agriculture

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Freshwater *Cladophora glomerata* Biomass as Promising Protein and Other Essential Nutrients Source for High Quality and More Sustainable Feed Production

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Abstract: A scientific justification, focused on the development of the sustainability of feed ingredients and farm animals' ecosystems, is required. Thus, it is crucial to search for alternative feed materials from natural sources for potential applications. The aim of this study was to evaluate the prospective utilization of freshwater *Cladophora glomerata* (*C. glomerata*) as an alternative source of protein and other essential nutrients in animals' feed. For this purpose, chemical analysis was performed on collected biomass samples from the Lithuanian rivers, Dubysa (B1), Šventoji (B2), Nevėžis (B3), and Jūra (B4). Microelements (Ca > K > N > P > Mg), trace elements (Zn > Cu), and heavy metals (Cr > Ni > Pb > Cd) have not exceeded permissible levels. The crude protein content of *C. glomerata* biomass ranged from 16% to 21.5% DM. The essential amino acid profile excelled, with the highest total amino acid amount of 140.99 g/kg in B4. The highest total presence of polyunsaturated fatty acids (11.71%) as well as the ratio of polyunsaturated to saturated fatty acids (0.22) was observed in B1. The lowest ratio of omega-6/omega-3 was in B1 (1.30). As a result of bioaccumulation, *C. glomerata* could serve as a source of proteins, as well as amino and fatty acids, implying that biomass could be an alternative and a beneficial component of animal feed.

Keywords: macroalgal biomass; *Cladophora glomerata*; amino acids; fatty acids; feed alternatives; sustainability

Citation: Nutautaitė, M.; Vilienė, V.; Raceviciūtė-Stupelienė, A.; Bliznikas, S.; Karosienė, J.; Koreivienė, J. Freshwater *Cladophora glomerata* Biomass as Promising Protein and Other Essential Nutrients Source for High Quality and More Sustainable Feed Production. *Agriculture* **2021**, *11*, 582. https://doi.org/10.3390/agriculture11070582

Academic Editors: Vito Laudadio and Vincenzo Tufarelli

Received: 16 June 2021
Accepted: 22 June 2021
Published: 24 June 2021

1. Introduction

The increasing population is directly correlated with the increasing consumption of animal products [1]. Therefore, a long-term strategy for the intensive, but sustainable, development of livestock farming is essential. Clearly, even according to optimistic predictions, a shortage of traditional protein feed components is inevitable. Intensified development of sustainable livestock farming, while applying the use of innovative or alternative feed additives, could contribute to the sustainability of any animals' ecosystem. For example, materials from aquatic ecosystems such as algal biomass as protein and other essential nutrients source could be applied in feed production [2–4]. This would help to alleviate the scarcity of fodder feed materials, and the incorporation of algal biomass into feed production would help to address another global issue: greenhouse gas emissions from livestock activities [5]. Since the cultivation of algae requires small areas and their reproduction is rapid, or they are simply found naturally in nature, the cultivation of fodder crops could be optimized.

It is necessary to search for and analyse the potential applications of alternative feed materials by ensuring their high quality, safety, and sustainability, taking into account anti-nutritional factors and potential toxicity. Algal biomass is characterized as a source of essential vitamins, minerals, proteins, polyunsaturated fatty acids, and antioxidants [6–8]. The high potential of algae derives from the fact that they are not as well-known as agricultural crops, that they can be grown in places where other plants cannot, and the productivity of algae exceeds higher plants many times. Since algae use solar resources more effectively, their ability to produce useful compounds and biomass is generally acknowledged, and they can be utilized to improve the nutritional value of food and feed [9]. Macroalgae, like microalgae, blend higher plant features with biotechnological properties unique to microbial cells. Namely, they can reproduce quickly in a liquid medium with ordinary nutritional requirements and still accumulate certain metabolites. *Cladophora* species, whether they are marine or freshwater, are ecologically and economically important macroalgae. These species provide essential ecosystem services, and their biomass is used for a variety of applications, including soil additives, fertilizers, plant growth biostimulants, food and animal feed, phycocolloids, nutraceuticals, pharmaceuticals, cosmetics, wastewater treatment, and renewable biofuel production [10,11]. *Cladophora* species are recommended as an important substitute for human food and animal feeding due to their high protein content [12]. Thus, in the food and feed industry, the species is used as a biomass with a low-calorie content and a high variety of nutrients, vitamins, and fibre [13].

The development of more sustainable feed materials and food supply chains and the sustainability of the farm animals' ecosystem require a scientific justification for the integration of alternative protein materials into new or existing animal feed. The specific *Cladophora glomerata* (*C. glomerata*) has a rich spectrum of bioactive components, which is reflected in its chemical composition. Therefore, such biomass would be adequate for use as a feed additive in the prism of today's issues. Filamentous green algae *C. glomerata* thrives and forms large communities in nutrient-rich water bodies, especially in slow-flowing rivers [14]. However, blooming caused by *C. glomerata* decreases biodiversity since the assemblage is mostly composed of only one green algae species, reducing the recreational value of water bodies, and creating a detrimental ecological and economic impact. Therefore, by harvesting excess macroalgal biomass from water bodies and integrating it into feed production, the waste would be adapted as a raw material, creating a sustainable production chain. Thus, the aim of this study was to evaluate the potential of freshwater *C. glomerata* biomass, from four different Lithuanian rivers, to be utilized as an alternative source of protein and other essential nutrients in animals' feed by conducting detailed biomass chemical analysis and defining profiles of amino and fatty acids.

2. Materials and Methods

2.1. C. glomerata Biomass Collection and Preparation

Freshwater *C. glomerata* biomass was collected in August–September 2019 from four Lithuanian rivers: Dubysa (N55°12′25.07″, E23°30′30.44″; B1), Šventoji (N55°39′20.14″, E25°10′18.39″; B2), Nevėžis (N55°5′46.52″, E23°46′55.57″; B3) and Jūra (N55°27′19.58″, E22°2′14.72″; B4) (Figure 1). The rivers were selected due to the presence of dense agglomerations of *C. glomerata*, usually covering over 50% of the river bottom area (Figure 1; Table 1). However, rivers differ by the catchment area and the water chemistry. Nutrient concentrations in the rivers were high: total nitrogen (TN) varied from 0.18 to 1.12 mg/L and total phosphorus (TP) from 0.012 to 0.073 mg/L (Table 1). The highest concentrations of nutrients and conductivity were determined in River Nevėžis, where *C. glomerata* was dominant among primary producers occupying a large area and building up a high quantity of excessive biomass. River Dubysa was distinguished by the lowest nutrient amount.

