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Study of the Effect of Mowing and Drying on the Lipid Composition of Grass Leaves in Permafrost Ecosystems

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Abstract: Mowing the plant shoots under hot, sunny, and dry conditions severely traumatizes the entire vegetative body, and the overall life cycle of the plant is altered. The purpose of the present research was to investigate the effects of mowing and drying on lipids, fatty acids (FA), sterols, and the systemic responses in leaves of plant material at three time points (24 h, 72 h, and leaves of new shoots after traumatic mowing in summer (1 July) and those subjected to cold hardening by autumn temperatures in September (aftergrass)) were analyzed for the first time. The leaves of five species of herbaceous plants growing in permafrost ecosystems were analyzed by HPTLC and GC-MS. It was established that fatty acids in the tissues of aftergrass leaves were characterized by higher values of the n-6/n-3 ratio than in summer grasses. It was demonstrated that exposure of leaves for 72 h in natural conditions in summer and at low temperatures in autumn in leaves of aftergrass resulted in significant changes in the composition of membrane phospholipids. The obtained findings indicate that leaves of aftergrass are the most valuable plant raw material in terms of FAs and phytosterols content compared to hay mowed in summer.

Keywords: plant raw material; fatty acids; sterols; lipids; mowing; mixture of herbs; pasture grass

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1. Introduction

Currently, the agricultural sector faces the challenge of increasing the yield of cultivated crops and the efficient use of nutrient resources for high-quality crop production, which can provide sufficient food for the rapidly growing population of the planet in a changing climate [1]. Plants grown for fodder experience the influence of extreme natural and climatic factors in the North-Eastern part of Russia, including the vast territory of the Yakutia cryolithozone, which has a total area of approximately 3.1 million km^2 [2]. These include a short growing season (up to 80–120 days of frost-free period per year), dry cold air with extremely low temperatures in winter (up to –60 °C), high temperatures in summer (up to +40 °C), and permafrost spread throughout the territory [3]. During short vegetation periods, plants are exposed to a high amount of solar radiation activity, moisture deficit, and short frosts on the soil surface in early summer and autumn. About 2000 species of higher vascular plants grow under the peculiar climatic conditions of the Yakutia cryolithozone [4]. Some of them play an important role as fodders for herbivorous farm animals (Yakut horse breed, aboriginal Yakutian cattle), whose meat and milk form the basis of the traditional diet of local residents.

The main chemical substances on which the fodder values of grasses depend are easily digestible forms of carbohydrates, proteins, and plant lipids. Plant lipids are recognized as the most important dietary components in the diet of humans and animals, providing a significant part of the energy requirements of their organisms and serving as a source of essential FAs, such as linoleic C18:2n-6 (LA) and alpha-linolenic C18:3n-3 (ALA) acids [5,6].

The specifics of seasonal growth and development of the main mass of herbaceous vegetation in the cryolithozone are that intensive growth occurs in the first half of summer. However, the northern meadow phytocenoses at this time are often flooded by flood waters and subjected to squeezing by animals and mowing (traumatic damage to shoots). Mowing the plant shoots in the hot, sunny, and dry weather severely traumatizes the entire plant body, and the entire vital process is altered. Mowed plant shoots begin to lose turgor, all metabolic processes are suspended, the protoplasm loses water, the mowed plant material is heated by high ambient temperatures, and nutrients are gradually lost. It has been found that mowing shoots at earlier phases of plant development (active growth) leads to higher losses of nutrients [7]. After mowing for a long exposure to natural conditions in hot and dry weather, water is removed from the tissues of the plant raw material, resulting in an increased concentration of dry substances, and a longer exposure to natural conditions leads to a reduction and loss of nutrients in the plant raw material. In experiments with *Avena sativa* L. growing under cryolithozone conditions, it was shown that a significant decrease in photosynthetic pigment content in the leaves of summer-vegetative oat plants during the dry period of July was related to adaptation of the photosynthetic apparatus to high insolation and moisture deficiency, when the photosynthetically active radiation flux density during daytime hours in July was up to 1200–1500 μmol/(m^2 s) with a total of 1 mm of rainfall per decade [8].

Autumn-vegetating herbaceous vegetation and winter-green cryo-fodder are cereals that retain up to 80% of their green mass under snow, especially in the sedges, cotton grasses, and some types of horsetails [9,10]. Another way to obtain green cryo-fodder is to sow annual grasses in late terms, which are also preserved by natural cold at the beginning of winter. Thus, frozen grass can accumulate a significant amount of nutrients [11].

Mowing is the primary means of forage collection in grasslands worldwide [12,13], and may affect the quantity and quality of forage yield [14,15]. During the stabling period, the main forage for farm animals is grass cut and air-dried (natural drying) with a moisture content of 15–17% or lower [16]. It has been shown that single mowing can increase forage yields in the short term [17,18], and prolonged mowing with high frequency can reduce forage quantity and quality by directly reducing the soil nutrient content [19].

After mowing green shoots, plant leaves are exposed to excessively high ambient temperatures, increased solar radiation, and drought to induce hyperosmotic stress [20]. Under these conditions, metabolic rearrangements, including those at the cell membrane and membrane lipid levels, occur in raw plant materials. Sterols are one of the main groups of membrane lipids that ensure the integrity of cell membranes [21], and their functional properties and state largely depend on the FAs composition of the lipid bilayer [22]. Earlier experiments with mowing grass shoots have shown that three- and five-fold mowing of grasses during the growing season reduces all FAs, particularly ALA [23]. The content of lipids and FAs decreases during the growing season; therefore, in all grains after 140 days of sampling, the amount of ALA in forage grasses decreases by a factor of three [24]. It was established earlier in our research that the later-season sowing of cereal crops at the onset of low hardening ambient temperatures promoted the accumulation of significant amounts of membrane phospholipids and essential polyunsaturated fatty acids (PUFA), such as LA and ALA, in their tissues. Yakutian horses fed on such fodder accumulated significant amounts of these acids in their tissues [11].

To evaluate the effect of mowing on the nutritive value of leaves, as well as on the state of cell membranes of leaf tissues after mowing, we performed a field experiment under the conditions of frozen ecosystems in Yakutia using GC-MS analysis of the FA composition and content of total lipids, phytosterols, and their esters, as well as the composition of neutral and polar lipids. The present study provides information on what happens to the lipids of grass leaves after mowing shoots at 24 h and 72 h under natural conditions, as well as in grass shoots that have grown back after mowing and have undergone cold hardening by low temperatures in permafrost ecosystems (aftergrass). It should be noted that in the applied aspect, the standard evaluation of the produced plant fodder in terms of the

content of fodder units, fiber, carbohydrates, proteins, and fats does not provide a complete picture of its value. In addition to the above parameters, the n-6/n-3 ratio, PUFA content and sterols, which can have positive biological effects as immunomodulators, metabolic regulators, and antioxidants are important [25,26].

The purpose of this study was to investigate the effects of mowing and drying on the content of lipids, FAs, sterols, and systemic responses in the leaves of plant raw material at three time points (24 h, 72 h, and aftergrass) and to obtain plant raw material with a high lipid content, which can be used in the diet of farm animals in the North.

