J.F.; data curation, L.S.; writing—original draft preparation, L.S.; writing—review and editing, S.C.B.; visualization, L.S.; supervision, L.S.; project administration, S.C.B.; funding acquisition, L.S.; S.C.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Gesellschaft für Angewandte Vitaminforschung (GVF) and the Society of Nutrition and Food Science (SNFS), to the topic "Healthy aging-The contribution of

adequate nutritional status with essential nutrients (Gesundes Altern—Der Beitrag eines adäquaten Ernährungsstatus mit essenziellen Nährstoffen)" as a master degree project by Annika Köhler.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the local Ethical Committee (Ethik-Kommission der Landesärztekammer Baden-Württemberg (F-2021-061; 27 April 2021) and was registered at ClinicalTrials.gov (NCT05120791).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

Acknowledgments: Many thanks to the participants and students who made the project possible, as well as the measurements of the SCFAs by Andreas Rings.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Rudnicka, E.; Napierała, P.; Podfigurna, A.; Męczekalski, B.; Smolarczyk, R.; Grymowicz, M. The World Health Organization (WHO) approach to healthy ageing. *Maturitas* **2020**, *139*, 6–11. [CrossRef] [PubMed]
2. Ogra, P.L. Ageing and its possible impact on mucosal immune responses. *Ageing Res. Rev.* **2010**, *9*, 101–106. [CrossRef] [PubMed]
3. Saffrey, M.J. Aging of the mammalian gastrointestinal tract: A complex organ system. *Age* **2014**, *36*, 1019–1032. [CrossRef] [PubMed]
4. Tan, B.L.; Norhaizan, M.E.; Liew, W.-P.-P.; Rahman, H.S. Antioxidant and Oxidative Stress: A Mutual Interplay in Age-Related Diseases. *Front. Pharmacol.* **2018**, *9*, 1162. [CrossRef] [PubMed]
5. Martins, D.A.; Custódio, L.; Barreira, L.; Pereira, H.; Ben-Hamadou, R.; Varela, J.; Abu-Salah, K.M. Alternative Sources of n-3 Long-Chain Polyunsaturated Fatty Acids in Marine Microalgae. *Mar. Drugs* **2013**, *11*, 2259–2281. [CrossRef] [PubMed]
6. Ryckebosch, E.; Bruneel, C.; Muylaert, K.; Foubert, I. Microalgae as an alternative source of omega-3 long chain polyunsaturated fatty acids. *Lipid Technol.* **2012**, *24*, 128–130. [CrossRef]
7. Torres-Tiji, Y.; Fields, F.J.; Mayfield, S.P. Microalgae as a future food source. *Biotechnol. Adv.* **2020**, *41*, 107536. [CrossRef]
8. Stiefvatter, L.; Lehnert, K.; Frick, K.; Montoya-Arroyo, A.; Frank, J.; Vetter, W.; Schmid-Staiger, U.; Bischoff, S.C. Oral Bioavailability of Omega-3 Fatty Acids and Carotenoids from the Microalgae *Phaeodactylum tricornutum* in Healthy Young Adults. *Mar. Drugs* **2021**, *19*, 700. [CrossRef]
9. Khan, M.I.; Shin, J.H.; Kim, J.D. The promising future of microalgae: Current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. *Microb. Cell Factories* **2018**, *17*, 36. [CrossRef]
10. Stark, K.D.; Van Elswyk, M.E.; Higgins, M.R.; Weatherford, C.A.; Salem, N., Jr. Global survey of the omega-3 fatty acids, docosahexaenoic acid and eicosapentaenoic acid in the blood stream of healthy adults. *Prog. Lipid Res.* **2016**, *63*, 132–152. [CrossRef]
11. Burdge, G. Metabolism of α-linolenic acid in humans. *Prostaglandins Leukot. Essent. Fat. Acids* **2006**, *75*, 161–168. [CrossRef]
12. Calder, P.C. n−3 Polyunsaturated fatty acids, inflammation, and inflammatory diseases. *Am. J. Clin. Nutr.* **2006**, *83*, 1505S–1519S. [CrossRef]
13. Neumann, U.; Louis, S.; Gille, A.; Derwenskus, F.; Schmid-Staiger, U.; Briviba, K.; Bischoff, S.C. Anti-inflammatory effects of Phaeodactylum tricornutum extracts on human blood mononuclear cells and murine macrophages. *J. Appl. Phycol.* **2018**, *30*, 2837–2846. [CrossRef]
14. Batsis, J.A.; Villareal, D.T. Sarcopenic obesity in older adults: Aetiology, epidemiology and treatment strategies. *Nat. Rev. Endocrinol.* **2018**, *14*, 513–537. [CrossRef] [PubMed]
15. Neumann, U.; Derwenskus, F.; Gille, A.; Louis, S.; Schmid-Staiger, U.; Briviba, K.; Bischoff, S.C. Bioavailability and Safety of Nutrients from the Microalgae Chlorella vulgaris, Nannochloropsis oceanica and Phaeodactylum tricornutum in C57BL/6 Mice. *Nutrients* **2018**, *10*, 965. [CrossRef]
16. Goiris, K.; Muylaert, K.; Fraeye, I.; Foubert, I.; De Brabanter, J.; De Cooman, L. Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. *J. Appl. Phycol.* **2012**, *24*, 1477–1486. [CrossRef]
17. Park, H.-A.; Hayden, M.M.; Bannerman, S.; Jansen, J.; Crowe-White, K.M. Anti-Apoptotic Effects of Carotenoids in Neurodegeneration. *Molecules* **2020**, *25*, 3453. [CrossRef]
18. Kim, S.M.; Jung, Y.-J.; Kwon, O.-N.; Cha, K.H.; Um, B.-H.; Chung, D.; Pan, C.-H. A Potential Commercial Source of Fucoxanthin Extracted from the Microalga Phaeodactylum tricornutum. *Appl. Biochem. Biotechnol.* **2012**, *166*, 1843–1855. [CrossRef]
19. Fung, A.; Hamid, N.; Lu, J. Fucoxanthin content and antioxidant properties of Undaria pinnatifida. *Food Chem.* **2013**, *136*, 1055–1062. [CrossRef] [PubMed]
20. Mohibbullah, M.; Haque, M.N.; Khan, M.N.A.; Park, I.-S.; Moon, I.S.; Hong, Y.-K. Neuroprotective effects of fucoxanthin and its derivative fucoxanthinol from the phaeophyte Undaria pinnatifida attenuate oxidative stress in hippocampal neurons. *J. Appl. Phycol.* **2018**, *30*, 3243–3252. [CrossRef]

21. Neumann, U.; Derwenskus, F.; Flaiz Flister, V.; Schmid-Staiger, U.; Hirth, T.; Bischoff, S.C. Fucoxanthin, A Carotenoid Derived from Phaeodactylum tricornutum Exerts Antiproliferative and Antioxidant Activities In Vitro. *Antioxidants* **2019**, *8*, 183. [CrossRef]
22. Grabowska, M.; Wawrzyniak, D.; Rolle, K.; Chomczyński, P.; Oziewicz, S.; Jurga, S.; Barciszewski, J. Let food be your medicine: Nutraceutical properties of lycopene. *Food Funct.* **2019**, *10*, 3090–3102. [CrossRef]
23. Rao, A.V.; Rao, L.G. Carotenoids and Human Health. *Pharm. Res.* **2007**, *55*, 207–216.
24. Chang, C.-S.; Chang, C.-L.; Lai, G.-H. Reactive oxygen species scavenging activities in a chemiluminescence model and neuroprotection in rat pheochromocytoma cells by astaxanthin, beta-carotene, and canthaxanthin. *Kaohsiung J. Med. Sci.* **2013**, *29*, 412–421. [CrossRef]
25. Joshi, Y.B.; Praticò, D. Vitamin E in aging, dementia, and Alzheimer's disease. *BioFactors* **2012**, *38*, 90–97. [CrossRef] [PubMed]
26. Gao, B.; Chen, A.; Zhang, W.; Li, A.; Zhang, C. Co-production of lipids, eicosapentaenoic acid, fucoxanthin, and chrysolaminarin by Phaeodactylum tricornutum cultured in a flat-plate photobioreactor under varying nitrogen conditions. *J. Ocean Univ. China* **2017**, *16*, 916–924. [CrossRef]
27. Stiefvatter, L.; Neumann, U.; Rings, A.; Frick, K.; Schmid-Staiger, U.; Bischoff, S.C. The Microalgae *Phaeodactylum tricornutum* Is Well Suited as a Food with Positive Effects on the Intestinal Microbiota and the Generation of SCFA: Results from a Pre-Clinical Study. *Nutrients* **2022**, *14*, 2504. [CrossRef]
28. Carballo, C.; Chronopoulou, E.G.; Letsiou, S.; Maya, C.; Labrou, N.E.; Infante, C.; Power, D.; Manchado, M. Antioxidant capacity and immunomodulatory effects of a chrysolaminarin-enriched extract in Senegalese sole. *Fish Shellfish Immunol.* **2018**, *82*, 1–8. [CrossRef] [PubMed]
29. Reis, B.; Gonçalves, A.T.; Santos, P.; Sardinha, M.; Conceição, L.E.C.; Serradeiro, R.; Pérez-Sánchez, J.; Calduch-Giner, J.; Schmid-Staiger, U.; Frick, K.; et al. Immune Status and Hepatic Antioxidant Capacity of Gilthead Seabream *Sparus aurata* Juveniles Fed Yeast and Microalga Derived β-glucans. *Mar. Drugs* **2021**, *19*, 653. [CrossRef] [PubMed]
30. Zhang, H.; Tang, Y.; Zhang, Y.; Zhang, S.; Qu, J.; Wang, X.; Kong, R.; Han, C.; Liu, Z. Fucoxanthin: A Promising Medicinal and Nutritional Ingredient. *Evid.-Based Complement. Altern. Med.* **2015**, *2015*, 1–10. [CrossRef]
31. Lafarga, T. Effect of microalgal biomass incorporation into foods: Nutritional and sensorial attributes of the end products. *Algal Res.* **2019**, *41*, 101566. [CrossRef]
32. Francezon, N.; Tremblay, A.; Mouget, J.-L.; Pasetto, P.; Beaulieu, L. Algae as a Source of Natural Flavors in Innovative Foods. *J. Agric. Food Chem.* **2021**, *69*, 11753–11772. [CrossRef]
33. Batista, A.P.; Niccolai, A.; Fradinho, P.; Fragoso, S.; Bursic, I.; Rodolfi, L.; Biondi, N.; Tredici, M.R.; Sousa, I.; Raymundo, A. Microalgae biomass as an alternative ingredient in cookies: Sensory, physical and chemical properties, antioxidant activity and in vitro digestibility. *Algal Res.* **2017**, *26*, 161–171. [CrossRef]
34. Xu, D.; Liu, H.; Yang, C.; Xia, H.; Pan, D.; Yang, X.; Yang, L.; Wang, S.; Sun, G. Effects of different delivering matrices of β-glucan on lipids in mildly hypercholesterolaemic individuals: A meta-analysis of randomised controlled trials. *Br. J. Nutr.* **2020**, *125*, 294–307. [CrossRef] [PubMed]
35. Lalia, A.Z.; Lanza, I.R. Insulin-Sensitizing Effects of Omega-3 Fatty Acids: Lost in Translation? *Nutrients* **2016**, *8*, 329. [CrossRef]
36. Shibabaw, T. Omega-3 polyunsaturated fatty acids: Anti-inflammatory and anti-hypertriglyceridemia mechanisms in cardiovascular disease. *Mol. Cell. Biochem.* **2020**, *476*, 993–1003. [CrossRef] [PubMed]
37. Mayer, C.; Côme, M.; Ulmann, L.; Zittelli, G.C.; Faraloni, C.; Nazih, H.; Ouguerram, K.; Chénais, B.; Mimouni, V. Preventive Effects of the Marine Microalga Phaeodactylum tricornutum, Used as a Food Supplement, on Risk Factors Associated with Metabolic Syndrome in Wistar Rats. *Nutrients* **2019**, *11*, 1069. [CrossRef] [PubMed]
38. Gora, A.H.; Rehman, S.; Kiron, V.; Dias, J.; Fernandes, J.M.O.; Olsvik, P.A.; Siriyappagouder, P.; Vatsos, I.; Schmid-Staiger, U.; Frick, K.; et al. Management of Hypercholesterolemia Through Dietary SS-glucans–Insights From a Zebrafish Model. *Front. Nutr.* **2022**, *8*, 797452. [CrossRef]
39. Toth, M.J.; Tchernof, A. Lipid metabolism in the elderly. *Eur. J. Clin. Nutr.* **2000**, *54* (Suppl. 3), S121–S125. [CrossRef]
40. Poli, A.; Corsini, A. Reversible and non-reversible cardiovascular risk in patients treated with lipid-lowering therapy: Analysis of SEAS and JUPITER trials. *Eur. J. Intern. Med.* **2010**, *21*, 372–373. [CrossRef]
41. Nakashima, A.; Yamada, K.; Iwata, O.; Sugimoto, R.; Atsuji, K.; Ogawa, T.; Ishibashi-Ohgo, N.; Suzuki, K. β-Glucan in Foods and Its Physiological Functions. *J. Nutr. Sci. Vitaminol.* **2018**, *64*, 8–17. [CrossRef] [PubMed]
42. Léveillé, P.; Chouinard-Watkins, R.; Windust, A.; Lawrence, P.; Cunnane, S.C.; Brenna, J.T.; Plourde, M. Metabolism of uniformly labeled 13 C-eicosapentaenoic acid and 13 C-arachidonic acid in young and old men. *Am. J. Clin. Nutr.* **2017**, *106*, 467–474. [CrossRef] [PubMed]
43. Fortier, M.; Tremblay-Mercier, J.; Plourde, M.; Chouinard-Watkins, R.; Vandal, M.; Pifferi, F.; Freemantle, E.; Cunnane, S.C. Higher plasma n-3 fatty acid status in the moderately healthy elderly in southern Québec: Higher fish intake or aging-related change in n-3 fatty acid metabolism? *Prostaglandins Leukot. Essent. Fat. Acids* **2010**, *82*, 277–280. [CrossRef]
44. Rees, D.; Miles, E.A.; Banerjee, T.; Wells, S.J.; Roynette, C.E.; Wahle, K.W.; Calder, P.C. Dose-related effects of eicosapentaenoic acid on innate immune function in healthy humans: A comparison of young and older men. *Am. J. Clin. Nutr.* **2006**, *83*, 331–342. [CrossRef]
45. Bauch, A.; Lindtner, O.; Mensink, G.B.M.; Niemann, B. Dietary intake and sources of long-chain n-3 PUFAs in German adults. *Eur. J. Clin. Nutr.* **2006**, *60*, 810–812. [CrossRef] [PubMed]

46. EPA & DHA Intake Recommendations | GOED Omega-3. Available online: https://goedomega3.com/intake-recommendations (accessed on 21 September 2022).
47. Harris, W.S.; Del Gobbo, L.; Tintle, N.L. The Omega-3 Index and relative risk for coronary heart disease mortality: Estimation from 10 cohort studies. *Atherosclerosis* **2017**, *262*, 51–54. [CrossRef]
48. Bresson, J.L.; Flynn, A.; Heinonen, M.; Hulshof, K.; Korhonen, H.; Lagiou, P.; Løvik, M.; Marchelli, R.; Martin, A.; Moseley, B.; et al. Scientific Opinion on the Substantiation of Health Claims Related to Undaria Pinnatifida (Harvey) Suringar and Maintenance or Achievement of a Normal Body Weight (ID 2345) Pursuant to Article 13(1) of Regulation (EC) No 1924/2006. *EFSA J.* **2009**, *7*, 1302. [CrossRef]
49. Bae, M.; Kim, M.-B.; Park, Y.-K.; Lee, J.-Y. Health benefits of fucoxanthin in the prevention of chronic diseases. *Biochim. Biophys. Acta BBA-Mol. Cell Biol. Lipids* **2020**, *1865*, 158618. [CrossRef]
50. Lee, A.-H.; Shin, H.-Y.; Park, J.-H.; Koo, S.Y.; Kim, S.M.; Yang, S.-H. Fucoxanthin from microalgae Phaeodactylum tricornutum inhibits pro-inflammatory cytokines by regulating both NF-κB and NLRP3 inflammasome activation. *Sci. Rep.* **2021**, *11*, 543. [CrossRef]
51. Claesson, M.J.; Jeffery, I.B.; Conde, S.; Power, S.E.; O'Connor, E.M.; Cusack, S.; Harris, H.M.B.; Coakley, M.; Lakshminarayanan, B.; O'Sullivan, O.; et al. Gut microbiota composition correlates with diet and health in the elderly. *Nature* **2012**, *488*, 178–184. [CrossRef]
52. Kumar, M.; Babaei, P.; Ji, B.; Nielsen, J. Human gut microbiota and healthy aging: Recent developments and future prospective. *Nutr. Health Aging* **2016**, *4*, 3–16. [CrossRef] [PubMed]
53. Fu, Y.; Wang, Y.; Gao, H.; Li, D.; Jiang, R.; Ge, L.; Tong, C.; Xu, K. Associations among Dietary Omega-3 Polyunsaturated Fatty Acids, the Gut Microbiota, and Intestinal Immunity. Available online: https://www.hindawi.com/journals/mi/2021/8879227/ (accessed on 18 February 2021).
54. Qi, Y.; Goel, R.; Kim, S.; Richards, E.M.; Carter, C.S.; Pepine, C.J.; Raizada, M.K.; Buford, T.W. Intestinal Permeability Biomarker Zonulin is Elevated in Healthy Aging. *J. Am. Med. Dir. Assoc.* **2017**, *18*, 810.e1–810.e4. [CrossRef]
55. Rico, M.; López, A.; Santana-Casiano, J.M.; Gonzàlez, A.G.; Gonzàlez-Dàvila, M. Variability of the phenolic profile in the diatom *Phaeodactylum tricornutum* growing under copper and iron stress. *Limnol. Oceanogr.* **2013**, *58*, 144–152. [CrossRef]
56. Müller, L.; Fröhlich, K.; Böhm, V. Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (αTEAC), DPPH assay and peroxyl radical scavenging assay. *Food Chem.* **2011**, *129*, 139–148. [CrossRef]
57. De Alencar, D.B.; Diniz, J.C.; Rocha, S.A.; Pires-Cavalcante, K.M.; De Lima, R.L.; De Sousa, K.C.; Freitas, J.O.; Bezerra, R.M.; Baracho, B.M.; Sampaio, A.H.; et al. Fatty acid composition from the marine red algae *Pterocladiella capillacea* (S. G. Gmelin) Santelices & Hommersand 1997 and *Osmundaria obtusiloba* (C. Agardh) R. E. Norris 1991 and its antioxidant activity. *An. Acad. Bras. Ciênc.* **2018**, *90*, 449–459. [CrossRef]
58. Meijer, B.; Seinen, M.L.; Hosman, T.; Linskens, R.K.; Kneppelhout, J.-K.; Peters, G.J.; Mulder, C.J.; Van Bodegraven, A.A.; De Boer, N.K. High inter-individual variability of serum xanthine oxidoreductase activity in IBD patients. *Nucleosides Nucleotides Nucleic Acids* **2018**, *37*, 317–323. [CrossRef] [PubMed]
59. Aranda, R.; Doménech, E.; Rus, A.D.; Real, J.T.; Sastre, J.; Vina, J.; Pallardó, F.V. Age-related increase in xanthine oxidase activity in human plasma and rat tissues. *Free Radic. Res.* **2007**, *41*, 1195–1200. [CrossRef] [PubMed]
60. Peng, J.; Yuan, J.-P.; Wu, C.-F.; Wang, J.-H. Fucoxanthin, a Marine Carotenoid Present in Brown Seaweeds and Diatoms: Metabolism and Bioactivities Relevant to Human Health. *Mar. Drugs* **2011**, *9*, 1806–1828. [CrossRef]
61. Lepretti, M.; Martucciello, S.; Burgos Aceves, M.A.; Putti, R.; Lionetti, L. Omega-3 Fatty Acids and Insulin Resistance: Focus on the Regulation of Mitochondria and Endoplasmic Reticulum Stress. *Nutrients* **2018**, *10*, 350. [CrossRef]
62. Guzmán, S.; Gato, A.; Lamela, M.; Freire-Garabal, M.; Calleja, J.M. Anti-inflammatory and immunomodulatory activities of polysaccharide from *Chlorella stigmatophora* and *Phaeodactylum tricornutum*. *Phytother. Res.* **2003**, *17*, 665–670. [CrossRef]
63. Tan, A.; Sullenbarger, B.; Prakash, R.; McDaniel, J.C. Supplementation with eicosapentaenoic acid and docosahexaenoic acid reduces high levels of circulating proinflammatory cytokines in aging adults: A randomized, controlled study. *Prostaglandins Leukot. Essent. Fat. Acids* **2018**, *132*, 23–29. [CrossRef]
64. Simopoulos, A.P. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. Pharmacother.* **2002**, *56*, 365–379. [CrossRef]
65. Smith, G.I.; Atherton, P.; Reeds, D.N.; Mohammed, B.S.; Rankin, D.; Rennie, M.J.; Mittendorfer, B. Dietary omega-3 fatty acid supplementation increases the rate of muscle protein synthesis in older adults: A randomized controlled trial. *Am. J. Clin. Nutr.* **2011**, *93*, 402–412. [CrossRef]
66. Smith, G.I.; Julliand, S.; Reeds, D.N.; Sinacore, D.R.; Klein, S.; Mittendorfer, B. Fish oil–derived n−3 PUFA therapy increases muscle mass and function in healthy older adults. *Am. J. Clin. Nutr.* **2015**, *102*, 115–122. [CrossRef] [PubMed]
67. Murphy, C.H.; Flanagan, E.M.; De Vito, G.; Susta, D.; Mitchelson, K.A.J.; Castro, E.D.M.; Senden, J.M.G.; Goessens, J.P.B.; Mikłosz, A.; Chabowski, A.; et al. Does supplementation with leucine-enriched protein alone and in combination with fish-oil-derived *n*–3 PUFA affect muscle mass, strength, physical performance, and muscle protein synthesis in well-nourished older adults? A randomized, double-blind, placebo-controlled trial. *Am. J. Clin. Nutr.* **2021**, *113*, 1411–1427. [CrossRef] [PubMed]

68. Kalstad, A.A.; Myhre, P.L.; Laake, K.; Tveit, S.H.; Schmidt, E.B.; Smith, P.; Nilsen, D.W.T.; Tveit, A.; Fagerland, M.W.; Solheim, S.; et al. Effects of n-3 Fatty Acid Supplements in Elderly Patients after Myocardial Infarction: A Randomized Controlled Trial. *Circulation* **2020**, *143*, 528–539. [CrossRef] [PubMed]
69. Sahni, S.; Dufour, A.B.; Fielding, R.A.; Newman, A.B.; Kiel, D.P.; Hannan, M.T.; Jacques, P.F. Total carotenoid intake is associated with reduced loss of grip strength and gait speed over time in adults: The Framingham Offspring Study. *Am. J. Clin. Nutr.* **2020**, *113*, 437–445. [CrossRef] [PubMed]
70. Xu, J.; Song, C.; Song, X.; Zhang, X.; Li, X. Carotenoids and risk of fracture: A meta-analysis of observational studies. *Oncotarget* **2016**, *8*, 2391–2399. [CrossRef]
71. Eftekharsadat, B.; Aghamohammadi, D.; Dolatkhah, N.; Hashemian, M.; Salami, H. Lower serum levels of alpha tocopherol and lycopene are associated with higher pain and physical disability in subjects with primary knee osteoarthritis: A case-control study. *Int. J. Vitam. Nutr. Res.* **2021**, *91*, 304–314. [CrossRef]
72. Power, R.; Nolan, J.M.; Prado-Cabrero, A.; Roche, W.; Coen, R.; Power, T.; Mulcahy, R. Omega-3 fatty acid, carotenoid and vitamin E supplementation improves working memory in older adults: A randomised clinical trial. *Clin. Nutr.* **2021**, *41*, 405–414. [CrossRef]
73. EFSA Panel on Dietetic Products; Nutrition and Allergies (NDA). Scientific Opinion on the Safety of 'Yeast Beta-glucans' as a Novel Food Ingredient. *EFSA J.* **2011**, *9*, 2137. [CrossRef]
74. Benzie, I.F.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76. [CrossRef] [PubMed]
75. Wendlinger, C.; Hammann, S.; Vetter, W. Various concentrations of erucic acid in mustard oil and mustard. *Food Chem.* **2014**, *153*, 393–397. [CrossRef] [PubMed]
76. Thurnhofer, S.; Vetter, W. A Gas Chromatography/Electron Ionization−Mass Spectrometry−Selected Ion Monitoring Method for Determining the Fatty Acid Pattern in Food after Formation of Fatty Acid Methyl Esters. *J. Agric. Food Chem.* **2005**, *53*, 8896–8903. [CrossRef]
77. HS-Omega-3 Index-Omegametrix. Available online: https://www.omegametrix.eu/hs_omega_3_index.php (accessed on 19 September 2022).
78. Stuetz, W.; McGready, R.; Cho, T.; Prapamontol, T.; Biesalski, H.; Stepniewska, K.; Nosten, F. Relation of DDT residues to plasma retinol, α-tocopherol, and β-carotene during pregnancy and malaria infection: A case–control study in Karen women in northern Thailand. *Sci. Total Environ.* **2006**, *363*, 78–86. [CrossRef]
79. Montoya-Arroyo, A.; Toro-González, C.; Sus, N.; Warner, J.; Esquivel, P.; Jiménez, V.M.; Frank, J. Vitamin E and carotenoid profiles in leaves, stems, petioles and flowers of stinging nettle (*Urtica leptophylla* Kunth) from Costa Rica. *J. Sci. Food Agric.* **2022**, *102*, 6340–6348. [CrossRef] [PubMed]
80. Kyle, U.G.; Bosaeus, I.; De Lorenzo, A.D.; Deurenberg, P.; Elia, M.; Gomez, J.M.; Heitmann, B.L.; Kent-Smith, L.; Melchior, J.-C.; Pirlich, M.; et al. Bioelectrical impedance analysis—Part I: Review of principles and methods. *Clin. Nutr.* **2004**, *23*, 1226–1243. [CrossRef] [PubMed]
81. Jones, S.E.; Kon, S.S.C.; Canavan, J.L.; Patel, M.S.; Clark, A.L.; Nolan, C.M.; Polkey, M.I.; Man, W.D.-C. The five-repetition sit-to-stand test as a functional outcome measure in COPD. *Thorax* **2013**, *68*, 1015–1020. [CrossRef] [PubMed]
82. McConnell, S.; Kolopack, P.; Davis, A. The Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC): A review of its utility and measurement properties. *Arthritis Care Res.* **2001**, *45*, 453–461. [CrossRef]
83. Haftenberger, M.; Heuer, T.; Heidemann, C.; Kube, F.; Krems, C.; Mensink, G.B. Relative validation of a food frequency questionnaire for national health and nutrition monitoring. *Nutr. J.* **2010**, *9*, 36. [CrossRef]

marine drugs

MDPI

Article

Effect of Previous Frozen Storage, Canning Process and Packing Medium on the Fatty Acid Composition of Canned Mackerel

Ricardo Prego [1], Marcos Trigo [2], Beatriz Martínez [3] and Santiago P. Aubourg [2,*]

[1] Department of Oceanography, Marine Research Institute (CSIC), 36208 Vigo, Spain
[2] Department of Food Technology, Marine Research Institute (CSIC), c/Eduardo Cabello, 6, 36208 Vigo, Spain
[3] Department of Food Technologies, CIFP Coroso, Avda. da Coruña, 174, 15960 Ribeira, Spain
* Correspondence: saubourg@iim.csic.es

Abstract: This study addressed the fatty acid (FA) composition of canned Atlantic mackerel (*Scomber scombrus*). In it, the effect of prior frozen storage (6 months at −18 °C), different packing media (water, brine, and sunflower, refined and extra virgin olive oils), and canning procedure was investigated. As a result, the canning procedure led to a decrease ($p < 0.05$) in saturated FA (STFA) levels, an increase ($p < 0.05$) in polyunsaturated FA (PUFA) and total ω3 FA values, and higher PUFA/STFA and ω3/ω6 ratio values. Concerning the packing medium effect, the great presence of C18:2ω6 in sunflower oil led to high PUFA and PUFA/STFA values and low ω3/ω6 ratios when compared to other packing media. However, the high presence of C18:1ω9 in both olive oils tested did not lead to remarkable increases ($p > 0.05$) of this FA presence. Additionally, the presence of total ω3 FAs, C20:5ω3 and C22:6ω3 did not provide differences in canned fish muscle as a result of using different packing media. In all canned samples, ω3/ω6 values were included in the 8.2–10.8 range. Prior frozen storage did not have a substantial effect ($p > 0.05$) on the FA group (STFA, monounsaturated FA, PUFA, total ω3 FA) and FA ratio (PUFA/STFA and ω3/ω6) values.

Keywords: Atlantic mackerel; frozen storage; packing medium; canning; fatty acids; polyunsaturated; ω3/ω6 ratio; EPA; DHA

Citation: Prego, R.; Trigo, M.; Martínez, B.; Aubourg, S.P. Effect of Previous Frozen Storage, Canning Process and Packing Medium on the Fatty Acid Composition of Canned Mackerel. *Mar. Drugs* **2022**, *20*, 666. https://doi.org/10.3390/md20110666

Academic Editors: Giuseppina Tommonaro and Annabella Tramice

Received: 16 September 2022
Accepted: 21 October 2022
Published: 25 October 2022

1. Introduction

Seafood consumption has increased in recent decades, providing a high content of important constituents for the human diet, such as nutritional and digestive proteins, lipid-soluble vitamins (namely, A and D), microelements (*I*, *F*, *Ca*, *Cu*, *Zn*, *Fe* and others) and highly unsaturated fatty acids [1]. In this context, marine lipids are now the subject of a great deal of attention due to their high content of polyunsaturated fatty acids (PUFAs); among PUFA compounds, special attention has been paid to ω3 fatty acids (FAs), i.e., eicosapentaenoic (C20:5ω3, EPA) and docosahexaenoic (C22:6ω3, DHA) acids), on the basis of their positive role in preventing certain human diseases [2,3]. In this context, it is now recognised that most Western countries do not consume adequate levels of FAs belonging to the ω3 series, with great attention on the ω3/ω6 ratio of foods included in the human diet [4,5]. In order to prevent relevant health disorders, ω3/ω6 ratios varying from 1/4 to 1/1 have been recommended depending on the disease under consideration [6].

However, seafood constitutes highly perishable products whose quality rapidly declines post-mortem as a result of processing and storage. Marine species, due to their chemical composition, pH close to neutrality and high-water content, are an excellent media for microbial growth and enzymatic reactions [7,8]. Such food deteriorates after death due to the development of different damage pathways [9]. Since marine lipid composition includes a high content of PUFA compounds, the development of lipid oxidation during processing is likely to occur, especially if thermal treatment and a fatty fish species are concerned. Consequently, important losses of unsaturated FAs are likely to be produced and lead to detrimental effects on nutritional and sensory values [10,11].

Among traditional technologies, canning represents one of the most important means of marine species preservation [12,13]. Recent FAO statistics (2010–2019) have reported that about 9.5–11.5% (ca. 16.3–17.5 million tons per year) of total world fishery production would correspond to marine species canning [14]. In this process, seafood is introduced in sealed hermetic containers in the company of different kinds of packing media (oil, brine, pickle, etc.) and typically encompasses a sterilisation procedure, in which the canned fish tissue is heated in a temperature range of 110–130 °C for a period of 25–120 min. The extensive heat treatment involved alters the nature of the raw material, so that a product with different characteristics is formed. As a result of heat treatment, both enzymes and bacteria should be permanently inactivated [15,16]. Thus, a wide range of fish and invertebrate species produce excellent canned products, supporting an important role in human nutrition. Unfortunately, most species destined for canning are caught in large quantities and canneries have to store the raw material before it is processed. Consequently, most of the problems with canned fish acceptance can be related to the quality of the raw material, which continuously changes during storage prior to processing [9,17]. Previous research accounts for studies focused on chemical changes and quality loss in canned seafood as a result of the thermal treatment involved, packing medium employed and prior storage conditions applied. Such studies have addressed biogenic amine formation [18], changes in physical properties [19,20], amino acid and protein profile modifications [21,22], lipid damage [23] and the presence of metals [24]. Furthermore, the preservative effect of an antioxidant addition in the packing medium has been proved [25,26]. Concerning the FA profile changes, most research has addressed the effect of packing media [17,27,28]; on the contrary, only a few studies have addressed the effect of thermal treatment (sterilisation) during canning, and the conditions of prior storage to canning [29,30].

In the present work, the FA composition of canned Atlantic mackerel (*Scomber scombrus*) was addressed. The effect of prior frozen storage (6 months at −18 °C), different packing media (water, brine, and sunflower, refined and extra virgin olive oils), and canning procedure was investigated. This study focused on FA groups (saturated FAs, STFAs, monounsaturated FAs, MUFAs, PUFAs, total ω3 FAs) and FA ratios (PUFA/STFA and ω3/ω6) in initial and canned mackerel muscle. According to their high nutritional significance, the evolution of the EPA and DHA contents was analysed. Changes in the moisture and water values of mackerel muscle were also evaluated.

2. Results and Discussion

2.1. Moisture and Lipid Content of Initial and Canned Mackerel Muscle

Moisture and lipid values (Table 1) corresponding to initial fish agree with those reported for mackerel and other fatty fish species [31–33]. A comparison of initial mackerel tissue and canned mackerel without having undergone a prior frozen storage period revealed a marked decrease in average moisture value in all kinds of canned samples (Table 1); differences were found to be significant ($p < 0.05$) in all cases, except for the brine-packed batch. A general decrease in average moisture value in canned fish was detected as a result of the prior 6-month frozen storage period (termed "6-canned"); this decrease was found to be significant ($p < 0.05$) in all batches except for canned samples packed with refined olive oil. For both 0-canned and 6-canned samples, no significant differences ($p > 0.05$) were detected as a result of the packing medium employed. The results showed that prior frozen storage and canning procedure led to a decrease in moisture content in canned fish. No effect of the packing medium was found on the observed decrease in moisture content.

Table 1. Moisture and lipid content ($g \cdot kg^{-1}$ muscle) * in initial and canned mackerel muscle including different packing media **.

Constituent	Packing Medium	Mackerel Muscle		
		Initial	**0-Canned**	**6-Canned**
Moisture	Water	690.4 C (19.1)	652.6 Ba (13.0)	596.6 Aa (17.1)
	Brine	690.4 B (19.1)	674.1 Ba (21.6)	620.5 Aa (9.3)
	Sunflower oil	690.4 C (19.1)	659.3 Ba (8.0)	622.7 Aa (15.5)
	Refined olive oil	690.4 B (19.1)	643.3 Aa (12.2)	619.8 Aa (20.0)
	Virgin olive oil	690.4 C (19.1)	658.2 Ba (15.5)	619.0 Aa (17.8)
Lipids	Water	71.1 A (8.5)	115.2 Bb (14.9)	153.5 Cb (9.5)
	Brine	71.1 A (8.5)	96.6 Bb (14.6)	156.9 Cb (12.5)
	Sunflower oil	71.1 B (8.5)	51.1 Aa (6.9)	64.8 ABa (15.8)
	Refined olive oil	71.1 A (8.5)	60.1 Aa (5.5)	61.3 Aa (10.3)
	Virgin olive oil	71.1 A (8.5)	62.3 Aa (11.0)	65.8 Aa (13.6)

* Average values of five replicates ($n = 5$); standard deviations are indicated in brackets. In each row, average values followed by different capital letters (A,B,C) denote significant differences ($p < 0.05$) as a result of prior frozen storage and canning procedure. In each column, average values followed by different lowercase letters (a,b) denote significant differences ($p < 0.05$) as a result of packing medium. ** Abbreviations: 0-canned (canned fish without prior frozen storage) and 6-canned (canned fish with prior 6-month frozen storage).

The evolution of the lipid content of canned fish indicated great differences according to the nature of the packing medium employed (Table 1). Thus, a comparison between initial and 0-canned fish revealed a substantial lipid increase ($p < 0.05$) with canning when aqueous media (i.e., water or brine) were employed. On the contrary, lower average values were detected in canned fish if applying an oily packing medium; differences were found to be significant ($p < 0.05$) if sunflower oil was used. A general increase in average lipid content in canned fish was detected as a result of the prior holding time, differences being significant ($p < 0.05$) for canned fish including aqueous packing media. For both 0-canned and 6-canned samples, fish corresponding to oil-packed samples showed lower ($p < 0.05$) lipid values than their counterparts from aqueous media. The results showed that the polar nature of packing medium has a marked effect on the lipid content of canned mackerel tissue.

In the present study, moisture and lipid contents in canned fish can be influenced by different damage mechanisms. On the one hand, protein denaturation during frozen storage, and especially during the canning procedure, can lead to a decrease in the water-holding capacity of fish muscle, so that a water release into the packing medium would be produced [9,34]. As a result of this moisture content decrease, lipids and other constituents would increase their relative content in canned muscle. On the other hand, the presence of an oily medium as a coating would partially extract the lipid fraction of the muscle, so that a lipid content decrease would be produced. This decreasing effect would not take place in fish canned with aqueous packing media and would explain the higher lipid content found in the current study in fish canned with aqueous packing when compared to their counterparts corresponding to oily packing.

Another aspect to be taken into account is the fact that protein has become degraded as a result of the canning procedure and may diffuse out of the muscle into the surrounding packing medium; this effect would be especially important in an aqueous-packing medium. This protein loss would lead to a relative increase in the lipid content in the muscle and could partly explain the lipid content increase detected in canned fish corresponding to both aqueous-packing media. Protein degradation loss from the canned muscle could be increased in fish previously subjected to frozen storage [7,34]. This fact would explain the general higher average lipid values observed in 6-canned fish when compared to their counterparts from the 0-canned batch.

In most cases, previous research has shown a moisture content decrease in fish muscle as a result of the canning procedure [35,36]. However, different and contradictory results have been shown for the lipid content depending on the use of an aqueous or an oily packing medium. Additionally, the content of both constituents has shown a marked dependency on the extraction degree of the coating medium before canned muscle analysis. Thus, a comparative packing study was carried out on Little Tunny (*Euthynnus alletteratus*) [37]; as a result, a decrease in moisture and lipid contents was detected in brine-canned fish, while fish canned in olive oil led to lower moisture values and no differences in the lipid content. A great absorption of packing oil into the fish muscle was reported during soybean-packed tuna (*Thunnus alalunga*) [21] and coconut-, sunflower- and groundnut-packed yellowfin tuna (*Thunnus albacares*) [38]; additionally, moisture loss in canned fish was detected in such studies. Fish packing with an aqueous medium led to a higher moisture content and a lower lipid value than fish that was packed by using an oily coating medium [28,39]. When applying a sunflower oil-packing medium, a moisture value decrease and a lipid content increase were detected in canned Coho salmon (*Oncorhynchus kisutch*) as a result of canning [40]. Recently, a substantial decrease in moisture content and increase in lipid value was observed in water-packed Chub mackerel (*Scomber colias*) by Malga et al. [35] as a result of the canning procedure.

Concerning the effect of prior fish-holding time, no effect of prior frozen storage time (0–15-month period at −18 °C) of sardine (*Sardina pilchardus*) was detected on the water and lipid contents of the corresponding canned product [41]. However, an increase in lipid content in canned sardine (*S. pilchardus*) was proved by increasing the prior holding time in ice [30]; on the contrary, no effect on moisture level was reported.

2.2. FA Composition of Initial Packing Oils and Initial Mackerel Muscle

The composition of initial oils employed as packing media is shown in Table 2. A very different FA composition was detected in sunflower oil when compared to both olive oils. The two major FAs in sunflower oil were C18:2ω6 and C18:1ω9 (ca. 55% and 31%, respectively); additionally, two relatively abundant FAs were C16:0 and C18:0. For both olive oils, the most abundant FA was C18:1ω9 (ca. 74%), followed by C16:0 (ca. 12–13%). Other relatively abundant FAs were C18:2ω6, C18:0 and C18:1ω7.

The values for FA groups and the ratios of initial packing oils are presented in Table 3. According to individual FA composition, the following decreasing sequence was detected for FA groups in sunflower oil: PUFA > MUFA > STFA. A different decreasing sequence was observed for both olive oils: MUFA > STFA > PUFA. Higher ($p < 0.05$) PUFA/STFA ratios were detected in sunflower oil than in both olive oils. Furthermore, ω3/ω6 ratio values and even total ω3 levels were found to be negligible in all initial oils. Total ω6 content was especially high in sunflower oil according to the great content on C18:2ω6.

Concerning the initial mackerel muscle, a very different FA composition was observed when compared to any of the packing oils used (Table 2). The most abundant FAs were C16:0, C18:1ω9 and C22:6ω3, other abundant FAs being C20:5ω3, C18:0, C16:ω7, C18:1ω7 and C14:0. Such FA composition agrees with previous research carried out on wild fatty fish species [31,33,36]. Each of the FA groups, STFA, MUFA and PUFA, revealed values included in the 30–36% range, with PUFA/STFA and ω3/ω6 ratios reaching valuable levels

round 0.9 and 9.0, respectively. Additionally, the total ω3 level was around 27%, EPA and DHA being the major components of this FA group (ca. 8.5% and 17.0%, respectively).

Table 2. Fatty acid (FA) composition (g·100 g^{-1} total FAs) * of initial mackerel muscle and initial oils employed as packing media.

FA	Initial Oil-Packing Medium			Initial Mackerel Muscle
	Sunflower Oil	Refined Olive Oil	Virgin Olive Oil	
14:0	0.12 (0.01)	0.05 (0.00)	0.00 (0.00)	4.15 (0.45)
15:0	0.00 (0.00)	0.04 (0.00)	0.00 (0.00)	0.62 (0.08)
16:0	7.37 (0.01)	12.66 (0.04)	12.51 (0.00)	22.53 (0.85)
16:1ω7	0.15 (0.00)	0.79 (0.01)	0.92 (0.02)	5.16 (0.79)
17:0	0.06 (0.00)	0.09 (0.01)	0.12 (0.01)	1.06 (0.17)
18:0	4.21 (0.00)	3.24 (0.03)	3.33 (0.00)	5.44 (0.31)
18:1ω9	31.00 (0.01)	74.54 (0.01)	74.25 (0.02)	21.95 (3.69)
18:1ω7	0.79 (0.00)	2.03 (0.01)	2.24 (0.01)	4.89 (0.05)
18:2ω6	55.08 (0.02)	5.95 (0.01)	5.99 (0.02)	1.24 (0.15)
20:1ω9	0.20 (0.02)	0.28 (0.03)	0.26 (0.00)	2.67 (0.25)
20:2ω6	0.06 (0.00)	0.08 (0.01)	0.12 (0.04)	0.37 (0.04)
20:4ω6	0.86 (0.02)	0.16 (0.01)	0.15 (0.01)	1.00 (0.06)
22:1ω9	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.47 (0.05)
20:5ω3	0.10 (0.06)	0.05 (0.04)	0.04 (0.00)	8.48 (1.16)
22:4ω6	0.04 (0.00)	0.09 (0.01)	0.07 (0.00)	0.42 (0.08)
24:1ω9	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.70 (0.11)
22:5ω3	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	1.83 (0.36)
22:6ω3	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	17.03 (1.72)

* Average values of five independent determinations (n = 5); standard deviations are indicated in brackets.

In order to better focus on possible changes in the FA composition of mackerel muscle, a discussion of the FA results is addressed to the FA groups (STFA, MUFA, PUFA, and total ω3) and FA ratios (PUFA/STFA and ω3/ω6) in the next sections. Additionally, and based on the great significance of EPA and DHA [2,3], changes in their contents are also discussed individually.

Table 3. Values * for fatty acid (FA) groups (g·100 g^{-1} total FAs) and ratios in initial oils employed as packing media **.

FA Group/Ratio	Initial Oil-Packing Medium		
	Sunflower Oil	**Refined Olive Oil**	**Virgin Olive Oil**
STFA	11.76 aA (0.03)	16.06 bB (0.08)	15.96 bB (0.01)
MUFA	32.14 aB (0.02)	77.63 bC (0.04)	77.67 bC (0.01)
PUFA	56.10 bC (0.01)	6.31 aA (0.04)	6.37 aA (0.01)
PUFA/STFA	4.77 b (0.01)	0.39 a (0.00)	0.40 a (0.00)
Total ω3	0.10 b (0.06)	0.03 a (0.01)	0.04 a (0.00)
Total ω6	56.00 b (0.06)	6.28 a (0.00)	6.33 a (0.01)
ω3/ω6 ratio	0.00 a (0.00)	0.00 a (0.00)	0.01 a (0.00)

* Average values of five independent determinations (n = 5); standard deviations are indicated in brackets. Abbreviations: STFA (saturated FAs), MUFA (monounsaturated FAs) and PUFA (polyunsaturated FAs). ** In each row, values followed by different lowercase letters (a,b) indicate significant differences ($p < 0.05$) among the three initial oils. For each initial oil, different capital letters (A,B,C) denote significant differences ($p < 0.05$) among FA groups (STFA, MUFA and PUFA).

2.3. Effect of Canning Procedure on the FA Composition of Canned Mackerel Muscle

The composition of the initial fish and the canned muscle without prior frozen storage (i.e., 0-canned fish) revealed substantial changes in the FA groups as a result of the canning procedure in all kinds of packed fish (Table 4). The different types of canned mackerel products showed lower ($p < 0.05$) STFA levels than the initial fish. On the contrary, a substantial increase ($p < 0.05$) in PUFA content was detected in all canned batches. Concerning the MUFA presence, no effect ($p > 0.05$) was implied for this FA group as a result of the canning procedure, except for the sunflower oil batch. According to this distribution of FA groups, all kinds of 0-canned fish showed an increase ($p < 0.05$) in PUFA/STFA ratio (Figure 1), all values being included in the 1.22–1.47 range.

Table 4. Determination * of fatty acid (FA) group values (g·100 g^{-1} FAs) in initial and canned mackerel muscle including different packing media **.

FA Group	Packing Medium	Mackerel Muscle		
		Initial	**0-Canned**	**6-Canned**
STFA	Water	33.79 B (0.34)	28.23 Aa (0.56)	29.89 Aa (2.72)
	Brine	33.79 B (0.34)	28.50 Aa (0.56)	28.32 Aa (0.91)
	Sunflower oil	33.79 B (0.34)	27.79 Aa (0.34)	28.94 Aa (0.78)
	Refined olive oil	33.79 B (0.34)	27.57 Aa (0.47)	28.43 Aa (1.35)
	Virgin olive oil	33.79 B (0.34)	29.30 Aa (2.35)	28.62 Aa (1.06)

Table 4. *Cont.*

FA Group	Packing Medium	Mackerel Muscle		
		Initial	**0-Canned**	**6-Canned**
MUFA	Water	35.85 A (2.82)	36.24 Ab (0.87)	32.59 Aab (3.77)
	Brine	35.85 A (2.82)	35.31 Aab (3.89)	35.96 Ab (1.13)
	Sunflower oil	35.85 B (2.82)	31.33 Aa (1.70)	31.47 Aa (1.36)
	Refined olive oil	35.85 A (2.82)	37.33 Ab (1.71)	34.31 Aab (1.72)
	Virgin olive oil	35.85 A (2.82)	35.36 Ab (0.56)	34.30 Ab (0.70)
PUFA	Water	30.36 A (2.04)	35.52 Ba (0.46)	37.51 Bab (2.26)
	Brine	30.36 A (2.04)	36.19 Bab (2.76)	35.72 Ba (1.30)
	Sunflower oil	30.36 A (2.04)	40.88 Bb (1.73)	39.59 Bb (2.06)
	Refined olive oil	30.36 A (2.04)	35.09 Ba (1.58)	37.26 Bab (1.46)
	Virgin olive oil	30.36 A (2.04)	35.32 Bab (2.32)	37.08 Bab (0.54)

* Average values of five replicates (n = 5); standard deviations are indicated in brackets. In each row, average values followed by different capital letters (A,B) denote significant differences ($p < 0.05$) as a result of prior frozen storage and canning procedure. For each FA group and in each column, average values followed by different lowercase letters (a,b) denote significant differences ($p < 0.05$) as a result of packing medium. ** Abbreviations: STFA (saturated FAs), MUFA (monounsaturated FAs), PUFA (polyunsaturated FAs), 0-canned (canned fish without prior frozen storage) and 6-canned (canned fish with prior 6-month frozen storage).

Related to the total ω3 FA content (Table 5), a comparison of average values revealed a general increase in all kinds of samples as a result of the canning procedure. This increase was found to be significant ($p < 0.05$) in all cases except for canned fish including brine as a packing medium. The analysis of the ω3/ω6 ratio showed an average increase with the thermal process in most cases (Figure 2), fish canned in sunflower oil being the only exception. All canned values were included in the 8.7–11.0 range, which according to nutritional recommendations can be considered as highly valuable [6].

According to the results obtained for the total ω3 FAs, the analysis of the EPA and DHA presence (Table 5) in fish muscle revealed a substantial increase in average values after the canning procedure. For EPA content, differences were found to be significant ($p < 0.05$) in canned fish including an aqueous filling medium (i.e., water or brine). In the case of DHA level, a significant increase ($p < 0.05$) was found in canned fish including any of the olive-oil filling media tested.

Previous research accounts for studies focused on the effect of the canning procedure on the FA composition of canned fish muscle. No effect of canning was detected in FA composition (individual FAs and FA groups) in brine-canned Little Tunny (*E. alletteratus*) [37]. As a result of canning, a decrease in MUFA content was detected by Naseri and Rezaei [17] in brine-canned sprat (*Clupeonella cultriventris*), while no differences were detected in STFA and PUFA levels; no effect on total ω3 and ω3/ω6 ratio was observed.

The canning procedure carried out on water-canned Atlantic mackerel (*S. scombrus*) [42] and brine-canned Chub mackerel (*S. colias*) [26,29] did not provide differences in the polyene index (PI) (calculated as the C22:6ω3 + C20:5ω3)/C16:0 FA ratio) of canned muscle. No remarkable effect on FA group contents and PI was detected in water-canned Chub mackerel

(*S. colias*) [35]; however, a substantial increase was observed for the ω3/ω6 ratio as a result of canning.

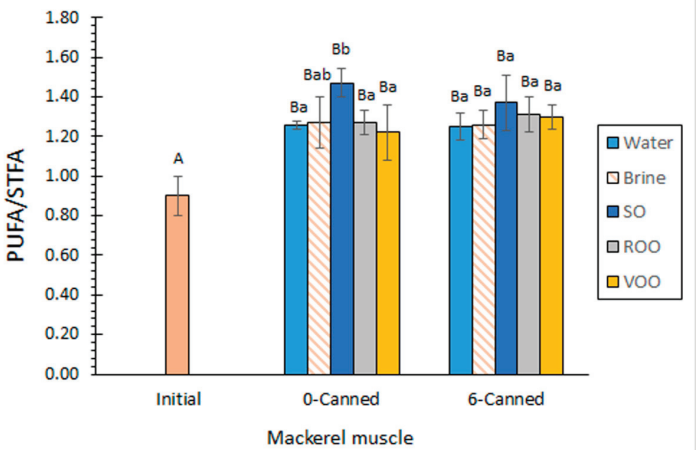


Figure 1. Determination of PUFA/STFA ratio in initial and canned mackerel muscle including different packing media. Average values of five replicates (n = 5); standard deviations are indicated by bars. For each packing medium, average values followed by different capital letters (A,B) denote significant differences ($p < 0.05$) as a result of canning procedure and prior frozen storage. For each prior holding condition, average values followed by different lowercase letters (a,b) denote significant differences ($p < 0.05$) as a result of packing medium. Abbreviations: SO (sunflower oil), ROO (refined olive oil), VOO (extra virgin olive oil), PUFA (polyunsaturated fatty acid), STFA (saturated fatty acid), 0-canned (canned fish without prior frozen storage) and 6-canned (canned fish with prior 6-month frozen storage).

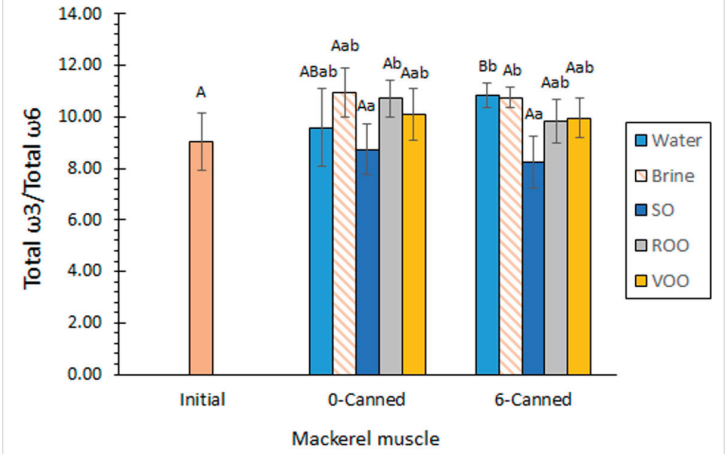


Figure 2. Determination of ω3/ω6 ratio in initial and canned mackerel muscle including different packing media. Average values of five replicates (n = 5); standard deviations are indicated by bars. For each packing medium, average values followed by different capital letters (A,B) denote significant differences ($p < 0.05$) as a result of canning procedure and prior frozen storage. For each prior holding condition, average values followed by different lowercase letters (a,b) denote significant differences ($p < 0.05$) as a result of packing medium. Abbreviations: SO (sunflower oil), ROO (refined olive oil), VOO (extra virgin olive oil), 0-canned (canned fish without prior frozen storage) and 6-canned (canned fish with prior 6-month frozen storage).

Table 5. Determination (g·100 g^{-1} total FAs) of EPA, DHA and total ω3 fatty acid values * in initial and canned mackerel muscle including different packing media **.

FA Group	Packing Medium	Mackerel Muscle		
		Initial	**0-Canned**	**6-Canned**
Total ω3	Water	27.33 A (3.04)	32.10 Ba (0.40)	34.33 Ba (3.85)
	Brine	27.33 A (3.04)	33.16 Aab (3.68)	32.67 Aa (1.19)
	Sunflower oil	27.33 A (3.04)	36.65 Bb (1.76)	33.45 ABa (3.09)
	Refined olive oil	27.33 A (3.04)	32.09 Ba (1.57)	33.82 Ba (1.54)
	Virgin olive oil	27.33 A (3.04)	32.13 Ba (1.38)	33.68 Ba (0.60)
EPA	Water	8.48 A (1.16)	11.32 Bb (0.67)	11.73 Ba (1.73)
	Brine	8.48 A (1.16)	11.32 Bb (0.81)	11.14 Ba (1.06)
	Sunflower oil	8.48 A (1.16)	10.61 Aa (1.19)	11.58 Aa (2.44)
	Refined olive oil	8.48 A (1.16)	9.57 Aab (2.10)	10.56 Aa (1.32)
	Virgin olive oil	8.48 A (1.16)	8.96 Aa (1.73)	10.68 Aa (1.50)
DHA	Water	17.03 A (1.72)	18.54 Aa (0.48)	19.92 Aa (2.96)
	Brine	17.03 A (1.72)	19.31 Aab (3.88)	18.96 Aa (1.90)
	Sunflower oil	17.03 A (1.72)	23.79 ABb (2.59)	19.30 Ba (1.86)
	Refined olive oil	17.03 A (1.72)	20.34 Bb (1.09)	20.98 Ba (1.11)
	Virgin olive oil	17.03 A (1.72)	21.15 Bb (1.57)	20.75 Ba (1.41)

* Average values of five replicates (n = 5); standard deviations are indicated in brackets. In each row, average values followed by different capital letters (A,B) denote significant differences ($p < 0.05$) as a result of prior frozen storage and canning procedure. In each column and for each ratio, average values followed by different lowercase letters (a,b) denote significant differences ($p < 0.05$) as a result of packing medium. ** Abbreviations: EPA (eicosapentaenoic acid) and DHA (docosahexaenoic acid).

2.4. Effect of Packing Medium on the FA Composition of Canned Mackerel Muscle

No effect ($p > 0.05$) of the packing medium could be detected on the STFA content in canned samples corresponding to both 0-canned and 6-canned processing conditions (Table 4). However, some differences could be outlined from the analysis of the MUFA and PUFA presence (Table 4). Canned fish including sunflower oil as a packing medium revealed the lowest average values for MUFA content; for canned fish without prior frozen storage, such differences were found to be significant ($p < 0.05$) by comparison to all other packing media except for brine-canned batch. For canned fish with a prior holding period, differences in MUFA content were only found to be significant ($p < 0.05$) by comparison to brine- and extra virgin olive oil-packed fish. In the case of PUFA presence, the highest average values were detected in canned fish corresponding to sunflower oil-packing medium (Table 4); differences with canned muscle corresponding to other packing

media were found to be significant ($p < 0.05$) when compared to water- and refined olive oil-packed (0-canned samples) and to brine-canned (6-canned samples) mackerel muscle.

According to the FA group distribution, the highest average values for the PUFA/STFA ratio were detected in canned fish including sunflower oil as a packing medium (Figure 1). For canned fish without prior storage, differences were found to be significant ($p < 0.05$) by comparison with all canned samples, except for those including brine as a packing medium; on the contrary, differences were not found to be significant ($p > 0.05$) when taking into account samples that were previously stored under frozen conditions.

Concerning the total ω3 FA value, scarce differences were observed as a result of the packing medium employed (Table 5). For canned samples without prior frozen storage, a higher ($p < 0.05$) level was detected in canned samples including sunflower oil as a packing medium when compared to their counterparts corresponding to water and both olive oil media. In the case of canned samples with prior storage, no significant differences ($p > 0.05$) were detected as a result of the packing medium used.

Average values for the ω3/ω6 ratio revealed the lowest average values in canned samples corresponding to sunflower oil packing (Figure 2); however, no significant differences were observed between sunflower-canned samples and initial ones. For 0-canned fish, differences were found to be significant ($p < 0.05$) by comparison to the refined olive oil-packed batch; in the case of 6-canned samples, differences were found to be significant ($p < 0.05$) by comparison to canned fish including aqueous media.

Concerning the two most valuable ω3 FAs (namely, EPA and DHA; Table 5), no differences ($p > 0.05$) were detected as a result of the packing medium employed in samples that were previously stored under frozen conditions; canned fish corresponding to both olive oil-packed conditions showed the lowest and highest average values for EPA and DHA, respectively. In the case of canned fish without a prior holding period, average EPA levels were higher in samples corresponding to aqueous packing media (i.e., water and brine), differences being significant ($p < 0.05$) by comparison to samples including sunflower oil and extra virgin olive oil. For 0-canned fish, the highest average values for DHA presence were detected in samples including any oily filling media; differences were found to be significant ($p < 0.05$) when compared to water-packed samples.

The present results can be considered the result of two opposite effects. On one side, the absorption of the packing oil into the canned muscle is likely to be produced and lead to an increase in FA content in the muscle of FAs that are present in the packing oil. On the other side, packing oil can act as an extracting system from fish muscle into the packing medium, so that a modification of the FA profile of canned fish can be expected. As expressed in the Material and Methods section, packing media were eliminated in the present study by wrapping the canned fish with filter paper before starting the canned muscle analysis.

The current results show associations between the FA composition of the packing oils with the FA composition of the canned mackerel muscle. Thus, the great presence of C18:2ω6 (Table 2) in sunflower oil led to high PUFA (Table 4) and PUFA/STFA (Figure 1) values and low ω3/ω6 ratios (Figure 2). However, the high presence of C18:1ω9 in both olive oils (Table 2) did not lead to substantial increases in the presence of this FA in the corresponding canned muscle (Table 4). Additionally, the presence of ω3 FAs, measured as total ω3 FA, EPA and DHA contents (Table 5), did not undergo remarkable decreases in fish muscle, even though such FAs are present in very low levels in the initial oils tested in this study. The results showed that a low effect of the packing oil composition was produced on the composition of the canned fish muscle in the current study. This result can be explained on the basis that packing oils were carefully extracted by wrapping the fish muscle with filter paper.

The present results show that no differences ($p > 0.05$) in FA composition were detected by the comparison of canned fish corresponding to both olive oils. It is inferred that the presence of preservative (namely, antioxidants) compounds in extra virgin olive oil [43] did not lead to a greater retention of PUFAs.

Previous research has shown a strong effect of the FA composition of the packing medium employed (i.e., oil packing) on the FA profile of canned fish. Thus, the fat composition of canned tuna (*T. alalunga*) tended to be similar to that of the soya bean oil used for packing [36]; as a result, canned tuna increased in C18:1ω9, C18:2ω6 and C18:3ω3 values, and showed remarkable decreases in C20:4ω6, EPA and DHA levels. A comparative study of two packing conditions (brine and olive oil) of Little Tunny (*E. alletteratus*) was carried out by Aubourg et al. [37]; as a result, a strong presence of the FAs of olive oil was detected in canned tuna packed under such oil medium and leading to higher levels of MUFAs (C18:1ω9, C16:1ω7 and C22:1ω11) and C18:2ω6, but a lower presence of PUFAs (C20:4ω6, EPA and DHA) and total ω3 FAs. Similarly, Ruiz-Roso et al. [27] detected a great influence of olive oil-packing medium in canned sardine (*S. pilchardus*); an increase in MUFA and PUFA presence, but a decrease in STFA, total ω3, EPA and DHA values was detected. Tarley et al. [28] carried out a comparative study of soybean oil and tomato sauce as packing media for sardine (*Sardinella brasiliensis*); as a result, higher levels of C18:2ω6 and C18:3ω3 were observed in soybean oil-canned sardine, while higher levels of EPA and DHA were found in sauce-packed fish. A lower PI was detected in sunflower oil-canned sprat (*C. cultriventris*) when compared to their counterparts packed in brine [17]; a comparison between muscle packed under both packing media revealed higher levels of DHA, EPA, STFAs, MUFAs, total ω3 FAs and ω3/ω6 ratios in brine canned sprat than in their counterparts packed in oil. Recently, Gómez-Limia et al. [44] observed the absorption of the oil used in the canning process in European eel (*Anguilla anguilla*); higher values of C18:1ω9 and MUFAs and decreases in STFA, PUFA and PUFA/STFA values were detected in olive oil-packed eel when compared to their counterparts corresponding to a sunflower oil batch.

2.5. Effect of Prior Frozen Storage on the FA Composition of Canned Mackerel Muscle

The effect of the prior holding step can be evaluated by a comparison of 0-canned and 6-canned samples. Thus, the analysis of the STFA, MUFA and PUFA contents did not provide significant differences ($p > 0.05$) as a result of the prior frozen storage (Table 4). A different trend according to the aqueous or oily packing condition employed could not be concluded. According to FA group results, no differences ($p > 0.05$) were detected for the PUFA/STFA value as a result of the prior frozen storage (Figure 1).

No significant differences ($p > 0.05$) were detected for the total ω3 FA values (Table 5), as well as for the ω3/ω6 ratio (Figure 2) as a result of the prior 6-month frozen period. The ω3/ω6 ratio showed a decreasing average value with frozen storage in canned samples corresponding to all packing media, except for those samples including water as a packing medium. According to the results observed for total ω3 FA values, the analysis of the EPA and DHA (Table 5) presence in canned fish did not reveal a significant effect ($p > 0.05$) related to the prior holding period.

Previous research concerning the effect of prior holding condition on the FA profile of canned fish can be considered scarce. An increased prior frozen storage time (0–15-month period) led to a PI decrease in brine-canned Chub mackerel (*S. colias*) that was explained on the basis of an increased development of the lipid oxidation mechanism [29]. On the contrary, and in agreement with the current study, no substantial effect was detected in the PI of brine-canned sardine (*S. pilchardus*) by increasing the prior holding time on ice [41]. Recently, Reblová et al. [30] detected remarkable decreases in PUFA/STFA and ω3/ω6 ratios in canned sardine (*S. pilchardus*) by increasing the prior chilling time (0–15-day period); however, no effect was observed for the presence of STFA, MUFA and PUFA groups in canned fish.

3. Materials and Methods

3.1. Initial Fish, Frozen Storage and Chemicals

Fresh Atlantic mackerel (*S. scombrus*) (110 specimens) (length and weight ranges: 27.5–31.0 cm and 215–255 g, respectively) were obtained at Vigo harbour (North-Western

Spain) in November 2020 and transported on ice to the laboratory within 20 min. Ten fish were taken, divided into five groups (two individuals per group), beheaded, eviscerated and filleted. Then, the white muscle was separated, pooled together within each group, minced, analysed independently (n = 5) and considered as initial fish.

On the same day, 50 fish were taken and divided into five groups (ten individuals per group). The fish were beheaded, eviscerated, filleted and subjected to the canning procedure (samples without prior frozen storage; 0-canned samples).

The remaining fish (50 specimens) were stored at −40 °C for 48 h and then kept frozen (−18 °C) for 6 months. After this time, individuals were thawed overnight (4 °C) and divided into five groups (ten individuals per group). Fish pieces were beheaded, eviscerated, filleted and subjected to the canning procedure (samples with prior frozen storage; 6-canned samples).

General solvents (chloroform, methanol and toluene) and chemicals (acetyl chloride and NaCl) used were of reagent grade and purchased from Merck (Darmstadt, Germany). Standards and other chemicals were as expressed in the related analytical procedure. Sunflower oil was obtained from Aceites Toledo, S. A. (Toledo, Spain). Olive oils were obtained from Aceites Carbonell S. A. (Alcolea, Córdoba, Spain); both olive oils (Virgen Extra Arbequina and Olive Oil) were prepared from olives obtained in Lleida (Spain).

3.2. Canning Process

At each canning time, 45-g portions of mackerel fillets were placed in small flat rectangular cans (105 × 60 × 25 mm; 150 mL). As packing media, water, brine (aq. 2% NaCl solution), sunflower oil, refined olive oil and extra virgin olive oil were employed, respectively. Packing media were added in order to fulfil the corresponding cans. Each can was prepared with a single fish.

The cans were vacuum-sealed and then subjected to the sterilisation process in a horizontal steam retort (115 °C, 45 min; F_o = 7 min) (CIFP Coroso, Ribeira, A Coruña, Spain). Once the heating time was completed, steam was cut off, and air was used to flush away the remaining steam. The cans were cooled at reduced pressure. Finally, they were stored at room temperature (20 °C) for 3 months.

A 3-month canned storage was carried out according to common practice employed in canneries. A minimum of 2 months is suggested as necessary by manufacturers in order to optimise fish palatability in commercial canned fish [28].

3.3. Sampling Procedure

At each sampling time, the cans were opened, and the liquid part was carefully drained off gravimetrically. Then, the mackerel muscle was separated, and the remaining packing medium was eliminated from the fish muscle by wrapping with filter paper.

The fish white muscle of two cans with the same packing medium was pooled together, minced and employed to carry out the different FA analyses. Cans corresponding to each packing medium were analysed by means of five replicates (n = 5).

3.4. Assessment of Moisture and Lipid Content in Mackerel Muscle

The moisture of the fish muscle of initial and canned samples was determined as the weight difference (1–2 g) before and after 4 h at 105 °C, according to the official method 950.46B [45]. The results were calculated as $g \cdot kg^{-1}$ of fish muscle.

The lipids of the fish muscle of initial and canned samples were extracted by the Bligh and Dyer [46] method, which employs a single-phase solubilisation of the lipids using a chloroform–methanol (1:1) mixture. Quantification was carried out according to Herbes and Allen [47]. The results were calculated as $g \cdot kg^{-1}$ of fish muscle.

3.5. Analysis of the FA Composition

Lipid extracts of fish muscle (initial and canned samples) and initial oil samples were converted into FA methyl esters (FAME) by using acetyl chloride in methanol and then

analysed by gas–liquid chromatography (GLC; Perkin Elmer 8700 chromatograph, Madrid, Spain) [48]. The quantitative response of the equipment was checked with a GLC quantitative standard (FAME Mix, Supelco, Inc., Bellefonte, PA, USA). Peaks corresponding to FAME were identified by a comparison of their retention times with those of a standard mixture (Qualmix Fish, Larodan, Malmo, Sweden). Peak areas were automatically integrated. Nonadecanoic FA (C19:0) (Sigma-Aldrich, St. Louis, MO, USA) was used as internal standard for quantitative purposes; for it, 100 μL (i.e., 40 μg C19:0) of a 0.4 mg·mL^{-1} solution in toluene was added to each sample before the methylation reaction with acetyl chloride. The content of each FA was calculated as g·100 g^{-1} of total FAs.

The results concerning the FA groups (STFA, MUFA, PUFA and total ω3 FA) and FA ratios (PUFA/STFA and ω3/ω6) were calculated on the basis of the quantification of the corresponding individual FAs mentioned in Table 2.

3.6. Statistical Analysis

The data (n = 5) obtained from moisture, lipid and FA determinations were subjected to a one-way ANOVA ($p < 0.05$) to investigate the differences resulting from canning procedure, packing medium and prior frozen storage (Statistica version 6.0, 2001; Statsoft Inc., Tulsa, OK, USA). A comparison of means was performed using a least-squares difference (LSD) method.

4. Conclusions

The present research checked the effect of the canning procedure and that of two common procedures carried out during canning (namely, packing medium addition and prior holding period). The FA composition of canned Atlantic mackerel showed a substantial effect of the canning procedure. A decrease ($p < 0.05$) in STFA levels, an increase ($p < 0.05$) in PUFA and total ω3 FA values and higher PUFA/STFA and ω3/ω6 ratio values were detected in canned fish. Concerning the effect of the packing medium, the great presence of C18:2ω6 in sunflower oil led to high PUFA and PUFA/STFA values and low ω3/ω6 ratios. However, the high presence of C18:1ω9 in both olive oils tested did not lead to remarkable increases ($p > 0.05$) in the presence of this FA in the corresponding canned mackerel muscle. Additionally, the contents of ω3 FAs, EPA and DHA did not provide remarkable differences as a result of the packing conditions used. Prior frozen storage did not have a substantial effect ($p > 0.05$) on FA group presence (STFA, MUFA, PUFA, total ω3) and FA ratio (PUFA/STFA and ω3/ω6) values.

The analysis of the FA composition of the resulting canned fish showed that the three factors maintained highly valuable FA contents, ω3/ω6 ratios being included in all cases in the 8.2–10.9 range and levels of EPA and DHA being included in 9.0–11.7 and 18.5–23.8 g·100 g^{-1} total FA ranges, respectively. According to the current nutritional recommendations, such scores can be considered highly valuable for human health and diet. On the basis of the great importance of canned seafood, further research ought to be carried out focused on the incidence of canning procedure, packing medium and prior holding time on other nutritional values (i.e., content on essential amino acids, vitamins, essential elements, etc.), as well as on the sensory and physical properties of canned fish related to quality.

Author Contributions: Conceptualization, R.P. and S.P.A.; methodology, M.T. and B.M.; data curation, M.T. and B.M.; writing—original draft preparation, S.P.A.; writing—review and editing, R.P. and S.P.A. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: Manuel Iglesias and Raquel Costa are acknowledged for their great technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Tilami, S.K.; Sampels, S. Nutritional value of fish: Lipids, proteins, vitamins, and minerals. *Rev. Fish. Sci.* **2018**, *26*, 242–253.
2. Minihane, A.; Armah, C.; Miles, E.; Madden, J.; Clark, A.; Caslake, M.; Calder, P. Consumption of fish oil providing amounts of eicosapentaenoic acid and docosahexaenoic acid that can be obtained from the diet reduces blood pressure in adults with systolic hypertension: A retrospective analysis. *J. Nutr.* **2016**, *146*, 516–523. [CrossRef] [PubMed]
3. Singer, P.; Richter, V.; Singer, K.; Löhlein, I. Analyses and declarations of omega-3 fatty acids in canned seafood may help to quantify their dietary intake. *Nutrients* **2021**, *13*, 2970. [CrossRef]
4. Uauy, R.; Valenzuela, A. Marine oils: The health benefits of *n*-3 fatty acids. *Nutrition* **2000**, *16*, 680–684. [CrossRef]
5. Komprda, T. Eicosapentaenoic and docosahexaenoic acids as inflammation-modulating and lipid homeostasis influencing nutraceuticals: A review. *J. Funct. Foods* **2012**, *4*, 25–38. [CrossRef]
6. Simopoulos, A.P. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. Pharm.* **2002**, *56*, 365–379. [CrossRef]
7. Sikorski, Z.; Kolakowski, E. Endogenous enzyme activity and seafood quality: Influence of chilling, freezing, and other environmental factors. In *Seafood Enzymes*; Haard, N., Simpson, B., Eds.; Marcel Dekker: New York, NY, USA, 2000; pp. 451–487.
8. Campos, C.; Gliemmo, M.; Aubourg, S.P.; Barros-Velázquez, J. Novel technologies for the preservation of chilled aquatic food products. In *Novel technologies in Food Science. Their Impact on Products, Consumer Trends and Environment*; McElhatton, A., do Amaral, P., Eds.; Springer, Science and Business, Inc.: Heidelberg, Germany, 2012; Chapter 13; pp. 299–324.
9. Aubourg, S.P. Review: Loss of quality during the manufacture of canned fish products. *Food Sci. Technol. Int.* **2001**, *7*, 199–215. [CrossRef]
10. García-Arias, M.T.; Álvarez-Pontes, E.; García-Linares, M.C.; García-Fernández, M.C.; Sánchez-Muniz, F.J. Cooking-freezing-reheating (CFR) of sardine (*Sardina pilchardus*) fillets. Effect of different cooking and reheating procedures on the proximate and fatty acid compositions. *Food Chem.* **2003**, *83*, 349–356. [CrossRef]
11. Tokur, B.; Korkmaz, K. Novel thermal sterilisation technologies in seafood processing. In *Innovative Technologies in Seafood Processing*; Özoğul, Y., Ed.; CRC Press: Boca Raton, FL, USA, 2020; pp. 303–322.
12. Horner, W. Canning fish and fish products. In *Fish Processing Technology*, 2nd ed.; Hall, G., Ed.; Blackie Academic and Professional, Chapman and Hall: London, UK, 1997; pp. 119–159.
13. Pitarch, J.L.; Vilas, C.; de Prada, C.; Palacín, C.G.; Alonso, A.A. Optimal operation of thermal processing of canned tuna under product variability. *J. Food Eng.* **2021**, *304*, 110594. [CrossRef]
14. FAO Inform. *Fishery and Aquaculture Statistics. Commodities, Yearbook 2019*; Food and Agriculture Organization of the United Nations: Rome, Italy, 2021; p. 41.
15. Banga, J.; Alonso, A.; Gallardo, J.M.; Pérez-Martín, R. Mathematical modelling and simulation of the thermal processing of anisotropic and non-homogeneous conduction-heated canned foods: Application to canned tuna. *J. Food Eng.* **1993**, *18*, 369–387. [CrossRef]
16. Lukoshkina, M.; Odoeva, G. Kinetics of chemical reactions for prediction of quality of canned fish during storage. *App. Bio-chem. Microb.* **2003**, *39*, 321–327. [CrossRef]
17. Naseri, M.; Rezaei, M. Lipid changes during long-term storage of canned sprat. *J. Aquat. Food Prod. Technol.* **2012**, *21*, 48–58. [CrossRef]
18. Evangelista, W.P.; Silva, T.M.; Guidi, L.R.; Tette, P.A.S.; Byrro, R.M.D.; Santiago-Silva, P.; Fernandes, C.; Gloria, M.B.A. Quality assurance of histamine analysis in fresh and canned fish. *Food Chem.* **2016**, *211*, 100–106. [CrossRef] [PubMed]
19. Lazos, E.S. Freshwater nase (*Chondrostoma nasus*): Thermally processed as a potential food resource. *J. Aquat. Food Prod. Technol.* **1997**, *6*, 45–63. [CrossRef]
20. Mohan, C.O.; Remya, S.; Ravishankar, C.N.; Vijayan, P.K.; Srinivasa Gopal, T.K. Effect of filling ingredient on the quality of canned yellowfin tuna (*Thunnus albacares*). *Int. J. Food Sci. Technol.* **2014**, *49*, 1557–1564. [CrossRef]
21. Castrillón, A.; Navarro, P.; García-Arias, M. Tuna protein nutritional quality changes after canning. *J. Food Sci.* **1996**, *61*, 1250–1253. [CrossRef]
22. Gómez-Limia, L.; Franco, I.; Martínez-Suárez, S. Effects of processing step, filling medium and storage on amino acid profiles and protein quality in canned European eels. *J. Food Comp. Anal.* **2021**, *96*, 103710. [CrossRef]
23. Nazari, F.; Goli, M. The effect of replacing oil with water and NaCl with KCl on soybean oil hydrolysis and oxidation in canned skipjack tuna fish at the end of the 18-month shelf life. *Food Sci. Biotechnol.* **2017**, *26*, 49–53. [CrossRef]
24. Lazarini, T.E.M.; Milani, R.F.; Yamashita, D.M.; Saron, E.S.; Morgano, M.A. Canned sardines commercialized in Brazil: Packaging and inorganic contaminants evaluation. *Food Pack. Shelf Life* **2019**, *21*, 100372. [CrossRef]
25. Naseri, M.; Rezaei, M.; Moieni, S.; Hosseini, H.; Eskandari, S. Effects of different filling media on the oxidation and lipid quality of canned silver carp (*Hypophthalmichthys molitrix*). *Int. J. Food Sci.* **2011**, *46*, 1149–1156. [CrossRef]
26. Barbosa, R.G.; Trigo, M.; Campos, C.A.; Aubourg, S.P. Preservative effect of algae extracts on lipid composition and rancidity development in brine–canned Atlantic chub mackerel (*Scomber colias*). *Eur. J. Lipid Sci. Technol.* **2019**, *121*, 1900129. [CrossRef]

27. Ruiz-Roso, B.; Cuesta, I.; Pérez, M.; Borrego, E.; Pérez-Olleros, L.; Varela, G. Lipid composition and palatability of canned sardines. Influence of the canning process and storage in olive oil for five years. *J. Sci. Food Agric.* **1998**, *77*, 244–250. [CrossRef]
28. Tarley, C.R.T.; Visentainer, J.V.; Matsushita, M.; de Souza, N.E. Proximate composition, cholesterol and fatty acids profile of canned sardines (*Sardinella brasiliensis*) in soybean oil and tomato sauce. *Food Chem.* **2004**, *88*, 1–6. [CrossRef]
29. Prego, R.; Fidalgo, L.G.; Saraiva, J.A.; Vázquez, M.; Aubourg, S.P. Impact of prior high-pressure processing on lipid damage and volatile amines formation in mackerel muscle subjected to frozen storage and canning. *LWT-Food Sci. Technol.* **2021**, *135*, 109957. [CrossRef]
30. Reblová, Z.; Aubourg, S.P.; Pokorný, J. The effect of different freshness of raw material on lipid quality and sensory acceptance of canned sardines. *Foods* **2022**, *11*, 1987. [CrossRef]
31. Saito, H.; Yamashiro, R.; Ishihara, K.; Xue, C. Lipids of three highly migratory fishes: *Euthynnus affinis*, *Sarda orientalis*, and *Elagatis bipinnulata*. *Biosci. Bitechnol. Biochem.* **1999**, *63*, 2028–2030. [CrossRef]
32. Aubourg, S.P.; Rodríguez, A.; Gallardo, J.M. Rancidity development during mackerel (*Scomber scombrus*) frozen storage: Effect of catching season and commercial presentation. *Eur. J. Lipid Sci. Technol.* **2005**, *107*, 316–323. [CrossRef]
33. Pirini, M.; Testi, S.; Ventrella, V.; Pagliarani, A.; Badiani, A. Blue-back fish: Fatty acid profile in selected seasons and retention upon baking. *Food Chem.* **2010**, *123*, 306–314. [CrossRef]
34. Sikorski, Z.; Kolakowska, A. Changes in protein in frozen stored fish. In *Seafood Proteins*; Sikorski, Z., Sun Pan, B., Shahidi., F., Eds.; Chapman and Hall: New York, NY, USA, 1994; pp. 99–112.
35. Malga, J.M.; Trigo, M.; Martínez, B.; Aubourg, S.P. Preservative effect on canned mackerel (*Scomber colias*) lipids by addition of octopus (*Octopus vulgaris*) cooking liquor in the packaging medium. *Molecules* **2022**, *27*, 739. [CrossRef]
36. García-Arias, M.T.; Sánchez-Muniz, F.J.; Castrillón, A.M.; Navarro, M.P. White tuna canning, total fat, and fatty acid changes during processing and storage. *J. Food Comp. Anal.* **1994**, *7*, 119–130. [CrossRef]
37. Aubourg, S.P.; Medina, I.; Gallardo, J.M.; Pérez-Martín, R. Effect of oil and brine canning and storage on Little Tunny (*Euthynnus allettteratus*) lipids. *Grasas Aceites* **1995**, *46*, 77–84. [CrossRef]
38. Mohan, C.O.; Remya, S.; Murthy, L.N.; Ravishankar, C.N.; Kumar, K.A. Effect of filling medium on cooking time and quality of canned yellowfin tuna (*Thunnus albacares*). *Food Cont.* **2015**, *50*, 320–327. [CrossRef]
39. Selmi, S.; Monser, L.; Sadok, S. The influence of local canning process and storage on pelagic fish from Tunisia: Fatty acids profile and quality indicators. *J. Food Proc. Preserv.* **2008**, *32*, 443–457. [CrossRef]
40. Rodríguez, A.; Carriles, N.; Gallardo, J.M.; Aubourg, S.P. Chemical changes during farmed Coho salmon (*Oncorhynchus kisutch*) canning: Effect of a preliminary chilled storage. *Food Chem.* **2009**, *112*, 362–368. [CrossRef]
41. Aubourg, S.P.; Medina, I. Quality differences assessment in canned sardine (*Sardina pilchardus*) by fluorescence detection. *J. Agric. Food Chem.* **1997**, *45*, 3617–3621. [CrossRef]
42. Barbosa, R.G.; Trigo, M.; Fett, R.; Aubourg, S.P. Impact of a packing medium with alga *Bifurcaria bifurcata* extract on canned Atlantic mackerel (*Scomber scombrus*) quality. *J. Sci. Food Agric.* **2018**, *98*, 3462–3467. [CrossRef] [PubMed]
43. Méndez, L.; Sacchi, R.; Medina, I.; Aubourg, S.P. Nutritional and preservative properties of polyphenol-rich olive oil: Effect on seafood processing and storage. In *Lipid Oxidation in Food and Biological Systems*; Bravo-Díaz, C., Ed.; Springer Nature Switzerland AG: Cham, Switzerland, 2022; pp. 455–477.
44. Gómez-Limia, L.; Cobas, N.; Franco, I.; Martínez-Suárez, S. Fatty acid profiles and lipid quality indices in canned European eels: Effects of processing steps, filling medium and storage. *Food Res. Int.* **2020**, *136*, 109601. [CrossRef]
45. AOAC. *Official Methods for Analysis of the Association of Analytical Chemistry*, 15th ed.; Association of Official Chemists, Inc.: Arlington, VA, USA, 1990; pp. 931–937.
46. Bligh, E.; Dyer, W. A rapid method of total extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917. [CrossRef]
47. Herbes, S.E.; Allen, C.P. Lipid quantification of freshwater invertebrates: Method modification for microquantitation. *Can. J. Fish. Aquat. Sci.* **1983**, *40*, 1315–1317. [CrossRef]
48. Christie, W.W. *Gas Chromatography of Lipids: A Practical Guide*; The Oily Press: Ayr, UK; Scotland, UK, 1989; pp. 87–134.

marine drugs

MDPI

Article

LASSO Regression with Multiple Imputations for the Selection of Key Variables Affecting the Fatty Acid Profile of *Nannochloropsis oculata*

Vasilis Andriopoulos [1,2] and Michael Kornaros [1,2,*]

[1] Laboratory of Biochemical Engineering & Environmental Technology (LBEET), Department of Chemical Engineering, University of Patras, 26504 Patras, Greece; billandri@upatras.gr
[2] Institute of Circular Economy and Environment (ICEE), University of Patras' Research and Development Center, 26504 Patras, Greece
* Correspondence: kornaros@chemeng.upatras.gr

Abstract: The marine microalga *Nannochloropsis oculata* has garnered significant interest as a potential source of lipids, both for biofuel and nutrition, containing significant amounts of C16:0, C16:1, and C20:5, n-3 (EPA) fatty acids (FA). Growth parameters such as temperature, pH, light intensity, and nutrient availability play a crucial role in the fatty acid profile of microalgae, with *N. oculata* being no exception. This study aims to identify key variables for the FA profile of *N. oculata* grown autotrophically. To that end, the most relevant literature data were gathered and combined with our previous work as well as with novel experimental data, with 121 observations in total. The examined variables were the percentages of C14:0, C16:0, C16:1, C18:1, C18:2, and C20:5, n-3 in total FAs, their respective ratios to C16:0, and the respective content of biomass in those fatty acids in terms of ash free dry weight. Many potential predictor variables were collected, while dummy variables were introduced to account for bias in the measured variables originating from different authors as well as for other parameters. The method of multiple imputations was chosen to handle missing data, with limits based on the literature and model-based estimation, such as using the software PHREEQC and residual modelling for the estimation of pH. To eliminate unimportant predictor variables, LASSO (Least Absolute Shrinkage and Selection Operator) regression analysis with a novel definition of optimal lambda was employed. LASSO regression identified the most relevant predictors while minimizing the risk of overfitting the model. Subsequently, stepwise linear regression with interaction terms was used to further study the effects of the selected predictors. After two rounds of regression, sparse refined models were acquired, and their coefficients were evaluated based on significance. Our analysis confirms well-known effects, such as that of temperature, and it uncovers novel unreported effects of aeration, calcium, magnesium, and manganese. Of special interest is the negative effect of aeration on polyunsaturated fatty acids (PUFAs), which is possibly related to the enzymatic kinetics of fatty acid desaturation under increased oxygen concentration. These findings contribute to the optimization of the fatty acid profile of *N. oculata* for different purposes, such as production of, high in PUFAs, food or feed, or production of, high in saturated and monounsaturated FA methyl esters (FAME), biofuels.

Keywords: de novo synthesis; heavy metals; lipid induction; nitrogen starvation; oxygen; parametric methods; phosphate starvation; polar lipids; statistical analysis; TAG

Citation: Andriopoulos, V.; Kornaros, M. LASSO Regression with Multiple Imputations for the Selection of Key Variables Affecting the Fatty Acid Profile of *Nannochloropsis oculata*. *Mar. Drugs* **2023**, *21*, 483. https://doi.org/10.3390/md21090483

Academic Editor: Ricardo Calado

Received: 6 August 2023
Revised: 28 August 2023
Accepted: 31 August 2023
Published: 2 September 2023

1. Introduction

Nannochloropsis oculata has emerged as a promising microalga for the production of high-value biomolecules, particularly long-chain polyunsaturated fatty acids (LC-PUFA), such as C20:5, n-3 or eicosapentaenoic acid (EPA) [1,2]. EPA, as a key component of the fatty acid (FA) profile, plays a vital role in human health and nutrition, offering benefits such as

cognitive enhancement, mental health improvement, and cardiovascular protection [3]. Additionally, under stressful conditions, such as nitrogen limitation, *N. oculata* can accumulate large amounts of saturated FAs, primarily C16:0 or palmitic acid, and monounsaturated FAs like C16:1 or palmitoleic acid, both of which are suitable for biodiesel production [4]. Therefore, adjustment of the cultivation conditions according to the end goal is critical for industrial scale production.

The FA composition of *N. oculata* can be affected by various factors, including temperature, light intensity, pH, and the type of reactor used for cultivation [1,5,6]. Temperature plays a significant role in the FA composition of *N. oculata*, with total lipid content increasing with temperature, while polyunsaturated fatty acid (PUFA) levels decrease and saturated fatty acid (SFA) levels increase [5]. Optimal growth and total lipid productivity for *N. oculata* have been reported at 20–25 °C, with the highest EPA production achieved between 14–20 °C [5,7].

Light intensity is another factor that can influence the FA profile of *N. oculata*. Su et al. reported that irradiance of 500 µmol photons m^{-2} s^{-1} resulted in the highest total lipid productivity [8]. In another study, Martínez-Macias et al. found that saturating light intensity reduced the levels of SFAs and increased the combined levels of monounsaturated fatty acids (MUFAs) and PUFAs, although the variation was marginal [6]. Additionally, the type of reactor used for cultivation had a great impact on the FA profile, as demonstrated by the same authors, who observed a decrease in PUFAs (20:5n-3) and (20:4n-3) when using a tubular reactor instead of an Erlenmeyer flask [6].

The pH and light period during cultivation have also been shown to impact the FA composition and antioxidant capacity of *N. oculata* [1]. The study revealed that optimal conditions for productivity and biomass composition varied, and that further investigation is necessary to better understand the effects of these variables on the FA profile. Additionally, a plethora of other factors including metal concentration [9], CO_2 levels [10], agitation [11], light color [12], and magnetic fields [13] have a demonstrated effect on the fatty acid content of *Nannochloropsis* and other microalgae. Evaluating the effects of these factors on the FA composition of *N. oculata* is crucial for optimizing its cultivation conditions and maximizing its potential as a source of biofuels and high-value biomolecules.