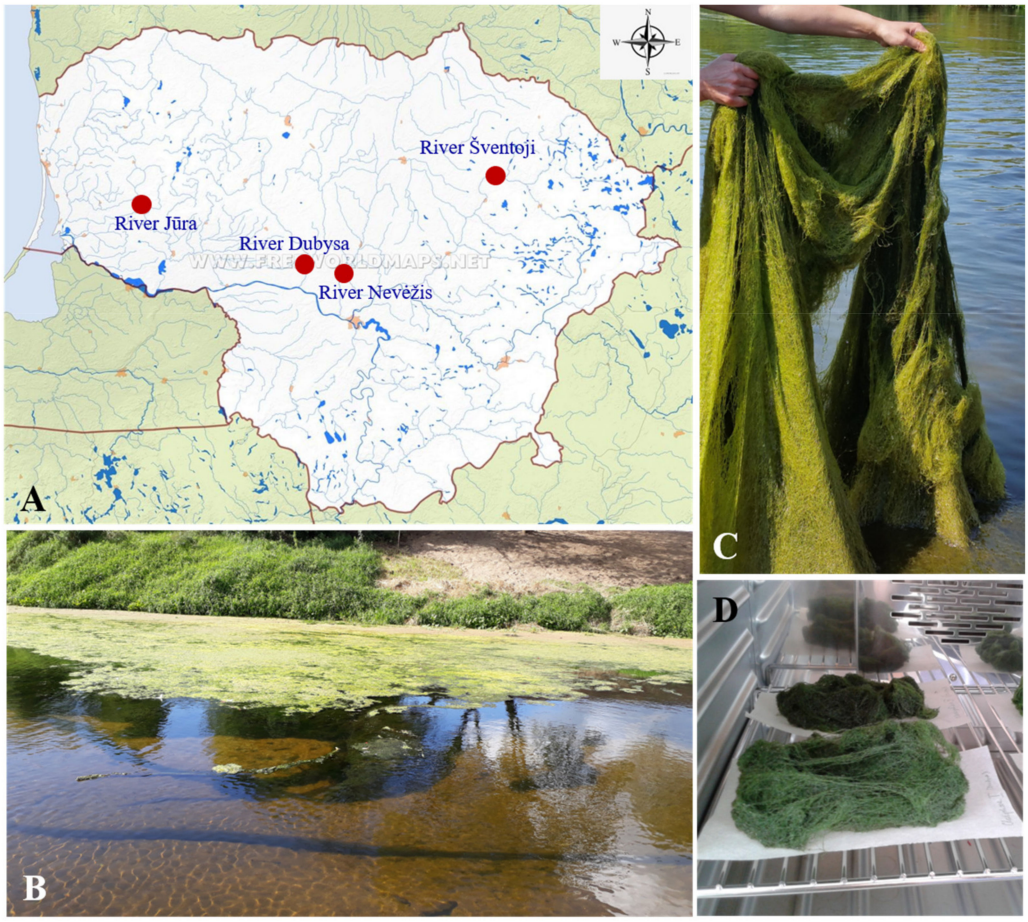

Figure 1. (**A**) The locations of *C. glomerata* biomass collection in the rivers of Lithuania; (**B**) Agglomerations of macroalgal *C. glomerata* biomass in River Nevėžis; (**C**) Collection of *C. glomerata* biomass; (**D**) Dried biomass of macroalgae.

Table 1. Characteristics of the rivers and the abundance of *Cladophora* macroalgae at the sampling sites.

River	* River length, km	* Catchment Area, km^2	* Average Annual Discharge at the Mouth, m^3/s	Current Velocity, m/s	TP, mg/L	TN, mg/L	Water Temperature, °C	pH	Conductivity, µS/cm	Bottom Coverage by *C. glomerata*, %
Dubysa (B1)	131	1972	14.2	0.11–0.57	0.012–0.035	0.18–0.40	16.4–20.1	8.41–8.80	535–581	40–90
Šventoji (B2)	246	6889	56.5	0.26–0.32	0.037–0.070	0.53–0.99	17.6–18.9	8.40–9.19	479–487	45–95
Jūra (B3)	172	3994	38	0.11–0.53	0.031–0.073	0.28–0.64	14.9–18.0	8.56–9.21	420–483	50–95
Nevėžis (B4)	209	6146	32.7	0.10–0.13	0.500–0.520	0.84–1.12	18.2–19.2	8.54–8.62	948–977	50–100

* Kilkus and Stonevičius [15].

Fresh macroalgal biomass was manually harvested from the river (Figure 1). Subsamples of about 1 kg of wet biomass were collected from up to 6 sites and intermixed altogether. Macroalgal biomass was several times carefully washed with tap water to remove sand and mud particles. Macrozoobenthos, macrophytes and other debris were

manually removed from the biomass. The samples were dried in an oven at 60 °C overnight and stored in closed plastic bags at room temperature until the analysis.

2.2. C. glomerata Biomass Chemical Analysis

Macroalgal biomass samples were analysed in accordance with the Commission regulation (EU) No 691/2013 of 19 July 2013 amending Regulation (EC) No 152/2009 as regards methods of sampling and analysis. Chemical analysis to determine individual elements in the dry biomass was performed in the accredited research laboratory following methods specified in Table 2.

Table 2. Methods by which each element was determined in *C. glomerata* biomass.

Element	Method	Element	Method
N	The Commission Directive 72/199/EEC	Zn Cu	LST EN ISO 15510:2017
P	Directive 71/393/EEC	Cr	
K Ca	Directive 71/250/EEC	Ni Cd	LST EN ISO 15550:2017
Mg	Directive 73/46/EEC	Pb	

C. glomerata samples from different Lithuanian rivers with each river's three replicates were grinded for further chemical analysis. The dry matter of algal biomass was determined by drying it in an oven at 105 °C until a constant weight and dry matter yield was calculated. Dried *C. glomerata* biomass samples were used for further laboratory analyses described below. Crude protein content was determined by the Kjeldahl method. A conversion factor of 6.25 was used to convert total nitrogen to crude protein [16]. Crude fat was extracted with petroleum ether (boiling range of 40–60 °C) by the Soxhlet extraction method. Crude ash was determined by incineration in a muffle furnace at 550 °C for 3 h (Commission Regulation (EC) No. 152/2009). Crude fibre was determined as the residue after sequential treatment with hot (100 °C) H_2SO_4 (conc. 1.25%) and hot (100 °C) NaOH (1.25%) according to the Weende method by the FiberCap system (Foss Tecator AB, Höganäs, Sweden).