2. Materials and Methods

2.1. Experimental Design and Plant Species Composition

This study was conducted between 2021 and 2022 in Central Yakutia (Russia, Republic of Sakha (Yakutia)). To assess the effect of traumatic damage to shoots (mowing) on the lipid composition of plant raw material, we conducted the following experiment: freshly harvested leaves of summer-vegetable grasses (species composition mainly consisted of Poaceae and one species in the family Cyperaceae (Table 1)) were used as control (Figure 1a), which were selected on 1 July and designated as the "fresh cut grass", then grass shoots were cut at 4–5 cm height and cut leaves were kept for 24 h and 72 h in natural conditions and designated as "hay after 24 h" (Figure 1b) and "hay after 72 h" (Figure 1c) (drying). Experiments to obtain aftergrass were as follows: wild grass shoots were also subjected to cutting (1 July) at a height of 4–5 cm from the ground to stimulate the establishment of young shoots; by sampling (29 September), grass shoots underwent the first and second phases of hardening at low temperatures of the Yakutian cryolithozone (Figure 1d). The voucher specimens were deposited in the herbarium of the Department of Experimental Plant Biology of Permafrost Ecosystems, Institute for Biological Problems of Cryolithozone, Siberian Branch of the Russian Academy of Sciences (Yakutsk, Russia. Specimens Nos. PKA/2021/YA/SE/1, PKA/2021/YA/SE/2, PKA/2021/YA/SE/3, PKA/2021/YA/SE/4 and PKA/2021/YA/SE/5). The average air temperature during the growing season (June–September) was 14.1 °C, the amount of precipitation from May to September was 122.5 mm. In May, the average amount of precipitation was 10.3 mm. In June, July, August, and September, the precipitation was 19.9, 20.2, 51, and 21.1 mm, respectively.

Table 1. Species composition of plants and their stages of development.

№	Species	Stages of Development (1 July)	Stages of Development (29 September)	Sample	Percentage of Each Species (%)
1	*Bromus inermis* Leyss.	Stem elongation	Dough development	leaves	30%
2	*Calamagrostis langsdorffii* (Link) Trin.	Tillering	Milk development	leaves	20%
3	*Beckmannia syzigachne* (Steud.) Fernald	Stem elongation	Dough development	leaves	20%
4	*Elytrigia repens* (L.) Nevski	Ear emergence	Milk development	leaves	20%
5	*Carex atherodes* Spreng.	Tillering	Milk development	leaves	10%

2.2. Plant Material

Plant samples were collected in the Republic of Sakha (Yakitia), Central Yakutia from 2021 to 2022. They were collected in steppe meadows at three locations, 8–10 samples from each (62°01′38″ N. 129°43′55″ E). The samples were collected in the morning (9:00–11:00). The species was authenticated by Prof. Klim A. Petrov (IBPC SB RAS, Yakutsk, Russia). A plant-shoot pruner (Instrum-Agro, Colibri, Hong Kong, China) was used for mowing. Herbs from each collection date were pooled to obtain samples with different growth periods. After combining the herb samples from each collection date, three total samples were collected from each growth period (Stem elongation, Tillering, Ear elongation, 1 July;

Dough development, Milk development, Dough development, 29 September). Flag leaves from cut shoots were selected for biochemical analysis. Leaf samples were collected from medium biomass (2–5 g) from a mixture of different grasses without a root system from a plot of 2 m^2 plot in triplicate. Leaf samples were immediately fixed with liquid nitrogen and transported to the laboratory in dewar flasks. Samples were stored in a freezer at –80 °C (Panasonic, Tokyo, Japan) before analysis. For biochemical studies, grass leaf samples fixed in liquid nitrogen were dried using a lyophilizer (VirTis, New York, NY, USA).

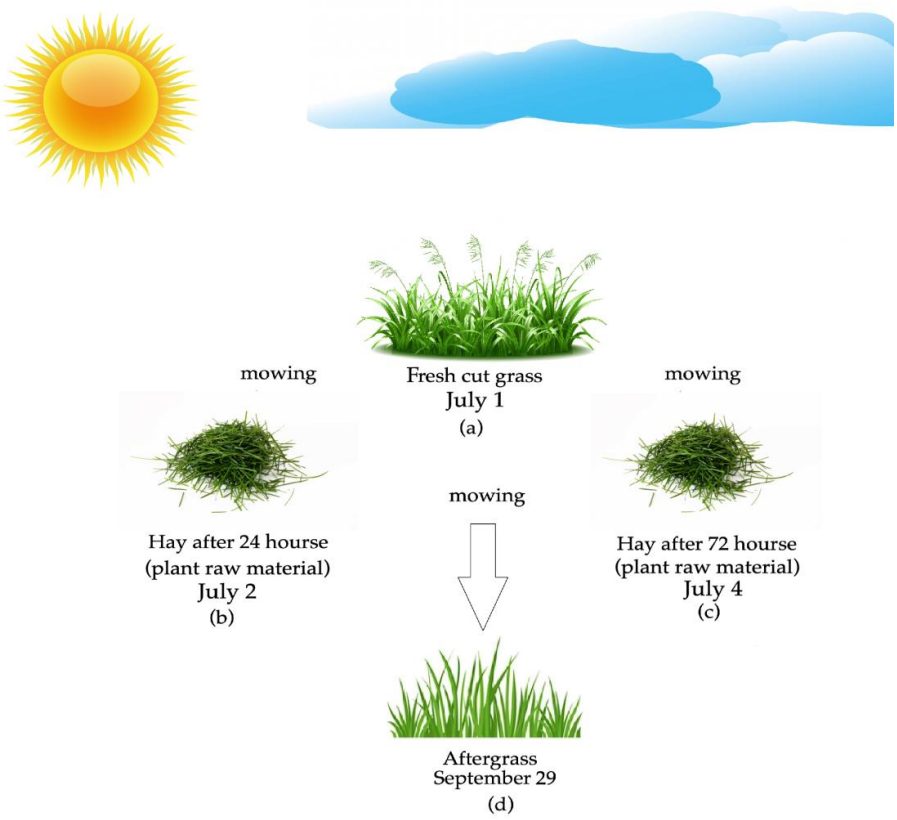

Figure 1. Scheme of a field experiment for evaluating the effect of post-mowing shoots on the lipids, FAs, and sterol profile of leaf plant raw materials.

Data on air temperature in the habitats of herbaceous plants were taken from an Internet resource (http://www.pogodaiklimat.ru/weather.php?id=24959 (access date: 31 March 2023)). Weather conditions during the years of the experiment were typical for Central Yakutia (Table 2).

Table 2. Meteorological parameters during the research period (Central Yakutia).

Sampling Date	Daily Average Air Temperature, °C *	Total Precipitation, mm **	Photoperiod, h
1 July (Fresh cut grass)	24.9 ± 8.9	9.1	19.0
2 July (Hay after 24 h)	23.0 ± 8.1	9.1	19.0
3 July	24.4 ± 7.2	9.3	19.0
4 July (Hay after 72 h)	26.4 ± 5.0	9.3	18.9
29 September (Aftergrass)	−0.2 ± 1.3	0.5	12.1

* Average data for 24 h prior to sampling; ** the amount within 10 days before sampling. Data taken from the sites "http://www.pogodaiklimat.ru/weather.php?id=24959 accessed on 10 June 2023)".