In the era of big data, the challenge of variable selection from large numbers of variables, often exceeding the number of observations, is a pressing issue in many scientific fields. Parametric methods, such as the Least Absolute Shrinkage and Selection Operator (LASSO) regression, have emerged as powerful tools to address this challenge [14]. LASSO regression, a type of linear regression that uses shrinkage, is particularly adept at handling high-dimensional data, as it performs both variable selection and regularization to enhance the prediction accuracy and the interpretability of the statistical model it produces. The LASSO algorithm's underlying principle revolves around striking a balance between the complexity of the model and its predictive capability. By adding a penalty term proportional to the absolute values of the regression coefficients, LASSO effectively encourages the reduction of less impactful coefficients to exactly zero. This property not only aids in selecting relevant variables but also guards against overfitting, a common concern in situations where the number of variables surpasses the available observations.

The versatility of LASSO finds application in a multitude of scientific domains. In the realm of genomics, LASSO aids in discerning the genetic markers that are truly relevant to certain traits or diseases from the noise in high-throughput genetic data [15]. In economics, it assists in untangling the intricate relationships between numerous economic indicators, contributing to more accurate predictive models [16]. Additionally, LASSO's utility extends to fields like climate science, where it aids in identifying the pivotal climatic variables driving specific environmental phenomena [17].

However, real-world datasets often contain missing values, which can introduce bias, reduce the efficiency of the statistical analyses, and lead to a loss of information. Multiple imputations, a flexible and widely applicable type of approach, have been proposed to handle missing data [18]. This method replaces each missing value with a set of plausible

values to generate multiple complete datasets. The variability between these imputations reflects the uncertainty about the right value to impute. Pooling methods such as Rubin's rule are then used to combine the results obtained from each of these datasets, providing a single estimate and confidence interval that incorporate both within-imputation and between-imputation variability, thus yielding valid statistical inferences that properly reflect the uncertainty due to missing values [18].

The goal of the current study is to identify the key growth variables influencing the FA profile of *N. oculata* using data obtained from different experiments conducted under various conditions and reactor types, as well as the relevant literature data. To comprehensively analyze the relationships between growth variables and the FA profile of *N. oculata*, we employed LASSO regression analysis and stepwise linear regression, inspired by the recently developed High Dimensional Selection with Interactions (HDSI) algorithm, which aims to incorporate interaction terms in LASSO regression [19]. Stepwise linear regression is a computationally intensive but powerful method for creating sparse models. The combination of LASSO regression with stepwise regression should provide a thorough examination of the complex relationships between growth variables and the FA profile of *N. oculata*, setting the stage for future optimization efforts. By employing this specific combination of advanced regression techniques, we aim to provide valuable insights into the modulation of *N. oculata* FA profile for different purposes, such as the production of high-value PUFA-rich biomass, suitable for food or feed, or rich in saturated and monounsaturated FAs biomass, with a lipid profile suitable for biofuels.

2. Results

The methodology followed is presented in Figure 1 and extensively discussed in the Materials and Methods section. Briefly, original data were combined with data from the literature. Additional variables were added to account for data origin, nitrogen source, and the temporal profile of selected variables. Upper and lower limits were chosen for missing data using in-house developed models and educated assumptions. LASSO analysis with multiple imputations for the missing data was performed to reduce the variables to only a fraction of their initial number (115) in order to assist the subsequent selection of significant interactions with stepwise linear regression. In total, two rounds of LASSO regression and two rounds of stepwise regression were performed. Refined sparse models were finally acquired with pooling of multiple coefficients with Rubin's rule. A full list of the variables included in the analysis, with their descriptions and coded names, is provided in the Supplementary Materials (S1).

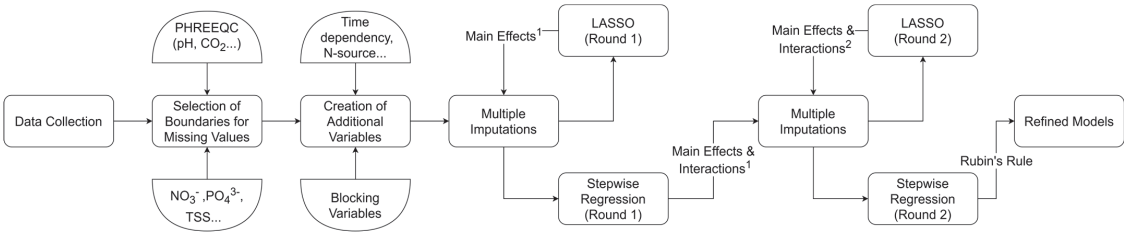


Figure 1. Methodology followed during the analysis presented in this article.

2.1. First Round of LASSO Regression

The results of the first round of LASSO regression (Tables 1–3) are presented in the paragraphs below.

For the percentage of C14:0 in total FAs (C14:0) (Table 1), its ratio to C16:0 (C14:0/C16:0), and its percentage in the biomass in terms of ash free dry weight (AFDW) (C14:0%AFDW), the selected variables were potassium concentration (cK), phosphate concentration (cPO4), CO_2 partial pressure (pCO2), 4-day average CO_2 partial pressure (pCO2_4DaysAv), temperature (T), 2-day average aeration rate in volumes of air per working volume per minute

or vvm (VVM_2DaysAv), 4-day average dissolved CO_2 concentration (CO2aq_4DaysAv), light intensity (LI), and 4-day average photon flux per volume (LV_4DaysAv). All of these variables had a positive effect, except for VVM_2DayAv and LV_4DaysAv.

Table 1. Results of the 1st round of LASSO regression for C14:0, C16:0, C16:1, C18:1, C18:2 and C20:5n3.

Variable *	C14:0	C16:0	C16:1	C18:1	C18:2	C20:5n3
cBOH3		−				+
cCa					−	
cCu				+		
cFe			+	−		
cK			−			
cMn			+	+	+	
cMg			+			
N-source NO3		+			−	
N-source Urea					+	
cNO3					+	
cNO3_2DaysAv				−		
cNO3_3DaysAv						+
cUrea_2DaysAv				−		
cUrea_4DaysAv					+	
cNa		−				
cNa_3DaysAv				+		
cPO4	+					
cPO4_4DaysAv					+	
cSO4						
cSi						+
T	+	+				−
T_2DaysAv						
T_3DaysAv			−			
T_4DaysAv					+	
Aeration		+	+	+	−	−
pH		+				
pH_4DaysAv		+				
CO2aq_4DaysAv						−
HCO3_2DaysAv			−			
LI		+				−
LV			−		+	
LV_3DaysAv				+		
LV_4DaysAv	−					
LP		+	+	+		−

* Positive signs indicate positive LASSO coefficients, whereas negative signs indicate negative LASSO coefficients.

Table 2. Results of the first round of LASSO regression for C14:0/C16:0, C16:1/C16:0, C18:1/C16:0, C18:2/C16:0, and C20:5n3/C16:0.

Variable *	C14:0/C16:0	C16:1/C16:0	C18:1/C16:0	C18:2/C16:0	C20:5n3/C16:0
cBOH3					+
cMn				+	
cMg		+			
cNO3_3DaysAv					+
cNa_4DaysAv					+
cPO4_4DaysAv				+	
cSi		+			+
S					+
T		+			−
Aeration					−
LI					−
LV		+			
LV_4DaysAv			+		
LP					−

* Positive signs indicate positive LASSO coefficients, whereas negative signs indicate negative LASSO coefficients.

Table 3. Results of the first round of LASSO regression for C14:0%AFDW, C16:0%AFDW, C16:1%AFDW, C18:1%AFDW, C18:2%AFDW, and C20:5n3%AFDW.

Variable *,**	C14:0%AFDW	C16:0%AFDW	C16:1%AFDW	C18:1%AFDW	C18:2%AFDW	C20:5n3%AFDW
cBOH3		−				+
cCo		−				
cCu					+	
cFe			+			
cK	+	+	+			
cMn					+	
N-source Urea					+	
cNO3_3DaysAv		−	+			
cNO3_4DaysAv				−		
cUrea_2DaysAv		−		−		
totalN_4DaysAv			+			
cPO4_4DaysAv		−	+	−		−
cSO4		−				
pCO2	+					
pCO2_2DaysAv				+		
pCO2_3DaysAv		+				
pCO2_4DaysAv	+		+			
T						−
T_3DaysAv		+				
Aeration		+	+	+		−
VVM						

Table 3. *Cont.*

Variable *,**	C14:0%AFDW	C16:0%AFDW	C16:1%AFDW	C18:1%AFDW	C18:2%AFDW	C20:5n3%AFDW
VVM_2DaysAv	−	−				
pH_2DaysAv					−	
pH_4DaysAv			+			
CO2aq_2DaysAv				+		
CO2aq_4DaysAv	+	+	+			
LI	+	+	+	+		
LV_4DaysAv				+	+	
LP		+				
DW_2DaysAv					+	
DW_4DaysAv		+	+	+		

* Positive signs indicate positive LASSO coefficients, whereas negative signs indicate negative LASSO coefficients; ** AFDW: Ash Free Dry Weight.

For the percentage of C16:0 in total FAs (C16:0) and its percentage in the biomass in terms of ash free dry weight (C16:0%AFDW), the selected variables were boron concentration (cBOH3), cobalt concentration (cCo), cK, nitrate as a nitrogen source (N-source NO3), 3-day average nitrate concentration (cNO3_3DaysAv), 2-day average urea concentration (cUrea_2DaysAv), sodium concentration (cNa), 4-day average phosphate concentration (cPO4_4DaysAv), sulfate concentration (cSO4), 3-day average CO_2 partial pressure (pCO2_3DaysAv), T, 3-day average temperature (T_3DaysAv), the presence of aeration (Aeration), VVM_2DaysAv, pH (pH), 4-day average pH (pH_4_DaysAv), CO2aq_4DaysAv, LI, light period (LP), and 4-day average biomass concentration in terms of ash free dry weight (DW_4DaysAv). cBOH3, cCo, NO3_3DaysAv, Urea_2DaysAv, cNa, cPO4_4DaysAv, cSO4 andVVM_2DaysAv had a negative effect, while the rest of the variables displayed a positive effect.

For the percentage of C16:1 in total FAs (C16:1), its ratio to C16:0 (C16:1/C16:0), and its percentage in the biomass in terms of ash free dry weight (C16:1%AFDW), the selected variables were iron concentration (cFe), cK, manganese concentration (cMn), magnesium concentration (cMg), cNO3_3DaysAv, 4-day total nitrogen concentration (totalN_4DaysAv), cPO4_4DaysAv, cSO4, silicon concentration (cSi), pCO2_4DaysAv, T, T_3DaysAv, Aeration, pH_4_DaysAv, CO2aq_4DaysAv, 2-day average dissolved bicarbonate concentration (HCO3_2DaysAv), LI, photon flux per volume (LV), LP, and DW_4DaysAv. All of these variables had a positive effect, except for cK, T_3DaysAv and HCO3_2DaysAv.

For the percentage of C18:1 in total FAs (C18:1), its ratio to C16:0 (C18:1/C16:0), and its percentage in the biomass in terms of ash free dry weight (C18:1%AFDW), the selected variables were copper concentration (cCu), cFe, cMn, 2-day average nitrate concentration (cNO3_2DaysAv), 4-day average nitrate concentration (cNO3_4DaysAv), Urea_2DaysAv, 3-day average sodium concentration (cNa_3DaysAv), 4-day average phosphate concentration (cPO4_4DaysAv), 2-day average CO_2 partial pressure (pCO2_2DaysAv), Aeration, 2-day average dissolved CO_2 concentration (CO2aq_2DaysAv), LI, 3-day average photon flux per volume (LV_3DaysAv), LV_4DaysAv, LP, and DW_4DaysAv. cFe, NO3_2DasAv, NO3_4DasAv, Urea_2DaysAv, and cPO4_dDaysAv had a negative effect, while the rest of the variables displayed a positive effect.

For the percentage of C18:2 1 in total FAs (C18:2), its ratio to C16:0 (C18:2/C16:0), and its percentage in the biomass in terms of ash free dry weight (C18:2%AFDW), the selected variables were calcium concentration (cCa), cCu, cMn, NsourceNO3, urea as a nitrogen source (NsourceUrea), nitrate concentration (cNO3), 4-day average urea concentration (cUrea_4DaysAv), cPO4_4DaysAv, 4-day average temperature (T_4DaysAv), Aeration, 2-day average pH (pH_2DaysAv), LV, LV_4DaysAv, and 2-day average biomass concentration

average biomass concentration in terms of ash free dry weight (DW_2DaysAv). All of these variables had a positive effect, except for cCa, NsourceNO3, Aeration, and pH_2DaysAv, which had a negative effect.

For the percentage of C20:5, n-3 in total FAs (C20:5n3), its ratio to C16:0 (C20:5n3/C16:0), and its percentage in the biomass in terms of ash free dry weight (C20:5n3%AFDW), the selected variables were cBOH3, cNO3_3DaysAv, 4-day average sodium concentration (cNa_4DaysAv), cPO4_4DaysAv, cSi, salinity (S), T, Aeration, CO2aq_4DaysAv, LI, and LP. T, Aeration, CO2aq_4DaysAv, LI, and LP had a negative effect, while the rest of the variables showed a positive effect.

In summary, the 1st round of LASSO regression removed most of the initial variables, with the remaining variables being related to well-established growth parameters, such as temperature, pH, CO_2 partial pressure, light intensity or nutrient concentration or less studied parameters such as metal concentration and light period. One variable that emerged as important is the presence of aeration, which exerted a negative effect on both C18:2 and C20:5, n-3. The full dataset of results of the 1st LASSO regression round is provided in the Supplementary Materials (S2).

2.2. First Round of Stepwise Regression

During the first round of stepwise regression, the variables that were selected and presented above were fitted to linear models with interactions in a stepwise manner. The algorithm starts from a model including all the main effects and interactions and excludes or adds terms one at a time. As the process continues, only the most significant terms remain. It is a computationally intensive process, but it is a powerful method that can handle multicollinearity better than simple linear regression and was thus preferred. It is also similar to the bootstrapping method used by Jain and Xu in their HDSI algorithm [19]. The first round of stepwise regression was only an intermediate stage, therefore its results are provided in the Supplementary Materials (S3).

2.3. 2nd Rounds of LASSO and Stepwise Regression

The second round of LASSO regression included the main effects and interactions present in the models resulting from the first round of stepwise regression. This resulted in a reduced number of main effects and interactions. The main effects that were present in the models, either individually or as part of an interaction, were included in the second and final round of stepwise regression. However, only the most notable interactions (as described in the Materials and Methods section) were included to prevent overfitting. The second round of stepwise regression further reduced the number of terms resulting in sparse interpretable models. Only the results for the final models (Tables 4–9) are presented below (and the full dataset in Supplementary Materials S3), while the results of the second round of LASSO are provided in the Supplementary Materials (S2).

C14:0 was notably influenced by T, exhibiting a positive effect (mean p-value < 0.05). A secondary factor (trimmed p-value < 0.05) was the positive interaction of T with cPO4. cPO4 also contributed to the model with a positive coefficient, albeit with a p-value exceeding 0.05.

Conversely, cPO4 was absent from the model for C14:0/C16:0, but its interaction with cK was notable (p-value < 0.05) with a positive coefficient. The only other term in this model, besides the intercept, was cK, which also had a positive coefficient.

The model for C14:0%AFDW was primarily influenced by variables related to aeration rate and CO_2 supply. Both pCO2_4DaysAv and VVM_2DaysAv exhibited notable negative effects (mean p-value < 0.05), while their interaction had a notable positive effect. CO2aq_4DaysAv and its interaction with pCO2_4DaysAv also had a negative effect (p-value >> 0.05).

Table 4. Stepwise regression results for the C14:0 fatty acid percentage, its ratio to C16:0 and its percentage in biomass in terms of AFDW.

C14:0			C14:0/C16:0			C14:0%AFDW		
	Estimate *	***p*-value** **		**Estimate** *	***p*-Value** **		**Estimate** *	***p*-Value** **
(Intercept)	$2.0 \times 10^{0} \pm 5.4 \times 10^{-1}$	**0.028 ± 0.018** †	(Intercept)	$1.4 \times 10^{-1} \pm 9.6 \times 10^{-2}$	0.133 ± 0.271 †	(Intercept)	$1.1 \times 10^{0} \pm 4.0 \times 10^{-2}$	**0.000 ± 0.000** †
cPO4	$8.5 \times 10^{2} \pm 3.6 \times 10^{3}$	0.943 ± 0.231	cK	$4.4 \times 10^{0} \pm 9.4 \times 10^{0}$	0.811 ± 0.383	pCO2_4DaysAv	$-4.1 \times 10^{0} \pm 1.1 \times 10^{0}$	**0.030 ± 0.150** †
T	$1.6 \times 10^{-1} \pm 4.6 \times 10^{-2}$	**0.001 ± 0.002** †	cK:cPO4	$9.6 \times 10^{4} \pm 1.2 \times 10^{4}$	**0.000 ± 0.000** †	VVM_2DaysAv	$-7.2 \times 10^{-2} \pm 8.8 \times 10^{-3}$	**0.000 ± 0.000** †
cPO4:T	$5.6 \times 10^{2} \pm 2.4 \times 10^{2}$	0.061 ± 0.230 †				CO2aq_4DaysAv	$-6.2 \times 10^{0} \pm 4.6 \times 10^{1}$	0.981 ± 0.136
						pCO2_4DaysAv:VVM_2DaysAv	$2.2 \times 10^{1} \pm 3.4 \times 10^{0}$	**0.000 ± 0.000** †
						pCO2_4DaysAv:CO2aq_4DaysAv	$-1.1 \times 10^{1} \pm 2.8 \times 10^{2}$	0.883 ± 0.317
R^2 ***	0.32 ± 0.02		R^2 ***	0.2 ± 0.03		R^2 ***	0.52 ± 0.01	

* Pooled standard deviation (Rubin's rule); ** Bold values indicate mean *p*-value value below 0.05, while the dagger symbol (†) indicates trimmed mean (80%) of *p*-value below 0.05; *** Adjusted values.

Table 5. Stepwise regression results for the C16:0 fatty acid percentage and its percentage in biomass in terms of AFDW.

C16:0			C16:0%AFDW		
	Estimate *	***p*-Value** **		**Estimate** *	***p*-Value** **
(Intercept)	$-6.9 \times 10^{0} \pm 7.5 \times 10^{0}$	0.197 ± 0.250	(Intercept)	$-8.0 \times 10^{0} \pm 3.6 \times 10^{0}$	0.118 ± 0.205 †
cK	$1.0 \times 10^{3} \pm 1.2 \times 10^{3}$	0.562 ± 0.494	cK	$1.4 \times 10^{3} \pm 2.4 \times 10^{2}$	**0.004 ± 0.058** †
NsourceNO3	$3.2 \times 10^{0} \pm 3.5 \times 10^{0}$	0.520 ± 0.496	NsourceNO3	$2.0 \times 10^{-1} \pm 7.9 \times 10^{-1}$	0.932 ± 0.247
cNO3_3DaysAv	$8.5 \times 10^{1} \pm 5.8 \times 10^{2}$	0.978 ± 0.146	cPO4_3DaysAv	$-2.7 \times 10^{4} \pm 4.2 \times 10^{3}$	**0.000 ± 0.000** †
cNa	$-3.4 \times 10^{0} \pm 6.2 \times 10^{0}$	0.749 ± 0.431	pCO2_3DaysAv	$2.9 \times 10^{-2} \pm 1.6 \times 10^{0}$	0.960 ± 0.194
cPO4_3DaysAv	$-1.2 \times 10^{2} \pm 3.6 \times 10^{3}$	0.990 ± 0.097	Aeration	$1.8 \times 10^{-1} \pm 7.0 \times 10^{-1}$	0.933 ± 0.245
Aeration	$5.4 \times 10^{0} \pm 6.4 \times 10^{0}$	0.553 ± 0.495	VVM_2DaysAv	$-4.5 \times 10^{-1} \pm 7.7 \times 10^{-2}$	**0.000 ± 0.000** †
VVM_2DaysAv	$-6.0 \times 10^{-1} \pm 4.2 \times 10^{-1}$	0.307 ± 0.461 †	pH	$2.1 \times 10^{-2} \pm 1.7 \times 10^{-1}$	0.984 ± 0.124
LI	$-2.6 \times 10^{-4} \pm 2.3 \times 10^{-3}$	0.987 ± 0.110	pH_4DaysAv	$-1.5 \times 10^{-1} \pm 3.4 \times 10^{-1}$	0.792 ± 0.399
LP	$2.3 \times 10^{0} \pm 3.4 \times 10^{0}$	0.673 ± 0.467	CO2aq_4DaysAv	$3.6 \times 10^{1} \pm 1.9 \times 10^{2}$	0.958 ± 0.200
DW_4DaysAv	$-1.7 \times 10^{-2} \pm 1.8 \times 10^{-1}$	0.990 ± 0.096	pCO2_3DaysAv:VVM_2DaysAv	$1.5 \times 10^{1} \pm 6.9 \times 100$	0.057 ± 0.224 †
NsourceNO3:T_3DaysAv	$5.1 \times 10^{-2} \pm 1.1 \times 10^{-1}$	0.824 ± 0.380	Aeration:LI	$2.0 \times 10^{-2} \pm 1.7 \times 10^{-3}$	**0.000 ± 0.000** †
cNO3_3DaysAv:cNa	$-3.2 \times 10^{3} \pm 2.4 \times 10^{3}$	0.263 ± 0.440 †			
cNO3_3DaysAv:VVM_2DaysAv	$-1.0 \times 10^{2} \pm 1.8 \times 10^{2}$	0.737 ± 0.440			
T:pH	$8.0 \times 10^{-2} \pm 1.1 \times 10^{-2}$	**0.000 ± 0.000** †			
Aeration:LI	$5.5 \times 10^{-3} \pm 9.3 \times 10^{-3}$	0.717 ± 0.449			

Table 5. *Cont.*

C16:0			C16:0%AFDW		
	Estimate *	***p*-Value ****		**Estimate ***	***p*-Value ****
Aeration:LP	$5.3 \times 10^{0} \pm 4.6 \times 10^{0}$	0.375 ± 0.483 †			
pH:LI	$1.3 \times 10^{-4} \pm 4.6 \times 10^{-4}$	0.922 ± 0.266			
R^2 ***	0.51 ± 0.00		R^2 ***	0.59 ± 0.01	

* Pooled standard deviation (Rubin's rule); ** Bold values indicate mean *p*-value value below 0.05, while the dagger symbol (†) indicates trimmed mean (80%) of *p*-value below 0.05; *** Adjusted values.

Table 6. Stepwise regression results for the C16:1 fatty acid percentage, its ratio to C16:0, and its percentage in AFDW biomass.

C16:1			C16:1/C16:0			C16:1%AFDW		
	Estimate *	***p*-Value ****		**Estimate ***	***p*-Value ****		**Estimate ***	***p*-Value ****
(Intercept)	$2.8 \times 10^{1} \pm 4.1 \times 10^{-1}$	**0.000 ± 0.000** †	(Intercept)	$1.3 \times 10^{0} \pm 1.7 \times 10^{-1}$	**0.000 ± 0.000** †	(Intercept)	$2.4 \times 10^{1} \pm 3.8 \times 10^{0}$	**0.000 ± 0.000** †
cNO3_3DaysAv	$-2.8 \times 10^{0} \pm 1.6 \times 10^{2}$	0.990 ± 0.098	cMn	$2.6 \times 10^{4} \pm 2.5 \times 10^{4}$	0.430 ± 0.488	cMg	$-1.1 \times 10^{1} \pm 3.5 \times 10^{1}$	0.907 ± 0.284
LV	$-1.8 \times 10^{-6} \pm 3.5 \times 10^{-5}$	0.997 ± 0.056	cMg	$3.4 \times 10^{0} \pm 6.2 \times 10^{0}$	0.767 ± 0.423	cNO3_3DaysAv	$-5.9 \times 10^{3} \pm 1.9 \times 10^{3}$	0.064 ± 0.243 †
cNO3_3DaysAv:LV	$-5.9 \times 10^{-1} \pm 2.0 \times 10^{-1}$	**0.008 ± 0.081** †	cNO3_3DaysAv	$-1.7 \times 10^{1} \pm 3.9 \times 10^{1}$	0.832 ± 0.372	cPO4_4DaysAv	$-1.5 \times 10^{3} \pm 5.7 \times 10^{3}$	0.937 ± 0.244
			totalN_4DaysAv	$-5.1 \times 10^{0} \pm 1.2 \times 10^{1}$	0.771 ± 0.415	cSi	$-8.0 \times 104 \pm 5.5 \times 10^{4}$	0.291 ± 0.435 †
			cPO4_4DaysAv	$2.7 \times 10^{2} \pm 6.8 \times 10^{2}$	0.861 ± 0.345	pH_4DaysAv	$-1.8 \times 10^{0} \pm 3.1 \times 10^{-1}$	**0.000 ± 0.000** †
			cSi	$9.7 \times 10^{3} \pm 5.5 \times 10^{3}$	0.233 ± 0.423 †	LI	$-1.7 \times 10^{-2} \pm 5.7 \times 10^{-3}$	0.063 ± 0.244 †
			pCO2_4DaysAv	$6.1 \times 10^{-4} \pm 1.1 \times 10^{-2}$	0.997 ± 0.055	DW_4DaysAv	$-4.6 \times 10^{-1} \pm 4.5 \times 10^{-1}$	0.483 ± 0.481
			T	$-1.7 \times 10^{-3} \pm 1.3 \times 10^{-2}$	0.964 ± 0.184	cNO3_3DaysAv:pH_4DaysAv	$5.8 \times 10^{2} \pm 2.0 \times 10^{2}$	0.069 ± 0.242 †
			T_3DaysAv	$4.0 \times 10^{-4} \pm 1.1 \times 10^{-2}$	0.948 ± 0.217	cSi:LI	$8.2 \times 10^{2} \pm 1.6 \times 10^{2}$	**0.000 ± 0.000** †
			Aeration	$-8.2 \times 10^{-3} \pm 4.1 \times 10^{-2}$	0.960 ± 0.195	pCO2_4DaysAv:DW_4DaysAv	$2.3 \times 10^{0} \pm 5.2 \times 10^{-1}$	**0.001 ± 0.001** †
			pH_4DaysAv	$6.7 \times 10^{-4} \pm 5.3 \times 10^{-3}$	0.984 ± 0.123			
			CO2aq_4DaysAv	$-6.3 \times 10^{-1} \pm 4.9 \times 10^{0}$	0.981 ± 0.136			
			HCO3_2DaysAv	$2.5 \times 10^{-3} \pm 3.1 \times 10^{-2}$	0.994 ± 0.078			
			cFe:cNO3_3DaysAv	$6.7 \times 10^{5} \pm 1.6 \times 10^{6}$	0.843 ± 0.363			
			cMg:LV	$-4.3 \times 10^{-4} \pm 7.7 \times 10^{-5}$	**0.000 ± 0.000** †			
			cNO3_3DaysAv:cPO4_4DaysAv	$3.7 \times 10^{5} \pm 3.7 \times 10^{5}$	0.449 ± 0.492			
			totalN_4DaysAv:HCO3_2DaysAv	$-3.6 \times 10^{1} \pm 1.0 \times 10^{2}$	0.880 ± 0.320			
			totalN_4DaysAv:LV	$-2.4 \times 10^{-3} \pm 2.8 \times 10^{-3}$	0.504 ± 0.489			
			T:Aeration	$-5.1 \times 10^{-3} \pm 4.9 \times 10^{-3}$	0.465 ± 0.497			
			T:pH_4DaysAv	$-3.9 \times 10^{-3} \pm 5.5 \times 10^{-3}$	0.318 ± 0.464 †			
			T_3DaysAv:pH_4DaysAv	$7.4 \times 10^{-4} \pm 5.7 \times 10^{-3}$	0.510 ± 0.496			
			Aeration:LP	$-7.5 \times 10^{-2} \pm 9.0 \times 10^{-2}$	0.578 ± 0.493			
R^2 ***	0.09 ± 0.01		R^2 ***	0.71 ± 0.00		R^2 ***	0.56 ± 0.02	

* Pooled standard deviation (Rubin's rule); ** Bold values indicate mean *p*-value value below 0.05, while the dagger symbol (†) indicates trimmed mean (80%) of *p*-value below 0.05; *** Adjusted values.

Table 7. Stepwise regression results for the C18:1 fatty acid percentage, its ratio to C16:0, and its percentage in biomass in terms of AFDW.

C18:1			C18:1/C16:0			C18:1%AFDW		
	Estimate *	***p*-Value ****		**Estimate ***	***p*-Value ****		**Estimate ***	***p*-Value ****
(Intercept)	$-2.1 \times 10^{0} \pm 1.4 \times 10^{0}$	0.189 ± 0.115	(Intercept)	$1.4 \times 10^{-2} \pm 5.9 \times 10^{-2}$	0.509 ± 0.321	(Intercept)	$2.7 \times 10{-}1 \pm 2.7 \times 10{-}1$	0.521 ± 0.129
cNO3_2DaysAv	$-5.7 \times 10^{2} \pm 7.2 \times 10^{2}$	0.464 ± 0.498	cNa_3DaysAv	$1.6 \times 10^{-1} \pm 1.4 \times 10^{-1}$	0.404 ± 0.480 †	cNO3_2DaysAv	$-1.9 \times 102 \pm 1.9 \times 102$	0.507 ± 0.499
cNO3_4DaysAv	$-5.0 \times 10^{2} \pm 6.7 \times 10^{2}$	0.527 ± 0.499	CO2aq_2DaysAv	$-3.2 \times 10^{-1} \pm 3.3 \times 10^{0}$	0.990 ± 0.095	cNO3_4DaysAv	$-1.8 \times 102 \pm 1.9 \times 102$	0.494 ± 0.499
cNa_3DaysAv	$8.4 \times 10^{0} \pm 3.2 \times 10^{0}$	**0.036 ± 0.179** †	cNO3_2DaysAv:LI	$-1.8 \times 10^{-1} \pm 1.8 \times 10^{-1}$	0.473 ± 0.499	pCO2_2DaysAv	$8.1 \times 10{-}2 \pm 4.4 \times 10{-}1$	0.964 ± 0.184
cPO4_4DaysAv	$5.1 \times 10^{1} \pm 9.8 \times 10^{2}$	0.997 ± 0.055	cNO3_4DaysAv:LI	$-1.5 \times 10^{-1} \pm 1.6 \times 10^{-1}$	0.527 ± 0.499	Aeration	$1.2 \times 100 \pm 2.9 \times 10{-}1$	**0.016 ± 0.006** †
pCO2_2DaysAv	$6.0 \times 10^{0} \pm 1.6 \times 10^{0}$	**0.000 ± 0.000** †	cNa_3DaysAv:LP	$6.6 \times 10^{-2} \pm 1.0 \times 10^{-1}$	0.685 ± 0.460	CO2aq_2DaysAv	$-1.4 \times 101 \pm 6.4 \times 101$	0.954 ± 0.209
CO2aq_2DaysAv	$-6.6 \times 10^{2} \pm 4.7 \times 10^{2}$	0.192 ± 0.379 †	Aeration:LV_4DaysAv	$1.3 \times 10^{-5} \pm 5.5 \times 10^{-7}$	**0.000 ± 0.000** †	LI	$4.1 \times 10{-}4 \pm 1.3 \times 10{-}3$	0.897 ± 0.303
LI	$-2.3 \times 10^{-5} \pm 4.4 \times 10^{-4}$	0.997 ± 0.057	Aeration:LP	$1.4 \times 10^{-1} \pm 3.2 \times 10^{-2}$	**0.005 ± 0.007** †	LV_4DaysAv	$-2.3 \times 10{-}5 \pm 6.8 \times 10{-}5$	0.897 ± 0.304
LV_3DaysAv	$-3.3 \times 10^{-5} \pm 1.9 \times 10^{-4}$	0.967 ± 0.178				DW_4DaysAv	$-1.0 \times 10{-}3 \pm 1.9 \times 10{-}2$	0.997 ± 0.055
DW_4DaysAv	$1.0 \times 10^{-2} \pm 1.3 \times 10^{-1}$	0.993 ± 0.080				pCO2_2DaysAv:LV_3DaysAv	$5.9 \times 10{-}4 \pm 9.8 \times 10{-}5$	**0.012 ± 0.100** †
cNa_3DaysAv:LV_3DaysAv	$1.0 \times 10^{-4} \pm 4.2 \times 10^{-4}$	0.913 ± 0.281				LV_3DaysAv:DW_4DaysAv	$2.0 \times 10{-}5 \pm 3.9 \times 10{-}5$	0.760 ± 0.427
Aeration:LV_4DaysAv	$3.3 \times 10^{-4} \pm 1.0 \times 10^{-4}$	0.087 ± 0.281 †				LV_4DaysAv:DW_4DaysAv	$4.2 \times 10{-}5 \pm 2.4 \times 10{-}5$	0.240 ± 0.427 †
Aeration:LP	$5.4 \times 10^{0} \pm 1.1 \times 10^{0}$	**0.000 ± 0.000** †						
R^2 ***	0.81 ± 0.00		R^2 ***	0.74 ± 0.00		R^2 ***	0.82 ± 0.00	

* Pooled standard deviation (Rubin's rule); ** Bold values indicate mean *p*-value value below 0.05, while the dagger symbol (†) indicates trimmed mean (80%) of *p*-value below 0.05; *** Adjusted values.

Table 8. Stepwise regression results for the C18:2 fatty acid percentage, its ratio to C16:0 and its percentage in biomass in terms of AFDW.

C18:2			C18:2/C16:0			C18:2%AFDW		
	Estimate *	***p*−Value ****		**Estimate ***	***p*-Value ****		**Estimate ***	***p*-Value ****
(Intercept)	$1.6 \times 10^{1} \pm 7.6 \times 10^{-1}$	**0.000 ± 0.000** †	(Intercept)	$7.4 \times 10^{-1} \pm 4.1 \times 10^{-2}$	**0.000 ± 0.000** †	(Intercept)	$4.4 \times 10^{-1} \pm 1.3 \times 10^{-1}$	0.191 ± 0.392 †
cCa	$-9.3 \times 10^{2} \pm 6.1 \times 10^{1}$	**0.000 ± 0.000** †	cCa	$-4.1 \times 10^{1} \pm 3.4 \times 10^{0}$	**0.000 ± 0.000** †	LV	$-3.2 \times 10^{-5} \pm 1.7 \times 10^{-5}$	0.887 ± 0.310
cPO4_4DaysAv	$3.3 \times 10^{3} \pm 6.7 \times 10^{3}$	0.795 ± 0.398	cMn	$2.0 \times 10^{4} \pm 1.2 \times 10^{4}$	0.270 ± 0.444 †	LV_4DaysAv	$-2.9 \times 10^{-5} \pm 5.0 \times 10^{-5}$	0.706 ± 0.453
Aeration	$-4.5 \times 10^{0} \pm 3.8 \times 10^{-1}$	**0.000 ± 0.000** †	cNO3	$6.2 \times 10^{1} \pm 7.2 \times 10^{0}$	**0.000 ± 0.000** †	DW_2DaysAv	$1.4 \times 10^{-1} \pm 5.6 \times 10^{-2}$	0.099 ± 0.284 †
cMn:T_4DaysAv	$4.4 \times 10^{4} \pm 6.3 \times 10^{3}$	**0.000 ± 0.000** †	cPO4_4DaysAv	$1.2 \times 10^{2} \pm 3.6 \times 10^{2}$	0.890 ± 0.313	cCa:pH_2DaysAv	$-2.7 \times 10^{-2} \pm 4.8 \times 10^{-1}$	0.997 ± 0.055
			Aeration	$-1.8 \times 10^{-}3 \pm 2.2 \times 10^{-2}$	0.993 ± 0.081	cMn:T_4DaysAv	$7.3 \times 10^{3} \pm 7.3 \times 10^{2}$	**0.000 ± 0.000** †
			cMn:T_4DaysAv	$1.7 \times 10^{2} \pm 3.9 \times 10^{2}$	0.837 ± 0.370	cMn:DW_2DaysAv	$2.5 \times 10^{3} \pm 1.0 \times 10^{4}$	0.940 ± 0.232
			Aeration:pH_2DaysAv	$-3.0 \times 10^{-2} \pm 3.2 \times 10^{-3}$	**0.007 ± 0.081** †	LV:DW_2DaysAv	$2.9 \times 10^{-5} \pm 8.2 \times 10^{-6}$	**0.003 ± 0.058** †
						LV_4DaysAv:DW_2DaysAv	$7.4 \times 10^{-6} \pm 1.5 \times 10^{-5}$	0.810 ± 0.392
R^2 ***	0.79 ± 0.00		R^2 ***	0.72 ± 0.01		R^2 ***	0.87 ± 0.02	

* Pooled standard deviation using Rubin's rule; ** Bold values indicate mean *p*−value value equal or below 0.05, while the dagger symbol (†) indicates trimmed mean (80%) of *p*-value equal or below 0.05; *** Adjusted values.

Table 9. Stepwise regression results for the C20:5n-3 fatty acid percentage and its ratio to C16:0.

C20:5n3			C20:5n3/C16:0		
	Estimate *	***p*-Value ****		**Estimate ***	***p*-Value ****
(Intercept)	$5.0 \times 10^{1} \pm 4.3 \times 10^{0}$	**0.000 ± 0.000** †	(Intercept)	$3.0 \times 10^{0} \pm 7.2 \times 10^{-1}$	**0.000 ± 0.000** †
cNO3_3DaysAv	$7.2 \times 10^{2} \pm 1.7 \times 10^{3}$	0.843 ± 0.363	cBOH3	$6.0 \times 10^{1} \pm 2.3 \times 10^{2}$	0.936 ± 0.238
cNa_4DaysAv	$-2.1 \times 10^{-1} \pm 3.9 \times 10^{0}$	0.997 ± 0.058	cNO3_3DaysAv	$-5.4 \times 10^{0} \pm 4.8 \times 10^{1}$	0.987 ± 0.111
S	$3.1 \times 10^{-3} \pm 5.8 \times 10^{-2}$	0.997 ± 0.057	T	$-5.4 \times 10^{-2} \pm 2.7 \times 10^{-2}$	**0.008 ± 0.058** †
T	$-7.4 \times 10^{-1} \pm 1.8 \times 10^{-1}$	**0.001 ± 0.002** †	Aeration	$-7.1 \times 10^{-1} \pm 1.9 \times 10^{-1}$	**0.047 ± 0.211** †
Aeration	$-1.1 \times 10^{1} \pm 3.5 \times 10^{0}$	**0.003 ± 0.001** †	LP	$-8.4 \times 10^{-1} \pm 8.1 \times 10^{-1}$	0.268 ± 0.441 †
LP	$-7.6 \times 10^{0} \pm 4.7 \times 10^{0}$	0.127 ± 0.307 †	cNO3_3DaysAv:cNa_4DaysAv	$-6.5 \times 10^{1} \pm 7.3 \times 10^{2}$	0.369 ± 0.477 †
cNO3_3DaysAv:S	$1.1 \times 10^{2} \pm 5.4 \times 10^{1}$	0.157 ± 0.363 †	cNO3_3DaysAv:S	$9.2 \times 10^{0} \pm 1.1 \times 10^{1}$	0.410 ± 0.492
T:LP	$-2.9 \times 10^{-2} \pm 1.4 \times 10^{-1}$	0.892 ± 0.303	T:LP	$7.0 \times 10^{-3} \pm 3.4 \times 10^{-2}$	0.600 ± 0.480
Aeration:LI	$-1.0 \times 10^{-2} \pm 8.0 \times 10^{-3}$	0.233 ± 0.351	Aeration:LI	$-2.1 \times 10^{-5} \pm 1.3 \times 10^{-4}$	0.975 ± 0.151
			Aeration:LP	$-3.2 \times 10^{-2} \pm 1.5 \times 10^{-1}$	0.953 ± 0.211
R^2 ***	0.42 ± 0.00		R^2 ***	0.42 ± 0.01	

* Pooled standard deviation using Rubin's rule; ** Bold values indicate mean *p*-value value equal or below 0.05, while the dagger symbol (†) indicates trimmed mean (80%) of *p*-value equal or below 0.05; *** Adjusted values.

Parameters with positive mean coefficients in the model for C16:0 included cK, NsourceNO3 and its interaction with T_3DaysAv, NO3_3DaysAv, Aeration, LP, the interactions between pH and both T (p-value < 0.05) and LI, as well as the interactions of Aeration with both LI and LP (trimmed mean p-value < 0.05). Negative influences were observed for cNa, cPO4_3DaysAv, VVM_2DaysAv (trimmed mean p-value < 0.05), LI, DW_4DaysAv, and the interactions of NO3_3DaysAv with both cNa (trimmed mean p-value < 0.05) and VVM_2DaysAv.

For C16:0%AFDW, the positive effect of cK, the negative effect of cPO4_3DaysAv, the negative effect of VVM_2DaysAv, and the positive effect of the interaction between Aeration and LI were notable (mean p-value < 0.05). A secondary factor (trimmed mean p-value < 0.05) was the positive effect of the interaction between pCO2_3DaysAv and VVM_2DaysAv.

The model for C16:1 contained, apart from an intercept, only NO3_3DaysAv and LV, both of which had a negative effect (p >> 0.05), as well as their interaction, which had a notable negative effect (mean p-value < 0.05).

Conversely, the model for C16:1/C16:0 contained the largest number of terms from all models presented in this article, the most notable of which were the negative interaction between cMg and LV (p-value < 0.05), the interaction between T and pH_4DaysAv (trimmed mean p-value < 0.05), which presented a negative effect, and cSi (trimmed mean p-value < 0.05), which had a positive effect. Other terms with a positive effect (p > 0.05) were cMn and cMg, cPO4_4DaysAv and its interaction with NO3_3DaysAv, pCO2_4DaysAv, T_3DaysAv and its interaction with pH_4DaysAv, pH_4DaysAv, HCO3_2DaysAv and the interaction between cFe and NO3_3DaysAv. The remaining terms had a negative effect (p > 0.05) and included totalN_4DaysAv as well as its interactions with both HCO3_2DaysAv and LV, T and its interaction with pH_4DaysAv, CO2aq_4DaysAv and the interaction between Aeration and LP.

C16:1%AFDW was influenced by many of the same parameters as C16:1/C16:0, like cMg and cPO4_4DaysAv, which, in that case, had negative effects (p-value > 0.05). cSi also appears with an opposite effect (negative), which is, akin to the case of C16:1/C16:0, moderately important (trimmed mean p-value < 0.05). NO3_3DaysAv and its interaction with the pH_4DaysAv presented moderately significant effects (trimmed mean p-value < 0.05), negative and positive respectively. pH_4DaysAv had a notable negative effect (mean p-value < 0.05), while the interactions between cSi and LI, and between pCO2_4DaysAv and DW_4DaysAv had strong positive effects (mean p-value < 0.05). LI and the DW_4DaysAv showed negative effects (p-value > 0.05).

The most important (mean p-value < 0.05) terms for C18:1 were NO3_3DaysAv, pCO2_2DaysAv, and the interaction between the Aeration and LP, all of which had a positive effect. The interaction between Aeration and LV_4DaysAv also had a positive effect (trimmed mean p-value < 0.05), while CO2aq_2DaysAv had a negative effect (trimmed mean p-value < 0.05). Other negative influences (p-value > 0.05) were those of NO3_2DaysAv and NO3_3DaysAv, CO2aq_2DaysAv, LI, and LV_3DaysAv. The remaining terms had positive effects (p-value > 0.05) and included cPO4_4DaysAv, DW_4DaysAv and the interaction between can_3DaysAve and LV_3DaysAv.

Aeration dominated the model of C18:1/C16:0 with its positive interactions (mean p-value < 0.05) with both LV_4DaysAv and LP, while cNa_3DaysAv also had a positive effect (trimmed mean p-value < 0.05). The interaction between cNa_3DaysAv and LP had a positive effect (p-value > 0.05), while negative influences (p-value > 0.05) were observed for CO2aq_2DaysAv and the interactions of cNO3_2DaysAv and cNO3_4DaysAv with LI.

Aeration was also important for C18:1%AFDW with a notable positive effect (mean p-value < 0.05), while the interaction of pCO2_2DaysAv with LV_3DaysAv was also notable. The interaction between LV_4DaysAv and DW_4DaysAv was moderately important (trimmed mean p-value < 0.05) with a positive effect. Other terms included in the model were cNO3_2DaysAv and cNO3_4DaysAv, both with a negative effect, LV_4DaysAv and DW_4DaysAv (both with negative effect), pCO2_3DaysAv (positive

effect), CO2aq_2DaysAv (negative effect), LI (positive effect), and the positive interactions of DW_4DaysAv with both LV_3DaysAv and LV_4DaysAv.

The model of C18:2 was primarily influenced by cCa, Aeration, and the interaction between cMn and T_4DaysAv, with the first two having a significant (mean p-value << 0.05) negative effect and the third showing a positive effect (mean p-value << 0.05). cPO4_4DaysAv was also included in the model and had a positive effect (p-value > 0.05).

The negative effects of cCa (mean p-value << 0.05) and the interaction between the Aeration and pH_2DaysAv (mean p-value < 0.05), as well as the positive effect of cNO3 (mean p-value << 0.05), were the most important effects in the model for C18:2/C16:0, while cMn had a moderately important positive effect (trimmed mean p-value < 0.05). cPO4_4DaysAv and the interaction between cMn and T_4DaysAv had positive effects, while Aeration had a negative effect, all with p-value larger than 0.05.

The interactions between cMn and T_4DaysAv (mean p-value << 0.05) and between LV and DW_2DaysAv (mean p-value < 0.05), both of which were positive, had the most notable effects on C18:2%AFDW. DW_2DaysAv was included in the model with a moderately positive effect (trimmed mean p-value < 0.05), while its interactions with both LV_4DaysAv and cMn also had a positive effect (p-value > 0.05). LV, LV_4DaysAv and the interaction between cCa and pH2DaysAv had negative effects (p-value > 0.05).

Similar to the case of C18:2, Aeration was a notable term for C20:5n3, with a negative effect (mean p-value < 0.05). The other notable term was T, also with a negative effect. LP had a moderately important negative effect (trimmed mean p-value < 0.05), while the interaction between NO3_3DaysAv and S had a positive effect (trimmed mean p-value < 0.05). NO3_3DaysAv and S had positive effects (p-value > 0.05), while cNa_4DaysAv, LP and its interaction with the T, and the interaction between Aeration and LI all had negative effects (p-value > 0.05).

Aeration and T had the most notable effects on C20:5n3/C16:0, both negative (mean p-value < 0.05), similarly to C20:5n3, while LP and the interaction between NO3_3DaysAv and cNa_4DaysAv had moderately important negative effects (trimmed mean p-value < 0.05). cBOH3, the interaction between NO3_3DaysAv and S, and the interaction between T and LP all had positive effects (p-value > 0.05). On the other hand, NO3_3DaysAv and the interactions of Aeration with LI and LP all had negative effects (p-value > 0.05).

The second rounds of LASSO and stepwise regression, which included the main effects and interactions derived from the initial models, yielded succinct and interpretable results. Aeration emerged as a pivotal factor consistently influencing the fatty acid composition across various species. It exhibited diverse effects, forming positive interactions with specific variables, such as LV_4DaysAv and LP, while also displaying negative interactions with others. This underscores the importance of aeration control in manipulating fatty acid profiles.

Mineral ions, notably calcium, magnesium, and potassium, played a discernible role in determining fatty acid composition. Their effects were evident through main effects as well as interactions, further highlighting their significance in lipid metabolism regulation. Temperature exhibited significant interactions with several parameters, often leading to shifts in fatty acid profiles. This suggests that temperature management could be a valuable strategy for manipulating lipid production in *N. oculata*.

Nitrogen, especially nitrate, emerged as an influential factor affecting fatty acid profiles. Its interactions with other variables, such as LV and pH_4DaysAv, demonstrated the intricate involvement of nitrogen in lipid synthesis pathways. Additionally, CO_2-related variables contributed to the models, indicating the relevance of CO_2 supply in lipid metabolism. The presence of both positive and negative effects underscores the complexity of CO_2's role in fatty acid production.

In conclusion, the refined models resulting from the second rounds of LASSO and stepwise regression emphasized the consistent significance of aeration, ion concentrations, temperature, nitrogen sources, and CO_2-related variables in shaping the fatty acid composition of *N. oculata*. These findings provide valuable insights into the potential manipulation

of lipid profiles for various applications, from biodiesel production to nutritional supplementation. The ensuing discussion will delve into the mechanistic underpinnings of these observed effects, connecting them to broader metabolic pathways and potential implications for bioprocess optimization.

3. Discussion

In the results section, coded names were used for the variables studied in this article. For the ease of readers focusing on the discussion, variables in this section will be addressed with their original names.

3.1. Multi-Level Effects of Temperature

Temperature had a positive effect on C14:0 and C16:0, with increasing temperatures and phosphate concentrations acting synergistically to increase the percentage of C14:0 in total FAs, while temperature seems to enhance the positive effect of increasing pH on C16:0. On the other hand, C16:1 was influenced by temperature in a non-trivial way, with some temperature related terms having a positive effect and others negative. The most significant of them, however, the interaction between temperature and the 4-day average pH, had a negative coefficient. On the other hand, there is a definitive strong negative effect of temperature on C20:5, n-3, both in terms of its percentage in total FAs and its ratio to C16:0. The positive effect of temperature on the accumulation of saturated FAs and its negative effect on PUFAs is well documented in *Nannochloropsis* [1,7,20], while exceptions exist [20]. However, a few remarks on the specific effects of temperature and its interplay with pH and CO_2 concentration can still be made.

K1 and K2 of the CO_2 equilibrium increase with temperature and salinity [21–23]. On the other hand, Henry constant for CO_2 decreases with temperature, which limits CO_2 transfer from the supplied air to the liquid, while salinity also has a negative but less pronounced effect [24,25]. As K1 and K2 increase, the CO_2 equilibrium shifts towards bicarbonate and carbonate, respectively, for a given value of pH. Conversely, for given values of K1 and K2, an increase in pH (and therefore a decrease in proton concentration) will shift the CO_2 equilibrium towards bicarbonate or carbonate, respectively. Microalgae like *Nannochloropsis* utilize carbon concentrating mechanisms, which equip the enzyme carbonic anhydrase, one of the fastest enzymes in nature, to rapidly convert bicarbonate to CO_2 upon demand in the chloroplast [26]. Thus, for saturated FAs accumulation, conditions favoring the shift of the equilibrium towards bicarbonate, such as high temperature (for example above 30 °C) and moderate pH (for example between 7 and 8.5), might be more important than those promoting the initial dissolution of CO_2 in water. On the other hand, increasing temperature might disproportionately increase the respiration rate of the cells comparatively to photosynthesis, and thus negatively impact total biomass and total lipid productivity [26]. Therefore, increasing light availability would be beneficial at high temperatures to increase the photosynthetic rate. No interaction was observed between temperature and light supply in any case. There were, however, positive interactions between the presence of aeration or CO_2 partial pressure and light intensity, light period, or the light flux per volume in the cases of C16:0 and C18:1. That highlights the importance of light under increased carbon supply for the accumulation of saturated and monosaturated FAs.

3.2. Aeration Has a Negative Effect on PUFAs

On the other hand, the interaction between aeration and light intensity or light period was negative for C20:5, n-3. Most importantly, the effect of aeration was significantly negative for both the C20:5, n-3 percentage in total FAs and its ratio to C16:0, while the presence of aeration also had a strong negative effect on C18:2. That could simply reflect the disproportionate accumulation of saturated or monosaturated FAs under stress conditions and increased carbon and light availability compared to the synthesis of PUFAs. Another possibility however could be that the absence of aeration itself has a positive effect on the

biosynthesis of PUFAs. A mechanistic explanation for that scenario could be related to the cascade of enzymes involved in PUFA synthesis. The desaturation of carbon bonds for the synthesis of unsaturated FAs is catalyzed by desaturases, enzymes that require molecular oxygen [27,28]. The more double bonds, the more desaturation steps are involved, and thus the more oxygen is required. Therefore, if desaturation is upregulated from increased oxygen concentration, which would be the case in a non-aerated autotrophic microalgal culture, its effect would be more important as the number of double bonds increase, which is compatible with the results presented in this article, with the effect of aeration being positive for saturated FAs, positive or slightly negative for C16:1 and C18:1 and significantly negative for C18:2 and C20:5, n-3. Readers not familiar with photosynthesis might find the increase in oxygen under non-aerated conditions nontrivial, but it is a well-established fact that dissolved oxygen levels rapidly rise in well illuminated microalgal cultures, which is a problem in large scale cultivation addressed with the use of costly equipment, such as degasser columns [29,30].

Dark or cold treatment, but not a combination of them, has also been shown to trigger de novo C20:5, n-3 biosynthesis in *N. oceanica* [31]. In this specific study, aeration was applied only at the control temperature setting (28–32 °C), while cultures in cold conditions (15 °C) were shaken manually twice per day (personal communication with corresponding author). Therefore, the observed positive effect of low temperature could be at least partially attributed to the same mechanism proposed above. The observed positive effect of darkness is also in agreement with our results, specifically with the negative effect of light period in PUFAs. The lack of positive interaction between low temperature and darkness further supports the idea that oxygen is responsible for the positive effect of decreasing temperature on PUFAs content, since in the absence of light there is no production of oxygen and thus only the positive effect of darkness remains, while increased oxygen solubility might also have a positive influence. These results are compatible with the observations of Harris and James, who were some of the first researchers to suggest that oxygen concentration directly affects fatty acid desaturation independently from temperature [32]. They observed an increase in FA desaturation in bulb tissue with either decreasing temperature or increased oxygen concentration, but the same was not observed in plant leaf tissue and *Chlorella vulgaris*. They also observed that in dark conditions increased oxygen concentrations enhanced FA desaturation also in leaf tissue and *C. vulgaris*. They concluded that the observed effect of low temperature in FA desaturation can be attributed to the increase in oxygen solubility.

While the effect of temperature is straightforward, since decreasing temperature increases oxygen solubility in water, the effects of aeration depend on the net oxygen mass balance in the culture. Ronda et al. measured the dissolved oxygen concentration and gamma-linolenic acid (C18:3, n-6) biomass content of *Arthrospira platensis* in a not-well illuminated bubble column photobioreactor [33]. They observed an increase in dissolved oxygen concentration and C18:3, n-6 with increasing aeration rate. They attributed the positive effect of aeration to C18:3, n-6 to the increased dissolved oxygen. This demonstrates the complicated interplay of aeration, illumination, and biomass concentration or reactor geometry, since if the light availability is not adequate to support photosynthesis in a significant portion of the reactor, the net oxygen mass balance is negative in comparison to the equilibrium concentration, in which case increased aeration results in a higher dissolved oxygen concentration. The same is observed in heterotrophic production of PUFAs from microalgae and yeast, where the negative effects of oxygen depletion are well established [28,34,35]. In the context of the present study, our analysis suggests, probably for the first time in microalgal research, that under light replete conditions, a termination of aeration induces biosynthesis of PUFAs by the increase in intracellular oxygen.

Another way that oxygen could influence the fatty acid profile is the enzymatic or non-enzymatic oxidation of PUFAs to oxylipins, a class of molecules that act as mediators and have bioactive properties beneficial to human health [36]. Oxidative conditions such as those present under elevated oxygen concentration and saturating light intensity could

enhance oxylipin synthesis, which could cause an observed reduction in the abundance of the respective fatty acid. While light intensity was negatively associated with PUFAs in the current study, the negative effect of the presence of aeration is not compatible with increased oxylipin synthesis, since an increase in PUFAs is observed under non-aerated conditions, which, as mentioned before, would lead to an increase in oxygen concentration. Regardless, the magnitude of the potential effect of oxylipins to the observed fatty acid profile and lipid concentration cannot yet be assessed due to the relative sparsity of information on the formation of oxylipins under different growth conditions in *Nannochloropsis oculata* or other microalgae. Future studies should focus on this very interesting class of molecules.

3.3. Cell Wall Stability Might Also Be Related to the Effects of Aeration

Another explanation for the negative effect of the presence of aeration on the content of PUFAs could be related to the shear stress caused by aeration. Polyunsaturated fatty acids are present in the cell membranes, which are attached to the cell wall via the cell cortex [37]. Calcium, which plays a role in cell wall stability in plants, had a significant negative effect of both C18:2 percentage in total FAs and its ratio to C16:0. Calcium starvation has been show to significantly increase the total lipid content of *Chlorella*, while the C18:2 percentage in total FAs decreased [38]. In freshwater microalgae, calcium can trigger homeoviscous adaptation by toughening the cell wall and leading to an increase in the cell membrane fluidity via accumulation of PUFAs [39]. The results presented here suggest that reduction of calcium concentration to levels lower than that of seawater (~0.01 M or 0.41 g L^{-1}) might have a similar effect for marine microalgae, since the maximum calcium concentration in the current study was that of seawater. Additionally, calcium is important in the signaling of nitrogen starvation via its presence on membrane sensor proteins. Reduction of calcium concentration might limit the response to nutrient starvation and thus the accumulation of saturated FAs, and, in return, lead to an increase in the relative abundance of PUFAs [40].

Another parameter that could be related to the stability of the cell wall is silicon, which was significant for C16:1. Silicon is an important element for diatoms, which rely on it to create their frustules. In non-diatom microalgae like Ochrophyta, which contain the genus *Nannochloropsis*, silicon either is a component of structures in the cell or is accumulated in a non-localized manner [27]. Potassium is another critical nutrient for microalgae, playing a role in various cellular processes including enzyme activation, osmoregulation, and pH regulation. Potassium concentration and its interaction with phosphate had strong positive effect on C14:0/C16:0 and the C16:0 biomass content, respectively. Potassium nitrate increases lipid accumulation in comparison to sodium nitrate [41,42]. These results suggest the important roles of silicon and potassium in biofuels production.

3.4. Novel Effects of Magnesium and Manganese

Other micronutrients that were highlighted as significant in this study were magnesium and manganese, the former with a positive effect on C16:1, particularly via its notable negative interaction with the light flux per volume, and the latter with its strong positive effect on all parameters related to C18:2, especially via its interaction with temperature. Magnesium is a component of chlorophyll and thus one of the most critical micronutrients in photoautotrophic organisms. It is also important for ribosome stability and an activator of ribulose biphosphate carboxylase (RuBisCO) among other enzymes. Magnesium deprivation led to the highest monounsaturated FA content in *Auxenochlorella protothecoides* in one study [43]. The interaction of magnesium and light appears with a moderate severity index in a multivariable study of mixotrophic cultivation of algae isolated from a lentic system [44]. In the current study, the observed negative interaction of magnesium concentration with the light flux per volume could indicate that magnesium concentration upregulates chlorophyll production, which could lead to excessive light uptake under high light availability. Indeed at magnesium limited conditions increase in its concentration leads to increase in chlorophyll content [38]. However, the data used in this study did not include magnesium deprived conditions, since magnesium is abundant in sea water

and added in adequate amounts in artificial sea water. Thus, the results of the current study might indicate a novel effect of magnesium at high concentrations. Manganese is an essential micronutrient for microalgae, as a component of PSII and enzyme cofactor, but is also considered a heavy metal. Manganese depletion can lead to an increase in polyunsaturated FAs in *Nannochloropsis oceanica* [9], which was not observed in the current study, despite the presence of manganese-depleted data points [45]. On the contrary, manganese had a (previously undocumented) strong positive effect on C18:2. Possible explanations are the upregulation of enzymes in the FA metabolism or upregulation of antioxidant enzymes like manganese superoxide dismutases [46], which could limit lipid peroxidation [28]. Lipid peroxidation increases with temperature, which, together with the strong positive interaction of manganese concentration with temperature, supports this idea [47].

3.5. Stressful Effects of Excessive Aeration Rate

An important parameter for saturated FAs and thus biodiesel production seems to be the aeration rate and its interplay with the partial CO_2 pressure. While the presence of aeration is necessary for saturated FAs accumulation, excessive aeration rate seems to have a negative impact, which is alleviated with increasing CO_2 partial pressure, as indicated by the positive interaction between aeration rate and the CO_2 partial pressure. This is observed for both the C14:0 and C16:0 biomass contents. These results are supplementary to those of Spolaore et al. who studied the effect of aeration rate between 0.02 and 0.25 vvm, under ambient CO_2 concentration, and reported the maximum of this range as the optimal value [48]. Additionally, those results agree with the data presented by Ajala and Alexander [11], who studied separately the effects of aeration and agitation on the proximate composition of *N. oculata* and other microalgae under a high CO_2 partial pressure (2% or 0.02 atm at ambient pressure). Specifically, they presented the effects of shaking, aeration, and stirring, which indicate that at moderate and high aeration rates, the increase in agitation rate either by shaking or stirring initially increased the lipid content and after a certain threshold had a negative effect. On the other hand, a rise of aeration rate from 0.15 to 2 vvm increased the lipid content. These results might also be relevant to the negative effect of aeration on PUFA content, since, as discussed, an increase in saturated FAs will result in the decrease in the relative PUFA content.

3.6. Nitrogen and Phosphate Stress Effects Agree with Well-Established Knowledge

The effects of nitrogen and phosphate concentration were mostly in line with the existing literature, with nitrate being the most important N-source but also the most prevalent in the dataset. Interestingly, the negative effect of nitrogen concentration was more pronounced for C16:1 and C18:1 than for C16:0. In *Nannochloropsis* C16:0, C16:1, and C18:1 are the main components of TAGs [49,50], where they accumulate under stress, especially under nitrogen starvation, via de novo synthesis through the acyl-CoA-dependent pathway, or via conversion of membranes [51]. On the other hand, C18:2 and C20:5, n-3 are mainly present in polar lipids [50], and they are accumulated under conditions requiring membrane plasticity, such as low temperatures, while they are negatively affected by nutrient limitation. However, PUFAs might also be synthesized de novo during nutrient starvation and transferred to TAGs [51–53]. Such an effect was not observed in the final results presented in this article, since all variables for the biomass content of C20:5, n3 were excluded after the second round of LASSO regression, while nitrate or phosphate concentrations were not included in the model for C18:2 biomass content.

However, the first round of LASSO (MinMSE) indicated a negative effect of all variables related to phosphate concentration on the biomass content in C20:5, n-3, which agrees with the findings of Matsui et al., who reported maximum accumulation of C20:5, n-3 at the initial stages of phosphate starvation [50]. Interestingly, in contrast to nitrate starvation, phosphate starvation seems to be more important for C16:0 than for C16:1 and C18:1, according to the final models. Shi et al. presented data showing a significant increase in

C16:0 and C18:1 FA percentages after day 2 and day 4 of phosphate starvation, respectively, while no noticeable change was observed for C16:1 [54]. Interestingly, the 3-day average phosphate concentration was included in our models for C16:0 FA percentage and biomass content, while the 4-day average appeared in the models for C16:1 and C18:1.

It could also be argued that the positive effect of biomass concentration on the biomass content in C18:2 might be related to nutrient starvation, since increasing biomass concentration will increase the consumption rate of resources. It seems to be more related, however, to the protective effects of cell shading under high light availability as it is indicated by the strong positive interaction with the light flux per volume, which has a negative coefficient in the model.

In conclusion, the results presented agree with well-known facts of the FA profile and content of *N. oculata*, such as the effects of temperature and nutrient starvation, while less well-established effects reported for other *Nannochloropsis* species, such as those of potassium and manganese, were demonstrated for the first time in *N. oculata*. Novel findings include the effect of magnesium on C16:1/C16:0, the effect of calcium on C18:2, and the negative effect of the presence of aeration in PUFA content, which could be attributed to different mechanisms, possibly due to an increase in dissolved oxygen concentration, similar to that occurring under low temperatures.

4. Materials and Methods

A total of 121 observations were used in the analysis presented in this paper, out of which 30 came from novel experiments, 24 came from our previous work, and 67 came from other writers. The full dataset is provided in the Supplementary Materials (S1).

The next sections describe the growth conditions and analyses performed to the novel experiments, the methodology for data collection and processing, and, finally, the statistical methods used to evaluate the effects of different predictor variables to the fatty acid profile.

4.1. Chemicals and Reagents

$NaNO_3$ (PanReac, Barcelona, Spain) and $NaH_2PO_4 \cdot 2H_2O$ (Honeywell, Tokyo, Japan) were used as nitrogen and phosphorus sources, respectively, while the trace elements Na_2EDTA (Sigma, St. Luis, MO, USA), $FeCl_3 \cdot 6H_2O$ (Acros Organics, Geel, Belgium), $CuSO_4 \cdot 5H_2O$ (Sigma), $ZnSO_4 \cdot 7H_2O$ (Sigma), $CoCl_2 \cdot 6H_2O$ (Fisher Scientific, Waltham, MA, USA), $MnCl_2 \cdot 4H_2O$ (Acros Organics), and $Na_2MoO_4 \cdot 2H_2O$ (Chem-Lab NV, Zedelgem, Belgium) were used for media preparation. Cyanocobalamin, Thiamine HCl, and Biotin were procured from Sigma-Aldrich. Chemicals used for analysis included ammonium bicarbonate (Sigma), HPLC-grade chloroform (Honeywell) and HPLC-grade methanol (Fisher Scientific).

4.2. Microalgal Species & Cultivation Conditions

Nannochloropsis oculata, was originally provided by the Laboratory of Zoology (Department of Biology, University of Patras, Greece) and was maintained as previously described [1,2]. Inoculum volume of 10 mL of the maintenance cultures was used to inoculate Erlenmeyer flasks of 500 mL capacity with 450 mL working volume. The medium used was four-times concentrated f/2 medium without silicate or vitamins. Initial pH was adjusted to 8 with addition of 1 N NaOH before the inoculation under a UV-sterilized laminar flow cabinet. Ambient air, filtered with a sterile 0.2 μm Whatman PTFE air filter, was provided at a rate of ~2.8 vvm, which also provided mixing, while light was supplied continuously from below at an intensity ~100 μmol m^{-2} s^{-1}, measured at the illuminated surface, by 6000 K white LED light bulbs. After 15 days, these cultures were collected and used to inoculate new cultures at various conditions at an initial chlorophyll concentration of 5–6 mg Chl a L^{-1}. Three sets of experiments were carried out.

The first set aimed to study the effects of light intensity, aeration, and salinity. The duration of this experiment was 7 days, temperature was set to 20 °C, and other conditions were:

1. Aeration, 350 μmol m^{-2} s^{-1} and 60 ppt (mg L^{-1}) salinity.
2. Aeration, 90 μmol m^{-2} s^{-1} and normal seawater salinity (~38 ppt).
3. Lack of aeration, 90 μmol m^{-2} s^{-1} and 60 ppt salinity.
4. Lack of aeration, 90 μmol m^{-2} s^{-1} and normal seawater salinity.

The same arrangement as that described for the pre-cultures was used, while the flasks subjected to non-aerated conditions were airtightly sealed with rubber stoppers.

The second set of experiments also had the same arrangement as that used for pre-cultures, targeted the effects of temperature, initial pH, and light period, and was complementary to a published article of our group [1]. Each day, the pH was adjusted to a specific setpoint with HCl or NaOH. The duration of this experiment was 5 days, light intensity was fixed to ~100 μmol m^{-2} s^{-1}, and the rest of the conditions were:

1. Initial pH 9.5, 20 °C, light period 12:12 (12 h light and 12 h dark).
2. Initial pH 6.5, 20 °C, light period 24:0 (continuous light).
3. Initial pH 8, 27.5 °C, light period 12:12.
4. Initial pH 8, 27.5 °C, light period 24:0.
5. Initial pH 6.5, 35 °C, light period 12:12.
6. Initial pH 9.5, 35 °C, light period 24:0.

The third experiment was a trial culture in a flat panel photobioreactor. Artificial sea water (ASW) with a brackish level of salinity enriched with 4 times concentrated f/2 medium [1] was used. The composition of the ASW (without the f/2 nutrients) was 2.71 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.67 g L^{-1} KCl, 0.33 g L^{-1} $CaCl_2 \cdot 2H_2O$ and 16.67 g L^{-1} NaCl. The reactor was continuously illuminated from one side with a 4000 K LED panel, with the light intensity at the inner illuminated surface measured at 195 ± 28 μmol m^{-2} s^{-1}, which corresponded to ~2249 μmol photons per second per m^3 of working volume. Aeration with ambient air was provided at the bottom of the reactor with a horizontal sparger at a rate of ~0.33 vvm. Ambient temperature had little variation during the day and was measured daily, while pH was also monitored daily. The experiment lasted for 10 days, while biomass was collected for fatty acid profiling at days 5 and 7. Total Suspended Solids (TSS), nitrate concentration and total phosphorus concentration were also monitored daily as described below.

4.3. Analytical Measurements

Nitrate and total phosphorus concentration was measured daily at the filtered growth medium obtained from the TSS measurement. Nitrates were measured spectroscopically at 220 and 275 nm [55], while total phosphorus was measured as orthophosphates with the ascorbic acid method after hydrolyzation under low pH [55].

4.4. Biomass Composition Analysis

4.4.1. Biomass Collection

At the end of each run, wet biomass was obtained via centrifugation at 3780× *g* for 7 min (Hermle, Z 366), washed with 0.5 M ammonium bicarbonate [56], freeze-dried (Telstar, LyoQuest), and stored in a desiccator. Ash content was then measured according to Standard Methods [55]. Subsequently, freeze-dried biomass was weighted and subjected to extraction and transesterification of FAs as described below.

4.4.2. Fatty Acid Profiling and Quantification

Fatty acids were converted to their respective methyl esters (FAMEs) with the one-step in situ transesterification method of Indarti et al., as modified by Levine et al. [57,58], and subsequent analysis was made on a GC (Agilent Technologies, 7890A) equipped with a detector (FID) and a capillary column (DB–WAX, 10 m × 0.1 mm × 0.1 μm), as previously described [59]. To quantify the produced FAMEs, a reference standard (FAMQ-005, Accustandard) and an internal standard solution (C17: 0, Sigma) were used [59].

4.5. Choice of Regression Techniques and Data Preparation

The goal of the research presented in this paper was the selection of important variables for the fatty acid profile of *Nannochloropsis oculata* in a non-excluding manner. To that end, all of the available literature data was collected, evaluated, and combined with our own data. The end result was a dataset with a very large number of predictor variables, which increased further in number due to the introduction of dummy variables with the one-hot method in order to take into account categorical variables and fixed effects, as well as the use of 2-, 3-, and 4-day averages for some numerical variables, like pH, to include the history of growth conditions of the model [60]. Therefore, a regression method that can handle many predictors with a relatively small number of observations was necessary. LASSO regression is ideal for that case, since it is a method that eliminates non-important variables leading to sparse models while considering the mean squared error. To take into account interaction terms, a hybrid approach inspired by others was used [19]. In this hybrid technique, LASSO regression was used to evaluate the significance of variables or their interactions and reduce their number, while stepwise linear regression was used both to identify significant interaction terms and to provide the final refined model with confidence intervals for coefficients.

In the following paragraphs, the methodology used in this paper is presented, starting with the selection of predictor and response variables, followed by the handling of missing data, the data preparation steps, the hybrid regression, and the final model evaluation.

4.5.1. One-Hot Encoding

One-hot encoding is a prevalent technique employed in the preprocessing of categorical data for machine learning algorithms. This method addresses the issue of categorical variables by transforming each category into a new binary feature, thereby enabling more effective handling by algorithms that require numerical input.

The one-hot encoding process operates by creating binary dummy variables for each category of the categorical variable. For a categorical variable with n categories, $n - 1$ binary features are created, each representing a single category. A given category is denoted by a '1' in its corresponding binary feature and '0' in all of the others. This transformation results in a binary vector for each category, where the vector length equals the number of categories in the original variable. To the n^{th} category, only zero values of the n - 1 dummy variables are assigned.

In the context of this study, one-hot encoding was utilized in two instances, one to account for the origin of the different datasets used, which were collected from various research groups, and the second to take into account the nitrogen source type (ammonium, nitrate or urea). Each research group or nitrogen source type was treated as a category within a categorical variable, and one-hot encoding was applied to this variable. This approach allowed for the preservation of crucial information about the data's origin or nitrogen source type without imposing an arbitrary ordinal structure that could potentially bias the subsequent analysis. The resulting binary features were then incorporated into the data used for regression in subsequent steps, enabling a comprehensive evaluation of the influence of each research group on the response variable.

4.5.2. Temporal Effects

For selected variables, for which the temporal profile was known or could be estimated, apart from the value at the day of biomass collection, 3 other variables were generated: the 2-, 3- and 4-day averages. In that way, a sense of evolution through time was incorporated into the analysis. The reasoning behind this step was that microalgae acclimate to environmental conditions, and each acclimation state might be influenced differently by environmental factors.

4.6. Handling of Missing Data

Handling missing data is a critical step in regression analysis, as the presence of missing values can lead to biased estimates, loss of efficiency, and complications in the model-building process. Various strategies exist for dealing with missing data, ranging from simple methods such as listwise deletion or mean imputation to more sophisticated techniques such as multiple imputation.

In the context of this study, missing data was addressed using multiple imputation, a statistically robust method that accounts for the uncertainty associated with missing values. Multiple imputation works by creating several different plausible imputed datasets and appropriately combining their results. This approach provides a more accurate estimate of the uncertainty due to missing data than single imputation methods, as it reflects the additional variance due to the imputation process.

The imputation process in this study was guided by model-based limits or educated assumptions. This means that the imputed values were not arbitrary but were based on logical assumptions about the relationships among variables or derived from statistical models built on the observed data. This approach ensures that the imputed values are plausible given the observed data and the assumptions made, thereby reducing the potential bias introduced by the imputation process.

Regression was performed with each imputed dataset and the results were subsequently combined. Below the methodology used to select limits for the imputation of different missing data types is presented.

4.6.1. Conversion of Cell and Optical Density to Biomass Concentration

Biomass concentration in terms of weight per volume of culture was used instead of optical density or cell count, since it is of higher practical importance when the microalgae production is considered and can be easily compared between different studies. Additionally, optical density can significantly vary with cultivation conditions and the state of the cell [61], while the same applies to the distribution of individual cell weight [62]. Due to interference of salt to the biomass concentration measurement in terms of dry weight (DW) [56], the ash-free dry weight (AFDW) was selected as a better alternative to DW [55].

In some cases, the biomass concentration was provided as a function of time, either in DW [8,63,64] or AFDW [1,2,65] basis. Original data presented in this article were in the AFDW basis. DW values were imputed as AFDW assuming 1% and 20% ash content for the upper and lower limits, respectively, while the AFDW values were repeated in each imputation dataset unchanged.

In one case, where optical density at 750 nm was provided along with the initial and final values of biomass concentration [7], the lower limit for biomass concentration was set by a linear relation between the initial and the final biomass concentration, while for the upper limit biomass, concentration was assumed to follow the same trend as the optical density. The initial and final biomass concentrations, used to calculate the time averaged biomass concentrations, were treated the same as described above, since they were provided by the authors.

In other cases, the cell density was provided as a function of time along with the final biomass concentration [6] or with information regarding the biomass productivity, which could be used to estimate the final biomass concentration [45,66]. In those cases, the initial biomass concentration was estimated using the cell count and the assumption that the average single cell weight was 10 pg for the lower limit and equal to the final biomass weight/cell count for the upper limit [67]. The temporal profile in both cases was assumed to be the same as the cell count temporal profile, with the lower case following a linear increase in cell weight from 10 pg to the final cell weight, and for the upper limit a constant cell weight was assumed.

The treatment of missing biomass concentration for the rest of the cases is explained in the Supplementary Materials (S4) [50,68,69].

4.6.2. Estimation of Nitrogen and Phosphate Concentrations

In all cases, the initial nitrogen concentration was known, while some authors also provided the temporal evolution of nitrogen concentration [1,7,63,65] or provided the final values or information on the time when it was depleted [2,8,45]. In most cases, nitrate was the nitrogen source. Data of nitrate consumption and biomass production were used to fit a second order polynomial model consumption of nitrate vs. biomass concentration change with R squared 0.97. Predictor variables for the model were initial nitrate and biomass concentrations. The data indicate that higher initial nitrate and biomass concentrations lead to higher nitrate uptake, which has also been observed for other species [70], and which is compatible with nitrate reductase upregulation in the presence of high nitrate concentrations [71]. The highest dNO_3dX value seems to be -0.0065 mg N-NO_3 g^{-1} AFDW. The model is presented in the Supplementary Materials (S4). The limits for imputation of nitrate concentration were therefore estimated using the initial nitrate concentration and the upper/lower limits of biomass concentration for the lower and upper limit for nitrate concentration respectively. In cases where ammonia or urea were the nitrogen source, the concentration was either provided by the authors [45,69] or, in one case, assumed to follow the same trend as nitrate [64].

Data on phosphate availability was more sparse, with less authors providing information on the temporal profile or the final values of phosphate concentration [1,2,45,50,65]. The lower limit for imputation was estimated assuming biomass phosphorus content 2.5% and using the upper biomass concentration limit, while the upper limit was estimated assuming 0.5% biomass phosphorus content and using the lower biomas concentration limit. The limits of 0.5 and 2.5% were chosen based on a study on the luxury uptake of phosphate by *Nannochloropsis salina* [72].

4.6.3. Estimation of pH and Inorganic Carbon

In this study, missing pH values were estimated using a two-step process: initial prediction and subsequent refinement. The equilibrium pH was initially approximated via the iPHREEQC COM module, which was interfaced with Matlab, utilizing the growth medium's chemical composition and temperature as inputs. The PHREEQC library llnl.dat was used, since it is the most suitable for marine systems [73]. Due to expected discrepancies between the predicted and the actual pH values, residual modelling was implemented as a refinement step. This involved calculating residuals by subtracting 10^(- predicted equilibrium pH) from 10^(- real pH) in cases where real pH was known. These residual values were then used to create a linear model in Matlab, with predictors dN, dP (representing the total consumption of nitrate and phosphate at a given time in mol L^{-1}, respectively) and the aeration rate in vvm. The resulting model demonstrated an R-squared value greater than 0.96, indicating a robust estimation of the deviation from the initial predictions, hence offering a more precise method for pH estimation. This model was utilized only in cases where ambient air was used for aeration. In cases where the CO_2 concentration in the air feed exceeded that of ambient air, the pH was either provided by the authors or presumed to be the equilibrium value calculated with PHREEQC. The residual model is provided in the Supplementary Materials (S4), while information on the iPHREEQC COM module can be found on the USGS website.

Using the partial pressure of CO_2 in the air feed and the medium composition and temperature, the concentration and speciation of inorganic carbon in the medium was also estimated using PHREEQC with the llnl.dat library, assuming equilibrium conditions. While at high biomass densities this might not be true, due to high carbon consumption [74], equilibrium conditions have been used to describe CO_2 mass transfer in lab scale microalgal cultivation with success in the past [75]. For the novel data presented that involved non-aerated conditions, inorganic carbon was assumed to be zero after 7 days of cultivation due to the estimated carbon content of produced biomass. For the other case where no aeration was applied, the lower limit was also set to zero while the upper limit was that in equilibrium with air, calculated with PHREEQC as in the rest of the cases [66].

4.6.4. Assumptions for Illumination Conditions

In all cases, the light intensity measured at the surface of the container used for cultivation was provided. The type of vessel as well as the working volume and orientation of the light source were also provided in most cases in the article or by the authors upon request. In one case, the type of reactor (cylindrical photobioreactor) was mentioned without any information on dimensions [63]. In that case, diameter was assumed to be between 5 and 10 cm, based on other examples of similar systems in the market and the literature. These boundaries were used to set the lower and upper limits for the illuminated surface, assuming illumination from one side of the reactor. Illumination related variables used for regression were the light intensity in µmol photons m^{-2} s^{-1} and the product of the light intensity with the illuminated surface divided by the working volume (µmol photons m^{-3} s^{-1}), which was used as a measure of the light availability in the culture. All of the variables considered in this article are detailed in the Supplementary Materials (S1).

4.6.5. Selection of Response Variables

Three types of response variables were considered: the percentage of a specific FA in total FAs, the ratio of that FA to C16:0 [76] that is usually the most abundant FA in *Nannochloropsis* [77], and the biomass content in the specific FA in terms of ash free dry weight [1].

4.7. Data Normalization

4.7.1. Z-Score Normalization

Regularization methods like LASSO regression require normalization of the predictor variables to ensure that all predictors contribute equally to the model, regardless of their original scales. Without normalization, a predictor could dominate the model, not because it is necessarily more important or informative but due to its magnitude. This is particularly crucial for LASSO regression, which applies a penalty to the coefficients of the predictors in order to perform variable selection and prevent overfitting. The penalty is based on the magnitude of the coefficients, so if the predictors are not on the same scale, the penalty could unfairly affect predictors with larger scales.

Z-score normalization is a good option for normalizing predictors because it not only brings all predictors onto the same scale (mean of 0 and standard deviation of 1) but also maintains the distribution and relationships in the data. This makes it easier for LASSO regression to fairly evaluate the importance of each predictor and make accurate predictions. The normalization of variables with the Z-score method has as follows:

$$\text{Xi normalized} = (\text{Xi} - \text{MEANi})/\text{SDi} \quad (1)$$

where:

Xi are the predictors;
MEANi are the mean values of all the observations for each predictor;
SDi are the standard deviation values for each predictor.

4.7.2. Centering of Response Variables

Centering of response variables is an important step in regularization methods like LASSO and Ridge regression. The primary reason for this is to ensure that the intercept term of the model is not penalized. Regularization methods work by adding a penalty term to the loss function that the model seeks to minimize. This penalty term discourages the model from assigning too much importance to any one predictor, which can help prevent overfitting. However, the intercept term in a linear model is not a coefficient that multiplies a predictor variable but rather represents the expected value of the response variable when all of the predictors are zero. Penalizing the intercept term could therefore lead to a model that is biased and does not fit the data well. By centering the response variable (subtracting the mean from each value), we ensure that the intercept term represents the mean of the

response variable, and we can safely apply the penalty to the other coefficients without affecting the intercept. This helps to maintain the interpretability of the model while still gaining the benefits of regularization. As already mentioned, centering is achieved simply by subtracting the mean value of the variable from each observation:

$$\text{Y centered} = \text{Y} - \text{MEANY} \quad (2)$$

where:

Y is the response variable;

MEANY is the mean value of the response variable.

4.8. LASSO Regression

LASSO (Least Absolute Shrinkage and Selection Operator) regression is a method used in regression analysis and machine learning to perform variable selection and regularization. The goal of LASSO regression is to obtain the subset of predictors that minimizes prediction error for a quantitative response variable. The technique works by imposing a constraint on the model parameters that causes regression coefficients for some variables to shrink toward zero. Variables with a regression coefficient equal to zero after the shrinkage process are excluded from the model. This property of LASSO regression makes it particularly useful for analyzing datasets with many predictors.

The LASSO method minimizes the residual sum of squares subject to the sum of the absolute value of the coefficients being less than a tuning parameter, λ. Mathematically, this can be represented as:

$$\text{minimize } (1/(2 \times \text{n})) \times \Sigma[\text{Y} - \text{B}_0 - \Sigma[\text{Bi} \times \text{Xi}]]^2 + \lambda \times \Sigma\,|\,\text{Bi}\,| \quad (3)$$

where:

n is the number of observations;

B_0 is the intercept term;

λ is a tuning parameter controlling the amount of shrinkage: the larger the value of λ, the greater the amount of shrinkage;

Bi are the parameter coefficients.

The first part of the equation, $(1/2 \times \text{n}) \times \Sigma[\text{Y} - \text{B}_0 - \Sigma[\text{Bi} \times \text{Xi}]]^2$, is the mean squared error, a measure of the model's prediction error. The second part, $\lambda \times \Sigma\,|\,\text{Bi}\,|$, is the L1 penalty, which imposes a cost on the size of the coefficients.

In MATLAB, LASSO regression can be implemented using the "lasso" function. The function takes as input a matrix X of predictor variables and a vector Y of the response variable, and returns a vector of coefficients B.

The minimization of the objective function in LASSO regression is typically achieved using a coordinate descent algorithm. This algorithm works by iteratively optimizing the objective function over one parameter at a time, holding all other parameters fixed. The algorithm continues until the change in the objective function is below a certain threshold, indicating that the solution has converged.

In the context of LASSO regression, the coordinate descent algorithm works by iteratively updating each regression coefficient Bi by minimizing the objective function with respect to Bi, holding all other coefficients fixed. This process is repeated until the coefficients converge to a solution.

The LASSO method has the advantage of producing simpler and interpretable models that involve only a subset of the predictors. However, the choice of the tuning parameter λ is crucial as it determines the level of penalty, and, hence, the number of predictors in the final model. Instead of using a single λ value, it is common practice to explore a range of λ values in order to identify the one that optimizes the model. Cross-validation is frequently employed in this process, with the optimal λ chosen as the one that minimizes prediction error. Another interpretation of optimal λ is the largest value of λ such that the mean cross-validated error is within one standard error of the minimum. This value of λ

is often chosen for model selection because it provides a model that is simpler (i.e., has fewer predictors) but whose predictive performance is within one standard error of the best performing model. This is in line with the principle of parsimony, or Occam's razor, which prefers simpler models when their performance is not significantly worse than more complex models.

4.9. Stepwise Regression with Selected Main Effects and Their Interactions

While LASSO regression is a powerful tool for parameter selection, it does not account for interactions between the different parameters examined. At the same time, interactions between the main effects might be of great importance and reveal novel aspects of the relationship between the studied variables. In the current article, a hybrid approach was followed to deal with these issues. Specifically, initially main effects were chosen with LASSO regression as described earlier, while a refinement step followed, during which the main effects chosen with LASSO and their interactions were fitted to a linear model using stepwise linear regression. This was performed in Matlab using the "stepwiselm" function and non-normalized data. Stepwise regression allows both for further variable selection with inclusion of significant interaction terms and comparison between selected variables via the *p*-value associated with each one. As in the case of LASSO regression, stepwise regression was also performed separately for each imputed dataset. In this case, however, the results were pooled using Rubin's rule, since within-imputation variance existed.

4.10. Rubin's Rule

Rubin's rule is a statistical method that is widely used in the analysis of multiple imputed datasets. The rule was proposed by Donald Rubin in 1987 and is designed to account for the uncertainty introduced by the imputation process when calculating estimates and their variances.

In the context of regression analysis, Rubin's rule is used to combine estimates from multiple imputed datasets to produce a single estimate that reflects both the within-imputation variance and the between-imputation variance. The within-imputation variance is the average of the variances of the estimates from each imputed dataset, while the between-imputation variance is a measure of the variability of the estimates across the different imputed datasets.

The total variance, according to Rubin's rule, is calculated as the sum of the within-imputation variance and a corrected form of the between-imputation variance. The correction factor accounts for the number of imputations. Specifically, the total variance (T) is calculated as follows:

$$T = W + (1 + 1/m) \times B$$

where W is the within-imputation variance, B is the between-imputation variance, and m is the number of imputations.

Once the total variance is calculated, the total standard deviation can be derived by taking the square root of the total variance. The total standard deviation is a measure of the total uncertainty of the estimate, taking into account both the uncertainty within each imputed dataset and the uncertainty between different imputed datasets.

4.11. Post Hoc Analysis

In our study, we employed a multi-step approach to identify significant main effects and interaction terms, and to generate sparse, interpretable models. This process involved the use of LASSO regression, stepwise linear regression, and the application of trimmed mean calculations.

Initially, we utilized LASSO regression as a screening tool to identify important main effects. This method is known for its effectiveness in dealing with high-dimensional data and its ability to perform variable selection. We used three distinct criteria in the LASSO regression: the model that minimizes the mean squared error (MSE), the model

that balances predictive accuracy and model simplicity (1SE), and a unique approach that we will refer to as "NoBlocking".

The "NoBlocking" criterion involves examining the lambda value at which all dummy variables, accounting for the fixed effects of the data origin, are excluded from the model. The rationale behind this approach is that the exclusion of these blocking variables indicates that the original categorical variable, in that case the data origin, is not a significant predictor in the presence of the other variables in the model. This method should provide a means of identifying the most influential predictors in the model.

Following the LASSO regression, we performed stepwise linear regression using all variables selected with the three criteria. This step was crucial in selecting important interaction terms, which can often provide additional insights into the relationships between variables that are not evident when considering main effects alone.

We then conducted a second round of LASSO regression using all the main effects and interactions present in the models from the previous step. The purpose of this step was to further reduce the number of variables and assist in the generation of sparse models. LASSO's ability to perform variable selection was again leveraged here to help simplify our models.

In each of these steps, we employed the use of trimmed mean calculations. For *p*-values, we focused on the mean rather than the trimmed mean. This is because a low mean *p*-value indicates the absence of large outliers and thus a narrower confidence interval, which is more significant. On the other hand, for variable coefficients, the trimmed mean was more important than the mean. This is because large outliers greater than zero can give the impression that a variable is significant, while in reality, it may have been excluded during LASSO regression.

Finally, we performed a second and final round of stepwise regression. For this step, we used the main effects that remained in the "NoBlocking" criterion, either individually or as part of an interaction, and the most important interactions, defined as those with a trimmed mean value of coefficients greater than zero. This final step resulted in sparse, interpretable models, providing us with a clear and concise understanding of the relationships between our variables.