2.3. C. glomerata Biomass Amino Acids Profile Analysis

The hydrolysis of algal biomass samples for amino acid analysis followed the procedures outlined in Commission Regulation (EC) No. 152/2009. The amino acid assay was performed by the AccQ Tag technology (Waters Corp., Milford, MA, USA). For amino acid analyses in samples, the Shimadzu low pressure gradient HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of solvent delivery module LC-10AT_{VP}, auto-injector SIL-10AD_{VP}, column oven CTO-10AC_{VP}, spectrofluorometric detector RF-10A_{XL}, system controller SCL-10A_{VP}, online degasser DGU-14A was used. For HPLC system control and data collection, the Workstation LC Solution (Shimadzu Corp., Kyoto, Japan) was used. Amino acid derivatives were separated on a Nova-Pak C18, 4 mm, 150 × 3.9 mm chromatography column (Waters Corp., Milford, MA, USA) at a temperature of 37 °C. For separation, 10 µL of each derivate was injected into the column. Separated derivatives were detected at the Ex 250 nm–Em 395 nm wavelength. A gradient flow was used for the separation of amino acid derivatives. The flow rate was set at 1 mL/min. The mobile phase consisted of eluent A (prepared from Waters AccQ Tag Eluent A concentrate by diluting 100 mL of concentrate to 1 L of ultrapure water), eluent B (acetonitrile) and eluent C (ultrapure water). Amino acids were identified by the retention times as compared to the retention times of the amino acids in the standard solution. The results were calculated by measuring the peak areas of the sample and the standard solution for each amino acid.

2.4. C. glomerata Biomass Fatty Acids Profile Analysis

Lipid extraction from biomass samples for further fatty acids analysis was performed with a chloroform-methanol (2:1) mixture as described by Folch et al. [17]. Exactly 0.5 g of algal biomass was taken, and 10 mL of extraction mixture was added. The extraction was carried out by leaving the mixture overnight at room temperature. The samples were filtered, then 20–40 mL of 0.74% KCl solution was added, shaken for 1–2 min, and left for 10–12 h to completely separate the layers. The lower layer was transferred via syringe to 20 mL tubes and evaporated at 50 °C on a vacuum thermostat. The resulting fat was methylated with freshly prepared 2% sodium methylate (NaOMe) solution according to Christopherson and Glass [18]. Then, 5 mL of NaOMe was added to the tube with fat, shaken and left for 1 h at room temperature. Subsequently, 7 mL of 1N HCl, 5 mL of hexane and 2 mL of H_2O were added. The tubes were stoppered and shaken for 1 min for better layer separation. Exactly 2 mL of the top layer was transferred to conical tubes and evaporated. The resulting mixtures of fatty acid methyl esters were analysed with a GC-2010 (Shimadzu Corp., Kyoto, Japan) gas chromatograph with FID detector (Shimadzu Corp., Kyoto, Japan). The Capillary column ATTM-FAME (30 m, ID: 0.25 mm) (Alltech Associates Inc., Deerfield, IL., USA) was used. The column temperature change was programmed from 150 °C to 240 °C. Inlet temperature—240 °C, detector temperature—240 °C. Carrier gas—nitrogen, flow rate—63.0 mL/min. Analysis time—60 min. Individual fatty acids were identified by retention times when compared to the retention times of fatty acids in a mixture of known composition. The fatty acid content (% of the total acid content) was determined using a chromatographic data processing program, GCsolution (Ver. 2.32; Shimadzu Corp., Kyoto, Japan).

The average amount of each fatty acid was used to calculate the total content of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids, PUFA/SFA, and omega-6/omega-3 (n6/n3) ratios.

2.5. Statistical Analysis

The research was a completely randomized design with 4 algal biomass samples with 3 replicates. Data analysis was performed by SPSS for Windows, version 25.0 (IBM Corp., Released 2017, Armonk, NY, USA). One-way analysis of variance (ANOVA) test post-hoc (Fisher's least significant difference test) was conducted to detect differences among *C. glomerata* biomass from different rivers in Lithuania. A calculated p value of less than 0.05 ($p < 0.05$) was considered statistically significant.

3. Results

3.1. Chemical Composition of C. glomerata Biomass

In our study, individual elements were identified to assess the overall chemical composition of freshwater *C. glomerata* biomass (Figures 2 and 3). The amount of macro elements varied from 1.46 to 4.15 mg/kg DM of nitrogen (N) and 0.16–0.49 mg/kg DM of phosphorus (P) in the *C. glomerata* biomass, with the highest values observed in B3 (Figure 2). Potassium (K) varied in the range of 3.25–6.00 mg/kg DM and the highest concentration was found in B2 biomass. Particularly high levels of up to 26.34 and 27.16 mg/kg DM of calcium (Ca) were found in biomasses B1 and B4 respectively, whereas the levels of magnesium macro-mineral (Mg) were very low (0.26–0.42 mg/kg DM) in all algal tested biomasses. For the other trace minerals tested, the highest concentration of zinc (Zn) was found in B3, while the highest concentration of copper (Cu) was found in B1 *C. glomerata* biomass (Figure 3).

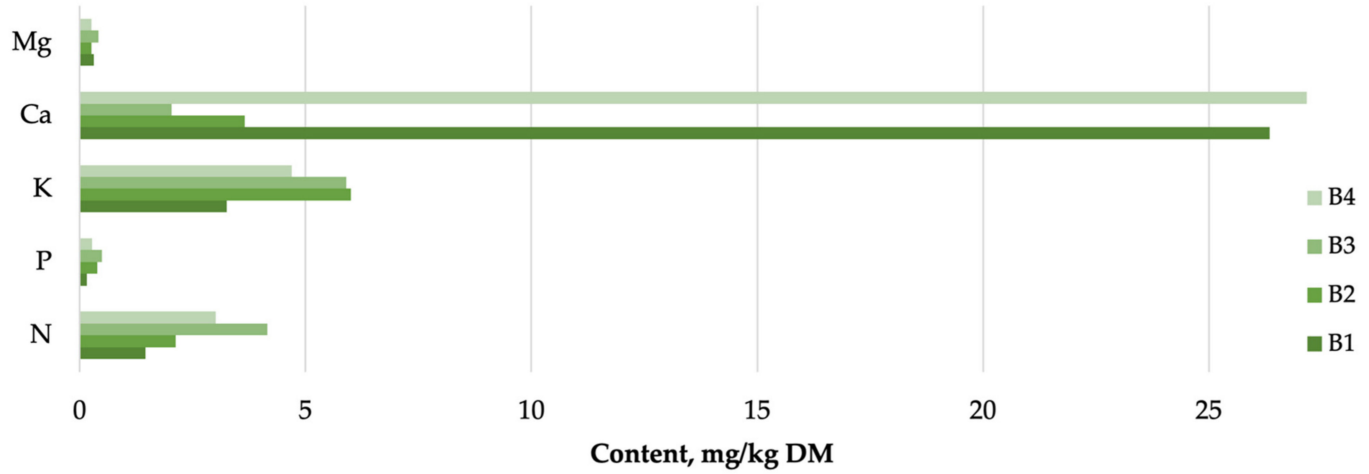

Figure 2. Separate macro elements content in *C. glomerata* biomass from different rivers in Lithuania (Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4)).

To determine potential toxicity and to define if macroalgal biomass is safe to use for animals' feed, the most important heavy metals and their contents were determined (Figure 3). The highest amount in terms of biomass was chromium (Cr) followed by nickel (Ni), whereas percentages of lead (Pb) and cadmium (Cd) were more than ten times lower.