The level of moisture supply of the territory was calculated by the Hydro-thermal Coefficient of Selyaninov (K):

$$K = R \times 10/\Sigma t,$$

where R is the sum of precipitation in millimeters for the study period, Σt is the sum of temperatures (°C) for the same time.

2.3. Determination of Lipids Content

For lipid extraction, 10 mL of a mixture of chloroform/methanol 2:1 was added to a weighed portion of lyophilized plant material (20 mg). Ionol (0.00125 g per 100 mL of the mixture) was added to the mixture as an antioxidant. The mixture was thoroughly mixed and left for 30 min until complete diffusion of the lipids into the solvent. The solution was transferred quantitatively to a separatory funnel through a filter. The mortar and filter were washed three times with the same solvent mixture. Water was added to separate the non-lipid components.

For lipid analysis, lower chloroform fractions were separated. Chloroform (high-purity grade, stabilized with 0.005% amylene) was removed from the lipid extract under vacuum using a UL-2000 rotary evaporator (Ulab, Shanghai, China).

Neutral and polar lipids were analyzed using HPTLC on silica gel plates 60 (10 × 10 cm) (Merck, Darmstadt, Germany), which were eluted with hexane and activated by heating at 140 °C for 20 min before analysis. Solutions of the test objects and standards were manually applied to the TLC plate in the form of tracks that were 8 mm wide. For the analysis of neutral lipids, the following mixture was used as the mobile phase: hexane–diethyl ether–glacial acetic acid (80:20:1 *v*/*v*/*v*), and for polar lipids chloroform–methanol–water (65:25:4 *v*/*v*/*v*) for [27].

Lipids were identified using standards for the target components and specific reagents for individual functional groups [28].

The amounts of NL and PL were determined densitometrically using a Sorbfil (Imid, Krasnodar, Russia). For this purpose, chromatograms were developed using 10% sulfuric acid in methanol, followed by heating at 140 °C. The plate was sprinkled with reagent using a sprayer (Lenchrom, Saint Petersburg, Russia) connected to a JAS 1202 compressor (JAS-AIR, Hong Kong, China), was dried, then it was transferred to a desiccator, and heated for 20 min at 140 °C. Calculation of the content of individual classes of lipids in chromatograms was carried out using the Sorbfil TLC View program using standard phosphatidylcholine (PC) solutions (Larodan, Solna, Sweden) and monogalactosyldiglycerides (MGDG) (Sigma, St. Louis, MO, USA) and Rf values from the literature [28].

2.4. Determination of FAs Content

Lipid extraction was performed as previously described. At the homogenization stage, a known amount of 10 μg of nonadecanoic acid (C19:0) was added to control the lipid extractability (%). Fatty acid methyl esters (FAMEs) were obtained using the Christie method [29]. TLC on glass plates with KSK silica gel (Reachem, Moscow, Russia) and benzene as the mobile phase was used for FAME purification. To visualize the FAME zone (Rf = 0.71–0.73), the 0.5-cm edge of the plate was treated with 10% sulfuric acid in methanol followed by heating up to 100 °C in an oven. The FAME zone was removed from the plate with a spatula from a dry untreated plate within the zones where FAME was found and eluted from the silica gel with *n*-hexane. The FAME analysis was performed by GC-MS using a 5973/6890N MSD/DS gas chromatograph–mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) with a quadrupole mass spectrometer as the detector. A quadrupole mass spectrometer was used as the detector. The method of ionization was electronic impact EI, ionization energy of 70 eV. The analysis was performed in the mode of the total ion current recording. An HP-INNOWAX capillary column (30 m × 250 μm × 0.50 μm) with a stationary phase (PEG) was used to separate the FAME mixture. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The evaporator temperature was 250 °C, the ion source temperature was 230 °C, and the detector temperature was 150 °C.

The temperature of the line connecting the chromatograph to the mass spectrometer was 280 °C. The scanning range was 41–450 amu. The volume of the injected sample was 1 µL. An injector split the flow as 5:1. The separation of the FAME mixture was carried out in isothermal mode at 200 °C. To identify FAs, the NIST 08 mass spectral library and the Christie FAME mass spectral archive were used [30]. In some cases, the calculation of the equivalent length of the carbon chain (ECL) was calculated.

The relative FAs content was determined using the method of internal normalization in weight percent (wt.%) of the total content in the test sample, taking into account the FAs response coefficient.

Absolute FA content was calculated using the internal standard method (C19:0) according to the following formula:

$$Cd = Cint.st. \times Sx/Sint.st,$$

The Cd—content of the component to be determined, Cint.st. is the known content of the internal standard, Sx, and Sint.st.—areas of the corresponding peaks in the chromatogram.

2.5. Separation of Sterols and Their Esters

Sterols and sterol esters were isolated and separated using a neutral lipid system (hexane:diethyl ether:acetic acid eluent (80:20:1, $v/v/v$)). To visualize the zone of sterols and their esters, the edge of the plate was treated with a 10% sulfuric acid solution in ethanol, followed by heating up to 140 °C on a hotplate. Zones containing sterol components were visualized as rose-blue patches. Next, sterols and their esters were extracted sequentially from a dry untreated plate within the zones where sterol components were found in chloroform and ethyl acetate. At each of these phases, the sample was placed in solvent on an ultrasonic bath (Bandelin Sonorex, Berlin, Germany) and centrifuged at 3000× g. The ethyl acetate fraction was taken with a 1 mL pipette, transferred to glass tubes, and then evaporated to dry in a nitrogen current to remove the solvent.

Silylation. For the analysis, trimethylsilyl derivatives of the target components were obtained by heating the sample in a drying cabinet (Binder, Tuttlingen, Germany) for 30 min at 70 °C with the addition of 200 µL BSTFA N,O-bis-(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (Fluka, New York, USA). Ergosterol (Sigma-Aldrich, Saint Louis, MO, USA) was used as the internal standard.

GC-MS Analysis. Sterols and their esters were analyzed by the gas chromatography using a 7777QQQ/7890N MSD/DS chromato–mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). The detector mass spectrometer was used in single quadrupole mode, the ionization method of was electronic impact EI, the ionization energy was 70 eV, and the full ionic current registration mode was used for the analysis. A HP5-MS capillary column (30 m × 250 µm × 0.25 µm) with a stationary phase of 5% phenyl–methyl–polysiloxane was used for separation. Helium was used as the carrier gas at a constant flow rate of 1 mL/min. The volume of the injected sample was 1 µL. The temperature was gradient: from 150 to 300 °C at a rate of 10 °C/min and incubated at this temperature for 23 min. Evaporator temperature was 250 °C, ion source temperature was 230 °C, detector temperature was 150 °C, line temperature between chromatograph and mass spectrometer was 280 °C. The scanning range was 50–700 a.e.m. An injector split the flow as 5:1.

Identification and Quantitative Analysis. Sterols and sterol esters were identified using target component standards by comparing their retention times (Table S1). NIST 08 and Wiley 7 mass spectra libraries were used as required. Quantitative analysis of the target components was performed using an external calibration method, considering the response of the internal standard.