5. Conclusions

The aim of this study was to investigate the available information on the fatty acid profile and content of *Nannochloropsis oculata* and identify key parameters for the abundance of selected fatty acids for this species. Novel data combined with information gathered from the literature was subjected to analysis using advanced regression methods and multiple imputation for missing data. This is probably the first time that LASSO regression has been used in the context of research on microalgal composition, and one of the first uses of a variation of the HDSI algorithm in general. Additionally, a novel approach to treat categorical variables in LASSO regression was introduced. The results agree with well-established facts regarding the effects of environmental conditions, such as temperature and pH, on the fatty acid content and profile of *N. oculata*, while novel observations were made, and the most important of these is the potential positive effect of the lack of aeration to the content in PUFAs, especially C20:5, n-3, the most valuable biomolecule produced by *Nannochloropsis*, with great importance to human nutrition and health. Additional highlights include the potentially significant (and previously undocumented for this species) effects of calcium, manganese, and magnesium. The research presented here paves the way for new experiments that will aim to investigate those effects, potentially contributing to the production of PUFAs and biofuels from *N. oculata* and other microalgal species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/md21090483/s1, Table S1: Variables and Data; Table S2: LASSO Regression Results; Table S3: Stepwise Regression Results; Table S4: Handling of Missing Data.

Author Contributions: Conceptualization, V.A. and M.K..; methodology, V.A.; validation, M.K.; formal analysis, V.A.; investigation, V.A.; resources, M.K.; writing—original draft preparation, V.A.; writing—review and editing, M.K.; supervision, M.K.; funding acquisition, M.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research has been co-financed by the European Union and Greek national funds through the Operational Program "Competitiveness, Entrepreneurship and Innovation 2014–2020 (EPAnEK)" in the context of the project entitled "Production of phenolic compounds from microalgae and characterization of biomass residue for utilization in fish farming facilities (AlgaPHESH, MIS 5045802)".

Institutional Review Board Statement: Not applicable.

Data Availability Statement: All data directly used to derive the results presented in this article is available in the Supplementary Materials. Additional data can be made available upon request.

Acknowledgments: Special thanks to Andreas Ioannou and Evangelos Birbas who designed, assembled, and prepared the flat panel photobioreactor used in the novel data presented in this article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Andriopoulos, V.; Lamari, F.N.; Hatziantoniou, S.; Kornaros, M. Production of Antioxidants and High Value Biomass from *Nannochloropsis oculata*: Effects of pH, Temperature and Light Period in Batch Photobioreactors. *Mar. Drugs* **2022**, *20*, 552. [CrossRef] [PubMed]
2. Andriopoulos, V.; Gkioni, M.D.; Koutra, E.; Mastropetros, S.G.; Lamari, F.N.; Hatziantoniou, S.; Kornaros, M. Total Phenolic Content, Biomass Composition, and Antioxidant Activity of Selected Marine Microalgal Species with Potential as Aquaculture Feed. *Antioxidants* **2022**, *11*, 1320. [CrossRef] [PubMed]
3. Tocher, D.R.; Betancor, M.B.; Sprague, M.; Olsen, R.E.; Napier, J.A. Omega-3 Long-Chain Polyunsaturated Fatty Acids, EPA and DHA: Bridging the Gap between Supply and Demand. *Nutrients* **2019**, *11*, 89. [CrossRef]
4. Vooren, G.V.; Grand, F.L.; Legrand, J.; Cuiné, S.; Peltier, G.; Pruvost, J. Investigation of Fatty Acids Accumulation in *Nannochloropsis oculata* for Biodiesel Application. *Bioresour. Technol.* **2012**, *124*, 421–432. [CrossRef]
5. Wei, L.; Huang, X.; Huang, Z. Temperature Effects on Lipid Properties of Microalgae *Tetraselmis subcordiformis* and *Nannochloropsis oculata* as Biofuel Resources. *Chin. J. Oceanol. Limnol.* **2014**, *33*, 99–106. [CrossRef]
6. Martínez-Macias, M.d.R.; Aguilar-Ruiz, R.J.; Nateras-Ramírez, O.; Sánchez-Machado, D.I.; López-Cervantes, J.; Dévora-Isiordia, G.E.; Álvarez-Sánchez, J.; Ríos-Vázquez, N.J. Influence of Different Reactor Types on *Nannochloropsis oculata* Microalgae Culture for Lipids and Fatty Acid Production. *JAOCS J. Am. Oil Chem. Soc.* **2021**, *98*, 993–1000. [CrossRef]
7. Aussant, J.; Guihéneuf, F.; Stengel, D.B. Impact of Temperature on Fatty Acid Composition and Nutritional Value in Eight Species of Microalgae. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 5279–5297. [CrossRef] [PubMed]
8. Su, C.H.; Chien, L.J.; Gomes, J.; Lin, Y.S.; Yu, Y.K.; Liou, J.S.; Syu, R.J. Factors Affecting Lipid Accumulation by *Nannochloropsis oculata* in a Two-Stage Cultivation Process. *J. Appl. Phycol.* **2011**, *23*, 903–908. [CrossRef]
9. Savvidou, M.G.; Boli, E.; Logothetis, D.; Lymperopoulou, T.; Ferraro, A.; Louli, V.; Mamma, D.; Kekos, D.; Magoulas, K.; Kolisis, F.N. A Study on the Effect of Macro- and Micro- Nutrients on *Nannochloropsis oceanica* Growth, Fatty Acid Composition and Magnetic Harvesting Efficiency. *Plants* **2020**, *9*, 660. [CrossRef]
10. Chiu, S.Y.; Kao, C.Y.; Tsai, M.T.; Ong, S.C.; Chen, C.H.; Lin, C.S. Lipid Accumulation and CO_2 Utilization of *Nannochloropsis oculata* in Response to CO_2 Aeration. *Bioresour. Technol.* **2009**, *100*, 833–838. [CrossRef]
11. Ajala, S.O.; Alexander, M.L. Evaluating the Effects of Agitation by Shaking, Stirring and Air Sparging on Growth and Accumulation of Biochemical Compounds in Microalgae Cells. *Biofuels* **2022**, *13*, 371–381. [CrossRef]
12. Sung, M.-G.; Han, J.-I.; Lee, B.; Chang, Y.K. Wavelength Shift Strategy to Enhance Lipid Productivity of *Nannochloropsis gaditana*. *Biotechnol. Biofuels* **2018**, *11*, 70. [CrossRef] [PubMed]
13. Chu, F.-J.; Wan, T.-J.; Pai, T.-Y.; Lin, H.-W.; Liu, S.-H.; Huang, C.-F. Use of Magnetic Fields and Nitrate Concentration to Optimize the Growth and Lipid Yield of *Nannochloropsis oculata*. *J. Environ. Manag.* **2020**, *253*, 109680. [CrossRef] [PubMed]
14. Tibshirani, R. Regression Shrinkage and Selection via the Lasso. *J. R. Stat. Soc. Ser. B* **1996**, *58*, 267–288. [CrossRef]
15. Deutelmoser, H.; Scherer, D.; Brenner, H.; Waldenberger, M.; Suhre, K.; Kastenmüller, G.; Bermejo, J.L. Robust Huber-LASSO for Improved Prediction of Protein, Metabolite and Gene Expression Levels Relying on Individual Genotype Data. *Brief. Bioinform.* **2021**, *22*, bbaa230. [CrossRef] [PubMed]
16. Uyen, P.H.; Uyen, V.T.L.; Hoa, L.T.; Trung, T.Q. LASSO Regression and Its Application in Forecasting Macro Economic Indicators: A Study on Vietnam's Exports. In *Prediction and Causality in Econometrics and Related Topics*; Ngoc Thach, N., Ha, D.T., Trung, N.D., Kreinovich, V., Eds.; Studies in Computational Intelligence; Springer International Publishing: Cham, Switzerland, 2022; pp. 575–585, ISBN 978-3-030-77094-5.

17. Rachmawati, R.N.; Sari, A.C. Yohanes Lasso Regression for Daily Rainfall Modeling at Citeko Station, Bogor, Indonesia. *Procedia Comput. Sci.* **2021**, *179*, 383–390. [CrossRef]
18. Rubin, D.B. Multiple Imputation After 18+ Years. *J. Am. Stat. Assoc.* **1996**, *91*, 473–489. [CrossRef]
19. Jain, R.; Xu, W. HDSI: High Dimensional Selection with Interactions Algorithm on Feature Selection and Testing. *PLoS ONE* **2021**, *16*, e0246159. [CrossRef]
20. Peng, X.; Meng, F.; Wang, Y.; Yi, X.; Cui, H. Effect of pH, Temperature, and CO_2 Concentration on Growth and Lipid Accumulation of *Nannochloropsis* Sp. MASCC 11. *J. Ocean. Univ. China* **2020**, *19*, 1183–1192. [CrossRef]
21. Millero, F.J.; Graham, T.B.; Huang, F.; Bustos-Serrano, H.; Pierrot, D. Dissociation Constants of Carbonic Acid in Seawater as a Function of Salinity and Temperature. *Mar. Chem.* **2006**, *100*, 80–94. [CrossRef]
22. Papadimitriou, S.; Loucaides, S.; Rérolle, V.M.C.; Kennedy, P.; Achterberg, E.P.; Dickson, A.G.; Mowlem, M.; Kennedy, H. The Stoichiometric Dissociation Constants of Carbonic Acid in Seawater Brines from 298 to 267 K. *Geochim. Et Cosmochim. Acta* **2018**, *220*, 55–70. [CrossRef]
23. Roy, R.N.; Roy, L.N.; Vogel, K.M.; Porter-Moore, C.; Pearson, T.; Good, C.E.; Millero, F.J.; Campbell, D.M. The Dissociation Constants of Carbonic Acid in Seawater at Salinities 5 to 45 and Temperatures 0 to 45 °C. *Mar. Chem.* **1993**, *44*, 249–267. [CrossRef]
24. Weiss, R.F. Carbon Dioxide in Water and Seawater: The Solubility of a Non-Ideal Gas. *Mar. Chem.* **1974**, *2*, 203–215. [CrossRef]
25. Bailey, N.; Papakyriakou, T.N.; Bartels, C.; Wang, F. Henry's Law Constant for CO_2 in Aqueous Sodium Chloride Solutions at 1 Atm and Sub-Zero (Celsius) Temperatures. *Mar. Chem.* **2018**, *207*, 26–32. [CrossRef]
26. Shene, C.; Chisti, Y.; Bustamante, M.; Rubilar, M. Effect of CO_2 in the Aeration Gas on Cultivation of the Microalga *Nannochloropsis oculata*: Experimental Study and Mathematical Modeling of CO_2 Assimilation. *Algal Res.* **2016**, *13*, 16–29. [CrossRef]
27. Borowitzka, M.A.; Beardall, J.; Raven, J.A. (Eds.) *The Physiology of Microalgae*; Springer International Publishing: Cham, Switzerland, 2016; ISBN 978-3-319-24943-8.
28. Sun, X.-M.; Geng, L.-J.; Ren, L.-J.; Ji, X.-J.; Hao, N.; Chen, K.-Q.; Huang, H. Influence of Oxygen on the Biosynthesis of Polyunsaturated Fatty Acids in Microalgae. *Bioresour. Technol.* **2018**, *250*, 868–876. [CrossRef]
29. Slegers, P.M.; van Beveren, P.J.M.; Wijffels, R.H.; van Straten, G.; van Boxtel, A.J.B. Scenario Analysis of Large Scale Algae Production in Tubular Photobioreactors. *Appl. Energy* **2013**, *105*, 395–406. [CrossRef]
30. Sforza, E.; Pastore, M.; Franke, S.M.; Barbera, E. Modeling the Oxygen Inhibition in Microalgae: An Experimental Approach Based on Photorespirometry. *New Biotechnol.* **2020**, *59*, 26–32. [CrossRef]
31. Chua, E.T.; Dal'Molin, C.; Thomas-Hall, S.; Netzel, M.E.; Netzel, G.; Schenk, P.M. Cold and Dark Treatments Induce Omega-3 Fatty Acid and Carotenoid Production in *Nannochloropsis oceanica*. *Algal Res.* **2020**, *51*, 102059. [CrossRef]
32. Harris, P.; James, A.T. The Effect of Low Temperatures on Fatty Acid Biosynthesis in Plants. *Biochem. J.* **1969**, *112*, 325–330. [CrossRef]
33. Ronda, S.R.; Bokka, C.; Ketineni, C.; Rijal, B.; Allu, P. Aeration Effect on *Spirulina platensis* Growth and γ-Linolenic Acid Production. *Braz. J. Microbiol.* **2012**, *43*, 12–20. [CrossRef] [PubMed]
34. Romero, A.M.; Jordá, T.; Rozès, N.; Martínez-Pastor, M.T.; Puig, S. Regulation of Yeast Fatty Acid Desaturase in Response to Iron Deficiency. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **2018**, *1863*, 657–668. [CrossRef] [PubMed]
35. Jiru, T.M.; Abate, D. Oleaginous Microorganisms, Diversity, Lipid Biosynthesis Pathway and Strain Improvement. *Webpub. J. Sci. Res.* **2014**, *2*, 55–65.
36. Linares-Maurizi, A.; Reversat, G.; Awad, R.; Bultel-Poncé, V.; Oger, C.; Galano, J.-M.; Balas, L.; Durbec, A.; Bertrand-Michel, J.; Durand, T.; et al. Bioactive Oxylipins Profile in Marine Microalgae. *Mar. Drugs* **2023**, *21*, 136. [CrossRef] [PubMed]
37. Goodenough, U.; Roth, R.; Kariyawasam, T.; He, A.; Lee, J.-H. Epiplasts: Membrane Skeletons and Epiplastin Proteins in Euglenids, Glaucophytes, Cryptophytes, Ciliates, Dinoflagellates, and Apicomplexans. *mBio* **2018**, *9*, 10–1128. [CrossRef]
38. Gorain, P.C.; Bagchi, S.K.; Mallick, N. Effects of Calcium, Magnesium and Sodium Chloride in Enhancing Lipid Accumulation in Two Green Microalgae. *Environ. Technol.* **2013**, *34*, 1887–1894. [CrossRef]
39. Shim, S.J.; Hong, M.E.; Chang, W.S.; Sim, S.J. Repeated-Batch Production of Omega-3 Enriched Biomass of *Chlorella sorokiniana* via Calcium-Induced Homeoviscous Adaptation. *Bioresour. Technol.* **2020**, *303*, 122944. [CrossRef]
40. Chen, H.; Wang, Q. Regulatory Mechanisms of Lipid Biosynthesis in Microalgae. *Biol. Rev.* **2021**, *96*, 2373–2391. [CrossRef]
41. Sharma, A.; Sahoo, P.; Singhal, S. Influence Of Different Nitrogen And Organic Carbon Sources On Microalgae Growth And Lipid Production. *IOSR J. Pharm. Biol. Sci.* **2015**, *10*, 48–53. [CrossRef]
42. Gour, R.S.; Bairagi, M.; Garlapati, V.K.; Kant, A. Enhanced Microalgal Lipid Production with Media Engineering of Potassium Nitrate as a Nitrogen Source. *Bioengineered* **2018**, *9*, 98–107. [CrossRef]
43. Polat, E.; Yüksel, E.; Altınbaş, M. Mutual Effect of Sodium and Magnesium on the Cultivation of Microalgae *Auxenochlorella protothecoides*. *Biomass Bioenergy* **2020**, *132*, 105441. [CrossRef]
44. Chiranjeevi, P.; Mohan, S.V. Critical Parametric Influence on Microalgae Cultivation towards Maximizing Biomass Growth with Simultaneous Lipid Productivity. *Renew. Energy* **2016**, *98*, 64–71. [CrossRef]
45. Roleda, M.Y.; Slocombe, S.P.; Leakey, R.J.G.; Day, J.G.; Bell, E.M.; Stanley, M.S. Effects of Temperature and Nutrient Regimes on Biomass and Lipid Production by Six Oleaginous Microalgae in Batch Culture Employing a Two-Phase Cultivation Strategy. *Bioresour. Technol.* **2012**, *129*, 439–449. [CrossRef] [PubMed]
46. Wolfe-Simon, F.; Starovoytov, V.; Reinfelder, J.R.; Schofield, O.; Falkowski, P.G. Localization and Role of Manganese Superoxide Dismutase in a Marine Diatom. *Plant Physiol.* **2006**, *142*, 1701–1709. [CrossRef] [PubMed]

47. Abbas, S.; Saeed, S.; Ammar, M.; Aman, S.; Shakeel, S.N. Effect of High Temperature on Activities and Lipid Production in Mutants of *Chlamydomonas reinhardtii*. *Int. J. Agric. Biol.* **2018**, *20*, 1560–8530.
48. Spolaore, P.; Joannis-Cassan, C.; Duran, E.; Isambert, A. Optimization of *Nannochloropsis oculata* Growth Using the Response Surface Method. *J. Chem. Technol. Biotechnol.* **2006**, *81*, 1049–1056. [CrossRef]
49. Xu, Y. Biochemistry and Biotechnology of Lipid Accumulation in the Microalga *Nannochloropsis oceanica*. *J. Agric. Food Chem.* **2022**, *70*, 11500–11509. [CrossRef]
50. Matsui, H.; Shiozaki, K.; Okumura, Y.; Ishikawa, M.; Waqalevu, V.; Hayasaka, O.; Honda, A.; Kotani, T. Effects of Phosphorous Deficiency of a Microalga *Nannochloropsis oculata* on Its Fatty Acid Profiles and Intracellular Structure and the Effectiveness in Rotifer Nutrition. *Algal Res.* **2020**, *49*, 101905. [CrossRef]
51. Janssen, J.H.; Wijffels, R.H.; Barbosa, M.J. Lipid Production in *Nannochloropsis gaditana* during Nitrogen Starvation. *Biology* **2019**, *8*, 5. [CrossRef]
52. Meng, Y.; Jiang, J.; Wang, H.; Cao, X.; Xue, S.; Yang, Q.; Wang, W. The Characteristics of TAG and EPA Accumulation in *Nannochloropsis oceanica* IMET1 under Different Nitrogen Supply Regimes. *Bioresour. Technol.* **2015**, *179*, 483–489. [CrossRef]
53. Janssen, J.H.; Lamers, P.P.; de Vos, R.C.H.; Wijffels, R.H.; Barbosa, M.J. Translocation and de Novo Synthesis of Eicosapentaenoic Acid (EPA) during Nitrogen Starvation in *Nannochloropsis gaditana*. *Algal Res.* **2019**, *37*, 138–144. [CrossRef]
54. Shi, Y.; Liu, M.; Ding, W.; Liu, J. Novel Insights into Phosphorus Deprivation Boosted Lipid Synthesis in the Marine Alga *Nannochloropsis oceanica* without Compromising Biomass Production. *J. Agric. Food Chem.* **2020**, *68*, 11488–11502. [CrossRef] [PubMed]
55. APHA. *Standard Methods for the Examination of Water and Wastewater*; American Public Health Association: Washington, DC, USA, 1998; ISBN 978-0-87553-235-6.
56. Zhu, C.J.; Lee, Y.K. Determination of Biomass Dry Weight of Marine Microalgae. *J. Appl. Phycol.* **1997**, *9*, 189–194. [CrossRef]
57. Levine, R.B.; Costanza-Robinson, M.S.; Spatafora, G.A. *Neochloris oleoabundans* Grown on Anaerobically Digested Dairy Manure for Concomitant Nutrient Removal and Biodiesel Feedstock Production. *Biomass Bioenergy* **2011**, *35*, 40–49. [CrossRef]
58. Indarti, E.; Majid, M.I.A.; Hashim, R.; Chong, A. Direct FAME Synthesis for Rapid Total Lipid Analysis from Fish Oil and Cod Liver Oil. *J. Food Compos. Anal.* **2005**, *18*, 161–170. [CrossRef]
59. Koutra, E.; Grammatikopoulos, G.; Kornaros, M. Microalgal Post-Treatment of Anaerobically Digested Agro-Industrial Wastes for Nutrient Removal and Lipids Production. *Bioresour. Technol.* **2017**, *224*, 473–480. [CrossRef]
60. Harris, D.; Harris, S. *Digital Design and Computer Architecture*; Morgan Kaufmann: Burlington, MA, USA, 2010; ISBN 978-0-08-054706-0.
61. Kandilian, R.; Lee, E.; Pilon, L. Radiation and Optical Properties of *Nannochloropsis oculata* Grown under Different Irradiances and Spectra. *Bioresour. Technol.* **2013**, *137*, 63–73. [CrossRef]
62. Usai, A.; Theodoropoulos, C.; Di Caprio, F.; Altimari, P.; Cao, G.; Concas, A. Structured Population Balances to Support Microalgae-Based Processes: Review of the State-of-Art and Perspectives Analysis. *Comput. Struct. Biotechnol. J.* **2023**, *21*, 1169–1188. [CrossRef]
63. Millán-Oropeza, A.; Torres -Bustillos, L.G.; Fernández-Linares, L. Simultaneous Effect of Nitrate (NO_3^-) Concentration, Carbon Dioxide (CO_2) Supply and Nitrogen Limitation on Biomass, Lipids, Carbohydrates and Proteins Accumulation in *Nannochloropsis oculata*. *Biofuel Res. J.* **2015**, *2*, 215–221. [CrossRef]
64. Gu, N.; Lin, Q.; Li, G.; Tan, Y.; Huang, L.; Lin, J. Effect of Salinity on Growth, Biochemical Composition, and Lipid Productivity of *Nannochloropsis oculata* CS 179. *Eng. Life Sci.* **2012**, *12*, 631–637. [CrossRef]
65. Aussant, J. Optimisation of Fatty Acid Production by Selected Microalgae for High-Value Applications. Ph.D. Thesis, University of Galway, Galway, Connacht, Ireland, 2018.
66. Andrew, A.R.; Yong, W.T.L.; Misson, M.; Anton, A.; Chin, G.J.W.L. Selection of Tropical Microalgae Species for Mass Production Based on Lipid and Fatty Acid Profiles. *Front. Energy Res.* **2022**, *10*, 912904. [CrossRef]
67. Brown, M.R.; Garland, C.D.; Jeffrey, S.W.; Jameson, I.D.; Leroi, J.M. The Gross and Amino Acid Compositions of Batch and Semi-Continuous Cultures of *Isochrysis* Sp. (Clone T.ISO), *Pavlova lutheri* and *Nannochloropsis oculata*. *J. Appl. Phycol.* **1993**, *5*, 285–296. [CrossRef]
68. Rasdi, N.W.; Qin, J.G. Effect of N:P Ratio on Growth and Chemical Composition of *Nannochloropsis oculata* and *Tisochrysis lutea*. *J. Appl. Phycol.* **2015**, *27*, 2221–2230. [CrossRef]
69. Gutierrez, J.; Kwan, T.A.; Zimmerman, J.B.; Peccia, J. Ammonia Inhibition in Oleaginous Microalgae. *Algal Res.* **2016**, *19*, 123–127. [CrossRef]
70. Rani, V.; Maróti, G. Assessment of Nitrate Removal Capacity of Two Selected Eukaryotic Green Microalgae. *Cells* **2021**, *10*, 2490. [CrossRef]
71. Sanz-Luque, E.; Chamizo-Ampudia, A.; Llamas, A.; Galvan, A.; Fernandez, E. Understanding Nitrate Assimilation and Its Regulation in Microalgae. *Front. Plant Sci.* **2015**, *6*, 899. [CrossRef]
72. Sforza, E.; Calvaruso, C.; La Rocca, N.; Bertucco, A. Luxury Uptake of Phosphorus in *Nannochloropsis salina*: Effect of P Concentration and Light on P Uptake in Batch and Continuous Cultures. *Biochem. Eng. J.* **2018**, *134*, 69–79. [CrossRef]
73. Lu, P.; Zhang, G.; Apps, J.; Zhu, C. Comparison of Thermodynamic Data Files for PHREEQC. *Earth-Sci. Rev.* **2022**, *225*, 103888. [CrossRef]
74. Zhao, S.; Ding, Y.-D.; Liao, Q.; Zhu, X.; Huang, Y. Experimental and Theoretical Study on Dissolution of a Single Mixed Gas Bubble in a Microalgae Suspension. *RSC Adv.* **2015**, *5*, 32615–32625. [CrossRef]

75. Fu, J.; Huang, Y.; Liao, Q.; Xia, A.; Fu, Q.; Zhu, X. Photo-Bioreactor Design for Microalgae: A Review from the Aspect of CO_2 Transfer and Conversion. *Bioresour. Technol.* **2019**, *292*, 121947. [CrossRef]
76. Jónasdóttir, S.H. Fatty Acid Profiles and Production in Marine Phytoplankton. *Mar. Drugs* **2019**, *17*, 151. [CrossRef] [PubMed]
77. Graeve, M.; Greenacre, M.J. The Selection and Analysis of Fatty Acid Ratios: A New Approach for the Univariate and Multivariate Analysis of Fatty Acid Trophic Markers in Marine Pelagic Organisms. *Limnol. Oceanogr. Methods* **2020**, *18*, 196–210. [CrossRef]

marine drugs

MDPI

Communication

Recovery of Fatty Acid and Volatile Flavor Compound Composition in Farmed Tiger Puffer (*Takifugu rubripes*) with a Fish Oil-Finishing Strategy

Lin Li [1,2,3], Feiran Zhang [1,3], Xiaoxue Meng [1,3], Xishuai Cui [1], Qiang Ma [1], Yuliang Wei [1,2], Mengqing Liang [1,2] and Houguo Xu [1,2,*]

[1] Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China
[2] Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China
[3] College of Fisheries and Life Sciences, Shanghai Ocean University, Shanghai 201306, China
* Correspondence: xuhg@ysfri.ac.cn

Abstract: Booming fish farming results in a relative shortage of fish oil (FO) supply, meaning that alternative oils are increasingly used in fish feeds, which leads to reduction of long-chain polyunsaturated fatty acids (LC-PUFAs) and other relevant changes in fish products. This study investigated the efficacy of an FO-finishing strategy in recovering the muscle quality of farmed tiger puffer. An eight-week feeding trial (growing-out period) was conducted with five experimental diets, in which graded levels (0 (control), 25, 50, 75, and 100%) of added FO were replaced by poultry oil (PO). Following the growing-out period was a four-week FO-finishing period, during which fish in all groups were fed the control diet. Dietary PO significantly decreased the muscle LC-PUFA content, whereas in general, the FO-finishing strategy recovered it to a level comparable with that of the group fed FO continuously. The recovery efficiency of EPA was higher than that of DHA. Dietary PO also led to changes of volatile flavor compounds in the muscle, such as butanol, pentenal, and hexenal, whereas the FO-finishing strategy mitigated the changes. In conclusion, the FO-finishing strategy is promising in recovering the LC-PUFA and volatile-flavor-compound composition in farmed tiger puffer after the feeding of PO-based diets.

Keywords: long-chain polyunsaturated fatty acid; muscle flavor; DHA recovery; feeding strategy

Citation: Li, L.; Zhang, F.; Meng, X.; Cui, X.; Ma, Q.; Wei, Y.; Liang, M.; Xu, H. Recovery of Fatty Acid and Volatile Flavor Compound Composition in Farmed Tiger Puffer (*Takifugu rubripes*) with a Fish Oil-Finishing Strategy. *Mar. Drugs* **2023**, *21*, 122. https://doi.org/10.3390/md21020122

Received: 30 December 2022
Revised: 8 February 2023
Accepted: 10 February 2023
Published: 13 February 2023

1. Introduction

Fish are the main source of n-3 long-chain polyunsaturated fatty acids (LC-PUFAs), mainly 22:6n-3 (docosahexaenoic acid, DHA) and 20:5n-3 (eicosapentaenoic acid, EPA) [1]. Aquaculture satisfies the growing global demand for fish, but also consumes an increasing share of the world's wild-fish fishery via the use of fishmeal and fish oil (FO) in fish feeds [2]. Therefore, increasing levels of alternative sources such as plant ingredients, livestock processing by-products, and single-cell materials are being used in fish feeds.

Poultry oil (PO) is a by-product of chicken processing and has a relatively low price and a large annual production. With high contents of 16:0, 18:1n-9, and 18:2n-6, PO is a potential lipid source for fish feeds. Partial or complete FO replacement by PO has proved feasible in diets of many aquaculture fish species [3–12]. It has been widely accepted that the fatty-acid profile of farmed fish generally reflects that of their diets [13]. Therefore, as expected, dietary replacement of FO by PO in the aforementioned studies led to decreased LC-PUFA content in the fish.

The FO-finishing strategy, namely, re-feeding FO for a period after long-term feeding with alternative oils, has been demonstrated to be an efficient way to restore the LC-PUFA content in farmed fish when alternative oils are used to replace FO in fish feeds [13]. This strategy has been practiced in sunshine bass (*Morone chrysops* × *M. saxatilis*) [14] and

rainbow trout (*Oncorhynchus mykiss*) [15] when PO is used in the feeds, but the LC-PUFA recovery efficiency is not consistent between species. Moreover, the effects of dietary PO on the volatile-flavor-compound composition in farmed fish, as well as their response to FO-finishing, have not been studied.

Volatile flavor compounds, which consist of aldehyde, alcohol, acid, ketone, phenol, and heterocyclic compounds containing nitrogen and sulfur, are an important component of fish flavor. Polyunsaturated fatty acids are major substrates of volatile-flavor-compound formation. This has been demonstrated in a number of fish species such as crucian carp (*Carassius auratus gibelio*), Nile tilapia (*Oreochromis niloticus*), gilthead seabream (*Sparus aurata*), tench (*Tinca tinca*), brown trout (*Salmo trutta*), turbot (*Psetta maxima*), rainbow trout, and Senegal sole (*Solea senegalensis*) [16–23]. These studies also suggest that the dietary lipid source significantly affects the muscle-volatile-flavor-compound composition via changes in fatty-acid metabolism.

With these considerations in mind, the present study was aimed at comprehensively evaluating the efficacy of FO-finishing in recovering LC-PUFA and volatile-flavor-compound composition in farmed tiger puffer, which is an important aquaculture species in Asia [24]. Results of this study will provide new insights into the manipulation of fish-product quality via the feeding strategy.

2. Results

2.1. Fatty-Acid Composition in Muscle and Liver

Fish grew normally during the whole experiment [25]. The average fish body weight at the end of the growing-out period and FO-finishing period was 41.99 g and 82.37 g, respectively. At the end of the growing-out period, with increasing levels of dietary PO, the contents of monounsaturated fatty acid (MUFA) and n-6 PUFA in the muscle significantly ($p < 0.05$) increased; that of n-3 PUFA significantly decreased; but the content of saturated fatty acid (SFA) was stable (Table 1, Figure 1). In particular, the DHA content decreased from 21.32% TFA in the FO group to 15.06% in the PO group (71% of that in the FO group), and accordingly, the EPA content decreased from 8.32% to 6.18% (74% of that in the FO group). However, the 22:5n-3 content slightly increased from the FO group to the PO group. After FO-finishing, the change in MUFA and PUFA caused by PO was substantially mitigated (Table 2, Figure 1). The DHA and EPA was recovered to be 85% and 94% of that in the control group continuously fed FO, respectively.

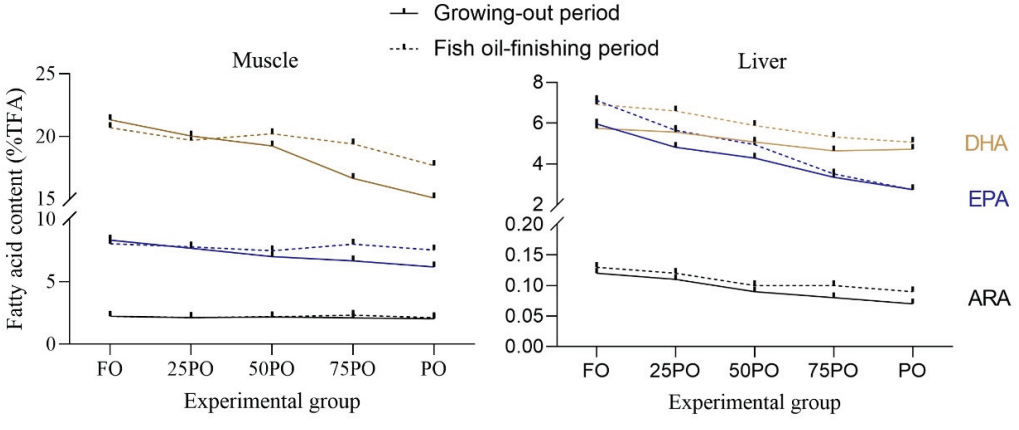


Figure 1. LC-PUFA contents in the muscle and liver of experimental tiger puffer.

Table 1. Fatty-acid compositions in the muscle at the end of the growing-out period (%TFA, mean ± standard error).

Fatty Acid	FO	25PO	50PO	75PO	PO
14:0	0.67 ± 0.05 ^{c}	0.56 ± 0.05 ^{bc}	0.50 ± 0.01 ^{abc}	0.42 ± 0.03 ^{ab}	0.36 ± 0.03 ^{a}
16:0	23.22 ± 0.25	22.79 ± 0.31	22.98 ± 0.06	22.54 ± 0.10	22.54 ± 0.25
18:0	12.39 ± 0.15	12.51 ± 0.18	12.13 ± 0.05	12.12 ± 0.28	11.92 ± 0.13
20:0	0.35 ± 0.15	0.42 ± 0.00	0.32 ± 0.00	0.25 ± 0.01	0.14 ± 0.03
∑SFA	36.63 ± 0.54 ^{b}	36.28 ± 0.18 ^{ab}	35.92 ± 0.09 ^{ab}	35.33 ± 0.34 ^{ab}	34.96 ± 0.20 ^{a}
16:1n-7	1.12 ± 0.13	1.02 ± 0.08	0.93 ± 0.04	0.90 ± 0.05	0.86 ± 0.04
17:1n-7	0.53 ± 0.02 ^{ab}	0.55 ± 0.03 ^{b}	0.47 ± 0.01 ^{ab}	0.45 ± 0.01 ^{ab}	0.44 ± 0.04 ^{a}
18:1n-9	11.15 ± 0.18 ^{a}	12.57 ± 0.08 ^{b}	13.94 ± 0.11 ^{c}	15.19 ± 0.11 ^{d}	16.34 ± 0.31 ^{e}
24:1n-9	0.23 ± 0.05	0.41 ± 0.05	0.24 ± 0.08	0.72 ± 0.35	0.31 ± 0.11
∑MUFA	13.02 ± 0.33 ^{a}	14.54 ± 0.19 ^{b}	15.57 ± 0.17 ^{b}	17.27 ± 0.27 ^{c}	17.95 ± 0.47 ^{c}
18:2n-6	10.82 ± 0.22 ^{a}	12.13 ± 0.30 ^{b}	12.86 ± 0.09 ^{b}	14.57 ± 0.20 ^{c}	16.04 ± 0.11 ^{d}
20:2n-6	0.68 ± 0.03 ^{a}	0.77 ± 0.01 ^{b}	0.78 ± 0.01 ^{b}	0.83 ± 0.02 ^{bc}	0.88 ± 0.01 ^{c}
20:4n-6	2.23 ± 0.08	2.12 ± 0.07	2.18 ± 0.04	2.10 ± 0.02	2.02 ± 0.04
∑n-6 PUFA	13.73 ± 0.17 ^{a}	15.01 ± 0.30 ^{b}	15.82 ± 0.08 ^{b}	17.50 ± 0.19 ^{c}	18.94 ± 0.13 ^{d}
18:3n-3	0.28 ± 0.05	0.24 ± 0.03	0.26 ± 0.00	0.24 ± 0.02	0.20 ± 0.01
20:5n-3	8.32 ± 0.04 ^{d}	7.67 ± 0.11 ^{c}	7.00 ± 0.02 ^{b}	6.66 ± 0.10 ^{ab}	6.18 ± 0.23 ^{a}
22:5n-3	4.89 ± 0.15	4.86 ± 0.05	4.81 ± 0.07	5.11 ± 0.13	5.22 ± 0.09
22:6n-3	21.32 ± 0.73 ^{b}	20.01 ± 0.59 ^{b}	19.22 ± 0.14 ^{b}	16.63 ± 0.44 ^{a}	15.06 ± 0.23 ^{a}
∑n-3 PUFA	34.81 ± 0.81 ^{c}	32.79 ± 0.53 ^{bc}	31.3 ± 0.06 ^{b}	28.64 ± 0.20 ^{a}	26.65 ± 0.54 ^{a}
∑n-3/∑n-6	2.54 ± 0.08 ^{c}	2.19 ± 0.07 ^{b}	1.98 ± 0.01 ^{b}	1.64 ± 0.03 ^{a}	1.41 ± 0.02 ^{a}

Data in a same row not sharing a same superscript letter are significantly different ($p < 0.05$). TFA: total fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; n-6 PUFA: n-6 poly-unsaturated fatty acid; n-3 PUFA: n-3 polyunsaturated fatty acid.

Table 2. Fatty-acid compositions in the muscle after fish oil-finishing (%TFA, mean ± standard error).

Fatty Acid	FO	25PO	50PO	75PO	PO
14:0	0.75 ± 0.02	0.80 ± 0.09	0.67 ± 0.02	0.62 ± 0.03	0.65 ± 0.01
16:0	23.39 ± 0.20 ^{b}	22.47 ± 0.39 ^{ab}	22.3 ± 0.30 ^{ab}	22.38 ± 0.14 ^{ab}	22.04 ± 0.19 ^{a}
18:0	12.61 ± 0.30	12.52 ± 0.44	12.80 ± 0.45	12.46 ± 0.13	12.73 ± 0.35
20:0	0.53 ± 0.01	0.54 ± 0.03	0.52 ± 0.06	0.44 ± 0.01	0.44 ± 0.02
∑SFA	37.28 ± 0.47	36.33 ± 0.74	36.28 ± 0.76	35.89 ± 0.10	35.86 ± 0.38
16:1n-7	1.11 ± 0.02	1.23 ± 0.12	1.05 ± 0.03	0.97 ± 0.06	0.99 ± 0.03
17:1n-7	0.63 ± 0.06	0.59 ± 0.03	0.63 ± 0.04	0.54 ± 0.00	0.55 ± 0.04
18:1n-9	10.69 ± 0.15 ^{a}	11.43 ± 0.26 ^{ab}	11.51 ± 0.25 ^{abc}	11.64 ± 0.08 ^{bc}	12.35 ± 0.11 ^{c}
24:1n-9	0.29 ± 0.03	0.34 ± 0.03	0.40 ± 0.02	0.37 ± 0.03	0.50 ± 0.09
∑MUFA	12.72 ± 0.19 ^{a}	13.59 ± 0.38 ^{ab}	13.59 ± 0.26 ^{ab}	13.52 ± 0.06 ^{ab}	14.39 ± 0.16 ^{b}
18:2n-6	10.81 ± 0.23 ^{a}	11.37 ± 0.09 ^{a}	11.54 ± 0.13 ^{ab}	12.19 ± 0.24 ^{bc}	12.79 ± 0.10 ^{c}
20:2n-6	0.70 ± 0.04 ^{a}	0.87 ± 0.04 ^{b}	0.85 ± 0.01 ^{b}	0.90 ± 0.03 ^{b}	0.92 ± 0.03 ^{b}
20:4n-6	2.23 ± 0.02	2.13 ± 0.10	2.19 ± 0.08	2.32 ± 0.06	2.12 ± 0.04
∑n-6 PUFA	13.74 ± 0.24 ^{a}	14.37 ± 0.02 ^{ab}	14.57 ± 0.07 ^{b}	15.41 ± 0.21 ^{c}	15.83 ± 0.16 ^{c}
18:3n-3	0.11 ± 0.11	0.29 ± 0.07	0.16 ± 0.08	0.21 ± 0.05	0.15 ± 0.08
20:5n-3	8.02 ± 0.29	7.78 ± 0.28	7.46 ± 0.24	7.99 ± 0.05	7.52 ± 0.17
22:5n-3	5.26 ± 0.10 ^{a}	5.63 ± 0.18 ^{ab}	5.71 ± 0.06 ^{ab}	5.63 ± 0.16 ^{ab}	6.02 ± 0.10 ^{b}
22:6n-3	20.69 ± 0.32 ^{b}	19.68 ± 0.42 ^{b}	20.19 ± 0.71 ^{b}	19.38 ± 0.20 ^{ab}	17.65 ± 0.23 ^{a}
∑n-3 PUFA	34.08 ± 0.43 ^{b}	33.38 ± 0.49 ^{ab}	33.52 ± 0.93 ^{ab}	33.22 ± 0.09 ^{ab}	31.33 ± 0.54 ^{a}
∑n-3/∑n-6	2.48 ± 0.04 ^{c}	2.32 ± 0.03 ^{bc}	2.30 ± 0.06 ^{bc}	2.16 ± 0.04 ^{ab}	1.98 ± 0.02 ^{a}

Data in a same row not sharing a same superscript letter are significantly different ($p < 0.05$). TFA: total fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; n-6 PUFA: n-6 poly-unsaturated fatty acid; n-3 PUFA: n-3 polyunsaturated fatty acid. Data in a same row not sharing a same superscript letter are significantly different ($p < 0.05$).

The liver fatty-acid composition showed a similar trend in response to PO supplementation, but the change was more drastic compared to the muscle except that the n-6 PUFA was stable among different groups (Table 3, Figure 1). In particular, at the end of the growing-out period, the DHA and EPA in the PO group was only 38% and 46% of that in the FO control group, respectively. Similar mitigating effects of the FO-finishing strategy were observed in the liver (Table 4, Figure 1). After FO-finishing, the DHA and EPA in the PO group was recovered to be 73% and 82% of that in the FO control group, respectively.

Table 3. Fatty-acid compositions in the liver at the end of the growing-out period (%TFA, mean ± standard error).

Fatty Acid	FO	25PO	50PO	75PO	PO
14:0	3.49 ± 0.09 ^{c}	3.20 ± 0.08 ^{c}	2.55 ± 0.02 ^{b}	2.29 ± 0.05 ^{b}	1.84 ± 0.02 ^{a}
16:0	19.51 ± 0.63	20.65 ± 0.24	19.15 ± 0.26	19.93 ± 0.38	19.58 ± 0.13
18:0	8.63 ± 0.76	9.47 ± 0.48	7.91 ± 0.19	9.14 ± 0.75	8.68 ± 0.63
20:0	0.52 ± 0.03 ^{c}	0.48 ± 0.01 ^{c}	0.35 ± 0.02 ^{b}	0.31 ± 0.01 ^{ab}	0.24 ± 0.01 ^{a}
∑SFA	32.15 ± 1.32	33.80 ± 0.66	29.96 ± 0.25	31.66 ± 1.03	30.34 ± 0.60
16:1n-7	8.53 ± 0.21 ^{b}	8.31 ± 0.23 ^{ab}	8.41 ± 0.12 ^{ab}	7.15 ± 0.40 ^{a}	7.32 ± 0.35 ^{ab}
18:1n-9	23.90 ± 0.18 ^{a}	26.54 ± 0.46 ^{b}	30.83 ± 0.47 ^{c}	34.43 ± 0.26 ^{d}	37.25 ± 0.57 ^{e}
20:1n-9	2.22 ± 0.22	2.03 ± 0.22	2.39 ± 0.04	1.98 ± 0.17	2.01 ± 0.08
22:1n-9	0.25 ± 0.00 ^{d}	0.24 ± 0.01 ^{cd}	0.21 ± 0.01 ^{bc}	0.18 ± 0.00 ^{ab}	0.17 ± 0.01 ^{a}
∑MUFA	34.90 ± 0.31 ^{a}	37.12 ± 0.63 ^{b}	41.85 ± 0.30 ^{c}	43.74 ± 0.51 ^{c}	46.74 ± 0.37 ^{d}
18:2n-6	12.26 ± 0.28	12.27 ± 0.12	12.84 ± 0.19	12.58 ± 0.35	12.74 ± 0.25
18:3n-6	0.19 ± 0.01 ^{d}	0.17 ± 0.00 ^{cd}	0.15 ± 0.00 ^{bc}	0.13 ± 0.01 ^{ab}	0.13 ± 0.01 ^{a}
20:2n-6	0.79 ± 0.03	0.83 ± 0.03	0.84 ± 0.03	0.76 ± 0.02	0.78 ± 0.05
20:4n-6	0.12 ± 0.00 ^{b}	0.11 ± 0.00 ^{b}	0.09 ± 0.01 ^{a}	0.08 ± 0.01 ^{a}	0.07 ± 0.01 ^{a}
∑n-6 PUFA	13.35 ± 0.31	13.37 ± 0.14	13.91 ± 0.23	13.54 ± 0.37	13.72 ± 0.31
20:3n-3	0.58 ± 0.03 ^{b}	0.58 ± 0.05 ^{b}	0.51 ± 0.01 ^{ab}	0.46 ± 0.03 ^{ab}	0.42 ± 0.01 ^{a}
20:5n-3	5.96 ± 0.25 ^{c}	4.82 ± 0.11 ^{b}	4.29 ± 0.14 ^{b}	3.35 ± 0.23 ^{a}	2.74 ± 0.07 ^{a}
22:5n-3	4.14 ± 0.13 ^{c}	3.48 ± 0.08 ^{b}	3.42 ± 0.14 ^{b}	2.85 ± 0.02 ^{a}	2.45 ± 0.03 ^{a}
22:6n-3	7.12 ± 0.31 ^{c}	5.65 ± 0.04 ^{b}	4.95 ± 0.23 ^{b}	3.51 ± 0.04 ^{a}	2.73 ± 0.06 ^{a}
∑n-3 PUFA	17.78 ± 0.69 ^{d}	14.52 ± 0.25 ^{c}	13.17 ± 0.33 ^{c}	10.17 ± 0.23 ^{b}	8.34 ± 0.12 ^{a}
∑n-3/∑n-6	1.33 ± 0.02 ^{e}	1.09 ± 0.01 ^{d}	0.95 ± 0.03 ^{c}	0.75 ± 0.01 ^{b}	0.61 ± 0.01 ^{a}

Data in a same row not sharing a same superscript letter are significantly different ($p < 0.05$). TFA: total fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; n-6 PUFA: n-6 poly-unsaturated fatty acid; n-3 PUFA: n-3 polyunsaturated fatty acid.

Table 4. Fatty-acid compositions in the liver after fish oil-finishing (%TFA, mean ± standard error).

Fatty Acid	FO	25PO	50PO	75PO	PO
14:0	3.49 ± 0.11 ^{b}	3.28 ± 0.03 ^{b}	3.14 ± 0.12 ^{ab}	2.79 ± 0.10 ^{a}	2.84 ± 0.07 ^{a}
16:0	20.06 ± 0.20	19.44 ± 0.14	20.12 ± 0.30	20.10 ± 0.11	19.53 ± 0.21
18:0	9.52 ± 0.39	9.55 ± 0.16	9.72 ± 0.20	10.35 ± 0.37	9.35 ± 0.64
20:0	0.55 ± 0.01 ^{c}	0.50 ± 0.01 ^{bc}	0.46 ± 0.01 ^{ab}	0.47 ± 0.02 ^{abc}	0.40 ± 0.03 ^{a}
∑SFA	33.63 ± 0.47	32.76 ± 0.13	33.44 ± 0.42	33.71 ± 0.53	32.13 ± 0.77
16:1n-7	8.19 ± 0.21	8.02 ± 0.09	7.73 ± 0.21	7.32 ± 0.35	7.69 ± 0.11
18:1n-9	23.86 ± 0.39 ^{a}	25.22 ± 0.37 ^{ab}	27.04 ± 0.23 ^{bc}	28.54 ± 0.76 ^{cd}	29.94 ± 0.64 ^{d}
20:1n-9	1.95 ± 0.10	2.02 ± 0.04	1.74 ± 0.13	1.66 ± 0.06	1.85 ± 0.05
22:1n-9	0.24 ± 0.01 ^{b}	0.23 ± 0.00 ^{b}	0.21 ± 0.01 ^{ab}	0.22 ± 0.01 ^{b}	0.19 ± 0.01 ^{a}
∑MUFA	34.24 ± 0.23 ^{a}	35.49 ± 0.29 ^{ab}	36.73 ± 0.55 ^{bc}	37.74 ± 0.52 ^{cd}	39.67 ± 0.63 ^{d}
18:2n-6	11.89 ± 0.18	12.16 ± 0.06	11.96 ± 0.09	11.85 ± 0.11	12.22 ± 0.33
18:3n-6	0.18 ± 0.01 ^{c}	0.18 ± 0.00 ^{bc}	0.16 ± 0.01 ^{ab}	0.15 ± 0.00 ^{a}	0.16 ± 0.00 ^{ab}
20:2n-6	0.76 ± 0.02	0.81 ± 0.01	0.77 ± 0.01	0.79 ± 0.03	0.76 ± 0.01
20:4n-6	0.13 ± 0.01 ^{b}	0.12 ± 0.01 ^{ab}	0.10 ± 0.01 ^{ab}	0.10 ± 0.00 ^{ab}	0.09 ± 0.01 ^{a}
∑n-6 PUFA	12.96 ± 0.19	13.27 ± 0.06	12.99 ± 0.09	12.90 ± 0.14	13.23 ± 0.33
20:3n-3	0.61 ± 0.01	0.61 ± 0.03	0.55 ± 0.01	0.54 ± 0.01	0.55 ± 0.03
20:5n-3	5.75 ± 0.10 ^{c}	5.56 ± 0.07 ^{bc}	5.08 ± 0.21 ^{ab}	4.64 ± 0.05 ^{a}	4.72 ± 0.17 ^{a}
22:5n-3	4.23 ± 0.17 ^{b}	4.15 ± 0.07 ^{ab}	3.88 ± 0.20 ^{ab}	3.72 ± 0.09 ^{ab}	3.55 ± 0.09 ^{a}
22:6n-3	6.92 ± 0.22 ^{c}	6.60 ± 0.16 ^{c}	5.89 ± 0.06 ^{b}	5.32 ± 0.11 ^{ab}	5.07 ± 0.10 ^{a}
∑n-3 PUFA	17.51 ± 0.49 ^{c}	16.92 ± 0.14 ^{c}	15.40 ± 0.14 ^{b}	14.22 ± 0.08 ^{ab}	13.88 ± 0.25 ^{a}
∑n-3/∑n-6	1.35 ± 0.02 ^{d}	1.28 ± 0.00 ^{c}	1.19 ± 0.01 ^{b}	1.10 ± 0.01 ^{a}	1.05 ± 0.02 ^{a}

Data in a same row not sharing a same superscript letter are significantly different ($p < 0.05$). TFA: total fatty acid; SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; n-6 PUFA: n-6 poly-unsaturated fatty acid; n-3 PUFA: n-3 polyunsaturated fatty acid.

2.2. Volatile Flavor Components in the Muscle

From all muscle samples, a total of 61 volatile flavor components were detected, of which 37 were successfully identified (Figures 2 and 3, Table S1). Most of these compounds were small-molecular alcohols, ketones, or aldehydes. At the end of the growing-out period, the PO group had lower abundance of 1-butanol, 3-methyl butanol, 2-pentenal (E) dimer, (E)-2-hexenal monomer, (E)-2-hexenal dimer, 4-heptenal (Z), 3-pentanone, 1-octen-

3-ol monomer, 1-octen-3-ol dimer, and octanal dimer, but higher abundance of ethanol, 1-pentanol dimer, and pentanal than the FO control group. At the end of the FO-finishing period, the difference between the PO and FO groups was smaller, but obvious differences in ethanol, 2-pentenal (E) dimer, (E)-2-hexenal monomer, (E)-2-hexenal monomer, (E)-2-hexenal dimer, 4-heptenal (Z), and octanal dimer could still be observed.

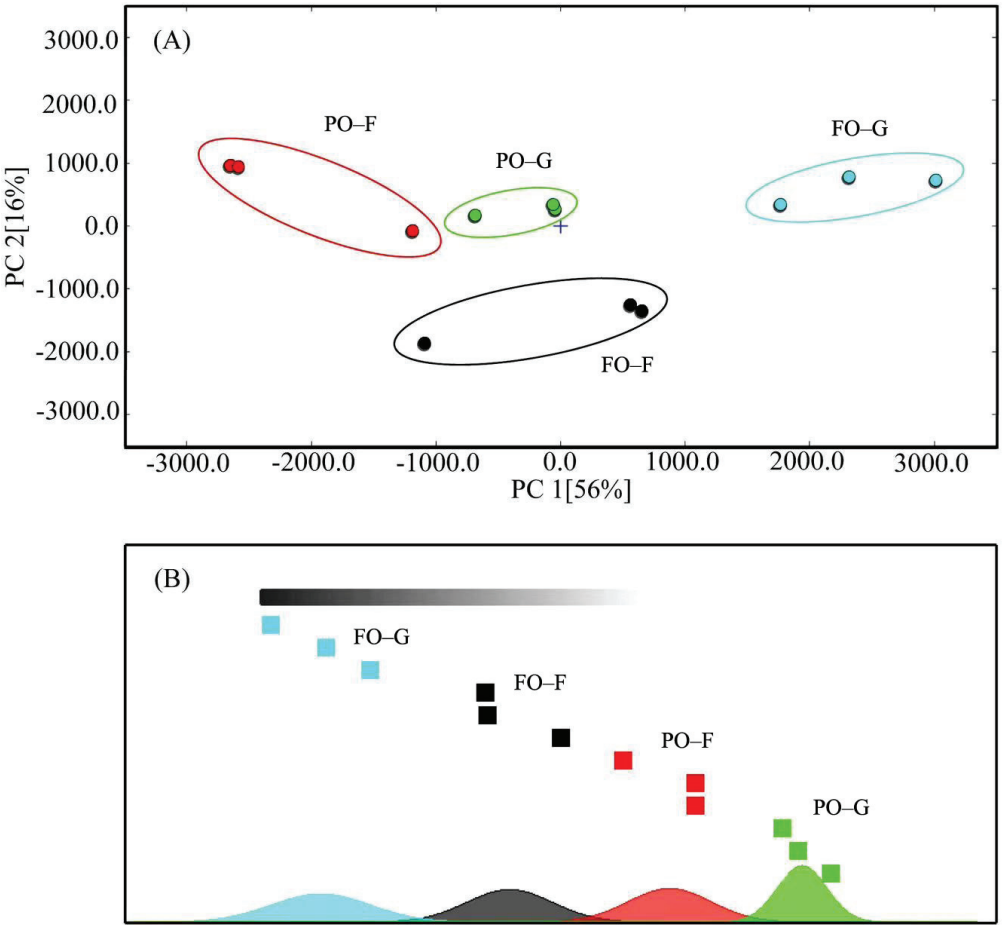


Figure 2. Principal component analysis (PCA) (**A**) and Euclidean distance (**B**) of volatile flavor compounds in the muscle. FO–G and PO–G: the fish oil and poultry oil group at the end of growing-out period, respectively; FO–F and PO–F: the fish oil and poultry oil group after fish oil-finishing, respectively.

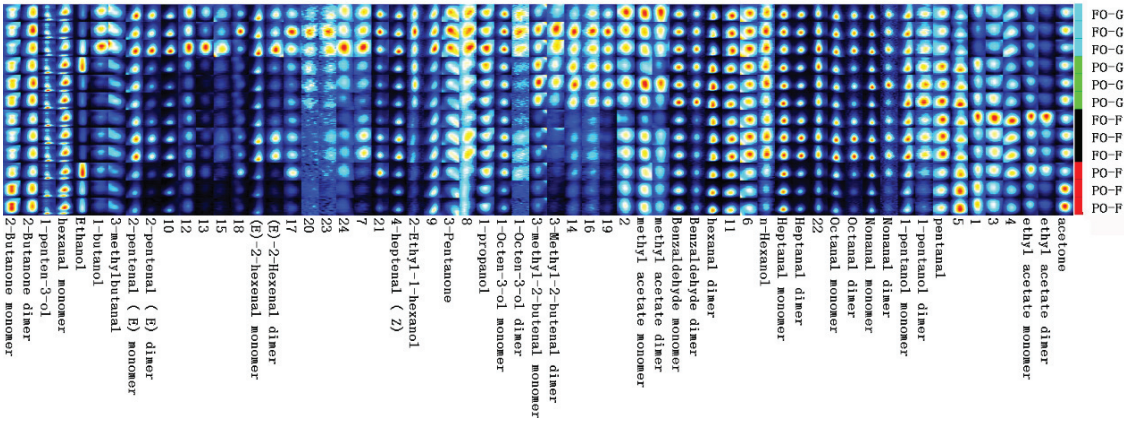


Figure 3. Gallery plot of the volatile flavor compounds in the muscle. The brightness indicates relative compound abundance. A column represents the signal peak of a certain volatile organic compound in different samples. A line represents all signal peaks of volatile organic compound selected from a certain sample. Compounds named as numbers were not successfully identified. The meanings of FO–G, PO–G, FO–F, and PO–F are the same as in Figure 3.

2.3. Determination of Muscle Odor with Electronic Nose

In general, the PCA (Figure S1) and LDA (Figure S2) in the muscle-odor analysis with electronic nose showed that at the end of the growing-out period, there was large distance between groups FO and PO, but this distance was substantially shortened at the end of the FO-finishing period.

3. Discussion

Poultry oil (PO) is increasingly used in fish feeds. However, a major concern of human consumers when fish oil (FO) is replaced by PO is the reduction of LC-PUFA in fish products. In the current study, increasing levels of dietary PO linearly decreased the n-3 LC-PUFA content in fish. At the end of the growing-out period, the muscle DHA content in the PO group was 70.6% of that in the FO control group. This ratio was higher than that observed in other fish species, namely, yellowtail kingfish (*Seriola lalandi*), 49.7% [6]; largemouth bass (*Micropterus salmoides*), 56.5% [7]; and European seabass (*Dicentrarchus labrax*), 30.5% [11]. The discrepancy could be mainly related to the fish species and most importantly, feeding duration. A longer period of PO feeding (12 weeks for largemouth bass; 16 weeks for European seabass) resulted in lower DHA content in fish muscle.

After FO-finishing, the DHA content in group PO was restored to be 85% of that in the FO control group. In most similar studies, an FO-finishing diet was able to restore the DHA content to between 70 and 90% of that in fish fed FO continuously [13]. However, in a study with smaller rainbow trout (initial body weight, 1.4 g), a 28-day FO-finishing period after 80 days of a growing-out feeding trial with PO completely restored the muscle DHA and EPA to levels comparable to those achieved via the continuous use of an FO-based diet [15]. Fish size is a clear determinant in the rate of fatty-acid turnover. Smaller fish have a higher rate of fatty-acid turnover and thus have higher capacity for restoring LC-PUFA. In another study on FO-finishing after PO feeding (50% FO replaced), 56 days of FO-finishing after 140 days of growing-out restored the muscle DHA, EPA, and ARA in sunshine bass to be 94%, 92%, and 100% of that in the FO control group, respectively [14]. These ratios were higher than those in the present study. It is clear that the duration of the finishing period is one of the most important factors influencing the final muscle LC-PUFA content. A longer finishing period resulted in higher restoration ratio of muscle LC-PUFA.

There are many studies regarding the use of an FO-finishing strategy after feeding diets based on other alternative oils such as rapeseed oil, palm oil, soybean oil, linseed

oil, coconut oil, sunflower oil, and olive oil [14,26–30]. Overall, the effect of alternative oil type on the efficiency of LC-PUFA recovery during the subsequent FO-finishing period appears to be complicated and species-specific. Relevant results in farmed fish species have been summarized in a recent review article [13]. In other marine fish species such as gilthead sea bream (*Sparus aurata*) and European sea bass, differences were observed in the degree of LC-PUFA recovery during the FO-finishing period when different alternative lipid sources such as rapeseed oil, soybean oil, and linseed oil were previously fed to fish [26–28]. Specific to tiger puffer, a previous study revealed that washing-out the PO in the muscle was easier compared to soybean oil, but more difficult compared to beef tallow [24]. In the liver, however, washing-out the PO was easier compared to rapeseed oil, but also more difficult compared to beef tallow. In another study regarding washing-out PO, it was found that washing-out the PO in sunshine bass was more difficult compared to washing-out grape oil and linseed oil [14]. The LC-PUFA restoration efficiency could be closely related to the efficiency of washing-out the characteristic fatty acids in different terrestrially-sourced oils. However, to date, no clear conclusion has been made by previous studies regarding the difference in washing-out efficiency among different individual fatty acids or different fatty-acid types. In addition, different terrestrially-sourced oils may lead to different lipid deposition levels in fish body, which could also affect the LC-PUFA restoration efficiency.

For tiger puffer, liver is also an edible part, particularly considering that nowadays tetrodotoxin is nearly undetectable in farmed fish [31]. Therefore, the restoration of LC-PUFA in the liver should also not be neglected when the FO-finishing strategy is practiced. Compared to muscle, the LC-PUFA restoration efficiency in the liver after FO-finishing was lower. This may be due to the fact that a limited number of fatty acids, such as DHA, EPA, and 16:0, tend to be selectively incorporated into muscle which is rich in polar lipids [32]. In other tissues with conservative fatty-acid profiles such as the neural tissue, in which LC-PUFA is vital for chemical communication/homeostasis, the LC-PUFA restoration efficiency could also be high, although no tissues other than muscle and liver were analyzed in this study.

Regarding the difference in efficiency of LC-PUFA restoration among individual fatty acids, EPA showed a higher efficiency than DHA in both muscle and liver of tiger puffer. This was different from most other similar studies, which showed that DHA is generally more completely restored, compared to EPA [13]. The present result could be related to the fact that the EPA content in tiger puffer is much lower than DHA, and thus is easier to be restored.

In addition to fatty-acid composition, the volatile-flavor-compound compositions also largely contribute to the fish-fillet quality. The identified volatile flavor compound in tiger puffer mainly consists of aldehyde, alcohol, ketone, phenol, and heterocyclic compounds containing nitrogen and sulfur. The volatile flavor compounds differentially abundant between the FO control group and the PO group were mainly flavor aldehydes and alcohols. Aldehydes have a lower odor threshold and a greater flavor effect on aquatic products, but alcohols have a higher odor threshold and a smaller contribution to flavor.

Many of the flavor aldehydes such as heptanal, octanal, and nonanal are products of the oxidation of oleic and linoleic acids [33–36]. At the end of the growing-out period, the volatile flavor compound profile was clearly different between the FO and PO groups. The high abundance of 1-pentanol dimer in the PO group may result in the flavors of mushroom, earth, and wax, which are generally considered "off tastes" in sensory-panel testing. The PO group also had higher abundance of pentanal, which could be derived from the oxidation of n-6 PUFA, and has almond, pungent, and malty flavors [35]. In contrast, in the muscle of the FO control group, the higher abundance of butanol, 2-pentenal, (E)-2-hexenal, and 4-heptenal (Z) would generate more flavor of butter, fruit (apple, orange, banana), cheese, boiled potato, and cooked fish. These compounds could also make the fillets taste sweeter and fattier. The adverse effects of PO supplementation on the volatile-flavor-compound profile of muscle were largely, but not completely, mitigated by the

FO-finishing. At the end of the FO-finishing period, obvious differences in ethanol, 2-pentenal (E) dimer, (E)-2-hexenal monomer, (E)-2-hexenal monomer, (E)-2-hexenal dimer, 4-heptenal (Z), and octanal dimer could still be observed. In particular, after FO finishing, the PO group still had a lower abundance of heptanal, octanal, and hexanol than the FO control group. Heptanal and octanal usually have fatty, dry fish, grass, and fruit flavors. Hexanol arises from lipoxygenase and hydroperoxide-lyase activities and has the taste of grass [37]. Lower abundance of these compounds indicates that the muscle from the PO group may have had less fishy flavor but also less grass flavor. In addition, for the FO control group, the abundance of many volatile compounds at week 8 was lower than that at week 12, indicating the decrease of volatile compounds with fish growth. Similar results were found in Atlantic salmon (*Salmo salar*) [38].

4. Materials and Methods

4.1. Experimental Diets

Five experimental diets were formulated. Fish oil (FO) was used as the sole added oil in the control diet. In other diets, added FO was replaced by poultry oil (PO, refined from duck skin, Shandong Haiding Agriculture and Animal Husbandry Co., Ltd., Jinan, China) at different levels, namely, 25%, 50%, 75%, and 100%. The five experimental diets were designated as FO (control), 25PO, 50PO, 75PO, and PO, respectively (Tables 5 and 6). The pellet feeds with a diameter of 2 mm were made with a laboratory-level single-screw pelleting machine and dried at 55 °C. The experimental diets were stored at −20 °C prior to use. The proximate composition analysis of experimental diets was performed according to the standard methods of the Association of Official Analytical Chemists (AOAC). In brief, the moisture content was measured by drying the samples to a constant weight at 105 °C; the protein content was assayed by measuring nitrogen content (N × 6:25) using the Kjeldahl method; the lipid content was assayed with petroleum ether extraction using the Soxhlet method; and the ash content was measured by incineration in a muffle furnace at 550 °C for 8 h.

Table 5. Formulation and proximate composition of the experimental diets (% dry matter basis).

Ingredients	FO	25PO	50PO	75PO	PO
Fish meal	42	42	42	42	42
Corn gluten meal	8	8	8	8	8
Soybean meal	14	14	14	14	14
Wheat meal	20.68	20.68	20.68	20.68	20.68
Brewer's yeast	5	5	5	5	5
Mineral premix [1]	0.5	0.5	0.5	0.5	0.5
Vitamin premix [1]	1	1	1	1	1
Monocalcium phosphate	1	1	1	1	1
L-ascorbyl-2-polyphosphate	0.2	0.2	0.2	0.2	0.2
Choline chloride	0.2	0.2	0.2	0.2	0.2
Betaine	0.3	0.3	0.3	0.3	0.3
Ethoxyquin	0.02	0.02	0.02	0.02	0.02
Mold inhibitor [2]	0.1	0.1	0.1	0.1	0.1
Soya lecithin	1	1	1	1	1
Fish oil [3]	6	4.5	3	1.5	0
Poultry oil	0	1.5	3	4.5	6
Proximate composition					
Crude protein	45.40	45.84	45.65	46.26	46.23
Crude lipid	9.31	10.06	9.99	10.08	9.89
Ash	9.41	9.47	9.56	9.56	9.47

[1] Mineral premix and vitamin premix, designed for marine fish, were purchased from Qingdao Master Biotech Co., Ltd., Qingdao, China. [2] Contained 50% calcium propionic acid and 50% fumaric acid. [3] Fish oil, purchased from Qingdao Surgreen Bioengineering Co., Ltd., is on-board processed anchovy oil.

Table 6. Fatty acid composition of fish oil, poultry oil, and experimental diets (%TFA).

Fatty Acid	Oil		Diet				
	Fish Oil	Poultry Oil	FO	25PO	50PO	75PO	PO
14:0	5.33	0.58	5.55	4.76	4.06	3.35	2.69
16:0	18.60	26.61	21.24	22.32	22.99	23.64	24.99
18:0	4.58	5.60	4.58	4.76	4.79	4.76	4.94
∑SFA	28.51	32.79	31.37	31.84	31.84	31.75	32.62
16:1n-7	5.34	2.91	6.17	5.74	5.15	4.75	4.60
18:1n-9	16.12	44.30	15.12	18.76	22.11	25.51	29.73
20:1n-9	1.48	0.42	0.97	0.85	0.71	0.57	0.46
∑MUFA	22.94	47.63	22.25	25.35	27.97	30.84	34.80
18:2n-6	12.28	15.11	13.25	14.20	14.41	14.63	15.11
20:2n-6	0.22	0.12	0.22	0.22	0.19	0.18	0.17
20:4n-6	0.55	0.21	0.68	0.69	0.60	0.58	0.52
22:2n-6	0.38	ND	0.33	0.28	0.24	0.17	0.12
∑n-6 PUFA	13.43	15.44	14.48	15.38	15.45	15.56	15.92
18:3n-3	1.69	0.69	1.52	1.38	1.28	1.12	1.03
20:5n-3	8.15	0.06	9.66	8.38	7.24	6.07	5.11
22:5n-3	0.82	0.03	1.17	1.05	0.98	0.85	0.77
22:6n-3	8.97	0.02	7.36	6.17	5.24	4.02	2.95
∑n-3 PUFA	19.63	0.80	19.70	16.98	14.74	12.06	9.87
∑n-3/∑n-6	1.46	0.05	1.36	1.10	0.95	0.77	0.62

TFA: total fatty acid; SFA: saturated fatty acid; MUFA: mono-unsaturated fatty acid; n-6 PUFA: n-6 poly-unsaturated fatty acid; n-3 PUFA: n-3 polyunsaturated fatty acid; ND: nondetectable.

4.2. Feeding Procedure and Sampling

Tiger puffer juveniles (average initial body weight, 12.3 ± 0.5 g; average body length, 6.5 ± 0.5 cm) were purchased from Hongqi Modern Fishery Industrial Park (Rizhao, China), and transported to the Yellow Sea Aquaculture Co., Ltd. (Yantai, China), where the feeding trial was conducted. At the beginning of the experiment, 600 randomly selected healthy fish were divided into 15 polyethylene tanks (0.7 × 0.7 × 0.4 m). Each tank was stocked with 40 fish. Each diet was randomly fed to triplicate tanks. Fish were hand-fed to apparent satiation three times daily (7:00, 12:00, and 18:00). The feeding trial lasted 12 weeks (Figure 4). Following the first 8-week growing-out period, during which the five experiment diets were normally fed to experimental fish, was a four-week FO-finishing period, during which fish in all five groups were fed the FO control diet.

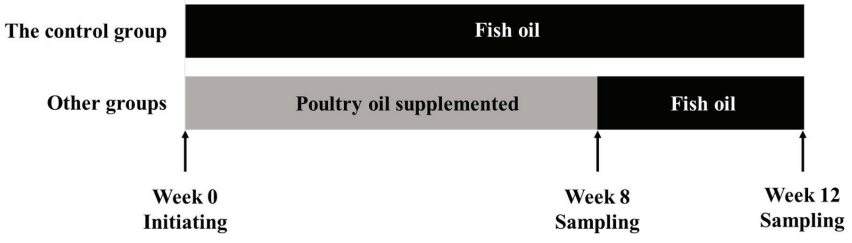


Figure 4. Experimental design and feeding periods.

Sampling was conducted at the end of both the growing-out period (week 8) and the FO-finishing period (week 12). Before sampling, fish were fasted for 24 h. In each sampling point, four fish were randomly selected from each tank, and the muscle and liver samples were collected. The samples were immediately frozen with liquid nitrogen and then stored at −76 °C before use. All sampling protocols, as well as all fish rearing practices, were reviewed and approved by the Animal Care and Use Committee of Yellow Sea Fisheries Research Institute.

4.3. Analysis of Fatty-Acid Composition

The fatty-acid compositions of oils, diets, muscles, and livers were analyzed with gas chromatography, as previously described [39]. Lipids in the samples were firstly extracted

with the chloroform methanol method. Fatty acids in the extracted oil were then saponified and methylated with KOH-methanol and HCL-methanol. Gas chromatograph (GC-2010 pro, Shimadzu, Kyoto, Japan) equipped with a quartz capillary column (SH-RT-2560, 100 m × 0.25 mm × 0.20 um) and a flame ionization detector was used in the analysis. The results are expressed as percentage of each fatty acid relative to the total fatty acids (%TFA).

4.4. Analysis of Volatile Organic Compounds in the Muscle

Two typical groups, FO and PO, was subjected to the analysis of volatile organic compounds in the muscle, which was conducted with gas chromatography−ion migration spectrometry (GC-IMS). A FlavourSpec® (G.A.S, Dordmund, Germany) platform equipped with a MXT-5 column (RESTEK, Bellefonte, PA, USA; 15 m × 0.53 mm × 1.0 μm) was used in this analysis. The column and IMS temperatures were 60 and 45 °C, respectively. High-purity nitrogen (purity, 99.999%) was used as the carrier gas. Three grams muscle samples were accurately weighed and placed in a 20 mL vial. The samples were incubated at 60 °C (500 r/min) for 15 min. The injection volume was 500 μL, and the temperature of the automatic injection needle was 85 °C. Built-in software VOCal and three tools including Reporter, Gallery Plot, and Dynamic PCA were used in the result report.

4.5. Electronic-Nose Analysis

The odor analysis for the muscle samples from groups FO and PO was performed with a PEN-3 portable electronic nose (Airsense, Schwerin, Germany). One gram of muscle sample was weighed and placed it in a 20 mL vial with lid and sealed. The samples were then heated at 50 °C in a water bath for 10 min, and finally subjected to electronic-nose analysis. Winmuster software was used for data collection and processing, generating results of principal component analysis (PCA) and linear discrimination method (LDA). Ten different metal-oxide sensors (W1C, benzene; W5S, nitrogen oxide; W3C, amine; W6S, hydride; W5C, short alkane; W1S, methyl; W1W, inorganic sulfide; W2S, alcohols; W2W, organic sulfide; and W3S, long chain alkanes) were used to detect the main volatile compounds in the samples.

4.6. Statistical Analyses

All data were analyzed by one-way ANOVA in SPSS 16.0. Multiple comparisons were performed using Tukey's test, and the significance level was decided when $p < 0.05$. The results are expressed as mean ± standard error.

5. Conclusions

In conclusion, results of this study suggested that the adverse effects of dietary poultry oil on fatty acid and volatile flavor compound compositions of farmed tiger puffer can be largely mitigated by a fish-oil-finishing strategy. However, despite the high efficiency of the fish-oil-finishing strategy in this study, the efficiency when there is a very long period of poultry-oil feeding remains unknown and warrants further study.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/md21020122/s1, Table S1: Volatile flavor compounds detected in the muscle; Figure S1: Principal component analysis (PCA) of response values in the analysis with electronic nose; Figure S2: Linear discriminant analysis (LDA) of response values in the analysis with electronic nose.

Author Contributions: Methodology, data curation, and writing—original draft, L.L.; data curation and formal analysis, F.Z. and X.M.; methodology, X.C.; software and formal analysis, Q.M. and Y.W.; funding acquisition and conceptualization, M.L.; conceptualization, funding acquisition, and writing—review and editing, H.X. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by Central Public-Interest Scientific Institution Basal Research Fund, CAFS/YSFRI (grant numbers 2022XT0403, 2020TD48, and 20603022022004), the Natural Science

Foundation of Shandong Province Outstanding Youth Foundation (grant number ZR2021YQ24), and China Agriculture Research System (grant number CARS-47).

Institutional Review Board Statement: The animal-study protocol was approved by the Animal Care and Use Committee of Yellow Sea Fisheries Research Institute (protocol code ACUC202105234235; date of approval, 23 May 2021).

Data Availability Statement: The data that support the findings of this study are included in the text and in the tables. Raw data for all figures are available from the corresponding author upon reasonable request.

Acknowledgments: We would like to thank Yingming Yang for his help in fish-rearing.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Alagawany, M.; Elnesr, S.S.; Farag, M.R.; El-Sabrout, K.; Alqaisi, O.; Dawood, M.A.O.; Soomro, H.; Abdelnour, S.A. Nutritional significance and health benefits of omega-3,-6 and-9 fatty acids in animals. *Anim. Biotechnol.* **2020**, *33*, 1678–1690. [CrossRef] [PubMed]
2. Tveteras, S.; Tveteras, R. The Global Competition for Wild Fish Resources between Livestock and Aquaculture. *J. Agric. Econ.* **2010**, *61*, 381–397. [CrossRef]
3. Rosenlund, G.; Obach, A.; Sandberg, M.G.; Standal, H.; Tveit, K. Effect of alternative lipid sources on long-term growth performance and quality of Atlantic salmon (*Salmo salar* L.). *Aquac. Res.* **2001**, *32*, 323–328. [CrossRef]
4. Liu, K.K.M.; Barrows, F.T.; Hardy, R.W.; Dong, F.M. Body composition, growth performance, and product quality of rainbow trout (*Oncorhynchus mykiss*) fed diets containing poultry fat, soybean/corn lecithin, or menhaden oil. *Aquaculture* **2004**, *238*, 309–328. [CrossRef]
5. Xue, M.; Luo, L.; Wu, X.; Ren, Z.; Gao, P.; Yu, Y.; Pearl, G. Effects of six alternative lipid sources on growth and tissue fatty acid composition in Japanese sea bass (*Lateolabrax japonicus*). *Aquaculture* **2006**, *260*, 206–214. [CrossRef]
6. Bowyer, J.N.; Qin, J.; Smullen, R.P.; Stone, D.A.J. Replacement of fish oil by poultry oil and canola oil in yellowtail kingfish (*Seriola lalandi*) at optimal and suboptimal temperatures. *Aquaculture* **2012**, *356*, 211–222. [CrossRef]
7. Yun, B.; Xue, M.; Wang, J.; Fan, Z.; Wu, X.; Zheng, Y.; Qin, Y. Effects of lipid sources and lipid peroxidation on feed intake, growth, and tissue fatty acid compositions of largemouth bass (*Micropterus salmoides*). *Aquac. Int.* **2012**, *21*, 97–110. [CrossRef]
8. Ahmad, W.A.R.W.; Stone, D.A.J.; Schuller, K.A. Dietary fish oil replacement with palm or poultry oil increases fillet oxidative stability and decreases liver glutathione peroxidase activity in barramundi (*Lates calcarifer*). *Fish Physiol. Biochem.* **2013**, *39*, 1631–1640. [CrossRef] [PubMed]
9. Friesen, E.; Balfry, S.K.; Skura, B.J.; Ikonomou, M.; Higgs, D.A. Evaluation of poultry fat and blends of poultry fat with cold-pressed flaxseed oil as supplemental dietary lipid sources for juvenile sablefish (*Anoplopoma fimbria*). *Aquac. Res.* **2013**, *44*, 300–316. [CrossRef]
10. Rombenso, A.N.; Trushenski, J.T.; Schwarz, M.H. Fish oil replacement in feeds for juvenile Florida Pompano: Composition of alternative lipid influences degree of tissue fatty acid profile distortion. *Aquaculture* **2016**, *458*, 177–186. [CrossRef]
11. Campos, I.; Matos, E.; Maia, M.R.G.; Marques, A.; Valente, L.M.P. Partial and total replacement of fish oil by poultry fat in diets for European seabass (*Dicentrarchus labrax*) juveniles: Effects on nutrient utilization, growth performance, tissue composition and lipid metabolism. *Aquaculture* **2019**, *502*, 107–120. [CrossRef]
12. Carvalho, M.; Montero, D.; Rosenlund, G.; Fontanillas, R.; Gines, R.; Izquierdo, M. Effective complete replacement of fish oil by combining poultry and microalgae oils in practical diets for gilthead sea bream (*Sparus aurata*) fingerlings. *Aquaculture* **2020**, *529*, 735696. [CrossRef]
13. Xu, H.; Turchini, G.M.; Francis, D.S.; Liang, M.; Mock, T.S.; Rombenso, A.; Ai, Q. Are fish what they eat? A fatty acid's perspective. *Prog. Lipid Res.* **2020**, *80*, 101064. [CrossRef]
14. Trushenski, J.T.; Lewis, H.A.; Kohler, C.C. Fatty acid profile of sunshine bass: I. Profile change is affected by initial composition and differs among tissues. *Lipids* **2008**, *43*, 629–641. [CrossRef] [PubMed]
15. Pares-Sierra, G.; Durazo, E.; Antonio Ponce, M.; Badillo, D.; Correa-Reyes, G.; Teresa Viana, M. Partial to total replacement of fishmeal by poultry by-product meal in diets for juvenile rainbow trout (*Oncorhynchus mykiss*) and their effect on fatty acids from muscle tissue and the time required to retrieve the effect. *Aquac. Res.* **2014**, *45*, 1459–1469. [CrossRef]
16. Liu, Y.; Jiao, J.; Shuang, G.; Li, J.; Limbu, S.M.; Fang, Q.; Li, Q.; Mei, L.; Zhen, Y. Dietary oils modify lipid molecules and nutritional value of fillet in Nile tilapia: A deep lipidomics analysis. *Food Chem.* **2019**, *277*, 515–523. [CrossRef] [PubMed]
17. Grigorakis, K.; Fountoulaki, E.; Giogios, I.; Alexis, M.N. Volatile compounds and organoleptic qualities of gilthead sea bream (*Sparus aurata*) fed commercial diets containing different lipid sources. *Aquaculture* **2009**, *290*, 116–121. [CrossRef]
18. Moreira, N.; Soares, S.; Valente, L.M.P.; Castro-Cunha, M.; Cunha, L.M.; Guedes de Pinho, P. Effect of two experimental diets (protein and lipid vegetable oil blends) on the volatile profile of Senegalese sole (*Solea senegalensis* Kaup, 1858) muscle. *Food Chem.* **2014**, *153*, 327–333. [CrossRef]

19. Serot, T.; Regost, C.; Prost, C.; Robin, J.; Arzel, J. Effect of dietary lipid sources on odour-active compounds in muscle of turbot (*Psetta maxima*). *J. Sci. Food Agric.* **2001**, *81*, 1339–1346. [CrossRef]
20. Timm-Heinrich, M.; Eymard, S.; Baron, C.P.; Nielsen, H.H.; Jacobsen, C. Oxidative changes during ice storage of rainbow trout (*Oncorhynchus mykiss*) fed different ratios of marine and vegetable feed ingredients. *Food Chem.* **2013**, *136*, 1220–1230. [CrossRef]
21. Turchini, G.M.; Mentasti, T.; Caprino, F.; Panseri, S.; Moretti, V.M.; Valfrè, F. Effects of dietary lipid sources on flavour volatile compounds of brown trout (*Salmo trutta* L.) fillet. *J. Appl. Ichthyol.* **2004**, *20*, 71–75. [CrossRef]
22. Turchini, G.M.; Moretti, V.M.; Mentasti, T.; Orban, E.; Valfrè, F. Effects of dietary lipid source on fillet chemical composition, flavour volatile compounds and sensory characteristics in the freshwater fish tench (*Tinca tinca* L.). *Food Chem.* **2007**, *102*, 1144–1155. [CrossRef]
23. Zhou, L.; Han, D.; Zhu, X.; Yang, Y.; Jin, J.; Xie, S. Effects of total replacement of fish oil by pork lard or rapeseed oil and recovery by a fish oil finishing diet on growth, health and fish quality of gibel carp (*Carassius auratus gibelio*). *Aquac. Res.* **2016**, *47*, 2961–2975. [CrossRef]
24. Liao, Z.; Sun, Z.; Bi, Q.; Gong, Q.; Sun, B.; Wei, Y.; Liang, M.; Xu, H. Application of the fish oil-finishing strategy in a lean marine teleost, tiger puffer (*Takifugu rubripes*). *Aquaculture* **2021**, *534*, 736306. [CrossRef]
25. Li, L.; Zhang, F.; Meng, X.; Cui, X.; Ma, Q.; Wei, Y.; Liang, M.; Xu, H. Fish oil replacement with poultry oil in the diet of tiger puffer (*Takifugu rubripes*): Effects on growth performance, body composition, and lipid metabolism. *Aquac. Nutr.* **2022**, *2022*, 2337933. [CrossRef]
26. Montero, D.; Robaina, L.; Caballero, M.J.; Gines, R.; Izquierdo, M.S. Growth, feed utilization and flesh quality of European sea bass (*Dicentrarchus labrax*) fed diets containing vegetable oils: A time-course study on the effect of a re-feeding period with a 100% fish oil diet. *Aquaculture* **2005**, *248*, 121–134. [CrossRef]
27. Turchini, G.M.; Francis, D.S.; De Silva, S.S. Modification of tissue fatty acid composition in Murray cod (*Maccullochella peelii peelii*, Mitchell) resulting from a shift from vegetable oil diets to a fish oil diet. *Aquac. Res.* **2006**, *37*, 570–585. [CrossRef]
28. Fountoulaki, E.; Vasilaki, A.; Hurtado, R.; Grigorakis, K.; Karacostas, I.; Nengas, I.; Rigos, G.; Kotzamanis, Y.; Venou, B.; Alexis, M.N. Fish oil substitution by vegetable oils in commercial diets for gilthead sea bream (*Sparus aurata* L.); effects on growth performance, flesh quality and fillet fatty acid profile: Recovery of fatty acid profiles by a fish oil finishing diet under fluctuating water temperatures. *Aquaculture* **2009**, *289*, 317–326. [CrossRef]
29. Karanth, S.; Sharma, P.; Pal, A.K.; Venkateshwarlu, G. Effect of different vegetable oils on growth and fatty acid profile of rohu (*Labeo rohita*, Hamilton); evaluation of a return fish oil diet to restore human cardio-protective fatty acids. *Asian-Australas. J. Anim. Sci.* **2009**, *22*, 565–575. [CrossRef]
30. Ng, W.K.; Chong, C.Y.; Wang, Y.; Romano, N. Effects of dietary fish and vegetable oils on the growth, tissue fatty acid composition, oxidative stability and vitamin E content of red hybrid tilapia and efficacy of using fish oil finishing diets. *Aquaculture* **2013**, *372*, 97–110. [CrossRef]
31. Katikou, P.; Gokbulut, C.; Kosker, A.R.; Campas, M.; Ozogul, F. An updated review of tetrodotoxin and its peculiarities. *Mar. Drugs* **2022**, *20*, 47. [CrossRef] [PubMed]
32. Sargent, J.R.; Tocher, D.R.; Bell, J.G. The lipids. In *Fish Nutrition*, 3rd ed.; Halver, J.E., Hardy, R.W., Eds.; Academic Press: Pittsburgh, PA, USA, 2002; Volume 4, pp. 181–257. ISBN 978-0-12-319652-1.
33. De Quirós, A.R.-B.; López-Hernández, J.; González-Castro, M.; De la Cruz-Garcia, C.; Simal-Lozano, J. Comparison of volatile components in fresh and canned sea urchin (*Paracentrotus lividus*, Lamarck) gonads by GC-MS using dynamic headspace sampling and microwave desorption. *Eur. Food Res. Technol.* **2001**, *212*, 643–647. [CrossRef]
34. Tao, N.; Wu, R.; Zhou, P.; Gu, S.; Wu, W. Characterization of odor-active compounds in cooked meat of farmed obscure puffer (*Takifugu obscurus*) using gas chromatography-mass spectrometry-olfactometry. *J. Food Drug Anal.* **2014**, *22*, 431–438. [CrossRef] [PubMed]
35. Mu, H.; Wei, Z.; Yi, L.; Liang, H.; Zhao, L.; Zhang, W.; Mai, K. Dietary fishmeal levels affect the volatile compounds in cooked muscle of farmed large yellow croaker *Larimichthys Crocea*. *Aquac. Res.* **2017**, *48*, 5821–5834. [CrossRef]
36. Wu, J.; Chen, X.; Chen, B.; Pan, N.; Qiao, K.; Wu, G.; Liu, Z. Collaborative analysis combining headspace-gas chromatography-ion mobility spectrometry (HS-GC-IMS) and intelligent (electronic) sensory systems to evaluate differences in the flavour of cultured pufferfish. *Flavour Fragr. J.* **2020**, *36*, 182–189. [CrossRef]
37. Salum, P.; Guclu, G.; Selli, S. Comparative evaluation of key aroma-active compounds in raw and cooked red mullet (*Mullus barbatus*) by aroma extract dilution analysis. *J. Agric. Food Chem.* **2017**, *65*, 8402–8408. [CrossRef] [PubMed]
38. Duan, Z.; Dong, S.; Sun, Y.; Dong, Y.; Gao, Q. Response of Atlantic salmon (*Salmo salar*) flavor to environmental salinity while culturing between freshwater and seawater. *Aquaculture* **2021**, *530*, 735953. [CrossRef]
39. Meng, X.; Bi, Q.; Cao, L.; Ma, Q.; Wei, Y.; Duan, M.; Liang, M.; Xu, H. Evaluation of necessity of cholesterol supplementation in diets of two marine teleosts, turbot (*Scophthalmus maximus*) and tiger puffer (*Takifugu rubripes*): Effects on growth and lipid metabolism. *Aquac. Nutr.* **2022**, *2022*, 4160991. [CrossRef]

marine drugs

MDPI

Article

Fatty Acids in Waste Tissues: The Nutraceutical Value of Gonads and Livers from the Moroccan *Hypophthalmichthys molitrix* and *Cyprinus carpio* Fishes

Giuseppina Tommonaro [1], Debora Paris [1], Giulia Guerriero [2,*], Fatima-Zahra Majdoubi [3], Gaetano Grieco [2,3], Carmine Iodice [1], Lucio Caso [1], Anouar Ouizgane [3], Aziz El Moujtahid [3,4], Sara El Ghizi [3], Meriem Bousseba [3], Mustapha Hasnaoui [3], Annalaura Iodice [1] and Annabella Tramice [1,*]

[1] National Research Council-Institute of Biomolecular Chemistry CNR-ICB, 80078 Pozzuoli, Italy; gtommonaro@icb.cnr.it (G.T.); dparis@icb.cnr.it (D.P.); ciodice@icb.cnr.it (C.I.); lucio.caso@icb.cnr.it (L.C.); annalaura94@live.com (A.I.)

[2] Comparative Endocrinology Laboratories (EClab), Department of Biology, University of Naples Federico II, 80126 Naples, Italy; grieco.eclab@gmail.com

[3] Environmental, Ecological and Agro-Industrial Engineering Laboratory (LGEEAI), Department of Biology, Faculty of Sciences and Techniques, University of Sultan Moulay Slimane, Beni Mellal 23000, Morocco; f.zmajdoubi@gmail.com (F.-Z.M.); az.elmoujtahid@gmail.com (A.E.M.); sara.elg2014@gmail.com (S.E.G.); meriembousseba@gmail.com (M.B.); m.hasnaoui@usms.ma (M.H.)

[4] Deraoua Fisheries Farm, National Hydrobiology and Fisheries Center, National Agency for Water and Forests, Rabat-Chellah 10002, Morocco

* Correspondence: giulia.guerriero@unina.it (G.G.); atramice@icb.cnr.it (A.T.)