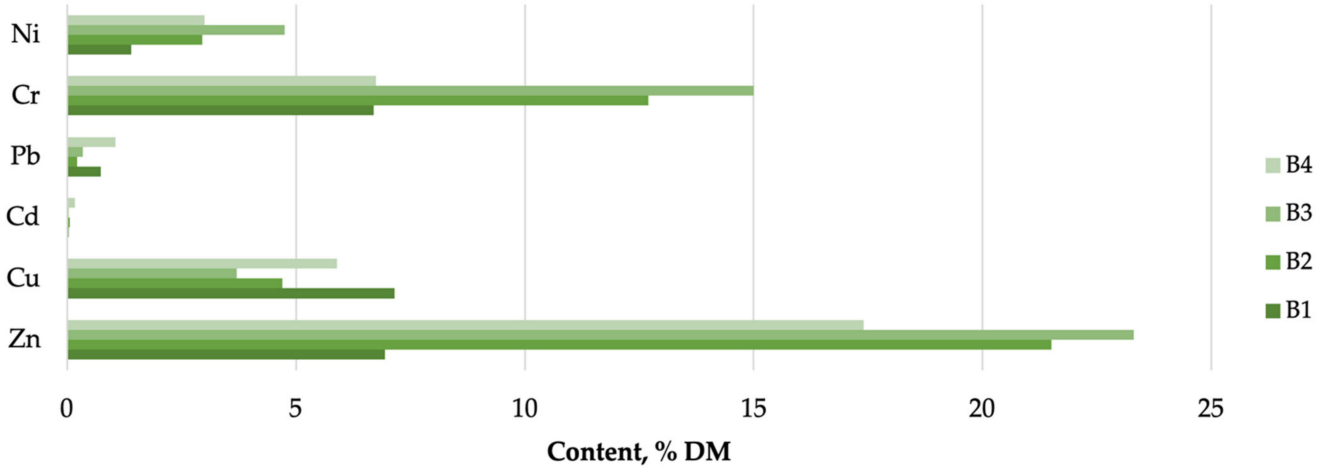

Figure 3. Separate trace elements and heavy metals content in *C. glomerata* biomass from different rivers in Lithuania (Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4)).

The chemical composition of *C. glomerata* biomass from different rivers in Lithuania is presented in Table 3. Foremost, the biomass samples that had already been dried after their collection from the rivers, were dried again to determine the dry matter (DM) content again, due to the moisture that may have formed during storage. The highest crude protein (CP) content was determined in B4 *C. glomerata* biomass ($p < 0.05$). Compared to B1, B2, and B3 samples, B4 had 14.87%, 25.74%, and 15.52%, respectively, higher CP content ($p < 0.05$). The highest content of crude fat (CF) was observed in B1 samples, significantly higher than in B2 and B3 ($p < 0.05$). After crude ash (CA) determination, this item statistically differed between all biomass samples ($p < 0.05$). However, the highest amount of CA was observed in B3 biomass and the lowest in B4 ($p < 0.05$). Crude fibre (CFB) content between all biomass samples statistically differed likewise, while the biggest difference was observed between B2 and B3, when B2 CFB content was 15.83% DM and B3 only 10.88% DM ($p < 0.05$).

Table 3. Chemical composition of *C. glomerata* biomass from different rivers in Lithuania.

	***C. glomerata* Biomass** [2,3,4]					
Item [1]	**B1**	**B2**	**B3**	**B4**	**SEM** [5]	***p* Value**
DM (% of dried samples)	94.95 [a]	92.63 [a]	95.19 [a]	91.12 [b]	0.35	0.000
			% DM			
CP	18.32 [a]	15.98 [a]	18.18 [a]	21.52 [b]	0.34	0.000
CF	0.35 [a]	0.18 [b]	0.19 [b]	0.31 [ab]	0.05	0.026
CA	48.45 [a]	39.05 [b]	49.83 [c]	36.96 [d]	0.23	0.000
CFB	13.97 [a]	15.83 [b]	10.88 [c]	13.14 [d]	0.19	0.000

Note: [1] DM, dry matter; CP, crude protein; CF, crude fat; CA, crude ash; CFB, crude fibre. [2] *C. glomerata* biomass from Lithuanian rivers, Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4). [3] Means with different superscript letters (a–d) in a row were significantly different ($p < 0.05$). [4] $n = 12$ (3 replicate samples from each river). [5] SEM, standard error of the means.

3.2. Amino Acids Profile of C. glomerata Biomass

In order to get a comprehensive profile of the amino acids in *C. glomerata,* the 16 most important and essential amino acids were identified (Table 4). In general, all data obtained after amino acids profiling of the collected macroalgal biomass significantly differed ($p < 0.05$). Except for one item, histidine content, where no significant differences between groups were found ($p > 0.05$). The total amount of amino acids was the highest in B4 (~141 g/kg) and was lower by 1/3 in B1. Glutamic acid, followed by aspartic acid and leucine, were the most prevalent amino acids in the *C. glomerata* profile from all rivers. In almost all cases, the highest concentration of a single amino acid was found in B4 biomass from the river Jūra (B4; $p < 0.05$). In terms of essential amino acids, the highest concentration of threonine was found in B4 biomass, half as large compared to B1 ($p < 0.05$). Valine content in B4 was higher than in B1, B2, and B3 biomass samples ($p < 0.05$). However, the highest concentration of the essential amino acid methionine was found in biomass B2 and compared to B4, this item was almost two times higher ($p < 0.05$). The highest concentration of the remaining essential amino acids, isoleucine, leucine, phenylalanine, and lysine, were also found in B4 *C. glomerata* biomass ($p < 0.05$), maintaining the trend. Regardless, amino acids which can be synthesized in the animal's body and are considered as "non-essential": the highest concentrations of aspartic acid, serine, glutamic acid, proline, glycine, alanine, tyrosine, and arginine, when comparing biomasses from the rivers, were determined in the B4 biomass samples again ($p < 0.05$). Overall, most of the amino acids were found in B4 biomass. Compared to the B1, B2, and B3 biomass profiles, the total amount of amino acids in B4 biomass was 37.63 g/kg, 22.74 g/kg, and 16.35 g/kg, respectively, higher ($p < 0.05$).