Statistical Processing. The experiments were performed with at least 3–4 independent repetitions (n = 3–4). The obtained data are presented as the arithmetic mean (M) and median (Me), and the spread of values was expressed as a standard deviation (±S.D.) or interquartile latitude [25%; 75%]. The normality of the distribution was checked using the Shapiro-Wilk criterion. To test for differences between the values following one-way

ANOVAs, the Newman-Keuls test was used. Differences were considered statistically significant at $p \leq 0.05$. Different letters are used in the figures and tables to indicate significant differences.

Statistical calculations were performed using the SigmaPlot 12.5 software package.

3. Results

3.1. Composition and Content of Lipids in Leaves of Plant Raw Material in Mowing and Drying

Using HPTLC, the composition of neutral lipids in the leaves of plant raw materials during mowing and drying was established. The results of the experiment revealed the composition of neutral lipids (NL) in the leaves after traumatic injury of shoots (mowing) and drying was revealed (Table 3). The content of 1,2-diacylglycerol (1,2-DAG) content was significantly higher in the leaves of fresh grass; after mowing, their amount significantly decreased when the leaves were aged for 24 h (drying). In the leaves of sedge (29 September), the 1,2-DAG content did not exceed 20% of the lipid sum, as in all variants of the experiment. A significant decrease in the content of free fatty acids (FFA) and triglycerides (TAG) was recorded in the leaves of plants exposed to natural conditions for 72 h. The decrease in fatty acids (FA) was significant from 24 h after the cutting. It was shown that the content of sterols, FA, and sterols ester + squalene + wax (SE + Sq + Wax) was higher in the leaves of shoots grown after mowing and subjected to cold hardening at low temperatures in the cryolithozone (aftergrass) than in the leaves of freshly mowed grass during the summer and hays.

Table 3. Composition of neutral lipids in leaves of grasses after mowing, drying, and in aftergrass (% of lipid structure).

Lipid Classes	Fresh Cut Grass	Hay after 24 h	Hay after 72 h	Aftergrass (29 September)
1,2-DAG	19.4 [17.3; 21.5] ^{A}	11.3 [9.9; 12.7] ^{B}	15.3 [12.8; 17.8] ^{AB}	14.5 [12.6; 16.4] ^{B}
Sterols	11.6 [9.5; 13.7] ^{C}	16.6 [15.2; 18] ^{B}	10.9 [9.2; 12.6] ^{C}	20.9 [18.5; 23.3] ^{A}
FFA	27.2 [24.7; 29.7] ^{A}	23 [21.5; 24.5] ^{A}	16 [15.1; 16.9] ^{C}	20.5 [20.1; 20.9] ^{B}
TAG	6.6 [6; 7.2] ^{B}	21.4 [19.8; 23] ^{A}	3.3 [2.4; 4.2] ^{C}	4.1 [3.3; 4.9] ^{C}
FA	14.4 [12; 16.8] ^{B}	2.9 [2.5; 3.3] ^{C}	3.4 [2.5; 4.3] ^{C}	20 [19.4; 20.6] ^{A}
SE + Sq + Wax	9.6 [9.1; 10.1] ^{B}	10.4 [9.3; 11.5] ^{B}	8.7 [8.1; 9.3] ^{B}	15.8 [14.6; 17.0] ^{A}

Note: 1,2-Diacylglycerol (1,2-DAG); Free fatty acids (FFA); Triglycerides (TAG); Fatty acid (FA); Sterols ester, Squalene, Wax (SE + Sq + Wax). Data are presented as Me [25%; 75%]. Values in the rows marked with the same letters were not significantly different at $p < 0.05$.

The next part of this study evaluated the absolute content of polar lipids (PL) after mowing and drying with leaf storage for 24 h, 72 h, and in herbage (Table 4). The amount of phosphatidylcholine (PC) in leaves stored for 72 h after mowing was markedly reduced (1.8-fold) compared with that in freshly cut grass. The leaves of the aftergrass plants were characterized by a higher content of PC than that in the summer grass leaves and compared with the leaves stored after mowing. The amount of phosphatidylinositol (PI) decreased 8.4-fold in leaves stored after mowing for 72 h compared to fresh cut grass. The phosphatidylserine (PS) content slightly increased in the leaves after 24 h of mowing. The amount of phosphatidylglycerol and diphosphatidylglycerol (PG + DPG) decreased 3.7-fold when the leaves were dried for 72 h during the summer. The amount of digalactosyldiglycerid (DGDG) also decreased after 72 h of drying of the leaves, and the amount of MGDG increased 2.6-fold in the aftergrass compared with that in fresh cut grass. The phosphatidylethanolamine (PE) content in the aftergrass was higher than that in the fresh cut grass. The lipid extracts contained lipids that could not be clearly separated using one-dimensional HPTLC, which prevented the identification of individual components (Figure S1).

Table 4. Composition of membrane lipids in leaves of grasses after mowing, drying, and in the aftergrass (mg/g DW).

Lipid Classes	Fresh Cut Grass	Hay after 24 h	Hay after 72 h	Aftergrass (29 September)
PS	4.6 [3.7; 5.5] ^{B}	6.5 [5.8; 7.1] ^{A}	5.2 [4.4; 6.0] ^{AB}	3.4 [2.8; 4.0] ^{B}
PI	10.1 [9.0; 11.2] ^{A}	4.3 [3.4; 5.2] ^{C}	1.2 [0.9; 1.5] ^{D}	7.2 [6.5; 8.0] ^{B}
PC	12.7 [11.5; 13.9] ^{B}	10.7 [9.7; 11.7] ^{C}	7.1 [6.6; 7.6] ^{D}	19.8 [18.4; 21.2] ^{A}
PG + DPG	25.3 [23.9; 26.7] ^{A}	10.5 [9.8; 11.1] ^{C}	7.0 [6.3; 7.7] ^{D}	13.8 [12.6; 15.0] ^{B}
DGDG	19.6 [17.6; 21.6] ^{B}	21.6 [20.6; 22.6] ^{B}	10.5 [9.0; 12.0] ^{C}	27.4 [25.7; 29.1] ^{A}
PE	8.6 [7.6; 9.6] ^{C}	13.3 [11.6; 14.9] ^{B}	6.6 [5.5; 7.7] ^{CD}	19.2 [17.3; 21.1] ^{A}
MGDG	6.7 [6.0; 7.5] ^{C}	15.7 [15.2; 16.1] ^{B}	8.5 [7.4; 9.6] ^{C}	17.8 [16.7; 18.9] ^{A}

Note: Phosphatidylcholine (PC); Phosphatidylinositol (PI); Phosphatidylglycerol + Diphosphatidyl-glycerol (PG + DPG); Digalactosyldiglycerid (DGDG); Phosphatidylethanolamine (PE); Monoga-lactosyldiglycerides (MGDG). Data are presented as Me [25%; 75%]. Values in the rows marked with the same letters were not significantly different at $p < 0.05$.