Abstract: Fishes are an important component of human nutrition, mainly acting as source of essential fatty acids in the prevention of cardiovascular disorders. The increase in their consumption has led to a growth of fishes waste; therefore, the disposal and recycling of waste has become a key issue to address, in accordance with circular economy principles. The Moroccan *Hypophthalmichthys molitrix* and *Cyprinus carpio* fishes, living in freshwater and marine environments, were collected at mature and immature stages. The fatty acid (FA) profiles of liver and ovary tissues were investigated by GC–MS and compared with edible fillet tissues. The gonadosomatic index, the hypocholesterolemic/hypercholesterolemic ratio, and the atherogenicity and thrombogenicity indexes were measured. Polyunsaturated fatty acids were found to be abundant in the mature ovary and fillet of both species, with a polyunsaturated fatty acids/saturated fatty acids ratio ranging from 0.40 to 1.06 and a monounsaturated fatty acids/polyunsaturated fatty acids ratio between 0.64 and 1.84. Saturated fatty acids were found to be highly abundant in the liver and gonads of both species (range 30–54%), as well as monounsaturated fatty acids (range 35–58%). The results suggested that the exploitation of fish wastes, such as the liver and ovary, may represent a sustainable strategy for the achievement of high value-added molecules with nutraceutical potential.

Keywords: *Hypophthalmichthys molitrix*; *Cyprinus carpio*; gonadosomatic index (GSI); fatty acids profile (FA); hypocholesterolemic/hypercholesterolemic ratio (h/H); atherogenicity index (AI); thrombogenicity index (TI)

Citation: Tommonaro, G.; Paris, D.; Guerriero, G.; Majdoubi, F.-Z.; Grieco, G.; Iodice, C.; Caso, L.; Ouizgane, A.; El Moujtahid, A.; El Ghizi, S.; et al. Fatty Acids in Waste Tissues: The Nutraceutical Value of Gonads and Livers from the Moroccan *Hypophthalmichthys molitrix* and *Cyprinus carpio* Fishes. *Mar. Drugs* **2023**, *21*, 188. https://doi.org/10.3390/md21030188

Academic Editor: Natalia V. Ivanchina

Received: 7 February 2023
Revised: 13 March 2023
Accepted: 15 March 2023
Published: 17 March 2023

1. Introduction

Fish represents an important food source for the countries bordering the Mediterranean, providing bioactive molecules such as fats, proteins, vitamins and minerals which are essential for human health [1,2]. A diet rich in fish promotes cardiovascular health, improves eyesight, prevents arthritis, diabetes, and cancer, protects the brain from diseases, and helps in weight loss [3]. Due to the key role of fish in protecting human health and ensuring a balanced diet, fisheries and aquaculture activities are being increasingly recognized for their essential contribution to global food security and nutrition in the twenty-first

century. Aquatic foods remain some of the most traded food commodities in the world, with 225 states and territories reporting some trading activity of fisheries and aquaculture products [4].

Although aquaculture is a limited activity in Morocco—with a low production not exceeding 0.1% of the national fish production—and in the Mediterranean area in general, recently, Morocco and the EU launched a project to strengthen the Moroccan aquaculture sector. In accordance with the objectives of the National Agency for the Development of Aquaculture (ANDA), the goal of this cooperation is centered on the diversification of the fish supply of Morocco and on increasing the fish yield of the country by about 199,000 tons per year [5]. The nine species currently cultured in Morocco belong to six major families of cold water and hot water: *Cyprinidae*, *Salmonidae*, *Esocidae*, *Centrarchidae*, *Cichlids* and *Anguilidae* that are either endemic or non-native to fresh or brackish waters [6]. Carp species (cyprinids) account for about 45% of the total world aquaculture production and are one of the most important fish on the world market, with habitats in rivers (basins) and also in areas largely occupied by marine flora and fauna such as seas, bays, and gulfs where rivers flow [7–9]. Compared with other large aquaculture species, such as salmon and shrimp, carp are recognized as an environmentally friendly fish because most are omnivorous (eating mollusks, crustaceans, insect larvae, and seeds) and therefore consume much less fishmeal and fish oil. This makes extensive cultivation highly favored, often in particularly difficult habitats or mixed marine and freshwater areas (such as the Caspian sea or the delta or estuary of rivers) [8–12].

Cyprinus carpio (common carp) is one of the most important cyprinid species; it is cultured in over 100 countries worldwide and accounts for up to 10% (over 3 million metric tons) of global annual freshwater aquaculture production [13,14]. This is related to its fast growth rate, easy cultivation in different geographic areas (ponds, canals, rivers, coasts), and its long-life, as well as high feed efficiency and high nutritive value [12,14,15]. Analogously, among the cultivated fishes, *Hypophthalmichthys molitrix* (silver carp) has attracted great attention for its growing production in many Asian countries such as China, Bangladesh, India, Russian Federation, and even in Iran where silver carp is a favorite meal for many people. Silver carp is no less important in the European Union where this species is widespread—produced in particular in Hungary, Romania, Croatia, and the Czech Republic [11,16]. Carp tissues are rich in minerals and vitamins, particularly phosphorous and vitamin B12, as well as having high levels of fatty acids, protein, and antioxidants. This unique nutrient profile makes carp highly desirable for people looking for a better dietary protein; it is also praised as one of the healthiest fish for human consumption [17]. In addition, aiming to promote an eco-friendly aquaculture with a focus on the growing amount of fish waste worldwide, special attention has recently been paid to reusing all fish tissue away from the food market since this represents a rich source of value-added compounds. It is not only fish tissue discarded after capture or fish lost between landing and transport to markets, but also fish tissue not used by the food industry [18].

In a previous study, it was described that waste viscera of common carp were utilized as raw material for the extraction of refined oils by ensilage and fishmeal processes; this carp oil produced a rich source of essential fatty acids of the ω-3 and ω-6 series [19]. Furthermore, not only silver carp meat but also eggs represent a valuable source of essential fatty acids for human nutrition [20]. However, the main therapeutic potential of fish consumption has been ascribed to the presence of polyunsaturated fatty acids (PUFAs) at high concentrations [21]. From a nutritional point of view, it is an established fact that farmed or freshwater fish tissues are a precious source of health-beneficial PUFAs, mainly eicosapentaenoic acid (EPA; C20:5 ω-3), docosahexaenoic acid (DHA; C22:6 ω-3), linoleic acid (LA; C18:2 ω-6), and arachidonic acid (AA; C20:4 ω-6). These ω-3 and ω-6 FAs have a pivotal role in the human diet in preventing many diseases [22–24]; in this sense, 2–3 servings of fish per week or the consumption of 250 mg of EPA and DHA per day are recommended by nutritionists [25]. The biological role of fish lipids and, in particular, of their fatty acids sets has been extensively described: lipids are important for fish development because

they represent a concentrated source of energy and are involved in many physiological functions (structural component for cellular membrane, precursor of vitamins, hormones, eicosanoids, thermal processes such as osmoregulation and immune response, sexual development) [24–28]. In addition, during ecological investigation, fatty acids—and in particular some polyunsaturated fatty acids—are considered very important biomolecules as trophic biomarkers in the marine food chain analyses of several ecological niches [23]. It is well known that the chemical nature and the percentage of fatty acids in fish tissues vary mainly with fish feeding, but other factors such as size or age, reproductive status, geographic location, season, and temperature influence the fat content and composition of fish muscle [14,24,29]. In a study carried out on gonadal tissues of *Cyprinus carpio*, it was reported that the lipids profile and their concentrations were significantly influenced by salinity and their amount increased with the development of maturity stages [30].

The principal aim of this study was to evaluate the total lipid content—focusing our attention on the analysis of the fatty acid sets—of liver and ovary tissues from immature and mature aquaculture Moroccan fishes of the species *Hypophthalmichthys molitrix* and *Cyprinus carpio* grown in the same conditions before their potential nutritional value was compared with that of their edible fillets [5]. Without making a distinction between material considered waste by the food industry and the edible fish portions, the lipid profiles were compared in order to describe the differences between the two species and the variations related to the stage of sexual maturity and to furnish important information from a nutritional point of view about the fatty acids content of tissues from specific parts of the fish body. Further, tissues were characterized by the hypocholesterolemic/hypercholesterolemic ratio (h/H), the index of atherogenicity (AI), and the index of thrombogenicity (TI). The nutraceutical value of these fish tissues was evaluated, taking into account in particular the possibility of using their gonads and livers (usually not edible parts) for farm food or pharmaceutical applications.

2. Results

2.1. Fatty Acids Profiles of Common and Silver Carp

The total lipids content and the fatty acid (FA) profiles of the liver, gonads, and fillet tissues of common and silver carp in two different sexual maturation stages were determined, and the results are reported in Table 1. An analysis of total lipid content in all tissues of both carp species showed that at the mature stage, gonads and liver tissues were richer in lipids than those at the immature stage; for mature common carp, the lipid content for 100 g of each tissue was 2.56 ± 0.48 g in the gonads and 1.27 ± 0.22 g in the liver; the corresponding immature tissue amounts were 0.88 ± 0.68 and 0.83 ± 0.08 g, respectively (Table 1). Similar results were also recorded for the mature and immature tissues of silver carp (Table 1). Fillet tissues showed the lowest amount of total lipid in both maturation stages, even though from the immature to the mature stage, the total lipid amount doubled (Table 1). In general, the GC-MS analyses of the FA content from common and silver carp tissues furnished clear information about the recovered fatty acids' distribution.

2.1.1. Saturated Fatty Acids Profile of Common and Silver Carp

Saturated fatty acids (SFAs) were highly abundant in all tissues, with a preferential accumulation into the liver (~44% in mature and immature common carp; 38% in mature carp; 54% in immature silver carp). However, at the mature stage, the liver content of SFA was almost similar for both species. In contrast, at the mature stage, the SFA amount represented a statistically discriminant parameter for the fillets of the two carps, as reported in Table 1; the SFA content in the fillet samples was 21.37 ± 4.45 mg × 100 g^{-1} of tissues for the mature common carp and a higher value for the corresponding silver carp fillet (65.03 ± 5.91 mg × 100 g^{-1} of tissues, $p = 0.01$).

Table 1. Total fat, total FA, SFA, MUFA, PUFA, ω-3 FA, and ω-6 FA concentrations (mean mg × 100 g^{-1} of tissue ± SE), PUFA/SFA and MUFA/SFA ratios of the gonads, liver, and fillet tissues of immature and mature common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmichthys molitrix*).

	Total Lipids (g/100 g)	Total FA [a] (g/100 g)	SFA [b] (mg/100 g)	MUFA [c] (mg/100 g)	PUFA [d] (mg/100 g)	ω-3 [e] FA (mg/100 g)	ω-6 [f] FA (mg/100 g)	PUFA/SFA	MUFA/SFA
common carp immature									
G	0.88 ± 0.68	0.109 ± 0.006	28.10 ± 1.22	41.14 ± 3.45	40.36 ± 2.14	17.06 ± 0.80	23.30 ± 1.34	1.44 ± 0.10	1.46 ± 0.14
L	0.83 ± 0.08	0.412 ± 0.006	187.80 ± 13.84	209.72 ± 10.01	14.56 ± 2.31	n.d.	14.56 ± 2.31	0.08 ± 0.01	1.12 ± 0.10
F	0.14 ± 0.07	0.059 ± 0.033	17.09 ± 9.05	29.64 ± 19.08	12.81 ± 5.66	4.70 ± 1.54	8.11 ± 4.12	0.75 ± 0.52	1.73 ± 1.44
silver carp immature									
G	0.25 ± 0.12	0.109 ± 0.033	26.43 ± 5.06	57.72 ± 16.45	25.05 ± 5.58 *	7.13 ± 0.97 **	17.92 ± 4.61	0.95 ± 0.28 *	2.18 ± 0.75
L	0.44 ± 0.18	0.135 ± 0.067	73.20 ± 27.91 **	52.62 ± 22.00 *	9.33 ± 5.00	n.d	9.33 ± 5.00	0.13 ± 0.08	0.72 ± 0.41
F	0.25 ± 0.06	0.021 ± 0.008	10.23 ± 3.61 *	6.50 ± 2.47	4.20 ± 1.65	1.60 ± 0.52 *	2.60 ± 1.13	0.41 ± 0.22	0.64 ± 0.33
common carp mature									
G	2.56 ± 0.48	0.581 ± 0.056	207.63 ± 16.61	193.25 ± 18.80	180.36 ± 26.92	119.84 ± 23.40	60.52 ± 3.52	0.87 ± 0.15	0.93 ± 0.12
L	1.27 ± 0.22	0.182 ± 0.058	79.79 ± 13.49	88.41 ± 23.37	14.64 ± 3.61	8.89 ± 2.01	5.74 ± 1.60	0.18 ± 0.05	1.11 ± 0.35
F	0.28 ± 0.12	0.067 ± 0.014	21.37 ± 4.45	22.38 ± 4.28	22.75 ± 5.35	13.57 ± 3.04	9.19 ± 2.31	1.06 ± 0.33	1.05 ± 0.30
silver carp mature									
G	0.98 ± 0.27	0.553 ± 0.055	170.30 ± 8.52	252.41 ± 19.79	130.96 ± 12.98 *	13.47 ± 0.67 **	117.48 ± 12.31 *	0.77 ± 0.08	1.48 ± 0.1 *
L	1.10 ± 0.47	0.215 ± 0.091	80.72 ± 39.48	124.79 ± 19.89 *	9.45 ± 5.03	n.d	9.45 ± 5.03 *	0.12 ± 0.08	1.55 ± 0.79
F	0.50 ± 0.08	0.210 ± 0.008	65.03 ± 5.91 *	119.43 ± 3.67 **	25.73 ± 3.33	12.01 ± 1.36	13.72 ± 1.98	0.40 ± 0.06	1.84 ± 0.1

G: gonad tissues; L: liver tissues; F: fillet tissues; [a] Total fatty acids recovered from different tissues. [b] Total SFA in tissues, including, C14:0 (myristic acid), C16:0 (palmitic acid), C17:0 (margaric acid), C18:0 (stearic acid), C20:0 (arachidic acid), C22:0 (behenic acid).[c] Total MUFAs in tissues, including, C16:1 ω-7 (palmitoleic acid), C17:1 (margaroleic acid), C18:1 ω-7 (vaccenic acid), C18:1 ω-9 (oleic acid), C20:1 ω-9 (gadoleic acid), and C22:1 (erucic acid). [d] Total PUFAs in tissues; the most abundant were evaluated, corresponding to ω-3, ω-6 fatty acids. [e] Total ω-3 fatty acids in tissues including C20:5 ω-3 (EPA) and C22:6 ω-3 (DHA); [f] Total ω-6 fatty acids, including 18:2 ω-6 (linoleic acid) and 20:4 ω-6 (arachidonic acid). The statistical significance of lipids was assessed in gonads, liver, and fillets via a Student's *t* test comparing the two fish species within the same growth stage. Abbreviations: * $p < 0.05$; ** $p < 0.01$; n.d.: not detected.

Moreover, at the immature stage, common carp showed a higher amount of SFA for the liver ($p = 0.007$) and fillet ($p = 0.03$) tissues.

The gonads of mature common and silver carps contained a high amount of SFA; in fact, 207.63 ± 16.61 (36% of total FA) and 170.30 ± 8.52 mg × 100 g^{-1} (31%) of tissues were recovered from the corresponding common and silver carps' tissues (Table 1).

Palmitic (C16:0) and stearic (C18:0) acids were stored preferentially into the mature gonad and immature liver of both species, as reported in Table S1.

2.1.2. Monounsaturated Fatty Acids Profile of Common and Silver Carp

Monounsaturated fatty acids (MUFAs) were the most abundant fatty acids: they were recovered preferentially into the livers of the mature (88.41 ± 23.37 mg × 100 g^{-1} of tissues, 48%) and immature stages (209.72 ± 10.01 mg × 100 g^{-1} of tissues, 51%) of common carp and similarly for the silver carp liver (124.79 ± 19.89 mg × 100 g^{-1} of mature tissues, 58%; 52.62 ± 22.00 mg × 100 g^{-1} of immature tissues, 38%).

MUFAs in the gonads were 193.25 ± 18.80 mg × 100 g^{-1} (33%) of mature tissues in the common carp and 252.41 ± 19.79 mg × 100 g^{-1} (46%) of the mature tissues in the silver carp; the corresponding values for immature tissues were lower (Table 1). Firstly, the oleic acid (C18:1ω-9) and then the vaccenic acid (C18:1ω-7) were the main recovered MUFAs.

Evaluating the edible tissues of the fillets, a preference for silver carp was recorded: silver carp mature fillet tissues contained the highest and most significant amount of MUFAs (119.43 ± 3.67 mg × 100 g^{-1} of tissues, 57%, $p = 0.0006$).

For the common carp, the mature gonads and immature liver values of oleic acid were higher than the corresponding tissues of the silver carp, as reported in Table S1.

For oleic and vaccenic acids, the mature gonad tissues of the common carp provided preferential results. In contrast, only the fillet tissue of the mature silver carp contained 85.47 ± 1.55 mg × 100 g^{-1} of tissues of oleic acid. (Table S1).

The MUFA/SFA ratio was also calculated for all tissues of the common and silver carp; as reported in Table 1, the values ranged from 0.64 ± 0.33 of silver carp immature fillet to 2.18 ± 0.75 of silver carp immature gonads. However, the gonad tissues of both carps showed values ~1 or above 1 regardless of the growth stage. For the fillet tissues of

this common carp, this ratio was always above 1, whereas in silver carp it occurred only at the mature stage. It was interesting to notice that at the mature stage, the gonads of the silver carp exhibited higher and more significant values ($p = 0.03$) than the corresponding common carp, as reported in Table 1.

2.1.3. Polyunsaturated Fatty Acids Profile of Common and Silver Carp

Polyunsaturated fatty acids (PUFAs) preferentially accumulated into the gonad tissues with an impressive difference between the two carp species: 180.36 ± 26.92 mg × 100 g^{-1} of tissues for the mature common carp and 130.93 ± 12.98 mg × 100 g^{-1} for the mature silver carp. In fact, 23–31% of PUFAs was present in the mature gonads of common and silver carp.

In total, 12–34% of PUFAs was recovered in the mature fillets of common and silver carp. Interestingly, the amount of PUFAs was higher in the mature tissues, with 22.75 ± 5.35 mg × 100 g^{-1} of tissues in common carp and 25.73 ± 3.33 mg × 100 g^{-1} of tissues in silver carp.

Liver tissues contained the lowest percentage of PUFAs (4–8%) for both carp species and between the two maturation stages.

On the basis of the proportions of the different fatty acids groups, the PUFA/SFA ratio (Table 1) varied from 0.08 to 1.44. The PUFA/SFA ratio was higher in the gonads and fillets of both carp species. In fact, in the common carp, the mature gonads and fillet tissues values were 0.87 ± 0.15 and 1.06 ± 0.33, respectively, and in the silver carp, the mature gonads and fillet tissues presented corresponding values of 0.77 ± 0.08 and 0.40 ± 0.06.

In Table 1, the ω-3 FA and ω-6 FA values for each tissue are reported. The major polyunsaturated fatty acids identified in Moroccan common and silver carp were C22:6 ω-3 (docosahexanoic acid, DHA, 4.13–14.37%), C20:5 ω-3 (eicosapentaenoic acid, EPA, 3.3–6.25%), C20:4 ω-6 (arachidonic acid, AA, 1.11–13.11%), and C18:2 ω-6 (linoleic acid, LA, 4.40–20.11%).

ω-3 FAs were significantly abundant in the mature gonads of common carp: 119.84 ± 23.40 mg × 100 g^{-1} of tissues ($p = 0.006$); in silver carp gonads, the value was 13.47 ± 0.67 mg × 100 g^{-1} of mature tissues. The mature fillets of common and silver carp contained similar amounts (Table 1), while the corresponding immature tissues were very low in ω-3 FAs.

In contrast, ω-6 FAs were significantly abundant in the mature gonads of silver carp with a value per 100 g of tissues of 117.48 ± 12.31 mg ($p = 0.04$); in common carp gonads, the values were 60.52 ± 3.52 mg of mature tissues and 23.30 ± 1.34 mg of immature tissues. Fillets of common carp contained ~10 mg in 100 g of mature and immature tissues; fillets of mature silver carp contained a value which was higher than the immature fillet amount.

Arachidonic (AA, C20:4 ω-6) and linoleic (LA, C18:2 ω-6, ω-9) acids were the principal ω-6 FAs detected. In particular, arachidonic acid was preferentially stored into the gonads of common carp, while in silver carp the gonads values were lower and only the mature fillet showed a value comparable to the common carp amount (Table S1). Linoleic acid was accumulated preferentially in the mature gonad tissues of silver carp.

2.2. Fat Quality Indices of Common and Silver Carp

The fat quality of all analyzed tissues was described by means of the following indexes: the hypocholesterolemic/hypercholesterolemic ratio (h/H), the index of atherogenicity (AI), and the index of thrombogenicity (TI). The results are reported in Figures 1–3. The h/H ratio ranged from 0.74 to 3.61 (Figure 1); in particular, the h/H ratio of the immature liver tissues of common carp were significantly higher ($p = 0.005$) than the corresponding value in silver carp.

The AI index ranged from 0.23 to 0.53 (Figure 2) and the TI values varied from 0.33 to 1.55 (Figure 3). It was interesting that at the mature stage, the fillet tissues of common and silver carp showed an h/H ratio of 3.44 ± 0.95 and 2.53 ± 0.26, respectively; the common carp mature and immature gonad values were 2.28 ± 0.33 and 3.26 ± 0.25. The AI values

of mature common and silver carp fillets were 0.28 ± 0.05 and 0.33 ± 0.03, respectively, and the corresponding TI values were 0.37 ± 0.11 and 0.63 ± 0.07. It is noteworthy that the AI values of the immature common and silver carp gonads were equally low and equal to ~0.3. The AI (p = 0.01) and TI (p = 0.03) indexes of the mature fillet of common carp were significantly low. The TI values were higher than 1 only for the liver tissues of both species.

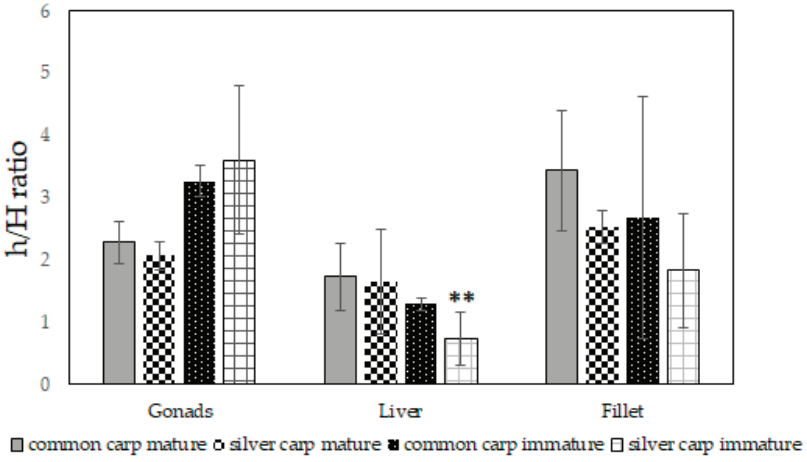


Figure 1. The hypocholesterolemic/hypercholesterolemic ratio (h/H) of immature and mature gonads, liver, and fillet tissues of common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmichthys molitrix*). For each tissue, significant discrepancies were evaluated with a Student's t test comparing the two fish species within the same growth stage. ** $p < 0.01$.

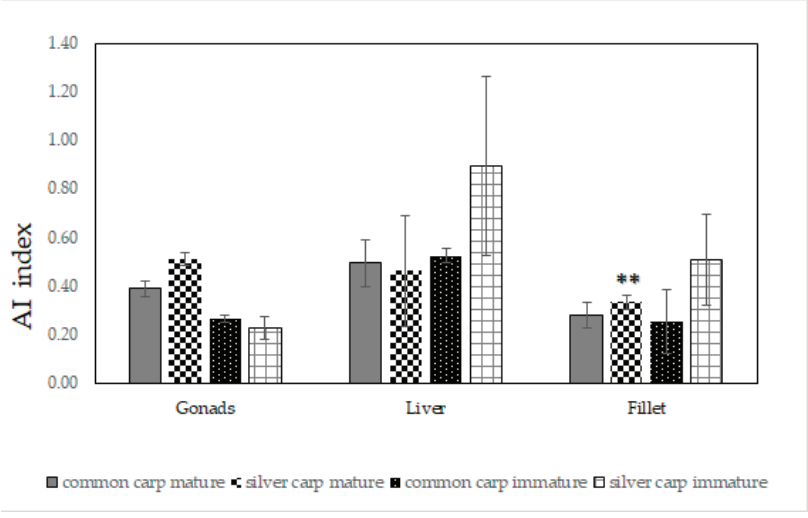


Figure 2. The index of atherogenicity (AI) of immature and mature gonads, liver, and fillet tissues of common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmichthys molitrix*). For each tissue, significant discrepancies were evaluated with a Student's t test comparing the two fish species within the same growth stage. ** $p < 0.01$.

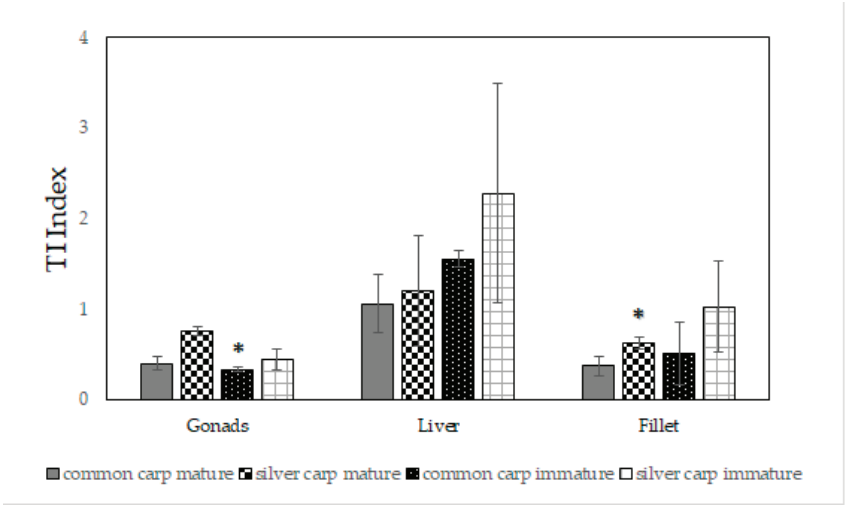


Figure 3. The index of thrombogenicity (TI) of immature and mature gonads, liver, and fillet tissues of common and silver carps. For each tissue, significant discrepancies were evaluated with a Student's *t* test comparing the two fish species within the same growth stage. * $p < 0.05$.

3. Discussion

It is well established that the consumption of fish or fish products containing bioactive lipids (MUFA, PUFA, lipid-soluble vitamins A, E, and D, natural antioxidants) has several health benefits, including a reduced risk of cardiovascular disease (CVD) and coronary heart diseases and prevention of cardiac arrhythmias, as well as anti-inflammatory and anti-thrombotic potency, and also efficient action to counter a plethora of diseases characterized by chronic inflammation (e.g., cancer) [31–33].

Fat quality in immature and mature Moroccan aquaculture *Hypophthalmichthys molitrix* and *Cyprinus carpio* tissues was described in this paper. Particular attention was devoted to those tissues normally not utilized in the production of human food such as the liver and gonads, but that could be used as feed for the production of super-fish or as a source for nutraceutical production, or even for industrial food preparation due to their relevant molecular profile. These fishes were collected from the Deroua Fisheries Station located in the Beni Mellal-Khenifra region, grown in the same earthen ponds, and equally naturally fed. The gonads and liver tissues of carp samples from mature (common carp GSI 18.34 ± 1.12; silver carp GSI 0.67 ± 0.26) and immature fishes (common carp GSI 7.08 ± 1.94; silver carp GSI 0.08 ± 0.03) (Table 2) were selected and investigated for their total lipid content and compared with fillets' lipid profiles.

The fatty acids profiles were calculated and examined, particularly their content of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) with attention to the amount of ω-3 and ω-6 fatty acids, the ratios PUFA/SFA and MUFA/SFA, the hypocholesterolemic/hypercholesterolemic ratio (h/H), and the indexes of atherogenicity (AI) and of thrombogenicity (TI).

Table 2. Biometrical measurements and maturity index (GSI, gonadal weight × 100)/(total weight) of ten immature and mature female carps, *Hypophthalmichthys molitrix* (silver carp) and *Cyprinus carpio* (common carp) collected at the Deroua Fisheries Station, Beni Mellal-Khenifra region (Morocco).

Female Fish (n = 10 Species)	Total Weight (g)	Length (cm)	Body Circumference (cm)	Gonadal Weight (g)	Liver Weight (g)	Fillet Weight (g)	GSI (%)
Immature *Hypophthalmichthys molitrix* (silver carp)	456.80 ± 70.20	36.01 ± 2.59	16.66 ± 1.13	0.52 ± 0.20	2.54 ± 0.54	19.62 ± 3.29	0.08 ± 0.03
Mature *Hypophthalmichthys molitrix* (silver carp)	1845.23 ± 573.06	53.50 ± 5.18	26.75 ± 3.73	8.23 ± 1.18	13.60 ± 4.87	53.08 ± 16.60	0.67 ± 0.29
Immature *Cyprinus carpio* (common carp)	804.28 ± 61.94	39.75 ± 0.78	24.13 ± 0.83	74.03 ± 6.59	11.58 ± 1.74	26.93 ± 4.65	7.08 ± 1.94
Mature *Cyprinus carpio* (common carp)	2800.07 ± 258.80	52.53 ± 2.48	39.33 ± 0.33	519.37 ± 80.10	12.07 ± 0.55	56.87 ± 8.03	18.34 ± 1.12

The important result was that those tissues considered as waste or normally not edible (gonads and livers) proved to be an important source of fatty acids and, properly dissecting carp viscera, it was possible to obtain extracts with different fatty acids profiles. It was interesting to note that in the passage from the immature to the mature (adult) stage, an increase in the lipidic content was observed for all tissues, but preferentially in the gonads and liver tissues of both carp species, the accumulation that resulted was remarkable. As reported in Table 1, the liver and the gonads of mature common carp contained, respectively, 2.56 and 1.27 g × 100 g^{-1} of tissues of total lipids, whereas in the immature stage, the corresponding values were 0.88 and 0.83 × 100 g^{-1} of tissues. We observed a mobilization of energy molecules of Moroccan common and silver carp during sexual maturation toward the liver and the gonads tissues, presumably in support of reproductive efforts. These results were in agreement with those previously reported; in fact, the liver constitutes the location for fat deposits in all fish species [34] and, during the maturation stage, a transfer of fats and also proteins to the reproductive sites has been extensively documented [35]. Furthermore, lipids are the main source of metabolic energy in marine fish for swimming, growth, and reproduction, and lipid levels vary in relation to the reproductive cycle [14,35,36].

On the other hand, as described before, fish lipid content and fatty acid (FA) profiles are greatly dependent on the growth conditions such as the feeding conditions, the seasonal variation, the geographical area of recovery, the reproductive status, and the farmed or wild origins [14,37–39]. Herein, the environmental (temperature, salinity, pH, geographical area) and feeding conditions were the same for all samples. At Deroua fish farm (Beni-Mellal, Morocco), phytoplankton constituted the basic diet of silver carp in ponds. The detritivorous carp (common carp) consumed essentially the organic matter contained in pond sediments. Therefore, the difference in fatty acids profiles depended on the reproductive status and on the distinct species.

Analyses of the total FA amounts recovered from the three selected tissues revealed some differences between the common and silver carp.

At the mature stage, gonads and liver tissues showed almost the same content of total FAs for both species, the total FA content of silver carp fillet tissues was 0.210 ± 0.008 g × 100 g^{-1}, which was higher than the value of 0.067 ± 0.008 g × 100 g^{-1} of the corresponding common carp fillets.

Interestingly, the composition of the FA mixtures depended on the tissues and maturation stage of the common and silver carp. In general, saturated fatty acids (SFAs) were recovered at 24–54% of the investigated carp tissues. In contrast, the content of total MUFAs ranged from 33 to 58% of the total FA; they were the most abundant fatty acids and the values here shown are higher than those previously reported [40].

The MUFAs content in mature silver carp was higher than in common carp for all tissues and in particular from the statistical analyses, the MUFA values of mature liver and fillet tissues of silver carp were favored.

These results appeared relevant especially when compared with data previously shown. Jorjani et al. (2015) [38] described the fatty acid profile of fillets from Iranian cultured common carp and silver carp, which were cultured in a semi-intensive manner with natural feeding: the MUFA contents of common and silver carp were evaluated to be 45% and 33% of the total FAs, respectively. In our analyses, the silver carp mature fillet tissues contained 19.43 $\pm$ 3.67 mg $\times$ 100 g^{-1} of tissues, which corresponded to 57 % of the total FAs [41].

The reason for the difference between our results regarding both SFA and MUFA values and the literature data is likely due to the different eating habits of the Moroccan fishes [14,41].

The MUFA/SFA ratio for the mature tissues of common and silver carp was generally above 1; this was interesting because this index was assessed in monitoring the fish growth in different seasonal conditions [14,42]. However, this ratio was a statistically discriminating parameter for both species: in fact, at the mature stage, the silver carp gonads showed higher values.

Aiming to propose a sustainable consumption of adult fishes' meat, common carp meat resulted in generally less fat, but the percentage of MUFAs in the mature fillets of silver carp was higher and with almost the same amount of PUFAs for both species. In fact, the PUFAs of Moroccan common and silver carps ranged from 3.53 to 36.82% of the total FAs, with 12–34% of PUFAs which were recovered in the mature fillets of common and silver carp. Evaluating the fillet tissues of both species, at the mature stage, the amount of total ω-3 FA was similar with a value of ~13 mg $\times$ 100 g^{-1} of tissues; analogous to the total ω-6 FA with a value of ~11 mg in 100 g of tissues. These results are of great relevance from a nutritional point of view because fillets represent the preferential edible part of fishes. The PUFAs content of fillets (values from 12.24 to 34.22%) and the PUFA/SFA ratio (0.40–1.06, Table 1) were comparable to values recovered from farmed carp species which were grown in different conditions [14,19,39]. These values are in agreement to those recommended by the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) [39,43]. In fact, according to the FAO, WHO, the British Department of Health (1994), and other authors and literature sources, PUFA/SFA ratios should preferably be within the range of 0.35 to 1.0 [44–47]. However, these values were around 1, or greater than 1 in the case of gonad tissues. Yeganeh et al. (2012) [14] reported that one of the marked differences between the farmed and wild common carp is the higher level of linoleic acid present in the farmed fish (about 15.3 vs. 3.1%). In the current analyses, linoleic acid was recovered in fillet tissues for ~3% for both species, similar to the wild species. In fact, this compound was present in the plant oils which are used in the feeding of farmed fish and accumulate largely unchanged in the lipids of marine fish [48]. In agreement with data previously reported for wild carp collected in different seasons [14,42], the fillets of both Moroccan species showed a good amount of arachidonic acid (AA, ~5 mg $\times$ 100 g^{-1} of mature tissues). The h/H ratio and AI and TI indices were calculated for the first time not only for fillets but also for the gonads and liver of both species, as shown in Figures 1–3. The results on lipid indices reported in this study are in agreement with those previously reported on the carp species [24,47,49,50].

The AI index describes the ability of pro-atherogenic activity or preventive anti-atherogenic effect (inhibiting the aggregation of plaques, diminishing the levels of cholesterol and phospholipids, and thus preventing coronary diseases). TI expresses the tendency to form clots in the blood vessels [50,51]. In all cases for the tissues of common and silver carp, AI was estimated to be below the recommended value of 1.0 [52] (Figure 2), with an interestingly low index of 0.28 and 0.33 for the common and silver carp fillets, respectively. Moreover, the h/H index was always above 1 for the mature and immature gonads and fillet of both species, and it ranged between 1.84 and 3.61. The TI values were higher than 1

only for the liver tissues of both species. These values below 1.0 provided evidence of good antithrombogenic properties. The TI values were determined from three saturated fatty acids: myristic (C14:0), palmitic (C16:0), and stearic (C18:0), although palmitic and stearic acid were the most abundant in our analyses.

In general, the low AI and TI values, as well as the high h/H index recorded for the silver and common carp tissues, had to be considered as important results because they are recommended in a healthy diet for the prevention of cardiovascular disorders [46,47,53]. It was worth noting that significantly lower AI and TI values were observed in in the fillet of mature common carp.

3.1. Fatty Acid Profile of Gonads of Common and Silver Carps

The fatty acids analysis of mature gonads tissues from common and silver carp revealed that they represent an important stock site of the total saturated fatty acids (SFAs). Table 1 shows that the highest value of SFAs was determined for the mature gonads of the common carp (207.63 $\pm$ 16.61 mg $\times$ 100 g^{-1} of tissues), while 170.30 $\pm$ 8.52 mg $\times$ 100 g^{-1} was the corresponding value of the silver carp mature gonads. The corresponding values at the immature stage were 13–15% lower.

As it has been described that MUFAs are involved in gonadal development [54], here we reported that the accumulation of MUFAs was greater in the mature gonads tissues of common and silver carp (193.25 $\pm$ 18.80 and 251.41 $\pm$ 19.79 mg $\times$ 100 g^{-1} of tissues, respectively), as a parameter of good fish living conditions. Furthermore, although the MUFA values of the mature gonads of the two carp species were high, they showed a significant difference favoring the silver carp tissues. In particular, while a prevalence of oleic and vaccenic acids was recorded in all tissues of both species, the gonad tissues of mature silver carp contained a noticeable amount of oleic acid. The importance of oleic acid is related to its properties in preventing cardiovascular diseases and its beneficial effect on cancer, autoimmune, and inflammatory diseases, in addition to its ability to facilitate wound healing [55,56]. High levels of oleic, and also arachidonic, acid have been reported as a characteristic property of freshwater fish oils [57].

A preferential and extremely important accumulation of PUFAs in the mature gonad tissues of Moroccan common and silver carps was recorded, although with some differences. A statistical comparison of the ω-3 and ω-6 values in both species, showed that ω-3 FAs were significantly abundant in the mature gonads of common carp, whereas ω-6 FAs were prevalent in the mature gonads of silver carp. The production of fish oils from carp viscera containing fatty acids of the ω-3 and ω-6 series has been previously reported [19]; further to this, the selection of the visceral tissues ensures the possibility of preparing oils with different ω-3 and ω-6 compositions.

The presence of PUFAs in the gonads of both mature species, and also an understanding of where and which FAs are allocated within the carp body (in particular the ω-3 and ω-6 FAs), could help to design efficient aquaculture systems and particularly to increase the ω-3 FAs in edible tissues and/or advise on how to reuse fish tissues for subsequent feeds rich in ω-3 FAs. In fact, it has been reported that more EPA, DHA, and in general PUFAs are present in fish fed with fish oil supplements compared with fish fed with vegetable oils [58]. EPA and DHA are needed for normal growth and development. They also affect retinal and brain phospholipid composition, intelligence quotient (IQ), and motor development [59]. The positive effect of ω-3 PUFAs on coronary heart diseases has been shown in many experimental studies in animals, humans, and tissue culture [60,61].

However, we recorded a preferential accumulation of AA in the gonad tissues of common carp. This is an important result due to the biological role of this compound. AA is a necessary FA because it is the precursor of prostaglandin and thromboxane, which will influence blood clot formation and its attachment to the endothelial tissue during wound healing. Moreover, it plays a key role in growth [62]. Evaluating the h/H ratio and AI and TI indices for gonad tissues, as shown in Figures 1–3, it was observed that the index (h/H) was always above 2 and the AI was estimated to be ~0.3 for common carp gonads. The TI

index was less than 1 for the mature and immature gonads of both carps (Figure 3). These data furnish clear clues on the possibility of better exploiting these tissues in the food or nutraceutical fields [63].

3.2. Fatty Acid Profile of Livers of Common and Silver Carps

The data recorded suggested that during the maturation stage, common and silver carp liver tissues lost a percentage of their fatty acids content (in particular common carp), although the total lipid content (saponificated and non-saponificated lipids) increased significantly In fact, if SFA values at the mature stage were low for both species, at the immature stage their values were remarkable: the second highest value of SFA was determined for the immature liver of common carp (187.80 $\pm$ 13.84 mg $\times$ 100 g^{-1} of tissues), which represented an important stock site of these compounds and which was statistically different from the corresponding value of silver carp liver (p = 0.008). These results were in agreement with data obtained from carp collected in other geographical areas [14]. Analogously, MUFAs decreased their values in the livers of mature common carp; instead, PUFAs values were generally low and similar in both maturation stages. These results suggested a redistribution of the lipid supplies during sexual maturation, meaning that they could be used with the aim to reuse these tissues or to monitor fish development.

4. Materials and Methods

4.1. Sample Collection and Preparation

Hypophthalmichthys molitrix (silver carp) and *Cyprinus carpio* (common carp) were recovered from the Deroua Fisheries Station located in the Beni Mellal-Khenifra region, a semi-arid climate zone in Morocco. The collection was conducted in April 2019. Fishes were both held in earthen ponds. Phytoplankton constituted the basic diet of silver carp in ponds. The detritivorous carp (common carp) consumed essentially the organic matter contained in pond sediments [64,65].

The breeding season in Morocco of the studied species, which is classified in arid and semi-arid zones, starts from April and extends to June when the water temperature in ponds varies between 20 °C and 26 °C. The seining of fish was performed smoothly in order to avoid fish stress. Female fish were recognized by their inflated and soft abdomen, inflated anus, and red pectoral fins. Mature silver carp were selected from 46 cm in length and up [66]; common carp female were selected from 54 cm in length and up [67] (see Mutethya et al., 2020). The mean of the biometrical data is reported in Table 2. The data include total weight, length, body circumference, gonadal weight, liver weight, fillet weight, and the gonadosomatic index (GSI, gonadal weight $\times$ 100)/total weight) reported as confirmation of fish maturity [68]. Our experiments were performed in strict accordance with European (Directive 2010/63) legislation on the care and use of animals for scientific purposes.

After being captured by seine nets, the specimens were anaesthetized by 2-phenoxyethanol and transported in an ice box at 4 °C within 3 h, and the biometric data of each dead fish were recorded: total weight, length, diameter, gonadosomatic index. Each sample was carefully subjected to abdominal dissection for the elimination of the viscera and for recovering its gonads, liver, and fillet tissues, and the collected materials were stored at −20 °C until molecular processing. All samples were treated under the same conditions. At the beginning of each lipid profile analysis, the aliquots of 10 samples for each species and maturity stage were permitted to equilibrate to room temperature, ground, and homogenized.

4.2. Lipid and Fatty Acid Analysis by Gas Chromatography-Mass Spectrometry (GC-MS)

Lipids were extracted from the carp tissues according to the Bligh and Dyer method [69]. A total of 50–100 mL of a solution of dichloromethane: methanol: water (10:20:7.5 $v/v/v$) was added to the tissues and lipid release was promoted by crumbling them in a mortar for 5–10 min as previously described [23].

The qualitative and quantitative characterizations of the total fatty acids recovered from the fish tissue organic extracts were determined by GC–MS on the corresponding fatty

acid methyl esters (FAMEs); they were obtained after the saponification of lipid extracts and following a modified AOAC Official Method, 991.39 [70], as previously reported [23]. The lipid fraction was saponificated and the free fatty acids were derivatized to fatty acid methyl esters (FAMEs) by methanolic sodium methoxide anhydrous (2–6 mL, 1 N) at 90 °C for 55 min [23,71]. All operations involving lipids or their constituent fatty acids were conducted in an atmosphere of pre-purified nitrogen. FAMEs were recovered by adding diethyl ether to the cooled reaction mixture. The FAME mixtures were redissolved in diethyl ether (EE, 2 mg/mL) and analyzed by GC–MS (scan mode) equipped with an ion-trap (Thermo Scientific, Waltham, MA, USA) on a 5% diphenylpolysiloxane column (OV-5 column, VF-5ms 30 × 0.25, Agilent Technologies, Middelburg, The Netherlands) able to separate FAMES, as reported in Figure S1. A Thermo Scientific™ PolarisQ ™ GC/MSn Benchtop Ion Trap Mass Spectrometer was used in EI (70 eV) under positive mode analysis (mass range 50–450). The elution of free fatty acid methyl esters required an increasing temperature gradient, as previously reported [23]. Samples of 2 µL were directly injected in split mode (1:10) and at a split flow of 10 mL min^{-1}, with a blink window of 3 min (with an inlet temperature of 270 °C, the transfer line set at 280 °C, and an ion-source temperature of 250 °C). The carrier gas was helium, which was used at a constant flow of 1.0 mL min^{-1}. FAMEs were identified by comparison of their retention time with those present in a mixture of 11 standard FAMEs (Figure S1, marine source, analytical standard, Sigma Aldrich) which was analyzed under the same conditions.

Aiming to quantitatively characterize each fish fatty acid mixture, for each GC–MS measurement, an internal standard was added to the FAMEs solution to be analyzed; a total of 50 µL of a 2.5 mg mL^{-1} solution of methyl tricosanoate (C23:0) was used, corresponding to 0.125 mg of internal standard per mL of solution to be analyzed (0.454 µg for each injection of 2 µL).

The equation:

$$CFA = [(m_{IS} \times A_{FA} \times RRF_{FA})/(1.04 \times A_{IS})]/V_{\text{-injection}}$$

was used to quantify all measured fatty acids, where CFA is the concentration of the fatty acid in mg × mL^{-1}, $V_{\text{-injection}}$ is the volume of each injected sample solution (2 µL), m_{IS} is the weight of the internal standard, A_{FA} is the fatty acid peak area in the GC spectrum, RRF_{FA} is the relative retention factor for each fatty acid [70], 1.04 is the correlation factor between the fatty acids and fatty acid methyl esters, and A_{IS} is the internal standard peak area in the GC spectrum. The RRF value accounts for the effective carbon number, and it was calculated according to previously published methods [70,71]: myristic acid, C14:0 (RRF = 1.08); palmitic acid, C16:0 (RRF = 1.05); palmitoleic acid, C16:1 n-7 (RRF = 1.05); stearic acid, C18:0 (RRF = 1.04); oleic acid, C18:1 n-9 (RRF = 1.03); eicosapentaenoic acid (EPA), C20:5 n-3 (RRF = 0.99); docosahexaenoic acid (DHA), C22:6 n-3 (RRF = 0.97); tricosanoic acid, C23:0 (RRF = 1.000).

As previously reported [23,70,72,73], the quantitation of individual FAs is based on the comparison of their peak areas, A_i, and the peak area of a suitable IS (internal standard), A_{IS}, which was in our case tricosanoic acid (23:0), the usual standard for the determination of PUFAs in fish extracts.

The response factors for each of the FAMEs not present in the standard mixture were estimated by comparison with the standard FAMEs which resembled them most closely in terms of chain length and number of double bonds. In any case, the validity of the theoretical relative RF (RRF) [24] was confirmed by Craske and Bannon [74] and Erder [75].

Furthermore, starting from CFA, it was possible to establish the total amount of each fatty acid (FA) as either free or saponificated fatty acid which was recovered in the EE extract of each fish species, as previously reported [23,70]; according to the weight of the frozen fillet fish tissue (m_{fis}), the mgs of each fatty acid free in 100 g of fish tissue were determined by the following formula:

$$[(\text{mg FA in 1 mg of EE extract} \times \text{total mg of EE extract})/\text{g } m_{fis}] \times 100$$

where g m_{fis} corresponds to the grams of frozen fish tissue analyzed. Three replicates of each sample were obtained. A total of 16 fatty acids were measured, with a limit of quantification (LOQ) of 0.3 mg/100 g of fish tissue.

4.3. Determination of Fat Quality Indices

The hypocholesterolemic/hypercholesterolemic ratio (h/H), the index of atherogenicity (AI), and the index of thrombogenicity (TI) were determined to describe the fat quality.

These factors were calculated using the following equations [49,50,76]:

$$\textbf{h/H} = \Sigma(\text{C18:1n 9, C18:1n 7, C18:2n 6, C18:3n 6, C18:3n 3, C20:3n 6, C20:4n 6, C20:5n 3, C22:4n 6, C22:5n 3, C22:6n 3})/\Sigma(\text{C14:0, C16:0})$$

$$\textbf{AI} = (\text{C12:0} + 4 \times \text{C14:0} + \text{C16:0})/((\text{n} - 6)\text{PUFA} + (\text{n} - 3)\text{PUFA} + \text{MUFA})$$

$$\textbf{TI} = (\text{C14:0} + \text{C16:0} + \text{C18:0})/(0.5 \times \text{MUFA} + 0.5 \times (\text{n} - 6)\text{PUFA} + 3.0 \times (\text{n-3})\text{PUFA} + (\text{n} - 3)\text{PUFA}/(\text{n} - 6)\text{PUFA}).$$

4.4. Statistical Analysis

The results obtained were presented as the means of measurements and the standard error (SE). Differences in the fatty acids values of the gonads, liver, and fillet tissues of fish studied were estimated with a Student's *t* test: for each lipid species, we assessed the differences between common and silver carp within the same growth stage, i.e., mature or immature with R software R core team (https://www.r-project.org/ accessed on 4 February 2023). p values < 0.05 were considered as statistically significant.

5. Conclusions

Carp are considered fish with great adaptability because they are able to grow not only in freshwater habitats but also in areas occupied by marine flora and fauna (ponds, rivers, sea coasts, etc.). An analysis of the fatty acid profiles of Moroccan common and silver carps, generally targeting the consumption of fishes at the mature stage, was reported in this study. The data suggested that the PUFA content of the edible parts of both carp species at the mature stage was interesting from a nutritional point of view, with AI and TI values below 1. Although there was a significant presence of biologically relevant FAs (EPA, DHA, AA) in both mature fillet species, the content of SFA in mature fillets and the PUFA/SFA ratio resulted in discriminating parameters for both carps, with the common carp values being preferable and better than those of the silver species.

The most important result of this study was that the fatty acid profiles of non-edible parts of Moroccan common and silver carps were determined: it revealed the possibility of using selected visceral tissues such as livers and gonads for the production of fish oil with a different composition or fishmeal from fisheries by-products. This would be extremely attractive as it would greatly help to reduce industrial fish waste materials, thereby promote environmental protection, economic growth, and human health.

In fact, the majority of worldwide fish oil production is mostly used in the aquaculture industry, while only a small proportion is used for the production of ω-3 PUFA-related products. Thus, the fishing of most species just for the production of fish oil is not a sensible or sustainable approach. Instead, reusing fish residues and side streams of processing, such as the head, liver, ovary, skin, trimes, etc., is considered a sustainable circular economy strategy, since fish by-products contain lipid ingredients and bioactive compounds.

Suffice it to say that PUFAs were present in the mature gonads of common and silver carp for 23–31% of the total FAs; ω-3 FAs were abundant in the mature gonads of common carp, whereas silver carp mature gonads contained ω-6 FAs preferentially.

In summary, our results provided some clues on the possibility of recycling fish body parts not used for food preparations as super-feeds and/or for obtaining fish extracts with high added-value and with different compositions, which can be employed for ap-

plications in human health and other industries (i.e., aquaculture, food, agrochemical, biotechnological, and pharmaceutical applications).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/md21030188/s1, Table S1. Principal SFA, MUFA, and ω-6 FA (mean mg × 100 g^{-1} of tissue ± SE), of gonads, liver and fillet tissues of immature and mature common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmichthys molitrix*).

Author Contributions: Conceptualization, AT., G.T. and G.G. (Giulia Guerriero); methodology, A.T., C.I., A.I., L.C. and G.T.; software, D.P.; validation, A.T., G.T. and G.G. (Giulia Guerriero); formal analysis, D.P.; investigation, A.T., G.G. (Giulia Guerriero) and G.T.; fishes resources F.-Z.M., G.G. (Gaetano Grieco), M.H., A.O., A.E.M., S.E.G. and M.B.; data curation, A.T. and D.P.; writing—original draft preparation, A.T., G.G. (Giulia Guerriero) and G.T.; writing—review and editing, A.T., G.G. (Giulia Guerriero) and G.T.; visualization, A.T.; supervision, A.T., G.G. (Giulia Guerriero) and G.T.; project administration, G.G. (Giulia Guerriero). All authors have read and agreed to the published version of the manuscript.

Funding: This research received no funding.

Data Availability Statement: The data presented in this study are available on request from the corresponding authors.

Acknowledgments: This work was realized in the framework of the Mediterranean and Middle East Universities Network Agreement (MUNA) between the University of Sultan Moulay Slimane and the University of Napoli Federico II, Naples (Italy) in collaboration with the National Research Council Institute of Biomolecular Chemistry, Pozzuoli, Italy. The authors thank the National Agency for Water and Forests in Morocco, all the members of the team of the fish farming station of Deroua, Beni-Mellal, and the laboratory of M. Hasnaoui and G. Guerriero for their logistic support.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Molina-Vega, M.; Gómez-Pérez, A.M.; Tinahones, F.J. Fish in the Mediterranean diet. In *The Mediterranean Diet: An Evidence-Based Approach*, 2nd ed.; Preedy, V.R., Watson, R.R., Eds.; Elsevier Science: Amsterdam, The Netherlands, 2020; pp. 275–284.
2. Parisi, C.; De Marco, G.; Labar, S.; Hasnaoui, M.; Grieco, G.; Caserta, L.; Inglese, S.; Vangone, R.; Madonna, A.; Alwany, M.; et al. Biodiversity Studies for Sustainable Lagoon: Thermophilic and Tropical Fish Species vs. Endemic Commercial Species at Mellah Lagoon (Mediterranean, Algeria). *Water* **2022**, *14*, 635. [CrossRef]
3. Ashraf, S.A.; Adnan, M.; Patel, M.; Siddiqui, A.J.; Sachidanandan, M.; Snoussi, M.; Hadi, S. Fish-Based Bioactives as Potent Nutraceuticals: Exploring the Therapeutic Perspective of Sustainable Food from the Sea. *Mar. Drugs* **2020**, *18*, 265. [CrossRef]
4. FAO. The State of World Fisheries and Aquaculture. Towards Blue Transformation. 2022. Available online: www.fao.org (accessed on 4 July 2022).
5. Moroccan-Aquaculture-Sector. 2022. Available online: www.moroccoworldnews.com (accessed on 7 December 2022).
6. Aba, M.; Belghyt, D.; Benabid, M. The main species of freshwater fish aquaculture interest in Morocco, current status and prospects. *Int. J. Fish. Aquat. Stud.* **2014**, *2*, 216–218.
7. Majdoubi, F.-Z.; Ouizgane, A.; Droussi, M.; Hasnaoui, M. The Effect of the Spawning Period on the Viability of Silver Carp (*Hypophthalmichthys molitrix*) Eggs. In *Recent Advances in Environmental Science from the Euro-Mediterranean and Surrounding Regions. EMCEI 2017. Advances in Science, Technology & Innovation (IEREK Interdisciplinary Series for Sustainable Development)*; Kallel, A., Ksibi, M., Ben Dhia, H., Khélifi, N., Eds.; Springer: Cham, Switzerland, 2018; pp. 1409–1411.
8. Jawdhari, A.; Mihăilescu, D.F.; Fendrihan, S.; Jujea, V.; Stoilov-Linu, V.; Negrea, B.-M. Silver Carp (*Hypophthalmichthys molitrix*) (Asian Silver Carp) Presence in Danube Delta and Romania—A Review with Data on Natural Reproduction. *Life* **2022**, *12*, 1582. [CrossRef] [PubMed]
9. Guilhermino, L.; Martins, A.; Lopes, C.; Raimundo, J.; Vieira, L.R.; Barboza, L.G.A.; Costa, J.; Antunes, C.; Caetano, M.; Vale, C. Microplastics in fishes from an estuary (Minho River) ending into the NE Atlantic Ocean. *Mar. Pollut. Bull.* **2021**, *173*, 113008. [CrossRef]
10. Roy, K.; Vrba, J.; Kaushik, S.J.; Mraz, J. Nutrient footprint and ecosystem services of carp production in European fishponds in contrast to EU crop and livestock sectors. *J. Clean. Prod.* **2020**, *270*, 122268. [CrossRef]
11. Fishbase. Trophic Ecosystem of Hypophthalmichthys Molitrix. Available online: www.fishbase.se/TrophicEco/EcosysList.php?ID=274&GenusName=Hypophthalmichthys&SpeciesName=molitrix (accessed on 17 November 2022).
12. Fishbase. Trophic Ecosystem of Cyprinus Carpio. Available online: www.fishbase.se/TrophicEco/EcosysList.php?ID=1450&GenusName=Cyprinus&SpeciesName=carpio (accessed on 17 November 2022).

13. Bostock, J.; McAndrew, B.; Richards, R.; Jauncey, K.; Telfer, T.; Lorenzen, K.; Little, D.; Ross, L.; Handisyde, N.; Gatward, I.; et al. Aquaculture: Global status and trends. *Philos. Trans. R. Soc. B Biol. Sci.* **2010**, *365*, 2897–2912. [CrossRef]
14. Yeganeh, S.; Shabanpour, B.; Hosseini, H.; Imanpour, M.R.; Shabani, A. Comparison of farmed and wild common carp (Cyprinus carpio): Seasonal variations in chemical composition and fatty acid profile. *Czech J. Food Sci.* **2012**, *30*, 503–511. [CrossRef]
15. Tokur, B.; Ozkütük, S.; Atici, E.; Ozyurt, G.; Ozyurt, C.E. Chemical and sensory quality changes of fish fingers, made from mirror carp (*Cyprinus carpio* L., 1758), during frozen storage (−18 °C). *Food Chem.* **2006**, *99*, 335–341. [CrossRef]
16. Buchtová, H.; Ježek, F. A new look at the assessment of the silver carp (*Hypophthalmichthys molitrix* Val.) as a food fish. *Czech J. Food Sci.* **2011**, *29*, 487–497. [CrossRef]
17. Organic Facts. Available online: www.organicfacts.net/health-benefits/animal-product/health-benefits-of-carp.html#comments-container (accessed on 7 December 2022).
18. Coppola, D.; Lauritano, C.; Esposito, F.P.; Riccio, G.; Rizzo, C.; de Pascale, D. Fish Waste: From Problem to Valuable Resource. *Mar. Drugs* **2021**, *19*, 116. [CrossRef]
19. Crexi, V.; Monte, M.L.; Soares, L.A.D.S.; Pinto, L.A.D.A. Production and refinement of oil from carp (*Cyprinus carpio*) viscera. *Food Chem.* **2009**, *119*, 945–950. [CrossRef]
20. Majdoubi, F.-Z.; Ouizgane, A.; Farid, S.; Mossetti, L.; Droussi, M.; Guerriero, G.; Hasnaoui, M. Fry Survival Rate as a Predictive Marker of Optimal Production of Silver Carp (*Hypophthalmichthys molitrix*, Valenciennes 1844): A Biostatistical Study in Deroua Fish Farm, Morocco. *Proc. Zool. Soc.* **2021**, *75*, 152–160. [CrossRef]
21. Khalili Tilami, S.; Sampels, S. Nutritional Value of Fish: Lipids, Proteins, Vitamins, and Minerals. *Rev. Fish. Sci. Aquac.* **2018**, *26*, 243–253. [CrossRef]
22. Swanson, D.; Block, R.; Mousa, S.A. Omega-3 Fatty Acids EPA and DHA: Health Benefits Throughout Life. *Adv. Nutr.* **2012**, *3*, 1–7. [CrossRef]
23. Tramice, A.; Trifuoggi, M.; Ahmad, M.F.; Lam, S.S.; Iodice, C.; Velotto, G.; Giarra, A.; Inglese, S.; Cupo, A.; Guerriero, G.; et al. Comparative Fatty Acid Profiling of Edible Fishes in Kuala Terengganu, Malaysia. *Foods* **2021**, *10*, 2456. [CrossRef]
24. Sobczak, M.; Panicz, R.; Eljasik, P.; Sadowski, J.; Tórz, A.; Żochowska-Kujawska, J.; Barbosa, V.; Dias, J.; Marques, A. Nutritional value and sensory properties of common carp (*Cyprinus carpio* L.) fillets enriched with sustainable and natural feed ingredients. *Food Chem. Toxicol.* **2021**, *152*, 112197. [CrossRef]
25. EFSA. Scientific Opinion of the Panel on Dietetic products, Nutrition and Allergies on a request from European Commission related to labelling reference intake values for n-3 and n-6 polyunsaturated fatty acids. *EFSA J.* **2009**, *1176*, 1–11.
26. Corsolini, S.; Borghesi, N. A comparative assessment of fatty acids in Antarctic organisms from the Ross Sea: Occurrence and distribution. *Chemosphere* **2017**, *174*, 747–753. [CrossRef]
27. Chapkin, R.S. Reappraisal of the essential fatty acids. In *Fatty Acids in Foods and Their Health Implications*, 3rd ed.; Chow, C.K., Ed.; CRC Press: Boca Raton, FL, USA, 2007; pp. 689–706.
28. Hwang, D. Dietary fatty acids and eicosanoids. In *Fatty Acids in Foods and Their Health Implications*, 1st ed.; Dekker, M., Chow, C.K., Eds.; CRC: Boca Raton, FL, USA, 1992; pp. 545–558.
29. Fei, S.; Chen, Z.; Xia, Y.; Liu, H.; Han, D.; Jin, J.; Zhu, X.; Xie, S. Effects of dietary arachidonic acid on reproduction performance, tissue fatty acid profile and gonadal steroidogenesis in female yellow catfish Pelteobagrus fulvidraco. *Aquac. Nutr.* **2021**, *27*, 700–711. [CrossRef]
30. Jahan, I.; Tiwari, V.; Verma, A.; Ranjan, A. Effect of salinity on lipid profile of *Cyprinus carpio* reared in inland saline water. *J. Environ. Biol.* **2020**, *41*, 228–233. [CrossRef]
31. Lordan, R.; Redfern, S.; Tsoupras, A.; Zabetakis, I. Inflammation and cardiovascular disease: Are marine phospholipids the answer? *Food Funct.* **2020**, *11*, 2861–2885. [CrossRef] [PubMed]
32. Lordan, R.; Tsoupras, A.; Zabetakis, I. Phospholipids of Animal and Marine Origin: Structure, Function, and Anti-Inflammatory Properties. *Molecules* **2017**, *22*, 1964. [CrossRef]
33. Tsoupras, A.; Lordan, R.; Shiels, K.; Saha, S.K.; Nasopoulou, C.; Zabetakis, I. In Vitro Antithrombotic Properties of Salmon (*Salmo salar*) Phospholipids in a Novel Food-Grade Extract. *Mar. Drugs* **2019**, *17*, 62. [CrossRef]
34. Hussain, B.; Mahboob, S.; Hassan, M.; Nadeem, S.; Sultana, T. Effect of maturation degree on fatty acid profile of different tissues in wild and farmed rohu (*Labeo rohita*). *Grasas Aceites* **2011**, *62*, 206–212. [CrossRef]
35. Nowosad, J.; Kucharczyk, D.; Łuczyńska, J.; Targońska, K.; Czarkowski, T.; Biłas, M.; Krejszeff, S.; Horváth, L.; Müller, T. Changes in European eel ovary development and body and ovary chemistry during stimulated maturation under controlled conditions: Preliminary data. *Aquac. Int.* **2014**, *23*, 13–27. [CrossRef]
36. Dhurmeea, Z.; Pethybridge, H.; Appadoo, C.; Bodin, N. Lipid and Fatty Acid Dynamics in Mature Female Al-Bacore Tuna (*Thunnus alalunga*) in the Western Indian Ocean. *PLoS ONE* **2018**, *13*, e0194558. [CrossRef] [PubMed]
37. Hong, H.; Zhou, Y.; Wu, H.; Luo, Y.; Shen, H. Lipid Content and Fatty Acid Profile of Muscle, Brain and Eyes of Seven Freshwater Fish: A Comparative Study. *J. Am. Oil Chem. Soc.* **2014**, *91*, 795–804. [CrossRef]
38. Böhm, M.; Schultz, S.; Koussoroplis, A.-M.; Kainz, M.J. Tissue-Specific Fatty Acids Response to Different Diets in Common Carp (*Cyprinus carpio* L.). *PLoS ONE* **2014**, *9*, e94759. [CrossRef]
39. Sobczak, M.; Panicz, R.; Eljasik, P.; Sadowski, J.; Tórz, A.; Żochowska-Kujawska, J.; Barbosa, V.; Domingues, V.; Marques, A.; Dias, J. Quality improvement of common carp (*Cyprinus carpio* L.) meat fortified with n-3 PUFA. *Food Chem. Toxicol.* **2020**, *139*, 111261. [CrossRef] [PubMed]

40. Lu, H.; Hong, H.; Luo, Y.; Desk, S. The seasonal fatty acids composition in different tissues of farmed common carp (*Cyprinus carpio*). *SDRP J. Food Sci. Technol.* **2016**, *1*, 11–18.
41. Jorjani, S.; Ghelichi, A.; Jorjani, H. Comparison of Chemical Compositions and Fatty Acid Profile of Cultured Common Carp (*Cyprinus carpio*) and Silver Carp (*Hypophthalmichthys molitrix*). In *Biology Education and Research in a Changing Planet*; Gnanamalar, S.D.E., Ed.; Springer: Singapore, 2015; pp. 167–172. [CrossRef]
42. Kmínková, M.; Winterová, R.; Kučera, J. Fatty acids in lipids of carp (*Cyprinus carpio*) tissues. *Czech J. Food Sci.* **2001**, *19*, 177–181. [CrossRef]
43. WHO/FAO Expert Consultation. Available online: www.foodpolitics.com/wp-content/uploads/FFA_summary_rec_conclsion.pdf (accessed on 8 December 2022).
44. Nutritional Aspects of Cardiovascular Disease. Report of the Cardiovascular Review Group Committee on Medical Aspects of Food Policy. Report on Health and Social Subjects 46. Lessons from DHA status regulation, our ancient diet, epidemiology and randomized controlled trials. *Rep. Health Soc. Subj.* **1994**, *46*, 1–186. [PubMed]
45. EFSA Panel on Dietetic Products, Nutrition, and Allergies. Dietary Reference Values for fats, including saturated fatty acids, polyunsaturated fatty acids, monounsaturated fatty acids, trans fatty acids, and cholesterol. *EFSA J.* **2009**, *3*, 107.
46. WHO/FAO Expert Consultation. Available online: https://www.who.int/publications/i/item/924120916X (accessed on 7 December 2022).
47. Ivanova, A.; Hadzhinikolova, L. Evaluation of nutritional quality of common carp (*Cyprinus carpio* L.) lipidsthrough fatty acid ratios and lipid indices. *Bulg. J. Agric. Sci.* **2015**, *21*, 180–185.
48. Grigorakis, K.; Alexis, M.N.; Taylor, K.D.A.; Hole, M. Comparison of wild and cultured gilthead sea bream (*Sparus aurata*); composition, appearance and seasonal variations. *Int. J. Food Sci. Technol.* **2002**, *37*, 477–484. [CrossRef]
49. Fehily, A.M.; Pickering, J.E.; Yarnell, J.W.G.; Elwood, P.C. Dietary indices of atherogenicity and thrombogenicity and ischemic heart disease risk: The Caerphilly Prospective Study. *Br. J. Nutr.* **1994**, *71*, 249–257. [CrossRef]
50. Bušová, M.; Kouřimská, L.; Tuček, M. Fatty acids profile, atherogenic and thrombogenic indices in freshwater fish common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) from market chain. *Cent. Eur. J. Public Health* **2020**, *28*, 313–319. [CrossRef]
51. Ulbricht, T.L.V.; Southgate, D.A.T. Coronary heart disease: Seven dietary factors. *Lancet* **1991**, *338*, 985–992. [CrossRef]
52. Fernandes, C.E.; Vasconcelos, M.A.D.S.; Ribeiro, M.D.A.; Sarubbo, L.A.; Andrade, S.A.C.; Filho, A.B.D.M. Nutritional and lipid profiles in marine fish species from Brazil. *Food Chem.* **2014**, *160*, 67–71. [CrossRef]
53. Zhang, X.; Ning, X.; He, X.; Sun, X.; Yu, X.; Cheng, Y.; Yu, R.-Q.; Wu, Y. Fatty acid composition analyses of commercially important fish species from the Pearl River Estuary, China. *PLoS ONE* **2020**, *15*, e0228276. [CrossRef] [PubMed]
54. Sargent, J. Origins and functions of egg lipids: Nutritional implications. In *Broodstock Management and Egg and Larval Quality*; Bromage, N.R., Roberts, R.J., Eds.; Blackwell Science: Oxford, UK, 1995; pp. 353–372.
55. Perdomo, L.; Beneit, N.; Otero, Y.F.; Escribano, Ó.; Díaz-Castroverde, S.; Gómez-Hernández, A.; Benito, M. Protective role of oleic acid against cardiovascular insulin resistance and in the early and late cellular atherosclerotic process. *Cardiovasc. Diabetol.* **2015**, *14*, 75. [CrossRef]
56. Sales-Campos, H.; Reis de Souza, P.; Crema Peghini, B.; Santana da Silva, J.; Ribeiro Cardoso, C. An Overview of the Modulatory Effects of Oleic Acid in Health and Disease. *Mini Rev. Med. Chem.* **2013**, *13*, 201–210.
57. Andrade, A.D.; Rubira, A.F.; Matsushita, M.; Souza, N.E. θ3 fatty acids in freshwater fish from south brazil. *J. Am. Oil Chem. Soc.* **1995**, *72*, 1207–1210. [CrossRef]
58. Aprodu, I.; Vasile, A.; Gurau, G.; Ionescu, A.; Paltenea, E. Evaluation of nutritional quality of the common carp (*Cyprinus carpio*) enriched in fatty acids. *Ann. Univ. Dunarea Jos Galati Fasc. VI Food Technol.* **2012**, *36*, 61–73.
59. Simopoulos, A.P. Evolutionary aspects of diet, the omega-6/omega-3 ratio and genetic variation: Nutritional implications for chronic diseases. *Biomed. Pharmacother.* **2006**, *60*, 502–507. [CrossRef]
60. Institute of Medicine; National Research Council. Annex 1. Dietary recommendations for fish consumption. In *A Framework for Assessing Effects of the Food System*; National Academies Press: Washington, DC, USA, 2015; pp. 287–302.
61. Connor, W.E. n-3 fatty acids in health and disease. In *Nutrition and Disease Update: Heart Disease*; Kritchevski, D., Carroll, K.K., Eds.; American Oil Chemists' Society: Champaign, IL, USA, 1994; pp. 7–42.
62. Sargent, J.; Tocher, D.; Bell, J. The lipids. In *Fish Nutrition*; Halver, J.E., Hardy, R.W., Eds.; Academic Press: San Diego, CA, USA, 2002; pp. 181–257.
63. Shavandi, A.; Hou, Y.; Carne, A.; McConnell, M.; Bekhit, A.E.-D.A. Marine Waste Utilization as a Source of Functional and Health Compounds. *Adv. Food Nutr. Res.* **2018**, *87*, 187–254. [CrossRef] [PubMed]
64. Farid, S.; Ouzgane, A.; Droussi, M.; Hasnaoui, M. Evolution des parametres zootechniques de la carpe argentee (*Hypophthalmichthys molitrix*) elevee sous climat semi-aride a la station de pisciculture deroua, Maroc. *J. Water Environ. Sci.* **2017**, *1*, 115–122.
65. Farid, S.; Ouizgane, A.; Majdoubi, F.-Z.; Hasnaoui, M.; Droussi, M. Diet of *Hypophthalmichthys molitrix*, *Ctenopharyngodon idella* and *Cyprinus carpio* in Ponds of Deroua Fisheries Station, Morocco. In Proceedings of the 4th Edition of International Conference on Geo-IT and Water Resources 2020, Geo-IT and Water Resources 2020, Al Hoceima, Morocco, 11–12 March 2020; pp. 1–6.
66. Sadouni, S.; Iguer-Ouada, M. Factors limiting successful reproduction in wild silver carp, *Hypophthalmichtys molitrix*, in Kherrata Reservoir, Algeria. *Fish. Aquat. Life* **2019**, *27*, 159–168. [CrossRef]

67. Mutethya, E.; Yongo, E.; Laurent, C.; Waithaka, E.; Lomodei, E. Population biology of common carp, Cyprinus carpio (*Linnaeus*, 1758), in Lake Naivasha, Kenya. *Lakes Reserv. Res. Manag.* **2020**, *25*, 326–333. [CrossRef]
68. Guerriero, G.; Ferro, R.; Ciarcia, G. Correlations between plasma levels of sex steroids and spermatogenesis during the sexual cycle of the chub, *Leuciscus cephalus* L. (Pisces: Cyprinidae). *Zool. Stud. Taipei* **2005**, *44*, 228–233.
69. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917. [CrossRef]
70. Cladis, D.P.; Kleiner, A.C.; Freiser, H.H.; Santerre, C.R. Fatty Acid Profiles of Commercially Available Finfish Fillets in the United States. *Lipids* **2014**, *49*, 1005–1018. [CrossRef]
71. Christie, W.W. Preparation of ester derivatives of fatty acids for chromatographic analysis. *Adv. Lipid Methodol.* **1993**, *2*, 69–111.
72. Tvrzická, E. Analysis of Fatty Acids in Plasma Lipoproteins by Gas Chromatography–Flame Ionization Detec-Tion: Quantitative Aspects. *Anal. Chim. Acta* **2002**, *465*, 337–350. [CrossRef]
73. Ackman, R.G.; Sipos, J.C. Application of Specific Response Factors in the Chromatographic Analysis of Methyl Esters of Fatty Acids with Flame Ionization Detectors. *J. Am. Oil Chem. Soc.* **1964**, *41*, 377. [CrossRef]
74. Craske, J.D.; Bannon, C.D. Gas liquid chromatography analysis of the fatty acid composition of fats and oils: A total system for high accuracy. *J. Am. Oil Chem. Soc.* **1987**, *64*, 1413–1417. [CrossRef]
75. Eder, K.; Reichlmayr-Lais, A.; Kirchgessner, M. Gas chromatographic analysis of fatty acid methyl esters: Avoiding discrimination by programmed temperature vaporizing injection. *J. Chromatogr. A* **1991**, *588*, 265–272. [CrossRef]
76. Fernández, M.; Ordóñez, J.A.; Cambero, I.; Santos, C.; Pin, C.; de la Hoz, L. Fatty acid compositions of selected varieties of Spanish dry ham related to their nutritional implications. *Food Chem.* **2007**, *101*, 107–112. [CrossRef]

marine drugs

MDPI

Article

Evaluation of Lipid Extracts from the Marine Fungi *Emericellopsis cladophorae* and *Zalerion maritima* as a Source of Anti-Inflammatory, Antioxidant and Antibacterial Compounds

Mariana Abraúl [1,2], Artur Alves [3], Sandra Hilário [3], Tânia Melo [1,2], Tiago Conde [1,2], Maria Rosário Domingues [1,2,*] and Felisa Rey [1,2,*]

[1] ECOMARE—Laboratory for Innovation and Sustainability of Marine Biological Resources, CESAM—Centre for Environmental and Marine Studies, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[2] LAQV-REQUIMTE, Mass Spectrometry Centre, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[3] CESAM—Centre for Environmental and Marine Studies, Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
* Correspondence: mrd@ua.pt (M.R.D.); felisa.rey@ua.pt (F.R.)

Abstract: Marine environments occupy more than 70% of the earth's surface, integrating very diverse habitats with specific characteristics. This heterogeneity of environments is reflected in the biochemical composition of the organisms that inhabit them. Marine organisms are a source of bioactive compounds, being increasingly studied due to their health-beneficial properties, such as antioxidant, anti-inflammatory, antibacterial, antiviral, or anticancer. In the last decades, marine fungi have stood out for their potential to produce compounds with therapeutic properties. The objective of this study was to determine the fatty acid profile of isolates from the fungi *Emericellopsis cladophorae* and *Zalerion maritima* and assess the anti-inflammatory, antioxidant, and antibacterial potential of their lipid extracts. The analysis of the fatty acid profile, using GC-MS, showed that *E. cladophorae* and *Z. maritima* possess high contents of polyunsaturated fatty acids, 50% and 34%, respectively, including the omega-3 fatty acid 18:3 *n*-3. *Emericellopsis cladophorae* and *Z. maritima* lipid extracts showed anti-inflammatory activity expressed by the capacity of their COX-2 inhibition which was 92% and 88% of inhibition at 200 μg lipid mL^{-1}, respectively. *Emericellopsis cladophorae* lipid extracts showed a high percentage of inhibition of COX -2 activity even at low concentrations of lipids (54% of inhibition using 20 μg lipid mL^{-1}), while a dose-dependent behaviour was observed in *Z. maritima*. The antioxidant activity assays of total lipid extracts demonstrated that the lipid extract from *E. cladophorae* did not show antioxidant activity, while *Z. maritima* gave an IC_{20} value of 116.6 ± 6.2 μg mL^{-1} equivalent to 92.1 ± 4.8 μmol Trolox g^{-1} of lipid extract in the DPPH•assay, and 101.3 ± 14.4 μg mL^{-1} equivalent to 106.6 ± 14.8 μmol Trolox g^{-1} of lipid extract in the ABTS$^{\bullet+}$ assay. The lipid extract of both fungal species did not show antibacterial properties at the concentrations tested. This study is the first step in the biochemical characterization of these marine organisms and demonstrates the bioactive potential of lipid extracts from marine fungi for biotechnological applications.

Keywords: antibacterial; anti-inflammatory; antioxidant activity; α-linolenic acid; COX-2; fatty acids; lipids; marine fungi; PUFA

Citation: Abraúl, M.; Alves, A.; Hilário, S.; Melo, T.; Conde, T.; Domingues, M.R.; Rey, F. Evaluation of Lipid Extracts from the Marine Fungi *Emericellopsis cladophorae* and *Zalerion maritima* as a Source of Anti-Inflammatory, Antioxidant and Antibacterial Compounds. *Mar. Drugs* **2023**, *21*, 199. https://doi.org/10.3390/md21040199

Academic Editors: Giuseppina Tommonaro and Annabella Tramice

Received: 20 February 2023
Revised: 17 March 2023
Accepted: 20 March 2023
Published: 23 March 2023

1. Introduction

The interest in the identification of new therapeutic agents has guided the exploration of natural environments throughout human history. Marine ecosystems occupy more than 70% of the earth's surface, presenting very diverse environments and singular conditions. Marine habitats have been colonized by highly differentiated organisms, and much of them

remain unexplored. However, technological advances have revealed that these habitats contain organisms that can be used as sources of diversified natural products.

Marine organisms are a source of bioactive compounds, being increasingly studied due to their beneficial properties for biotechnological applications [1–4]. From 2016 to 2020, 7547 new natural products of marine origin have been described [5]. In this search for new natural compounds, microorganisms have stood out for their potential due to their ability to be cultivated on a large scale, producing high amounts of primary and secondary metabolites at low cost and with low environmental impact [1,6].

Marine fungi are an ecologically diverse group found in nearly every marine habitat explored, such as decaying coastal wood, algae, marine animals, coral reef, sea garbage, mangrove plants, or sediments [7–11]. These organisms play relevant ecological roles contributing to phytoplankton population cycles, carbon pumps, and nutrient cycling [12]. Several fungal species are used for different purposes, such as food, medical or industrial applications [13,14]. Enzymes (e.g., polysaccharidases, lipases, proteases) extracted from marine fungi have been studied due to their specific activity, even under less favorable conditions (e.g., high salinity, high pressure, acid, and alkaline pH) [13]. Despite the importance of their metabolites for scientific advances, the exploitation of marine fungi to produce bioactive compounds is limited [15,16]. However, marine fungi represent the marine group with the highest number of new compounds analyzed in recent years, illustrating the progressive interest in the identification of bioactive compounds produced by these organisms [5,17]. These compounds included molecules from eight groups: terpenes, sterols, alkaloids, ethers, phenols, lactones, peptides, and others that do not fit into these groups [3,15]. Several molecules produced by marine fungi have been identified for their anti-inflammatory, antibacterial, antiviral, and anticancer properties [15,17,18]. Fungi represent cheap and easily available sources of bioactive compounds, such as lipids, enzymes, or organic acids [19,20]. These marine organisms could be used as bio-factories to produce compounds with bioactivity for biotechnology industries.

Inflammatory diseases are one of the most common pathologies nowadays, so it is necessary to find new sources of anti-inflammatory agents. A recent review of marine fungi identified 133 anti-inflammatory metabolites, polyketides, and terpenoids, the chemical classes with a high number of reported molecules [17]. However, the anti-inflammatory potential of lipids of fungal origin has been poorly studied, even though strong anti-inflammatory and anti-thrombotic properties have been found in lipid fractions of the entomopathogenic fungus *Beauveria bassiana* (Bals.-Criv.) Vuill. [21].

Inflammation and oxidative processes are closely linked to many diseases and disorders (e.g., cardiometabolic disease, obesity, depression) [22–24], as the inflammatory response can lead to increased oxidative stress. Then, the balance of both processes is important to maintain human health. The imbalance between the production of reactive oxygen species (ROS), either generated by endogenous (e.g., mitochondria) or exogenous (e.g., environmental factors, pollutants, radiations) sources [25], and the endogenous antioxidant compounds can lead to oxidative stress, causing damage to fatty acids, DNA and proteins as well as other cellular components [25]. ROS imbalance has been associated with several disorders in human health, such as rapid aging, cancer, cardiovascular, inflammatory, and neurodegenerative diseases [26,27]. Therefore, the search for alternative sources of natural antioxidants for the pharma, cosmetic, and food industries has been increasing in the last few years.

Natural compounds with antioxidant activity have been explored in marine organisms [28–30]. These compounds are interesting natural ingredients for several fields, such as food preservatives, functional ingredients to inhibit the action of ROS, preventing the oxidative damage associated with several diseases [31], or cosmetics to protect against oxidative injuries associated with UV irradiation and photoaging [32]. The antioxidant activity of methanolic extracts from marine fungi has been identified in several species, such as *Cladosporium cladosporioides* (Fresen.) G.A. de Vries and *Curvularia trifolii* (Kauffman) Boedijn from corals [33], and *Cladosporium rubrum* T. Vicente, M. Gonçalves & A. Alves and

Penicillium lusitanum M. Gonçalves, L. Santos, B.M.V. Silva, A.C. Abreu, T.F.L. Vicente, A.C. Esteves & A. Alves from algae and seawater, respectively [19]. Moreover, some compounds, such as polysaccharides isolated from marine filamentous fungi (e.g., *Penicillium* sp.) have also been identified as antioxidant agents [34]. However, the activity of lipid extracts from marine fungi has been scarcely investigated.

The objective of the present study was to characterize the fatty acid profile of two species of marine fungi and to screen the bioactive potential of their lipid extracts as anti-inflammatory, antioxidant, and antibacterial agents. The model species for this study were the fungi *Emericellopsis cladophorae* M. Gonçalves, T. Vicente & A. Alves, and *Zalerion maritima* (Linder) Anastasiou, which have been associated with interesting properties [19,35]. *Emericellopsis cladophorae* belongs to the family *Bionectriaceae*. This species has been recognized to have antibacterial, antioxidant, and cytotoxic bioactivities [19]. A recent study with *E. cladophorae* using untargeted metabolomics and genome sequencing determined the biosynthetic potential of this fungal species to produce bioactive metabolites [36]. *Zalerion maritima* belongs to the family *Lulworthiaceae*. This species has been shown to produce extracellular enzymatic activities (e.g., amylases, cellulase, xylanase), and mycelium extracts exhibited antibacterial activity against Gram-positive bacteria [19]. However, the lipid composition of these fungal species has been overlooked.

2. Results

2.1. Fatty Acid Profiles

The lipid content in *E. cladophorae* and *Z. maritima* was 3.30 ± 2.64 and 1.60 ± 0.40 μg lipid mg^{-1} wet weight, respectively. The percentage of phospholipids in total lipid extracts was 6.16% ± 2.92 and 29.01% ± 13.31 in *E. cladophorae* and *Z. maritima*, respectively.

Fatty acid profiles of the lipid extracts of *E. cladophorae* and *Z. maritima* are summarized in Table 1. This analysis revealed that the most abundant fatty acids in *E. cladophorae* samples were 18:2 *n*-6 (45.38 ± 4.79%), 18:1 *n*-9 (18.35 ± 0.59%), 16:0 (15.97 ± 0.73%), 18:0 (13.51 ± 5.55%) and 18:3 *n*-3 (4.22 ± 0.69%) (Table 1). The fatty acids with the highest relative abundance in the *Z. maritima* samples were 16:0 (27.64 ± 2.72%), 18:0 (26.43 ± 9.34%), 18:2 *n*-6 (24.51 ± 7.32%), 18:1 *n*-9 (11.07 ± 3.20%) and 18:3 *n*-3 (7.95 ± 1.47%) (Table 1). In both fungal species, these fatty acids represented more than 97% of the total fatty acids. The odd-chain fatty acids 15:0 and 17:0 were identified in trace abundances (~0.2%) in both species.

Emericellopsis cladophorae presented a high relative abundance of unsaturated fatty acids (~69%), containing 49.85% ± 5.45 polyunsaturated fatty acids (PUFA) and 18.99 ± 0.58% of monounsaturated fatty acids (MUFA), while saturated fatty acids (SFA) accounted for 31.16 ± 5.10% (Table 1). *Zalerion maritima* has a high abundance of SFA (55.37 ± 11.75%), with 11.78 ± 3.40% and 32.55 ± 8.36% of MUFA and PUFA, respectively (Table 1).

2.2. Anti-Inflammatory Activity

The anti-inflammatory potential of *E. cladophorae* and *Z. maritima* lipid extracts was evaluated by the inhibition of human cyclooxygenase-2 (COX-2) activity assay. Lipid extracts of *E. cladophorae* showed high inhibition of COX-2 activity even at low concentrations (Figure 1). The extract of this fungal species showed inhibition of COX-2 activity of 53.9 ± 2.4%, 80.3 ± 2.0%, 78.5 ± 1.9%, and 91.7 ± 0.6% with concentrations of lipid extracts of 20, 60, 125 and 200 μg mL^{-1}, respectively (Figure 1). The lipid extract required to inhibit 60% (IC_{60}) of COX-2 activity was 45.38 ± 10.68 μg mL^{-1}. The anti-inflammatory activity of *Z. maritima* lipid extracts exhibited a dose-dependent behavior with an increment in the inhibition of COX-2 activity at increasing lipid concentrations (Figure 1). The inhibition of COX-2 activity was 12.3 ± 4.3%, 49.4 ± 11.2%, 63.9 ± 0.6%, and 88.2 ± 0.2% at concentrations of 20, 60, 125, and 200 μg lipid mL^{-1}, respectively (Figure 1). In this fungal species, the IC_{60} was 124.44 ± 1.55 μg lipid mL^{-1}.

Table 1. Relative abundance (%) of fatty acids identified in lipid extracts of *Emericellopsis cladophorae* and *Zalerion maritima*. Values represent mean ± standard deviation (n = 4).

Fatty Acid	*Emericellopsis cladophorae*	*Zalerion maritima*
14:0	0.16 ± 0.01	0.43 ± 0.06
15:0	0.08 ± 0.01	0.09 ± 0.01
16:0	15.97 ± 0.73	27.64 ± 2.72
16:1	0.07 ± 0.03	—
16:1 *n*-7	0.40 ± 0.04	0.19 ± 0.09
16:2 *n*-4	0.08 ± 0.02	—
17:0	0.07 ± 0.01	0.12 ± 0.02
18:0	13.51 ± 5.55	26.43 ± 9.34
18:1 *n*-9	18.35 ± 0.59	11.07 ± 3.20
18:1	0.17 ± 0.02	0.35 ± 0.10
18:2 *n*-6	45.38 ± 4.79	24.51 ± 7.32
18:3 *n*-3	4.22 ± 0.69	7.95 ± 1.47
20:0	0.29 ± 0.11	0.33 ± 0.05
20-methyl-heneicosanoate (iso)	—	0.30 ± 0.09
20:1	—	0.17 ± 0.10
20:2 *n*-6	0.17 ± 0.04	0.12 ± 0.07
22:0	0.61 ± 0.04	—
24:0	0.48 ± 0.07	0.33 ± 0.10
SFA	31.16 ± 5.10	55.37 ± 11.75
MUFA	18.99 ± 0.58	11.78 ± 3.40
PUFA	49.85 ± 5.45	32.55 ± 8.36

Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

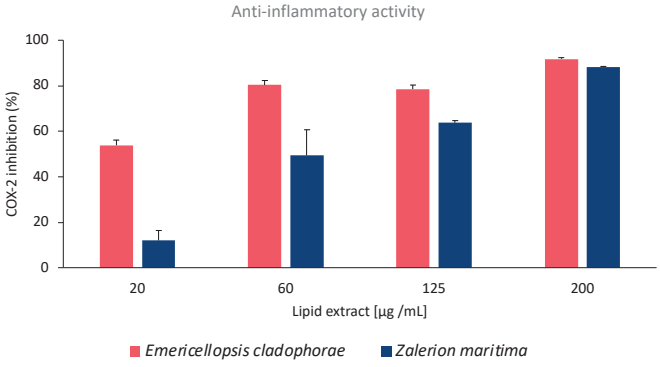


Figure 1. Inhibition of COX-2 activity (expressed as percentage of inhibition, %) as a function of the concentration of the lipid extracts of *Emericellopsis cladophorae* and *Zalerion maritima*. Results are averages of three assays (n = 3) ± standard deviation.