3.3. Fatty Acids Profile of C. glomerata Biomass

The fatty acids profile of *C. glomerata* biomass determined in our research is presented in Table 5. Palmitic (C16:0), elaidic (C18:1 n9), and myristic (C14:0) acids were dominant in the *Cladophora* biomass profile among the 33 fatty acids determined. Fatty acids with all or predominantly single bonds were calculated. In terms of total saturated fatty acids (SFA) in different algal biomass was above 50% of the total fatty acids content ($p < 0.05$). To be more precisely, the highest total SFA content was calculated in B2 biomass, lower, but slightly similar in B3 and B4, and the lowest amount in B1 *C. glomerata* samples ($p < 0.05$). After calculating the total monounsaturated fatty acids (MUFA) content, some significant differences between the analysed biomasses were obtained. The highest total MUFA content was found in B2 algal biomass. Compared to B1, B3 and B4, MUFA content was respectively higher by 0.46%, 1.05%, and 0.36% in B2 biomass ($p < 0.05$). Another calculated group of fatty acids is polyunsaturated fatty acids (PUFA). Most of these fatty acids were calculated from the B1 *C. glomerata* biomass. Comparing the total amount of PUFA among other algal biomasses, the lowest content was found in B2, slightly more, but very similarly between both in B3 and B4 biomasses ($p < 0.05$). An important lipid index PUFA/SFA

ratio was calculated. *C. glomerata* biomasses collected from different Lithuanian rivers and analysed during our study showed that the PUFA/SFA ratio significantly differs between all biomasses, except between B3 and B4 (the same ratio was obtained ($p > 0.05$)). After calculating the PUFA/SFA ratio in B1 and B2 algal biomasses, this indicator was found to be two times higher in B1 samples than in B2 ($p < 0.05$).

Table 4. Amino acids profile of *C. glomerata* biomass from different rivers in Lithuania.

	***C. glomerata* Biomass** [1,2,3]					
Item (g/kg DM)	**B1**	**B2**	**B3**	**B4**	**SEM** [4]	***p* Value**
Aspartic acid	10.90 [a]	13.63 [b]	14.18 [c]	15.97 [c]	0.63	0.001
Threonine	3.49 [a]	6.52 [b]	6.28 [b]	6.77 [b]	0.42	0.001
Serine	4.14 [a]	6.21 [b]	6.29 [c]	7.25 [c]	0.34	0.001
Glutamic acid	14.16 [a]	16.90 [b]	17.53 [c]	19.50 [c]	0.48	0.000
Proline	5.98 [a]	5.92 [a]	6.40 [a]	7.22 [b]	0.25	0.006
Glycine	8.06 [a]	9.23 [b]	9.54 [c]	11.46 [c]	0.32	0.000
Alanine	8.49 [a]	8.12 [a]	10.09 [b]	10.73 [b]	0.24	0.000
Valine	8.39 [a]	8.48 [a]	8.90 [a]	10.42 [b]	0.39	0.006
Methionine	1.94 [b]	4.14 [a]	2.24 [b]	2.08 [b]	0.21	0.000
Isoleucine	5.94 [a]	6.17 [a]	6.80 [a]	7.69 [b]	0.30	0.004
Leucine	9.71 [a]	9.64 [b]	10.81 [c]	12.01 [d]	0.31	0.002
Tyrosine	1.73 [a]	1.71 [a]	2.09 [b]	2.35 [b]	0.14	0.009
Phenylalanine	6.37 [a]	6.93 [a]	7.39 [a]	8.50 [b]	0.35	0.004
Histidine	3.04	3.01	3.33	3.68	0.28	0.077
Lysine	5.76 [a]	5.85 [a]	6.46 [a]	7.88 [b]	0.26	0.001
Arginine	5.25 [a]	5.79 [a]	6.31 [a]	7.47 [b]	0.20	0.000
Total	103.36 [a]	118.25 [b]	124.64 [c]	140.99 [d]	3.88	0.001

Note: [1] *C. glomerata* biomass from Lithuanian rivers, Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4). [2] Means with different superscript letters (a–d) in a row were significantly different ($p < 0.05$). [3] $n = 12$ (3 replicate samples from each river). [4] SEM, standard error of the means.

Individual omega-3 (n3) fatty acids were identified and evaluated. The highest content of α-linolenic acid (C18:3 n3) was found in B1 algal biomass (4.29%), 2 times less in B2 (1.91%) ($p < 0.05$). In the remaining *C. glomerata* biomasses B3 and B4, this amount was observed to be very similar (2.90% and 2.96%, respectively). However, it was lower compared to B1 ($p < 0.05$), but higher compared to B2 ($p < 0.05$). Another n3 essential fatty acid identified in our study is eicosapentaenoic acid (C20:5 n3). The highest content of the mentioned fatty acid (C20:5 n3) was found in B4 macroalgal biomass. The content of the remaining biomasses (B1; B2; B3) was lower and differed statically from B4 ($p < 0.05$). However, the last identified n3 fatty acid, docosapentaenoic acid (C22: 5 n3), was observed only in B1 biomass. The maximum total amount of n3 fatty acids was calculated in B1 *C. glomerata* biomass. Compared to B1 and others, the total amount of n3 was 2.40%, 1.50%, and 0.99% lower in B2, B3, and B4, respectively ($p < 0.05$). The following omega-6 (n6) fatty acids were identified in our study: linoleic acid (C18:2 n6), arachidonic acid (C20:4 n6), docosadienoic acid (C22:2 n6). Linoleic acid content varied significantly across all algal biomasses ($p < 0.05$). However, the largest significant difference was found between B1 and B2 biomasses, when almost two times higher concentration of C18:2 n6 was observed in B1 compared to B2 ($p < 0.05$). Arachidonic acid (C20:4 n6) was defined in all biomass samples, except B2. However, other analysed biomasses revealed statistically significant differences, where most of C20:4 n6 was identified in B4 algal biomass, less in B3, and the least in B1 ($p < 0.05$). No statistically significant data were obtained after identifying docosadienoic acid (C22:2 n6), as in B2 and B4 samples, such an acid was not identified at all, and the content found in the remaining biomasses (B1; B3) was identical ($p > 0.05$). The total amount of n6 was calculated, and significant differences were obtained between all analysed biomasses. The largest difference was found between B1 and B2, where the total n6 content in B1 was 6.62% and only 3.80% in B2 ($p < 0.05$); lower amounts were found in

B3 and B4, which were 6.00% and 5.51%, respectively ($p < 0.05$). Ultimately, the n6/n3 ratio was calculated: in B3 biomass, this indicator was slightly higher compared to B1, B2 and B4 ($p < 0.05$), but comparing B1 and B4 this ratio was nearly the same ($p > 0.05$).

Table 5. Fatty acids profile of *C. glomerata* biomass from different rivers in Lithuania.

