

Article

The Promotive Effect of *Cyanobacteria* and *Chlorella* sp. Foliar Biofertilization on Growth and Metabolic Activities of Willow (*Salix viminalis* L.) Plants as Feedstock Production, Solid Biofuel and Biochar as C Carrier for Fertilizers via Torrefaction Process

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Abstract: The effect of foliar application of *Cyanobacteria* and *Chlorella* sp. monocultures on physiological activity, element composition, development and biomass weight of basket willow (*Salix viminalis* L.) and the possibility to prepare biofuel from it in the fortification process was studied. Triple foliar plant spraying with non-sonicated monocultures of *Cyanobacteria* (*Anabaena* sp. PCC 7120, *Microcystis aeruginosa* MKR 0105) and *Chlorella* sp. exhibited a considerably progressive impact on metabolic activity and development of plants. This biofertilization increased cytomembrane impermeability, the amount of chlorophyll in plants, photosynthesis productivity and transpiration, as well as degree of stomatal opening associated with a decreased concentration of intercellular CO₂, in comparison to control (treatments with water, Bio-Algeen S90 or with environmental sample). The applied strains markedly increased the element content (N, P, K) in shoots and the productivity of crucial growth enzymes: alkaline or acid phosphorylase, total dehydrogenases, RNase and nitrate reductase. Treatments did not affect energy properties of the burnt plants. These physiological events were associated with the improved growth of willow plants, namely height, length and amount of all shoots and their freshly harvested dry mass, which were increased by over 25% compared to the controls. The effectiveness of these treatments depended on applied monoculture. The plant spraying with *Microcystis aeruginosa* MKR 0105 was a little more effective than treatment with *Chlorella* sp. and *Anabaena* sp. or the environmental sample. The research demonstrate that the studied *Cyanobacteria* and *Chlorella* sp. monocultures have prospective and useful potential in production of *Salix viminalis* L., which is the basic energy plant around the word. In this work, a special batch reactor was used to produce torrefaction material in an inert atmosphere: nitrogen, thermogravimetric analysis and DTA analysis, like Fourier-transform infrared spectroscopy. The combustion process of *Salix viminalis* L. with TG-MS analysis was conducted as well as study on a willow torrefaction process, obtaining 30% mass reduction with energy loss close to 10%. Comparing our research results to other types of biomasses, the isothermal temperature of 245 °C during thermo-chemical conversion of willow for the carbonized solid biofuel production from *Salix viminalis* L. biomass fertilized with *Cyanobacteria* and *Chlorella* sp. is relatively low. At the end, a SEM-EDS analysis of ash from torrefied *Salix viminalis* L. after carbonization process was conducted.

Keywords: *Salix viminalis* L.; *Cyanobacteria*; *Chlorella* sp.; physiological activity; growth; torrefaction; biofuels