2.3. Antioxidant Activity

The total lipid extracts of *E. cladophorae* did not show antioxidant activity. Antioxidant activity was observed in *Z. maritima* lipid extracts, requiring concentrations of lipid extracts of 116.63 ± 6.21 and 101.30 ± 14.37 µg mL^{-1} to inhibit 20% (IC_{20}) of DPPH•and $ABTS^{\bullet+}$ radicals, respectively (Table 2). The Trolox equivalent was calculated for DPPH•and $ABTS^{\bullet+}$ assays, with values being 92.13 ± 4.82 and 106.58 ± 14.75 µmol Trolox g^{-1} lipid extract, respectively (Table 2).

Table 2. Inhibition concentration (IC) of lipid extracts (μg mL^{-1}) providing 20% of inhibition (IC_{20}) after 120 min of DPPH• and ABTS•$^{+}$ radical scavenging activity, and the corresponding Trolox equivalent units (TE) (μmol of Trolox g^{-1} lipid) in samples from *Zalerion maritima*. Results are averages of three assays (n = 3) ± standard deviation.

	IC_{20} μg mL^{-1}	TE μmol Trolox g^{-1} Lipid Extract
DPPH•	116.63 ± 6.21	92.13 ± 4.82
ABTS•$^{+}$	101.30 ± 14.37	106.58 ± 14.75

2.4. Antibacterial Activity

The total lipid extract of *E. cladophorae* and *Z. maritima* did not show bacteriostatic or bactericidal effects on both bacteria tested, *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 6538). The log UFC mL^{-1} values were identical between the different concentrations of lipid extract tested and the control (Figure 2).

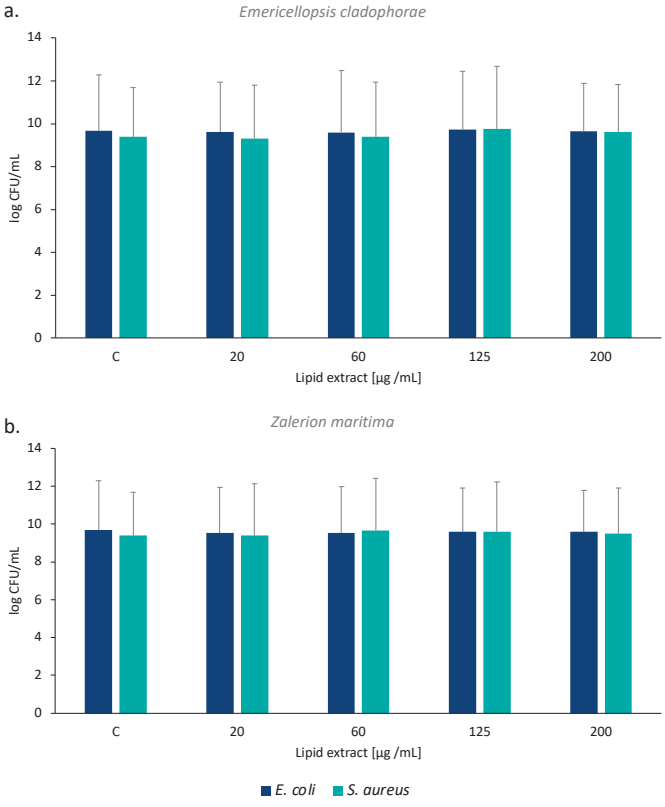


Figure 2. Effects of (**a**) *Emericellopsis cladophorae* and (**b**) *Zalerion maritima* lipid extracts on the growth of *Escherichia coli* and *Staphylococcus aureus*, expressed as log CFU mL^{-1}. Values are averages of three assays (n = 3) ± standard deviation.

3. Discussion

The lipid yield of *E. cladophorae* was higher than that of *Z. maritima*. However, the percentage of phospholipids in the lipid extracts of *Z. maritima* was higher than that in *E. cladophorae*, representing about a fourth of the total lipids in the former species. Marine fungi exhibit elevated lipid content, with a lipid: carbohydrate: protein ratio of c. 13:5:1, on

average [37]. Several species of fungi have been recognized as oleaginous microorganisms due to the high lipid content, which can represent up to 86% of dry weight in some species [38]. The lipid content in fungi is influenced by biotic (e.g., species strain) and abiotic factors (e.g., nitrogen and carbon sources, C/N ratio, temperature, pH) [6,38]. Then, the yield of triacylglycerols (TAG) and the fatty acid composition are modeled by growth conditions and may vary between isolates of fungi, as it was encompassed in several strains [6]. Additionally, the lipid composition changes according to mycelia age. Young mycelia presented a higher proportion of polar lipids (e.g., glycolipids, sphingolipids, and phospholipids) and PUFA; however, an increment in neutral lipid (e.g., TAG) was observed in aged mycelia [39]. A study comparing thirteen marine fungal species isolated from the water column and sediments identified phospholipids as the main polar lipid category, with phosphatidylcholine (PC) and phosphatidylethanolamine (PE) as the most abundant phospholipid classes [40].

Emericellopsis cladophorae displayed a high amount of PUFA, with a relative abundance of almost 50%. However, in *Z. maritima*, the most abundant fatty acid group was SFA. Studies in marine fungi have identified the fatty acid 18:2 *n*-6 as the most abundant PUFA, with a relative abundance between 15–55% [40,41]. Similar results have been obtained in the present study. The α-linolenic acid (18:3 *n*-3, ALA) was the second most abundant PUFA in both *E. cladophorae* and *Z. maritima* species, with a relative abundance higher than that observed in other species [41]. However, the fatty acid profile of the same strain can differ according to the age of the cultures, showing that the stationary phase may provide a more reproducible fatty acid composition than younger cultures [41]. The most abundant PUFA identified in *E. cladophorae* and *Z. maritima* (i.e., 18:2 *n*-6 and 18:3 *n*-3) are important fatty acids in marine food webs since they are precursors of essential fatty acids such as arachidonic acid (20:4 *n*-6, ARA), eicosapentaenoic acid (20:5 *n*-3, EPA) and docosahexaenoic acid (22:6 *n*-3, DHA) [42]. ALA has also been noted for playing essential roles in human health, such as brain development [43], cardiovascular-protective agents [44], or skin lipid modulators improving the skin barrier [45]. Marine fungi can be an alternative source of ingredients of omega-3 fatty acids, namely the essential fatty ALA, for healthy and nutritional products.

The evaluation of COX-2 activity and expression is one of the most common assays in screenings for anti-inflammatory potential. The COX-2 enzyme is selectively induced by pro-inflammatory mediators during inflammation, such as cytokines, mitogens, carcinogens, or oncogenes. This enzyme catalyzes prostaglandin biosynthesis from 20:4 *n*-6, whose availability is dependent on phospholipase A2 (PLA2) expression and/or activity [46]. Prostaglandins are eicosanoids with important roles as pro-inflammatory signaling molecules. Thus, the inhibition of the COX-2 enzyme promotes the suppression of prostaglandins and, consequently, an anti-inflammatory effect. The lipid extracts of *E. cladophorae* showed a high (54%) inhibition of COX-2 activity with the lowest concentration tested (20 μg lipid mL^{-1}) while inhibited 90% of COX-2 activity with the highest concentration tested (200 μg lipid mL^{-1}). Fatty acid analysis of *E. cladophorae* and *Z. maritima* identified omega-3 fatty acids in their lipid extracts, which play a key role as regulators of the inflammatory process. These molecules compete with ARA to produce eicosanoids, suppressing the production of pro-inflammatory mediators [47], and acting as antagonist agents. The beneficial effect of ALA (18:3 *n*-3) against inflammatory-related diseases has been suggested in several studies. Exposure of macrophages to the fatty acid 18:3 *n*-3 promoted a high increase in oxylipins derived from this fatty acid, suggesting that ALA may dampen the inflammatory phenotype of M1-like macrophages [48]. Consumption of diets rich in ALA contributed to potentially beneficial alterations in the plasma oxylipin profiles by reducing oxylipins that induce inflammation [49]. ALA isolated from *Actinidia polygama* fruit downregulates the inflammatory iNOS, COX-2, and TNF- α gene expressions and modulates the anti-inflammatory response [50]. Further studies to elucidate the lipid molecules in which 18:3 *n*-3 is esterified will reveal the potential of these fungal lipid extracts as anti-inflammatory agents in therapeutic treatments.

The lipid extracts of *E. cladophorae* did not show antioxidant activity in the present study. This result is in accordance with the low antioxidant activity obtained in a previous study using methanolic mycelial extracts, which also contain lipids [19]. The differences in antioxidant activity found between the present study (no activity) and that observed in the previous study (low activity of methanolic extracts) could be related to different extract compositions. Methanolic extracts can contain other molecules, like phenolics, that could contribute to the low activity observed. The antioxidant activity of *E. cladophorae* has been related to phenolic, ortho-phenols, and flavonoid compounds [19], as observed in other filamentous fungi [51,52]. Additionally, the differences can also be associated with physiological effects related to the life stage or culture conditions [51,53], as microorganisms can produce different compounds throughout their life stages or under different growth conditions [6,38]. Other extracts from *E. cladophorae*, such as culture filtrate extracts, showed antibacterial activity against the Gram-positive bacterium *Kocuria rhizophila*, while culture medium extracts reduced between 30% and 70% the viability of Vero cells in cytotoxicity assays [19].

The results of the antioxidant scavenging activity of *Z. maritima* lipid extracts against DPPH radicals suggest a higher antioxidant activity than other strains of marine fungi analyzed previously [19]. Studies evaluating the antioxidant activity of other filamentous fungi achieved an IC_{50} in ABTS assays using concentrations of methanolic or ethanol extracts between 2 and 13 mg mL^{-1} [51,52]. However, the present study showed that lipid extracts of *Z. maritima* achieved an IC_{20} in ABTS assays with a concentration of 101 µg mL^{-1}. These results suggest a higher antioxidant capacity of this marine species than that of filamentous fungi isolated from other sources. Marine organisms are recognized to have a high proportion of PUFA. These fatty acids are associated with several beneficial properties, such as antioxidant activity [54]. A study screening the antioxidant activity of lipid extracts from seven microalgae determined that higher antioxidant activity was associated with a higher concentration of PUFA [55]. Omega-3 PUFA, such as 18:3 *n*-3, act as antioxidants by regulating the antioxidant signaling pathways of cell membranes [54]. The higher relative abundance of 18:3 *n*-3 in *Z. maritima* than in *E. cladophorae* may contribute to the antioxidant activity of the former species.

Several factors have led to the development of antimicrobial resistance microbes, such as the misuse and overuse of antibiotics. The identification of new antimicrobial drugs to combat antibiotic-resistance microorganisms (e.g., *S. aureus*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*) is urgently needed. The lipids identified in marine organisms have been shown to be a natural source of effective agents against a wide spectrum of microorganisms [4,56]. The lipid extracts of *E. cladophorae* and *Z. maritima* did not show antibacterial activity against *E. coli* ATCC 25922 (Gram-negative) and *S. aureus* ATCC 6538 (Gram-positive) bacteria. Although *E. cladophorae* showed activity against the Gram-positive bacterium *K. rhizophila* in a previous study [19], this was based on culture medium extracts and may be associated with secondary metabolites secreted by the fungus. In fact, species of *Emericellopsis* have been shown to produce metabolites with antimicrobial properties, such as nonribosomal peptides [57].

PUFA have been recognized for their bioactivity and healthy benefits. However, the biological activities recorded in the lipid extracts of *E. cladophorae* and *Z. maritima* can be related to different lipid classes or species or by a synergistic effect of different lipids present in their lipidome. An in-depth characterization of lipid extracts using lipidomics tools would shed some light on the structure of bioactive lipids and be useful for understanding the mechanisms of action and the structure-activity relationship.

4. Materials and Methods

4.1. Reagents

Potato Dextrose Broth and Potato Dextrose Agar were purchased from Merck (Darmstadt, Germany), and sea salt from Sigma-Aldrich (St. Louis, MO, USA). Dichloromethane (CH_2Cl_2) and methanol (MeOH) were purchased from Fisher Scientific Ltd. (Loughborough,

UK), and *n*-hexane was purchased from Carlo Erba Reagents (Cornaredo, MI, Italy). All the solvents were of high-performance liquid chromatography (HPLC) grade. Milli-Q water was used as ultrapure water (Synergysup®, Millipore Corporation, Billerica, MA, USA). 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) was purchased from Aldrich (Milwaukee, WI); 2,20-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS•$^{+}$) was obtained from Fluka (Buchs, Switzerland); 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The cyclooxygenase (COX-2) inhibitory screening assay was performed using a commercial kit, Cayman test kit-701080, from Cayman Chemical Company (Ann Arbor, MI, USA). All the other reagents and chemicals used were of the highest grade of purity commercially available.

4.2. Fungal Strains

Two fungal strains isolated from the Portuguese coast were used in this study. The *E. cladophorae* strain (MUM 19.33) was identified by Gonçalves et al. [8]. It was isolated from the green macroalga *Cladophora* sp., in the estuary of Ria de Aveiro [8]. The *Z. maritima* strain (CMG 67) was previously identified by Gonçalves et al. [58]. This strain was isolated from wood blocks of *Pinus pinaster*, which were submerged three meters deep in a marina in Ria de Aveiro for one year [58].

4.3. Strain Cultivation

The fungal strains were cultivated in Potato Dextrose Broth (PDB) containing 3% sea salt, which was prepared and distributed in 50 mL portions over seven 250 mL Erlenmeyer flasks, autoclaved at 120 °C for 20 min at 1 bar pressure. Three plugs (± 5 mm), taken from cultures of *E. cladophorae* and *Z. maritima* actively growing in Potato Dextrose Agar (PDA) (Merck, Darmstadt, Germany) containing 3% sea salt, were introduced into each Erlenmeyer for inoculation. Fungal cultures were incubated at 25 °C for 14 days.

After mycelium growth, the biomass was collected by gravitational filtration through filter paper and placed into 50 mL tubes. Four biological replicas ($n = 4$) of *E. cladophorae* and *Z. maritima* biomass were obtained and immediately stored at −80 °C for further analysis.

4.4. Lipid Extraction

The frozen mycelium was macerated with liquid nitrogen using mortar and pestle. The total lipids were extracted from the mycelium using the Bligh and Dyer method [59] with some modifications. Briefly, the biomass was mixed with 2.5 mL of methanol and 1.25 mL of dichloromethane and homogenized for 2 min, followed by sonication for 15 min. Subsequently, the mixture was incubated on ice for 60 min in an orbital shaker (Stuart, SSL2) at 100 rpm. During the incubation period, it was sonicated for 15 min after 30 min and 60 min. After this period, 1.25 mL of dichloromethane was added to the mixture, and it was homogenized for 2 min and centrifugated (Centurion Scientific, Pro-Analytical C4000R, Stoughton, UK) at 3000 rpm for 10 min. The organic phase was recovered in a new glass tube. The biomass was reextracted by adding 2.5 mL of methanol and 2.5 mL of dichloromethane to the pellet, followed by homogenization and centrifugation at 3000 rpm for 10 min. The organic phase was collected and combined with the first in the previous tube. After drying, 2.5 mL of methanol, 2.5 mL of dichloromethane, and 2.25 mL of Milli-Q water were added. Then the mixture was vortexed for 2 min and centrifuged at 3000 rpm for 10 min. The organic phase was collected, and the aqueous phase was reextracted by adding 1.8 mL of dichloromethane, homogenized for 1 min, and centrifugated at 3000 rpm for 10 min. The organic phase was collected into the respective tube and dried under a nitrogen stream. The lipid extracts were transferred to amber vials, dried, weighed, and stored at −20 °C for further analysis. The total lipid extract was quantified by gravimetry.

4.5. Phospholipid Quantification

The amount of phospholipid was quantified according to Bartlett and Lewis [60]. Lipid extracts were dissolved in 300 µL of dichloromethane, and 10 µL of each sample was

transferred into a glass tube, in duplicate, and dried under a nitrogen stream. A volume of 125 μL of perchloric acid (70%) was added, and the samples were placed in a heating block (Stuart, SBH200D/3) at 180 °C for 60 min to promote phospholipid hydrolysis. Phosphate standards (0 to 1.5 μg mL^{-1}) were prepared for the calibration curve. To all samples and standards were added 825 μL of Milli-Q water, 125 μL of ammonium molybdate ($NaMoO_4$ H_2O, 2.5%), and 125 μL of ascorbic acid (10%), followed by a 1-min vortex homogenization between additions. Both samples and standards were incubated in a water bath at 100 °C for 10 min. After cooling, 200 μL of samples and standards were transferred to a 96-well reading plate, and absorbance was measured at 797 nm on a microplate UV-Vis spectrophotometer. The conversion factor 775/31 (25) was used to estimate the phospholipid amount in the samples.

4.6. Fatty Acid Analysis through Gas Chromatography—Mass Spectrometry (GC-MS)

4.6.1. Transesterification

Fatty acids of total lipid extracts were transmethylated to obtain the fatty acid methyl esters (FAME). Total lipid extracts were resuspended in dichloromethane, and a volume corresponding to 8 μg of phospholipids was transferred to a glass tube previously washed with *n*-hexane. The solvent was evaporated under a nitrogen stream, and 1 mL of methylated C19:0 internal standard (0.99 μg mL^{-1}), prepared in *n*-hexane, and 200 μL of a methanolic solution of potassium hydroxide (2.0 M), were added to the samples. The mixture was homogenized for 2 min, and then 2 mL of a saturated aqueous sodium chloride solution (1 g mL^{-1}) was added. After centrifugation at 2000 rpm for 5 min, a volume of 600 μL of the organic phase containing the FAME was collected in a new glass tube and dried under a nitrogen stream.

4.6.2. Gas Chromatography—Mass Spectrometry (GC-MS)

Analysis of the FAME was performed by gas chromatography (Agilent 8860 GC System, Santa Clara, CA, USA) coupled to mass spectrometry (GC-MS) in a GC 5977B Network Mass Selective Detector system equipped with an electronic impact source that operates at 70 eV and at a temperature of 230 °C. The column used was DB-FFAP (Agilent 123-3232, 30 m × 320 μm × 0.25 μm).

Samples were resuspended in 100 μL of *n*-hexane, and a volume of 2 μL was injected in splitless mode using a G 4513 A autosampler with the injector at 220 °C and the detector at 230 °C. The temperature program started with a temperature of 58 °C for 2 min, with a linear increase of 25 °C min^{-1} until 160 °C, followed by another increase of 2 °C min^{-1} until 210 °C and, finally, an increase of 20 °C min^{-1} until 225 °C. This temperature was maintained for 15 min. The carrier gas (helium) was maintained at a constant flow rate of 1.4 mL min^{-1}. The mass spectra acquisition was performed in full scan mode in the range of *m/z* 50–550.

4.6.3. Identification and Integration

Fatty acids from samples of *E. cladophorae* and *Z. maritima* were identified using Agilent MassHunter Qualitative10.0 software and the NIST14L library. Retention times and mass spectra from the samples were compared with commercial standards of fatty acid methyl esters (Supelco 37, Component FAME Mix, ref. 47885-U, Sigma-Aldrich, Darmstadt, Germany). The amounts of fatty acids were calculated as relative abundance (%) using the area of each peak obtained from integration with the Agilent MassHunter Qualitative10.0 software and considering the sum of all relative areas of the identified fatty acids.

4.7. Determination of Anti-Inflammatory Activity of Lipid Extracts

The anti-inflammatory potential of fungal lipid extracts was assessed using a commercial human cyclooxygenase (COX-2) inhibitor screening assay kit—Cayman test kit-701080 (Cayman Chemical Company, Ann Arbor, MI, USA). This assay measures the amount of prostaglandin F2α generated from arachidonic acid (20:4 *n*-6, ARA) in the cyclooxyge-

nase reaction. This assay was carried out according to the instructions provided by the manufacturer. Lipid extracts of *E. cladophorae* and *Z. maritima* were dissolved in 10 µL of DMSO to obtain the final reaction concentrations of 20, 60, 125, and 200 µg of lipid mL^{-1}. Positive and negative controls were provided by the assay kit protocol. The positive control used inactivated COX-2 enzyme, and the negative control used the enzyme with 100% initial activity without any inhibitor. The assay was performed in three replicates (n = 3) of lipid extracts by fungal species. Interferences were considered by subtracting COX-2 inhibition from the blank assays. The results were expressed as a percentage of inhibited COX-2 activity. The prostanoid produced was quantified by spectrophotometry (415 nm, Multiskan GO 1.00.38, Thermo Scientific, Hudson, NH, USA) and processed with the software SkanIT version 3.2 (Thermo Scientific, Waltham, MA, USA).

4.8. Determination of Antioxidant Activity of Lipid Extracts

4.8.1. DPPH Radical Scavenging Activity Assay

The antioxidant scavenging activity against the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) was evaluated using the Magalhães et al. method [61] with modifications [55,62]. An ethanolic solution of DPPH•(250.0 µM) was prepared. This concentration presented an absorbance of ~0.9 measured at 517 nm using a UV-Vis spectrophotometer controlled by the SkanIT software version 3.2. The stock solution was used to prepare the standard solutions (37.5, 50.0, 75.0, 100.0, and 112.50 µM in ethanol). The radical stability was evaluated after the addition of 150 µL of ethanol and 150 µL of DPPH•diluted solution in a 96-well microplate which was incubated for 120 min at room temperature, with absorbance measured at 517 nm every 5 min. The antioxidant activity of the samples was tested using three different concentrations of lipid extracts for each fungal species (20, 60, and 125 µg mL^{-1} in ethanol). The Trolox standards (5.0, 12.5, 25.0, and 37.5 µM in ethanol) were prepared using a Trolox stock solution of 1000 µM. The antioxidant scavenging potential was evaluated using a volume of 150 µL of lipid extracts at different concentrations and 150 µL of Trolox standard solutions, which were placed in the 96-well microplate and followed by the addition of 150 µL of DPPH•diluted solution. To assess the stability of the DPPH•radical, we evaluated that the absorbance variation of this solution was not greater than 10% by placing 150 µL of the DPPH•solution and 150 µL of ethanol. During the incubation with the extracts and Trolox standards, blanks were performed by replacing the DPPH•solution with 150 µL of ethanol. Radical reduction was monitored by measuring the decrease in absorbance during the reaction, quantifying the radical scavenging, accompanied by a color change. The absorbance was read every 5 min for 120 min on the UV-Vis plate spectrophotometer. All measurements were performed in triplicate.

The antioxidant activity of the lipid extracts was determined as the percentage of DPPH radical inhibition according to Equation (1):

$$\text{Inhibition } (\%) = \left[\frac{\text{Abs DPPH} - (\text{Abs Samples} - \text{Abs Control})}{\text{Abs DPPH}} \right] \times 100 \quad (1)$$

The concentration of the sample capable of reducing 20% of the DPPH•radical after 120 min (IC_{20}) was calculated by linear regression using samples concentrations and the inhibition curve percentage. Activity is expressed as Trolox equivalents (TE, µmol Trolox g^{-1} of lipid extract), according to Equation (2):

$$\text{TE} \left(\mu\text{mol g}^{-1} \right) = \left[\frac{\text{IC}_{20} \text{ Trolox} \left(\mu\text{mol L}^{-1} \right)}{\text{IC}_{20} \text{ Samples} \left(\mu\text{g mL}^{-1} \right)} \right] \times 1000 \quad (2)$$

4.8.2. ABTS Radical Scavenging Activity Assay

In the 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical cation ($ABTS^{\bullet+}$) assay, an ethanolic solution of $ABTS^{\bullet+}$ of 7000 µM initial concentration was prepared,

which was used to prepare the standard solutions (25.0, 50.0, 75.0 and 100.0 μM in ethanol). A calibration curve was constructed to calculate the ABTS•$^+$ concentration corresponding to the absorbance of ~0.9, which was chosen for the samples assay. Standards were prepared by adding 150 μL of ethanol and then 150 μL of ABTS•$^+$ solution to the wells, and the absorbance was read at 734 nm after 2 min on a UV-Vis plate spectrophotometer [63,64].

Three lipid extract concentrations were prepared for each fungal species (20, 60, and 125 μg mL^{-1} in ethanol). The Trolox standards (5.0, 12.5, 25.0, and 37.5 μM in ethanol) were prepared in the same way as described above. The antioxidant potential of the extracts (150 μL) and the Trolox standards activity (150 μL) were evaluated after they were placed with 150 μL of the ABTS•$^+$ radical. Since the lipid extracts can absorb in the same wavelength, blanks of each extract concentration were prepared along with 150 μL of ethanol. To assess the stability of the ABTS•$^+$ radical and absorbance variation, controls were performed to assess the ABTS•$^+$ decay, as was done for the DPPH•assay. Radical reduction was monitored by measuring the decrease in absorbance during the reaction, quantifying the radical scavenging accompanied by a color change. The absorbance was read every 5 min for 120 min on the UV-Vis plate spectrophotometer. All measurements were performed in triplicate.

The antioxidant activity of the samples was determined as the percentage of ABTS radical inhibition according to Equation (3):

$$\text{Inhibition } (\%) = \left[\frac{\text{Abs ABTS} - (\text{Abs Samples} - \text{Abs Control})}{\text{Abs ABTS}} \right] \times 100 \quad (3)$$

The concentration of sample capable of reducing 20% of the ABTS•$^+$ radical after 120 min (IC_{20}) was calculated by linear regression using samples concentrations and the inhibition curve percentage. Activity was expressed as Trolox equivalents (TE, μmol Trolox g^{-1} of lipid extract), according to Equation (4):

$$\text{TE} \left(\mu\text{mol g}^{-1} \right) = \left[\frac{\text{IC}_{20} \text{ Trolox} \left(\mu\text{mol L}^{-1} \right)}{\text{IC}_{20} \text{ Samples} \left(\mu\text{g mL}^{-1} \right)} \right] \times 1000 \quad (4)$$

4.9. Determination of Antibacterial Activity of Lipid Extracts

The antibacterial activity of lipid extracts was tested against two bacterial species, a Gram-positive and a Gram-negative, specifically *Staphylococcus aureus* ATCC 6538 and *Escherichia coli* ATCC 25922, respectively. The inoculum for the antibacterial tests was obtained by growing the bacteria in tryptic soy broth (TSB) at 37 °C and 150 rpm overnight (approx. 18 h). Afterward, the culture was diluted in 0.9% NaCl solution and adjusted to a concentration of 10^8 colony-forming units (CFUs) per mL. The assays were performed in 96-well plates and in triplicate. Lipid extracts in DMSO were mixed with Mueller Hinton Broth and distributed 150 μL per well to obtain the final concentrations of 20, 60, 125, and 200 μg mL^{-1}. Each well was then inoculated with 50 μL of the bacterial suspension. Two controls were included, namely, a bacterial inoculum only and a control containing the bacterial inoculum and the same amount of DMSO added to the bacterial culture with lipid extracts. The bacterial suspensions were incubated at 37 °C and 120 rpm for 24 h. After incubation, bacterial cultures were serially diluted in 0.9% NaCl and plated in Mueller Hinton agar using the drop plate method. After incubation at 37 °C for 18 h, viable counts were determined as log CFU mL^{-1} and compared with the control with DMSO. Extracts were considered to have bacteriostatic or bactericidal effects if a decrease of <3-log and ≥3-log in CFU mL^{-1}, respectively, was observed.

5. Conclusions

The fatty acid analysis allowed the identification of a high proportion of PUFA in the composition of both fungal species (50% in *E. cladophorae*, 33% in *Z. maritima*), including interesting fatty acids such as α-linolenic acid (18:3 *n*-3). Lipid extracts of *E. cladophorae*

and *Z. maritima* demonstrated a high potential as anti-inflammatory agents. *Emericellopsis cladophorae* showed a high percentage of inhibited COX-2 activity even at low concentrations of lipids (20 μg lipid mL^{-1} achieved 54% of COX-2 inhibition activity), while *Z. maritima* lipid extracts displayed a dose-dependent behavior in the inhibition of COX-2 activity. Lipid extracts of *Z. maritima* demonstrated antioxidant scavenging activity against DPPH• and $ABTS^{\bullet+}$ with concentrations of 116.6 ± 6.2 and 101.3 ± 14.4 μg mL^{-1}, respectively to achieve an IC_{20} in both antioxidant assays. The lipid concentrations of *E. cladophorae* and *Z. maritima* used in the antibacterial assay did not show antibacterial properties against *E. coli* and *S. aureus*.

Fungi can act as cheap bio-factories of bioactive molecules, which could be cultivated on a large scale. The high proportion of PUFA in the marine fungi *E. cladophorae* and *Z. maritima* could be explored to produce food-grade lipids for food, fed, and nutraceutical ingredients. Additionally, lipid extracts of these fungal species have potential clinical applications, such as in the treatment of inflammation, wound healing, and skin diseases. This study contributes to the bioprospection of marine fungi as promising sources of natural compounds with interesting properties, which can be relevant in biotechnological applications.

Author Contributions: Conceptualization, A.A., M.R.D. and F.R.; methodology, M.A., S.H., T.M., T.C. and F.R.; validation, A.A., T.M., M.R.D. and F.R.; formal analysis, M.A., A.A., T.M., T.C. and F.R.; investigation, all authors; resources, A.A. and M.R.D.; data curation, M.A., T.M., T.C. and F.R.; writing—original draft preparation, M.A. and F.R.; writing—review and editing, F.R.; visualization, M.A. and F.R.; funding acquisition, A.A. and M.R.D. All authors have read and agreed to the published version of the manuscript.

Funding: The authors thank the University of Aveiro, Fundação para a Ciência e Tecnologia (FCT), and Ministério da Ciência Tecnologia e Ensino Superior (MCTES) for the financial support to the research units CESAM [UIDB/50017/2020 + UIDP/50017/2020 + LA/P/0094/2020] and LAQV-REQUIMTE (UIDP/50006/2020 + UIDB/50006/2020) through national funds and, where applicable, co-funded by ERDF, within Portugal 2020 Partnership Agreement and Compete 2020, and to the Portuguese Mass Spectrometry Network (RNEM, LISBOA-01-0145-FEDER-402-022125). The authors acknowledge FCT/MCTES for individual funding in the scope of the Individual Call to Scientific Employment Stimulus (CEECIND/00580/2017 to Felisa Rey, CEECIND/01578/2020 to Tânia Melo), and the PhD grant to Sandra Hilário (SFRH/BD/137394/2018) and to Tiago Conde (2020.05678.BD). The authors are thankful to the COST Action EpiLipidNET, CA19105-Pan-European Network in Lipidomics and EpiLipidomics.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be made available on request.

Acknowledgments: This work is a contribution of the Marine Lipidomics laboratory. The authors thank Cátia Fidalgo for her support in the antibacterial assay.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Debbab, A.; Aly, A.H.; Lin, W.H.; Proksch, P. Bioactive Compounds from Marine Bacteria and Fungi. *Microb. Biotechnol.* **2010**, *3*, 544–563. [CrossRef] [PubMed]
2. Deshmukh, S.K.; Prakash, V.; Ranjan, N. Marine Fungi: A Source of Potential Anticancer Compounds. *Front. Microbiol.* **2018**, *8*, 2536. [CrossRef] [PubMed]
3. Hasan, S.; Ansari, M.; Ahmad, A.; Mishra, M. Major Bioactive Metabolites from Marine Fungi: A Review. *Bioinformation* **2015**, *11*, 176–181. [CrossRef] [PubMed]
4. Alves, E.; Dias, M.; Lopes, D.; Almeida, A.; Domingues, M.D.R.; Rey, F. Antimicrobial Lipids from Plants and Marine Organisms: An Overview of the Current State-of-the-Art and Future Prospects. *Antibiotics* **2020**, *9*, 441. [CrossRef] [PubMed]
5. Carroll, A.R.; Copp, B.R.; Davis, R.A.; Keyzers, R.A.; Prinsep, M.R. Marine Natural Products. *Nat. Prod. Rep.* **2022**, *39*, 1122. [CrossRef]
6. Athenaki, M.; Gardeli, A.; Diamantopoulou, P.; Tchakouteu, S.; Sarris, D.; Philippoussis, A.; Papanikolaou, S. Lipids from Yeasts and Fungi: Physiology, Production and Analytical Considerations. *J. Appl. Microbiol.* **2017**, *124*, 336–367. [CrossRef]

7. Gonçalves, M.; Aleixo, A.; Vicente, F.; Esteves, A.; Alves, A. Three New Species of *Neocamarosporium* Isolated from Saline Environments: *N. aestuarinum* Sp. Nov., *N. endophyticum* Sp. Nov. and *N. halimiones* Sp. Nov. *Mycosphere* **2019**, *10*, 608–621. [CrossRef]
8. Gonçalves, M.F.M.; Vicente, T.F.L.; Esteves, A.C.; Alves, A. Novel Halotolerant Species of *Emericellopsis* and *Parasarocladium* Associated with Macroalgae in an Estuarine Environment. *Mycologia* **2020**, *112*, 154–171. [CrossRef]
9. Marchese, P.; Garzoli, L.; Young, R.; Allcock, L.; Barry, F.; Tuohy, M.; Murphy, M. Fungi Populate Deep-sea Coral Gardens as Well as Marine Sediments in the Irish Atlantic Ocean. *Environ. Microbiol.* **2021**, *23*, 4168–4184. [CrossRef]
10. Abdel-Wahab, M.A.; Jones, E.B.G.; Bahkali, A.H.A.; El-Gorban, A.M. Marine Fungi from Red Sea Mangroves in Saudi Arabia with *Fulvocentrum rubrum* sp. nov. (Torpedosporales, Ascomycota). *Nova_Hedwig.* **2019**, *108*, 365–377. [CrossRef]
11. Gladfelter, A.S.; James, T.Y.; Amend, A.S. Marine Fungi. *Curr. Biol.* **2019**, *29*, R191–R195. [CrossRef]
12. Amend, A.; Burgaud, G.; Cunliffe, M.; Edgcomb, V.P.; Ettinger, C.L.; Gutiérrez, M.H.; Heitman, J.; Hom, E.F.Y.; Ianiri, G.; Jones, A.C.; et al. Fungi in the Marine Environment: Open Questions and Unsolved Problems. *mBio* **2019**, *10*, e01189-18. [CrossRef] [PubMed]
13. Le Strat, Y.; Ruiz, N.; Fleurence, J.; Pouchus, Y.-F.; Déléris, P.; Dumay, J. Marine Fungal Abilities to Enzymatically Degrade Algal Polysaccharides, Proteins and Lipids: A Review. *J. Appl. Phycol.* **2022**, *34*, 1131–1162. [CrossRef]
14. Savidov, N.; Gloriozova, T.A.; Poroikov, V.V.; Dembitsky, V.M. Highly Oxygenated Isoprenoid Lipids Derived from Fungi and Fungal Endophytes: Origin and Biological Activities. *Steroids* **2018**, *140*, 114–124. [CrossRef] [PubMed]
15. Hu, Y.; Chen, J.; Hu, G.; Yu, J.; Zhu, X.; Lin, Y.; Chen, S.; Yuan, J. Statistical Research on the Bioactivity of New Marine Natural Products Discovered during the 28 Years from 1985 to 2012. *Mar. Drugs* **2015**, *13*, 202–221. [CrossRef] [PubMed]
16. Overy, D.; Rämä, T.; Oosterhuis, R.; Walker, A.; Pang, K.-L. The Neglected Marine Fungi, *Sensu Stricto*, and Their Isolation for Natural Products' Discovery. *Mar. Drugs* **2019**, *17*, 42. [CrossRef]
17. Xu, J.; Yi, M.; Ding, L.; He, S. A Review of Anti-Inflammatory Compounds from Marine Fungi, 2000–2018. *Mar. Drugs* **2019**, *17*, 636. [CrossRef]
18. Wiese, J.; Imhoff, J.F. Marine Bacteria and Fungi as Promising Source for New Antibiotics. *Drug. Dev. Res.* **2019**, *80*, 24–27. [CrossRef]
19. Gonçalves, M.F.M.; Paço, A.; Escada, L.F.; Albuquerque, M.S.F.; Pinto, C.A.; Saraiva, J.A.; Duarte, A.S.; Rocha-Santos, T.A.P.; Esteves, A.C.; Alves, A. Unveiling Biological Activities of Marine Fungi: The Effect of Sea Salt. *Appl. Sci.* **2021**, *11*, 6008. [CrossRef]
20. Ferreira, J.A.; Lennartsson, P.R.; Edebo, L.; Taherzadeh, M.J. Zygomycetes-Based Biorefinery: Present Status and Future Prospects. *Bioresour. Technol.* **2013**, *135*, 523–532. [CrossRef]
21. Tsoupras, A.; Kouvelis, V.N.; Pappas, K.M.; Demopoulos, C.A.; Typas, M.A. Anti-Inflammatory and Anti-Thrombotic Properties of Lipid Bioactives from the Entomopathogenic Fungus *Beauveria bassiana*. *Prostaglandins Other Lipid Mediat.* **2022**, *158*, 106606. [CrossRef] [PubMed]
22. Codoñer-Franch, P.; Valls-Bellés, V.; Arilla-Codoñer, A.; Alonso-Iglesias, E. Oxidant Mechanisms in Childhood Obesity: The Link between Inflammation and Oxidative Stress. *Transl. Res.* **2011**, *158*, 369–384. [CrossRef]
23. Steven, S.; Frenis, K.; Oelze, M.; Kalinovic, S.; Kuntic, M.; Bayo Jimenez, M.T.; Vujacic-Mirski, K.; Helmstädter, J.; Kröller-Schön, S.; Münzel, T.; et al. Vascular Inflammation and Oxidative Stress: Major Triggers for Cardiovascular Disease. *Oxidative Med. Cell. Longev.* **2019**, *2019*, 7092151. [CrossRef] [PubMed]
24. Rawdin, B.J.; Mellon, S.H.; Dhabhar, F.S.; Epel, E.S.; Puterman, E.; Su, Y.; Burke, H.M.; Reus, V.I.; Rosser, R.; Hamilton, S.P.; et al. Dysregulated Relationship of Inflammation and Oxidative Stress in Major Depression. *Brain Behav. Immun.* **2013**, *31*, 143–152. [CrossRef]
25. Krumova, K.; Cosa, G. Chapter 1. Overview of Reactive Oxygen Species. In *Comprehensive Series in Photochemical & Photobiological Sciences*; Nonell, S., Flors, C., Eds.; Royal Society of Chemistry: Cambridge, UK, 2016; Volume 1, pp. 1–21. ISBN 978-1-78262-038-9.
26. Prasad, S.; Gupta, S.C.; Tyagi, A.K. Reactive Oxygen Species (ROS) and Cancer: Role of Antioxidative Nutraceuticals. *Cancer Lett.* **2017**, *387*, 95–105. [CrossRef]
27. Giorgi, C.; Marchi, S.; Simoes, I.C.M.; Ren, Z.; Morciano, G.; Perrone, M.; Patalas-Krawczyk, P.; Borchard, S.; Jędrak, P.; Pierzynowska, K.; et al. Mitochondria and Reactive Oxygen Species in Aging and Age-Related Diseases. *Int. Rev. Cell Mol. Biol.* **2018**, *340*, 209–344. [CrossRef] [PubMed]
28. Lee, S.; Lee, Y.S.; Jung, S.H.; Kang, S.S.; Shin, K.H. Anti-Oxidant Activities of Fucosterol from the Marine Algae *Pelvetia siliquosa*. *Arch. Pharm. Res* **2003**, *26*, 719–722. [CrossRef] [PubMed]
29. Terme, N.; Boulho, R.; Kucma, J.-P.; Bourgougnon, N.; Bedoux, G. Radical Scavenging Activity of Lipids from Seaweeds Isolated by Solid-Liquid Extraction and Supercritical Fluids. *OCL* **2018**, *25*, D505. [CrossRef]
30. Ha, A.W.; Na, S.J.; Kim, W.K. Antioxidant Effects of Fucoxanthin Rich Powder in Rats Fed with High Fat Diet. *Nutr. Res. Pract.* **2013**, *7*, 475. [CrossRef]
31. Lordan, S.; Ross, R.P.; Stanton, C. Marine Bioactives as Functional Food Ingredients: Potential to Reduce the Incidence of Chronic Diseases. *Mar. Drugs* **2011**, *9*, 1056–1100. [CrossRef]
32. Pallela, R. Antioxidants from Marine Organisms and Skin Care. In *Systems Biology of Free Radicals and Antioxidants*; Laher, I., Ed.; Springer: Berlin/Heidelberg, Germany, 2014; pp. 3771–3783. ISBN 978-3-642-30017-2.
33. Couttolenc, A.; Medina, M.E.; Trigos, Á.; Espinoza, C. Antioxidant Capacity of Fungi Associated with Corals and Sponges of the Reef System of Veracruz, Mexico. *Electron. J. Biotechnol.* **2022**, *55*, 40–46. [CrossRef]

34. Sun, H.-H.; Mao, W.-J.; Chen, Y.; Guo, S.-D.; Li, H.-Y.; Qi, X.-H.; Chen, Y.-L.; Xu, J. Isolation, Chemical Characteristics and Antioxidant Properties of the Polysaccharides from Marine Fungus *Penicillium* sp. F23-2. *Carbohydr. Polym.* **2009**, *78*, 117–124. [CrossRef]
35. Paço, A.; Duarte, K.; da Costa, J.P.; Santos, P.S.M.; Pereira, R.; Pereira, M.E.; Freitas, A.C.; Duarte, A.C.; Rocha-Santos, T.A.P. Biodegradation of Polyethylene Microplastics by the Marine Fungus *Zalerion maritimum*. *Sci. Total Environ.* **2017**, *586*, 10–15. [CrossRef]
36. Gonçalves, M.F.M.; Hilário, S.; Van de Peer, Y.; Esteves, A.C.; Alves, A. Genomic and Metabolomic Analyses of the Marine Fungus *Emericellopsis cladophorae*: Insights into Saltwater Adaptability Mechanisms and Its Biosynthetic Potential. *J. Fungi* **2022**, *8*, 31. [CrossRef]
37. Thomas, S.; Lengger, S.K.; Bird, K.E.; Allen, R.; Cunliffe, M. Macromolecular Composition and Substrate Range of Three Marine Fungi across Major Cell Types. *FEMS Microbes* **2022**, *3*, xtab019. [CrossRef]
38. Subramaniam, R.; Dufreche, S.; Zappi, M.; Bajpai, R. Microbial Lipids from Renewable Resources: Production and Characterization. *J. Ind. Microbiol. Biotechnol.* **2010**, *37*, 1271–1287. [CrossRef] [PubMed]
39. Fakas, S.; Makri, A.; Mavromati, M.; Tselepi, M.; Aggelis, G. Fatty Acid Composition in Lipid Fractions Lengthwise the Mycelium of *Mortierella isabellina* and Lipid Production by Solid State Fermentation. *Bioresour. Technol.* **2009**, *100*, 6118–6120. [CrossRef]
40. Gutiérrez, M.; Vera, J.; Srain, B.; Quiñones, R.; Wörmer, L.; Hinrichs, K.; Pantoja-Gutiérrez, S. Biochemical Fingerprints of Marine Fungi: Implications for Trophic and Biogeochemical Studies. *Aquat. Microb. Ecol.* **2020**, *84*, 75–90. [CrossRef]
41. Cooney, J.J.; Doolittle, M.M.; Grahl-Nielsen, O.; Haaland, I.M.; Kirk, P.W. Comparison of Fatty Acids of Marine Fungi Using Multivariate Statistical Analysis. *J. Ind. Microbiol.* **1993**, *12*, 373–378. [CrossRef]
42. Kelly, J.; Scheibling, R. Fatty Acids as Dietary Tracers in Benthic Food Webs. *Mar. Ecol. Prog. Ser.* **2012**, *446*, 1–22. [CrossRef]
43. Pandohee, J. Alpha-Linolenic Acid. In *Nutraceuticals and Health Care*; Elsevier: Amsterdam, The Netherlands, 2022; pp. 279–288. ISBN 978-0-323-89779-2.
44. Yuan, Q.; Xie, F.; Huang, W.; Hu, M.; Yan, Q.; Chen, Z.; Zheng, Y.; Liu, L. The Review of Alpha-linolenic Acid: Sources, Metabolism, and Pharmacology. *Phytother. Res.* **2022**, *36*, 164–188. [CrossRef]
45. Simard, M.; Tremblay, A.; Morin, S.; Martin, C.; Julien, P.; Fradette, J.; Flamand, N.; Pouliot, R. α-Linolenic Acid and Linoleic Acid Modulate the Lipidome and the Skin Barrier of a Tissue-Engineered Skin Model. *Acta Biomater.* **2022**, *140*, 261–274. [CrossRef] [PubMed]
46. Alexanian, A.; Sorokin, A. Cyclooxygenase 2: Protein-Protein Interactions and Posttranslational Modifications. *Physiol Genom.* **2017**, *49*, 667–681. [CrossRef]
47. Conde, T.A.; Zabetakis, I.; Tsoupras, A.; Medina, I.; Costa, M.; Silva, J.; Neves, B.; Domingues, P.; Domingues, M.R. Microalgal Lipid Extracts Have Potential to Modulate the Inflammatory Response: A Critical Review. *IJMS* **2021**, *22*, 9825. [CrossRef] [PubMed]
48. Pauls, S.D.; Rodway, L.A.; Winter, T.; Taylor, C.G.; Zahradka, P.; Aukema, H.M. Anti-Inflammatory Effects of α-Linolenic Acid in M1-like Macrophages Are Associated with Enhanced Production of Oxylipins from α-Linolenic and Linoleic Acid. *J. Nutr. Biochem.* **2018**, *57*, 121–129. [CrossRef] [PubMed]
49. Caligiuri, S.P.B.; Aukema, H.M.; Ravandi, A.; Pierce, G.N. Elevated Levels of Pro-Inflammatory Oxylipins in Older Subjects Are Normalized by Flaxseed Consumption. *Exp. Gerontol.* **2014**, *59*, 51–57. [CrossRef] [PubMed]
50. Ren, J.; Chung, S.H. Anti-Inflammatory Effect of α-Linolenic Acid and Its Mode of Action through the Inhibition of Nitric Oxide Production and Inducible Nitric Oxide Synthase Gene Expression via NF-KB and Mitogen-Activated Protein Kinase Pathways. *J. Agric. Food Chem.* **2007**, *55*, 5073–5080. [CrossRef]
51. Hameed, A.; Hussain, S.A.; Yang, J.; Ijaz, M.U.; Liu, Q.; Suleria, H.A.R.; Song, Y. Antioxidants Potential of the Filamentous Fungi (*Mucor circinelloides*). *Nutrients* **2017**, *9*, 1101. [CrossRef]
52. Smith, H.; Doyle, S.; Murphy, R. Filamentous Fungi as a Source of Natural Antioxidants. *Food Chem.* **2015**, *185*, 389–397. [CrossRef]
53. Papanikolaou, S.; Galiotou-Panayotou, M.; Chevalot, I.; Komaitis, M.; Marc, I.; Aggelis, G. Influence of Glucose and Saturated Free-Fatty Acid Mixtures on Citric Acid and Lipid Production by *Yarrowia lipolytica*. *Curr. Microbiol.* **2006**, *52*, 134–142. [CrossRef]
54. Oppedisano, F.; Macrì, R.; Gliozzi, M.; Musolino, V.; Carresi, C.; Maiuolo, J.; Bosco, F.; Nucera, S.; Caterina Zito, M.; Guarnieri, L.; et al. The Anti-Inflammatory and Antioxidant Properties of n-3 PUFAs: Their Role in Cardiovascular Protection. *Biomedicines* **2020**, *8*, 306. [CrossRef] [PubMed]
55. Conde, T.A.; Neves, B.F.; Couto, D.; Melo, T.; Neves, B.; Costa, M.; Silva, J.; Domingues, P.; Domingues, M.R. Microalgae as Sustainable Bio-Factories of Healthy Lipids: Evaluating Fatty Acid Content and Antioxidant Activity. *Mar. Drugs* **2021**, *19*, 357. [CrossRef]
56. Bartolomeu, M.; Vieira, C.; Dias, M.; Conde, T.; Couto, D.; Lopes, D.; Neves, B.; Melo, T.; Rey, F.; Alves, E.; et al. Bioprospecting Antibiotic Properties in Photodynamic Therapy of Lipids from *Codium tomemtosum* and *Chlorella vulgaris*. *Biochimie* **2022**, *203*, 32–39. [CrossRef]
57. Kuvarina, A.E.; Gavryushina, I.A.; Kulko, A.B.; Ivanov, I.A.; Rogozhin, E.A.; Georgieva, M.L.; Sadykova, V.S. The Emericellipsins A–E from an Alkalophilic Fungus *Emericellopsis alkalina* Show Potent Activity against Multidrug-Resistant Pathogenic Fungi. *JoF* **2021**, *7*, 153. [CrossRef]

58. Gonçalves, M.F.M.; Abreu, A.C.; Hilário, S.; Alves, A. Diversity of Marine Fungi Associated with Wood Baits in the Estuary Ria de Aveiro, with Descriptions of *Paralulworthia halima*, comb. nov., *Remispora submersa*, sp. nov., and *Zalerion pseudomaritima*, sp. nov. *Mycologia* **2021**, *113*, 664–683. [CrossRef]
59. Bligh, E.G.; Dyer, W.J. A Rapid Method of Total Lipid Extraction and Purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917. [CrossRef]
60. Bartlett, M.E.; Lewis, D.H. Spectrophotometric Determination of Phosphate Esters in the Presence and Absence of Orthophosphate. *Anal. Biochem.* **1970**, *36*, 159–167. [CrossRef] [PubMed]
61. Magalhães, L.M.; Segundo, M.A.; Reis, S.; Lima, J.L.F.C. Automatic Method for Determination of Total Antioxidant Capacity Using 2,2-Diphenyl-1-Picrylhydrazyl Assay. *Anal. Chim. Acta* **2006**, *558*, 310–318. [CrossRef]
62. Rey, F.; Cartaxana, P.; Melo, T.; Calado, R.; Pereira, R.; Abreu, H.; Domingues, P.; Cruz, S.; Domingues, R.M. Domesticated Populations of *Codium tomentosum* Display Lipid Extracts with Lower Seasonal Shifts than Conspecifics from the Wild—Relevance for Biotechnological Applications of This Green Seaweed. *Mar. Drugs* **2020**, *18*, 188. [CrossRef] [PubMed]
63. Magalhães, L.M.; Barreiros, L.; Maia, M.A.; Reis, S.; Segundo, M.A. Rapid Assessment of Endpoint Antioxidant Capacity of Red Wines through Microchemical Methods Using a Kinetic Matching Approach. *Talanta* **2012**, *97*, 473–483. [CrossRef] [PubMed]
64. Ozgen, M.; Reese, R.N.; Tulio, A.Z.; Scheerens, J.C.; Miller, A.R. Modified 2,2-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS) Method to Measure Antioxidant Capacity of Selected Small Fruits and Comparison to Ferric Reducing Antioxidant Power (FRAP) and 2,2′-Diphenyl-1- Picrylhydrazyl (DPPH) Methods. *J. Agric. Food Chem.* **2006**, *54*, 1151. [CrossRef]

marine drugs

MDPI

Article

Effect of Nitrogen, Salinity, and Light Intensity on the Biomass Composition of *Nephroselmis* sp.: Optimization of Lipids Accumulation (Including EPA)

Savvas Giannis Mastropetros [1], Konstantina Tsigkou [1], Yannis Cladas [2], Arun Kumar Priya [3,4] and Michael Kornaros [1,*]

[1] Laboratory of Biochemical Engineering & Environmental Technology (LBEET), Department of Chemical Engineering, University of Patras, 26504 Patras, Greece; savvasgiannismas@gmail.com (S.G.M.); ktsigkou@chemeng.upatras.gr (K.T.)
[2] Department of Animal Production, Fisheries & Aquaculture, University of Patras, Mesolonghi, 30200 Nea Ktiria, Greece; jkladas@upatras.gr
[3] Department of Chemical Engineering, KPR Institute of Engineering and Technology, Coimbatore 641407, India; a.k.priya@kpriet.ac.in
[4] Project Prioritization, Monitoring & Evaluation and Knowledge Management Unit, ICAR-Indian Institute of Soil & Water Conservation (ICAR-IISWC), Dehradun 248195, India
* Correspondence: kornaros@chemeng.upatras.gr

Abstract: Microalgal biomass is characterized by high protein, carbohydrates, and lipids concentrations. However, their qualitative and quantitative compositions depend not only on the cultivated species but also on the cultivation conditions. Focusing on the microalgae's ability to accumulate significant fatty acids (FAs) amounts, they can be valorized either as dietary supplements or for biofuel production, depending on the accumulated biomolecules. In this study, a local isolate (*Nephroselmis* sp.) was precultured under autotrophic conditions, while the Box–Behnken experimental design followed using the parameters of nitrogen (0–250 mg/L), salinity (30–70 ppt) and illuminance (40–260 μmol m^{-2} s^{-1}) to evaluate the accumulated biomolecules, with an emphasis on the amount of FAs and its profile. Regardless of the cultivation conditions, the FAs of C14:0, C16:0, and C18:0 were found in all samples (up to 8% *w/w* in total), while the unsaturated C16:1 and C18:1 were also characterized by their high accumulations. Additionally, the polyunsaturated FAs, including the valuable C20:5n3 (EPA), had accumulated when the nitrogen concentration was sufficient, and the salinity levels remained low (30 ppt). Specifically, EPA approached 30% of the total FAs. Therefore, *Nephroselmis* sp. could be considered as an alternative EPA source compared to the already-known species used in food supplementation.

Keywords: *Nephroselmis* sp.; microalgae; biomass composition; lipids accumulation; dietary supplement; eicosapentaenoic acid (EPA); Box–Behnken experimental design

Citation: Mastropetros, S.G.; Tsigkou, K.; Cladas, Y.; Priya, A.K.; Kornaros, M. Effect of Nitrogen, Salinity, and Light Intensity on the Biomass Composition of *Nephroselmis* sp.: Optimization of Lipids Accumulation (Including EPA). *Mar. Drugs* **2023**, *21*, 331. https://doi.org/10.3390/md21060331

Academic Editors: Giuseppina Tommonaro and Annabella Tramice

Received: 11 May 2023
Revised: 24 May 2023
Accepted: 26 May 2023
Published: 28 May 2023

1. Introduction

The majority of environments and habitats worldwide, such as seawater, brackish or freshwater, rocks, land, and soil, among others, are appropriate for microalgae growth. According to the literature, they may grow when attached to substrates, form mats, or float [1]. Microalgae have exhibited a wide range of cultivation and growth conditions (temperature, nutrient availability, pH level, light intensity, and CO_2 presence) and morphology and size. For example, species such as *Dunaliella salina* are able to tolerate and adapt to high salinity levels at a significantly wide temperature range of 0–35 °C [1,2]. It is known that, aside from their critical role in the cycles of carbon and nitrogen on Earth, they are also produced and used commercially, either in the food and pharmaceutical industries or as biofuels [3,4]. Due to the high diversity of microalgae and their various growth requirements, the isolation and selection of local strains seem to be an effective alternative

for their utilization, especially in the case of outdoor cultivation. As the climate of a location can significantly impinge on the microalgal population and growth dynamics, their screening and growth investigation could enhance their positive impact on high-added value compound accumulation [5,6].

The Mediterranean region is known for exhibiting ideal climate conditions for microalgae growth, especially in the south due to the warm temperatures, which usually do not fall below 15 °C [7]. According to the literature, several microalgal and cyanobacterial species have been isolated in Greek environments and habitats by various research groups [6,8–11], usually targeting and taking advantage of the sturdy local isolates to enhance value-added compound production [6]. More specifically, 15 *Dunaliella* strains were isolated by Lortou et al. [9] from Greek saltworks, resulting in nine different species, which were *D. minutissima*, *D. parva*, *D. asymmetrica*, *D. minuta*, *D. terricola*, *D. viridis*, *D. granulata*, *D. bioculata*, and *D. polymorpha*. On the other hand, Prasinophyceae strains were isolated by Tzovenis et al. [11] from a Greek coastal lagoon. Both the *Tetraselmis* and *Pyramimonas* local isolates were presented as good alternatives for polyunsaturated FAs (PUFAs) production. Additionally, according to Gkelis et al. [10], 29 strains were isolated from freshwaters in Greece and were classified as Chroococcales, Synechococcales, and Nostocales, and reported the following strains, such as *Nostoc oryzae*, *Chlorogloeopsis fritschii,* and *Synechococcus* cf. *nidulans*. Finally, according to Hotos et al. [6], one dinoflagellate (*Amphidinium carterae*), three cyanobacteria (*Phormidium* sp., *Anabaena* sp., and *Cyanothece* sp.), and five Chlorophyta species (*Asteromonas gracilis*, *Dunaliella* sp., *Tetraselmis marina*, *Tetraselmis* sp., and *Nephroselmis* sp.) were collected from Western Greece lagoons, while their identification, morphological observation, and characterization followed.

Especially for the genus *Nephroselmis*, it is worth mentioning that it was established by Stein in 1878 on the basis of freshwater species and was named *Nephroselmis olivacea* Stein. Over time, more than 10 *Nephroselmis* species have been identified, originating either from freshwater or marine environments [6,12,13]. The general characteristics of the specific genus are as follows: (a) a flattened cell body with two flagella of different sizes (one short one at the front and one longer one at the back) that move in different directions; (b) scales on the surface, the second of which is rod-shaped; (c) two to four layers of scales on the cell surface, the second of which has an unclear shape; and (d) flagellar with a root system, which includes a rhizoplast and three microtubular roots [14]. A fairly wide range of *Nephroselmis* sp. cultivation conditions and carbon sources have been tested, targeting the enhancement of biomass production and added-value compound accumulation, mainly FAs and pigments [8,15–17].

Focusing on lipids accumulation, their classification refers to polar (phospholipids and glycolipids) and neutral (acylglycerols and free FAs) lipids. Their content, described as the dry weight of biomass, may vary between 1.5 and 75%, depending on the species, culture medium, and cultivation conditions. Moreso, FAs are carboxylic acids with chains of 4 to 36 C atoms and various levels of saturation (mono-, di- or even poly-saturated) [18]. Their production can be strongly influenced and manipulated due to specific (stress) condition applications. Specifically, PUFAs, mainly C20:5n3 (EPA) and C22:6n3 (DHA), have been widely recognized as essential compounds for both nutrition and health due to their participation in the metabolic pathways responsible for resolving inflammation. Furthermore, EPA is essential for several biological regulation functions, including cardiovascular disease, arrhythmia, atherosclerosis, and cancer. EPA is a PUFA that is heavily involved in the food and nutritional supplement industry, and thus its isolation from autotrophic microorganisms, such as these of the *Nephroselmis* genus, could minimize the production costs in combination with a sustainable downstream process [19,20]. Due to their importance in human wellness and health, there is high market interest worldwide, indicating that the omega-3 market size in 2019 was estimated at USD 2.49 billion, with the potential for further expansion [19].

Regarding biomass production enhancement and added-value compound accumulation, it is widely known that different cultivation strategies should be used. For instance,

it was mentioned in the literature that a two-fold biomass productivity increase led to an overall biodiesel decrease of around 40%. Such an example significantly indicates the need for cultivation conditions manipulation [21,22]. More specifically, optimal conditions should be provided if the main target is biomass accumulation, while alterations to the cultivation conditions should be applied, for instance, nutrient depletion, cultivation systems, or growth modes, if the study focuses on valuable compound accumulation. For this reason, two-stage cultivation systems have been proposed in order to separate the two aforementioned phases and effectively exploit the microalgal biomass [21,23] compared to the one-stage systems. While pointing out two-stage cultivation strategies, several types have been proposed in the literature regarding inducer additions, nutrient starvation, metabolic switch, irradiation, or multi-stress applications [21].

The aim of the current study is the evaluation of a local isolate of the genus *Nephroselmis* regarding its adaptation to a range of salinity values, light intensity, and concentration of available nitrogen. This green alga was cultivated under different conditions, as they resulted from an experimental design using the Box–Behnken methodology. The biomass of *Nephroselmis* sp. gathered research interest due to the variety of the FAs content and, specifically, the existence of the nutritionally valuable EPA. It was collected after five days, and the production of FAs (including EPA), as well as proteins, carbohydrates, and total pigments, was investigated. From the above, a primary conclusion can be drawn about whether the strain under study warrants being used to recover healthy fats and whether its protein and carbohydrate contents make it intrinsically valuable as a nutritional supplement.

2. Results and Discussion

2.1. Biomass Adaptation and Lipids Concentration

To determine the concentration of each cellular component in the culture medium (proteins, carbohydrates, FAs, pigments), it was first necessary to measure the concentration of the produced biomass and analyze its composition on a dry weight basis. Table 1 presents the biomass concentration values of all the experimental scenarios resulting from the experimental design. The biomass concentration values were not statistically different after the analysis of variance and *t*-test comparison, with a significance level of 0.05. Since the substrates were inoculated by a parent autotrophic culture aiming to contain an initial biomass concentration of 100 mg L^{-1}, it was observed that *Nephroselmis* sp. did not show new growth, even in the cases of nitrogen and light sufficiency. It was inferred that the local isolate used the five days spent in the new flasks as a period of adaptation to the new conditions studied. The microorganisms of the genus *Nephroselmis* have been reported regarding their lag phase ranging from 2 to 10 days, depending on the growth conditions [24]. The particular local species was also examined by Hotos et al., who observed a similar behavior during its autotrophic growth, as it took four to five days to begin the exponential phase of growth [8]. The concentration values found below 100 mg L^{-1} can be attributed both to the experimental error and the lysis of some cells, especially in cultures without a nitrogen source and/or under inadequate light. It is worth mentioning that the growth rate of microalgae is limited under nutrient and light stress; however, with the appropriate carbon-to-nitrogen ratio in the substrate, valuable biomolecules, such as FAs, can accumulate. These FAs are no longer a component of the cellular membranes but form triglycerides [25].

The FAs reached a maximum concentration of 15 mg L^{-1} (Figure 1), while their production under the tested ranges of nitrogen concentration, salinity, and light intensity can be predicted by the equations in Table 2. In the same table, it can be seen that only the parameter of nitrogen played a significant role statistically. The most encouraging results, which were related to FAs accumulation, were found in cultures that had a nitrogen content between 210 and 220 mg L^{-1} and light intensity of 150 to 170 μmol m^{-2} s^{-1} when the salinity of the substrate was adjusted to 30 ppt. Nitrogen deficiency did not favor the FAs' synthesis as expected, and therefore, it can be assumed that *Nephroselmis* sp. consumed

the intracellular lipids (initially around 20% of the dry biomass [6]) for energy during its adaptation period to the second cultivation stage. Microalgae store organic carbon for their long-term energy needs in the form of FAs. For instance, palmitic acid can be oxidized in the mitochondria to produce energy according to the reaction described by Sorgüven et al. [26]:

$$\text{Palmitic acid} + \text{ATP} + \text{CoA} \rightarrow \text{Palmitoyl-S-CoA} + \text{AMP} + \text{Ppi}$$

$$\text{Palmitoyl-S-CoA} + 23\ O_2 + 131\ \text{Pi} + 131\ \text{ADP} \rightarrow \text{CoA} + 131\ \text{ATP} + 16CO_2 + 146\ H_2O$$

Table 1. Acquired data after the Box–Behnken experimental design and presentation of the biomass concentration after 5 days of cell acclimation. The standard deviations arose from the differences between the two replicate cultures, and values that share a letter are not significantly different ($p = 0.05$).

Run	Nitrogen (mg L^{-1})	Salinity (ppt)	Light (μmol m^{-2} s^{-1})	Biomass (mg/L) (Average ± SD)
1	0	30	150	92.9 ± 22.5 ^{A}
2	250	30	150	117.4 ± 8.1 ^{A}
3	0	70	150	96.3 ± 8.6 ^{A}
4	250	70	150	109.8 ± 5.6 ^{A}
5	0	50	40	103.2 ± 1.4 ^{A}
6	250	50	40	97.2 ± 9.4 ^{A}
7	0	50	260	106.2 ± 11.4 ^{A}
8	250	50	260	113.2 ± 9.8 ^{A}
9	125	30	40	98.4 ± 0.9 ^{A}
10	125	70	40	96.4 ± 6.1 ^{A}
11	125	30	260	111.1 ± 0.3 ^{A}
12	125	70	260	127.1 ± 26.5 ^{A}
13	125	50	150	108.0 ± 20.9 ^{A}
14	125	50	150	114.4 ± 1.4 ^{A}
15	125	50	150	101.7 ± 2.2 ^{A}

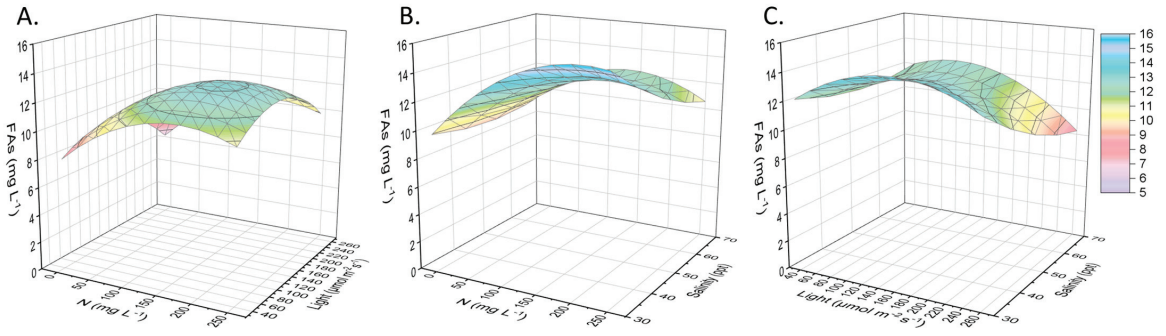


Figure 1. Presentation of the surface plots regarding the FAs production as a function of the initial nitrogen concentration, light intensity, and salinity. FAs (mg L^{-1}) related to (**A**) nitrogen concentration (mg L^{-1}) and light intensity (μmol of photons m^{-2} s^{-1}), given salinity of 50 ppt. (**B**) Nitrogen concentration (mg L^{-1}) and salinity (ppt), given light intensity of 110 μmol of photons m^{-2} s^{-1}. (**C**) Salinity (ppt) and light intensity (μmol of photons m^{-2} s^{-1}), given nitrogen concentration of 125 mg L^{-1}.

Table 2. Equation presentation for the description of biomass compounds production in the tested range of nitrogen (0–250 mg L^{-1}), light intensity (40–260 μmol of photons m^{-2} s^{-1}), and salinity (30–70 ppt). The equations are provided in their analytical form, considering all parameters, as well as in a simplified form, taking into account only the parameters of statistical significance, as derived by Minitab 19.

Biomass Compounds	Equation (All Parameters)	Simplified Equation (Only Parameters of Statistical Significance)
Proteins (mg/L)	$28.7 + 1.05\times10^{-1}N - 0.88\times10^{-1}S - 1.32\times10^{-1}L - 2.58\times10^{-4}N^2 + 0.66\times10^{-3}S^2 + 3.39\times10^{-4}L^2 - 0.51\times10^{-4}NS + 0.14\times10^{-4}NL - 2.17\times10^{-4}SL$	$28.7 + 1.05\times10^{-1}N - 1.32\times10^{-1}L - 2.58\times10^{-4}N^2 + 3.39\times10^{-4}L^2$
Carbohydrates (mg/L)	$52.5 - 2.17\times10^{-1}N - 6.15\times10^{-1}S - 2.05\times10^{-2}L + 6.52\times10^{-4}N^2 + 4.39\times10^{-3}S^2 + 1.38\times10^{-4}L^2 - 0.48\times10^{-4}NS + 1.71\times10^{-4}NL + 1.65\times10^{-4}SL$	$52.5 - 2.17\times10^{-1}N - 6.15\times10^{-1}S - 2.05\times10^{-2}L + 6.52\times10^{-4}N^2$
Pigments (mg/L)	$11.32 + 2.07\times10^{-2}N - 2.69\times10^{-1}S - 3.93\times10^{-2}L - 0.48\times10^{-4}N^2 + 2.01\times10^{-3}S^2 + 0.08\times10^{-4}L^2 + 0.64\times10^{-4}NS - 0.61\times10^{-4}NL + 2.19\times10^{-4}SL$	$11.32 + 2.07\times10^{-2}N - 2.69\times10^{-1}S - 3.93\times10^{-2}L - 0.48\times10^{-4}N^2 + 2.01\times10^{-3}S^2 + 0.08\times10^{-4}L^2 - 0.61\times10^{-4}NL + 2.19\times10^{-4}SL$
FAs (mg/L)	$7.20 + 6.86\times10^{-2}N - 1.03\times10^{-1}S + 6.81\times10^{-2}L - 1.28\times10^{-4}N^2 + 1.79\times10^{-3}S^2 - 0.17\times10^{-3}L^2 - 5.23\times10^{-4}NS + 0.13\times10^{-4}NL - 4.86\times10^{-4}SL$	$7.20 + 6.86\times10^{-2}N$
EPA (% of FAs)	$51.3 + 4.47\times10^{-2}N - 1.15S - 1.87\times10^{-1}L - 0.93\times10^{-4}N^2 + 9.09\times10^{-3}S^2 + 4.53\times10^{-4}L^2 - 0.83\times10^{-3}NS - 0.92\times10^{-4}NL + 3.62\times10^{-4}SL$	$51.3 - 1.15S - 1.87\times10^{-1}L + 9.09\times10^{-3}S^2 + 4.53\times10^{-4}L^2$

N: nitrogen (mg L^{-1}), *L*: light intensity (μmol of photons m^{-2} s^{-1}), and *S*: salinity (ppt).

In another case, when *Chlorella protothecoides* was studied, a decrease in the fatty acid content was also observed during the adaptation period from the first to the second stage of cultivation. This particular species spent 36 days in the lag phase in the substrate with limited nitrate concentration. As a result, FAs fell from 20% to below 15% of the dry biomass weight during that time [27]. *Nephroselmis* sp. has exhibited similar behavior in conditions of nitrogen deficiency but in terms of cellular pigment (chlorophylls and carotenoids) reduction [16]. Given the fixed nitrogen concentration value (Figure 1), a preference for low salinity levels was observed, and a region of optimal light intensity appeared around 150 μmol m^{-2} s^{-1} (for lipids production). It has been previously reported that the biomass lipids of *Nephroselmis* genus microalgae also reached 18.8% w/w when grown under 120 μmol of photons m^{-2} s^{-1} [15].

Furthermore, similar to the total FAs, EPA also appeared to accumulate in low salinity levels and light irradiation. It almost reached 30% of the total FAs when the cultures had light equal to 60 μmol m^{-2} s^{-1} and when the initial nitrogen concentration exceeded 100 mg L^{-1} (Figure 2). Microalgae are able to accumulate n−3 PUFAs up to 10–20% of their dry biomass, accompanied by low proliferation rates due to the nutrients' limitations [28].

Some photosynthetic microorganisms produce EPA or docosahexaenoic acid (DHA), while others contain both valuable FAs. Regarding the EPA, which concerns the present work, it has been observed that a normal content in microalgal biomass did not exceed 5% *w/w*. Indicatively, *Phaeaodactylum tricornutum* yielded 2.2%, *Monodus subterranues* accumulated 2.3 to 3.2% on a dry weight basis, and *Nannochloropsis gaditana* produced biomass with 2.84% of EPA [29–31].

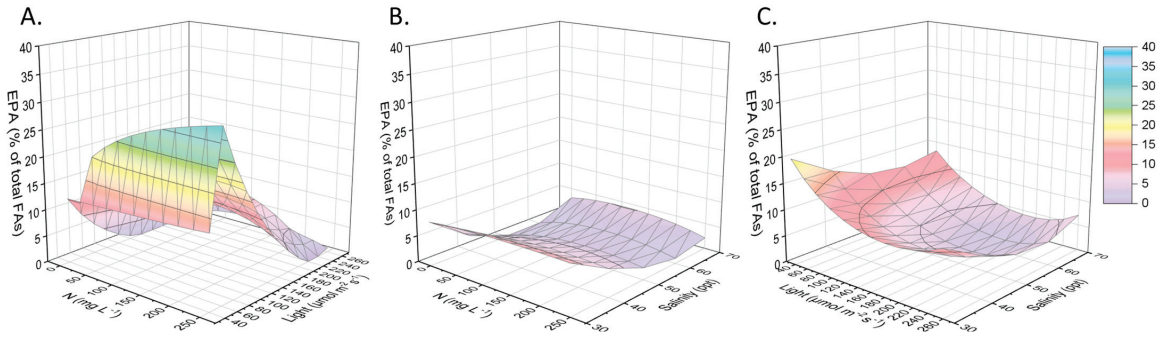


Figure 2. Presentation of the surface plots regarding the EPA production as a function of the initial nitrogen concentration, light intensity, and salinity. EPA (% of total FAs) related to (**A**) nitrogen concentration (mg L^{-1}) and light intensity (μmol of photons m^{-2} s^{-1}), given salinity of 50 ppt. (**B**) Nitrogen concentration (mg L^{-1}) and salinity (ppt), given light intensity of 110 μmol of photons m^{-2} s^{-1}. (**C**) Salinity (ppt) and light intensity (μmol of photons m^{-2} s^{-1}), given nitrogen concentration of 125 mg L^{-1}.

The large variety of FAs (Figure 3) in the biomass of *Nephroselmis* sp. can be divided into saturated and unsaturated molecules. As for the saturated FAs, myristic acid (C14:0), palmitic acid (C16:0), and stearic acid (C18:0) were mainly detected. The first constituted 20% of the total profile, while the second could correspond to 30%, particularly when nitrogen was not supplied to the substrate. Conversely, saturated C18:0 appeared in smaller quantities (<10 %). Looking at the unsaturated FAs, the ones that stood out were palmitoleic acid (C16:1), oleic acid (C18:1), and eicosapentaenoic acid (EPA). One in five FAs was C16:1, while C18:1 appeared at a frequency of about 1 in 10 in every experimental culture. Finally, the EPA was shown to be increased (23.7%) in the case of *Nephroselmis* sp. being provided to a medium with a nitrogen concentration of 125 mg L^{-1} at low salinity levels and under the minimum tested illumination. A large percentage was also occupied when exposed to higher salinity, equal to 70 ppt. In this case, it was measured to be 15.2%. From Figure 3, it was also found that high light intensities (260 μmol m^{-2} s^{-1}) did not enhance the accumulation of EPA, which only in the case of zero nitrogen and average salinity approached 11% among the other FAs.

By comparing the FAs profile of the local isolate with that of *Nephroselmis* sp. KGE2, both similarities and differences were distinguished, concluding that the properties of the substrate determined the qualitative and quantitative compositions of the lipid load of the biomass [17]. When *Nephroselmis* sp. KGE2 was cultivated in livestock wastewater, the FAs profile also showed C16:0 in a percentage close to 30%. Contrary to the case of the local isolate, both C14:0 and C18:0 were not found. On the other hand, the cultures from the livestock wastewater effluent exhibited linolelaidic acid (C18:2), which was the most abundant (around 20%) among the other unsaturated FAs, while in the present work, it did not exceed 3% of the total. Finally, the local *Nephroselmis* sp. accumulated α-linolenic acid (C18:3n3) up to 8.3%, whilst *Nephroselmis* sp. KGE2 had a similar content of γ-linolenic acid [17].

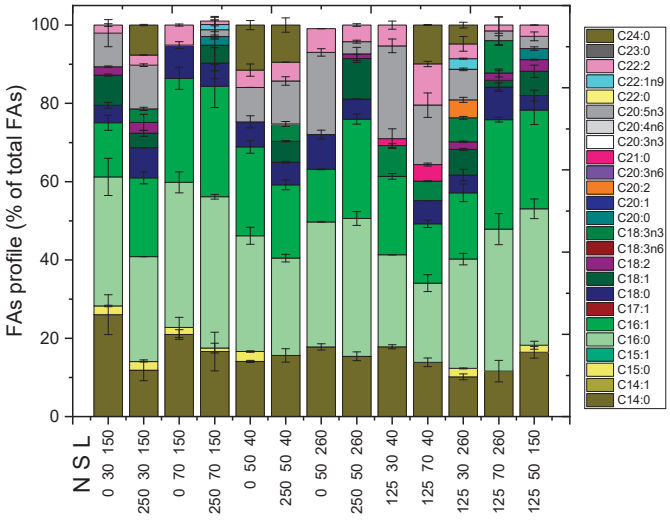


Figure 3. Qualitative profile presentation of the FAs detected, expressed as percentages of the total. N, S, and L on the x-axis stand for nitrogen (mg L^{-1}), salinity (ppt), and light (μmol photons m^{-2} s^{-1}).

2.2. Other Compounds of Interest

The local isolate, Nephroselmis sp., was first grown autotrophically in a complete medium and then inoculated in substrates with different combinations of nitrogen availability, salinity, and light supply. A biomass analysis was carried out in terms of its components for all the examined cases in order to determine the protein, carbohydrate, and pigment contents alongside the lipids. The equations, which describe the various compounds' content in biomass, are presented in Table 2 and take into account the cases of using all the parameters and only the significant ones. After biomass drying and analysis, it was found that sometimes the carbohydrates or proteins were the main cellular component in direct dependence on the cultivation conditions. Proteins approached 40% of the dry biomass, forming a protein concentration in the culture medium equal to 32 mg L^{-1} when Nephroselmis sp. was exposed to low salinity (30 ppt) and low light radiation (40 μmol m^{-2} s^{-1}) in a substate with approximately 200 mg N L^{-1} (Figure 4). Nitrogen is necessary for the production of proteins intracellularly, given that it is also an important component of amino acids. It has been established that the lack of nitrogen affects the photosynthetic activity of microalgae, and the reduction in the growth rate comes together with the low protein content of the biomass. Apart from the available nitrogen, light intensity affects protein synthesis; however, the positive or negative effects of the increase in light radiation depend on the cultivated species. When the light supply was enhanced, Chlorella vulgaris, Desmodesmus sp., and Scenedesmus obliquus produced more proteins, while protein formation by Botryococcus braunii was inhibited [32,33]. Additionally, excessive salinity induces the production of both membrane transport proteins and some plasma proteins in the halotolerant species, such as Chlamydomonas reinhardtii and Dunaliella salina [34].

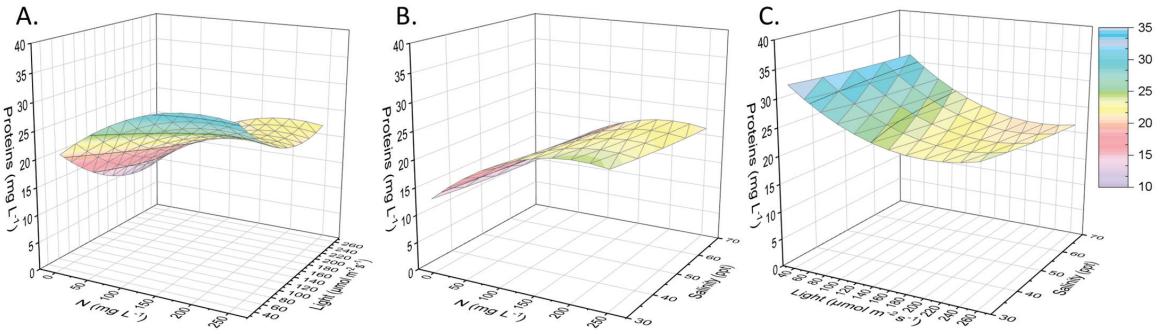


Figure 4. Surface plots regarding proteins as a function of the initial nitrogen concentration, light intensity, and salinity. (**A**) Proteins (mg L^{-1}) depending on nitrogen concentration (mg L^{-1}) and light intensity (μmol of photons m^{-2} s^{-1}), given salinity of 50 ppt. (**B**) Proteins (mg L^{-1}) depending on nitrogen concentration (mg L^{-1}) and salinity (ppt), given light intensity of 110 μmol of photons m^{-2} s^{-1}. (**C**) Proteins (mg L^{-1}) depending on salinity (ppt) and light intensity (μmol of photons m^{-2} s^{-1}), given nitrogen concentration of 125 mg L^{-1}.

In contrast, intracellular carbohydrates were favored by nitrogen stress and high light intensity. Aside from nutrient starvation, the international literature confirms high salinity and excessive illuminance as methods for the accumulation of intracellular sugars [35]. More specifically, the sugars reached 45% (40 mg L^{-1}), being the predominant compound (Figure 5). Nephroselmis sp., as eukaryotic microalgae, produces chlorophyll b in addition to chlorophyll a and carotenoids, and no genes for phycobilins are expressed. As shown in Figure 6, when the local isolate was adapted to an environment with sufficient nitrogen (around 200 mg L^{-1}) and little light, the total pigments increased intracellularly to 7% *w/w* (6 mg L^{-1}). When the light was in excess, photosynthetic pigments (chlorophyll and carotenoids) normally decreased, while the secondary carotenoids increased in some chlorophytes for photoprotection purposes [36]. Species belonging to Chlorophyta produced lutein and zeaxanthin, apart from b-carotene. Nephroselmis sp., in particular, was characterized by a high content of zeaxanthin that can represent more than 50% of the total carotenoids [37].

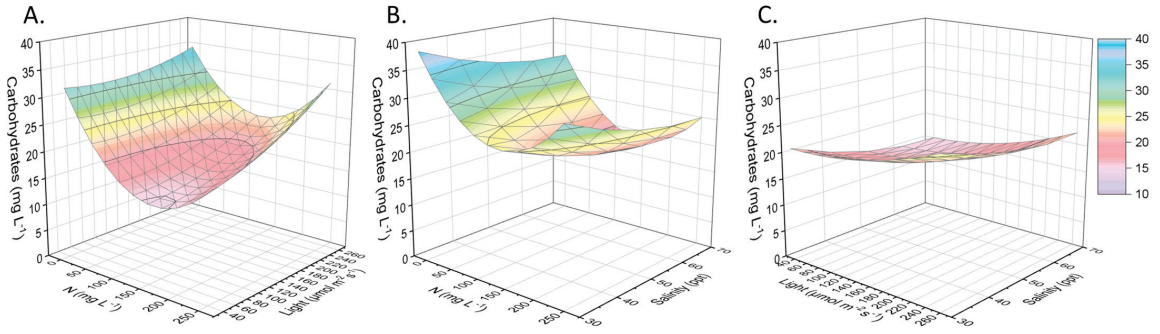


Figure 5. Presentation of the surface plots regarding carbohydrate production as a function of the initial nitrogen concentration, light intensity, and salinity. Carbohydrates (mg L^{-1}) related to (**A**) nitrogen concentration (mg L^{-1}) and light intensity (μmol of photons m^{-2} s^{-1}), given salinity of 50 ppt. (**B**) Nitrogen concentration (mg L^{-1}) and salinity (ppt), given light intensity of 110 μmol of photons m^{-2} s^{-1}. (**C**) Salinity (ppt) and light intensity (μmol of photons m^{-2} s^{-1}), given nitrogen concentration of 125 mg L^{-1}.

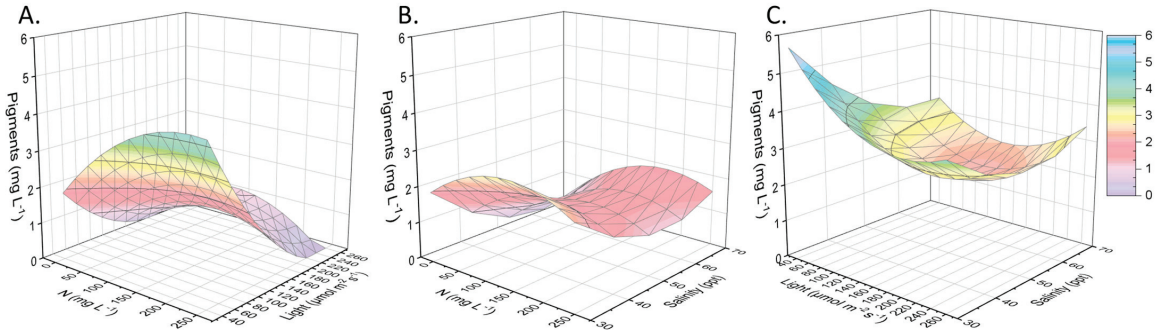


Figure 6. Presentation of the surface plots regarding pigments production (chlorophyll a, chlorophyll b and total carotenoids) production as a function of the initial nitrogen concentration, light intensity, and salinity. Pigments (mg L^{-1}) related to (**A**) nitrogen concentration (mg L^{-1}) and light intensity (μmol of photons m^{-2} s^{-1}), given salinity of 50 ppt. (**B**) Nitrogen concentration (mg L^{-1}) and salinity (ppt), given light intensity of 110 μmol of photons m^{-2} s^{-1}. (**C**) Salinity (ppt) and light intensity (μmol of photons m^{-2} s^{-1}), given nitrogen concentration of 125 mg L^{-1}.

3. Materials and Methods

3.1. Nephroselmis sp. Cultivation Conditions

The local isolate *Nephroselmis* sp., obtained from the saltworks (Messolonghi, Western Greece) [6], grew autotrophically, and a two-stage cultivation mode followed. The experimental conditions are based on preliminary works [6,8]. The first cultivation was carried out in a 6 L conical flask. Walne's medium was used to produce a total of 1.2 g biomass (200 mg L^{-1}) from an initial *Nephroselmis* sp. inoculum of 300 mg (50 mg L^{-1}). Cell proliferation was autotrophic under the illumination of 100 μmol of photons m^{-2} $s^{-1,}$ and the aeration was regulated at a flow rate of 1.5 L_{air} min^{-1} $L_{culture}$ so that the medium was saturated in carbon dioxide to avoid oxygen accumulation.

3.2. Second Stage Cultivation: Experimental Design and Conditions

Regarding the added-value compounds accumulation in the second stage, a three-level Box–Behnken experimental design was applied to three variables (nitrogen, salinity, and light intensity) using Minitab 19. The aforementioned variables were adjusted to 0–250 mg N L^{-1}, a salinity of 30–70 ppt, and light intensity of 40–160 μmol m^{-2} s^{-1} in 15 tests (in duplicate) of 400 mL each, as described in detail in Table 1. For the presented diagrams and behavior prediction of each compound (or compounds group) of interest, two types of equations are provided in Table 2. Such equations refer to the case of taking into consideration all the parameters tested (and their combinations), as well as the case of using only the parameters of statistical significance (simplified form), as derived from the Pareto charts, which are presented in Supplementary Materials.

3.3. Analytical Methods

3.3.1. Determination of Harvested Biomass, Total Solids, Volatile Solids, and Moisture

The harvested microalgal biomass was primarily washed, freeze-dried (Telstar, LyoQuest, Madrid, Spain), and estimated as a dry cell weight per volume of culture, according to the standard methods [38] for total suspended solids (TSS) determination, while the measurement of the compounds of interest followed. The calculation of total solids (TS), volatile solids (VS), and moisture was accomplished according to the standard methods [38].

3.3.2. FAs Determination

The FAs measurement was conducted on samples of 10–40 mg dry biomass, after their in-situ transesterification reaction, in the presence of the H_2SO_4 catalyst and the use of

5 mL of 100:10:1 CH_3OH:$CHCl_3$:H_2SO_4 solution. The samples were incubated for 2 h at 90 °C, and the addition of 2 mL H_2O and 2 mL of 4:1 C_6H_{14}:$CHCl_3$ followed for the stop of the transesterification reaction and the FAMEs extraction, respectively. The analysis was performed using gas chromatography with a flame ionization detector and He as the carrier gas (Agilent Technologies, 7890A, Wilmington, DE ,USA); however, more information regarding the process can be found in Koutra et al. [39].

3.3.3. Determination of Pigments, Proteins, and Carbohydrates Production

Additionally, the extraction of dry biomass by N, N′-dimethylformamide was accomplished for the pigments determination (chlorophyll a, b, and total carotenoids). The calculations for the pigments can be found in the work of Mastropetros et al. [40]. The protein content was quantified by the semi-micro Kjeldahl method [38], and their conversion to proteins was followed by multiplying the total Kjeldahl nitrogen by a factor of 6.25. Finally, the carbohydrates were determined colorimetrically through the Dubois method [41].

3.4. Analysis of Variance

The collected biomass from the second stage of cultivation was statistically analyzed through analysis of variance (ANOVA), while t-tests were conducted for comparison purposes. The statistical analysis and the data grouping according to their statistical difference were carried out via the Minitab 19 software (Minitab LLC, State College, PA, USA).

4. Conclusions

The results of the present study strengthened the position of microalgae in the field of nutritional biomass production and, more specifically, in the recovery of valuable FAs. The microalgae of the *Nephroselmis* genus are not widely examined regarding the accumulation of FAs and other biomass compounds, such as proteins, carbohydrates, and pigments, compared to other more common species. The local isolate, *Nephroselmis* sp., produced up to 15 mg L^{-1} of FAs, of which up to 30% may comprise polyunsaturated EPA. Regardless of the cultivation conditions, the FAs of the local microalgae were mainly C14:0, C16:0, C16:1, and C20:5n3 (EPA). Their amount increased in the presence of nitrogen (210 to 220 mg L^{-1}), at low salinity levels (30 ppt), and an illumination ranging from 150 to 170 μmol m^{-2} s^{-1}. Nitrogen deficiency did not favor the accumulation of FAs, as an adaptation of the cells to the new batches required lipids consumption for their energy purposes. Finally, *Nephroselmis* sp. was characterized by a high protein content when the nitrogen was adequate; however, proteins were replaced by intracellular sugars in the absence of nitrogen. The pigments from this species were maximized after five days in media with low salinity under a light intensity that was limited below 60 μmol m^{-2} s^{-1}.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/md21060331/s1, Figure S1: Pareto charts for the statistical significance of the nitrogen concentration, salinity level, and illuminance regarding the derived equations for (a) proteins, (b) carbohydrates, (c) pigments, (d) FAs, and (e) EPA production.