	***C. glomerata* Biomass** [2,3,4,5]					
Item [1] **(% of the Total Fatty Acids Content)**	**B1**	**B2**	**B3**	**B4**	**SEM** [6]	***p* Value**
C10:0	n/d	0.37 [a]	n/d	0.24 [b]	0.02	0.000
X	0.75 [a]	0.56 [b]	0.88 [c]	0.69 [a]	0.02	0.000
C12:0	0.25 [a]	0.40 [b]	0.80 [c]	0.30 [d]	0.01	0.000
X	0.64 [ab]	0.50 [a]	n/d	0.61 [b]	0.03	0.000
X	5.03 [a]	3.59 [b]	5.58 [a]	5.03 [c]	0.04	0.000
X	0.19 [b]	0.35 [a]	0.22 [b]	n/d	0.02	0.002
C14:0	8.86 [a]	12.48 [b]	9.70 [c]	11.21 [d]	0.06	0.000
X	0.80 [a]	0.76 [a]	0.84 [a]	0.62 [b]	0.02	0.001
C15:0	0.99 [a]	0.18 [b]	0.30 [c]	0.82 [d]	0.04	0.000
iC15:0	5.95 [a]	4.83 [b]	7.65 [c]	5.60 [d]	0.07	0.000
C16:0	33.54 [a]	37.11 [b]	32.65 [c]	32.89 [d]	0.08	0.000
iC16:0	0.80 [b]	0.50 [a]	0.76 [b]	0.40 [c]	0.02	0.000
trans-C16:1 n7	1.13 [b]	0.81 [a]	1.18 [b]	1.03 [b]	0.05	0.002
C16:1 n9	1.71 [a]	1.00 [b]	1.88 [c]	1.17 [d]	0.06	0.000
C16:1 n7	5.31 [a]	6.89 [b]	6.63 [c]	7.88 [d]	0.03	0.000
C17:0	n/d	0.49 [a]	0.50 [a]	0.29 [b]	0.03	0.000
iC17:0	0.49 [a]	0.18 [b]	0.58 [a]	0.23 [c]	0.03	0.000
C18:0	2.07 [a]	2.52 [b]	2.39 [c]	3.25 [d]	0.04	0.000
C18:1 n9	12.72 [a]	14.53 [b]	11.16 [c]	12.33 [d]	0.05	0.000
C18:1 n7	6.06 [a]	5.16 [b]	6.12 [a]	5.16 [c]	0.05	0.000
trans-C18:2 n6	1.43 [a]	n/d	1.11 [b]	n/d	0.03	0.000
cis-trans-C18:2 n6	n/d	0.74 [a]	n/d	1.35 [b]	0.02	0.000
trans-cis-C18:2 n6	0.57 [b]	0.31 [a]	0.56 [b]	0.45 [c]	0.02	0.000
C18:2 n6	4.15 [a]	2.75 [b]	3.83 [c]	3.33 [d]	0.04	0.000
C18:3 n3	4.29 [a]	1.91 [b]	2.90 [c]	2.96 [c]	0.05	0.000
C20:1 n9	0.59 [a]	n/d	0.37 [b]	n/d	0.01	0.000
C20:4 n6	0.27 [a]	n/d	0.30 [a]	0.38 [b]	0.01	0.000
C20:5 n3	0.58 [a]	0.77 [a]	0.68 [a]	1.13 [b]	0.09	0.003
C22:1 n9	0.22	n/d	n/d	0.24	0.02	0.264
C22:2 n6	0.20	n/d	0.20	n/d	0.01	0.932
C22:5 n3	0.22	n/d	n/d	n/d	n/d	n/d
C24:0	n/d	0.30	0.24	0.21	0.04	0.067
C24:1	0.20	n/d	n/d	0.21	0.01	0.393
SFA	52.94 [a]	59.37 [b]	55.57 [c]	55.43 [c]	0.11	0.000
MUFA	27.93 [a]	28.39 [b]	27.34 [c]	28.03 [a]	0.10	0.000
PUFA	11.71 [a]	6.48 [b]	9.58 [c]	9.59 [c]	0.11	0.000
PUFA/SFA	0.22 [a]	0.11 [b]	0.17 [c]	0.17 [c]	0.00	0.000
Total n3	5.08 [a]	2.68 [b]	3.58 [c]	4.09 [d]	0.07	0.000
Total n6	6.62 [a]	3.80 [b]	6.00 [c]	5.51 [d]	0.04	0.000
n6/n3	1.30 [a]	1.42 [b]	1.68 [c]	1.35 [a]	0.02	0.000
Total X	7.42 [a]	5.76 [b]	7.51 [c]	6.95 [d]	0.02	0.000

Note: [1] SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; X, unidentified fatty acids. [2] *C. glomerata* biomass from Lithuanian rivers, Dubysa (B1); Šventoji (B2); Nevėžis (B3); Jūra (B4). [3] n/d, not defined. [4] Means with different superscript letters (a–d) in a row were significantly different ($p < 0.05$). [5] $n = 12$ (3 replicate samples from each river). [6] SEM, standard error of the means.

During our study, "X" unidentified fatty acids were obtained. A total of five fatty acids were unidentified with a total of 7.42%, 5.76%, 7.51%, and 6.95% in B1, B2, B3, and B4, respectively.

4. Discussion

Algal biomass possesses biologically active ingredients which contain polysaccharides, proteins, polyphenols, pigments, mineral elements, and polyunsaturated fatty acids. Macroalgae are well-known for their high concentrations of biologically active compounds. Because of the need to rapidly adapt to changing environmental conditions, a variety of secondary metabolites, which are not found in other organisms, are produced [6]. The following compounds protect algal cells from stressful conditions such as UV radiation, sudden changes in temperature, or changes in nutrient concentrations [7]. Several studies have found a link between *C. glomerata* and high nutrient inputs [19]. Moreover, *Cladophora* species can survive in extremely saline environments (up to 100‰ of salinity) [20]. *Cladophora* macroalgae contains a high amount of carbohydrates, minerals, proteins and is featured by high moisture (around 90%) [21]. So, according to its nutritive value, theoretically, it could be used for animal nutrition. However, it should be taken into account that the composition of *Cladophora* macroalgal biomass is strongly dependent on environmental variables (weather conditions, season, method of algae cultivation, and collection). Because the content of compounds such as proteins, amino acids, lipids, and elements in *Cladophora* species is similar to that of plants used as feed materials, these algae could be used as a valuable feed additive [8].

4.1. Macro- and Micronutrients in Algal Biomass

Recent interdisciplinary studies of *C. glomerata* and *Ulva flexuosa* also suggest the suitability of freshwater macroscopic green algae as they are a rich source of macro- and micronutrients and other bioactive substances [12,22]. Mineral elements such as iodine, zinc, iron, copper, calcium, magnesium, sodium, and potassium are abundant in algal biomass. Compared to other feed materials, such as edible land plants, the content of these elements in algae can be found to be as much as 40% higher [23]. Therefore, given the potential mineral deficiency, traditional feed additives can be successfully replaced by algal culture additives [24,25]. Our results indicate that *C. glomerata* can also serve as a carrier of microelements for animals' diets. The macronutrient distribution in *C. glomerata* algal biomass from different Lithuanian rivers was as follows Ca > K > N > P > Mg and of the trace elements—Zn > Cu (Figure 2). This is in agreement with the results of Messsyasz et al. [12], where calcium content in collected freshwater *C. glomerata* biomass from Oporzyn lake in Poland was the highest as well. Moreover, Michalak et al. [26] demonstrated that *Cladophora* biomass enriched with microelement ions through biosorption can be used as a valuable feed additive for various animal breeds and can partially substitute for inorganic salts.