3.2. Influence of Mowing and Drying on the Composition and Content of FAs in Plant Raw Material Leaves

The GC-MS method was used to study changes in the FAs composition of lipids in fresh grass leaves, leaves after shoot cutting for 24 h and 72 h (drying), and in the leaves of the aftergrass. The relative percentages of total FAs and absolute FAs content in terms of DW are presented (Table 5). Between 13 and 15 FAs were detected in the leaves. The FAs composition varied slightly, e.g., only C12:0-i acid was detected in freshly cut leaves, which had a minimum content of 0.20% of sum. The palmitic acid content (C16:0) in the leaves did not differ significantly between fresh leaves and leaves taken for analysis after 24 h; however, the content of this acid decreased significantly with time, which was reflected in the saturated FAs (SFA) composition when leaves were aged for 72 h under natural cryolithozone conditions (Table 5). C16:0 content increased in the leaves of aftergrass, that is, in the leaves that underwent cold hardening by low temperatures of permafrost ecosystems, compared to the summer values. Among other SFAs, stearic acid (C18:0) dominated, the content of which significantly decreased during mowing and increased in the leaves of the aftergrass plant. In general, the total FAs content significantly decreased at 72 h of leaf residence after mowing, and in the aftergrass, it did not differ significantly from that in the fresh grass leaves. Physiologically important FAs, such as LA and ALA, decreased 2.7- and 2.9-fold, respectively, at the same leaf exposure, compared to summer values. The relative content of LA content did not change during the experiments. The relative ALA content was lowest in the aftergrass plant. In general, the relative content of saturated FAs decreased and that of unsaturated FAs increased in leaves after mowing. In aftergrass leaves, the relative content of saturated FA was higher than that of unsaturated FAs.

Table 5. FAs content in leaves of plant raw materials after mowing, drying, and aftergrass (% of sum and μg/g DW).

Fatty Acids	Fresh Cut Grass		Hay after 24 h		Hay after 72 h		Aftergrass (29 September)	
	% of Sum	μg/g DW	% of Sum	μg/g DW	% of Sum	μg/g DW	% of Sum	μg/g DW
C12:0-i	0.20 ± 0.16	3.18 ± 2.51	-	-	-	-	-	-
C14:0	1.07 ± 0.25 ^{a}	17.37 ± 5.94 ^{B}	2.56 ± 1.94 ^{a}	36.55 ± 17.63 ^{A}	1.06 ± 0.38 ^{a}	5.64 ± 3.01 ^{C}	1.71 ± 0.33 ^{a}	32.43 ± 14.86 ^{A}
C15:0	0.24 ± 0.05 ^{b}	3.90 ± 0.54 ^{B}	0.06 ± 0.00 ^{c}	0.83 ± 0.67 ^{C}	0.82 ± 0.00 ^{a}	4.37 ± 2.85 ^{B}	0.68 ± 0.16 ^{a}	12.86 ± 7.78 ^{A}
C16:0	19.19 ± 2.94 ^{b}	312.78 ± 74.28 ^{B}	28.88 ± 4.44 ^{a}	413.01 ± 68.52 ^{B}	21.38 ± 0.63 ^{b}	113.33 ± 24.58 ^{C}	27.88 ± 2.87 ^{a}	529.81 ± 22.61 ^{A}
C16:1n-9	0.32 ± 0.05	5.14 ± 0.97	-	-	-	-	-	-
C16:1n-7	0.26 ± 0.07 ^{c}	4.19 ± 0.96 ^{C}	0.51 ± 0.00 ^{b}	7.28 ± 1.88 ^{B}	0.78 ± 0.10 ^{a}	4.16 ± 2.12 ^{BC}	1.26 ± 0.53 ^{a}	23.85 ± 9.14 ^{A}
C16:1n-5	1.01 ± 0.11 ^{a}	16.44 ± 3.13 ^{B}	1.02 ± 0.62 ^{a}	14.57 ± 3.74 ^{B}	1.56 ± 0.05 ^{a}	8.25 ± 1.72 ^{C}	1.42 ± 0.28 ^{a}	26.93 ± 2.37 ^{A}
C17:0	0.35 ± 0.10 ^{b}	5.64 ± 1.95 ^{B}	0.28 ± 0.12 ^{b}	3.97 ± 1.38 ^{B}	0.36 ± 0.07 ^{b}	1.88 ± 0.42 ^{C}	0.69 ± 0.14 ^{a}	13.07 ± 3.78 ^{A}
C18:0	14.69 ± 3.84 ^{b}	239.50 ± 45.37 ^{B}	8.13 ± 1.64 ^{c}	116.32 ± 12.65 ^{C}	3.81 ± 0.32 ^{d}	20.19 ± 3.93 ^{D}	24.23 ± 2.47 ^{a}	460.41 ± 32.19 ^{A}
C18:1n-9	1.67 ± 0.19 ^{c}	27.22 ± 7.82 ^{B}	3.45 ± 0.83 ^{ab}	49.27 ± 6.89 ^{A}	4.17 ± 0.42 ^{a}	22.10 ± 3.12 ^{B}	3.09 ± 0.05 ^{b}	58.62 ± 9.65 ^{A}
C18:1n-7	0.52 ± 0.15 ^{c}	8.43 ± 4.97 ^{B}	1.33 ± 0.43 ^{a}	19.02 ± 3.15 ^{A}	0.97 ± 0.07 ^{b}	5.12 ± 0.98 ^{B}	1.13 ± 0.26 ^{a}	21.46 ± 6.98 ^{A}
C18:2n-6	12.54 ± 2.01 ^{a}	204.46 ± 30.13 ^{A}	15.27 ± 2.13 ^{a}	218.36 ± 45.32 ^{A}	14.47 ± 0.84 ^{a}	76.67 ± 18.18 ^{B}	10.81 ± 2.13 ^{ab}	205.32 ± 50.22 ^{A}
C18:3n-3	46.30 ± 6.55 ^{a}	754.65 ± 131.74 ^{A}	35.42 ± 3.59 ^{b}	506.49 ± 131.65 ^{AB}	48.92 ± 1.21 ^{a}	259.27 ± 55.25 ^{C}	24.76 ± 5.72 ^{c}	470.52 ± 87.52 ^{AB}
C20:0	0.71 ± 0.17 ^{b}	11.51 ± 2.42 ^{B}	2.00 ± 0.42 ^{a}	28.57 ± 5.96 ^{A}	0.83 ± 0.06 ^{b}	4.40 ± 1.12 ^{C}	1.37 ± 0.33 ^{a}	26.02 ± 9.01 ^{A}
C22:0	0.96 ± 0.18 ^{b}	15.60 ± 2.16 ^{B}	2.36 ± 0.83 ^{a}	33.75 ± 8.45 ^{A}	1.42 ± 0.35 ^{ab}	7.53 ± 2.92 ^{C}	1.40 ± 0.34 ^{ab}	26.64 ± 8.99 ^{A}

Note: "-"—acid not found; The table shows the average values from three biological replicates (M) and their standard deviations (±S.D.). Different letters (lowercase letters are for % of SUM; uppercase letters are for μg/g DW) located horizontally indicate the significant differences ($p \leq 0.05$).

The experiment revealed that the amount of polyunsaturated FAs (PUFAs) decreased after 72 h of leaf exposure following mowing; eventually, these changes were reflected in the total content of all FAs. Reliable results were obtained only when converted to g DW, because the relative FAs content did not reflect real changes in the composition and content of these acids in the leaves after mowing at different 24 h and 72 h soaking times. The ratio of n-6/n-3 increased at 24 h of leaf curing and in the leaves of the cotyledons, compared with the summer values (Figure 2). The contents of all SFAs decreased after 72 h in vivo (Figure 2). The sum of unsaturated FAs (USFAs) also decreased after 72 h of leaf exposure.