Author Contributions: Conceptualization, S.G.M., K.T. and M.K.; methodology, S.G.M. and K.T; software, S.G.M., Y.C., A.K.P. and M.K.; validation, S.G.M., K.T., A.K.P. and M.K.; formal analysis, S.G.M. and K.T.; investigation, S.G.M. and A.K.P.; resources, M.K.; data curation, S.G.M. and K.T.; writing—original draft preparation, S.G.M. and K.T.; writing—review and editing, S.G.M., K.T., Y.C. and M.K.; visualization, S.G.M.; supervision, M.K.; project administration, M.K.; funding acquisition, M.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by the research program "ALGAVISION: Isolation and culture of local phytoplankton species aiming to mass production of antibacterial substances, fatty acids, pigments and antioxidants" (MIS 5048496), funded by the General Secretariat of Research and Technology of the Greek Government.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Available on request.

Acknowledgments: We would like to acknowledge G. Hotos and his associates for their contribution regarding the green algae *Nephroselmis* sp. supply.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Borowitzka, M.A. Biology of microalgae. In *Microalgae in Health and Disease Prevention*; Elsevier: Amsterdam, The Netherlands, 2018; pp. 23–72.
2. Liu, Y.; Yildiz, I. The effect of salinity concentration on algal biomass production and nutrient removal from municipal wastewater by *Dunaliella salina*. *Int. J. Energy Res.* **2018**, *42*, 2997–3006. [CrossRef]
3. Eltanahy, E.; Torky, A. *Microalgae as Cell Factories: Food and Feed-Grade High-Value Metabolites*; The Royal Society of Chemistry Burlington House: London, UK, 2021. [CrossRef]
4. Sakarika, M.; Kornaros, M. *Chlorella vulgaris* as a green biofuel factory: Comparison between biodiesel, biogas and combustible biomass production. *Bioresour. Technol.* **2019**, *273*, 237–243. [CrossRef] [PubMed]
5. Lee, K.; Eisterhold, M.L.; Rindi, F.; Palanisami, S.; Nam, P.K. Isolation and screening of microalgae from natural habitats in the midwestern United States of America for biomass and biodiesel sources. *J. Nat. Sci. Biol. Med.* **2014**, *5*, 333. [CrossRef] [PubMed]
6. Hotos, G.; Avramidou, D.; Mastropetros, S.G.; Tsigkou, K.; Kouvara, K.; Makridis, P.; Kornaros, M. Isolation, identification, and chemical composition analysis of nine microalgal and cyanobacterial species isolated in lagoons of Western Greece. *Algal Res.* **2023**, *69*, 102935. [CrossRef]
7. Singh, J.; Saxena, R.C. An introduction to microalgae: Diversity and significance. In *Handbook of Marine Microalgae*; Elsevier: Amsterdam, The Netherlands, 2015; pp. 11–24.
8. Hotos, G.N.; Avramidou, D. The Effect of Various Salinities and Light Intensities on the Growth Performance of Five Locally Isolated Microalgae [*Amphidinium carterae*, *Nephroselmis* sp., *Tetraselmis* sp.(var. red pappas), *Asteromonas gracilis* and *Dunaliella* sp.] in Laboratory Batch Cu. *J. Mar. Sci. Eng.* **2021**, *9*, 1275. [CrossRef]
9. Lortou, U.; Panou, M.; Papapanagiotou, G.; Florokapi, G.; Giannakopoulos, C.; Kavoukis, S.; Iakovou, G.; Zalidis, G.; Triantafyllidis, K.; Gkelis, S. Beneath the Aegean Sun: Investigating Dunaliella Strains' Diversity from Greek Saltworks. *Water* **2023**, *15*, 1037. [CrossRef]
10. Gkelis, S.; Panou, M.; Konstantinou, D.; Apostolidis, P.; Kasampali, A.; Papadimitriou, S.; Kati, D.; Di Lorenzo, G.M.; Ioakeim, S.; Zervou, S.-K. Diversity, cyanotoxin production, and bioactivities of cyanobacteria isolated from freshwaters of Greece. *Toxins* **2019**, *11*, 436. [CrossRef]
11. Tzovenis, I.; Fountoulaki, E.; Dolapsakis, N.; Kotzamanis, I.; Nengas, I.; Bitis, I.; Cladas, Y.; Economou-Amilli, A. Screening for marine nanoplanktic microalgae from Greek coastal lagoons (Ionian Sea) for use in mariculture. *J. Appl. Phycol.* **2009**, *21*, 457–469. [CrossRef]
12. Nakayama, T.; Suda, S.; Kawachi, M.; Inouye, I. Phylogeny and ultrastructure of *Nephroselmis* and *Pseudoscourfieldia* (Chlorophyta), including the description of *Nephroselmis anterostigmatica* sp. nov. and a proposal for the Nephroselmidales ord. nov. *Phycologia* **2007**, *46*, 680–697. [CrossRef]
13. Yamaguchi, H.; Suda, S.; Nakayama, T.; Pienaar, R.N.; Chihara, M.; Inouye, I. Taxonomy of *Nephroselmis viridis* sp. nov.(Nephroselmidophyceae, Chlorophyta), a sister marine species to freshwater *N. olivacea*. *J. Plant Res.* **2011**, *124*, 49–62. [CrossRef]
14. Suda, S. Light Microscopy and Electron Microscopy of *Nephroselmis spinosa* sp. nov.(Prasinophyceae, Chlorophyta) 1. *J. Phycol.* **2003**, *39*, 590–599. [CrossRef]
15. Ji, M.K.; Yun, H.S.; Hwang, B.S.; Kabra, A.N.; Jeon, B.H.; Choi, J. Mixotrophic cultivation of *Nephroselmis* sp. using industrial wastewater for enhanced microalgal biomass production. *Ecol. Eng.* **2016**, *95*, 527–533. [CrossRef]
16. Coulombier, N.; Nicolau, E.; Le Déan, L.; Barthelemy, V.; Schreiber, N.; Brun, P.; Lebouvier, N.; Jauffrais, T. Effects of nitrogen availability on the antioxidant activity and carotenoid content of the microalgae *Nephroselmis* sp. *Mar. Drugs* **2020**, *18*, 453. [CrossRef] [PubMed]
17. Ahn, Y.; Park, S.; Ji, M.-K.; Ha, G.-S.; Jeon, B.-H.; Choi, J. Biodiesel production potential of microalgae, cultivated in acid mine drainage and livestock wastewater. *J. Environ. Manag.* **2022**, *314*, 115031. [CrossRef] [PubMed]
18. D'Alessandro, E.B.; Antoniosi Filho, N.R. Concepts and studies on lipid and pigments of microalgae: A review. *Renew. Sustain. Energy Rev.* **2016**, *58*, 832–841. [CrossRef]
19. Oliver, L.; Dietrich, T.; Marañón, I.; Villarán, M.C.; Barrio, R.J. Producing omega-3 polyunsaturated FAs: A review of sustainable sources and future trends for the EPA and DHA market. *Resources* **2020**, *9*, 148. [CrossRef]
20. Chen, G.-Q.; Jiang, Y.; Chen, F. Fatty acid and lipid class composition of the eicosapentaenoic acid-producing microalga, *Nitzschia laevis*. *Food Chem.* **2007**, *104*, 1580–1585. [CrossRef]
21. Aziz, M.M.A.; Kassim, K.A.; Shokravi, Z.; Jakarni, F.M.; Liu, H.Y.; Zaini, N.; Tan, L.S.; Islam, A.B.M.S.; Shokravi, H. Two-stage cultivation strategy for simultaneous increases in growth rate and lipid content of microalgae: A review. *Renew. Sustain. Energy Rev.* **2020**, *119*, 109621. [CrossRef]

22. Nagarajan, S.; Chou, S.K.; Cao, S.; Wu, C.; Zhou, Z. An updated comprehensive techno-economic analysis of algae biodiesel. *Bioresour. Technol.* **2013**, *145*, 150–156. [CrossRef]
23. Liyanaarachchi, V.C.; Premaratne, M.; Ariyadasa, T.U.; Nimarshana, P.H.V.; Malik, A. Two-stage cultivation of microalgae for production of high-value compounds and biofuels: A review. *Algal Res.* **2021**, *57*, 102353. [CrossRef]
24. Park, S.; Ahn, Y.; Pandi, K.; Ji, M.-K.; Yun, H.-S.; Choi, J.-Y. Microalgae cultivation in pilot scale for biomass production using exhaust gas from thermal power plants. *Energies* **2019**, *12*, 3497. [CrossRef]
25. Udayan, A.; Pandey, A.K.; Sirohi, R.; Sreekumar, N.; Sang, B.-I.; Sim, S.J.; Kim, S.H.; Pandey, A. Production of microalgae with high lipid content and their potential as sources of nutraceuticals. *Phytochem. Rev.* **2022**, 1–28. [CrossRef] [PubMed]
26. Sorgüven, E.; Özilgen, M. Thermodynamic efficiency of synthesis, storage and breakdown of the high-energy metabolites by photosynthetic microalgae. *Energy* **2013**, *58*, 679–687. [CrossRef]
27. Wang, T.; Tian, X.; Liu, T.; Wang, Z.; Guan, W.; Guo, M.; Chu, J.; Zhuang, Y. A two-stage fed-batch heterotrophic culture of *Chlorella protothecoides* that combined nitrogen depletion with hyperosmotic stress strategy enhanced lipid yield and productivity. *Process Biochem.* **2017**, *60*, 74–83. [CrossRef]
28. Guedes, A.C.; Amaro, H.M.; Barbosa, C.R.; Pereira, R.D.; Malcata, F.X. Fatty acid composition of several wild microalgae and cyanobacteria, with a focus on eicosapentaenoic, docosahexaenoic and α-linolenic acids for eventual dietary uses. *Food Res. Int.* **2011**, *44*, 2721–2729. [CrossRef]
29. Lu, C.; Acién Fernández, F.G.; Cañizares Guerrero, E.; Hall, D.O.; Molina Grima, E. Overall assessment of *Monodus subterraneus* cultivation and EPA production in outdoor helical and bubble column reactors. *J. Appl. Phycol.* **2002**, *14*, 331–342. [CrossRef]
30. Camacho-Rodríguez, J.; Cerón-García, M.C.; González-López, C.V.; Fernández-Sevilla, J.M.; Contreras-Gómez, A.; Molina-Grima, E. A low-cost culture medium for the production of *Nannochloropsis gaditana* biomass optimized for aquaculture. *Bioresour. Technol.* **2013**, *144*, 57–66. [CrossRef] [PubMed]
31. Cerón-García, M.C.; Fernández-Sevilla, J.M.; Sánchez-Mirón, A.; García-Camacho, F.; Contreras-Gómez, A.; Molina-Grima, E. Mixotrophic growth of *Phaeodactylum tricornutum* on fructose and glycerol in fed-batch and semi-continuous modes. *Bioresour. Technol.* **2013**, *147*, 569–576. [CrossRef]
32. Nzayisenga, J.C.; Farge, X.; Groll, S.L.; Sellstedt, A. Effects of light intensity on growth and lipid production in microalgae grown in wastewater. *Biotechnol. Biofuels* **2020**, *13*, 4. [CrossRef]
33. Ruangsomboon, S. Effect of light, nutrient, cultivation time and salinity on lipid production of newly isolated strain of the green microalga, *Botryococcus braunii* KMITL 2. *Bioresour. Technol.* **2012**, *109*, 261–265. [CrossRef]
34. Sithtisarn, S.; Yokthongwattana, K.; Mahong, B.; Roytrakul, S.; Paemanee, A.; Phaonakrop, N.; Yokthongwattana, C. Comparative proteomic analysis of *Chlamydomonas reinhardtii* control and a salinity-tolerant strain revealed a differential protein expression pattern. *Planta* **2017**, *246*, 843–856. [CrossRef]
35. González-Fernández, C.; Ballesteros, M. Linking microalgae and cyanobacteria culture conditions and key-enzymes for carbohydrate accumulation. *Biotechnol. Adv.* **2012**, *30*, 1655–1661. [CrossRef] [PubMed]
36. Mulders, K.J.M.; Lamers, P.P.; Martens, D.E.; Wijffels, R.H. Phototrophic pigment production with microalgae: Biological constraints and opportunities. *J. Phycol.* **2014**, *50*, 229–242. [CrossRef] [PubMed]
37. Coulombier, N.; Nicolau, E.; Le Déan, L.; Antheaume, C.; Jauffrais, T.; Lebouvier, N. Impact of Light Intensity on Antioxidant Activity of Tropical Microalgae. *Mar. Drugs* **2020**, *18*, 122. [CrossRef] [PubMed]
38. APHA. *Standard Methods for the Examination of Water and Wastewater*, 22nd ed.; Stand Methods 2012; Byrd Prepess Springfield: Washington, DC, USA, 2012; ISBN 9780875532356.
39. Koutra, E.; Kopsahelis, A.; Maltezou, M.; Grammatikopoulos, G.; Kornaros, M. Effect of organic carbon and nutrient supplementation on the digestate-grown microalga, *Parachlorella kessleri*. *Bioresour. Technol.* **2019**, *294*, 122232. [CrossRef]
40. Mastropetros, S.G.; Koutra, E.; Amouri, M.; Aziza, M.; Ali, S.S.; Kornaros, M. Comparative assessment of nitrogen concentration effect on microalgal growth and biochemical characteristics of two chlorella strains cultivated in digestate. *Mar. Drugs* **2022**, *20*, 415. [CrossRef] [PubMed]
41. Dubois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.T.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350–356. [CrossRef]

marine drugs

MDPI

Review

Extraction of *Nannochloropsis* Fatty Acids Using Different Green Technologies: The Current Path

Sérgio Cruz Sousa [1,2,*], Ana Cristina Freitas [1,†], Ana Maria Gomes [1,*] and Ana P. Carvalho [1,2]

[1] CBQF—Centro de Biotecnologia e Química Fina, Laboratório Associado, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Diogo Botelho 1327, 4169-005 Porto, Portugal; apcarvalho@ucp.pt (A.P.C.)
[2] REQUIMTE/LAQV—Instituto Superior de Engenharia, Instituto Politécnico do Porto, Rua Dr. António Bernardino de Almeida, 431, 4200-072 Porto, Portugal
* Correspondence: sdsousa@ucp.pt (S.C.S.); amgomes@ucp.pt (A.M.G.)
† Dr. Cristina Freitas had passed away.

Abstract: *Nannochloropsis* is a genus of microalgae widely recognized as potential sources of distinct lipids, particularly polyunsaturated fatty acids (PUFA). These may be obtained through extraction, which has conventionally been performed using hazardous organic solvents. To substitute such solvents with "greener" alternatives, several technologies have been studied to increase their extraction potential. Distinct technologies utilize different principles to achieve such objective; while some aim at disrupting the cell walls of the microalgae, others target the extraction per se. While some methods have been utilized independently, several technologies have also been combined, which has proven to be an effective strategy. The current review focuses on the technologies explored in the last five years to extract or increase extraction yields of fatty acids from *Nannochloropsis* microalgae. Depending on the extraction efficacy of the different technologies, distinct types of lipids and/or fatty acids are obtained accordingly. Moreover, the extraction efficiency may vary depending on the *Nannochloropsis* species. Hence, a case-by-case assessment must be conducted in order to ascertain the most suited technology, or tailor a specific one, to be applied to recover a particular fatty acid (or fatty acid class), namely PUFA, including eicosapentaenoic acid.

Keywords: green extraction; disruption; polar lipids; polyunsaturated fatty acids; eicosapentaenoic acid

Citation: Sousa, S.C.; Freitas, A.C.; Gomes, A.M.; Carvalho, A.P. Extraction of *Nannochloropsis* Fatty Acids Using Different Green Technologies: The Current Path. *Mar. Drugs* **2023**, *21*, 365. https://doi.org/10.3390/md21060365

Academic Editors: Giuseppina Tommonaro and Annabella Tramice

Received: 21 May 2023
Revised: 9 June 2023
Accepted: 12 June 2023
Published: 19 June 2023

1. Introduction

Microalgae have long been known to be a source of several compounds with quite interesting properties. As such, microalgae or its extracts have diverse applications in different areas, such as human nutrition, feed in aquaculture, biofertilizers, treatment of effluents, and even in human health [1–3]. Bioactive compounds present in microalgae include lipids, namely polyunsaturated fatty acids (PUFA), which are probably the most studied amongst compounds, sterols, pigments, proteins, enzymes, vitamins and other compounds with biological activity [2–4].

Nannochloropsis is a genus of microalgae comprising seven described species, wherein individuals are characterized by being non-motile, presenting a spherical morphology and diameters ranging from 2 to 8 µm [2,5,6]. Furthermore, these microalgae are also widely recognized for presenting high PUFA contents, particularly that of the omega-3 fatty acid eicosapentaenoic acid (EPA, C20:5n3) [7–12]. As all microalgae, *Nannochloropsis* have different composition lipids, which may be divided into polar (phospholipids, glycolipids, and betaine lipids) and non-polar (acylglycerols, sterols and free fatty acids) [13,14]. The PUFA present in these microalgae are mainly located in polar lipids, namely phospholipids, while their content in neutral triacylglycerols (TAG) is lower [8,15]. This is an advantage, since omega-3 PUFA are more stable and possess higher bioavailability when in the form of polar lipids (particularly phospholipids) [12,16].

In order to obtain the compounds of interest from microalgae cells, present in the cell wall itself or intracellularly, extraction must be performed [3,17]. The main objective of extraction techniques is to obtain a high yield of the desired compounds without jeopardizing quality and activity, as well as to preserve co-products, minimize the amount of energy spent and waste generation, optimize the process (operational temperature, pressure, carrying capacity, side reactions and separations) and be scalable [18].

Traditionally, fatty acids were obtained from microalgae via the conventional solvent extraction techniques. These techniques include solid–liquid and liquid–liquid extractions, in which organic solvents, such as hexane, toluene, dichloromethane, acetone and others are utilized [18,19]. However, nowadays, there is a generalized opinion that the solvents used in the extraction processes should be safe, inexpensive and nontoxic [18,20,21]. Hence, recent studies have focused on the development of extraction technologies to obtain microalgae extracts without utilizing (or minimizing the use of) toxic organic solvents, or by substitution by "greener" solvents, such as ethanol and deep eutectic solvents [3,18,22,23]. Nonetheless, non-aggressive extraction conditions must be utilized, to not have a detrimental impact on the compounds present in the microalgae cells, such as the degradation of lipids, which may occur at high temperatures [24–26].

The extraction of biologically active compounds (namely fatty acids) from microalgae, such as *Nannochloropsis*, has been performed utilizing several different technologies which include ultrasounds [8,27], microwaves [28,29], super- and subcritical fluids [30,31], and high pressure [32], among others.

Extraction processes may entail a very important step, that is, the pretreatment of the biomass, to increase/improve the extraction yield, obtained by disrupting the cell wall [18]. Cell wall rigidity can inhibit conventional organic solvents, such as hexane, to enter the cell, preventing or hindering the contact between the solvent and the intracellular compounds [24]. As such, pretreatment makes the bioactive compounds present in the cells more bioavailable [26]. Cell wall disruption techniques can be of mechanical, chemical, physical or enzymatic nature, such as high pressure homogenization, alkaline lysis, ultrasonication, and enzymatic hydrolysis [33–36]. *Nannochloropsis* microalgae have been described as possessing a rigid, robust, cell wall [7,32], resulting from its composition, consisting of an outer hydrophobic trilaminar sheat algaenan-based layer and an inner cellulose-based layer (linked by struts to the plasma membrane), which hinder the extraction of intracellular compounds [2,7,20,26,35]. Thus, pretreatments to disrupt cell wall are desired/required to increase extraction yields.

The current review provides an overview of the main extraction technologies utilized in the last five years to extract fatty acids from *Nannochloropsis* microalgae (Figure 1), as well as the principles/mechanisms responsible for the extraction effectiveness of each of those technologies.

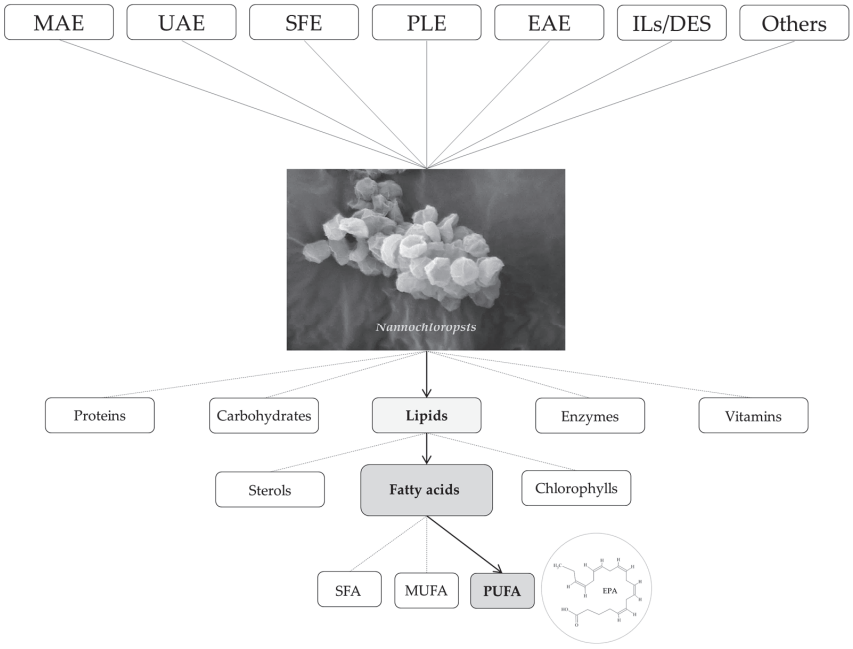


Figure 1. Technologies recently used to extract fatty acids from *Nannochloropsis*.

2. Extraction Technologies

2.1. Microwave-Assisted Extraction (MAE)

Microwave technology can, as previously mentioned, be used to enhance yields of bioactive compounds by disrupting cell walls and is used as a complement in the extraction methodology.

Microwaves are the alternating current signals with frequencies varying from 0.3 to 300 GHz, which transform electromagnetic energy into heat with the polarity of compounds. Polar compounds will realign in the direction of the electric field and, when the microwave field alters, they will rotate in high speed. If ions are present, ions will migrate as the electric field alters. Electromagnetic energy is then transformed into heat by the friction between the compounds or the ions [37]. Microwave heating is a non-contact heat source, heating the overall target reactants simultaneously as compared to conductive heating [38]. The heat source can penetrate into the biomaterial, interacting with polar molecules, such as water, and heat the entire sample uniformly [39]. The increase in temperature will cause the evaporation of water molecules, which will apply pressure on the cell walls. This will rupture the cell walls, which will release the intracellular components into the medium. The utilization of microwaves also facilitates extraction as hydrogen bounds are disrupted and dissolved ions, by migration, increase the penetration of solvent into the matrix [18]. Microwave-assisted extraction is considered to be a rapid and cost-effective method of obtaining bioactive compounds [18,37,39], and has been frequently used to extract different compounds from microalgae.

Regarding the extraction of fatty acids from *Nannochloropsis*, microwaves were usually applied at a frequency of 2.5 GHz, potencies below 800 W, temperatures lower than 100 °C and during a maximum of 30 min. One example, in which the highest potency was utilized, is the study of Quesada-Salas et al. (2021) [29], which compared MAE with UAE (ultrasound-assisted extraction) or bead milling to extract lipids from two *Nannochloropsis* species, namely *N. gaditana* and *N. oceanica*. The authors studied the temperature (between 50 and 100 °C) and time (between 5 and 25 min) of treatment, finding differences in the impact according to the species. By using quadratic models to predict the lipid extraction

yields, the authors found that although all parameters impacted the lipid yields from both species, some parameters impacted them differently. Regarding temperature, it positively correlated with *N. gaditana* lipid extraction, while showing no interaction with time. In *N. oceanica*, temperature presented a positive effect on lipid yield only to a certain threshold, after which it would present a negative effect, possibly due to lipid degradation at higher temperatures. Concerning the interaction with time, it was found that in *N. oceanica*, they do interact, however, the behavior was distinct depending on the specific temperature. The study also showed that distinct disruption technologies may change the fatty acid profile (namely that of EPA) of the extract of a specific species, and that this effect is species-dependent, since it was only observed regarding *N. oceanica*. Concerning the differences between technologies, MAE presented the highest yield (including EPA) from *N. oceanica*, while UAE was the most efficient for lipid extraction from *N. gaditana*, presenting lipid recoveries of 49 and 21.7% dry weight (dw), respectively.

Another study regarding *Nannochloropsis* in which MAE was utilized, in this case at low potencies ($\leq$100 W), was that of Zghaibi et al. (2020) [40]. In that research work, microwaves were associated with brine (NaCl), and temperature (between 60 and 100 °C) and extraction time (between 1 and 30 min) were evaluated for the impact on lipid extraction from *Nannochloropsis* sp. Results showed the highest PUFA percentage (44.1% dw, corresponding to 25.2 mg g^{-1}) was when temperature was the highest, which authors speculated could have resulted from a larger extension of ruptured cell walls (in which high amounts of PUFA are present). Regarding time, the authors found a similar result, with longer extraction times presenting higher amounts of those fatty acids, which was also observed for omega-3 fatty acids and monounsaturated fatty acids (MUFA). Those results were corroborated via scanning electron microscopy, which revealed larger cell damages in cells exposed to higher temperature and/or longer extraction time.

Table 1 presents other studies performed within the last five years in which microwaves have been applied either as a single treatment testing different potencies, extraction times (up to 30 min) and temperatures (up to 100 °C), or in combination with other technologies, namely ultrasounds [41] or ionic liquids [42], to obtain lipids from *Nannochloropsis* microalgae.

Table 1. Recent studies utilizing microwave-assisted extraction to extract lipids and fatty acids from *Nannochloropsis* microalgae.

MW/IL/US	Species	Solvent	Operational Conditions			Yield						References
						Lipid	Fatty Acids					
			Used	Tested	Optimum		SFA	MUFA	PUFA	Omega-3	EPA	
MW + IL	*N. oceanica*	[TMam] [Cl]	1.65 g IL 2.45 GHz 700 W	60–90 °C; 1–25 min	90 °C; 25 min	—	—	—	—	—	37.92 mg g^{-1} FAME	[43]
MW + IL	*N. oceanica*	[TMam] [Cl]	2.45 GHz 700 W	0.5–2.5 g IL; 60–100 °C; 5–30 min	1.65 g IL; 88.2 °C; 24.7 min	19.6% (dw)	35.8 mg g^{-1} FAME	35.2 mg g^{-1} FAME	51.1 mg g^{-1} FAME	39.4 mg g^{-1} FAME	19.6 g g^{-1} biomass; 37.9 mg g^{-1} FAME	[28]
MW	*N. gaditana*	$CHCl_3$:MeOH (2:1)	≤100 W	50–100 °C 5–25 min	100 °C; 5 min	17.7% (dw)	—	—	—	—	—	[29]
	N. oceanica				91 °C; 25 min	49% (dw)	—	—	—	—	—	
MW	*Nannochloropsis* sp.	Brine (NaCl) solution	2.45 GHz ≤800 W	60–100 °C 1–30 min	100 °C; 30 min	16.1% (dw)	~26% (TFA)	~23% (TFA)	44.1% (TFA); 25.2 mg g^{-1} (dw)	41.4% (TFA); 23.6 mg g^{-1} (dw)	31.5% (TFA); 17.9 mg g^{-1} (dw)	[40]
MW	*Nannochloropsis* sp.	Brine (NaCl) solution	2.45 GHz ≤ 800 W	1–35% (*w*/*v*) NaCl; 5–25% solid loading; 60–100 °C; 5–30 min	10% (*w*/*v*) NaCl; 5% solid loading; 100 °C; 30 min	16.1% (dw)	~27% (TFA)	~24% (TFA)	44.5% (TFA)	43% (TFA)	43% (TFA)	[44]
MW + IL	*Nannochloropsis* sp.	MeOH:[EMIM] [$MeSO_4$]	2.45 GHz 700 W	4–12 (Alga:MeOH ratio); 0.5–1 (MeOH:IL ratio); 5–25 min	1:4 (Alga:MeOH ratio); 1:0.5 (MeOH:IL ratio); 25 min	42.2% (dw)	10.2% (ww)	18.7% (ww)	9.3% (ww)	—	—	[42]
MW	*N. oculata*	$CHCl_3$:MeOH (2:1)	300 W	40–80 °C; 1–10 min	300 W; 80 °C; 1 min	33.6%	30.9% (TFA)	32.1% (TFA)	36.6% (TFA)	29.8% (TFA)	29.8% (TFA)	[45]
MW + US	*Nannochloropsis* sp.	MeOH	—	2:1–2:3 (Alga:MeOH) ratio; 100–140 W (US and MW); 3–7 min	2:3 (Alga:MeOH) ratio; 140 W; 7 min	FAME–48.2%	—	—	—	—	—	[41]

MW—microwaves; IL—ionic liquid; US—ultrasounds; SFA—saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids; EPA—eicosapentaenoic acid; TFA—total fatty acids; FAME—fatty acid methyl esters; [TMam] [Cl]—tetramethyl ammonium chloride; [EMIM] [$MeSO_4$]—1-ethyl-3-methylimmidazolium methyl sulphate; $CHCl_3$—chloroform; MeOH—methanol; dw—dry weight; ww—wet weight.

2.2. Ultrasound-Assisted Extraction (UAE)

Ultrasound-assisted extraction is another technology utilized to disrupt cell walls, and consequently increase extraction yields, which is sometimes used simultaneously with MAE.

This technology is based on the cavitation phenomenon. When a liquid is submitted to ultrasounds, cavitation bubbles are generated, which can create implosive collapse [46]. The intense sonication of liquid generates soundwaves that propagate into the liquid, resulting in alternate high-pressure and low-pressure cycles. Cavitation is the phenomenon resulting from the violent collapse of small vacuum bubbles, generated in the low-pressure cycle, during the high-pressure cycle. During cavitation, shearing forces are formed around the cells by the high pressure and high speed liquid jets, resulting in the cell structure being "mechanically" broken, thereby improving material transfer [39]. This reduces particle size and increases the contact between the solvent and the compounds to be extracted [18]. The enhancement in extraction yields is attributed to the microstreaming and heightened mass transfer by cavitation and bubbles collapse, which result in the destruction of the cells [47]. This technology can, as previously mentioned, improve extraction yields and reduce the amount of solvent utilized, and the extraction time and costs, as there is a reduction in the temperature needed for the extraction process [47,48].

Concerning *Nannochloropsis*, ultrasounds were commonly utilized at frequencies of 20 or 37 kHz, and potencies between 100 and 140 W. Regarding other parameters, amplitude, temperature and time were extremely variable. Similar to MAE, the latest research works have explored ultrasounds' association with other technologies and solvents (Table 2). One such example is the study of Guo et al. (2022) [27], in which ultrasounds were associated with a switchable hydrophilicity solvent (N, N, N′, N′-tetraethyl-1,3-propanediamine) to rupture the cell wall of *N. oceanica*. Exposure of the microalga to the solvent per se was sufficient to damage the cell wall, by reducing its thickness from 141 to 68.6 nm, and to originate lipid leakage. Nonetheless, applying ultrasounds further increased the extent of the damage, and consequently of the extraction yield. Indeed, ultrasonication of the solvent/biomass mixture presented a synergistic effect, which lead to a disruption of the cell wall, allowing for a high lipid extraction efficiency (98.2%). However, a differential fatty acids profile was obtained, particularly in PUFA, where in comparison with the conventional Bligh and Dyer (1959) [49] method (which utilizes a solvent mixture of chloroform/methanol, 1:2, *v*/*v*), the combined extraction process decreased the percentage of C18:2 and C18:3 in the extract, although EPA was increased.

In turn, Blanco-Llamero et al. (2021) [50] combined ultrasounds with commercial enzymes (namely a mixture of carbohydrases (Viscozyme®), cellulase (Celluclast®), and protease (Alcalase®)) to disrupt the cell wall of *N. gaditana*, to extract lipids. The authors found that ultrasounds were able to increase the extraction efficiency of the enzymes, and that an ultrasound-assisted enzymatic treatment (with the combination of all three enzymes), in which enzymatic activity was performed in an ultrasound bath, was able to yield the highest amount of lipids (28.9% dw), doubling the amount obtained with non-pretreated biomass (~14.5% dw). That treatment was also the one in which the extract presented the highest amount of polar lipids (45.66% glycolipids and 2.51% phosphatidylethanolamine), in comparison with the non-pretreated biomass (35.42% glycolipids and 1.79% phosphatidylethanolamine).

Ultrasounds were also studied by Figueiredo et al. (2019) [8] to increase the extraction efficiency of ethanol, with the aim of obtaining EPA-enriched lipid extracts from *N. oceanica*. The study showed that, by utilizing ultrasounds, the amount of EPA extracted using ethanol could be increased. Moreover, comparison between applying ultrasounds with a bath or with a probe showed that the latter was more efficient, allowing for the amount of PUFA and EPA extracted to be increased by 47 and 35%, respectively, when compared to extraction solely with ethanol.

This technology has also been associated with others, such as low-temperature hydrothermal liquefaction [51] or microwaves [41], as presented in Table 2.

Table 2. Extraction of lipids and fatty acids from *Nannochloropsis* using ultrasound-assisted extraction reported within the last five years.

US/other	Species	Solvent	Operational Conditions			Yield						References
						Lipid	Fatty Acids					
			Used	Tested	Optimum		SFA	MUFA	PUFA	Omega-3	EPA	
US	*N. gaditana*	$CHCl_3$:MeOH (2:1)	130 W; 20 kHz	50–80% amplitude; 10–30 min	80% amplitude; 30 min	21.7% (dw)	—	—	—	—	—	[29]
	N. oceanica					45.4% (dw)	—	—	—	—	—	
US + Switchable hydrophilicity solvent	*N. oceanica*	TEPDA	0.5 W/mL, 20 kHz, room temperature	30–180 min	120 min	98.2% (wet weight)	—	—	—	—	—	[27]
US + Celluclast + Viscozyme	*N. gaditana*	—	140 W; 37 kHz	2–6 h (ultrasounds)/pH 4–8; 35–55 °C (enzymatic pretreatment)	55 °C, pH 5.0, 6 h	24.2%	—	—	—	—	—	[50]
US + Celluclast + Viscozyme + Alcalase						28.9%	—	—	—	—	—	
US	*N. oceanica*	EtOH	130 W; 20 kHz; 70% amplitude; 8 min	Ultrasound probe; ultrasound bath	Ultrasound probe	60.3% (extract dw)	27.4 mg g^{-1} (dw)	24.6 mg g^{-1} (dw)	21.8 mg g^{-1} (dw)	—	15.4 mg g^{-1} (dw)	[8]
US + Low-temperature hydrothermal liquefaction	*Nannochloropsis* sp.	Dichloromethane	100 W; 60 min	30–90 s (sonication); 210–250 °C (HTL)	90-s sonication time, 250 °C	28.9% (dw)	—	—	—	—	—	[51]
—	*N. gaditana*	2-MeTHF:EtOH (1:3)	37 kHz; 50 °C; 30 min	EtOH; 2-MeTHF; hexane:EtOH (3:4); 2-MeTHF:EtOH (1:1, 1:2, 1:3, and 1:4)	2-MeTHF:ethanol (1:3)	16.3% (dw)	—	—	—	—	—	[23]
US + MW	*Nannochloropsis* sp.	MeOH	—	MeOH (10–30 mL); Ultrasounds and Microwaves Power (100–140 W); reaction time (3–7 min)	30 mL MeOH, 140 W (microwaves), 140 W (ultrasounds), 7 min	22.8% (dw)	66.5% (TFA)	20.7 (TFA)	12.7% (TFA)	—	—	[41]

US—ultrasounds; MW—microwaves; SFA—saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids; EPA—eicosapentaenoic acid; TFA—total fatty acids; EtOH—ethanol; MeOH—methanol; 2-MeTHF—2-Methyltetrahydrofuran; TEPDA—N, N, N′, N′-tetraethyl-1,3-propanediamine; $CHCl_3$—chloroform; dw—dry weight.

2.3. Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction (SFE), together with pressurized liquid extraction (PLE), is probably the most widely employed extraction technique for obtaining bioactive components from natural sources [52]. Supercritical fluid extraction utilizes the solvents above their critical pressures and temperatures [18,52]. As the solvent power of a supercritical fluid is a function of density, it can be varied by changing the extraction pressure and temperature, enabling it to be suitable as an extraction solvent [39]. In the conditions utilized in SFE, supercritical fluids (SCFs) possess particular physicochemical properties between gases and liquids, generally acquiring higher density than a gas, yet maintaining similar viscosities and diffusivities [52]. The density of the SCF is like that of a liquid, and it can be altered by changing the temperature and pressure. The low viscosity and high diffusivity of SCFs generates better transport properties, when compared to liquids [18]. Although different solvents can be utilized, the most utilized solvent is carbon dioxide (CO_2), due to its moderate critical pressure (7.4 MPa) and low critical temperature (31.1 °C) [39]. Supercritical CO_2 (SC-CO_2) has several advantages, as it has mild critical conditions, and is nontoxic, nonflammable, nonexplosive and noncorrosive. Additionally, it is easily available and cheap, and is easily separated from the extract, inert to the product. Carbon dioxide, being a gas at room temperature, can be easily removed from the extract, when compared to other extraction techniques [18,52]. Another advantage is that the properties of SCFs can be adjusted with pressure and temperature changes, which directly influences density, making the technique very selective, which is a major advantage when the objective is the extraction of compounds from complex matrices. This technique has also the advantage of the possibility of, during decompression, performing fractioning just by utilizing two or more decompression steps, which is useful to separate components in the extract [52].

In addition to the abovementioned advantages, like every technique, SFE also has disadvantages. The main disadvantage of SC-CO_2 is its low polarity. This property limits the compounds that can be extracted using this technique, which alone may not be able to extract polar compounds. However, this issue can be overcome by the addition of cosolvents (modifiers), which are employed at small proportions (1–10%), during the extraction process [52]. The cosolvents are solvents with higher polarity, which changes the polarity of the SCF and thus increasing the solvating power, thereby increasing the range of compounds that can be extracted [18,52]. This was the case in Askari et al.'s (2022) [30] study, in which SC-CO_2, in combination with *n*-hexane, was utilized as cosolvent to extract lipids from *N. oculata*. The authors assessed the impact of the cosolvent's presence, as well as that of temperature (between 35 and 75 °C) and pressure (between 150 and 550 bar), on the lipid and PUFA yields, finding that lipid yield and extraction kinetics were directly correlated with both temperature and pressure, independent of the other condition. This means that lipid yield and extraction kinetics increased with temperature at low or high pressure, and a similar behavior was observed regarding pressure in relation to temperature. The study also revealed that the presence of the cosolvent positively impacted both the amount of lipids extracted, as well as the rate at which they were obtained. Furthermore, lacing SC-CO_2 with *n*-hexane also resulted in a distinct fatty acid profile of the extract, doubling the amount of EPA, and increasing total PUFA content to nearly double of the saturated fatty acids (SFA), which was previously 1.5-fold higher than PUFA.

Leone et al. (2019) [53] explored the use of SC-CO_2 to extract omega-3 fatty acids from *Nannochloropsis* sp., pre-treated mechanically with diatomaceous earth. Results showed that, according to the pressure and temperature of the process, extraction selectivity could be tailored towards obtaining more EPA or docosahexaenoic acid (DHA). Specifically, when utilizing the highest pressures (550 bar) and temperatures (75 °C), EPA yield was increased, while the highest DHA recoveries were obtained at milder conditions (400 bar and 50 °C). The impact of the CO_2 flow rate on both EPA and DHA yields was also assessed, and the authors determined that they were directly correlated, since an increase in both omega-3 fatty acids yields (increase of ~45 and 70%, respectively) was observed when the flow rate was doubled.

Further examples of SFE (utilized at pressures in the range of 100–550 bar and temperatures varying from 40 up to 150 °C), alone or in combination with other strategies, applied to *Nannochloropsis* microalgae for lipids extraction, focusing on fatty acids, are presented in Table 3.

Table 3. Examples of supercritical fluid extraction of lipids and fatty acids from *Nannochloropsis*.

SF/PL/DEG	Species	Co-Solvent	Operational Conditions			Yield						References
						Lipid	Fatty Acids					
			Used	Tested	Optimum		SFA	MUFA	PUFA	Omega-3	EPA	
SF	*N. oculata*	*n*-hexane	200 mL min^{-1} (fr)	150–550 bar; 35–75 °C; 0–300 min; 0–3% co-solvent	550 bar; 75 °C; 150 min; 3% co-solvent	0.262 g g^{-1} (dw)	34.4% (TFA)	52.5% (TFA)	13.7% (TFA)	—	7.4% (TFA)	[30]
SF + PL	*Nannochloropsis* sp.	Ethanol	35 MPa; 50 °C; 10% co-solvent; 0.15 kg h^{-1} (fr)	—	—	—	—	—	—	—	61.9%	[12]
SF	*N. oculata*	Ethanol	—	0–80% water content; 100–200 bar; 100–150 °C; 80–160 min	40% water content; 150 °C; 150 bar; 120 min	0.253 g g^{-1} (dw)	59% (TFA)	35.6% (TFA)	5% (TFA)	—	—	[54]
SF	*N. maritima*	Ethanol	80 min; 0.02 kg h^{-1} (fr)	100–300 bar; 40–60 °C	300 bar; 40 °C	—	—	—	—	—	8.5% (total lipids)	[55]
SF + DEG	*Nannochloropsis* sp.	—	100 min	100–550 bar; 50–75 °C; 7.24–14.48 g min^{-1} (fr)	550 bar; 75 °C; 14.48 g min^{-1} (fr)–EPA 400 bar; 50 °C; 14.48 g min^{-1} (fr)–DHA	18.39 mg g^{-1} (dw)	4.74 mg g^{-1} (dw)	5.89 mg g^{-1} (dw)	6.92 mg g^{-1} (dw)	—	5.69 mg g^{-1} (dw)	[53]
SF + DEG	*N. gaditana*	—	100 min; 50 or 65 °C	250–550 bar; 7.24–14.48 g min^{-1} (fr)	250 bar; 65 °C; 7.24 g min^{-1} (fr)	34.61 mg g^{-1} (dw)	—	—	—	—	11.50 mg g^{-1} (dw)	[56]
SF	*N. oculata*	Ethanol	50 °C; 25 g min^{-1} (fr)	250–750 bar; 0–240 min	450 bar; 240 min	20%	—	—	—	—	—	[57]

SF—supercritical fluid; PL—pressurized liquid; DEG—diatomaceous earth grinding; SFA—saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids; EPA—eicosapentaenoic acid; DHA—docosahexaenoic acid; TFA—total fatty acids; fr—flow rate; dw—dry weight.

2.4. Pressurized Liquid Extraction (PLE)

Pressurized liquid extraction (PLE), also referred to as pressurized fluid extraction (PFE), pressurized hot-solvent extraction (PHSE) or accelerated solvent extraction (ASE), is an extraction technology based on the utilization of pressurized solvents at high temperatures, although always below their critical points, under conditions that maintain the solvents in the liquid state during the extraction process. When water is utilized as the extraction solvent, the general principles and instrumental requirements are the same, although other important parameters have significant influence, and the technology can be denominated as subcritical water extraction (SCWE), superheated water extraction (SHWE) or pressurized hot-water extraction (PWE) [52].

Pressurized liquid extraction conditions provide an enhanced mass-transfer rate, an increased solubility of the compounds to be extracted, and a decrease in solvent viscosity and surface tension [23,52]. The lower solvent viscosity and surface tension will allow the solvent to penetrate more easily into the matrix, reaching deeper areas and increasing the surface contact, which will improve the mass transfer to the solvent, resulting in an increased extraction rate [52]. As previously mentioned, when water is utilized as the solvent, the extraction is also affected by the dielectric constant (ε) of water. When water is heated at high temperatures while remaining in the liquid state, ε, which is a measure of the polarity of the solvent, is significantly reduced [31,52]. If this value is decreased to values close to the ones of organic solvents (when heated), water can be presented as a useful alternative. Even though this may not be possible for all applications, SWE can be seen as the "greenest" of the PLEs [52].

When compared to the conventional extraction processes, PLE presents numerous advantages, such as higher selectivity, shorter extraction times, faster extraction processes and smaller amounts of organic solvents [18,52]. Additionally, the possibility of automation is a further advantage of this technique, since it helps to reduce variations between extractions, which increases reproducibility [52].

Pertaining to pressurized liquids for extraction of fatty acids from *Nannochloropsis*, Blanco-Llamero and Señoráns (2021) [23] utilized the technology in combination with bio-based solvents to achieve less environmentally detrimental alternatives to extract omega-3 fatty acids from *N. gaditana*. The authors tested 2-methyltetrahydrofuran (2-MeTHF) as the extraction solvent, as well as its combination with ethanol, in a 1:3 (2-MeTHF:ethanol) ratio. Results showed that the combination of solvents yielded the highest lipid amount (16.32% dw) and that PLE with ethanol per se originated the extract with the highest glycolipids content (42.99% of extract), which the authors attributed to the higher polarity of the solvent. Nonetheless, the fatty acids profiles revealed that, concerning PUFA and EPA percentages (~48 and 36% of total fatty acids (TFA), respectively), no significant differences were obtained when the extract was obtained using ethanol as the only solvent, or when in combination with 2-MeTHF.

Regarding the specific case of water, Eikani et al. (2019b) [58] found that, when extracting lipids from *N. salina*, in comparison with the conventional Folch et al. (1957) method [59] (with corresponding solvent mixture of chloroform/methanol, 2:1, *v/v*), the extract possessed lower PUFA amount (11.41 and 19.98% of TFA, respectively). The authors ascribed the difference to a thermal degradation that may have had occurred due to the high temperatures utilized in SCWE, and to a possible thermal oxidation, which could have converted those PUFA to lower double bond PUFA, or even to saturated ones. In a distinct study [31], the same research group reported that utilizing ethanol as a solvent modifier in SCWE yielded an extract with higher lipid and PUFA contents. The authors also found that presence of the cosolvent allowed the extraction process to be performed at lower temperatures (90 °C), which may have decreased the extension of the abovementioned phenomena, or even prevent them from occurring.

Further examples of the technology applied recently to *Nannochloropsis* can be found in Table 4.

Table 4. Studies concerning *Nannochloropsis* extractions of lipids and fatty acids via pressurized liquid extraction.

Species	Co-Solvent	Operational Conditions			Yield						References
					Lipid	Fatty Acids					
		Used	Tested	Optimum		SFA	MUFA	PUFA	Omega-3	EPA	
N. salina	Ethanol	20 bar; 120 min	90–150 °C; 25–75% (water ethanol); 1–4 mL min^{-1} (fr)	90 °C; 75% (water ethanol); 4 mL min^{-1} (fr)	33.9% (dw)	44.26% (TFA)	41.35% (TFA)	14.39% (TFA)	—	—	[31]
N. salina	—	20 bar; 120 min	150–200 °C; 1–4 mL min^{-1} (fr); 1–4 g_{sample}	175 °C; 4 mL min^{-1} (fr); 1 g_{sample}	19.52% (dw)	35.48% (TFA)	53.11% (TFA)	11.41% (TFA)	—	—	[58]
N. gaditana	—	—	156.1–273.9 °C; 6.6–23.4 min; 33–117 g_{sample} L^{-1}	236.54 °C; 13.95 min; 60.5 g_{sample} L^{-1}	13.4% (dw)	—	—	—	—	15.04% (FAME)	[25]
N. gaditana	—	10 min	hexane or ethanol	hexane; 120 °C	17.6% (dw)	13.4% (TFA)	20.0% (TFA)	66.5% (TFA)	55.9% (FAME)	53% (FAME)	[11]
N. gaditana	—	120 °C; 15 min	Solvents	2-MeTHF:ethanol (1:3)	46.1% (dw)	20.17% (TFA)	27.86% (TFA)	51.94% (TFA)	39.73% (TFA)	38.54%	[23]

SFA—saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids; EPA—eicosapentaenoic acid; TFA—total fatty acids; FAME—fatty acid methyl esters; 2-MeTHF—2-Methyltetrahydrofuran; fr—flow rate; dw—dry weight.

2.5. Enzyme-Assisted Extraction (EAE)

Enzyme-assisted extraction (EAE) is yet another technology/technique which has been utilized to obtain fatty acids from microalgae. As previously mentioned, microalgae possess cell walls which, dependent on their composition, may hinder the access of the extraction solvent to the intracellular compounds. In this sense, there is the need to rupture or, at least, disrupt the cell wall so that extraction of such compounds may be achieved. The algaenan/cellulose wall of *Nannochloropsis* is particularly resistant to chemical or mechanical treatments [60] and in many cases there is a need to apply combinations of these to increase the extraction potential as described in the previous sections. A rather promising alternative strategy to overcome this constraint is the use of enzymes which, according to their nature, may hydrolyze the cell wall structural components. This will damage the cell wall integrity, thereby providing easier access of the extraction solvent to the intracellular compounds, as well as promoting their leakage [61,62]. In this sense, for *Nannochloropsis* microalgae, a cellulase can be applied, envisioning the degradation of the inner cellulose-based layer. The main treatment parameters which impact the enzyme, and consequently extraction efficiency, are the enzyme dosage, pH, temperature, time, and the homogenization (agitation) speed [20,61]. The combination of distinct enzymes, which is a strategy utilized to increase extraction yields [20], must be carefully evaluated, since their interaction may have an antagonistic effect, opposite to the desired synergistic one [61].

Recently, Zhao et al. (2022) [20] tested the activity of several enzymes, namely cellulase, laccase, pectinase, mannanase and xylanase on *N. oceanica*, as a pretreatment for ethanol extraction of lipids. After determining the two enzymes that yielded the highest lipid amounts (cellulase and laccase), whose extracts also presented the highest EPA contents, the authors combined the two enzymes. Optimization of enzymatic treatment was performed for enzyme dosage, enzymes ratio, buffer pH, temperature and time. The extract obtained through the combination of the two enzymes in a cellulase:lacase (2.5:1) ratio presented the highest EPA content, which increased 1.5-fold, compared with that obtained from untreated cells. Regarding the lipid classes, betaine lipids and free fatty acids were the ones positively impacted (greatly increased) by the enzymes pretreatment, while neutral lipids, phospholipids and glycolipids' contents decreased.

Qiu et al. (2019) [63] also assessed the impact of four enzymes, namely cellulase, hemicellulase, papain and pectinase, on lipid extraction from wet *Nannochloropsis* biomass, in an enzyme-assisted three phase partitioning, in which *tert*-butanol was utilized as the extraction solvent. The authors assessed the impact of several parameters, such as enzyme type and slurry/*tert*-butanol ratio, among others. In accordance with the findings of the abovementioned study of Zhao et al. (2022) [20], and with the known cellulose-based inner layer of the cell wall, the enzyme that presented the best result was cellulase. Extraction using the cellulase-assisted three phase partitioning yielded an extract with increased PUFA and EPA contents (19.91 and 13.98% of TFA, respectively), in comparison with those obtained without enzymatic hydrolysis (16.23 and 12.95% of TFA, respectively). Moreover, under the optimized conditions (20% ammonium sulphate, 6–7 pH, 1:2 slurry/*tert*-butanol ratio, 70 °C for 2 h), the cellulase-assisted three phase partitioning extraction system was able to extract 90% of total fatty acids after two extraction cycles, and it was demonstrated that it was scalable, as it presented similar results in a laboratory scale of 20 L. In this sense, the enzyme-assisted three phase partitioning was revealed to be a promising strategy to obtain fatty acids from wet *Nannochloropsis*.

Further studies pertaining to EAE, with more in-depth explanation of the parameters and associated ranges assessed therein, are presented in Table 5.

Table 5. Enzyme-assisted extraction applied to *Nannochloropsis* to obtain lipids and fatty acids.

Enzymes	Species	Solvent (Post-Enzymes)	Operational Conditions			Yield						References
						Lipid	Fatty Acids					
			Used	Tested	Optimum		SFA	MUFA	PUFA	Omega-3	EPA	
Laccase and cellulase	*N. oceanica*	EtOH	—	Laccase:cellulase (4:1, 2:1, 1:1,1:2, 1:4); pH (4.2–5.8); T (40–60 °C); time (1.5–24 h)	1:2.5 (laccase:cellulase); pH 5; 45 °C; 6 h	26.9%	—	—	—	—	20.7 g 100 g^{-1}	[20]
Cellulase, hemicellulase, papain and pectinase	*N. oculata*	—	200 U enzyme; pH 5.5; 45 °C; 12 h	Combinations	Mixture of all four	221.4 mg g^{-1} (dw)	—	—	—	—	—	[64]
Cellulase, hemicellulase, papain and pectinase	*Nannochloropsis* sp.	*tert*-butanol	200 U enzyme; pH 5.0; 50 °C; 4 h	Cellulase; hemicellulase; papain; pectinase	cellulase	88.70% (ww)	53% (TFA)	25.69% (TFA)	19.91% (TFA)	13.98% (TFA)	13.98% (TFA)	[35]
Cellulase and mannase	*Nannochloropsis* sp.	n-hexane:2-propanol (3:2)	13.8 mg g^{-1} (cellulase), 1.5 mg g^{-1} (mannase); pH 4.4; 53 °C; 24 h	Cellulase; mannase; cellulase/mannase	cellulase/mannase	73% (total lipids)	—	—	—	—	—	[60]

SFA—saturated fatty acids; MUFA—monounsaturated fatty acids; PUFA—polyunsaturated fatty acids; EPA—eicosapentaenoic acid; TFA—total fatty acids; EtOH—ethanol; ww—wet weight; dw—dry weight.

2.6. Ionic Liquids (ILs) and Deep Eutectic Solvents (DES)

Recently, there has been a demand for solvents able to extract lipids, among other compounds, from distinct "matrices", including microalgae, without having such a detrimental environmental impact as the conventionally utilized organic solvents. This has prompted researchers to explore other types of solvents, which include ionic liquids (ILs) and deep eutectic solvents (DES).

Ionic liquids are a class of solvents which, as aforementioned, have been studied as alternatives to the conventionally used solvents to extract several compounds from microalgae. The ILs are solutions of salts that present melting temperatures below 100 °C, some of which may still even be liquid (molten) at room temperature, and their composition comprises both anions and cations, hence their designation [7,65–67]. These solvents' properties can be manipulated by combination and permutation of the anions and cations comprised therein, which endow solvents with distinct polarity, thermal stability, hydrophobicity and viscosity, that can be tailored according to the specific goal for which they are intended [3,65,67]. Furthermore, within ILs there is a subclass denominated switchable solvents, of which there are two types, namely switchable polarity solvents (SPS) and switchable hydrophilicity solvents (SHS), which can reversibly change the characteristics in response to a stimulus/trigger [66–68].

Although ILs have been considered in "green" extractions, there are significant environmental concerns regarding the utilization of these solvents due to the inefficient biodegradation and the potential use and production of toxic reagents in the synthesis of some ILs [67,68]. Nevertheless, they have been studied with regard to lipid extraction from microalgae, as in Shankar et al. (2019) [69], in which protic ILs (a subtype of ILs) have been utilized to extract lipids from *N. oculata*. The authors found that, in comparison with the conventional Bligh and Dyer (1959) method [49], extraction via ILs (in combination with a posterior microwaves treatment), in particular butyrolactam hexanoate, increased lipid yield by 34.9%, with a lower content of pigments, which is a positive trait when fatty acid extraction is concerned. Moreover, the study revealed that extraction was more efficient when biomass was hydrated, which is also favorable to the implementation of the technology, since a drying step is circumvented.

As previously mentioned, DES (and natural DES, which are of a natural origin) are another type of solvent which have attracted the attention of researchers [3,70,71]. These comprise hydrogen-bond acceptors (HBA) (organic salts, such as choline chloride) and hydrogen-bond donors (HBD) (e.g., sugars or organic acids) [3,70]. Moreover, they present several properties which confer a "greener" status, in comparison with ILs and conventional organic solvents, which include biodegradability, low toxicity or lack thereof, easy synthesis and safety (nonvolatility and nonflammability). Despite the difficulty of separation from the extracted compounds, due to nonvolatility, since they are nontoxic, DES, and specifically natural DES, may be directly incorporated in food products, which also benefits the production as a purification step will not be required [70,71]. Similar to the abovementioned regarding ILs, switchable DES have also been developed to address cases in which separation of the compounds from the solvents are required [71].

Concerning *Nannochloropsis*, Cai et al. (2021) [71] studied the impact of DES on the extraction of lipids from *Nannochloropsis* sp., utilizing a three-phase partitioning system, and comparing the extraction with that performed using *t*-butanol. The authors found that the amount of lipids extracted using the DES, as well as the PUFA and EPA contents of such extracts, were higher (1.12 and 5.59-fold, respectively) than in the extract obtained using *t*-butanol. Moreover, DES was shown to retain considerable reusability, only losing 10% of lipid recovery after the fifth cycle was utilized.

Recent reports of lipid extraction from *Nannochloropsis* in which ILs or DES were studied are presented in Table 6.

Table 6. *Nannochloropsis* lipids extracted using ionic liquids or deep eutectic solvents.

Species	IL or DES	Parameters		Lipid Yield	References
		Tested	Optimum		
Nannochloropsis sp.	Tetramethylguanidine:menthol	1:1–5:1 (HBA:HBD molar ratio); 50–90 °C; 30–110 min; 5–40% (ammonium sulfate); 20:1–40:1 (liquid:solid ratio)	3:1 (HBA:HBD molar ratio); 80 °C; 90 min; 20% (ammonium sulfate); 35:1 (liquid:solid ratio)	127 mg g^{-1} (dw)	[71]
N. oceanica	[EMIM]Cl	0.2–3 g IL	2 g IL	13.9%	[72]
N. oculata	Lactam- or ammonium carboxylate-based	ILs	butyrolactam hexanoate	13.5% (dw)	[69]
Nannochloropsis sp.	Cholinium amino acid-based	ILs	cholinium arginate	98.6% (total lipids)	[7]
N. gaditana	[EMIM] [$MeSO_4$]	65–95 °C; 5–25 min; 1:4–1:12 (wet alga:MeOH); 1:0.5–1:1 (MeOH:IL)	14 min; 1:4 (wet alga:MeOH); 1:0.5 (MeOH:IL)	41.2% (dw)	[42]

IL—ionic liquid; DES—deep eutetic solvent; [EMIM] [$MeSO_4$]—1-ethyl-3-methylimmidazolium methyl sulfate; [EMIM]Cl—1-ethyl-3-methyl imidazolium chloride; HBA—hydrogen-bond acceptor; HBD—hydrogen-bond donor; MeOH—methanol; dw—dry weight.

An overview of the advantages and drawbacks of the different extraction technologies addressed so far is presented in Figure 2.

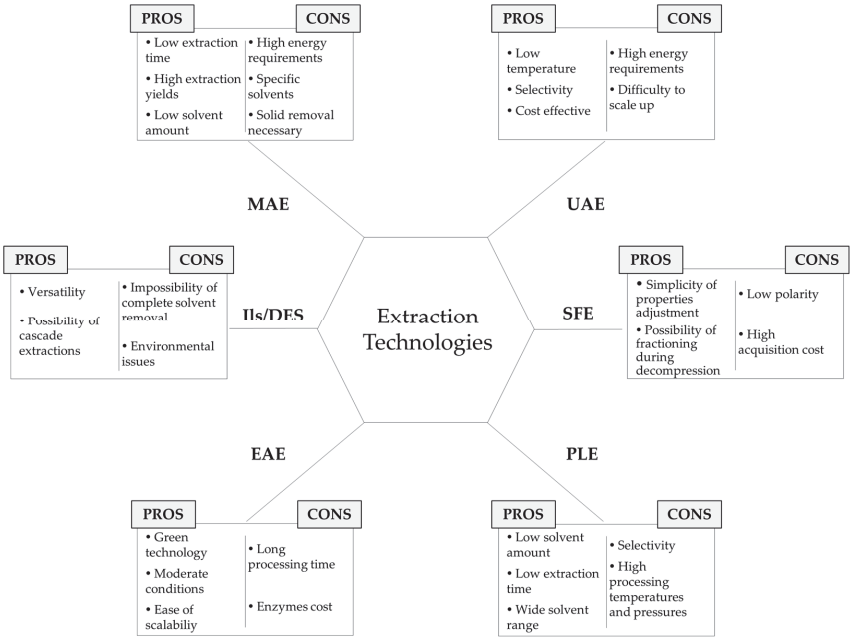


Figure 2. Pros and cons of the distinct extraction technologies.

2.7. Others

In addition to the aforementioned technologies, a myriad of other solutions, some more innovative than others, have been studied to extract lipids, including fatty acids, from *Nannochloropsis* microalgae. Wang et al. (2018) [73] explored the effect of screw extrusion on *N. oceanica* cell integrity and lipid recovery, and found that the treatment increased the amount of fatty acids, including PUFA, subsequently extracted using hexane. Moreover, a balance between screw speed and feed moisture was shown to be critical to achieve the highest yields. Quesada-Salas et al. (2021) [29] also assessed, among other technologies, the impact of mechanical disruption by bead milling on *N. oceanica* and *N. gaditana* cells, followed by lipid extraction using a chloroform/methanol solvent mixture. The study revealed no effect of the flow rate variation (between high and low) on lipid yield, however, bead size was shown to significantly impact the extraction, with smaller beads being able to disrupt over two times the number of cells disrupted by the larger beads. Bead milling did not significantly change the fatty acid composition of the extracts of *N. gaditana*, although an increase in unsaturated fatty acids (UFA) and EPA was observed in the *N. oceanica* extracts.

Chemical methods have also been applied to enhance the extraction of fatty acids from *Nannochloropsis*. Potassium hydroxide (KOH) was utilized by Park et al. (2020) [74] to assist the solvent extraction of lipids from *N. oceanica*. The study showed that inclusion of KOH in the extraction process enabled the removal chlorophyll from the extract, which in turn resulted in an increased amount of fatty acid methyl esters (FAME). This resulted in an extract more suited for biodiesel production, and which could further be separated from the EPA comprised therein, so that it could be utilized in other products. One other chemical approach is osmotic shock. Halim et al. (2021) [75], aiming to extract lipids from *N. gaditana*, explored hypotonic osmotic shock as a method to induce cell rupture. Opposite to the expected/desired outcome, exposure of the marine microalga biomass to a fresh water hypotonic solution did not rupture the cells. Nonetheless, the authors found that

there was indeed some damage to the cell walls, which enhanced the extent of the rupture resulting from the subsequent disruption techniques, in that specific case, high pressure homogenization, that allowed for a higher lipid yield.

Physical processes are likely the most studied regarding fatty acids extraction from *Nannochloropsis*. Lorente et al. (2018) [76] explored steam explosion as a pretreatment to diminish structural integrity of the cells, and consequently enhance lipid extraction from *N. gaditana*. The technology was able to disrupt the cell walls, and increase the amount of lipids extracted using hexane as extraction solvent by 8-fold, thereby resulting in a yield of ca. 80% as compared to the conventional Bligh and Dyer (1959) method [49]. Matos et al. (2019) [77] utilized non-thermal plasma to induce the rupture of *N. gaditana* cell walls, and found that the pretreatment to double the lipid yield, in comparison to untreated biomass, was similar to the obtained yield when microwaves were applied instead. The study also revealed that the fatty acids profile of the plasma-treated extract was distinct from the untreated, since the amount of omega-3 PUFA was decreased, while the SFA increased, which could be beneficial when biodiesel production is envisioned. Hydrodynamic cavitation is one other technology, which was utilized by Setyawan et al. (2018) [78] for lipid extraction from *Nannochloropsis* sp. The study showed that the yield was dependent on several parameters, namely the level and number of cavitation, microalga concentration, specific energy, and temperature. However, the authors did not determine the fatty acids profiles (which should be further explored), and therefore it is not possible to assess to what end such treatment may be indicated. In turn, hydrothermal disintegration was applied by Kröger et al. (2018) [26] to *N. oculata*, which obtained a 2-fold increase in lipid yield when compared with direct extraction, as well as an increase in C18:1 fatty acid while C18:0 and C18:3 contents were decreased (unusually, EPA contents were not reported). Teymouri et al. (2018) [79] studied flash hydrolysis to enhance the extraction of lipids from *Nannochloropsis* sp. and found that the treatment did increase the amount of lipids extracted. However, regarding lipid profile, it was determined that PUFA content decreased, with EPA being 4-fold higher in raw biomass which, therefore, resulted in a biodiesel-oriented extract. A shear-assisted process also was assessed by Kwak et al. (2020) [80] regarding lipid extraction from *Nannochloropsis* sp., and results showed that high lipid and EPA yields were achieved with minimal volume of extraction solvent. The technology was regarded as a viable alternative to several two-step extraction systems since it presented the lowest specific energy consumption. An extraction technology based on pulsed electric fields (PEF), namely high voltage electric discharge (HVDE), was utilized by Zhang et al. (2020) [6] to obtain bio-molecules from *N. oculata*, which included lipids. The authors found no significant differences between treated and untreated samples in regard to lipid yield, however, the highest EPA content was found in the extract from an untreated sample. In this sense, this technology was not indicated toward obtaining that specific omega-3 fatty acid. Nonetheless, HVDE treatment did increase the yields of palmitic (C16:0) and palmitoleic (C16:1n7) acids. Pulsed electric fields is a promising technology, worth of being more extensively explored to extract fatty acids from *Nannochloropsis*, since it has already been demonstrated to be efficient in increasing lipid extraction yields in other microalgae [81–84], and it was also utilized to obtain other compounds from microalgae of the *Nannochloropsis* genus [85,86]. Within physical processes which may be applied to extract *Nannochloropsis* fatty acids, high hydrostatic pressure has recently been reported to, independently or in combination with other technologies [32,87–89], enhance the extraction of PUFA, and specifically EPA [32,89], thus supporting the use of greener solvents than the conventional organic ones.

An altogether distinct approach was that of Halim et al. (2019) [90], which explored a mechanism of autolytic self-ingestion to decrease the thickness of the cell walls of *Nannochloropsis* microalgae (*Nannochloropsis* sp. and *Nannochloropsis gaditana*). The treatment consisted of a thermally coupled dark-anoxia incubation, which lead to the anaerobic metabolism being activated and the consequent consumption of sugar reserves. This resulted in the reduction of the polysaccharides comprised in the cellulose-based layer of

the cell wall, whose thickness was then decreased to half. The process weakened the cells, which were then easier to rupture in subsequent treatments, as those previously mentioned for such purpose (in that specific case, high pressure homogenization).

3. Future Trends

The future of *Nannochloropsis* fatty acids extraction must, and certainly will, include efforts to comply with the principles defined in the 2030 Agenda for Sustainable Development [91]. In this regard, one issue that must be addressed is the economic impact of the extraction processes, which should be pondered when extraction conducted via a specific technology is studied. The energy requirements of a particular technology have a great impact on the overall cost of an extraction process, and the costs associated with the acquisition of the equipment for industrial production are also an important issue. This shows that the scalability of a process to an industrial setting entails several challenges of economic, environmental and sustainability natures. Oil separation and water removal are downstream processes whose improvement may significantly impact/reduce production costs. The possibility of reusing the solvents utilized in the extraction is another cost-reduction strategy, which will also decrease the environmental impact of the extraction process. Environmental concerns also guide the future of fatty acids extraction, as greener solvents as well as technologies that are able to increase the extraction yields of the conventionally utilized solvents are explored. The latter will allow to decrease the amount of solvent utilized in the extraction process, which will improve it in terms of costs and also regarding (by decreasing) its environmental impact. As previously mentioned, natural DES are considered promising solvents due to their sustainability traits. Modifications to DES and ILs may also increase their biodegradability and hence increase the opportunities to utilize such solvents. One other stage of the process that may be improved is the cell wall rupture, which is also an energy-costly process, and alternatives, such as auto-lysis, are being presented as the future path. Lastly, genetic engineering has been able to generate strains with increased fatty acid production which, consequently, will increase the yields of the extraction processes, thereby reducing the overall impact of obtaining similar amounts of the compounds.

4. Conclusions

Throughout the present review several technologies were described, which were (and still are) explored with the aim of reducing the negative environmental and health impacts of utilizing the conventional organic solvents in fatty acids extraction. The studies demonstrated that many technologies have the ability to improve fatty acids yields by either rupturing the cells and/or improving solvent penetration. Hence, the use of such technologies may allow the use of different "greener" solvents that have previously been dismissed due to lower extraction yields. Nonetheless, it was observed that some technologies are more prone to extract a specific type of fatty acids, and that in some cases a specific technology presented different impact on yields according to the *Nannochloropsis* species. One may therefore conclude that particular attention must be paid to the application of the technology, and that its impact on a specific fatty acid from a specific microalga must be assessed independently, in a case-by-case approach. It can also be inferred that combination of technologies may be the future path, since it allows for increased yields, as well as the recovery and separation of different compounds (such as distinct fatty acids), from a single sequential extraction procedure.

Author Contributions: Conceptualization, S.C.S., A.C.F., A.M.G. and A.P.C.; resources, A.C.F., A.M.G. and A.P.C.; writing—original draft preparation, S.C.S.; writing—review and editing, S.C.S., A.C.F., A.M.G. and A.P.C.; supervision, A.C.F., A.M.G. and A.P.C.; project administration, A.C.F., A.M.G. and A.P.C.; funding acquisition, A.C.F., A.M.G. and A.P.C. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by National Funds through project Norte-01-0145-FEDER-000011-RL1-QUALIFOOD, FCT (Fundação para a Ciência e a Tecnologia) through projects UID/Multi/50016/2019 and SFRH/BD/105304/2014, and by FCT/MEC (PIDDAC), project reference IF/00588/2015.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Jesus Raposo, M.F.; Morais, R.M.S.C.; Morais, A.M.M.B. Health Applications of Bioactive Compounds from Marine Microalgae. *Life Sci.* **2013**, *93*, 479–486. [CrossRef] [PubMed]
2. Zanella, L.; Vianello, F. Microalgae of the Genus *Nannochloropsis*: Chemical Composition and Functional Implications for Human Nutrition. *J. Funct. Foods* **2020**, *68*, 103919. [CrossRef]
3. Raj, T.; Morya, R.; Chandrasekhar, K.; Kumar, D.; Soam, S.; Kumar, R.; Patel, A.K.; Kim, S.H. Microalgae Biomass Deconstruction Using Green Solvents: Challenges and Future Opportunities. *Bioresour. Technol.* **2023**, *369*, 128429. [CrossRef]
4. Cuellar-Bermudez, S.P.; Aguilar-Hernandez, I.; Cardenas-Chavez, D.L.; Ornelas-Soto, N.; Romero-Ogawa, M.A.; Parra-Saldivar, R. Extraction and Purification of High-Value Metabolites from Microalgae: Essential Lipids, Astaxanthin and Phycobiliproteins. *Microb. Biotechnol.* **2015**, *8*, 190–209. [CrossRef] [PubMed]
5. Chua, E.T.; Schenk, P.M. A Biorefinery for Nannochloropsis: Induction, Harvesting, and Extraction of EPA-Rich Oil and High-Value Protein. *Bioresour. Technol.* **2017**, *244*, 1416–1424. [CrossRef] [PubMed]
6. Zhang, R.; Marchal, L.; Lebovka, N.; Vorobiev, E.; Grimi, N. Two-Step Procedure for Selective Recovery of Bio-Molecules from Microalga *Nannochloropsis oculata* Assisted by High Voltage Electrical Discharges. *Bioresour. Technol.* **2020**, *302*, 122893. [CrossRef] [PubMed]
7. Chua, E.T.; Brunner, M.; Atkin, R.; Eltanahy, E.; Thomas-Hall, S.R.; Schenk, P.M. The Ionic Liquid Cholinium Arginate Is an Efficient Solvent for Extracting High-Value *Nannochloropsis* sp. Lipids. *ACS Sustain. Chem. Eng.* **2019**, *7*, 2538–2544. [CrossRef]
8. Figueiredo, A.R.P.; da Costa, E.; Silva, J.; Domingues, M.R.; Domingues, P. The Effects of Different Extraction Methods of Lipids from *Nannochloropsis oceanica* on the Contents of Omega-3 Fatty Acids. *Algal Res.* **2019**, *41*, 101556. [CrossRef]
9. Wang, X.; Fosse, H.K.; Li, K.; Chauton, M.S.; Vadstein, O.; Reitan, K.I. Influence of Nitrogen Limitation on Lipid Accumulation and EPA and DHA Content in Four Marine Microalgae for Possible Use in Aquafeed. *Front. Mar. Sci.* **2019**, *6*, 95. [CrossRef]
10. Kim, S.Y.; Kwon, Y.M.; Kim, K.W.; Kim, J.Y.H. Exploring the Potential of *Nannochloropsis* sp. Extract for Cosmeceutical Applications. *Mar. Drugs* **2021**, *19*, 690. [CrossRef]
11. Castejón, N.; Señoráns, F.J. Simultaneous Extraction and Fractionation of Omega-3 Acylglycerols and Glycolipids from Wet Microalgal Biomass of *Nannochloropsis gaditana* Using Pressurized Liquids. *Algal Res.* **2019**, *37*, 74–82. [CrossRef]
12. Jiménez Callejón, M.J.; Robles Medina, A.; Macías Sánchez, M.D.; González Moreno, P.A.; Navarro López, E.; Esteban Cerdán, L.; Molina Grima, E. Supercritical Fluid Extraction and Pressurized Liquid Extraction Processes Applied to Eicosapentaenoic Acid-Rich Polar Lipid Recovery from the Microalga *Nannochloropsis* sp. *Algal Res.* **2022**, *61*, 102586. [CrossRef]
13. Brennan, B.; Regan, F. In-Situ Lipid and Fatty Acid Extraction Methods to Recover Viable Products from *Nannochloropsis* sp. *Sci. Total Environ.* **2020**, *748*, 142464. [CrossRef] [PubMed]
14. Jiménez Callejón, M.J.; Robles Medina, A.; González Moreno, P.A.; Esteban Cerdán, L.; Orta Guillén, S.; Molina Grima, E. Simultaneous Extraction and Fractionation of Lipids from the Microalga *Nannochloropsis* sp. for the Production of EPA-Rich Polar Lipid Concentrates. *J. Appl. Phycol.* **2020**, *32*, 1117–1128. [CrossRef]
15. Huang, Y.; Zhang, D.; Xue, S.; Wang, M.; Cong, W. The Potential of Microalgae Lipids for Edible Oil Production. *Appl. Biochem. Biotechnol.* **2016**, *180*, 438–451. [CrossRef] [PubMed]
16. Servaes, K.; Maesen, M.; Prandi, B.; Sforza, S.; Elst, K. Polar Lipid Profile of *Nannochloropsis oculata* Determined Using a Variety of Lipid Extraction Procedures. *J. Agric. Food Chem.* **2015**, *63*, 3931–3941. [CrossRef]
17. Gomes, T.A.; Zanette, C.M.; Spier, M.R. An Overview of Cell Disruption Methods for Intracellular Biomolecules Recovery. *Prep. Biochem. Biotechnol.* **2020**, *50*, 635–654. [CrossRef]
18. Michalak, I.; Chojnacka, K. Algal Extracts: Technology and Advances. *Eng. Life Sci.* **2014**, *14*, 581–591. [CrossRef]
19. Sales, R.; del Carmen Cerón-García, M.; Navarro-López, E.; González-López, C.V.; Tsuzuki, M.Y.; Acién-Fernández, F.G.; Alarcón-López, F.J.; Molina-Grima, E. Processing *Nannochloropsis gaditana* Biomass for the Extraction of High-Value Biocompounds. *J. Appl. Phycol.* **2020**, *32*, 3113–3122. [CrossRef]
20. Zhao, K.; Zhang, L.; Zhang, M.; Tian, H.; He, D.; Zheng, J. Response Surface Optimization of Enzyme Pretreatment Improves Yield of Ethanol-Extracted Lipids from *Nannochloropsis oceanica*. *Eur. J. Lipid Sci. Technol.* **2022**, *124*, 2100200. [CrossRef]
21. Jiménez Callejón, M.J.; Robles Medina, A.; Macías Sánchez, M.D.; Esteban Cerdán, L.; González Moreno, P.A.; Navarro López, E.; Hita Peña, E.; Grima, E.M. Obtaining Highly Pure EPA-Rich Lipids from Dry and Wet *Nannochloropsis gaditana* Microalgal Biomass Using Ethanol, Hexane and Acetone. *Algal Res.* **2020**, *45*, 101729. [CrossRef]

22. Sánchez-Camargo, A.d.P.; Pleite, N.; Mendiola, J.A.; Cifuentes, A.; Herrero, M.; Gilbert-López, B.; Ibáñez, E. Development of Green Extraction Processes for *Nannochloropsis gaditana* Biomass Valorization. *Electrophoresis* **2018**, *39*, 1875–1883. [CrossRef] [PubMed]
23. Blanco-Llamero, C.; Señoráns, F.J. Biobased Solvents for Pressurized Liquid Extraction of *Nannochloropsis gaditana* Omega-3 Lipids. *Mar. Drugs* **2021**, *19*, 107. [CrossRef] [PubMed]
24. Alhattab, M.; Kermanshahi-Pour, A.; Brooks, M.S.L. Microalgae Disruption Techniques for Product Recovery: Influence of Cell Wall Composition. *J. Appl. Phycol.* **2019**, *31*, 61–88. [CrossRef]
25. Ho, B.C.H.; Kamal, S.M.M.; Danquah, M.K.; Harun, R. Optimization of Subcritical Water Extraction (SWE) of Lipid and Eicosapentaenoic Acid (EPA) from *Nannochloropsis gaditana*. *BioMed Res. Int.* **2018**, *2018*, 8273581. [CrossRef]
26. Kröger, M.; Klemm, M.; Nelles, M. Hydrothermal Disintegration and Extraction of Different Microalgae Species. *Energies* **2018**, *11*, 450. [CrossRef]
27. Guo, H.; Cheng, J.; Mao, Y.; Qian, L.; Yang, W.; Park, J.Y. Synergistic Effect of Ultrasound and Switchable Hydrophilicity Solvent Promotes Microalgal Cell Disruption and Lipid Extraction for Biodiesel Production. *Bioresour. Technol.* **2022**, *343*, 126087. [CrossRef] [PubMed]
28. Rezaei Motlagh, S.; Harun, R.; Awang Biak, D.R.; Hussain, S.A.; Omar, R.; Khezri, R.; Elgharbawy, A.A. Ionic Liquid-Based Microwave-Assisted Extraction of Lipid and Eicosapentaenoic Acid from *Nannochloropsis oceanica* Biomass: Experimental Optimization Approach. *J. Appl. Phycol.* **2021**, *33*, 2015–2029. [CrossRef]
29. Quesada-Salas, M.C.; Delfau--bonnet, G.; Willig, G.; Préat, N.; Allais, F.; Ioannou, I. Optimization and Comparison of Three Cell Disruption Processes on Lipid Extraction from Microalgae. *Processes* **2021**, *9*, 369. [CrossRef]
30. Askari, M.; Jafari, A.; Esmaeilzadeh, F.; Khorram, M.; Mohammadi, A.H. Kinetic Study on *Nannochloropsis oculata*'s Lipid Extraction Using Supercritical CO_2 and n-Hexane for Biodiesel Production. *ACS Omega* **2022**, *7*, 23027–23040. [CrossRef]
31. Eikani, M.H.; Khandan, N.; Feyzi, E. Increased Bio-Oil Yield from *Nannochloropsis salina* through Tuning the Polarity of Subcritical Water. *J. Mol. Liq.* **2019**, *277*, 163–169. [CrossRef]
32. Gallego, R.; Bueno, M.; Chourio, A.M.; Ibáñez, E.; Saldaña, M.D.A.; Herrero, M. Use of High and Ultra-High Pressure Based-Processes for the Effective Recovery of Bioactive Compounds from *Nannochloropsis oceanica* Microalgae. *J. Supercrit. Fluids* **2021**, *167*, 105039. [CrossRef]
33. He, Y.; Wang, X.; Wei, H.; Zhang, J.; Chen, B.; Chen, F. Direct Enzymatic Ethanolysis of Potential *Nannochloropsis* Biomass for Co-Production of Sustainable Biodiesel and Nutraceutical Eicosapentaenoic Acid. *Biotechnol. Biofuels* **2019**, *12*, 78. [CrossRef] [PubMed]
34. Angles, E.; Jaouen, P.; Pruvost, J.; Marchal, L. Wet Lipid Extraction from the Microalga *Nannochloropsis* sp.: Disruption, Physiological Effects and Solvent Screening. *Algal Res.* **2017**, *21*, 27–34. [CrossRef]
35. Qiu, Y.; Frear, C.; Chen, S.; Ndegwa, P.; Harrison, J.; Yao, Y.; Ma, J. Accumulation of Long-Chain Fatty Acids from *Nannochloropsis salina* Enhanced by Breaking Microalgae Cell Wall under Alkaline Digestion. *Renew. Energy* **2020**, *149*, 691–700. [CrossRef]
36. Gkioni, M.D.; Andriopoulos, V.; Koutra, E.; Hatziantoniou, S.; Kornaros, M.; Lamari, F.N. Ultrasound-Assisted Extraction of *Nannochloropsis oculata* with Ethanol and Betaine: 1,2-Propanediol Eutectic Solvent for Antioxidant Pigment-Rich Extracts Retaining Nutritious the Residual Biomass. *Antioxidants* **2022**, *11*, 1103. [CrossRef]
37. Pan, J.; Muppaneni, T.; Sun, Y.; Reddy, H.K.; Fu, J.; Lu, X.; Deng, S. Microwave-Assisted Extraction of Lipids from Microalgae Using an Ionic Liquid Solvent [BMIM][HSO_4]. *Fuel* **2016**, *178*, 49–55. [CrossRef]
38. Teo, C.L.; Idris, A. Enhancing the Various Solvent Extraction Method via Microwave Irradiation for Extraction of Lipids from Marine Microalgae in Biodiesel Production. *Bioresour. Technol.* **2014**, *171*, 477–481. [CrossRef]
39. Mubarak, M.; Shaija, A.; Suchithra, T.V. A Review on the Extraction of Lipid from Microalgae for Biodiesel Production. *Algal Res.* **2015**, *7*, 117–123. [CrossRef]
40. Zghaibi, N.; Omar, R.; Kamal, S.M.M.; Biak, D.R.A.; Harun, R. Kinetics Study of Microwave-Assisted Brine Extraction of Lipid from the Microalgae *Nannochloropsis* sp. *Molecules* **2020**, *25*, 784. [CrossRef]
41. Martinez-Guerra, E.; Howlader, M.S.; Shields-Menard, S.; French, W.T.; Gude, V.G. Optimization of Wet Microalgal FAME Production from *Nannochloropsis* sp. under the Synergistic Microwave and Ultrasound Effect. *Int. J. Energy Res.* **2018**, *42*, 1934–1949. [CrossRef]
42. Wahidin, S.; Idris, A.; Yusof, N.M.; Kamis, N.H.H.; Shaleh, S.R.M. Optimization of the Ionic Liquid-Microwave Assisted One-Step Biodiesel Production Process from Wet Microalgal Biomass. *Energy Convers. Manag.* **2018**, *171*, 1397–1404. [CrossRef]
43. Motlagh, S.R.; Khezri, R.; Harun, R.; Radiah, D.; Biak, A.; Hussain, S.A.; Chee, C.Y.; Kheawhom, S. Kinetic and Thermodynamic Studies of Eicosapentaenoic Acid Extraction from *Nannochloropsis oceanica* Using Tetramethyl Ammonium Chloride and Microwave Irradiation. *PLoS ONE* **2022**, *17*, e0267626. [CrossRef] [PubMed]
44. Zghaibi, N.; Omar, R.; Kamal, S.M.M.; Biak, D.R.A.; Harun, R. Microwave-Assisted Brine Extraction for Enhancement of the Quantity and Quality of Lipid Production from Microalgae *Nannochloropsis* sp. *Molecules* **2019**, *24*, 3581. [CrossRef]
45. De Moura, R.R.; Etges, B.J.; dos Santos, E.O.; Martins, T.G.; Roselet, F.; Abreu, P.C.; Primel, E.G.; D'Oca, M.G.M. Microwave-Assisted Extraction of Lipids from Wet Microalgae Paste: A Quick and Efficient Method. *Eur. J. Lipid Sci. Technol.* **2018**, *120*, 1700419. [CrossRef]
46. Han, F.; Pei, H.; Hu, W.; Zhang, S.; Han, L.; Ma, G. The Feasibility of Ultrasonic Stimulation on Microalgae for Efficient Lipid Accumulation at the End of the Logarithmic Phase. *Algal Res.* **2016**, *16*, 189–194. [CrossRef]

47. Adam, F.; Abert-Vian, M.; Peltier, G.; Chemat, F. "Solvent-Free" Ultrasound-Assisted Extraction of Lipids from Fresh Microalgae Cells: A Green, Clean and Scalable Process. *Bioresour. Technol.* **2012**, *114*, 457–465. [CrossRef] [PubMed]
48. Parniakov, O.; Apicella, E.; Koubaa, M.; Barba, F.J.; Grimi, N.; Lebovka, N.; Pataro, G.; Ferrari, G.; Vorobiev, E. Ultrasound-Assisted Green Solvent Extraction of High-Added Value Compounds from Microalgae *Nannochloropsis* spp. *Bioresour. Technol.* **2015**, *198*, 262–267. [CrossRef]
49. Bligh, E.; Dyer, W. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917. [CrossRef] [PubMed]
50. Blanco-Llamero, C.; García-García, P.; Señoráns, F.J. Combination of Synergic Enzymes and Ultrasounds as an Effective Pretreatment Process to Break Microalgal Cell Wall and Enhance Algal Oil Extraction. *Foods* **2021**, *10*, 1928. [CrossRef] [PubMed]
51. Saber, M.; Golzary, A.; Wu, H.; Takahashi, F.; Yoshikawa, K. Ultrasonic Pretreatment for Low-Temperature Hydrothermal Liquefaction of Microalgae: Enhancing the Bio-Oil Yield and Heating Value. *Biomass Convers. Biorefin.* **2018**, *8*, 509–519. [CrossRef]
52. Herrero, M.; Sánchez-Camargo, A.d.P.; Cifuentes, A.; Ibáñez, E. Plants, Seaweeds, Microalgae and Food by-Products as Natural Sources of Functional Ingredients Obtained Using Pressurized Liquid Extraction and Supercritical Fluid Extraction. *TrAC Trends Anal. Chem.* **2015**, *71*, 26–38. [CrossRef]
53. Leone, G.P.; Balducchi, R.; Mehariya, S.; Martino, M.; Larocca, V.; Di Sanzo, G.; Iovine, A.; Casella, P.; Marino, T.; Karatza, D.; et al. Selective Extraction of ω-3 Fatty Acids from *Nannochloropsis* sp. Using Supercritical CO_2 Extraction. *Molecules* **2019**, *24*, 2406. [CrossRef] [PubMed]
54. Jafari, A.; Esmaeilzadeh, F.; Mowla, D.; Sadatshojaei, E.; Heidari, S.; Wood, D.A. New Insights to Direct Conversion of Wet Microalgae Impregnated with Ethanol to Biodiesel Exploiting Extraction with Supercritical Carbon Dioxide. *Fuel* **2021**, *285*, 119199. [CrossRef]
55. Mouahid, A.; Seengeon, K.; Martino, M.; Crampon, C.; Kramer, A.; Badens, E. Selective Extraction of Neutral Lipids and Pigments from *Nannochloropsis salina* and *Nannochloropsis maritima* Using Supercritical CO_2 Extraction: Effects of Process Parameters and Pre-Treatment. *J. Supercrit. Fluids* **2020**, *165*, 104934. [CrossRef]
56. Molino, A.; Martino, M.; Larocca, V.; Di Sanzo, G.; Spagnoletta, A.; Marino, T.; Karatza, D.; Iovine, A.; Mehariya, S.; Musmarra, D. Eicosapentaenoic Acid Extraction from *Nannochloropsis gaditana* Using Carbon Dioxide at Supercritical Conditions. *Mar. Drugs* **2019**, *17*, 132. [CrossRef] [PubMed]
57. Obeid, S.; Beaufils, N.; Camy, S.; Takache, H.; Ismail, A.; Pontalier, P.Y. Supercritical Carbon Dioxide Extraction and Fractionation of Lipids from Freeze-Dried Microalgae *Nannochloropsis oculata* and *Chlorella vulgaris*. *Algal Res.* **2018**, *34*, 49–56. [CrossRef]
58. Eikani, M.H.; Khandan, N.; Feyzi, E.; Ebrahimi, I.M. A Shrinking Core Model for *Nannochloropsis salina* Oil Extraction Using Subcritical Water. *Renew. Energy* **2019**, *131*, 660–666. [CrossRef]
59. Folch, J.; Lees, M.; Sloane Stanley, G.H. A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues. *J. Biol. Chem.* **1957**, *55*, 497–509. [CrossRef]
60. Maffei, G.; Bracciale, M.P.; Broggi, A.; Zuorro, A.; Santarelli, M.L.; Lavecchia, R. Effect of an Enzymatic Treatment with Cellulase and Mannanase on the Structural Properties of *Nannochloropsis* Microalgae. *Bioresour. Technol.* **2018**, *249*, 592–598. [CrossRef] [PubMed]
61. Zuorro, A.; Maffei, G.; Lavecchia, R. Optimization of Enzyme-Assisted Lipid Extraction from *Nannochloropsis* Microalgae. *J. Taiwan Inst. Chem. Eng.* **2016**, *67*, 106–114. [CrossRef]
62. Kumar, R.R.; Rao, P.H.; Arumugam, M. Lipid Extraction Methods from Microalgae: A Comprehensive Review. *Front. Energy Res.* **2015**, *3*, 61. [CrossRef]
63. Qiu, C.; He, Y.; Huang, Z.; Li, S.; Huang, J.; Wang, M.; Chen, B. Lipid Extraction from Wet *Nannochloropsis* Biomass via Enzyme-Assisted Three Phase Partitioning. *Bioresour. Technol.* **2019**, *284*, 381–390. [CrossRef] [PubMed]
64. He, Y.; Zhang, B.; Guo, S.; Guo, Z.; Chen, B.; Wang, M. Sustainable Biodiesel Production from the Green Microalgae *Nannochloropsis*: Novel Integrated Processes from Cultivation to Enzyme-Assisted Extraction and Ethanolysis of Lipids. *Energy Convers. Manag.* **2020**, *209*, 112618. [CrossRef]
65. Zhou, J.; Wang, M.; Saraiva, J.A.; Martins, A.P.; Pinto, C.A.; Prieto, M.A.; Simal-Gandara, J.; Cao, H.; Xiao, J.; Barba, F.J. Extraction of Lipids from Microalgae Using Classical and Innovative Approaches. *Food Chem.* **2022**, *384*, 132236. [CrossRef]
66. Imbimbo, P.; D'Elia, L.; Liberti, D.; Olivieri, G.; Monti, D.M. Towards Green Extraction Methods from Microalgae Learning from the Classics. *Appl. Microbiol. Biotechnol.* **2020**, *104*, 9067–9077. [CrossRef] [PubMed]
67. Orr, V.C.A.; Rehmann, L. Ionic Liquids for the Fractionation of Microalgae Biomass. *Curr. Opin. Green Sustain. Chem.* **2016**, *2*, 22–27. [CrossRef]
68. Tripathy, D.B.; Gupta, A.; Mishra, A.; Quraishi, M.A.; Luqman, M.; Farhan Khan, M. Chapter 6—Switchable Solvents as Alternative Solvents for Green Chemistry. In *Green Sustainable Process for Chemical and Environmental Engineering and Science*; Inamuddin, Boddula, R., Asiri, A., Eds.; Elsevier: Amsterdam, The Netherlands, 2022; pp. 109–131; ISBN 978-0-12-819850-6.
69. Shankar, M.; Chhotaray, P.K.; Gardas, R.L.; Tamilarasan, K.; Rajesh, M. Application of Carboxylate Protic Ionic Liquids in Simultaneous Microalgal Pretreatment and Lipid Recovery from Marine *Nannochloropsis* sp. and *Chlorella* sp. *Biomass Bioenergy* **2019**, *123*, 14–24. [CrossRef]
70. Mehariya, S.; Fratini, F.; Lavecchia, R.; Zuorro, A. Green Extraction of Value-Added Compounds Form Microalgae: A Short Review on Natural Deep Eutectic Solvents (NaDES) and Related Pre-Treatments. *J. Environ. Chem. Eng.* **2021**, *9*, 105989. [CrossRef]

71. Cai, C.; Chen, X.; Li, F.; Tan, Z. Three-Phase Partitioning Based on CO_2-Responsive Deep Eutectic Solvents for the Green and Sustainable Extraction of Lipid from *Nannochloropsis* sp. *Sep. Purif. Technol.* **2021**, *279*, 119685. [CrossRef]
72. Rezaei Motlagh, S.; Harun, R.; Biak, D.R.A.; Hussain, S.A. Microwave Assisted Extraction of Lipid from *Nannochloropsis gaditana* Microalgae Using [EMIM]Cl. *IOP Conf. Ser. Mater. Sci. Eng.* **2020**, *778*, 012164. [CrossRef]
73. Wang, M.; Cheng, H.; Chen, S.; Wen, S.; Wu, X.; Zhang, D.; Yuan, Q.; Cong, W. Microalgal Cell Disruption via Extrusion for the Production of Intracellular Valuables. *Energy* **2018**, *142*, 339–345. [CrossRef]
74. Park, J.Y.; Kim, M.C.; Cheng, J.; Yang, W.; Kim, D.K. Extraction of Microalgal Oil from *Nannochloropsis oceanica* by Potassium Hydroxide-Assisted Solvent Extraction for Heterogeneous Transesterification. *Renew. Energy* **2020**, *162*, 2056–2065. [CrossRef]
75. Halim, R.; Papachristou, I.; Kubisch, C.; Nazarova, N.; Wüstner, R.; Steinbach, D.; Chen, G.Q.; Deng, H.; Frey, W.; Posten, C.; et al. Hypotonic Osmotic Shock Treatment to Enhance Lipid and Protein Recoveries from Concentrated Saltwater *Nannochloropsis* slurries. *Fuel* **2021**, *287*, 119442. [CrossRef]
76. Lorente, E.; Hapońska, M.; Clavero, E.; Torras, C.; Salvadó, J. Steam Explosion and Vibrating Membrane Filtration to Improve the Processing Cost of Microalgae Cell Disruption and Fractionation. *Processes* **2018**, *6*, 28. [CrossRef]
77. Matos, Â.P.; Teixeira, M.S.; Corrêa, F.M.P.S.; Machado, M.M.; Werner, R.I.S.; Aguiar, A.C.; Cubas, A.L.V.; Sant'Anna, E.S.; Moecke, E.H.S. Disruption of *Nannochloropsis gaditana* (Eustigmatophyceae) Rigid Cell Wall by Non-Thermal Plasma Prior to Lipid Extraction and Its Effect on Fatty Acid Composition. *Braz. J. Chem. Eng.* **2019**, *36*, 1419–1428. [CrossRef]
78. Setyawan, M.; Budiman, A.; Mulyono, P. Sutijan Optimum Extraction of Algae-Oil from Microalgae Using Hydrodynamic Cavitation. *Int. J. Renew. Energy Res.* **2018**, *8*, 451–458. [CrossRef]
79. Teymouri, A.; Adams, K.J.; Dong, T.; Kumar, S. Evaluation of Lipid Extractability after Flash Hydrolysis of Algae. *Fuel* **2018**, *224*, 23–31. [CrossRef]
80. Kwak, M.; Kim, D.; Kim, S.; Lee, H.; Chang, Y.K. Solvent Screening and Process Optimization for High Shear-Assisted Lipid Extraction from Wet Cake of *Nannochloropsis* sp. *Renew. Energy* **2020**, *149*, 1395–1405. [CrossRef]
81. Bensalem, S.; Lopes, F.; Bodénès, P.; Pareau, D.; Français, O.; Le Pioufle, B. Understanding the Mechanisms of Lipid Extraction from Microalga *Chlamydomonas reinhardtii* after Electrical Field Solicitations and Mechanical Stress within a Microfluidic Device. *Bioresour. Technol.* **2018**, *257*, 129–136. [CrossRef]
82. Silve, A.; Papachristou, I.; Wüstner, R.; Sträßner, R.; Schirmer, M.; Leber, K.; Guo, B.; Interrante, L.; Posten, C.; Frey, W. Extraction of Lipids from Wet Microalga *Auxenochlorella protothecoides* Using Pulsed Electric Field Treatment and Ethanol-Hexane Blends. *Algal Res.* **2018**, *29*, 212–222. [CrossRef]
83. Zbinden, M.D.A.; Sturm, B.S.M.; Nord, R.D.; Carey, W.J.; Moore, D.; Shinogle, H.; Stagg-Williams, S.M. Pulsed Electric Field (PEF) as an Intensification Pretreatment for Greener Solvent Lipid Extraction from Microalgae. *Biotechnol. Bioeng.* **2013**, *110*, 1605–1615. [CrossRef]
84. Pagels, F.; Pereira, R.N.; Amaro, H.M.; Vasconcelos, V.; Guedes, A.C.; Vicente, A.A. Continuous Pressurized Extraction versus Electric Fields-Assisted Extraction of Cyanobacterial Pigments. *J. Biotechnol.* **2021**, *334*, 35–42. [CrossRef] [PubMed]
85. Parniakov, O.; Barba, F.J.; Grimi, N.; Marchal, L.; Jubeau, S.; Lebovka, N.; Vorobiev, E. Pulsed Electric Field and PH Assisted Selective Extraction of Intracellular Components from Microalgae *Nannochloropsis*. *Algal Res.* **2015**, *8*, 128–134. [CrossRef]
86. Grimi, N.; Dubois, A.; Marchal, L.; Jubeau, S.; Lebovka, N.I.; Vorobiev, E. Selective Extraction from Microalgae *Nannochloropsis* sp. Using Different Methods of Cell Disruption. *Bioresour. Technol.* **2014**, *153*, 254–259. [CrossRef] [PubMed]
87. Kojima, Y.; Shimizu, A. Effect of High Hydrostatic Pressure Treatment with Room-Temperature Ionic Liquid 1-Ethyl-3-Methylimidazolium Acetate-Dimethyl Sulfoxide Mixture on Lipid Extraction from *Chlorella vulgaris*. *High Press. Res.* **2022**, *42*, 105–120. [CrossRef]
88. Xu, J.; Zhao, F.; Su, X. Direct Extraction of Lipids from Wet Microalgae Slurries by Super-High Hydrostatic Pressure. *Algal Res.* **2021**, *58*, 102412. [CrossRef]
89. Bueno, M.; Gallego, R.; Chourio, A.M.; Ibáñez, E.; Herrero, M.; Saldaña, M.D.A. Green Ultra-High Pressure Extraction of Bioactive Compounds from *Haematococcus pluvialis* and *Porphyridium cruentum* Microalgae. *Innov. Food Sci. Emerg. Technol.* **2020**, *66*, 102532. [CrossRef]
90. Halim, R.; Hill, D.R.A.; Hanssen, E.; Webley, P.A.; Blackburn, S.; Grossman, A.R.; Posten, C.; Martin, G.J.O. Towards Sustainable Microalgal Biomass Processing: Anaerobic Induction of Autolytic Cell-Wall Self-Ingestion in Lipid-Rich: *Nannochloropsis* slurries. *Green Chem.* **2019**, *21*, 2967–2982. [CrossRef]
91. United Nations Development Programme. *Transforming Our World: The 2030 Agenda for Sustainable Development 2015*; United Nations Development Programme: New York, NY, USA, 2015.

marine drugs

MDPI

Article

Characterization of Some Dermato-Cosmetic Preparations with Marine Lipids from Black Sea Wild Stingray

Magdalena Mititelu [1,†], Monica Licu [2,†], Carmen Elena Lupu [3,†], Sorinel Marius Neacșu [4,*], Gabriel Olteanu [1,*], Gabriela Stanciu [5,*], Doina Drăgănescu [6], Carmen-Nicoleta Oancea [7], Ștefan Sebastian Busnatu [8], Lucian Hîncu [9], Maria Viorica Ciocîlteu [10] and Dumitru Lupuleasa [4]

1 Department of Clinical Laboratory and Food Safety, Faculty of Pharmacy, University of Medicine and Pharmacy Carol Davila, 020956 Bucharest, Romania; magdalena.mititelu@umfcd.ro
2 Department of Medical Psychology, Faculty of Medicine, "Carol Davila" University of Medicine and Pharmacy, 050474 Bucharest, Romania; monica.licu@umfcd.ro
3 Department of Mathematics and Informatics, Faculty of Pharmacy, "Ovidius" University of Constanta, 6 Capitan Aviator Al. Serbanescu Street, Campus, C Block, 900001 Constanta, Romania; clupu@univ-ovidius.ro
4 Department of Pharmaceutical Technology and Bio-Pharmacy, Faculty of Pharmacy, Carol Davila University of Medicine and Pharmacy, 020945 Bucharest, Romania; dumitru.lupuliasa@umfcd.ro
5 Department of Chemistry and Chemical Engineering, Ovidius University of Constanta, 900527 Constanta, Romania
6 Department of Pharmaceutical and Computer Physics, Faculty of Pharmacy, Carol Davila University of Medicine and Pharmacy, 020956 Bucharest, Romania; doina.draganescu@umfcd.ro
7 Department of Biochemistry, Faculty of Medicine, University of Medicine and Pharmacy from Craiova, 200345 Craiova, Romania; carmen.oancea@umfcv.ro
8 Department of Cardio-Thoracic Pathology, Faculty of Medicine, "Carol Davila" University of Medicine and Pharmacy, 050474 Bucharest, Romania; stefan.busnatu@umfcd.ro
9 Department of Drug Industry and Pharmaceutical Biotechnologies, Faculty of Pharmacy, University of Medicine and Pharmacy Carol Davila, 020956 Bucharest, Romania; lucian.hincu@umfcd.ro
10 Department of Analytical and Instrumental Chemistry, Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova, Petru Rares Street, no. 2–4, 200638 Craiova, Romania; maria.ciocilteu@umfcv.ro
* Correspondence: neacsusorinelmarius@gmail.com (S.M.N.); gabriel.olteanu@mst.umfcd.ro (G.O.); gstanciu@univ-ovidius.ro (S.G.)
† These authors contributed equally to this work.