Green algae, especially *Cladophora* species, are widely regarded as the best bioindicators of nutrient and heavy metal toxicity in aquatic bodies. As a result, the concentrations of the most important heavy metals (Cr > Ni > Pb > Cd) were determined. The analysis revealed that the concentrations of the main heavy metals in *C. glomerata* did not exceed the recommended maximum levels. As a result, no potential toxicity could be observed for animals regarding heavy metal concentrations in algal biomass from different Lithuanian rivers, which was in line with the Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed.

Generally, macroalgae has a high ash content, as well as micro- and macro-elements, essential minerals, and trace elements that are required by all living organisms [6]. When comparing the amount of crude ash in traditional and alternative protein feed materials, fishmeal (25.95%) has the highest amount, followed by *Cladophora* meal (21.14%), rice bran (17.17%), soybean meal (8.26%), and cassava powder (1.97%) [27]. During our study, the crude ash content in *C. glomerata* from Lithuanian rivers varied from 36.96% to 49.83%, whereas the crude fibre content constituted about 13.46%. This result is similar to those of Messyasz et al. [12] and Michalak et al. [26], who found 39.25% of ash and 15.6–19.6% of crude fibre in *Cladophora* from lakes in Poland. Finally, algal biomass with a higher

fibre content could be adapted to rabbit diets because rabbits can achieve good growth performance on high-fibre diets due to their unique digestive physiology [28].

4.2. Proteins and Amino Acids in Algal Biomass

In addition to mineral elements, freshwater macroalgae can serve as a source of protein in animals' nutrition. In general, crude protein in algae dry matter can reach 44% but commonly does not exceed 5% [29]. Compared to other traditional protein sources used in feed production, the amount of crude protein found in soybean meal reaches from 40% to 49%. Considering the global challenges we are facing today with livestock and the predicted inevitable deficiency of traditional protein materials in animals' feed production in the future, freshwater macroalgae could be a solution as the protein content in *Cladophora* biomass ranges from 10% to 25% and it is comparable to other feed materials [8]. Similarly, crude protein in our analysed *C. glomerata* biomass from Lithuanian rivers ranged from 16% to 21.5%. However, *Cladophora* species are more commonly used as aquaculture feed additives than as livestock feed additives. It could be used as a fish meal replacement or as a protein source in aquaculture. For example, Anh et al. [27] indicated that replacement of fishmeal protein (60.08% crude protein in DM) with different levels of seaweed *Cladophora* meal from 10% to 50% in *Penaeus monodon* post larvae diets had significant effects on growth performance, feed efficiency and stress resistance. Thus, the use of *Cladophora* in animal feed production would contribute to the development of more sustainable livestock in the first place. Moreover, there are two advantages to using harvested wild freshwater *C. glomerata* biomass: (i) it will allow to save on expenses for algal biomass cultivation that requires special conditions and complex large pond systems; (ii) the removal of excessive waste biomass from natural water bodies allow to increase the biodiversity and the recreational value of aquatic ecosystems.

Amino acids, especially exogenous amino acids, used in various types of feed additives can increase animals' nutrient digestibility, compensate for nutrient deficiencies, and improve feed quality and final animal production composition [4]. Regardless of whether an amino acid is termed essential or non-essential, animals need sufficient amounts of all amino acids to meet their metabolic needs. Based on the total amount of amino acids, the highest total concentration was found in the *C. glomerata* biomass collected from the Lithuanian river Jūra (141 g/kg). Messyasz et al. [12] found that the amino acid content in *Cladophora* biomass indicates a very interesting new material which could be potentially used in animal feed as an alternative feed supplement.

Each amino acid's properties are determined by the structure of its chain, and thus, its carbon skeletons cannot be synthesized by higher animals. Eight of them (threonine, valine, isoleucine, leucine, phenylalanine, lysine, methionine) are considered nutritionally essential [30]. During our study, the essential amino acids totally accounted for 41.6–55.4 g/kg (~40% of the total amount of amino acid determined) in *C. glomerata* biomass and the concentration was the highest in samples from the River Jūra. The branched chain essential amino acids (isoleucine, leucine, and valine) act as tissue protein building blocks (accounting for 35% of the essential amino acids in muscle) and perform many indispensable metabolic functions [31]. Another essential amino acid, methionine, is one of the most limiting amino acids, playing a critical role in the body's protein synthesis [32]. Methyl groups of methionine are essential in animal nutrition and are involved in their metabolism. It should be noted that animals cannot synthesize them and must therefore obtain them from their daily ration [33]. The maximum amount of methionine in our study was observed in algal biomass collected from the Šventoji River (4.14 g/kg). Methionine constituted 3.5% of the total amino acid content in *Cladophora* biomass, like in the widely accepted additive *Spirulina platensis* [34], and was two times higher than in soybean meal. It is important to note that natural source macroalgal biomass is a promising source for replacing artificially grown *Spirulina* additives with a more sustainable animal feed material. Altogether, the amino acid arrangement in individual green algae is very similar: threonine improves plant

generative development; serine is required for chlorophyll synthesis. Proline improves plant generative development and regulates water management in the cell [6].

Research on protein nutrition has mainly focused on the dietary composition of essential amino acids which are not synthesized in animals' cells [35–37]. However, non-essential amino acids can be synthesized by the animal's body and do not need to be provided in the diet, but they still play an important role. Non-essential amino acids play an important and comprehensive role in whole-body metabolism and functions, according to scientific research and emerging evidence [38–41]. In our study, the highest concentrations of aspartic acid, serine, glutamic acid, proline, glycine, alanine, tyrosine, and arginine were determined in River Jūra macroalgal biomass samples. In terms of feed formation, all non-essential amino acids found in the algal biomass we studied would be useful in the mammalian diet, according to Elango et al. [35] and Wu [41]. However, higher concentrations of alanine and arginine are not recommended for carnivores, ferrets, minks, and young animals. The amino acid requirements of animals' diets are determined first by the animal's species, developmental stage, physiological status, small intestinal microbiota, general pathological state, and even environmental factors [31,42–44].

In general, according to the total amount of amino acids and according to the concentrations of individual amino acids, *C. glomerata* biomass can be used from any Lithuanian river we analysed. The majority of algae species contain all essential amino acids. However, one should take into account that their concentrations and mutual proportions are conditional on the season of occurrence [4].

4.3. Lipids and Fatty Acids in Algal Biomass

Lipids are high energy-dense compounds when compared to proteins, carbohydrates, or any other nutrient found in food and feed [2]. Algal biomass can potentially be used as a feed ingredient and can also be fed directly to livestock. For example, algal biomass can be digested by cattle, swine, and sheep [45]. Considering *C. glomerata* chemical composition indicators, the amount of crude fat is significantly lower, particularly in our study, where it varied from 0.19% to 0.35%. In this case, the amount of crude fat can be offset in feed production by supplementing the feed with other fat-rich additives, such as vegetable oils.