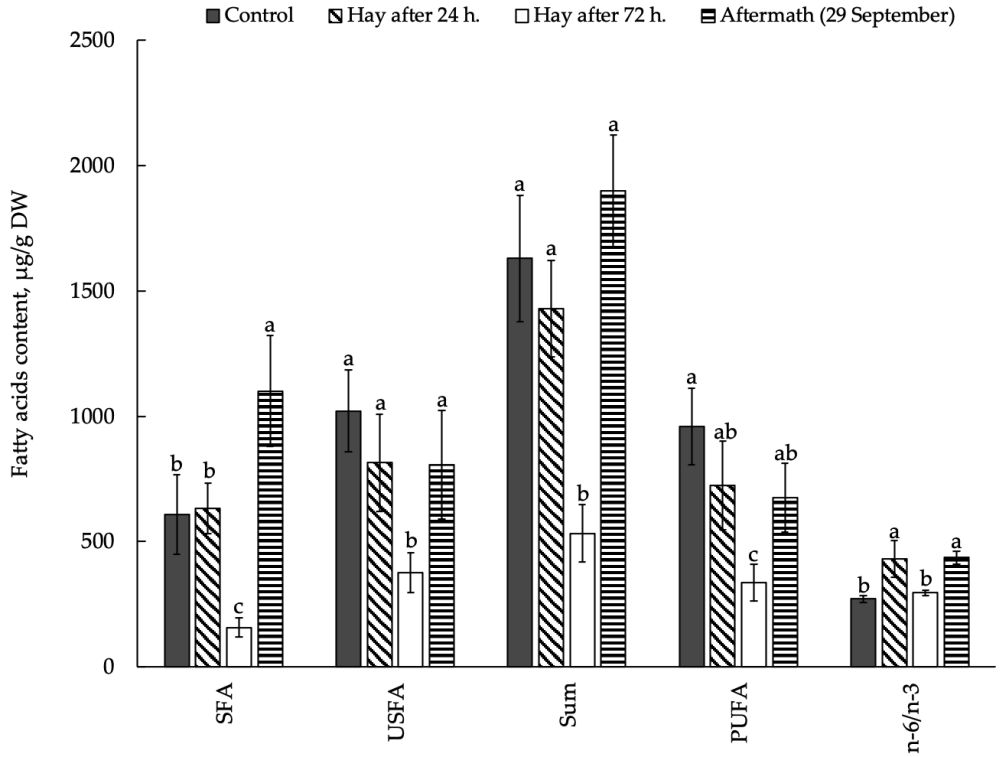

Figure 2. Saturated (SFA), unsaturated (USFA), sum FAs (Sum), polyunsaturated (PUFA), and n-6/n-3 ratios in plant raw material leaves after mowing, drying, and in aftergrass. The bars represent standard deviations. Means ± SDs, $n = 3$; the different letters indicate significant differences ($p \leq 0.05$).

3.3. Sterols and Their Esters in Plant Raw Material Leaves in Mowing and Drying

The absolute content of sterols increased during mowing and drying for 24 h; then, at a longer drying time of 72 h, the amount of all sterols decreased markedly compared to that in the fresh cut grass. Accumulation of sterol components occurred in the leaves of the aftergrass plants (Figure 3). The amount of free sterols significantly exceeded the content of bound sterols (fatty acid esters) in grass leaves in all experimental variants.

Using GC-MS, sterols, such as cholesterol, campesterol, stigmasterol, and β-sitosterol, were detected in the sterol profile of grass leaves (Figures 4 and S2). B-sitosterol dominated the sterol composition, and its content was higher than that of the other sterols in the leaves of all samples. The amount of β-sitosterol increased markedly when leaves were aged for 24 h after mowing compared to fresh cut grass; a longer exposure time of 72 h resulted in a marked decrease in the content of this sterol compared to fresh cut grass. The content of β-sitosterol in the aftergrass leaves was higher than that in the fresh cut grass variant. The content of sterols such as campesterol and stigmasterol after mowing first increased during leaf-holding for 24 h, then significantly decreased during 72 h of leaf-holding in natural conditions and in leaves of newly grown shoots in autumn, that is, in the aftergrass. All these changes affected the total sterols content; therefore, 24 h of leaf exposure after mowing

resulted in an increase in this index compared to the fresh cut grass. With a longer exposure of leaves to natural conditions (72 h), the number of sterols was minimal compared to the other variants of the experiment. The sum of all sterols in the leaves of the aftergrass was significantly higher than that in the fresh cut grass and at 72 h of exposure.

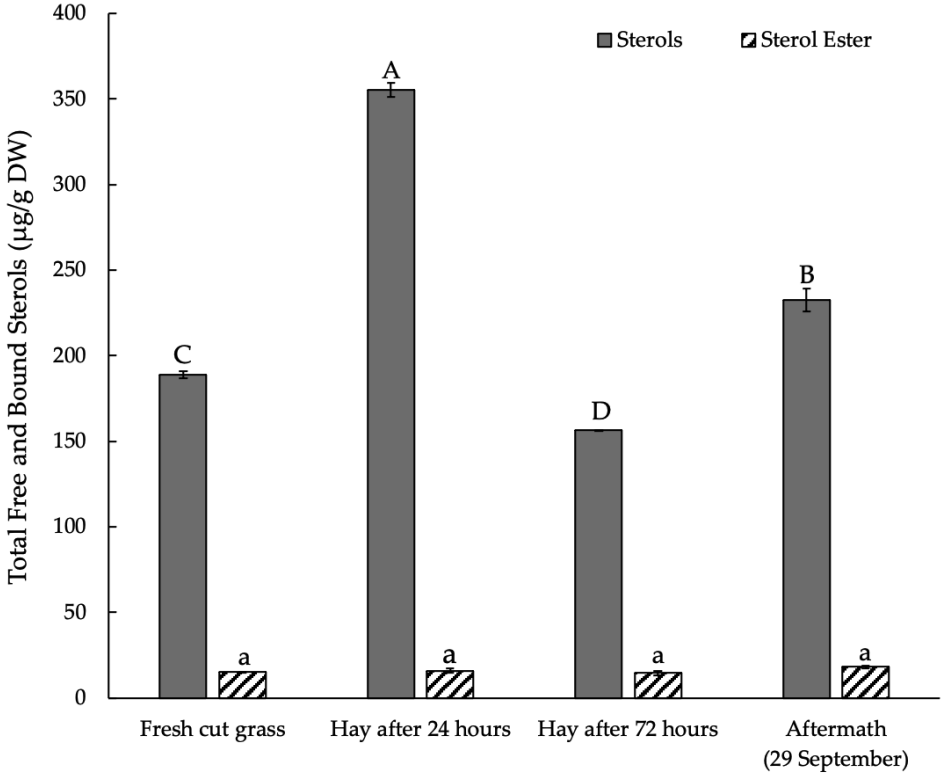

Figure 3. Total content of free and bound sterols in leaves of plant raw material after mowing, drying, and in aftergrass. The bars represent standard deviations. Means ± SDs, $n = 3$; the different letters indicate significant differences ($p \leq 0.05$).