Citation: Mititelu, M.; Licu, M.; Lupu, C.E.; Neacșu, S.M.; Olteanu, G.; Stanciu, G.; Drăgănescu, D.; Oancea, C.-N.; Busnatu, Ș.S.; Hîncu, L.; et al. Characterization of Some Dermato-Cosmetic Preparations with Marine Lipids from Black Sea Wild Stingray. *Mar. Drugs* **2023**, *21*, 408. https://doi.org/10.3390/md21070408

Academic Editors: Giuseppina Tommonaro and Annabella Tramice

Received: 30 June 2023
Accepted: 17 July 2023
Published: 19 July 2023

Abstract: The traditional knowledge about the therapeutic and nutritional value of fish has been unanimously recognized among the population since ancient times. So, thanks to the therapeutic virtues of these marine animals, it was possible to develop therapies for certain pathologies as well as the use of bioactive compounds as adjunctive therapies incorporated into the treatment regimen of patients. In the present study, stingray liver oil from wild species collected from the Romanian coast of the Black Sea was isolated and analyzed. Fatty acid analysis was performed by gas chromatography. The analysis of the distribution of fatty acids in the composition of stingray liver oil indicates a ratio of 2.83 of omega 3 fatty acids to omega 6, a ratio of 1.33 of polyunsaturated fatty acids to monounsaturated fatty acids, an iodine index of 111.85, and a total percentage of 68.98% of unsaturated fatty acids. Stingray liver oil was used to evaluate the healing action after preparing a fatty ointment. According to the experimental data, a complete regeneration capacity of the wounds was noted in 12 days without visible signs. Four emulgels with stingray liver oil were formulated and analyzed from a rheological and structural point of view in order to select the optimal composition, after which the anti-inflammatory effect on inflammation caused in laboratory rats was studied and an anti-inflammatory effect was found significant (a maximum inhibitory effect of 66.47% on the edemas induced by the 10% kaolin suspension and 65.64% on the edemas induced by the 6% dextran solution).

Keywords: healing effect; polyunsaturated fatty acids; stingray liver oil; fatty ointment; emulgels; anti-inflammatory effect

1. Introduction

The common stingray (*Dasyatis pastinaca*) is a member of the class Chondrichthyes (which includes mostly large cartilaginous fish and prey fish) and is related to the shark. Cartilaginous fish are also among the oldest vertebrates and possess a complex digestive system [1].

Stingray liver oil is particularly valuable due to its high content of monounsaturated and polyunsaturated fatty acids. The main ingredients in stingray liver oil are monounsaturated fatty acids, omega-3 fatty acids, important amounts of docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA), vitamin A, vitamin E, vitamin D, and minerals. The common stingray (*Dasyatis pastinaca*) is a cartilaginous fish related to the shark. It lives along shores with warmer water. It is also found in the Black Sea, approaching the coast when the water reaches 12 °C, looking for a place on the sandy bottoms, and retreating to the depths when the water cools. The weapon of a stingray is represented by one or more sharp spikes located at the end of the tail, through which it spreads a dangerous poison when it stings the victim. It feeds on small fish, mollusks, and crustaceans. The meat of stingrays is not eaten. The liver represents approximately 23% of the female and 11% of the male's body weight and contains 52–70% fat. A quality oil is extracted from it, rich in anti-rickets vitamins (A, D, and E) and polyunsaturated fatty acids (omega 3, 6, 9, etc.), also used in the healing of external wounds [2–4].

In the specialized literature, there are limited studies on *Dasyatis pastinaca*, although the therapeutic effects of the liver of marine animals are multiple and indisputable [5–12]. Lipid fractions isolated from *Dasyatis zugei* with petroleum ether and diethyl ether appear to have analgesic and anti-inflammatory properties highlighted in animal model studies [13]. *Dasyatis jenkinsii*, also known as the sharp-nosed stingray, belongs to the class *Elasmobranchii* and has therapeutic virtues in the treatment of inflammatory diseases and arthritis [14].

Stingray skin is a growing trend today; most of the big fashion designers use it to make accessories that delight fashion and luxury lovers all over the world. In the fashion industry, stingray skin is considered one of the most unique materials. With its special texture, stingray skin gives any fashion product a special quality, easily standing out in the crowd. In addition to the visual appeal that this leather possesses, accessories made of stingray leather are more resistant to abrasion, scratches, and high temperatures that could otherwise compromise some leather products. In the past, stingray skin was used for the handles of swords and tools because it aided grip and was resistant to damage from moisture or sweat. In the 21st century, stingray skin enjoys popularity among fashion lovers, not only because of its attractive design but also because of the superior quality of the product itself [15].

Depending on the width, stingray skin can be used for wallets, watch straps or other small accessories, belts, handbags, shoes, and other Western clothing items, interior decoration, automobiles, or other upholstery [15].

Marine animals represent reservoirs of bioactive compounds with impressive therapeutic virtues [16–18]. Fish liver oil, rich in polyunsaturated fatty acids, presents real health benefits, as reported throughout history in the specialized literature [19–25].

The effect of fatty acids in fish oil is not only limited internally, through food intake or the administration of supplements, but also externally, through skin applications. Their application in skin diseases includes photoaging, dermatitis, cancer, healing (scarring properties), and melanogenesis [26–29].

Burns, skin wounds, chronic wounds, and ulcers have affected and continue to affect millions of people around the world. The processes at the cellular and molecular level that occur in the inflammatory phase of wound healing are initiated and greatly amplified by pro-inflammatory cytokines, whose synthesis and activity can be modulated by polyunsaturated fatty acids [30,31]. Fatty acids have been shown to be essential in skin tissue reconstruction processes [32]. Animal studies have demonstrated the beneficial effect of the fatty acids present in cod liver oil on the prevention or healing of skin wounds, both topically and internally (orally as a supplement or by injection) [33–42]. Fish liver oil

stimulates angiogenesis, thus promoting wound healing. Compared to olive oil, fish liver oil has faster tissue repair activity [33].

The utilization of bioactive compounds from marine species for therapeutic purposes must also take into account the harvesting areas because the marine environment can be degraded in certain regions due to the increased level of pollution [43]. Various contaminants from the marine environment (heavy metals, pesticides, and microplastics) can also be found in marine organisms in concentrations that may endanger the safety of the consumer [44,45].

The purpose of this study is the characterization and use of stingray liver oil for the production of dermato-cosmetic preparations (ointments and emulgels) and the analysis of the healing and anti-inflammatory effects on laboratory animals. Stingray liver oil is a less studied marine oil that can be exploited as a valuable bioactive by-product resulting from the processing of stingray specimens exploited on farms to obtain leather products.

2. Results

2.1. Characteristics of Stingray Liver Oil

Table 1 shows the main physico-chemical properties of the obtained stingray liver oil. The oil is in the form of a clear, oily liquid with a characteristic odor and orange-reddish color.

Table 1. Values of examined parameters relative to stingray liver oil.

Parameter	Value ± SD
Iodine value (g I_2/100 g fatty acids)	111.85 ± 0.66
Acid value (mg KOH/g sample)	4.93 ± 0.33
Saponification value (mg KOH/g sample)	179.07 ± 0.25
Peroxide index (mEq O_2/kg)	0.8 ± 0.55
Density at 20 °C (g/mL)	0.921 ± 0.33
Refractive index	1.479 ± 0.55

SD—standard deviation.

Table 2 and Figure 1 show the distribution of fatty acids in the extracted and analyzed stingray liver oil. The predominant fraction is represented by polyunsaturated fatty acids (39.45%), while the total of unsaturated fatty acids represents approximately 69%, which explains the high iodine index (high degree of unsaturation of the analyzed oil).

Table 2. Percent distribution of fatty acids in the total lipid extract isolated from stingray liver oil.

Fatty Acid	mg/g ± SD (%)
C 10:0	3.42 ± 0.23
C 12:0	0.06 ± 0.13
C 14:0	4.23 ± 0.45
C 14:1	2.12 ± 0.22
C 15:0	0.16 ± 0.16
C 16:0	10.15 ± 1.84
C 16:1	12.23 ± 1.52
C 16:1ω-7	6.12 ± 0.81
C 17:0	1.15± 0.12
C 17:1	0.23 ± 0.18
C 18:0	8.85 ± 0.65
C 18:1	15.46 ± 1.73
C 18:1ω-7	4.75 ± 0.33
C 18:1ω-9	9.81 ± 0.74
C 18:2ω-6	2.18 ± 0.52
C 18:3	1.15 ± 0.25
C 18:3ω-3	2.12 ± 0.14
C 20:1	0.19 ± 0.38

Table 2. *Cont.*

Fatty Acid	mg/g ± SD (%)
C 20:3	0.08 ± 0.04
C 20:4ω-6	2.68 ± 0.55
C 20:5ω-3	3.86 ± 0.28
C 22:1	0.07 ± 0.14
C 22:5ω-3	1.49 ± 0.16
C 22:6ω-3	6.44 ± 0.31
Σ saturated fatty acids	31.02
Σ ω-3	13.91
Σ ω-6	4.86
Σ monounsaturated fatty acids	29.53
Σ polyunsaturated fatty acids	39.45
ω-3/ω-6	2.86
Polyunsaturated fatty acids/Saturated fatty acids	1.27
Polyunsaturated fatty acids/Monounsaturated fatty acids	1.33

SD—standard deviation.

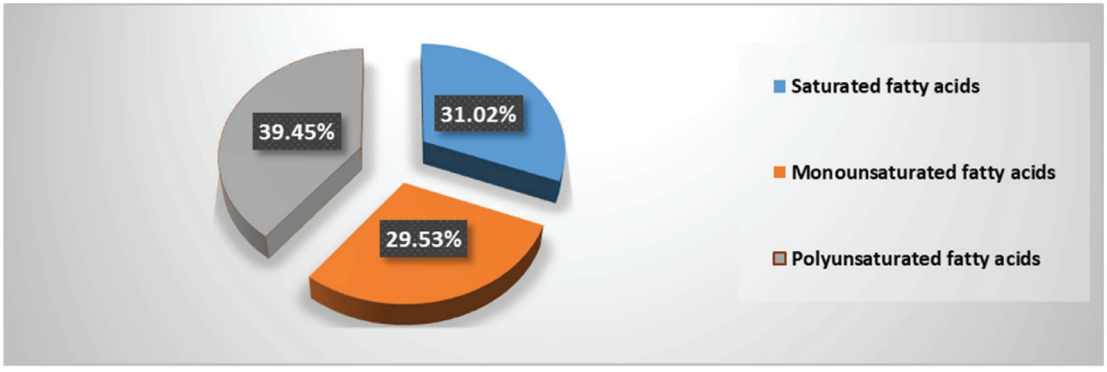


Figure 1. Distribution of different fractions of fatty acids in stingray liver oil.

2.2. Characteristics of Stingray Liver Oil Ointment

The results obtained from the analysis of the fatty ointment with stingray liver oil are shown in Table 3 and Figure 2. The ointment is homogeneous, stable, has a pleasant effect when applied to the skin, and has a pH compatible with the skin.

Table 3. Characteristics of catfish liver oil ointment.

Parameter	Result
Appearance	Homogeneous appearance, yellow-orange color, characteristic smell
pH	6.0
Thermal stability	Good stability, the samples remained homogeneous at the subjected temperature steps without separating into several phases
Viscosity	285 mPa/s

In addition, the ointment shows high extension values immediately after preparation, proof that it has a suitable consistency and a high stretching capacity. Over time, the creams maintain their consistency and stretchability well (Figure 2).

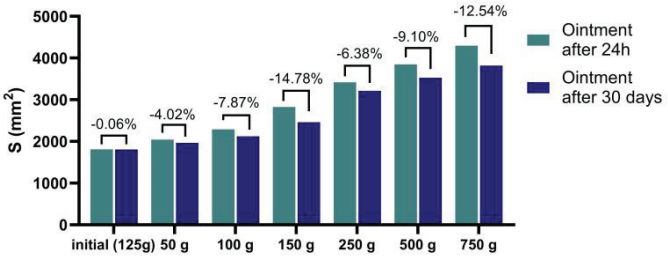


Figure 2. The stretching surface depending on the applied mass.

2.3. Evaluation of the Healing Action of Stingray Liver Oil Ointment

The evolution of wound healing is presented in Figure 3. According to the experimental data, an accelerated healing process can be noted in the group of animals treated with the stingray liver oil ointment.

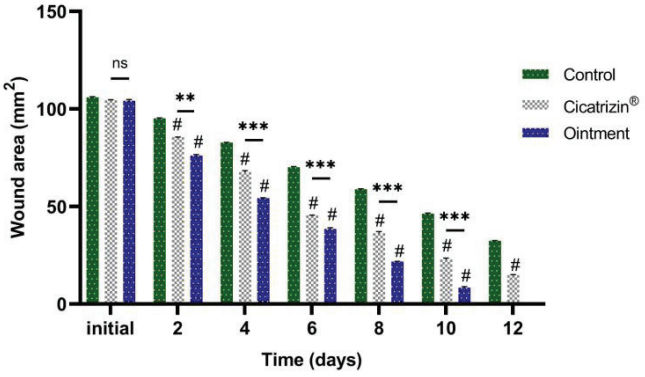


Figure 3. Evolution of the wound surface: average wound area (mm^2) ± SD (standard deviation). The data were represented as mean ± SD and analyzed using ANOVA. # $p < 0.001$ has statistical significance versus the control group, while ** $p < 0.01$ and *** $p < 0.001$ refer to statistical significance between the group treated with the stingray liver oil ointment and the group treated with Cicatrizin®.

All treatment groups were compared to the control group, to each other (treated with the stingray liver oil ointment and treated with Cicatrizin®), and to each other between treatments (days).

No statistical differences were found between the three groups at the initial moment. In the control group, there was a statistically significant decrease ($p \leq 0.001$) between days 4 and 10. A significant decrease ($p \leq 0.001$) in the group treated with the stingray liver oil ointment and the group treated with Cicatrizin® was seen on Day 2.

According to the data presented in Figure 4, a strong healing effect can be noted in the case of the treatment with stingray liver oil ointment (tested group), with a good evolution over time, significantly increased compared to the wound healing process in the untreated control group, and clearly superior to the reference group treated with Cicatrizin®.

After 8 days of treatment, a cure of 79.08% can be noted in the case of animals treated with the prepared ointment, 65.23% in the case of animals treated with Cicatrizin®, and 44.44% in the case of untreated animals (Figure 5).

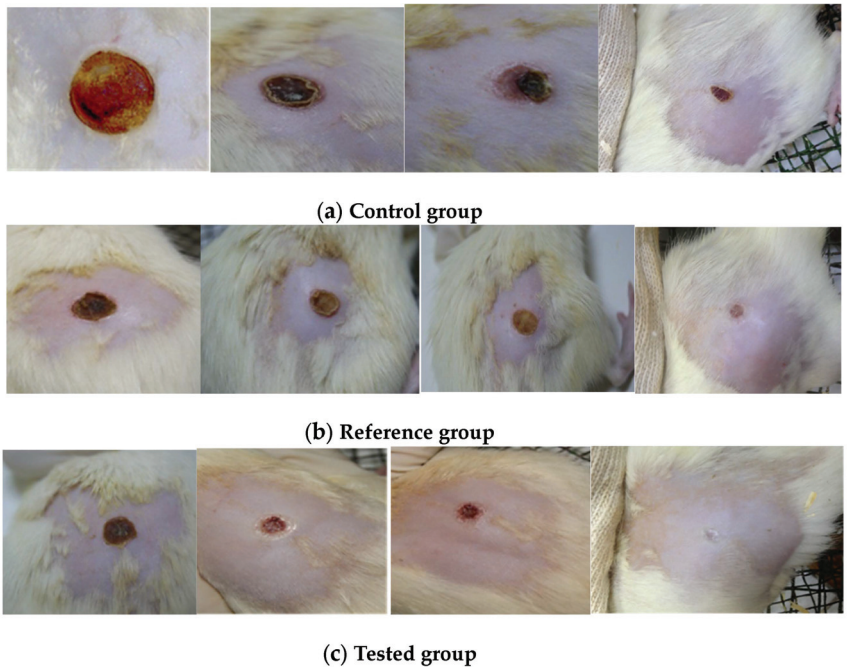


Figure 4. The evolution of the wounds at different times (initially, after 2 days, after 4 days, and after 8 days): (**a**) the untreated group (control); (**b**) the group treated with Cicatrizin® (reference); (**c**) the group treated with stingray liver oil ointment (tested).

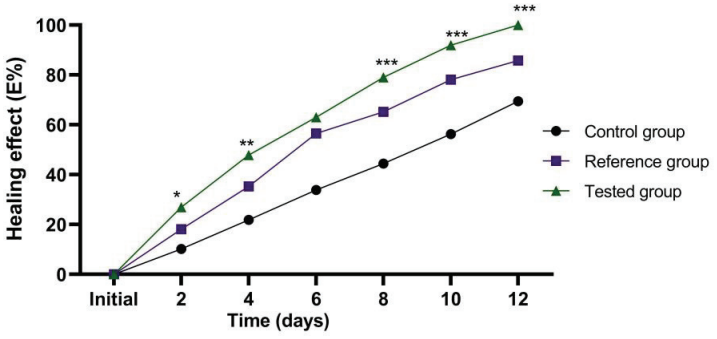


Figure 5. The evolution of the healing effect (E%) during the treatment. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ refer to statistical significance between the reference group and the tested group.

The post hoc analysis revealed a greater difference between Days 2 and 12 assessed in the study between the tested group and the control group.

2.4. Characteristics of Catfish Liver Oil Emulgels

The results obtained from the analysis of the emulgels with stingray liver oil are shown in Table 4 and Figure 6. According to the results obtained, the prepared formulas are stable and homogeneous (Table 4), but the best spreading surface was noted for Formula C (Figure 6), with good maintenance over time.

Table 4. Emulgels characteristics.

Characteristic	Formula A	Formula B	Formula C	Formula D
Initial macroscopic characteristics	appearance: homogenous; color: yellowish; smell: specific	appearance: homogenous; color: yellow; smell: specific	appearance: homogenous; color: orange-yellow; smell: specific	appearance: homogenous; color: orange-yellow; smell: specific
Macroscopic characteristics after 30 days	appearance: homogenous; color: yellowish; smell: specific	appearance: homogenous; color: yellow; smell: specific	appearance: homogenous; color: orange-yellow; smell: specific	appearance: homogenous; color: orange-yellow; smell: specific
Initial pH	6.4	6.1	5.8	5.6
pH after 30 days	6.2	6.0	5.7	5.6
Initial thermal stability	Good stability without a tendency to separate	Good stability without a tendency to separate	Good stability without a tendency to separate	Good stability without a tendency to separate
Thermal stability after 30 days	Good stability without a tendency to separate	Good stability without a tendency to separate	Good stability without a tendency to separate	Good stability without a tendency to separate

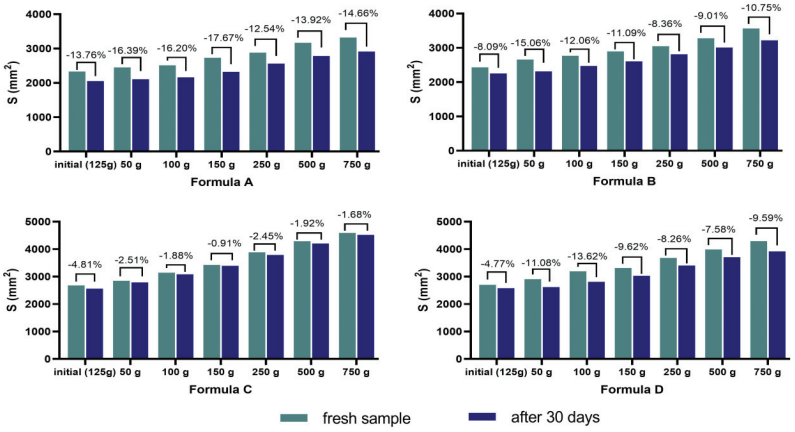


Figure 6. Spreading area for the emulgels prepared: Formula A, Formula B, Formula C and Formula D.

The increase in shear stress with the shear rate indicates a non-Newtonian behavior of the emulgels (Figure 7).

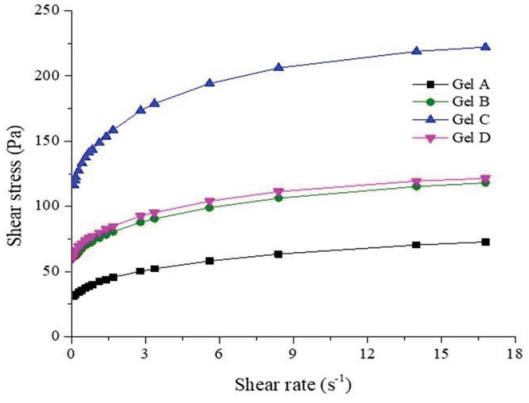


Figure 7. The rheograms for emulgels analyzed at 33 °C.

The rheological characteristics of the prepared emulgels are presented in Tables 5 and 6. Table 5 shows the flow parameters and viscosity of the analyzed emulgels.

Table 5. Herschel-Bulkley model flow parameters obtained through stationary shear analysis at 33 °C.

Gel/Flow Parameters	Yield Stress (Pa) (τ_0 - Pa)	Consistency Index (K - Pa·s^n)	Flow Index (n)	Viscosity at 0.3 rpm ($\eta_{0.3}$ - Pa·s) Initial	Viscosity at 0.3 rpm ($\eta_{0.3}$ - Pa·s) after 30 Days
Formula A	27.318	21.081	0.35	499.200	482.100
Formula B	47.233	33.738	0.29	872.700	810.200
Formula C	77.154	66.884	0.27	553.000	432.800
Formula D	53.366	35.114	0.28	861.600	755.600

Table 6. The coefficient values of rheological models tested at 33 °C.

Emulgel/Rheological Model	Casson	Herschel-Bulkley
Formula A	0.832	0.992
Formula B	0.975	0.997
Formula C	0.919	0.995
Formula D	0.969	0.996

The internal morphology of the emulgels is presented in Figure 8. According to the results of the rheological and morphological analysis, the optimal formula for emulsifying stingray liver oil in a gel base is Formula C.

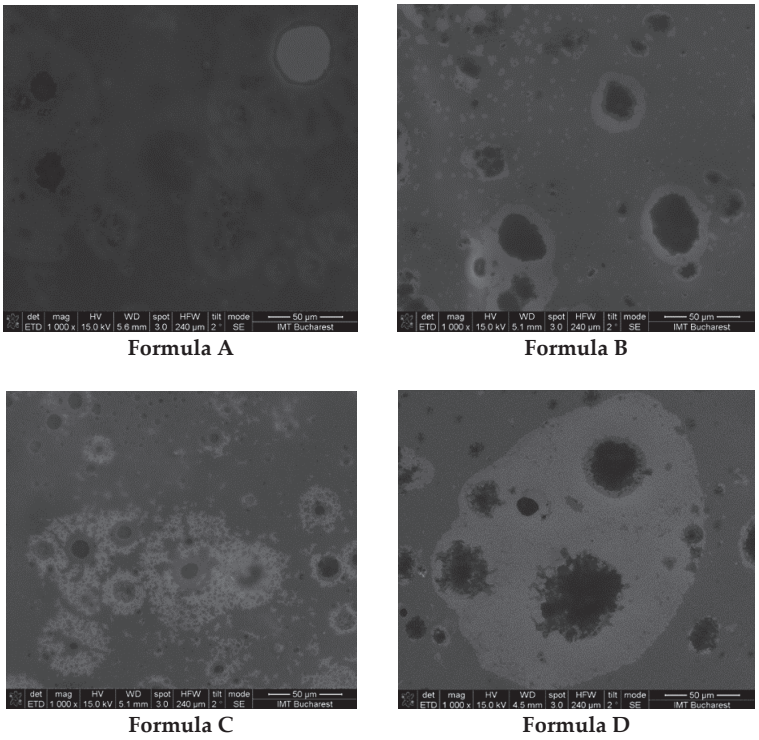


Figure 8. SEM images from the emulgels prepared: Formula A, Formula B, Formula C and Formula D.

2.5. Evaluation of the Anti-Inflammatory Action of Stingray Liver Emulgel (Formula C)

The experimental results of the evaluations of the anti-inflammatory effect carried out on the emulgel prepared according to Formula C (Table 4) are presented in Figures 9–12. They show the evolution of the edema inhibition effect (I%) over time for the preparations used (emulgel Formula C and Diclofenac gel) compared to the untreated control group.

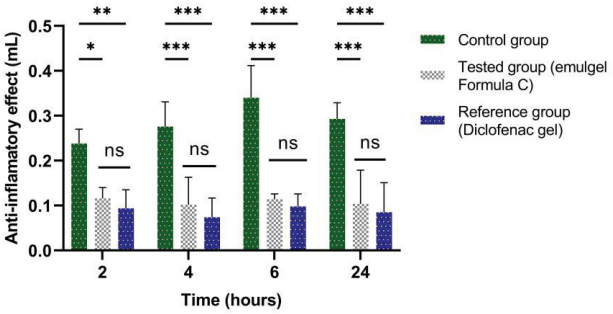


Figure 9. The anti-inflammatory effect of stingray liver oil emulgel on inflammatory edema induced with 10% kaolin suspension. The data were represented as mean ± SD and analyzed using ANOVA. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the control group, while ns: $p > 0.05$ statistical significance between the reference group and the tested group.

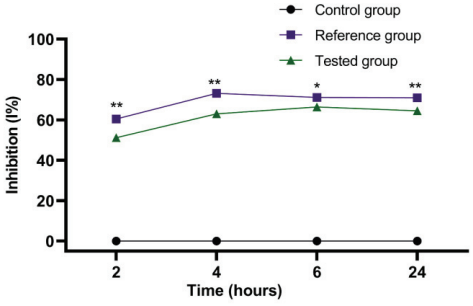


Figure 10. The evolution of the inhibition effect (I%) on inflammatory edema induced with 10% kaolin suspension during the treatment. * $p < 0.05$ and ** $p < 0.01$ refer to statistical significance between the reference group and the tested group.

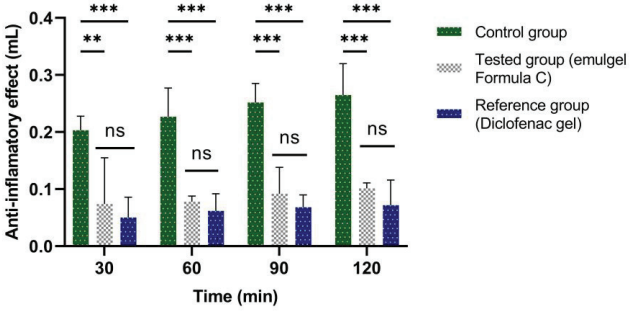


Figure 11. The anti-inflammatory effect of stingray liver oil emulgel on inflammatory edema induced with a 6% dextran solution. The data were represented as mean ± SD and analyzed using ANOVA. ** $p < 0.01$, and *** $p < 0.001$ versus the control group, while ns: $p > 0.05$ statistical significance between the reference group and the tested group.

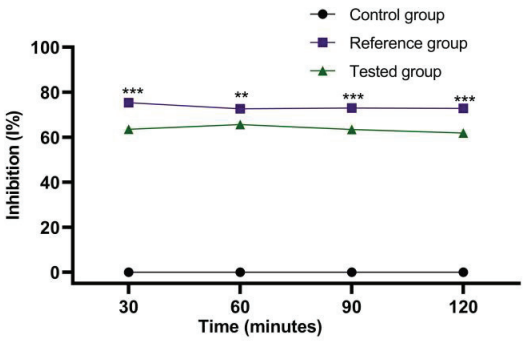


Figure 12. The evolution of the inhibition effect (I%) on inflammatory edema induced with a 6% dextran solution during the treatment. ** $p < 0.01$ and *** $p < 0.001$ refer to statistical significance between the reference group and the tested group.

The anti-inflammatory effect of stingray liver oil emulgel on inflammatory edema is significantly different from the control group but not from the reference group (Figures 9 and 10).

It can be noted from Figure 10 that there is a significant anti-inflammatory effect for the stingray liver oil emulgel, with the edema inhibition effect being between 51.26% and 66.47% on the edemas induced by the 10% kaolin suspension. The inhibitory effect of Diclofenac gel was between 60.5% and 73.19% and exhibited a maximum percent at a time period of 4 h.

In the case of edemas induced by the 6% dextran solution (Figure 12), the edema inhibition effect for emulgel was also significant, between 61.89% and 65.64%, while for Diclofenac gel chosen as a reference, the inhibitory effect was between 72.69% and 75.37%.

3. Discussion

The extraction technique of stingray liver oil was not aimed at obtaining a high extraction yield but at the application of a method that preserves the bioactive principles of the lipid composition and does not contaminate the final lipid fraction with toxic solvents in the event that the oil is also used for oral administration. About 260 g of the final oily fraction was extracted from 500 g of stingray liver. The value of the peroxide index (Table 1) indicates reduced oxidative degradation during the extraction process. The main valuable components of the lipid fraction are unsaturated fatty acids (especially omega 3 and omega 6 essential fatty acids), fat-soluble vitamins (A, D, and E), compounds with increased therapeutic potential, and compounds that are easily oxidizable. That is why it is recommended to condition the oil in hermetically sealed brown glass bottles in a cool (8–15 °C) and dark place.

The results from the analysis of fatty acids in the composition of stingray liver oil indicate a predominance of unsaturated fatty acids (Figure 1), as well as a predominance of omega 3 fatty acids compared to omega 6 fatty acids (Table 2). As with other lipids of marine origin, such as cod liver oil, salmon liver oil, and total lipid extract from mussels, the results indicate an increased amount of polyunsaturated fatty acids and a super-unit ratio of omega 3 to omega 6 acids (characteristic of marine fats) [46,47].

Omega 3 fatty acids, which are anti-inflammatory, unsaturated fatty acids, and fat-soluble vitamins in the composition of the oil have an emollient, restructuring, and antioxidant effect on the epidermis. There are clinical studies that indicate fish oil generally has regenerative, anti-inflammatory, and protective effects on the epidermis. Positive effects on certain dermatological conditions (dermatitis, eczema) were noted in the case of marine lipids [48–51].

Treatment of animals with stingray liver oil healing ointment resulted in complete healing in all animals after 12 days of treatment (Figure 3). On the 6th day of treatment, the

primary crust formed in the region of the wounds began to detach. After eight days, the crust was completely detached in all treated animals, and the remaining wound was covered with fine granular tissue (Figure 4C). After 10 days, the healing finished almost completely without very visible signs. Through the complex chemical composition presented by the healing ointment with stingray liver oil, it heals wounds on the skin and reduces inflammation. Healing and regeneration of the damaged tissues were achieved quickly and almost completely after only 10 days without obvious signs.

The cicatrizing ointment with stingray liver oil used in the study was well tolerated by the skin and presented a cicatrizing action on the experimental lesions produced in rats that was more effective than that of the Cicatrizin® ointment taken as a reference. Cicatrizin® ointment was chosen because it has a complex composition and contains natural plant extracts that are recognized for their healing effects. In the case of animals treated with Cicatrizin® ointment, a fine, dry crust was formed, which completely detached after 10 days of treatment, and healing occurred after 13–14 days. After the regeneration process, obvious traces remained. In the control group, the healing of the wounds was much more difficult, and the remaining marks were much more obvious, with the tissue under the crust remaining inflamed for a long time. The evolution of the healing effect over time indicates significant differences between the groups studied since the first administrations (Figure 5); thus, after six days, the wounds of the group tested with ointment had healed by 63.05%, the control group had healed by 33.87%, and the reference group had healed by 56.51%. After ten days of treatment, the percentage of wound healing was 91.94% in the group tested with ointment, 78.16% in the reference group treated with Cicatrizin®, and only 56.32% in the untreated control group.

The rheological characteristics of emulgels made with stingray liver oil depend on the concentration of the lipid fraction emulsified in the gel base. The formula with the best rheological parameters and characteristics (spreading capacity, flow parameters, plasticity, uniformity of distribution of emulsified particles) is Formula C. That is why Formula C was chosen to test its anti-inflammatory action.

The stingray liver oil proved to have a significant anti-inflammatory effect with a maximum at six hours of treatment in the case of edemas induced by the 10% kaolin suspension (66.47%) compared to the Diclofenac gel chosen as a reference, which registered a maximum effect after four hours of treatment at 73.19% (Figure 10). In the case of edema induced by the 6% dextran solution (Figure 12), the maximum effect of the emulgel with stingray liver oil was recorded after 60 minutes of treatment (65.64%), while with the reference gel, the maximum effect was recorded after 30 minutes of treatment (75.37%).

Along with vegetable products that represent important sources of antioxidants and fibers [52,53], ingredients with beneficial effects in the metabolic syndrome and especially in cardiovascular diseases, marine lipids are also important sources of antioxidants and polyunsaturated fatty acids with beneficial effects in many diseases, including the metabolic syndrome and cardiovascular diseases [54,55]. As a result, it is recommended to capitalize on the therapeutic potential of marine lipids both in medicinal formulas for internal and external use.

4. Materials and Methods

4.1. Extraction of Black Sea Stingray (Dasyatis pastinaca) Liver Oil

The laboratory technology used for the extraction of stingray liver oil sought to obtain a high-quality oil without toxic impurities from potentially toxic solvents and without the use of high temperatures that degrade the valuable compounds in the oil (polyunsaturated fatty acids, fat-soluble vitamins, etc.).

Stingray liver taken from six wild specimens fished in the Black Sea in August 2022 was used for extraction (Figure 13). After washing, the liver was subjected to freezing at −20 °C. The frozen liver was minced and heated in a water bath. The obtained oily fraction was washed 2–3 times with warm distilled water using a separatory funnel, after which it was subjected to the fractionation operation by cooling to 2 °C when the saturated

triglyceride fraction settled (Figure 14). The liquid oily part was separated by decantation and filtration from the sedimented triglyceride fraction. The oil thus obtained was stored in the dark and at low temperatures (8–15 °C).

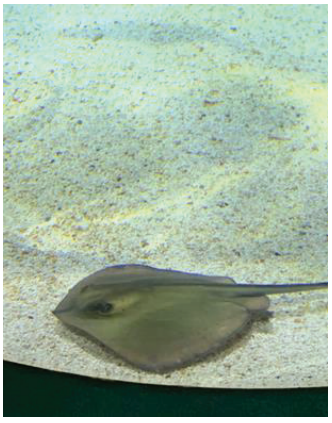


Figure 13. Black Sea common stingray (*Dasyatis pastinaca*).

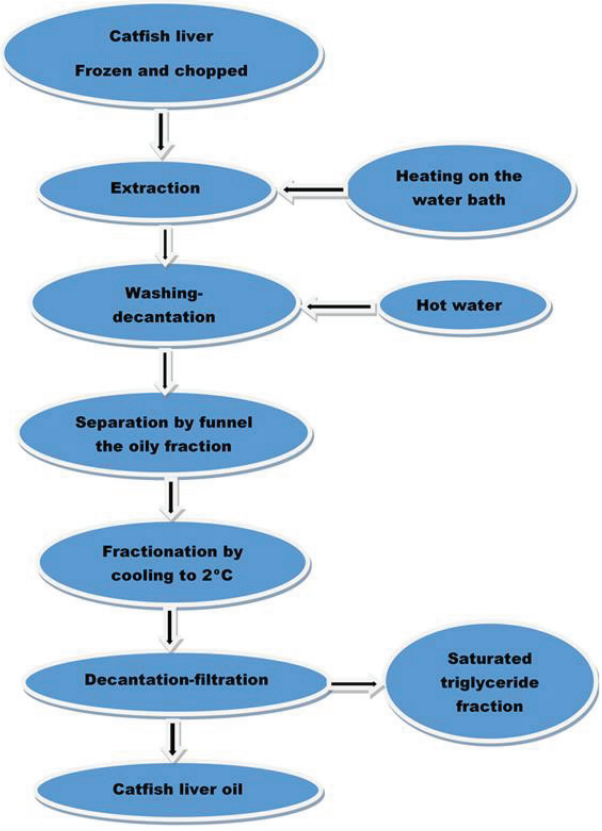


Figure 14. Technological scheme for obtaining stingray (catfish) liver oil.

4.2. Analysis of Black Sea Stingray (Dasyatis pastinaca) Liver Oil

The stingray liver oil was subjected to the following analyses: determination of the density at 20 °C, the refractive index, the acidity index, the iodine index, the saponification index, and the peroxide index according to the provisions of the Romanian Pharmacopoeia Edition X [56].

For the analysis of fatty acids from catfish liver oil, an internal standard (C 23:0 methyl ester; Nuchek Prep Inc., Elysian, M.N., USA) was added, and the mixture was dried under a nitrogen atmosphere and then subjected to hydrolysis using a 7.9% KOH solution in methanol. After cooling, the samples were treated with a 20% boron fluoride solution in methanol. Fatty acid methyl esters were detected with a gas chromatograph 17 Shimadzu GC (Kyoto, Japan) using helium as a carrier gas. The peak area was processed using Shimadzu Class GC-10 software (Version No., company name, Kyoto, Japan) [57].

All samples were tested three times, and the results were expressed as means ± SD (standard deviation). A standard mix of fatty acids and methyl esters (Nuchek Prep Inc., Elysian, MN, USA) was used to calibrate gas chromatography and determine response factors (Figure 15). All reagents used (Sigma-Aldrich, Schnelldorf, Germany) were of analytical purity.

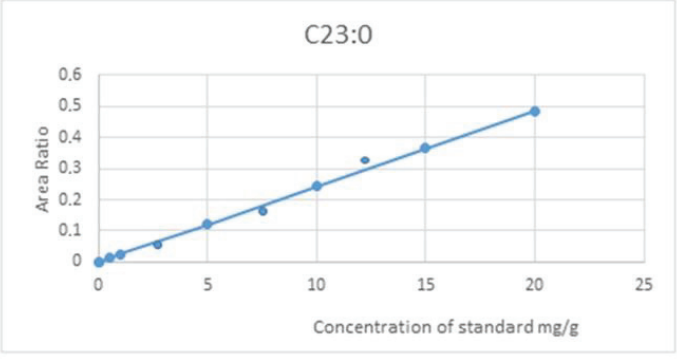


Figure 15. Calibration curve for the analysis of fatty acids in stingray liver oil (Y = 0.0243x + 0.0001).

4.3. Formulation of Ointment with Stingray (Dasyatis pastinaca) Liver Oil

The ointment with stingray liver oil was prepared according to the following formula: stingray liver oil (30 g), lanolin (15 g), and petroleum jelly (up to 100 g). The lanolin and petroleum jelly were liquefied in a water bath, then the stingray liver oil was added and homogenized until completely cooled.

Cosmetic lanolin was used (MAYAM brand). With an emollient and strong moisturizing action, active substances are transported into the skin to protect and repair dry, scaly, cracked, or itchy skin. This is a classic ingredient for pharmaceutical preparations, ointments, and traditional creams. Raw lanolin is anhydrous (does not contain water) and is obtained by processing sheep's wool. It is secreted by the sebaceous glands in the sheep's skin and is present on the wool threads. MAYAM lanolin is obtained from wool not affected by pesticides. The composition of petroleum jelly is a mixture of solid and liquid carbohydrates. Vaseline is obtained during the processing of petroleum fractions with a low boiling point, and its invention dates back to the middle of the 19th century. Petroleum jelly melts at 60 °C, dissolves in ether and chloroform, and is miscible with all oils except castor. At the same time, it does not dissolve in water or alcohol, so when applied to the skin, it washes off with difficulty. Cosmetic petroleum jelly is used in the manufacture of many ointments and creams. Cosmetic petroleum jelly from the Vaseline manufacturer was used for the preparation.

The ointment was subjected to additional analyses for characterization: appearance analysis, pH determination, thermal stability determination, viscosity determination, and spreadability determination.

The appearance was analyzed by a magnifying glass examination (4.5×) of a sample of ointment spread in a thin layer on a microscopic slide.

The determination of the pH was carried out after the processing of the preparations, namely the extraction of the samples with distilled water (1:5) and the measurement of the pH of the aqueous phase that separates after heating in a water bath at 60 °C and homogenization for 10 min. The Radelkis pH meter (Budapest, Hungary) was used to determine the pH.

The determination of the thermal stability was performed by keeping the samples in two temperature conditions (at 2 °C and at 40 °C for 8 h) as follows: 5 g of the sample from the ointment are introduced into weighing vials equipped with lids, and the vials are kept in the oven and in the refrigerator at the mentioned temperatures, after which the appearance of the samples, which must be kept homogeneous, is examined.

Viscosity was determined using a VEVOR NDJ-9S Digital Rotary Viscometer (Kansas City, USA).

The spreadability of the ointment was examined 28 h after preparation by measuring the spreading diameter of 1 g of sample placed between two 20 × 20 cm glass plates after 1 min using the Ojeda Arbussa method [58]. The upper plate mass was standardized at 125 g. The spreading areas reached by the sample were subsequently placed over the sample at 1 min intervals with weights of 50 g, 100 g, 150 g, 250 g, 500 g, and 750 g measured in millimeters. The determinations were repeated 30 days after the preparation of the ointment. The results were expressed in terms of the spreading area as a function of the applied mass according to the following equation:

$$S_i = d_i^2 \, (\pi/4) \quad (1)$$

in which:

- Si is the spreading area (mm^2) resulting from the applied mass "i" (g), and
- di is the mean diameter (mm) reached by the sample.

4.4. Testing the Healing Action of Stingray (Dasyatis pastinaca) Liver Oil Ointment

For this experiment, three groups of 10 male Wistar rats weighing 200 ± 10 g were used. The animals were kept in laboratory conditions for 2 days to get used to their new habitat (experimental room temperature of 22 ± 2 °C, humidity of 40–50%). The diet consisted of feeding at 8:00 and 17:00 and drinking water ad libitum from bottles.

Studies on laboratory animals were approved by the Scientific Research Ethics Commission of Carol Davila University of Medicine and Pharmacy, established under the Animal Protection (Code of Ethical Conduct 372/11.10.2022) Animal Welfare Act 1999 [59,60]. The clinical studies were carried out in compliance with the legislative norms.

The animals had shaved hair on their backs. After anesthesia with ether, wounds were produced by means of a device consisting of a metal disk with a diameter of 1 cm that was heated in water with 5% NaCl at 105 °C. The heated disk was applied to the dorsal shaved area and maintained for 10 s [61]. The animals were distributed by the randomization method into groups of 10 animals and were treated as follows:

Group 1—control group, untreated;

Group 2—the reference group treated with the cicatrizing ointment from the Romanian pharmaceutical market, Cicatrizin®, produced by the company Tis Farmaceutic S.A., which contains natural plant extracts in the composition (mallow, St. John's wort, chamomile, and calendula);

Group 3—the tested group treated with the stingray liver oil ointment.

The treatment was applied twice a day for 12 days. Wound development was observed every two days by measuring the treated areas (in mm^2) compared to those of the untreated

control group and the Cicatrizin®-treated group (reference group). During the study, the clinical condition of the animals was monitored.

The healing effect (E%) was calculated according to the following formula:

$$E\ \% = (A_i - A_{i+1}/A_i) \times 100 \quad (2)$$

where,

A_i—the average wound surface at the initial moment (i);

A_{i+1}—the average wound surface at the moment i + 1.

All determinations were performed in triplicate, and the results were expressed as mean ± SD (standard deviation). Statistical evaluation of clinical results was performed by Student's t test (t test) and analysis of variance (ANOVA) [62,63].

4.5. Formulation of Emulgels with Stingray (Dasyatis pastinaca) Liver Oil

Four emulgel formulas were prepared according to the composition shown in Table 7.

Table 7. Emulgel compositions.

Components	Formula A	Formula B	Formula C	Formula D
Carbopol 940	1 g	1 g	1 g	1 g
Glycerin	5 g	5 g	5 g	5 g
Triethanolamine	0.5 g	0.5 g	0.5 g	0.5 g
Tween 80	0.5 g	0.5 g	0.5 g	0.5 g
Stingray liver oil	3 g	5 g	7 g	10 g
Purified water	until 100 g	until 100 g	until 100 g	until 100 g

Carbopol 940 (Merck, Darmstadt, Germany) was hydrated using purified water (conductivity below 0.05 µS/cm; obtained in a system SGW Ultraclear UV PlusTM, Darmstadt, Germany) provided in the formula (Table 1) for at least 24 h in the presence of glycerin (purity over 99%; Merck, Germany) used as a dispersing agent. Triethanolamine (purity over 99%; Triethanolamine from Carl Roth GmbH + Co. K.G., Germany) was used for neutralization at the end of this interval. Catfish liver oil was emulsified in a gel base in the presence of the emulsifying agent Tween 80 (Merck, Darmstadt, Germany).

The prepared emulgels were subjected to analyses to assess their stability: appearance analysis, pH determination, thermal stability determination, viscosity determination, and spreadability determination, according to the techniques presented in the analysis of the previously prepared fatty ointment.

The rheological characteristics were analyzed using the Multi-Visc Rheometer rotational viscometer (Fungilab, Maharashtra, India) through stationary shear analysis at 33 °C ± 0.1 °C (close to skin temperature). Maintaining the temperature during the analysis was performed using a ThermoHaake P5 Ultrathermostat (Huston, USA). The three emulgel samples taken in the analysis were sheared at a shear rate specific to the TR 10 standard spindle, from 0.08 to 16.8 s^{-1}, corresponding to a rotational speed between 0.3 and 60 rpm. Upward rheograms of shear stress as a function of shear rate were obtained. The rheological data were further analyzed by applying different shear stress (τ) versus shear rate ($\dot{\gamma}$) models as follows: Casson (Equation (3)) and Herschel-Bulkley (Equation (4)):

$$\tau^{0.5} = {\tau_0}^{0.5} + \eta^{0.5} \cdot \dot{\gamma}^{0.5} \quad (3)$$

$$\tau = \tau_0 + K \cdot \dot{\gamma}^{n} \quad (4)$$

where,

η is plastic viscosity (Pa·s), τ_0 is yield stress (Pa), K is consistency index (Pa·s^n), and n is flow index (dimensionless) [64,65]. The flow parameters were determined using table Curve 2D software (Version No.5.01, Systat Software GmbH, Erkrath, Germany).

For internal morphology analysis, the emulgel samples were measured using a scanning electron microscope (SEM, Thermo Fisher Scientific, GmbH, Dreieich, Germany). SEM images were obtained using electron beam scanning at an accelerating voltage of 15 KV and a magnification of 1000×.

4.6. Testing the Anti-Inflammatory Action of Stingray (Dasyatis pastinaca) Liver Oil Emulgel

For this experiment, six groups of 10 male Wistar rats weighing 230 ± 15 g were used. The animals were kept in laboratory conditions for 2 days to get used to their new habitat (experimental room temperature of 22 ± 2 °C, humidity of 40–50%). The diet consisted of feeding at 8:00 and 17:00 and drinking water ad libitum from bottles.

The tests were carried out using two experimental methods of acute inflammation: the edema induced in the rat's paw with a 10% kaolin suspension and the one with a 6% dextran solution.

By injecting kaolin into the rat's paw, the formation of prostaglandins is stimulated, causing local inflammation and edema [66]. Dextran-induced edema is mainly due to the release of histamine and serotonin and is called anaphylactoid edema.

Edema was induced by the intra-plantar injection of 0.1 mL of 10% kaolin suspension and 0.2 mL of dextran solution.

For each edematous agent, three groups of 10 male Wistar rats were used. One group constituted the control group; one group was treated with the stingray liver oil emulgel formula C (the best formula according to the rheological evaluations); and one group was treated with Diclofenac gel (10 mg/g) produced by the Fiterman company. All animals were administered the edematous agent. On the paw in which the edema was induced, the test preparation was applied uniformly in a thin layer of ~0.25 g gel.

Group 1—control group, untreated;

Group 2—the reference group treated with Diclofenac gel (10 mg/g) produced by the Fiterman company;

Group 3—the tested group treated with the catfish liver oil emulgel (Figure 16).

Figure 16. Stingray liver oil emulgel (Formula C).

The evaluation of the anti-inflammatory effect of the ointment taken in the study was compared to the preparation of Diclofenac gel (10 mg/g produced by the Fiterman company) existing on the Romanian pharmaceutical market, applied to the paw with edema under the same conditions previously detailed.

The determinations were made against control groups (untreated individuals).

The volume of the rat's paw was measured with a plethysmometer, Ugo Basile 7140 (Gemonio, Italy). After the intra-plantar injection of the edematous agent, further measurements were performed at intervals of 2 h, 4 h, 6 h, and 24 h (for the edematous agent, 10% kaolin suspension) and at intervals of 30 min, 60 min, 90 min, and 120 min from the induction of edema (for the edematous agent, 6% dextran solution).

The average value of anti-inflammatory edema (expressed in mL) and the percentage of edema inhibition effect for each batch were calculated according to the formula:

$$\text{Edema inhibition effect (I)} \% = (\text{X control} - \text{X treatment agent/X control}) \times 100 \quad (5)$$

where,

X treatment agent represents the average value of the edema produced by the tested gel (Diclofenac gel or emulgel Formula C);

X control represents the average value of the edema produced in the control group in the same time interval after the administration of the edematous agent.

5. Conclusions

Stingray liver oil obtained through a process that does not alter the bioactive compounds in its composition represents a resource with significant therapeutic potential in the context of its capitalization as a by-product obtained from catfish breeding farms that pursue the exploitation of the skin of the stingray in the leather goods industry. Similar to all lipids of marine origin, stingray liver oil is an important source of polyunsaturated fatty acids and can be used in the development of therapeutic formulas for internal or external use. Having a significant healing and anti-inflammatory action and a composition rich in valuable nutritional principles, stingray liver oil represents a valuable resource for the dermato-cosmetics industry of bioactive natural products. Clinical studies indicate an increased therapeutic potential of stingray liver oil and good versatility for its use in various dermato-cosmetic preparations.

Author Contributions: Conceptualization, M.M., M.L. and C.E.L.; methodology, S.M.N., G.O. and S.G.; software, Ș.S.B. and M.V.C.; validation, S.M.N., G.O. and S.G.; formal analysis, M.M., M.L., C.E.L. and L.H.; investigation, D.D., C.-N.O., M.V.C. and D.L.; resources, Ș.S.B., D.L. and C.-N.O.; data curation, L.H.; writing—original draft preparation, S.M.N., G.O. and S.G.; writing—review and editing, S.M.N., G.O. and S.G.; visualization, D.D.; supervision, D.L.; project administration, M.M., M.L. and C.E.L.; funding acquisition, L.H. and Ș.S.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: The animal study protocol was approved by the Scientific Research Ethics Commission of Carol Davila University of Medicine and Pharmacy (protocol code 372 and date of approval 11 October 2022).

Data Availability Statement: There are no data available for this publication.

Acknowledgments: Publication of this paper was supported by the University of Medicine and Pharmacy Carol Davila, through the institutional program Publish not Perish.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bouchaâla, E.; BouAli, M.; Ben Ali, Y.; Miled, N.; Gargouri, Y.; Fendri, A. Biochemical Characterization and Molecular Modeling of Pancreatic Lipase from a Cartilaginous Fish, the Common Stingray (*Dasyatis pastinaca*). *Appl. Biochem. Biotechnol.* **2015**, *176*, 151–169. [CrossRef] [PubMed]
2. Sathivel, S. Production, Process Design and Quality Characterization of Catfish Visceral Oil. Ph. D. Thesis, Louisiana State University and Agricultural & Mechanical College, Baton Rouge, LA, USA, 2001. Available online: https://digitalcommons.lsu.edu/gradschool_disstheses/434 (accessed on 15 March 2023).
3. Sathivel, S.; Yin, H.; Prinyawiwatkul, W.; King, J.M. Comparison of chemical and physical properties of catfish oils prepared from different extraction processes. *J. Food Sci.* **2009**, *74*, E70–E76. [CrossRef] [PubMed]
4. Sathivel, S.; Prinyawiwatkul, W.; King, J.M.; Grimm, C.C.; Lloyd, S. Oil production from catfish viscera. *J. Am. Oil Chem. Soc.* **2003**, *80*, 377–382. [CrossRef]
5. Toti, E.; Oliver Chen, C.Y.; Palmery, M.; Valencia, D.V.; Peluso, I. Non-Provitamin A and Provitamin A Carotenoids as Immunomodulators: Recommended Dietary Allowance, Therapeutic Index, or Personalized Nutrition? *Oxidative Med. Cell. Longev.* **2018**, *2018*, 4637861. [CrossRef]
6. Yin, K.; Agrawal, D.K. Vitamin D and Inflammatory Diseases. *J. Inflamm. Res.* **2014**, *7*, 69. [CrossRef]
7. Veldurthy, V.; Wei, R.; Oz, L.; Dhawan, P.; Jeon, Y.H.; Christakos, S. Vitamin D, Calcium Homeostasis and Aging. *Bone Res.* **2016**, *4*, 16041. [CrossRef]
8. Gruenwald, J.; Graubaum, H.J.; Harde, A. Effect of Cod Liver Oil on Symptoms of Rheumatoid Arthritis. *Adv. Ther.* **2002**, *19*, 101–107. [CrossRef]

9. Galarraga, B.; Ho, M.; Youssef, H.M.; Hill, A.; McMahon, H.; Hall, C.; Ogston, S.; Nuki, G.; Belch, J.J.F. Cod Liver Oil (n-3 Fatty Acids) as an Non-Steroidal Anti-Inflammatory Drug Sparing Agent in Rheumatoid Arthritis. *Rheumatology* **2008**, *47*, 665–669. [CrossRef]
10. Baxter-Jones, A.D.G.; Faulkner, R.A.; Forwood, M.R.; Mirwald, R.L.; Bailey, D.A. Bone Mineral Accrual from 8 to 30 Years of Age: An Estimation of Peak Bone Mass. *J. Bone Miner. Res.* **2011**, *26*, 1729–1739. [CrossRef]
11. Downie, L.E.; Vingrys, A.J. Oral Omega-3 Supplementation Lowers Intraocular Pressure in Normotensive Adults. *Transl. Vis. Sci. Technol.* **2018**, *7*, 1. [CrossRef]
12. Cartolano, F.D.C.; Dias, G.D.; Miyamoto, S.; Damasceno, N.R.T. Omega-3 Fatty Acids Improve Functionality of High-Density Lipoprotein in Individuals with High Cardiovascular Risk: A Randomized, Parallel, Controlled and Double-Blind Clinical Trial. *Front. Nutr.* **2022**, *8*, 767535. [CrossRef] [PubMed]
13. Ravitchandirane, V.; Yogamoorthi, A. Studies on the Analgesic and Anti-Inflammatoryproperties of Crude Extracts of Sting Ray, *Dasyatis zugei* (Muller and Henle 1841). *Biosci. Biotechnol. Res. Asia* **2008**, *5*, 343–348.
14. Ravitchandirane, V.; Yogamoorthi, A.; Thangaraj, M. Pharmacological Investigation and Spectral Characterization of Bioactive Compounds from Crude Extracts of Sting Ray, *Dasyatis jenkinsii* (Annandale, 1909). *Chin. J. Nat. Med.* **2014**, *11*, 500–505. [CrossRef] [PubMed]
15. Pan American Leathers, Inc. Stingray Skin Guide. Available online: www.panamleathers.com (accessed on 7 February 2023).
16. Mititelu, M.; Stanciu, G.; Drăgănescu, D.; Ioniță, A.C.; Neacșu, S.M.; Dinu, M.; Stefanvan Staden, R.-I.; Moroșan, E. Mussel Shells, a Valuable Calcium Resource for the Pharmaceutical Industry. *Mar. Drugs* **2022**, *20*, 25. [CrossRef]
17. Mititelu, M.; Moroșan, E.; Nicoară, A.C.; Secăreanu, A.A.; Musuc, A.M.; Atkinson, I.; Cusu, J.P.; Nițulescu, G.M.; Ozon, E.A.; Sarbu, I.; et al. Development of immediate release tablets containing calcium lactate synthetized from Black Sea mussel shells. *Mar. Drugs* **2022**, *20*, 45. [CrossRef]
18. Mititelu, M.; Ioniţă, A.C.; Moroşan, E. Research regarding integral processing of mussels from Black Sea. *Farmacia* **2014**, *62*, 625–632.
19. Rodríguez-Cruz, M.; Serna, D.S. Nutrigenomics of ω-3 Fatty Acids: Regulators of the Master Transcription Factors. *Nutrition* **2017**, *41*, 90–96. [CrossRef]
20. Fodor, J.G.; Helis, E.; Yazdekhasti, N.; Vohnout, B. "Fishing" for the Origins of the "Eskimos and Heart Disease" Story: Facts or Wishful Thinking? *Can. J. Cardiol.* **2014**, *30*, 864–868. [CrossRef]
21. Gahche, J.J.; Bailey, R.L.; Potischman, N.; Dwyer, J.T. Dietary Supplement Use Was Very High among Older Adults in the United States in 2011–2014. *J. Nutr.* **2017**, *147*, 1968–1976. [CrossRef]
22. Villani, A.M.; Crotty, M.; Cleland, L.G.; James, M.J.; Fraser, R.J.; Cobiac, L.; Miller, M.D. Fish Oil Administration in Older Adults: Is There Potential for Adverse Events? A Systematic Review of the Literature. *BMC Geriatr.* **2013**, *13*, 41. [CrossRef]
23. Hardy, M.S.; Kekic, A.; Graybill, N.L.; Lancaster, Z.R. A Systematic Review of the Association between Fish Oil Supplementation and the Development of Asthma Exacerbations. *SAGE Open Med.* **2016**, *4*. [CrossRef]
24. Balk, E.M.; Lichtenstein, A.H. Omega-3 Fatty Acids and Cardiovascular Disease: Summary of the 2016 Agency of Healthcare Research and Quality Evidence Review. *Nutrients* **2017**, *9*, 865. [CrossRef]
25. Eslick, G.D.; Howe, P.R.C.; Smith, C.; Priest, R.; Bensoussan, A. Benefits of Fish Oil Supplementation in Hyperlipidemia: A Systematic Review and Meta-Analysis. *Int. J. Cardiol.* **2009**, *136*, 4–16. [CrossRef]
26. Porojnicu, A.C.; Bruland, Ø.S.; Aksnes, L.; Grant, W.B.; Moan, J. Sun Beds and Cod Liver Oil as Vitamin D Sources. *J. Photochem. Photobiol. B* **2008**, *91*, 125–131. [CrossRef]
27. Chee, K.M.; Gong, J.X.; Good Rees, D.M.; Meydanl, M.; Ausman, L.; Johnson, J.; Siguel, E.N.; Schaefer, E.J. Fatty Acid Content of Marine Oil Capsules. *Lipids* **1990**, *25*, 523–528. [CrossRef]
28. Breiden, B.; Sandhoff, K. The Role of Sphingolipid Metabolism in Cutaneous Permeability Barrier Formation. *Biochim. Biophys. Acta* **2014**, *1841*, 441–452. [CrossRef]
29. Huang, T.H.; Wang, P.W.; Yang, S.C.; Chou, W.L.; Fang, J.Y. Cosmetic and Therapeutic Applications of Fish Oil's Fatty Acids on the Skin. *Mar. Drugs* **2018**, *16*, 256. [CrossRef]
30. Baum, C.L.; Arpey, C.J. Normal Cutaneous Wound Healing: Clinical Correlation with Cellular and Molecular Events. *Dermatol. Surg.* **2005**, *31*, 674–686. [CrossRef]
31. Calder, P.C. Omega-3 Polyunsaturated Fatty Acids and Inflammatory Processes: Nutrition or Pharmacology? *Br. J. Clin. Pharmacol.* **2013**, *75*, 645–662. [CrossRef]
32. Kiecolt-Glaser, J.K.; Glaser, R.; Christian, L.M. Omega-3 Fatty Acids and Stress-Induced Immune Dysregulation: Implications for Wound Healing. *Mil. Med.* **2014**, *179*, 129–133. [CrossRef]
33. Shingel, K.I.; Faure, M.P.; Azoulay, L.; Roberge, C.; Deckelbaum, R.J. Solid Emulsion Gel as a Vehicle for Delivery of Polyunsaturated Fatty Acids: Implications for Tissue Repair, Dermal Angiogenesis and Wound Healing. *J. Tissue Eng. Regen. Med.* **2008**, *2*, 383–393. [CrossRef]
34. Arantes, E.L.; Dragano, N.; Ramalho, A.; Vitorino, D.; de-Souza, G.F.; Lima, M.H.M.; Velloso, L.A.; Araújo, E.P. Topical Docosahexaenoic Acid (DHA) Accelerates Skin Wound Healing in Rats and Activates GPR120. *Biol. Res. Nurs.* **2016**, *18*, 411–419. [CrossRef] [PubMed]
35. Wu, D.C.; Goldman, M.P. A Topical Anti-Inflammatory Healing Regimen Utilizing Conjugated Linolenic Acid for Use Post-Ablative Laser Resurfacing of the Face: A Randomized, Controlled Trial. *J. Clin. Aesthetic Dermatol.* **2017**, *10*, 12–17.

36. Komprda, T.; Sládek, Z.; Vícenová, M.; Simonová, J.; Franke, G.; Lipový, B.; Matejovičová, M.; Kacvinská, K.; Sabliov, C.; Astete, C.E.; et al. Effect of Polymeric Nanoparticles with Entrapped Fish Oil or Mupirocin on Skin Wound Healing Using a Porcine Model. *Int. J. Mol. Sci.* **2022**, *23*, 7663. [CrossRef] [PubMed]
37. Rodrigues, H.G.; Vinolo, M.A.R.; Sato, F.T.; Magdalon, J.; Kuhl, C.M.C.; Yamagata, A.S.; Pessoa, A.F.M.; Malheiros, G.; dos Santos, M.F.; Lima, C.; et al. Oral Administration of Linoleic Acid Induces New Vessel Formation and Improves Skin Wound Healing in Diabetic Rats. *PLoS ONE* **2016**, *11*, e0165115. [CrossRef]
38. Pereira, L.M.; Hatanaka, E.; Martins, E.F.; Oliveira, F.; Liberti, E.A.; Farsky, S.H.; Curi, R.; Pithon-Curi, T.C. Effect of Oleic and Linoleic Acids on the Inflammatory Phase of Wound Healing in Rats. *Cell Biochem. Funct.* **2008**, *26*, 197–204. [CrossRef]
39. Cardoso, C.R.B.; Souza, M.A.; Ferro, E.A.V.; Favoreto, S.; Pena, J.D.O. Influence of Topical Administration of N-3 and n-6 Essential and n-9 Nonessential Fatty Acids on the Healing of Cutaneous Wounds. *Wound Repair Regen.* **2004**, *12*, 235–243. [CrossRef]
40. Lu, Y.; Tian, H.; Hong, S. Novel 14,21-Dihydroxy-Docosahexaenoic Acids: Structures, Formation Pathways, and Enhancement of Wound Healing. *J. Lipid Res.* **2010**, *51*, 923–932. [CrossRef]
41. Tian, H.; Yao, X.; Zeng, R.; Sun, R.; Tian, H.; Shi, C.; Li, L.; Tian, J.; Yang, K. Safety and Efficacy of a New Parenteral Lipid Emulsion (SMOF) for Surgical Patients: A Systematic Review and Meta-Analysis of Randomized Controlled Trials. *Nutr. Rev.* **2013**, *71*, 815–821. [CrossRef]
42. Peng, Y.C.; Yang, F.L.; Subeq, Y.M.; Tien, C.C.; Chao, Y.F.C.; Lee, R.P. Lipid Emulsion Enriched in Omega-3 PUFA Accelerates Wound Healing: A Placebo-Controlled Animal Study. *World J. Surg.* **2018**, *42*, 1714–1720. [CrossRef]
43. Mititelu, M.; Moroşan, E.; Neacsu, S.M.; Ioniţă, E.I. Research regarding the pollution degree from romanian Black Sea coast. *Farmacia* **2018**, *66*, 1059–1063. [CrossRef]
44. Ioniţă, A.C.; Mititelu, M.; Moroşan, E. Analysis of heavy metals and organic pollutants from some Danube river fishes. *Farmacia* **2014**, *62*, 299–305.
45. Mititelu, M.; Nicolescu, T.O.; Ioniţă, C.A.; Nicolescu, F. Study of Heavy Metals and Organic Polluants from Some Fisches of Danube River. *J. Environ. Prot. Ecol.* **2012**, *13*, 869–874.
46. Jacobsen, C.; Warncke, S.A.; Hansen, S.H.; Sørensen, A.-D.M. Fish Liver Discards as a Source of Long-Chain Omega-3 Polyunsaturated Fatty Acids. *Foods* **2022**, *11*, 905. [CrossRef]
47. Malcorps, W.; Newton, R.W.; Sprague, M.; Glencross, B.D.; Little, D.C. Nutritional Characterisation of European Aquaculture Processing By-Products to Facilitate Strategic Utilisation. *Front. Sustain. Food Syst.* **2021**, *5*, 720595. [CrossRef]
48. Mititelu, M.; Udeanu, D.I.; Nedelescu, M.; Neacsu, S.M.; Nicoară, A.C.; Oprea, E.; Ghica, M. Quality Control of Different Types of Honey and Propolis Collected from Romanian Accredited Beekeepers and Consumer's Risk Assessment. *Crystals* **2022**, *12*, 87. [CrossRef]
49. Sawada, Y.; Saito-Sasaki, N.; Nakamura, M. Omega 3 Fatty Acid and Skin Diseases. *Front. Immunol.* **2021**, *11*, 623052. [CrossRef]
50. Stupin, A.; Mihalj, M.; Kolobarić, N.; Šušnjara, P.; Kolar, L.; Mihaljević, Z.; Matić, A.; Stupin, M.; Jukić, I.; Kralik, Z.; et al. Anti-Inflammatory Potential of n-3 Polyunsaturated Fatty Acids Enriched Hen Eggs Consumption in Improving Microvascular Endothelial Function of Healthy Individuals—Clinical Trial. *Int. J. Mol. Sci.* **2020**, *21*, 4149. [CrossRef]
51. Paduraru, D.N.; Coman, F.; Ozon, E.A.; Gherghiceanu, F.; Andronic, O.; Ion, D.; Stanescu, M.; Bolocan, A. The use of nutritional supplement in romanian patients—Attitudes and beliefs. *Farmacia* **2019**, *67*, 1060–1065. [CrossRef]
52. Ioniță-Mîndrican, C.-B.; Ziani, K.; Mititelu, M.; Oprea, E.; Neacșu, S.M.; Moroșan, E.; Dumitrescu, D.-E.; Roșca, A.C.; Drăgănescu, D.; Negrei, C. Therapeutic Benefits and Dietary Restrictions of Fiber Intake: A State of the Art Review. *Nutrients* **2022**, *14*, 2641. [CrossRef]
53. Stoicescu, I.; Lupu, E.C.; Radu, M.D.; Popescu, A.; Mihai, S. High-Performance Liquid Chromatography—Diode Array Detection (HPLC-DAD) Method for the Determination of Phenolic Compounds of Water Chesnut (*Trapa natans* L.). *Anal. Lett.* **2022**, *55*, 2147–2159. [CrossRef]
54. Méndez, L.; Dasilva, G.; Taltavull, N.; Romeu, M.; Medina, I. Marine Lipids on Cardiovascular Diseases and Other Chronic Diseases Induced by Diet: An Insight Provided by Proteomics and Lipidomics. *Mar. Drugs* **2017**, *15*, 258. [CrossRef]
55. Carrera, I.; Corzo, L.; Naidoo, V.; Martínez-Iglesias, O.; Cacabelos, R. Cardiovascular and lipid-lowering effects of a marine lipoprotein extract in a high-fat diet-induced obesity mouse model. *Int. J. Med. Sci.* **2023**, *20*, 292–306. [CrossRef]
56. Mititelu, M.; Neacsu, S.M.; Oprea, E.; Dumitrescu, D.-E.; Nedelescu, M.; Drăgănescu, D.; Nicolescu, T.O.; Rosca, A.C.; Ghica, M. Black Sea Mussels Qualitative and Quantitative Chemical Analysis: Nutritional Benefits and Possible Risks through Consumption. *Nutrients* **2022**, *14*, 964. [CrossRef]
57. National Drug Agency. *Romanian Pharmacopoeia*, 10th ed.; Editura Medicală: Bucharest, Romania, 1993; pp. 67–68, 335, 419–421, 921–922.
58. Popovici, I.; Lupuleasa, D. *Tehnologie Farmaceutică*, 2nd ed.; Polirom: Iaşi, Romania, 2017; Volume 2, ISBN 978-973-46-6920-2.
59. AVMA. *Guidelines for the Euthanasia of Animals*; American Veterinary Medical Association: Schaumburg, IL, USA, 2013.
60. OECD. Guidelines for Testing of Chemicals. In *Acute Oral Toxicities Up and Down Procedure*; OECD: Paris, France, 2001; Volume 425, pp. 1–26.
61. Mihele, D.; Manea, S.; Raiciu, D.; Udeanu, D. The cicatrizing action of some new gemmotherapy products. *Farmacia* **2006**, *6*, 24–28.
62. Mishra, P.; Singh, U.; Pandey, C.M.; Mishra, P.; Pandey, G. Application of student's t-test, analysis of variance, and covariance. *Ann. Card. Anaesth.* **2019**, *22*, 407–411. [CrossRef]

63. Leahu, A.; Lupu, E.C. Statistical simulation and prediction in software reliability. *Analele Univ. Ovidius Constanţa Ser. Mat.* **2008**, *16*, 81–90.
64. Atyim, P.; Olah, N.K.; Osser, G.; Toma, C.C.; Morgovan, C.; Atyim, E. Practical Investigation of Gels Containing Aristolochia (*Aristolochia clematitis*) Extract. *Stud. UBB Chem.* **2017**, *62*, 153–164. [CrossRef]
65. Puri, A.; Nguyen, H.X.; Banga, A.K. Microneedle-mediated intradermal delivery of epigallocatechin-3-gallate. *Int. J. Cosmet. Sci.* **2016**, *38*, 512–523. [CrossRef]
66. Castardo, J.C.; Prudente, A.S.; Ferreira, J.; Guimarães, C.L.; Monache, F.D.; Filho, V.C.; Otuki, M.F.; Cabrini, D.A. Anti-inflammatory effects of hydroalcoholic extract and two biflavonoids from *Garcinia gardneriana* leaves in mouse paw oedema. *J. Ethnopharmacol.* **2008**, *118*, 405–411. [CrossRef]

marine drugs

MDPI

Article

Growth Behavior, Biomass Composition and Fatty Acid Methyl Esters (FAMEs) Production Potential of *Chlamydomonas reinhardtii*, and *Chlorella vulgaris* Cultures

Itzel Y. López-Pacheco [1,2], Victoria Guadalupe Ayala-Moreno [3], Catherinne Arlette Mejia-Melara [3], José Rodríguez-Rodríguez [1], Sara P. Cuellar-Bermudez [1,2], Reyna Berenice González-González [1,2], Karina G. Coronado-Apodaca [1,2], Leonardo I. Farfan-Cabrera [1], Georgia María González-Meza [1,2], Hafiz M. N. Iqbal [1,2,*] and Roberto Parra-Saldívar [1,2,*]

1 Tecnologico de Monterrey, School of Engineering and Sciences, Monterrey 64849, Mexico; a00824134@tec.mx (I.Y.L.-P.); jrr@tec.mx (J.R.-R.); sara.cuellar@tec.mx (S.P.C.-B.); reyna.g@tec.mx (R.B.G.-G.); karina.coronado@tec.mx (K.G.C.-A.); farfanl@tec.mx (L.I.F.-C.); georgia.gonzalez@tec.mx (G.M.G.-M.)
2 Tecnologico de Monterrey, Institute of Advanced Materials for Sustainable Manufacturing, Monterrey 64849, Mexico
3 Francisco Morazán Department, Escuela Agrícola Panamericana, Zamorano, Km 30 Carretera de Tegucigalpa a Danlí, Valle del Yeguare, Municipio de San Antonio de Oriente, Tegucigalpa 11101, Honduras; victoria04ayala@outlook.com (V.G.A.-M.); kathy.11mejia@gmail.com (C.A.M.-M.)
* Correspondence: hafiz.iqbal@tec.mx (H.M.N.I.); r.parra@tec.mx (R.P.-S.)

Abstract: The production of biomolecules by microalgae has a wide range of applications in the development of various materials and products, such as biodiesel, food supplements, and cosmetics. Microalgae biomass can be produced using waste and in a smaller space than other types of crops (e.g., soja, corn), which shows microalgae's great potential as a source of biomass. Among the produced biomolecules of greatest interest are carbohydrates, proteins, lipids, and fatty acids. In this study, the production of these biomolecules was determined in two strains of microalgae (*Chlamydomonas reinhardtii* and *Chlorella vulgaris*) when exposed to different concentrations of nitrogen, phosphorus, and sulfur. Results show a significant microalgal growth (3.69 g L^{-1}) and carbohydrates (163 mg g^{-1}) increase in *C. reinhardtii* under low nitrogen concentration. Also, higher lipids content was produced under low sulfur concentration (246 mg g^{-1}). It was observed that sulfur variation could affect in a negative way proteins production in *C. reinhardtii* culture. In the case of *C. vulgaris*, a higher biomass production was obtained in the standard culture medium (1.37 g L^{-1}), and under a low-phosphorus condition, *C. vulgaris* produced a higher lipids concentration (248 mg g^{-1}). It was observed that a low concentration of nitrogen had a better effect on the accumulation of fatty acid methyl esters (FAMEs) (C16-C18) in both microalgae. These results lead us to visualize the effects that the variation in macronutrients can have on the growth of microalgae and their possible utility for the production of microalgae-based subproducts.

Keywords: microalgae; fatty acid methyl esters; proteins; biorefinery

Citation: López-Pacheco, I.Y.; Ayala-Moreno, V.G.; Mejia-Melara, C.A.; Rodríguez-Rodríguez, J.; Cuellar-Bermudez, S.P.; González-González, R.B.; Coronado-Apodaca, K.G.; Farfan-Cabrera, L.I.; González-Meza, G.M.; Iqbal, H.M.N.; et al. Growth Behavior, Biomass Composition and Fatty Acid Methyl Esters (FAMEs) Production Potential of *Chlamydomonas reinhardtii*, and *Chlorella vulgaris* Cultures. *Mar. Drugs* **2023**, *21*, 450. https://doi.org/10.3390/md21080450

Academic Editors: Giuseppina Tommonaro and Annabella Tramice

Received: 17 June 2023
Revised: 2 August 2023
Accepted: 8 August 2023
Published: 15 August 2023

1. Introduction

Microalgae biomass can be useful for the production of many materials, including bioplastics, food supplements, biofuels, UV-blockers, antimicrobial bio-compounds, and animal feed, among others [1]. In general, microalgae biomass has been reported to produce and accumulate carbohydrates, proteins, lipids, and fatty acids (FAs) [2], which makes it an interesting source of renewable raw material for a wide range of applications. Moreover, microalgae are considered a sustainable source of materials, since these microorganisms can be applied in bioremediation processes and convert waste into value-added products in a circular economy scheme [3]. For example, microalgae have been employed to reduce CO_2 emissions via bio-fixation processes because they can capture CO_2 through

the photosynthetic pathway. In this process, microalgae use CO_2 from the atmosphere or other sources like flue gas streams for biomass production; it has been determined that producing 1 g of microalgae biomass represents the capture of 1.8 g of CO_2 [4–6]. Thus, environmental benefits are also involved with the use of microalgae biomass as feedstock for some processes.

Another bioremediation application is the use of microalgae for wastewater treatment processes. In this respect, researchers have reported the removal of water pollutants, including up to 90% of COD, 100% nitrogen, and 50% phosphorous, producing not only good-quality effluents but also biomass that is able to be employed for the production of biofuels, bioplastic, and food sources, among other applications [7]. For example, *Chlorella* biomass from a swine wastewater phycoremediation process exhibited higher production of lipids—including triacylglycerol—in comparison to that obtained in the standard medium [8]; these lipids can be used in bioplastic production.

Microalgae use depends on its biomass composition in terms of macromolecules (proteins, lipids, and carbohydrates) and active compounds, which in turn depends on microalgae strains and microalgae culture conditions [9]. In the case of carbohydrate content, some microalgae strains can accumulate >25% dry wt of carbohydrates such as *Scenedesmus*, *Dunaliella*, and *Chlorella* (Chlorophyta) [10]. In the case of proteins, *Galdieria sulphuraria* (Rhodophyta, Cyanidiophyceae) can accumulate >60% dry wt [11], while *Chlorella vulgaris* can accumulate 48% dry wt [12]. Regarding lipids, microalgae can produce a yield per hectare that is at least 7 times higher than other oil crops (i.e., soybean, sunflower, corn) [13]. *Chlorella* sp. (Chlorophyta), *Phaeodactylum tricornutum* (Bacillariophyta), and *Botryococcus braunii* (Chlorophyta) can produce >20% dry wt of lipids [14,15].