Fatty acids are considered one of the most important algae components, especially polyunsaturated fatty acids (PUFA), which are crucial for human and animal health. Overall, *Cladophora* species extracts can implement an antimicrobial function which may be attributed to the general presence of fatty acids [7,46]. Several research papers indicate that macroalgae (both marine and freshwater) are rich in saturated (SFA) and unsaturated fatty acids (UFA) [12,46–48]. During our study, the total SFA amount in different algal biomass was more than 50% of the total fatty acids. However, Messyasz et al. [12] found less SFA (32.70%) in *C. glomerata*. It is necessary to note that the lipid content in *Cladophora* species' biomass can vary depending on salinity—the higher the salinity, the lower the fat content is [20]. Since the absorption of SFA from the digestive system is hard, unsaturated fatty acids such as monounsaturated fatty acids (MUFA) and PUFA release more energy [49]. MUFA observed in *C. glomerata* biomass analysed by us accounted for 27.34–28.39% of the total fatty acids content.

Indispensable PUFA must be obtained from the diet since mammals are unable to synthesize them. PUFAs, or long-chain highly unsaturated fatty acids with an omega-3 configuration, in particular, are known as "functional feed and food" elements [2]. Given PUFAs' fundamental role in metabolism, it's no wonder that they are linked to a variety of health benefits, e.g., antibacterial [50,51], anti-inflammatory [52], antioxidant [53], prevention of cardiac diseases [54], and tumour progression inhibition [55]. In general, fish and their oils or meals are the main commercial sources of PUFAs, but their suitability for human or animal consumption has been questioned from a biosafety perspective. It raised the demand to investigate alternative sources of high quality PUFAs such as macroalgae. Consequently, marine and freshwater macroalgae such as *Cladophora* species have been studied as alternative potential sources, as many of them could easily be cultivated in

different water sources (sea, rivers, lakes etc.) on a large scale [2,4,12,51,56]. In our study of freshwater *C. glomerata* biomass from different rivers in Lithuania, PUFA levels ranged between 6.48% and 11.71% of the total fatty acid content. In comparison, Pereira et al. [56] detected almost two-fold more PUFAs in the marine biomass of *C. albida*.

An important lipid index, the PUFA/SFA ratio, is used to assess the impact of diet on cardio-vascular health [57]. It is hypothesized that all PUFAs in the diet will lower low-density lipoprotein cholesterol (LDL-C) and serum cholesterol levels, whereas all SFAs lead to high serum cholesterol levels [57]. As a result, the higher the ratio, the more advantageous the impact. The highest PUFA/SFA ratio observed in our studies for *C. glomerata* biomass was 0.22 and was about 1.69 times higher compared to plant oils such as palm stearin (PUFA/SFA = 0.13 [58]).

Although analysed *C. glomerata* macroalgae do not have as many lipids compared to microalgae or terrestrial plants (for example, linseed or rapeseed), they may have advantageous lipid quality, particularly fatty acids composition, that can compensate for the nutritional value. We calculated the essential omega-3 and omega-6 fatty acid content in the current study, which are two important components of PUFA. Docosapentaenoic acid, eicosapentaenoic acid, and -linolenic acid are omega-3 fatty acids that perform critical functions in both human and animal organisms [59]. In our study, the highest content of α-linolenic acid was found in *C. glomerata* biomass from the Lithuanian River Dubysa (4.29%). The highest content of eicosapentaenoic acid was in the River Jūra (1.13%). Docosapentaenoic acid was found only in Dubysa's *C. glomerata* biomass (0.22%). Other research has shown that the use of algal biomass in the diet of laying hens has a positive impact on the nutritional properties of the egg yolk by increasing the content of essential omega-3 fatty acids [3]. However, based on our research results and to improve the content of omega-3 fatty acids in animal nutrition and the final product, *C. glomerata* extracts could be combined with vegetable oils such as rapeseed oil, which is particularly high in omega-3. For instance, Fredriksson et al. [60] presented results showing that combining algae with rapeseed oil in laying chickens' diets increased omega-3 fatty acids by 15% and reduced omega-6 acids content by 8% in the egg yolk. Considering the omega-6 fatty acid content in the *C. glomerata* biomass studied in our experiment, the total omega-6 fatty acid content varied from 2.75% to 4.62%. Taking in conjunction the omega-3 and omega-6 results obtained from freshwater *C. glomerata* from different Lithuanian rivers, it can be assumed that use of algal biomass can be successfully applied as feed additive or raw material.

Since long chain omega-3 and omega-6 PUFAs are synthesized by the same enzymes, an increase in one of these essential fatty acids usually means a reduction in the other fatty acids due to competition for the same metabolic enzymes [61]. As a result, the health-promoting benefits of these essential fatty acids are dependent on maintaining a proper balance of omega-6 and omega-3 PUFAs [62]. However, deficits of essential unsaturated fatty acids, as well as an improper ratio of omega-6 to omega-3, have been linked to a variety of diseases. It is important to maintain a low omega-6 and omega-3 ratio in humans and animals' diets to reduce inflammation. Nowadays, the perfect ratio of omega-6 to omega-3 is considered to be 1 or 4:1 [63]. The lowest mentioned ratio in *C. glomerata* biomass analysed in our study was determined in these rivers, Dubysa (1.30) and Jūra (1.35). Higher ratios were found in the biomass of the Šventoji (1.42) and Nevėžis (1.68) rivers. These results fall within the limits of perfect omega-6 omega-3 and are therefore suitable for use as animal nutritional supplements. Based on research by other scientists, feed additives with such a ratio would play an important role in improving the immune response and productivity of animals, as well as the nutritional value of PUFA-enriched final animal production [64,65].

5. Conclusions

According to the determined chemical composition, freshwater *C. glomerata* microalgal biomass from the Lithuanian rivers Dubysa, Šventoji, Nevėžis, and Jūra constitute an important source for sustainable commercial applications. The biomass contains macro-

and trace elements within the recommended limits and is safe for applications in animal nutrition due to low levels of heavy metals. However, chemical analysis should be performed after each biomass collection, as toxicity can be induced by several environmental factors and temporary pollution. As a result of bioaccumulation, freshwater *C. glomerata* may potentially serve as a source of protein, essential amino acids, and fatty acids, implying that they could be a beneficial component of animal feed.

Author Contributions: Conceptualization, M.N. and V.V.; methodology, S.B.; software, M.N.; validation, A.R.-S., J.K. (Jūratė Karosienė) and J.K. (Judita Koreivienė); formal analysis, M.N.; investigation, S.B.; resources, J.K. (Jūratė Karosienė) and J.K. (Judita Koreivienė); data curation, V.V.; writing—original draft preparation, M.N.; writing—review and editing, V.V. and A.R.-S.; visualization, M.N.; supervision, V.V. 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.

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

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