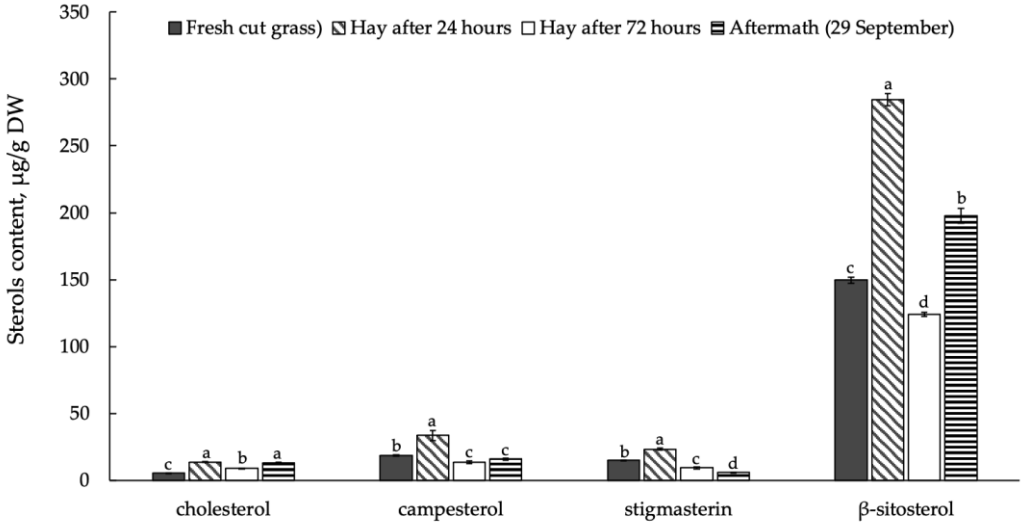

Figure 4. Sterol profile of plant raw material leaves after mowing, drying, and in aftergrass. The bars represent standard deviations. Means ± SDs, $n = 3$; the different letters indicate significant differences ($p \leq 0.05$).

The qualitative composition of the main types of bound sterols in grass leaves included β-sitosterol and cholesterol esters (Figure 5). The cholesterol content was higher in sterol

esters than in β-sitosterol. In aftergrass leaves, the pool of cholesterol in sterol esters was higher than that on other dates of the experiments. The β-sitosterol content was lower in the 72 h hay than in the fresh grass and 24 h hay. In general, a higher content of sterol esters was found in the leaves of the aftergrass than in the leaves after mowing.

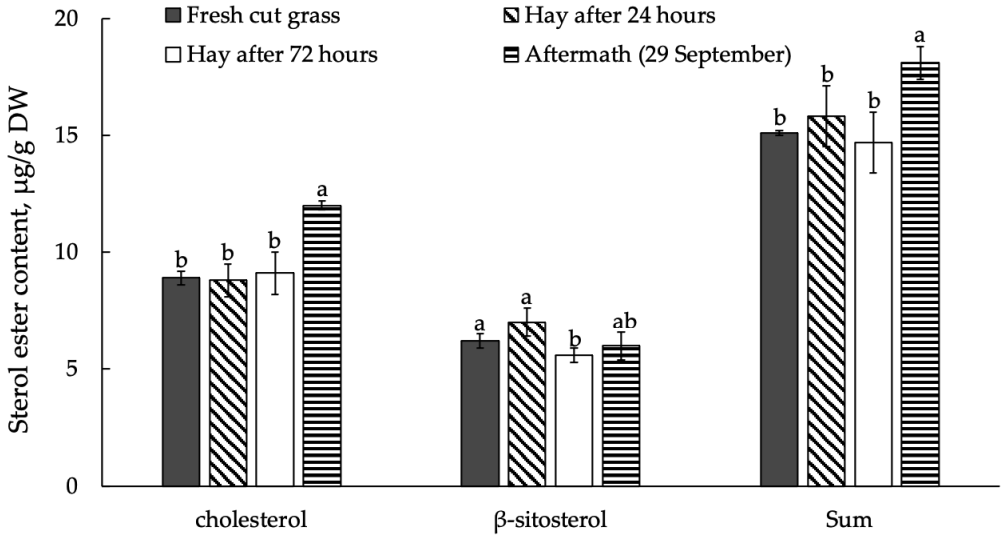

Figure 5. Contents of sterol esters in the leaves of plant raw material after mowing, drying and in aftergrass. The bars represent standard deviations. Means ± SDs, n = 3; different letters indicate significant differences ($p \leq 0.05$).

4. Discussion

Forage lands (meadow phytocenoses) are agricultural lands covered with herbaceous vegetation dominated by mesophilic perennial grasses. They are mainly used for mowing grass for green fodder, hay, haylage, and silage (hayfields) or for grazing cattle and horses (pastures). In Central Yakutia, the first half of the summer is characterized by very hot and dry weather (Table 2). The main amount of precipitation during the warm season occurs in the second half of summer and autumn. Thus, in May-June in Central Yakutia, 50 mm of precipitation falls, in July and August at 85 mm [31]. Thus, during the investigation period, the hydrothermal moisture coefficient amounted to K = 0.3. According to the classification of humid zones, the area where the fieldwork was carried out corresponded to the dry zone (<0.4).

Therefore, one of the reasons for the low productivity of cattle in Yakutia is the extremely low content of nutrients and biologically active substances in harvested hay [10].

As mentioned above, there is a loss of water in the cut leaves of grasses, and plant cells begin to experience various types of stresses (hyperosmotic stress, high air temperature, and drought). Under osmotic stress, the unbound water content in the cell changes. The inflow and retention of water inside a cell largely depends on the vacuolar membrane [32].

Traumatic damage to shoots (mowing) causes various regeneration mechanisms in plants, which leads to wound healing and restoration of lost aboveground plant organs through the growth of dormant (axillary) buds of perennial grasses. Previously, it was shown that new shoots of cereals (oats, bromegrass) grown after mowing had higher contents of LA and ALA in leaves than in control plants [11].

Our experiments revealed that free fatty acids (FFAs) dominated in the neutral lipid fraction, and the content remained high even in leaves after 72 h of exposure under natural conditions. Normally, the amount of FFAs in higher plants is low, and FFAs rarely accumulate in healthy tissues. FFAs are the intermediates in the synthesis of other metabolites. As fat-soluble anions, they can increase proton conductivity [33]. Most likely, the increase in FFAs content was related to the loss of large amounts of water in plant cells and protection against drought. A marked increase in TAG was detected in the leaves of grasses sampled 24 h after mowing. During that time, very hot and dry weather was

recorded in Central Yakutia, reaching +31.1 °C during the daytime (Table 2). Mowing of the plant shoots disrupts almost all their metabolic processes. The content of free water in the plant raw material decreases sharply after mowing due to the cessation of water from the root system with a simultaneous increase in its evaporation into the environment, which leads to a change in the lipid composition towards a greater accumulation of TAG. Normally, TAG does not accumulate in significant amounts in the vegetative tissues of plants [34], but various stress conditions such as drought [35], high or low temperature, and nutrient deficiency [36] can induce TAG production, especially in leaves, so it is likely that lipid droplets begin to accumulate in the form of TAG in the grasses studied in response to stress conditions, as we observed in hay leaves after 24 h of mowing (Table 3).