Triacylglycerols are non-polar lipids formed by three fatty acids esterified with glycerol. They are common molecules for energy storage in microalgae cells and the most common dietary fat for some organisms, such as humans [16]. Different FAs in microalgae have been identified, such as short-chain fatty acids, medium-chain fatty acids, and long-chain fatty acids. Palmitic (C16:0), stearic (C18:0), palmitoleic (C16:1), oleic (C18:1), linoleic (C18:2), and linolenic (C18:3) acids are examples of long-chain fatty acids found in microalgae [17]. Also, polyunsaturated fatty acids (PUFAs) have been found, including docosapentaenoic acid (DPA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), which are very important compounds in the food industry [18]. Microalgae biomass, especially its FAs content, has demonstrated potential for biodiesel production, and particular varieties can be useful in determining possible biodiesel characteristics [19] but also for bio-based lubricants [20], the latter being more sought and valued in recent decades.

In this manner, several applications exist for microalgae biomass and different biomolecules. For example, carbohydrates can be used to produce bioethanol, biobutanol, biomethane, bioplastics, food supplements, animal feed, and fertilizers [21–23]. Proteins can be used in human food, protein supplements [24], and stabilizers for food, cosmetic, and pharmaceutical products [25]. Lipids can be used for bioenergy, since the fatty acids in microalgal biomass produce high-quality biodiesel [26]. Also, lipids can act as surfactants or emulsifiers in cosmetics to provide additional properties. For example, adding PUFAs in cosmetic formulations provides anti-inflammatory and antioxidant properties [27]. Recently, the use of microalgae for the obtention of lubricant oils such as metalwork fluids has been explored. For this application, microalgae should produce long-carbon-chain ($C > 16$) FAs [28]. Moreover, microalgae cultures have been considered as a viable option for such macromolecules, since their production can be more sustainable [29].

In this context, microalgae biomass can be used to obtain specific compounds according to the desired application. Thus, different strategies have been investigated to obtain higher yields of certain biomolecules in microalgae biomass. For example, some researchers have evaluated pH variations [30], different carbon sources [31], variations in culture medium composition [32], and different photoperiods and light intensity modifications [33]. A simple technique consists of modifying the culture medium by reducing the supply of some macroelements; typically, nitrogen and phosphorus are the elements that are most

commonly varied, causing an increment in carbohydrates, proteins, and lipids. This strategy has demonstrated good potential; thus, varying other elements like sulfur should be further investigated (as they are in this article). This study evaluated the growth and biomass composition of two microalgae strains under different macronutrient concentrations. It was desirable to carry out this experimentation because in most articles, these macroelements (nitrogen, phosphorus, and sulfur) do not vary in the same microalga. Therefore, this study shows that the variation in these macroelements can impact the production of macromolecules of interest, which could be interesting for the industrial sector. The total carbohydrate, protein, and lipid production from *Chlamydomonas reinhardtii* and *Chlorella vulgaris* (Chlorophyta) was determined in response to different nitrogen, phosphorus, and sulfur concentrations. Moreover, the FAMEs production of these two microalgae strains was evaluated, which makes this article novel, because there are very few that evaluate the specific production of fatty acids by increasing or reducing the concentration of macroelements in the culture medium. The effect of varying the nitrogen, phosphorus, and sulfur concentrations in the culture medium was analyzed in detail to maximize the biomolecules yield derived from microalgae biomass.

2. Results and Discussion

2.1. Growth Behavior of Chlamydomonas reinhardtii and Chlorella vulgaris with Different Nitrogen, Phosphorus, and Sulfur Concentrations

In standard medium concentration [Control], *C. reinhardtii* showed cell growths of 2.08 g L^{-1}, 2.54 g L^{-1}, and 2.51 g L^{-1} at the 5th, 10th, and 15th culture days, respectively (Figure 1a–c). These results are comparable to those obtained by Morales-Sánchez et al. (2020) [34], where *Chlamydomonas malina* FT89.6 PG5 (Roscoff Culture Collection No. 2488) (https://roscoff-culture-collection.org/rcc-strain-details/2488, accessed on 10 June 2023) reached approximately 4 g L^{-1} at the 10th culture day. *C. vulgaris* culture showed a better microalgae growth at medium concentration of all macroelements [Control]; it reached 0.86 g L^{-1} of biomass growth at the 10th and 1.37 g L^{-1} at the 15th culture day (Figure 1d–f). These results are similar to those found by Josephine et al. [35], where >1 g L^{-1} was obtained at the 15th culture day of *C. vulgaris*.

C. reinhardtii presented a better growth at low nitrogen concentration [N^-] (3.69 g L^{-1} at 10th day) and at high phosphorus concentration [P^+] (3.12 g L^{-1} at 10th day) (Figure 1a,b). In this manner, an increment of at least 0.40 g L^{-1} can be obtained by modifying the concentration of nitrogen [N^-,N^+] or phosphorous [P^+] in comparison to the medium concentration [Control] after ten days of cultivation. It was shown that high concentration of nitrogen [N^+] and sulfur [S^+] had a noticeable negative effect on *C. vulgaris* growth, where 0.98 g L^{-1} and 0.66 g L^{-1} were obtained at the 15th culture day, respectively. However, at high nitrogen [N^+] concentration, *C. vulgaris* showed the tendency to increase microalgae growth after the 10th culture day, similar to the low nitrogen [N^-] concentration curve. These results are related to those found with *C. reinhardtii*, since both microalgae had better growth in low-nitrogen treatments [N^-]. Also, the inhibition effect on microalgae growth caused by high nitrogen loads in culture medium has been demonstrated [36].

Other studies have reported that low-nitrogen conditions in the culture medium increased cell growth. For instance, *Isochrysis galbana* (Haptophyta, Coccolithophyceae) presented enhanced cell growth at the nitrogen concentration of 144 mg L^{-1} in comparison to the nitrogen concentration of 288 mg L^{-1} [36]. Similarly, *Graesiella emersonii* (formerly *Chlorella emersonii*) (Chlorophyta) microalgae reached a higher cell growth under low-nitrogen conditions than under nitrogen abundance [37].

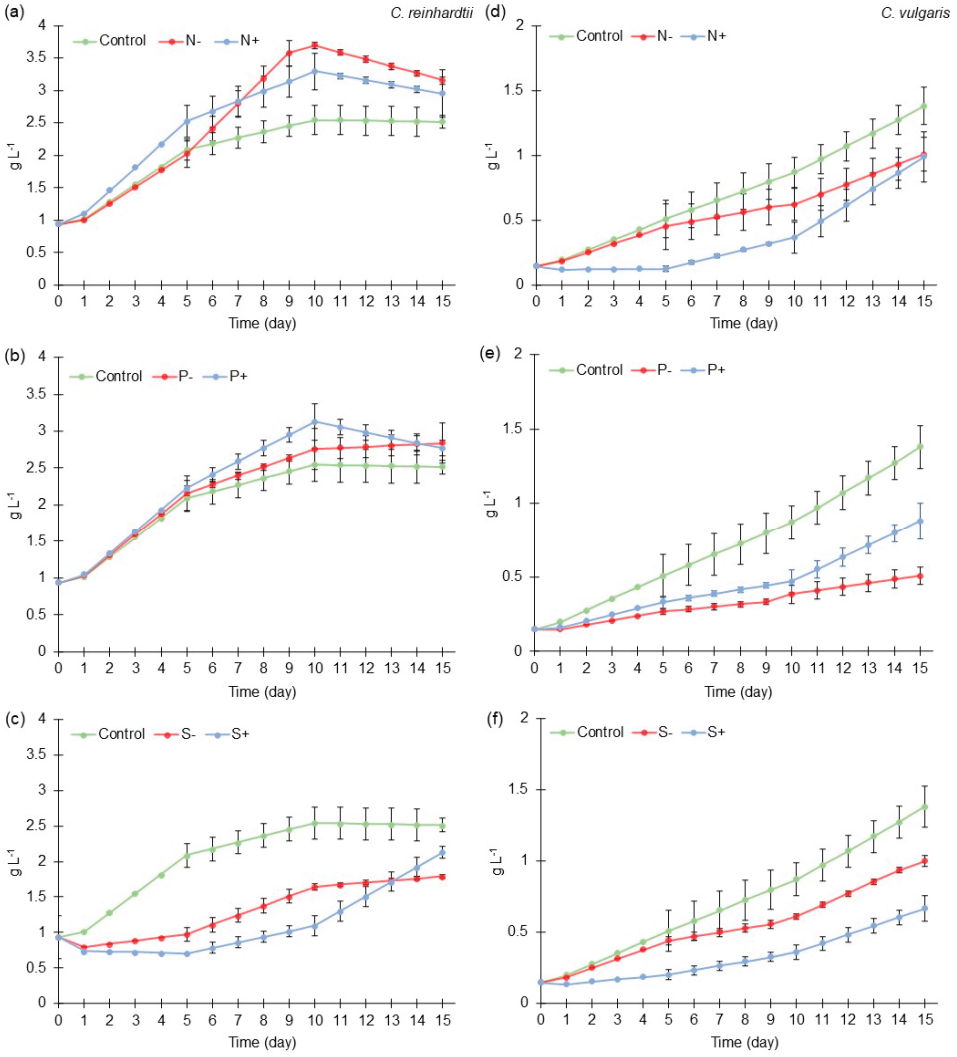


Figure 1. Growth behavior (g L^{-1}) of *Chlamydomonas reinhardtii* and *Chlorella vulgaris* at different nitrogen, phosphorus, and sulfur concentrations. (**a**) Cell growth of *Chlamydomonas reinhardtii* grown at different nitrogen concentrations. (**b**) Cell growth of *Chlamydomonas reinhardtii* grown at different phosphorus concentrations. (**c**) Cell growth of *Chlamydomonas reinhardtii* grown at different sulfur concentrations. (**d**) Cell growth of *Chlorella vulgaris* grown at different nitrogen concentrations. (**e**) Cell growth of *Chlorella vulgaris* grown at different phosphorus concentrations. (**f**) Cell growth of *Chlorella vulgaris* grown at different sulfur concentrations. All points were sampled by triplicate. The samples were taken every day.

The phosphorous concentration in the *C. reinhardtii* culture had a strong effect as well; optimal results were obtained under the high concentration [P^+]. This result is in agreement with those reported by Lovio Fragoso et al. (2019) [38], who cultivated *Chaetoceros muelleri* (Bacillariophyta) under high-phosphorus conditions, showing a slight increase in cell growth. On the other hand, for *C. vulgaris*, it was shown that lower concentration [P^-] has a more negative effect on microalgae growth than the effect obtained with high concentration [P^+]. The same effect was reported by Li et al. (2022) [39], who cultivated *Tetradesmus*

obliquus (formerly *Scenedesmus obliquus*) (Chlorophyta) under low-phosphorus condition (0.02 mg L^{-1}); experiments under low-concentration conditions showed lower values in terms of cell growth when compared to those obtained under high concentrations of phosphorus (2 mg L^{-1}) [39]. In this experiment, an exponential phase was not shown in any culture, so they can be maintained for a longer number of days.

Regarding sulfur, *C. reinhardtii* growth was negatively affected by this macronutrient, since 1.79 g L^{-1} and 2.12 g L^{-1} of microalgae biomass were obtained under low [S^-] and high [S±] sulfur concentrations at the 15th day, respectively (Figure 1c). Also, it was shown that high concentration of sulfur [S^+] had a noticeable negative effect on *C. vulgaris* growth, which was 0.66 g L^{-1} at the 15th culture day. Sulfur deprivation decreases photosynthetic activity in microalgae; thus, the low results for cell growth could be related to this phenomenon. In the case of high sulfur treatment, Mao et al. (2020) [40] reported a similar growth behavior, in which the variation in sulfur concentration affected microalgae growth during the first days of culture, subsequently increasing cell growth, until adapting a similar behavior to that presented by the control.

Also, it can be shown that at the 10th day of most microalgae cultures [Control, N^-, N^+, P^+, P^-, S^-], the exponential phase is reached in *C. reinhardtii* culture. The exponential phase of this microalgae can be reached usually at the 8th or 10th day of culture, even in light stress, salinity stress, or nutrient stress conditions. This behavior could be related to nutrients depletion and less light penetration because of its high cell density [34]. However, this behavior could imply that this microalga can be useful for obtaining large amounts of microalgae biomass in short times.

From ANOVA–Tukey pairwise analysis of *C. reinhardtii* growth, it was determined that at low nitrogen [N^-] concentration, a higher microalgae biomass was produced in all the experiments at the 10th day of culture (3.69 g L^{-1}). In the case of *C. vulgaris* culture, it was determined by the ANOVA–Tukey pairwise analysis that at medium [Control] concentration of macroelements, a higher microalgae biomass was produced in all the experiments at the 15th day of culture (1.37 g L^{-1}). These results demonstrate that the growth behavior of microalgae with different nitrogen, phosphorus, and sulfur concentrations depends on the microalgae strain; however, some similar trends were found. In the case of *Chlorella vulgaris*, it can be concluded that the variations made negatively affected cell growth, because the variation was excessive for the culture to adapt in such a short time. However, since the culture did not reach a stationary phase, it was not discarded that in later days the behavior would have changed.

2.2. Carbohydrates Content of Chlamydomonas reinhardtii and Chlorella vulgaris Grown with Different Nitrogen, Phosphorus, and Sulfur Concentrations

C. reinhardtii produced a higher concentration of carbohydrates with medium macroelements concentration [Control] in most microalgae cultures (Figure 2a–c), except in the cases of low nitrogen [N^-] and sulfur [S^-] concentration at the 5th culture day. Regarding *C. vulgaris*, an improved production of carbohydrates was obtained with lower macroelements concentrations in most of the microalgae cultures (Figure 2d–f).

At medium macroelements concentration [Control], *C. reinhardtii* obtained 108 mg g^{-1}, 155 mg g^{-1}, and 175 mg g^{-1} of carbohydrates at the 5th, 10th, and 15th culture days, respectively. On the other hand, *C. vulgaris* at medium macroelements concentration obtained 276 mg g^{-1}, 226 mg g^{-1}, and 151 mg g^{-1} of carbohydrates at the 5th, 10th, and 15th culture days, respectively. These results can be compared to those obtained by Morales-Sánchez et al. (2020) [34], where *Chlamydomonas malina* (Chlorophyta) RCC2488 reached approximately 200 mg g^{-1} at the 10th culture day.

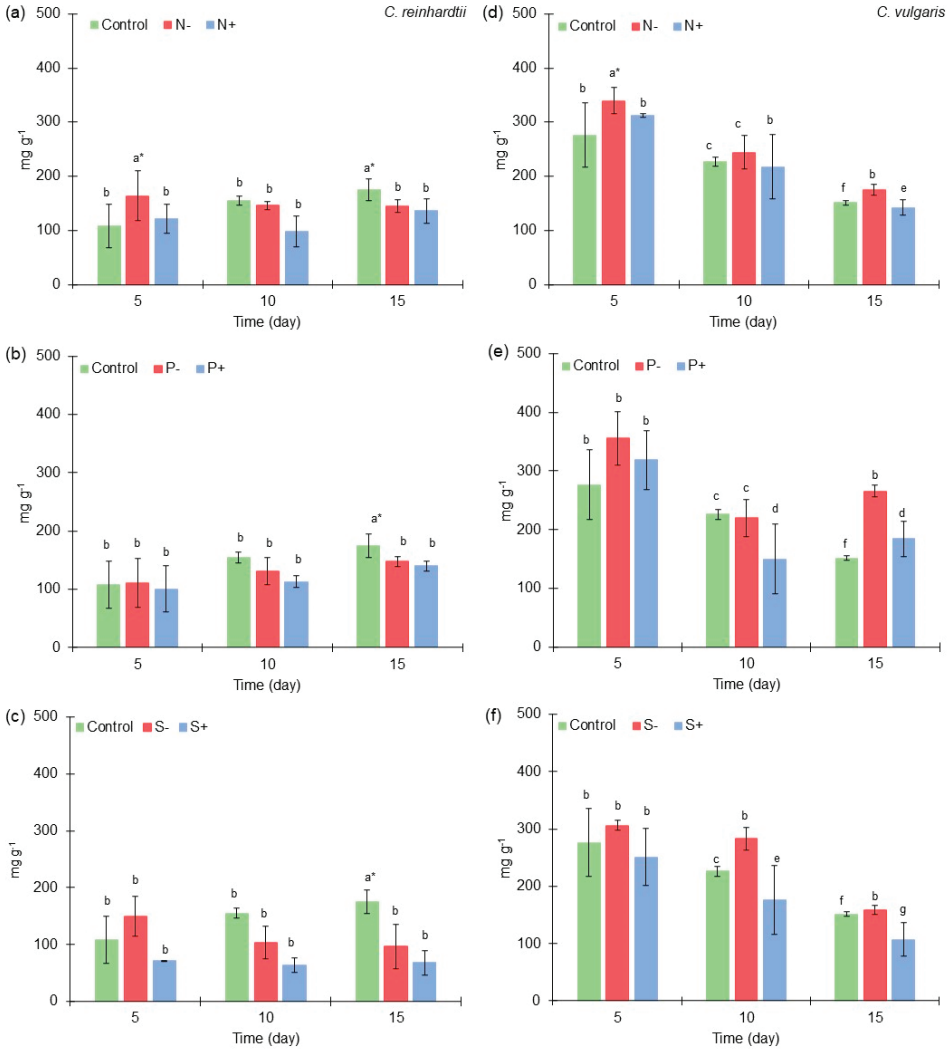


Figure 2. Carbohydrates content (mg g^{-1}) of *Chlamydomonas reinhardtii* and *Chlorella vulgaris* grown at different nitrogen, phosphorus, and sulfur concentrations. (**a**) Carbohydrates content of *Chlamydomonas reinhardtii* grown at different nitrogen concentrations. (**b**) Carbohydrates content of *Chlamydomonas reinhardtii* grown at different phosphorus concentrations. (**c**) Carbohydrates content of *Chlamydomonas reinhardtii* grown at different sulfur concentrations. (**d**) Carbohydrates content of *Chlorella vulgaris* grown at different nitrogen concentrations. (**e**) Carbohydrates content of *Chlorella vulgaris* grown at different phosphorus concentrations. (**f**) Carbohydrates content of *Chlorella vulgaris* grown at different sulfur concentrations. The results of the ANOVA–Tukey pairwise analysis carried out for each microalga in all the variations in macroelements also could be observed by different groups expressed by letters (symbol * = express the treatment where a higher production was obtained throughout the experiment). All points were sampled by triplicate. The samples were taken every five days.

At low nitrogen [N^-] and sulfur [S^-] concentration at the 5th culture day in *C. reinhardtii* culture, 163 mg g^{-1} (50% greater than medium concentration) and 149 mg g^{-1} (37%

greater than medium concentration) were obtained, respectively. The effect of nitrogen and sulfur deprivation in enhancing the carbohydrate content in microalgae biomass has been reported by different authors and using different microalgae strains. For example, in the case of *C. reinhardtii* CC-124, an increase of up to 4.3-fold was obtained [41], while *Vischeria calaminaris* (formerly *Eustigmatos calaminaris)* (Ochrophyta, Eustigmatophyceae) under nitrogen limitation conditions accumulated >80% of carbohydrates [42]. It is important to note, that the variation in some macroelements affects carbohydrates content in *C. reinhardtii* in a negative way, especially high concentrations of nitrogen, phosphorus, and sulfur.

In the experiments on nitrogen variations in *C. vulgaris* culture, a greater amount of carbohydrates was obtained at low nitrogen concentration [N^-], where 339 mg g^{-1} (22% greater than medium [Control] concentration), 244 mg g^{-1} (8% greater than control), and 174 mg g^{-1} of carbohydrates (15% greater than control) were obtained at the 5th, 10th, and 15th culture days, respectively. These results can be compared to those obtained by Cheng et al. (2017) [43], where *Chlorella* sp. growing in standard medium had an accumulation of 20–40% dry wt of carbohydrates. In their study, it was found that nitrogen limitation enhances carbohydrates accumulation in *Chlorella* sp. biomass, such as in *Tetradesmus obliquus* (formerly *Scenedesmus obliquus*) (Chlorophyta), where 43% dry wt of carbohydrates was obtained compared to the 25% dry wt reached under standard conditions.

Also, a greater amount of carbohydrates was obtained under low phosphorus concentration [P^-] in *C. vulgaris* culture, but only on days 5 and 15 of the microalgae culture, when 355 mg g^{-1} (28% greater than control) and 265 mg g^{-1} of carbohydrates (>50% greater than control) were obtained, respectively. It was shown in previous studies that low phosphorus concentrations could enhance the production of carbohydrate. For example, in *Chlorella* spp. culture, >50% of carbohydrates were obtained under phosphorus limitation [44]. Also, similar to this study, the limitation of phosphorus caused a greater accumulation of carbohydrates compared to results obtained under nitrogen limitation conditions [44]. Also, in *Scenedesmus*. LX1 culture, the carbohydrates production increased at least 20% under low-nutrient conditions (nitrogen/phosphorus) compared to that obtained in high-nutrient conditions [45].

At low sulfur concentrations [S^-] in *C. vulgaris* culture, the same behavior was observed that was obtained with low nitrogen and phosphorus concentration, since 306 mg g^{-1} (10% greater than control) and 282 mg g^{-1} of carbohydrates (25% greater than control) were reached at the 5th and 10th culture days, respectively. These results are similar to those found by Wang et al. (2022) [46], where *Chlorella sorokiniana* (Chlorophyta) grow under sulfur limitation conditions by obtaining >50% of carbohydrates compared to control conditions. Another microalga, *Chlorococcum infusionum* (*Chlorococcum humicola*) (Chlorophyta), reached a higher concentration of carbohydrates when grown in low-sulfur conditions, up to 10%, in comparison to standard conditions [47].

According to the ANOVA–Tukey pairwise analysis results of carbohydrates in *C. reinhardtii* culture, it was determined that the control [Medium] and low nitrogen [N^-] concentration conditions produced a higher carbohydrates content in all the experiments at the 15th day of culture (175 mg g^{-1}) and at the 5th day of culture (163 mg g^{-1}), respectively. In the case of the ANOVA–Tukey pairwise analysis of carbohydrates in *C. vulgaris* culture, it was determined that at low phosphorus [P^-] concentration, a higher carbohydrates content was produced in all the experiments at the 5th day of culture (355 mg g^{-1}). As it was possible to observe in the case of *C. reinhardtii*, a higher concentration was obtained in the control, a result which is given at the 15th day of culture, which leads us to consider that this microalga tends to accumulate a greater amount of carbohydrates over time. However, in the case of low nitrogen concentration [N^-], a greater amount of carbohydrates can be obtained on the 5th day of culture. Therefore, if its production is sought, its harvest can be considered at this time.

In this way, it can be shown that the deprivation of these macroelements could enhance the production of carbohydrates in both microalgae; however, it is important to remark that

each strain has a different behavior under these conditions. The increase in carbohydrates in microalgae biomass due to nutrients limitation conditions could be related to the fact that cells storing energy in the form of carbohydrates use less energy than they do by storing it in lipids (50% less ATP and 45% less NADPH in TAG synthesis process) [48]. Nitrogen and phosphorus limitation affects photosynthetic activity in microalgae by reducing the chlorophyll content and using absorbed energy capacity [49]. The accumulation of energy as carbohydrates guarantees the availability of energy for DNA replication and general cell metabolism [50], which is confirmed because under nitrogen limitations, microalgae increase their gene expression of starch-degrading enzymes [51].

Under phosphorus limitation conditions, the exchange of triose phosphate (glyceraldehyde-3-phosphate; product of photosynthetic pathway) could be reduced by the lack of cytoplasmic orthophosphate, generating an accumulation of precursors (e.g., glucose-6-phosphate) of carbohydrates production such as starch biosynthesis [52,53]. The sulfur deprivation in microalgae culture also could enhance carbohydrates biosynthesis by redirecting metabolic carbon flux to increase energy storage biomolecules production. Some enzymes (starch synthase and glycogen branching enzyme) related to carbohydrates synthesis could be significantly up-regulated under sulfur deprivation conditions, enhancing in this way the formation of amylose and amylopectin in microalgae cells [54].

2.3. Protein Content of Chlamydomonas reinhardtii and Chlorella vulgaris Grown with Different Nitrogen, Phosphorus, and Sulfur Concentrations

C. reinhardtii produced a higher concentration of proteins with medium macroelements concentration [Control] in most microalgae cultures, except in the cases of low nitrogen [N^-] and high phosphorus [P^+] concentration at the 5th culture day and low phosphorus [P^-] concentration at the 15th culture day (Figure 3a–c). On the other hand, *C. vulgaris* exhibited an improved production of proteins under high macroelements concentrations in some microalgae cultures (Figure 3d–f).

At medium macroelements concentration [Control] in *C. reinhardtii*, 123 mg g^{-1}, 94 mg g^{-1}, and 103 mg g^{-1} were obtained at the 5th, 10th, and 15th culture days, respectively. These results are similar to those reported by Rosa et al. (2023) [55], where *C. reinhardtii* obtained approximately 50 mg g^{-1} to 150 mg g^{-1} of proteins under standard conditions. In a medium-concentration treatment, *C. vulgaris* obtained 289 mg g^{-1}, 193 mg g^{-1}, and 184 mg g^{-1} of proteins at the 5th, 10th, and 15th culture days, respectively. These results are in agreement with those reported by Ma et al., 2021 [56], who cultivated *C. sorokiniana* FZU60 under batch conditions and produced 400 mg g^{-1} of proteins after three days of culture.

At low nitrogen [N^-] and high phosphorus [P^+] concentration at the 5th culture day, *C. reinhardtii* obtained 130 mg g^{-1} (5% greater than medium concentration) and 145 mg g^{-1} (17% greater than medium concentration), respectively. These results are similar to those found by Kamalanathan et al. (2016) [57], where *C. reinhardtii* obtained a higher amount of protein in conditions without nitrogen in the first five days of culture. The increase in proteins at low nitrogen [N^-] concentration could be related to the use of intracellular nitrogen stored in macromolecules such as chlorophyll. Similar to these results, *C. reinhardtii* culture under nitrogen limitation for the first cycles of cell division used the nitrogen found in the cells, and therefore, a slight increase ($\leq$13%) in the protein content was observed in the culture [58].

Other authors have reported that *C. reinhardtii* at high phosphorus [P^+] concentrations increased protein accumulation, which is in agreement with the results found in our work. Particularly, higher accumulation was reported of proteins involved in ribosome structure and synthesis, as well as in DNA and RNA metabolism [59]. Moreover, it has been demonstrated that this type of treatment [P^+] leads to a reduction in the production of carbohydrates in the cell [59]. Similar to our study, the microalgae culture with a high phosphorus concentration had a 26% lower carbohydrates content than the control condition.

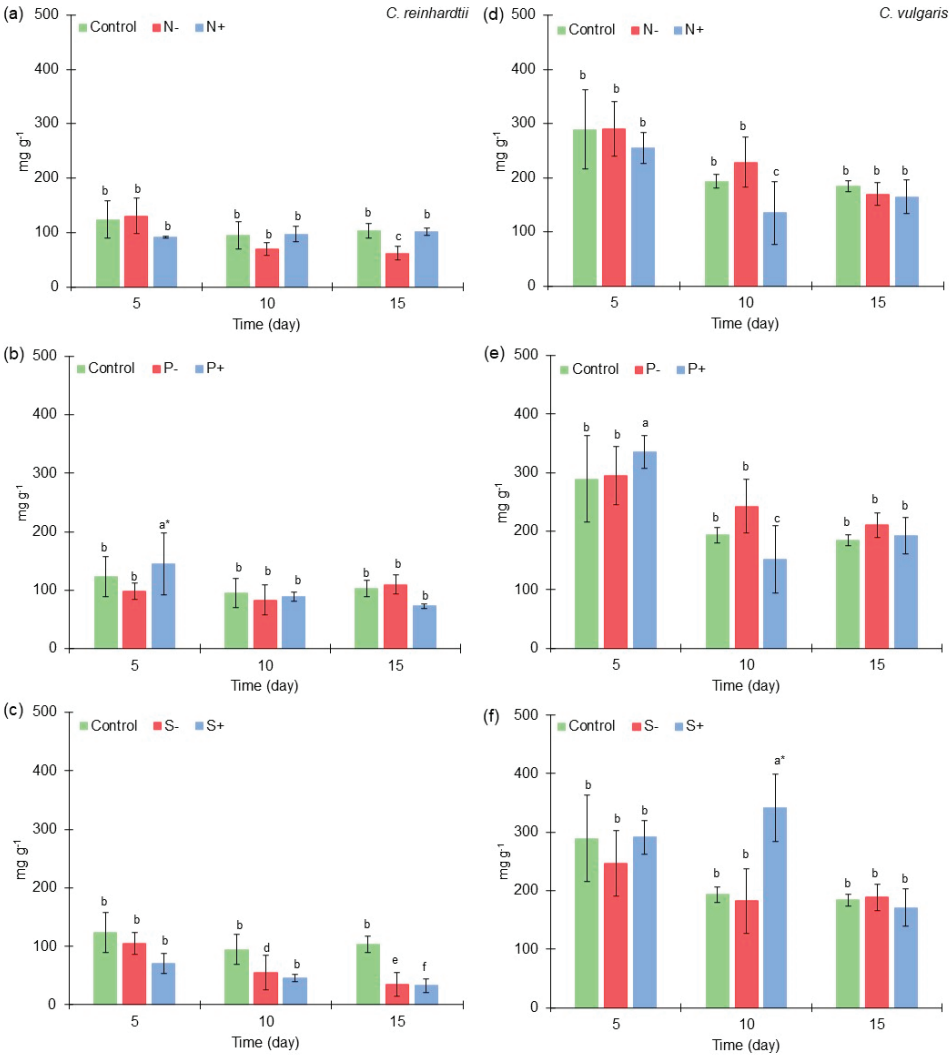


Figure 3. Protein content (mg g^{-1}) of *Chlamydomonas reinhardtii* and *Chlorella vulgaris* grown at different nitrogen, phosphorus, and sulfur concentrations. (**a**) Protein content of *Chlamydomonas reinhardtii* grown at different nitrogen concentrations. (**b**) Protein content of *Chlamydomonas reinhardtii* grown at different phosphorus concentrations. (**c**) Protein content of *Chlamydomonas reinhardtii* grown at different sulfur concentrations. (**d**) Protein content of *Chlorella vulgaris* grown at different nitrogen concentrations. (**e**) Protein content of *Chlorella vulgaris* grown at different phosphorus concentrations. (**f**) Protein content of *Chlorella vulgaris* grown at different sulfur concentrations. The results of the ANOVA–Tukey pairwise analysis carried out for each microalga in all the variations in macroelements also could be observed by different groups expressed by letters (symbol * = express the treatment where a higher production was obtained throughout the experiment). All points were sampled by triplicate. The samples were taken every five days.

In the experiments on nitrogen variations, *C. vulgaris* obtained a greater amount of proteins at low nitrogen concentration [N^-], where 228 mg g^{-1} (18% greater than medium [Control] concentration) was obtained at the 10th culture day. Under nitrogen limita-

tion, microalgae can up-regulate proteins related to lipids (1-Acylglycerol-3-phosphate O-acyltransferase and Glycerol-3-phosphate O-acyltransferase) and carbohydrates (e.g., Citrate synthase, Aconitase, α-Ketoglutarate dehydrogenase, Succinic dehydrogenase, Fumarase) synthesis [60]. Also, at high phosphorus concentration [P^+] of *C. vulgaris* culture, an increase in proteins content was observed, since it reached 335 mg g^{-1} (16% greater than medium concentration) at the 5th culture day. These results agree with those found in *C. reinhardtii* experiments.

Also, at low phosphorus concentration [P^-] of *C. vulgaris* culture, a greater amount of proteins was obtained, but only on days 10 and 15 of the microalgae culture, with 242 mg g^{-1} (25% greater than control) and 210 mg g^{-1} of proteins (14% greater than control), respectively. In *Chlorella* sp. culture, it was found that phosphorus limitation could enhance protein production more than high phosphorus concentration [61], and these results are similar to those found in this study. This behavior could be related to microalgae's capacity to preserve high levels of proteins involved in P's storage as intracellular polyphosphate and its assimilation [59].

It is important to note that the variation in sulfur affects proteins content in *C. reinhardtii* in a negative way, especially in the high-concentration [S^+] condition, since 70 mg g^{-1} (43% less than medium concentration), 45 mg g^{-1} (51% less than medium concentration), and 32 mg g^{-1} of proteins (>60% less than medium concentration) were obtained at the 5th, 10th, and 15th culture days, respectively. In addition, high sulfur concentration [S^+] resulted in 341 mg g^{-1} (>50% greater than control) at the 10th culture day of *C. vulgaris* culture. Contrarily, *C. vulgaris* at low sulfur [S^-] concentration produced a protein content decrease, which can be related to the affectation in biosynthesis of the sulfur amino acids such as cysteine and methionine [62]. Also, sulfur is a macronutrient related to nuclear transcripts encoding proteins involved in photosynthetic activity; therefore, its variation in culture medium has a negative impact on the protein content of the biomass [63].

From the ANOVA–Tukey pairwise analysis of protein content in *C. reinhardtii* culture, it was determined that at high phosphorus [P^+] concentration, a higher protein content was produced in all the experiments at the 5th day of culture (145 mg g^{-1}). On the other hand, from the ANOVA–Tukey pairwise analysis of protein in *C. vulgaris* culture, it was determined that at high sulfur [S^+] and high phosphorus [P^+] concentration was produced a higher protein content in all the experiments at the 10th day of culture (341 mg g^{-1}) and at the 5th day of culture (335 mg g^{-1}), respectively.

2.4. Lipid Content of Chlamydomonas reinhardtii and Chlorella vulgaris Grown with Different Nitrogen, Phosphorus, and Sulfur Concentrations

C. reinhardtii at medium macroelements concentration [Control] produced 84 mg g^{-1}, 154 mg g^{-1}, and 127 mg g^{-1} of lipids at the 5th, 10th, and 15th culture days (Figure 4a–c), respectively. These results are similar to those obtained with *C. reinhardtii* cc849, for which 150 mg g^{-1} of lipids was reached at control conditions. Also, it was observed that lipids increased in microalgae biomass at least 50% after nitrogen deprivation [64]. This behavior was also found in this study, since low nitrogen [N^-] concentration produced 128 mg g^{-1} (52% greater than medium [Control] concentration). On the other hand, *C. vulgaris* obtained an improved production of lipids with low macroelements concentrations in some microalgae cultures (Figure 4d–f). At medium macroelements concentration [Control], 121 mg g^{-1} and 169 mg g^{-1} were obtained at the 10th and 15th culture days, respectively. In other studies, *C. vulgaris* has had a lipid accumulation of 9 to 12% dry wt [65], which are similar results to those found here.

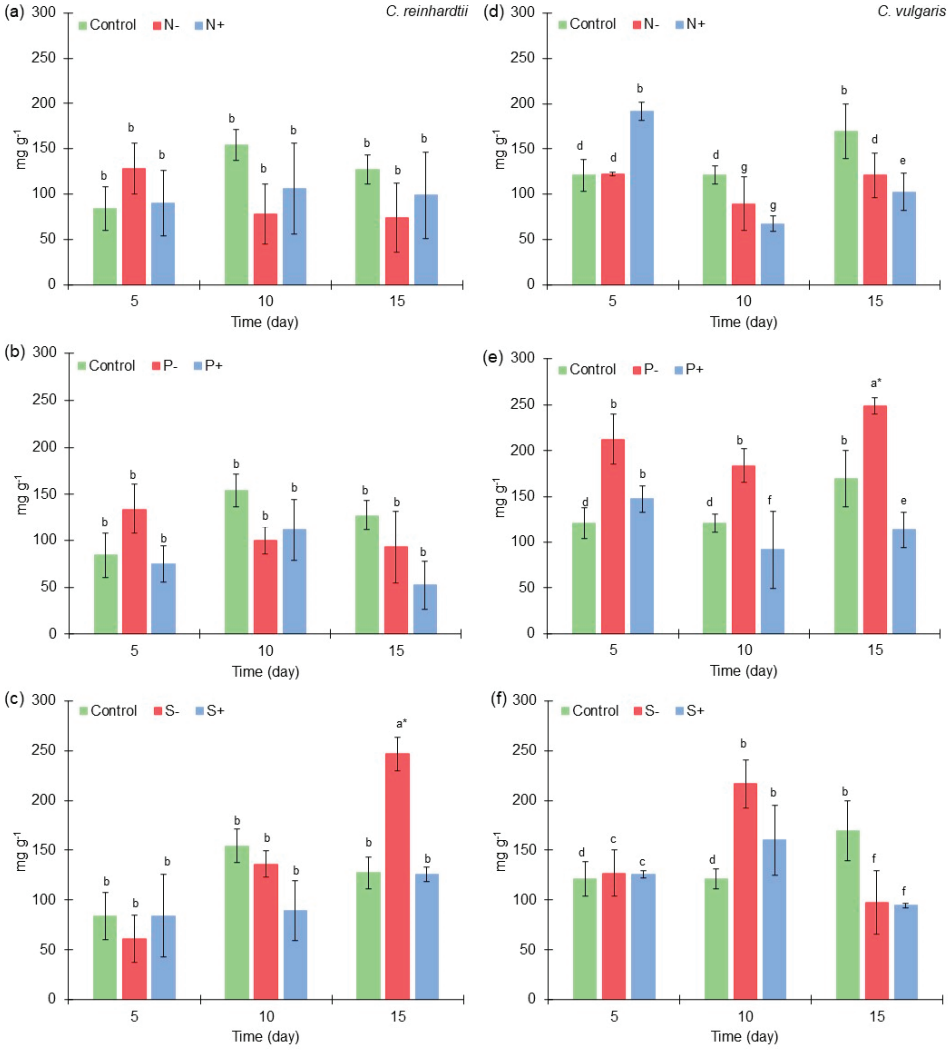


Figure 4. Lipid content (mg g^{-1}) of *Chlamydomonas reinhardtii* and *Chlorella vulgaris* grown at different nitrogen, phosphorus, and sulfur concentrations. (**a**) Lipid content of *Chlamydomonas reinhardtii* grown at different nitrogen concentrations. (**b**) Lipid content of *Chlamydomonas reinhardtii* grown at different phosphorus concentrations. (**c**) Lipid content of *Chlamydomonas reinhardtii* grown at different sulfur concentrations. (**d**) Lipid content of *Chlorella vulgaris* grown at different nitrogen concentrations. (**e**) Lipid content of *Chlorella vulgaris* grown at different phosphorus concentrations. (**f**) Lipid content of *Chlorella vulgaris* grown at different sulfur concentrations. The results of the ANOVA–Tukey pairwise analysis carried out for each microalga in all the variations in macroelements also could be observed by different groups expressed by letters (symbol * = express the treatment where a higher production was obtained throughout the experiment). All points were sampled by triplicate. The samples were taken every five days.

At low nitrogen concentration [N^{-}], a slightly higher amount of lipids was obtained in *C. vulgaris*, but only on day 5 of the microalgae culture, when122 mg g^{-1} was reached. Similarly, *Graesiella emersonii* (formerly *Chlorella emersonii*) produced more lipids under nitrogen limitation conditions; however, a complete nitrogen deprivation reduced lipid

production [37]. Hence, it is important to determine the optimal level of nitrogen limitation to improve lipid production in microalgae biomass depending on the microalgae strain. In this case, it is possible that with a concentration slightly higher than that considered in the low range, an increase in lipids could be observed.

The response of lipid production in microalgae culture under low-nitrogen conditions is related to the overexpression of genes involved in the TAG pathway. In *Tetradesmus bernardii* (Chlorophyta), it was observed that genes encoding diacylglycerol acyltransferase (DGAT2 and DGAT1) were up-regulated under low nitrogen concentration, as well as acetyl-coenzyme A carboxylase [66]. Also, in this condition, an overexpression of genes related to the biosynthesis of palmitoyl-acyl carrier protein (β-ketoacyl-acyl-carrier-protein synthase [KASII]) could be observed in *Scenedesmus acutus* [67].

At low phosphorus [P^-] concentration at the 5th culture day in *C. reinhardtii*, a higher production of lipids (134 mg g^{-1}) was obtained (37% greater than medium concentration). Similar to *C. reinhardtii*, in the experiments on phosphorus variations, *C. vulgaris* obtained a greater amount of lipids under low phosphorus concentration [P^-], reaching values of 212 mg g^{-1} (>50% greater than medium [Control] concentration), 183 mg g^{-1} (34% greater than medium concentration), and 248 mg g^{-1} (45% greater than medium concentration) at the 5th, 10th, and 15th culture days, respectively. This behavior is similar to that found in other microalgae such as *Scenedesmus* sp., which at lower phosphorus concentration produced an increase of approximately 50% in lipid content. The phosphorus limitation enhances the expression of genes related to fatty acid biosynthesis such as 3-ketoacyl-CoA synthase and 2-enoyl-CoA reductase [68]. In addition, lipids production could be associated with the low production of carbohydrates presented under the low phosphorus treatment [P^-], since a similar carbohydrate content to that obtained by the control was observed on certain sampling days (Figure 2); contrarily, the production of lipids showed an evident increase regardless of the sampling day.

In the case of low sulfur [S^-] concentration at the 15th culture day, 246 mg g^{-1} (>50% greater than control culture) was obtained in *C. reinhardtii* culture. *C. reinhardtii* CC-124 shows an increase in lipids during sulfur deprivation of approximately 35% [69]. These results are similar to those found by Gómez-De la Torre et al. (2023) [70], who reported a higher lipid content in sulfur and phosphorus limitation conditions, showing that the concentration of both macroelements in microalgae culture is highly relevant for the production of lipids. Also, 216 mg g^{-1} (>50% greater than control) was obtained under low concentration [S^-] conditions at the 10th culture day in *C. vulgaris*. This behavior could be related to the overexpression of genes encoding acyltransferases related to TAG production such as phospholipid diacylglycerol acyltransferase (PDAT1) and diacylglycerol acyltransferase (DGTT1) [71]. In addition, it was determined that sulfur limitation could affect acetyl-CoA metabolomics flow, which in turn may increase the production of fatty acids and genes related to fatty acid desaturase and phosphatidic acid phosphatase [54].

From the ANOVA–Tukey pairwise analysis of lipids production in *C. reinhardtii* culture, it was determined that at low sulfur [S^-] concentration, a higher lipids content was produced in all the experiments at the 15th day of culture (246 mg g^{-1}). On the other hand, from the ANOVA–Tukey pairwise analysis of lipid production in *C. vulgaris* culture, it was determined that at low phosphorus [P^-] concentration, a higher lipids content was produced in all the experiments at the 15th day of culture (248 mg g^{-1}).

2.5. FAMEs Accumulation of Chlamydomonas reinhardtii and Chlorella vulgaris Grown with Different Nitrogen, Phosphorus, and Sulfur Concentrations

C. reinhardtii in medium concentration of macronutrients [Control] and after 15 days of cultivation produced 20 ± 3 mg g^{-1} (31% of total fatty acids) of palmitic acid (C16:0), 12 ± 1.5 mg g^{-1} (17%$_{TFA}$) of oleic acid (C18:1n9c), 13 ± 2.5 mg g^{-1} (16%$_{TFA}$) of linoleic acid (C18:2n6c), and 15 ± 2.5 mg g^{-1} (18%$_{TFA}$) of α-linoleic acid (C18:3n3) (Figure 5a–c). These results are similar to those found by Zheng et al., 2022 [72], where *C. reinhardtii* obtained 21%$_{TFA}$ of palmitic acid, 9%$_{TFA}$ of oleic acid, and 17%$_{TFA}$ of linoleic acid.

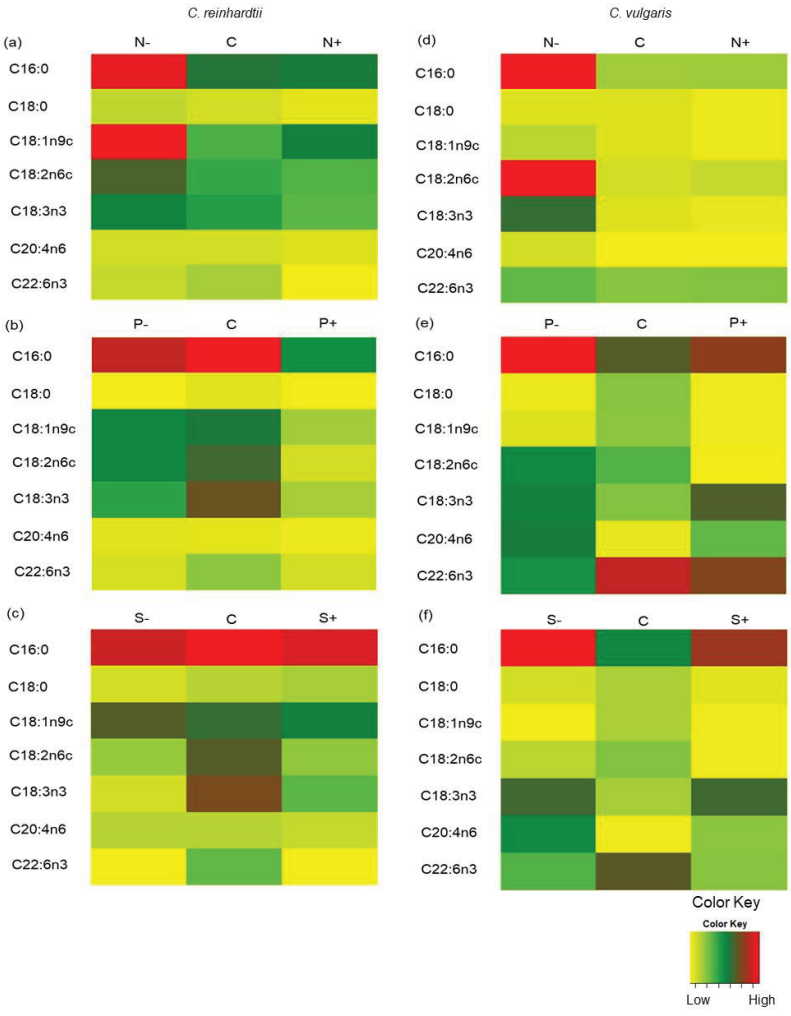


Figure 5. Heatmap of FAMEs (mg g^{-1}) in *Chlamydomonas reinhardtii* and *Chlorella vulgaris* grown at different nitrogen, phosphorus, and sulfur concentrations. (**a**) FAMEs production of *Chlamydomonas reinhardtii* grown at different nitrogen concentrations. (**b**) FAMEs production of *Chlamydomonas reinhardtii* grown at different phosphorus concentrations. (**c**) FAMEs production of *Chlamydomonas reinhardtii* grown at different sulfur concentrations. (**d**) FAMEs production of *Chlorella vulgaris* grown at different nitrogen concentrations. (**e**) FAMEs production of *Chlorella vulgaris* grown at different phosphorus concentrations. (**f**) FAMEs production of *Chlorella vulgaris* grown at different sulfur concentrations. All points were sampled by triplicate. The samples were taken at 15th culture day.

At low nitrogen concentration [N^-] in *C. reinhardtii* culture, 34 ± 8 mg g^{-1} [$28\%_{TFA}$] of palmitic acid, 36 ± 10 mg g^{-1} [$27\%_{TFA}$] of oleic acid, 23 ± 5 mg g^{-1} [$19\%_{TFA}$] of linoleic acid, and 19 ± 3 mg g^{-1} [$16\%_{TFA}$] of α-linoleic acid were produced at the 15th culture day, the highest result in the whole experiment with this microalga strain. In the literature, similar results can be found. For example, *Phaeodactylum tricornutum* produced a higher percentage of α-linoleic acid and palmitic acid under a low-nitrogen condition [73]. *Chlamydomonas malina* FT89.6 PG5 (Roscoff Culture Collection No. 2488) also showed the same behavior under low-nitrogen condition, since it accumulated a slightly higher amount of FAMEs [34].

On the other hand, *C. vulgaris* under low nitrogen concentration [N^-] conditions produced 35 ± 12 mg g^{-1} of palmitic acid (5-fold greater than control), 6 ± 1.2 mg g^{-1} of oleic acid (2-fold greater than control), 34 ± 12 mg g^{-1} of linoleic acid (8-fold greater than control), 21 ± 8 mg g^{-1} of α-linoleic acid (6-fold greater than control), and 3.8 ± 0.75 mg g^{-1} of arachidonic acid (2-fold greater than control) at the 15th culture day (Figure 5d). Similar to *C. reinhardtii*, in this treatment, the highest result in the whole experiment was obtained. This behavior was observed in *Vischeria calaminaris* (formerly *Eustigmatos calaminaris*) culture, which, under low nitrogen concentration, produced higher C16 and C20 fatty acids [42]. These results suggest that decreasing the concentration of nitrogen might be a more feasible strategy to increase the percentage of fatty acids produced in microalgae, in comparison to strategies related to the concentration of phosphorus and sulfur.

In low phosphorus [P^-] conditions, *C. reinhardtii* produced 5% more arachidonic acid (C20:4n6) than control conditions. This result is in agreement with other studies performed with *C. reinhardtii* culture, in which phosphorus stress generated longer-chain fatty acids (C18:0, C18:1n9c, C:20) compared to control conditions [69]. Similarly, under low phosphorus [P^-] conditions in *C. vulgaris* culture (Figure 5e), higher concentrations of palmitic acid (44% greater), linoleic acid (42% greater), α-linoleic acid (>50% greater), and arachidonic acid (>50% greater) were observed in comparison to control. The increment can be related to the overexpression of genes involving in fatty acid biosynthesis (e.g., 3-oxoacyl-ACP reductase, 3-ketoacyl-CoA synthase), especially in the case of palmitic and arachidonic acid production [68]. *C. vulgaris* at high phosphorus concentration [P^+], higher concentrations of palmitic (13% greater), α-linoleic acid (>50% greater), and arachidonic acid (>50% greater) were obtained compared to control.

Regarding sulfur treatments, low concentration [S^-] resulted in 23%$_{TFA}$ of oleic acid (10% more than control) in *C. reinhardtii* culture. Also, 2-fold greater control of arachidonic acid was obtained at low sulfur concentration. It has been demonstrated that high sulfur concentration causes negative effects on FAMEs production, except in the case of stearic acid, since it obtained >20% production compared to control. The increase in FAMEs concentration under low sulfur concentration could be related to the overexpression of genes related to acetyl-CoA carboxylase, 3-ketoacyl-ACP synthase, and 3-ketoacyl-ACP reductase production, which are involved in fatty acid synthesis [40].

C. vulgaris culture observed an increase in palmitic acid (>50% greater), linoleic acid (>50% greater), and arachidonic acid (>50% greater) compared to control at low sulfur concentration [S^-] (Figure 5e). These results are similar to those found with *Chlorella vulgaris* under sulfur-deprived medium, where an increase in palmitic, oleic, and linoleic acid were observed [70]. In addition, at high sulfur concentration [S^+], higher concentrations of palmitic acid (49% greater) and α-linoleic acid (>50% greater) were determined compared to the control.

3. Materials and Methods

3.1. Reagents and Equipment

The reagents used for culture medium preparation and to perform the carbohydrates, lipids, and FAMEs analysis were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Lowry Protein Assay Kit was purchased from Thermo Fisher Scientific (Rockford, IL, USA). The spectrophotometric measurements were taken with an absorbance microplate reader from BGM Labtech (Fluorstar omega, 415-0470, Ortenberg, Germany).

3.2. Inoculum Culture Conditions

The strains *Chlamydomonas reinhardtii* and *Chlorella vulgaris* were purchased from UTEX (UTEX, Austin, TX, USA). BG11 culture medium was used to perform the experiments with both microalgae strains. The BG11 culture medium composition was as follows: $NaNO_3$ 1.5 g L^{-1}, K_2HPO_4 40 mg L^{-1}, $CaCl_2{\cdot}2H_2O$ 36 mg L^{-1}, $MgSO_4{\cdot}7\ H_2O$ 75 mg L^{-1}, Citric Acid$\cdot H_2O$ 6 mg L^{-1}, $C_6H_8FeNO_7$ 6 mg L^{-1}, $Na_2EDTA{\cdot}2H_2O$ 1 mg L^{-1}, Na_2CO_3 20 mg L^{-1}, H_3BO_3 2.86 mg L^{-1}, $MnCl_2{\cdot}4H_2O$ 1.81 mg L^{-1}, $ZnSO_4{\cdot}7H_2O$ 0.22 mg L^{-1}, $Na_2MoO_4{\cdot}2H_2O$

0.39 mg L^{-1}, $CuSO_4 \cdot 5H_2O$ 0.079 mg L^{-1}, and $Co(NO_3)_2 \cdot 6H_2O$ 0.04 mg L^{-1}. The stock cultures were kept at 21 ± 1 °C with continuous light at 100 µmol photons m^{-2} s^{-1} and with 1.5 L m^{-2} min^{-1} of aeration (OPTIMA 4.5-Watt pump with an air filter of 0.20 µm).

3.3. Growth Measure of Chlamydomonas reinhardtii and Chlorella vulgaris with Different Nitrogen, Phosphorus, and Sulfur Concentrations

For each experiment, 100 mL *Chlamydomonas reinhardtii* and *Chlorella vulgaris* inocula, with an absorbance of 1 at the wavelength of 750 nm, were cultivated separately in lab glass bottles containing 900 mL of BG11. Before inoculation, the biomass was washed twice with bidistilled water. The cultures were maintained under the same conditions mentioned in the previous section. A factorial design was used for this research and applied to each experiment set (Table 1). Cell growth and biomass composition of *Chlamydomonas reinhardtii* and *Chlorella vulgaris* were evaluated under three different nitrogen, phosphorus, and sulfur concentrations. Nitrogen: low, 80 mg L^{-1} N; medium, 240 mg L^{-1} N; and high, 640 mg L^{-1} N. Phosphorus: low, 4 mg L^{-1} P; medium, 7 mg L^{-1} P; and high, 17 mg L^{-1} P. Sulfur: low, 8 mg L^{-1} S; medium, 20 mg L^{-1} S; and high 52 mg L^{-1} S. The foregoing values were obtained during 15 culture days. The medium concentration was taken as a control, since this is the standard concentration used in the BG11 medium.

Table 1. Experimental design to evaluate the effect of the nutrient concentration (nitrogen, phosphorus, and sulfur) on growth behavior and biomass composition in *Chlamydomonas reinhardtii* and *Chlorella vulgaris* culture.

RunEst	Run	Blocks	Concentration	Nutrient
11	1	2	Low	S
17	2	2	High	S
18	3	2	High	P
12	4	2	Low	P
15	5	2	Med	P
16	6	2	High	N
13	7	2	Med	N
10	8	2	Low	N
14	9	2	Med	S
24	10	3	Med	P
20	11	3	Low	S
19	12	3	Low	N
22	13	3	Med	N
25	14	3	High	N
26	15	3	High	S
23	16	3	Med	S
21	17	3	Low	P
27	18	3	High	P
8	19	1	High	S
6	20	1	Med	P
3	21	1	Low	P
4	22	1	Med	N
1	23	1	Low	N
5	24	1	Med	S
7	25	1	High	N
2	26	1	Low	S
9	27	1	High	P

Nutrients: Nitrogen (Low [N^-]: 80 mg L^{-1} N; Med [Control]: 240 mg L^{-1} N; High [N^+]: 640 mg L^{-1} N). Phosphorus (Low [P^-]: 4 mg L^{-1} P; Med [Control]: 7 mg L^{-1} P; High [P^+]: 17 mg L^{-1} P). Sulfur (Low [S^-]: 8 mg L^{-1} S; Med [Control]: 20mg L^{-1} S; High [S^+]: 52 mg L^{-1} S). Factorial design has been carried out for each type of microalgae strain (*Chlamydomonas reinhardtii* and *Chlorella vulgaris*).

Prior to the experiments, an equation of relationship between absorbance (750 nm) and dry weight (g L^{-1}) of each strain was defined, as shown in Equations (1) and (2). Samples of 1 mL were obtained every day to assess the biomass growth curve by spectrophotometry.

The dry weight was determined by filtering 10 mL of serial algal culture dilutions using pre-weighed Whatman GF/C glass microfiber filters. The filter holding the algal biomass was dried at 100 °C for 2 h. Then, filters with the algal biomass were cooled at room temperature inside a vacuum desiccator and weighed gravimetrically.

$$C.reinhardtii \text{ g L}^{-1} = 3.9218(Abs_{750}) - 0.2085 \; R^2 = 0.996 \quad (1)$$

$$C.vulgaris \text{ g L}^{-1} = 1.0444(Abs_{750}) - 0.1672 \; R^2 = 0.991 \quad (2)$$

3.4. Biomass Characterization of Chlamydomonas reinhardtii and Chlorella vulgaris Grown with Different Nitrogen, Phosphorus, and Sulfur Concentrations

The cultures of *Chlamydomonas reinhardtii* and *Chlorella vulgaris* were sampled by taking 15 mL of the culture on days 5, 10, and 15. Samples were stored at 4 °C until further analysis. To perform the carbohydrate, protein, and lipid assay, samples were centrifuged at 6000 rpm and 4 °C for 20 min. Then, the supernatant was discharged, and the biomass was washed twice and resuspended in distilled water.

3.5. Total Carbohydrates Content of Chlamydomonas reinhardtii and Chlorella vulgaris Grown with Different Nitrogen, Phosphorus, and Sulfur Concentrations

Carbohydrate content was quantified using the sulfuric acid–UV assay described by López-legarda et al. [74]. Briefly, 300 µL of the washed biomass was taken before adding 1 mL of concentrated sulfuric acid; then, the mixture was incubated in an ice bath for two minutes. Then, the absorbance was measured at 315 nm using a microplate reader from BGM Labtech. Finally, the absorbance readings were used to obtain the concentration of carbohydrates (mg L^{-1}) by using a glucose standard curve.

3.6. Total Protein Content of Chlamydomonas reinhardtii and Chlorella vulgaris Grown with Different Nitrogen, Phosphorus, and Sulfur Concentrations

The Modified Lowry Protein Assay Kit was used to determine the total protein produced by *Chlamydomonas reinhardtii* and *Chlorella vulgaris*. Firstly, 1 mL of Lowry's reagent was added to 200 µL of washed biomass solution. Secondly, the tube was vortexed. After 10 min, 100 µL of Folin reagent was added, and the mixture was vortexed again. After 30 min of reaction in the dark, the absorbance of the samples was measured at 750 nm in an absorbance microplate reader from BGM Labtech. Finally, the protein concentration was obtained using a BSA calibration curve.

3.7. Total Lipid Content of Chlamydomonas reinhardtii and Chlorella vulgaris Grown with Different Nitrogen, Phosphorus, and Sulfur Concentrations

The lipid content produced by *Chlamydomonas reinhardtii* and *Chlorella vulgaris* was quantified using the sulfo-phospho-vanillin assay described by Mishra et al. (2014) [75]. Briefly, 2 mL of sulfuric acid (98%) was added to 100 µL of the concentrated washed microalgae biomass (10:1). Then, it was heated for 10 min at 100 °C. Next, it was cooled in an ice bath for 5 min. After that, 5 mL of phospho-vanillin reagent was added, and the sample was incubated for 15 min at 37 °C. Finally, the absorbance was measured at 530 nm in an absorbance microplate reader from BGM Labtech. The lipid concentration was obtained using a canola oil calibration curve.

3.8. FAMEs of Chlamydomonas reinhardtii and Chlorella vulgaris Grown with Different Nitrogen, Phosphorus, and Sulfur Concentrations

Chloroform, methanol, and distilled water were added in ratios of 1:2:0.4 ($v/v/v$), respectively, into 150 mg of dried biomass. The resulting mixture was mixed for 30 s and then centrifuged at 4800 rpm for 10 min at 4 °C. After centrifugation, the lower layer was carefully recovered and filtered using a PTFE syringe filter (0.45 µm pore size, Thermo Fisher Scientific, Waltham, MA, USA). Then, it was transferred into a pre-weighed glass

tube. The remaining chloroform was dried in a concentrator at 45 °C for 2 h at 15 mm Hg. The extracted lipids were gravimetrically weighted to estimate the total lipid content according to the Bligh and Dyer method with some modifications [76].

Fatty acids were determined through derivatization to fatty acid methyl esters (FAMEs) in closed vial by using (2 mL) of methanol at 7% with sulfuric acid using triundecanoin (C11:0) as internal standard. Mixture was heated at 80 °C for 1.5 h. Immediately after reaching room temperature, sample was extracted with 3 mL of hexane while manually shaking for 1 min. The upper phase formed was recovered, and the inferior phase was extracted again with 3 mL of hexane. Recovered phases were placed in a volumetric flask and brought to 10 mL with hexane.

Fatty acid profile was analyzed by a gas chromatograph with a mass spectrometer (Perkin Elmer, Clarus 600/560D, Bridgeport, CT, USA) by using an HP-88 capillary column (100 m, 0.25 mm × 0.20 μm). The carrier gas was helium at a constant flow of 1 mL/min. The initial oven temperature was 140 °C (held for 5 min), and it was increased by 4 °C/min until it reached the final temperature 240 °C (held for 15 min). Split ratio was 1:10, injector temperature was 265 °C, mass spectrometer analysis used EI ion source, electron energy was 70 eV, the temperature of source and interface were 210 °C, and range of m/z = 30–550. For FAMEs identification, the retention time of a typical chromatogram of the 37-component standard reference for FAME was used, and the quantification was realized used the standard internal method, using triundecanoin as the internal standard (AOAC Official Method 996.06).

3.9. Data Analysis

All data were analyzed by Minitab 21 (Minitab Inc., PA, USA). They were analyzed by an ANOVA with a Tukey pairwise comparison for each type of microalga ($\alpha = 0.05$). The heatmaps for FAMEs expression were created with R-studio using ggplot2 package.

4. Conclusions

The results found in this study allow us to visualize the impact of three macronutrients' (nitrogen, phosphorus, and sulfur) variation on the production of biomolecules of interest in the same microalgae strain (*Chlamydomonas reinhardtii* and *Chlorella vulgaris*). It was shown that some microalgae (*C. reinhardtii*) could produce more biomass under low nitrogen concentration (3.69 g L^{-1}) than standard conditions. It was shown that low concentrations of nitrogen and phosphorus could enhance carbohydrates and lipids in microalgae culture. In addition, surprisingly, the impact that sulfur variation can have on the production of biomolecules in microalgae was found, especially in the case of lipids and proteins. Interestingly, it was determined that low concentration of nitrogen had a better effect on accumulation of some FAMEs (e.g., palmitic, oleic, and linoleic acid) in both microalgae. These results could be useful for increasing productivity of some biomolecules in large-scale microalgae cultures, especially in those intended for the production of FAMEs (C16-C:18).

Author Contributions: Conceptualization, I.Y.L.-P.; methodology, V.G.A.-M., C.A.M.-M. and I.Y.L.-P.; formal analysis, I.Y.L.-P. and J.R.-R.; investigation, I.Y.L.-P., J.R.-R. and S.P.C.-B.; data curation, I.Y.L.-P., S.P.C.-B. and R.B.G.-G.; writing—original draft preparation, I.Y.L.-P.; writing—review and editing, I.Y.L.-P., S.P.C.-B., R.B.G.-G., K.G.C.-A., L.I.F.-C., G.M.G.-M., H.M.N.I. and R.P.-S.; visualization, I.Y.L.-P., H.M.N.I. and R.P.-S.; supervision, H.M.N.I. and R.P.-S.; project administration, H.M.N.I. and R.P.-S.; funding acquisition, H.M.N.I. and R.P.-S. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the Consejo Nacional de Humanidades, Ciencias y Tecnologías (CONAHCYT). This research was funded by Tecnologico de Monterrey project entitled "Exploring and optimizing CO_2 bio fixation process for microalgae lipids production to formulate green metalworking fluids—for cleaner manufacturing processes" (ID: I023—IAMSM002—C4-T2—E). Tec challenge-based projects call 2022.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data related to this work is given in this manuscript.

Acknowledgments: The authors would like to gratefully acknowledge the Latin American and Caribbean Water Center from Tecnológico de Monterrey for the laboratory facilities. The authors would like to acknowledge the funding provided by Tecnologico de Monterrey through the Bioprocess Research Chair [0020209I13]. This work was partially supported by Consejo Nacional de Humanidades, Ciencias y Tecnologías [CONAHCYT] Mexico under the Sistema Nacional de Investiga-dores [SNI] program awarded to Hafiz M.N. Iqbal [CVU: 735340], Roberto Parra–Saldivar [CVU: 35753], and Karina G. Coronado-Apodaca [CVU: 625883]. The Technology Scholarship to I. Lopez [CVU: 859227] from CONAHCYT is also thankfully acknowledged.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Khan, M.I.; Shin, J.H.; Kim, J.D. The promising future of microalgae: Current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. *Microb. Cell Fact.* **2018**, *17*, 36. [CrossRef] [PubMed]
2. Hossain, S.M.Z. Biochemical Conversion of Microalgae Biomass into Biofuel. *Chem. Eng. Technol.* **2019**, *42*, 2594–2607. [CrossRef]
3. López-Pacheco, I.Y.; Silva-Núñez, A.; García-Perez, J.S.; Carrillo-Nieves, D.; Salinas-Salazar, C.; Castillo-Zacarías, C.; Afewerki, S.; Barceló, D.; Iqbal, H.N.M.; Parra-Saldívar, R. Phyco-remediation of swine wastewater as a sustainable model based on circular economy. *J. Environ. Manag.* **2021**, *278*, 111534. [CrossRef] [PubMed]
4. Adamczyk, M.; Lasek, J.; Skawińska, A. CO_2 Biofixation and Growth Kinetics of *Chlorella vulgaris* and *Nannochloropsis gaditana*. *Appl. Biochem. Biotechnol.* **2016**, *179*, 1248–1261. [CrossRef]
5. Dineshbabu, G.; Uma, V.S.; Mathimani, T.; Deviram, G.; Arul Ananth, D.; Prabaharan, D.; Uma, L. On-site concurrent carbon dioxide sequestration from flue gas and calcite formation in ossein effluent by a marine cyanobacterium *Phormidium valderianum* BDU 20041. *Energy Convers. Manag.* **2017**, *141*, 315–324. [CrossRef]
6. Hariz, H.B.; Takriff, M.S.; Mohd Yasin, N.H.; Ba-Abbad, M.M.; Mohd Hakimi, N.I.N. Potential of the microalgae-based integrated wastewater treatment and CO_2 fixation system to treat Palm Oil Mill Effluent (POME) by indigenous microalgae; *Scenedesmus* sp. and *Chlorella* sp. *J. Water Process Eng.* **2019**, *32*, 100907. [CrossRef]
7. Srimongkol, P.; Sangtanoo, P.; Songserm, P.; Watsuntorn, W.; Karnchanatat, A. Microalgae-based wastewater treatment for developing economic and environmental sustainability: Current status and future prospects. *Front. Bioeng. Biotechnol.* **2022**, *10*, 1048819. [CrossRef]
8. Kuo, C.M.; Chen, T.Y.; Lin, T.H.; Kao, C.Y.; Lai, J.T.; Chang, J.S.; Lin, C.S. Cultivation of *Chlorella* sp. GD using piggery wastewater for biomass and lipid production. *Bioresour. Technol.* **2015**, *194*, 326–333. [CrossRef]
9. Ahmad, A.; Hassan, S.; Banat, F. An overview of microalgae biomass as a sustainable aquaculture feed ingredient: Food security and circular economy. *Bioengineered* **2022**, *13*, 9521–9547. [CrossRef]
10. Gouda, M.; Tadda, M.A.; Zhao, Y.; Farmanullah, F.; Chu, B.; Li, X.; He, Y. Microalgae Bioactive Carbohydrates as a Novel Sustainable and Eco-Friendly Source of Prebiotics: Emerging Health Functionality and Recent Technologies for Extraction and Detection. *Front. Nutr.* **2022**, *9*, 806692. [CrossRef]
11. Sui, Y.; Vlaeminck, S.E. Effects of salinity, pH and growth phase on the protein productivity by *Dunaliella salina*. *J. Chem. Technol. Biotechnol.* **2019**, *94*, 1032–1040. [CrossRef]
12. Baldisserotto, C.; Sabia, A.; Giovanardi, M.; Ferroni, L.; Maglie, M.; Pancaldi, S. Chlorophyta microalgae as dietary protein supplement: A comparative analysis of productivity related to photosynthesis. *J. Appl. Phycol.* **2022**, *34*, 1323–1340. [CrossRef]
13. Udayan, A.; Pandey, A.K.; Sirohi, R.; Sreekumar, N.; Sang, B.-I.; Sim, S.J.; Kim, S.H.; Pandey, A. Production of microalgae with high lipid content and their potential as sources of nutraceuticals. *Phytochem. Rev.* **2022**. [CrossRef] [PubMed]
14. Ferreira, G.F.; Ríos Pinto, L.F.; Maciel Filho, R.; Fregolente, L.V. A review on lipid production from microalgae: Association between cultivation using waste streams and fatty acid profiles. *Renew. Sustain. Energy Rev.* **2019**, *109*, 448–466. [CrossRef]
15. Deshmukh, S.; Kumar, R.; Bala, K. Microalgae biodiesel: A review on oil extraction, fatty acid composition, properties and effect on engine performance and emissions. *Fuel Process. Technol.* **2019**, *191*, 232–247. [CrossRef]
16. Bayly, G.R. Lipids and disorders of lipoprotein metabolism. In *Clinical Biochemistry: Metabolic and Clinical Aspects*; Elsevier: Amsterdam, The Netherlands, 2014; pp. 702–736.
17. Maltsev, Y.; Maltseva, K. Fatty acids of microalgae: Diversity and applications. *Rev. Environ. Sci. Bio/Technol.* **2021**, *20*, 515–547. [CrossRef]
18. Babu, S.S.; Gondi, R.; Vincent, G.S.; JohnSamuel, G.C.; Jeyakumar, R.B. Microalgae Biomass and Lipids as Feedstock for Biofuels: Sustainable Biotechnology Strategies. *Sustainability* **2022**, *14*, 15070. [CrossRef]
19. Akubude, V.C.; Nwaigwe, K.N.; Dintwa, E. Production of biodiesel from microalgae via nanocatalyzed transesterification process: A review. *Mater. Sci. Energy Technol.* **2019**, *2*, 216–225. [CrossRef]
20. Farfan-Cabrera, L.I.; Franco-Morgado, M.; González-Sánchez, A.; Pérez-González, J.; Marín-Santibáñez, B.M. Microalgae Biomass as a New Potential Source of Sustainable Green Lubricants. *Molecules* **2022**, *27*, 1205. [CrossRef]

21. Moreira, J.B.; Vaz, B.d.S.; Cardias, B.B.; Cruz, C.G.; Almeida, A.C.A.d.; Costa, J.A.V.; Morais, M.G.d. Microalgae Polysaccharides: An Alternative Source for Food Production and Sustainable Agriculture. *Polysaccharides* **2022**, *3*, 441–457. [CrossRef]
22. Lopez-Pacheco, I.Y.; Rodas-Zuluaga, L.I.; Cuellar-Bermudez, S.P.; Hidalgo-Vázquez, E.; Molina-Vazquez, A.; Araújo, R.G.; Martínez-Ruiz, M.; Varjani, S.; Barceló, D.; Iqbal, H.M.N.; et al. Revalorization of Microalgae Biomass for Synergistic Interaction and Sustainable Applications: Bioplastic Generation. *Mar. Drugs* **2022**, *20*, 601. [CrossRef]
23. De Carvalho Silvello, M.A.; Severo Gonçalves, I.; Patrícia Held Azambuja, S.; Silva Costa, S.; Garcia Pereira Silva, P.; Oliveira Santos, L.; Goldbeck, R. Microalgae-based carbohydrates: A green innovative source of bioenergy. *Bioresour. Technol.* **2022**, *344*, 126304. [CrossRef] [PubMed]
24. Amorim, M.L.; Soares, J.; Coimbra, J.S.D.R.; Leite, M.D.O.; Albino, L.F.T.; Martins, M.A. Microalgae proteins: Production, separation, isolation, quantification, and application in food and feed. *Crit. Rev. Food Sci. Nutr.* **2021**, *61*, 1976–2002. [CrossRef] [PubMed]
25. Bertsch, P.; Böcker, L.; Mathys, A.; Fischer, P. Proteins from microalgae for the stabilization of fluid interfaces, emulsions, and foams. *Trends Food Sci. Technol.* **2021**, *108*, 326–342. [CrossRef]
26. Morales, M.; Aflalo, C.; Bernard, O. Microalgal lipids: A review of lipids potential and quantification for 95 phytoplankton species. *Biomass Bioenergy* **2021**, *150*, 106108. [CrossRef]
27. De Luca, M.; Pappalardo, I.; Limongi, A.R.; Viviano, E.; Radice, R.P.; Todisco, S.; Martelli, G.; Infantino, V.; Vassallo, A. Lipids from Microalgae for Cosmetic Applications. *Cosmetics* **2021**, *8*, 52. [CrossRef]
28. Farfan-Cabrera, L.I.; Rojo-Valerio, A.; Calderon-Najera, J.D.D.; Coronado-Apodaca, K.G.; Iqbal, H.M.N.; Parra-Saldivar, R.; Franco-Morgado, M.; Elias-Zuñiga, A. Microalgae Oil-Based Metal Working Fluids for Sustainable Minimum Quantity Lubrication (MQL) Operations—A Perspective. *Lubricants* **2023**, *11*, 215. [CrossRef]
29. Wang, Y.; Tibbetts, S.; McGinn, P. Microalgae as Sources of High-Quality Protein for Human Food and Protein Supplements. *Foods* **2021**, *10*, 3002. [CrossRef]
30. Cheng, C.-L.; Lo, Y.-C.; Huang, K.-L.; Nagarajan, D.; Chen, C.-Y.; Lee, D.-J.; Chang, J.-S. Effect of pH on biomass production and carbohydrate accumulation of *Chlorella vulgaris* JSC-6 under autotrophic, mixotrophic, and photoheterotrophic cultivation. *Bioresour. Technol.* **2022**, *351*, 127021. [CrossRef]
31. Cheirsilp, B.; Torpee, S. Enhanced growth and lipid production of microalgae under mixotrophic culture condition: Effect of light intensity, glucose concentration and fed-batch cultivation. *Bioresour. Technol.* **2012**, *110*, 510–516. [CrossRef]
32. Salman, J.M.; Grmasha, R.A.; Stenger-Kovács, C.; Lengyel, E.; Al-sareji, O.J.; AL-Cheban, A.M.A.A.; Meiczinger, M. Influence of magnesium concentrations on the biomass and biochemical variations in the freshwater algae, *Chlorella vulgaris*. *Heliyon* **2023**, *9*, e13072. [CrossRef] [PubMed]
33. Beigbeder, J.-B.; Lavoie, J.-M. Effect of photoperiods and CO_2 concentrations on the cultivation of carbohydrate-rich *P. kessleri* microalgae for the sustainable production of bioethanol. *J. CO_2 Util.* **2022**, *58*, 101934. [CrossRef]
34. Morales-Sánchez, D.; Schulze, P.S.C.; Kiron, V.; Wijffels, R.H. Production of carbohydrates, lipids and polyunsaturated fatty acids (PUFA) by the polar marine microalga *Chlamydomonas malina* RCC2488. *Algal Res.* **2020**, *50*, 102016. [CrossRef]
35. Josephine, A.; Kumar, T.S.; Surendran, B.; Rajakumar, S.; Kirubagaran, R.; Dharani, G. Evaluating the effect of various environmental factors on the growth of the marine microalgae, *Chlorella vulgaris*. *Front. Mar. Sci.* **2022**, *9*, 954622. [CrossRef]
36. Zarrinmehr, M.J.; Farhadian, O.; Heyrati, F.P.; Keramat, J.; Koutra, E.; Kornaros, M.; Daneshvar, E. Effect of nitrogen concentration on the growth rate and biochemical composition of the microalga, *Isochrysis galbana*. *Egypt. J. Aquat. Res.* **2020**, *46*, 153–158. [CrossRef]
37. Chin, G.J.W.L.; Andrew, A.R.; Abdul-Sani, E.R.; Yong, W.T.L.; Misson, M.; Anton, A. The effects of light intensity and nitrogen concentration to enhance lipid production in four tropical microalgae. *Biocatal. Agric. Biotechnol.* **2023**, *48*, 102660. [CrossRef]
38. Lovio Fragoso, J.; Hayano Kanashiro, C.; Lopez Elias, J. Effect of different phosphorus concentrations on growth and biochemical composition of *Chaetoceros muelleri*. *Lat. Am. J. Aquat. Res.* **2019**, *47*, 361–366. [CrossRef]
39. Li, M.; Li, Y.; Zhang, Y.; Xu, Q.; Iqbal, M.S.; Xi, Y.; Xiang, X. The significance of phosphorus in algae growth and the subsequent ecological response of consumers. *J. Freshw. Ecol.* **2022**, *37*, 57–69. [CrossRef]
40. Mao, X.; Lao, Y.; Sun, H.; Li, X.; Yu, J.; Chen, F. Time-resolved transcriptome analysis during transitions of sulfur nutritional status provides insight into triacylglycerol (TAG) and astaxanthin accumulation in the green alga *Chromochloris zofingiensis*. *Biotechnol. Biofuels* **2020**, *13*, 128. [CrossRef]
41. Cakmak, T.; Angun, P.; Ozkan, A.D.; Cakmak, Z.; Olmez, T.T.; Tekinay, T. Nitrogen and sulfur deprivation differentiate lipid accumulation targets of *Chlamydomonas reinhardtii*. *Bioengineered* **2012**, *3*, 343–346. [CrossRef]
42. Krzemińska, I.; Szymańska, M.; Ciempiel, W.; Piasecka, A. Auxin supplementation under nitrogen limitation enhanced oleic acid and MUFA content in *Eustigmatos calaminaris* biomass with potential for biodiesel production. *Sci. Rep.* **2023**, *13*, 594. [CrossRef] [PubMed]
43. Cheng, D.; Li, D.; Yuan, Y.; Zhou, L.; Li, X.; Wu, T.; Wang, L.; Zhao, Q.; Wei, W.; Sun, Y. Improving carbohydrate and starch accumulation in *Chlorella* sp. AE10 by a novel two-stage process with cell dilution. *Biotechnol. Biofuels* **2017**, *10*, 75. [CrossRef] [PubMed]
44. Michelon, W.; Da Silva, M.L.B.; Mezzari, M.P.; Pirolli, M.; Prandini, J.M.; Soares, H.M. Effects of Nitrogen and Phosphorus on Biochemical Composition of Microalgae Polyculture Harvested from Phycoremediation of Piggery Wastewater Digestate. *Appl. Biochem. Biotechnol.* **2016**, *178*, 1407–1419. [CrossRef]

45. Zhuang, L.-L.; Azimi, Y.; Yu, D.; Wu, Y.-H.; Hu, H.-Y. Effects of nitrogen and phosphorus concentrations on the growth of microalgae *Scenedesmus*. LX1 in suspended-solid phase photobioreactors (ssPBR). *Biomass Bioenergy* **2018**, *109*, 47–53. [CrossRef]
46. Wang, Y.; Xu, H.; Yang, J.; Zhou, Y.; Wang, X.; Dou, S.; Li, L.; Liu, G.; Yang, M. Effect of sulfur limitation strategies on glucose-based carbohydrate production from *Chlorella sorokiniana*. *Renew. Energy* **2022**, *200*, 449–456. [CrossRef]
47. Narchonai, G.; Arutselvan, C.; LewisOscar, F.; Thajuddin, N. Enhancing starch accumulation/production in *Chlorococcum humicola* through sulphur limitation and 2,4-D treatment for butanol production. *Biotechnol. Rep.* **2020**, *28*, e00528. [CrossRef]
48. Subramanian, S.; Barry, A.N.; Pieris, S.; Sayre, R.T. Comparative energetics and kinetics of autotrophic lipid and starch metabolism in chlorophytic microalgae: Implications for biomass and biofuel production. *Biotechnol. Biofuels* **2013**, *6*, 150. [CrossRef]
49. Fattore, N.; Bellan, A.; Pedroletti, L.; Vitulo, N.; Morosinotto, T. Acclimation of photosynthesis and lipids biosynthesis to prolonged nitrogen and phosphorus limitation in *Nannochloropsis gaditana*. *Algal Res.* **2021**, *58*, 102368. [CrossRef]
50. Bišová, K.; Zachleder, V. Cell-cycle regulation in green algae dividing by multiple fission. *J. Exp. Bot.* **2014**, *65*, 2585–2602. [CrossRef]
51. Tan, K.W.M.; Lin, H.; Shen, H.; Lee, Y.K. Nitrogen-induced metabolic changes and molecular determinants of carbon allocation in *Dunaliella tertiolecta*. *Sci. Rep.* **2016**, *6*, 37235. [CrossRef]
52. Geigenberger, P. Regulation of Starch Biosynthesis in Response to a Fluctuating Environment. *Plant Physiol.* **2011**, *155*, 1566–1577. [CrossRef] [PubMed]
53. Ran, W.; Wang, H.; Liu, Y.; Qi, M.; Xiang, Q.; Yao, C.; Zhang, Y.; Lan, X. Storage of starch and lipids in microalgae: Biosynthesis and manipulation by nutrients. *Bioresour. Technol.* **2019**, *291*, 121894. [CrossRef] [PubMed]
54. Xu, H.; Yang, J.; Wang, X.; Peng, Q.; Han, Y.; Liu, X.; Liu, K.; Dou, S.; Li, L.; Liu, G.; et al. Starch accumulation dynamics and transcriptome analysis of *Chlorella sorokiniana* during transition of sulfur nutritional status. *Front. Mar. Sci.* **2022**, *9*, 986400. [CrossRef]
55. Rosa, R.M.; Machado, M.; Vieira Vaz, M.G.M.; dos Santos, R.L.; do Nascimento, A.G.; Araújo, W.L.; Nunes-Nesi, A. Urea as a source of nitrogen and carbon leads to increased photosynthesis rates in *Chlamydomonas reinhardtii* under mixotrophy. *J. Biotechnol.* **2023**, *367*, 20–30. [CrossRef] [PubMed]
56. Ma, R.; Zhang, Z.; Tang, Z.; Ho, S.-H.; Shi, X.; Liu, L.; Xie, Y.; Chen, J. Enhancement of co-production of lutein and protein in *Chlorella sorokiniana* FZU60 using different bioprocess operation strategies. *Bioresour. Bioprocess.* **2021**, *8*, 82. [CrossRef]
57. Kamalanathan, M.; Pierangelini, M.; Shearman, L.A.; Gleadow, R.; Beardall, J. Impacts of nitrogen and phosphorus starvation on the physiology of *Chlamydomonas reinhardtii*. *J. Appl. Phycol.* **2016**, *28*, 1509–1520. [CrossRef]
58. Schmollinger, S.; Mühlhaus, T.; Boyle, N.R.; Blaby, I.K.; Casero, D.; Mettler, T.; Moseley, J.L.; Kropat, J.; Sommer, F.; Strenkert, D.; et al. Nitrogen-Sparing Mechanisms in *Chlamydomonas* Affect the Transcriptome, the Proteome, and Photosynthetic Metabolism. *Plant Cell* **2014**, *26*, 1410–1435. [CrossRef]
59. Plouviez, M.; Abyadeh, M.; Hasan, M.; Mirzaei, M.; Paulo, J.A.; Guieysse, B. The proteome of *Chlamydomonas reinhardtii* during phosphorus depletion and repletion. *Algal Res.* **2023**, *71*, 103037. [CrossRef]
60. Shang, C.; Zhu, S.; Wang, Z.; Qin, L.; Alam, M.A.; Xie, J.; Yuan, Z. Proteome response of *Dunaliella parva* induced by nitrogen limitation. *Algal Res.* **2017**, *23*, 196–202. [CrossRef]
61. Estevam, B.R.; Pinto, L.F.R.; Filho, R.M.; Fregolente, L.V. Growth and Metabolite Production in *Chlorella* sp.: Analysis of Cultivation System and Nutrient Reduction. *BioEnergy Res.* **2022**. [CrossRef]
62. Salbitani, G.; Perrone, A.; Rosati, L.; Laezza, C.; Carfagna, S. Sulfur Starvation in Extremophilic Microalga *Galdieria sulphuraria*: Can Glutathione Contribute to Stress Tolerance? *Plants* **2022**, *11*, 481. [CrossRef] [PubMed]
63. Irihimovitch, V.; Yehudai-Resheff, S. Phosphate and sulfur limitation responses in the chloroplast of *Chlamydomonas reinhardtii*. *FEMS Microbiol. Lett.* **2008**, *283*, 1–8. [CrossRef]
64. Xu, L.; Cheng, X.; Wang, Q. Enhanced Lipid Production in *Chlamydomonas reinhardtii* by Co-culturing with *Azotobacter chroococcum*. *Front. Plant Sci.* **2018**, *9*, 741. [CrossRef]
65. Yadavalli, R.; Ratnapuram, H.; Motamarry, S.; Reddy, C.N.; Ashokkumar, V.; Kuppam, C. Simultaneous production of flavonoids and lipids from *Chlorella vulgaris* and *Chlorella pyrenoidosa*. *Biomass Convers. Biorefinery* **2022**, *12*, 683–691. [CrossRef]
66. Gao, B.; Wang, F.; Huang, L.; Liu, H.; Zhong, Y.; Zhang, C. Biomass, lipid accumulation kinetics, and the transcriptome of heterotrophic oleaginous microalga *Tetradesmus bernardii* under different carbon and nitrogen sources. *Biotechnol. Biofuels* **2021**, *14*, 4. [CrossRef]
67. Sirikhachornkit, A.; Suttangkakul, A.; Vuttipongchaikij, S.; Juntawong, P. De novo transcriptome analysis and gene expression profiling of an oleaginous microalga *Scenedesmus acutus* TISTR8540 during nitrogen deprivation-induced lipid accumulation. *Sci. Rep.* **2018**, *8*, 3668. [CrossRef] [PubMed]
68. Yang, F.; Xiang, W.; Li, T.; Long, L. Transcriptome analysis for phosphorus starvation-induced lipid accumulation in *Scenedesmus* sp. *Sci. Rep.* **2018**, *8*, 16420. [CrossRef] [PubMed]
69. Çakmak, Z.E.; Ölmez, T.T.; Çakmak, T.; Menemen, Y.; Tekinay, T. Induction of triacylglycerol production in *Chlamydomonas reinhardtii*: Comparative analysis of different element regimes. *Bioresour. Technol.* **2014**, *155*, 379–387. [CrossRef] [PubMed]
70. Gómez-De la Torre, A.E.; Ochoa-Alfaro, A.E.; Rocha-Uribe, A.; Soria-Guerra, R.E. Effects of sulfur and phosphorus concentration on the lipid accumulation and fatty acid profile in *Chlorella vulgaris* (Chlorophyta). *Folia Microbiol.* **2023**, *68*, 453–463. [CrossRef]

71. Boyle, N.R.; Page, M.D.; Liu, B.; Blaby, I.K.; Casero, D.; Kropat, J.; Cokus, S.J.; Hong-Hermesdorf, A.; Shaw, J.; Karpowicz, S.J.; et al. Three Acyltransferases and Nitrogen-responsive Regulator Are Implicated in Nitrogen Starvation-induced Triacylglycerol Accumulation in *Chlamydomonas*. *J. Biol. Chem.* **2012**, *287*, 15811–15825. [CrossRef]
72. Zheng, S.; Zou, S.; Feng, T.; Sun, S.; Guo, X.; He, M.; Wang, C.; Chen, H.; Wang, Q. Low temperature combined with high inoculum density improves alpha-linolenic acid production and biochemical characteristics of *Chlamydomonas reinhardtii*. *Bioresour. Technol.* **2022**, *348*, 126746. [CrossRef] [PubMed]
73. Wang, X.; Fosse, H.K.; Li, K.; Chauton, M.S.; Vadstein, O.; Reitan, K.I. Influence of Nitrogen Limitation on Lipid Accumulation and EPA and DHA Content in Four Marine Microalgae for Possible Use in Aquafeed. *Front. Mar. Sci.* **2019**, *6*, 95. [CrossRef]
74. López-legarda, X.; Taramuel-gallardo, A.; Arboleda-, C. Comparison of methods using sulfuric acid for determination of total sugars. *Rev. Cuba. Quím.* **2017**, *29*, 180–198.
75. Mishra, S.K.; Suh, W.I.; Farooq, W.; Moon, M.; Shrivastav, A.; Park, M.S.; Yang, J. Rapid quantification of microalgal lipids in aqueous medium by a simple colorimetric method. *Bioresour. Technol.* **2014**, *155*, 330–333. [CrossRef] [PubMed]
76. Bligh, E.G.; Dyer, W.J. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **1959**, *37*, 911–917. [CrossRef]

MDPI
St. Alban-Anlage 66
4052 Basel
Switzerland
www.mdpi.com

Marine Drugs Editorial Office
E-mail: marinedrugs@mdpi.com
www.mdpi.com/journal/marinedrugs

Academic Open
Access Publishing

mdpi.com

ISBN 978-3-0365-9089-9