Phospholipids are major constituents of cell membranes and are also important signaling molecules that regulate plant growth and development, as well as cellular responses to environmental changes [37]. In our experiments, we found that mowing leaves and drying them for 24 h and 72 h under natural conditions reduced the amount of PI, PC, and the sum of PG + DPG. The increase in the amount of PC, DGDG, PE, MGDG, and the decrease in PI and PG + DPG in leaves of aftergrass is probably because young shoots after mowing are exposed to low air temperatures, which leads to changes in the composition of membrane lipids, that is, there is an increase in the content of some lipids and a decrease in the amount of others. Plastid membranes consist of glycolipids including MGDG, DGDG, and phospholipid PG [38]. The decrease in the amount of PG + DPG and DGDG in the leaves of grasses after mowing for 72 h was probably due to the fact that the photosynthetic apparatus was disassembled and the photosynthetic pigments and plastid membranes were destroyed. During membrane remodeling, the proportion of bilayer lipids (PC, PI, PG, and DGDG) decreased, which confirmed the presence of membrane packing defects [39]. Under the action of low temperatures in plant cells, ultrastructural changes are associated with the growth of chloroplast size and modification of mitochondrial forms [40]. Apart from PC and PE, plant mitochondrial membranes contain PG, PI, and the mitochondrial-specific lipid DGP [41,42]. In our experiments, a decrease in PC, PI, and DPG during 72 h of leaf drying in the summer after mowing and an increase in PC in the leaves of the holly leaves in the fall indirectly indicate significant changes in mitochondria under the action of these stressors.

After harvesting, grass is dried for various periods to achieve a higher dry matter content and enhance silage fermentation [43,44]. Grass wilting is associated with oxidative losses of PUFAs, mainly ALA, whose proportion decreases in the total GI with a simultaneous increase in the proportion of C16:0 [45,46]. In our experiments, the amount of C16:0 decreased sharply, as well as the amount of LA and ALA, and the greatest decrease in these acids was observed during 72 h of leaf exposure in natural conditions; at this time in the Yakutia cryolithozone, there are very hot and dry weather conditions (Table 1). The n-6/n-3 ratio is an important parameter for evaluating the nutritional quality of lipids in animal feeds [47]; this ratio was higher in the lipids of aftergrass leaves than in summer-vegetated grass leaves (Figure S2), confirming its high nutritive value for animals in northern conditions.

Herbal phytosterols are environmentally friendly feed additives that stimulate growth and positively influence the immune status of animals. In addition, sterols are vital membrane components, responsible for both structural and regulatory functions in many key plant cellular processes [48]. Analysis of the sterol profile (Table S1) showed that at 24 h of leaf exposure after mowing, there is an increase in all sterols, mainly due to β-sitosterol; this sterol is a precursor in the synthesis of another "stress sterol"—stigmasterin [49]. It is believed that in higher plants, the content of β-sitosterol increases and campesterol decreases under cold stress [50]. In our experiments, we found that the amount of β-sitosterol increased by 1.3 times in the leaves of cold-hardened aftergrass, and the amount of campesterol decreased by 1.2 times compared with summer grass leaves. It is known that sterols are present in the membranes of plant mitochondria at a higher percentage (about 3–10%) than in the membranes of chloroplasts [51], which confirms the assumption,

based on the results obtained, of the effect of low September quenching temperatures on ultrastructural changes in mitochondrial membranes in aftergrass leaves.

Sterol esters do not have charged groups and, therefore, cannot be integrated into bilayer membranes in significant amounts. In this regard, sterol esters (SE) and TAG usually act as depots for the storage of sterols, FAs, and 1,2-DAG [52]. Most likely, the sterol esters in the leaves of aftergrass are the components in which the depot of cholesterol is stored, as its amount in SE was much higher than the amount of β-sitosterol.

It was also revealed that in September, despite the identical species composition (Table 1), the studied plant species had different developmental phases compared with July. All these facts indicate that not only do the low hardening air temperatures in September affect the composition of lipids, FAs, and sterols in the leaves of the aftergrass, but the developmental phases of plants at this time also have a certain influence. For example, it was shown earlier that the shoots of perennial herbaceous plants of the cryolithozone (*Psathyrostachys juncea* Tzvel., *Elymus sibiricus* L., *Bromopsis inermis* Leys) grown after mowing, and those that passed cold hardening had different developmental phases and, as a result, different contents of photosynthetic pigments [3].

Generally, the climate of the cryolithozone of Yakutia in the autumn period is favorable for the production of green cryofeed (aftergrass), as low ambient temperatures preserve the green mass of plants mowed in the middle of the summer period.

Thus, the natural cold, an unlimited and cheap resource of the cryolithozone, can be used to obtain aftergrass, plant raw materials with a high content of biologically active compounds (phytosterols and FAs), their subsequent isolation, and the creation of a biologically active supplement to the diet of many farm animals, which may be of interest in the field of agricultural plant biotechnology.

5. Conclusions

Data were obtained on the accumulation of lipid components in the leaves of grasses after their mowing and drying in the middle of the growing season and after cold hardening by low temperatures of the cryolithozone (aftergrass), as well as the effect of mowing shoots with leaf exposure for 24 h and 72 h (drying) under natural conditions on the amount of lipids, FAs, and sterols. As a result of the studies, it was found that mowing grass shoots with a leaf exposure of 72 h (drying) led to a more pronounced decrease in lipid components than with a shorter exposure (24 h). Aftergrass leaves contained a higher content of free sterols, saturated FAs, and the ratio of n-6/n-3 than fresh cut grass leaves. The absolute content of all FAs in aftergrass leaves and fresh leaves of summer grasses did not differ significantly from each other, indicating that such plant food is more valuable than grass leaves cut and dried under natural conditions in summer. Such plant fodder provides energy for livestock and wild animals in the North (Yakutian horses, aboriginal Yakutian cows) fed by it. The experimental data obtained in the present work can be used to obtain plant feeds with high lipid and PUFAs contents under conditions of a sharp continental climate. The natural cold of the cryolithozone is an unlimited and inexpensive resource for obtaining plant raw materials with a high content of sterols and FAs (aftergrass leaves), as shown in this study. We believe that the subsequent isolation of biologically active compounds and the creation of such raw plant materials as an additional supplement to the diet of many farm animals may be of great prospect in the field of agricultural plant biotechnology.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13092252/s1, Figure S1: Typical TLC chromatogram of neutral lipids (daylight) (A) and polar lipids (daylight) (B) of grass leaves obtained by one-dimensional TLC; Figure S2: GC chromatogram of the sample; Table S1: Chromatographic retention time (RT, min) and characteristic ions (m/z) in the mass-spectra of the identified sterol components.

Author Contributions: Conceptualization, V.V.N. and K.A.P.; methodology, V.V.N., L.V.D. and K.A.P.; software, V.V.N., L.V.D. and N.V.S.; validation, L.V.D. and N.V.S.; formal analysis, V.V.N., L.V.D.

and N.V.S.; investigation, V.V.N. and K.A.P.; resources, K.A.P. and L.V.D.; data curation, V.V.N. and L.V.D.; writing—original draft preparation, V.V.N.; writing—review and editing, V.V.N., L.V.D. and K.A.P.; visualization, V.V.N.; supervision, L.V.D.; project administration, V.V.N. and K.A.P.; funding acquisition, V.V.N. and K.A.P. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data used to support the findings of this study can be made available by the corresponding author upon request.

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