1. Introduction

The element content is the one of the most important factors in the development and metabolic activity of plants, which is a key issue in organic and integrated farming producing biofuel for energy needs. Prevention of nutrient deficiency is very important in the case of organic energy biomass cultivation (e.g., *Salix viminalis* L.), which should be conducted on poor quality soil and not compete with food production. Proper plant feeding which is friendly to the environment counteracts dangerous disturbances in physiological activities of plants which can cause a lower yield of biomass. Several authors [1–3] demonstrated that one of the sustainable methods of effective fertilization may be treating plants with *Cyanobacteria Nostoc muscorum*, which absorbs nitrogen from the air and can then pass it and other compounds to plants, thus improving their value as biofuel by increasing their energy properties. The nitrate reductase, nitrogenase and other metabolites contained in this *Cyanobacteria*, when sprayed on plants, could increase plant growth. Moreover, peptides and amino acids found in the filtrate of these microorganisms can enhance plant development and their biomass yield. This biofertilization is non-toxic to living organisms and the environment, causes less soil contamination and toxicity, is an alternative to artificial fertilizers and pesticides, and protects plants against diseases [1]. Moreover, it is cost-effective compared to chemical compounds and conventional agriculture methods [2,4]. It has been also noted that *Cyanobacteria*, green algae and *Bacillariophyceae* contain numerous macronutrients and micronutrients, hormones (cytokinins, auxins, gibberellins), biostimulators, amino acids, polyamines, vitamins and a number of other supplementary metabolites which can promote growth of plants and energy biomass yield [4–9]. In addition to the profitable use of algae in energy crops proposed in this study, the possibility of absorbing CO₂ is another advantage of their use in the production of biomass, which is being developed in current research [10,11]. According to Karthikeyanb et al. [12] and Nain et al. [13], these organisms can also improve the sorption complex, structure and porosity of soil by secreting mucus and polysaccharides. Additionally, some strains of *Cyanobacteria* can fix atmospheric dinitrogen (N=N) and transform it into ammonium, the available form to plants, consequently increasing the energy biomass yield, as stated in wheat, rice, gillyflower and vines [3,12,14–18]. In soil it can convert insoluble phosphorus into the forms accessible to plants, thereby increasing their energy biomass yield and assimilability. These algae are also able to synthesize some compounds that prevent the development of pathogenic microflora [19–21] and can enhance symbiosis with other numerous organisms [22]. The extract of seaweeds induced various beneficial changes in plants, causing increased biomass yield, enhanced nutrient downloading, resistance to low temperature and stress, higher post-harvest shelf life and germination of seeds, and more effective protection against phytopathogens [23]. Due to the mentioned properties, *Cyanobacteria* and algae are the focus of interest in relation to organic crop production, including cultivation of energy plants, which are important economically and are grown on poor quality soil, often contaminated with toxic pollutants and not suitable for food production. Moreover, the cultivation of energy plants on poor soil does not compete with food production which must be performed on high-quality aerials covering only 52% of arable regions. Although the mentioned data concerning the biology of these microorganisms is generally known, their influence on metabolic activity and growth of particular plant species is different and thus still needs elucidation. So far, there is scant information on the possible use of microalgae to increase the growth, development, chemical composition and energy properties of willow (*Salix viminalis* L.) in terms of its suitability for energy production. There is also no information indicating the possibility of torrefaction of willow biomass fertilized with microalgae and the quality of the biofuel obtained from this thermo-chemical conversion. The presented research is the first study which demonstrates the influence of the chosen strains of *Cyanobacteria* and *Chlorella* sp. on the growth, metabolic activity and energy properties of willow and the quality of carbonized willow obtained from it, bearing the fact that this plant is a recommended source of renewable energy around the world.

The purpose of the presented experiments was to check the impact of foliar application of monocultures of *Cyanobacteria* (*Microcystis aeruginosa* MKR 0105, *Anabaena* sp. PCC 7120) and green algae (*Chlorella* sp.) on the development, metabolic activity and energy properties of basket willow, as well as the possibility of obtaining sustainable biofuel from it in the torrefaction process. The proposed thermo-chemical conversion of biomass plays a key role in modern energy production due to several advantages: improving caloric value, increasing grind ability, decreasing costs of fuel storage thanks to obtaining hydrophobic characteristics, and the possibility of directly replacing coal in power plants by solid carbonized fuel without additional costs.

2. Materials and Methods

2.1. Plant Material

Willow (*Salix viminalis* L.) plants were derived from woody cuttings that were rooted in accordance with the procedure used in the commercial production of this species. Woody cuttings were placed at the end of April, in 5 liter pots filled with poor soil and the obtained plants were then cultivated individually without transplanting on open fields under natural conditions and watered with tap water. Every treatment was performed in three series and in three repetitions which consisted of 10 plants. The series and repetitions were deployed in a randomized block design and located in Skiernewice, Central Poland. The element content in soil is shown in Table 1.

Table 1. Element content in soil.

N	P	K	Ca	Mg	Fe	Mn	Cu	Zn	B	Dry Mass
mg kg ⁻¹ Dry Weight										%
1.01	930	3829	25,734	1553	656	45.1	23.5	29.0	60.1	25.3

The strains of *Cyanobacteria* (*Microcystis aeruginosa* MKR 0105, *Anabaena* sp. PCC 7120) and algae (*Chlorella* sp.) were cultured using ATCC Medium 616 (own standard product), at a temperature of 27 °C and with light emitted by FAREL lamps (PHILIPS FAREL, Piła, Poland), 18 W, using the method developed by Romanowska-Duda et al. [24]. Before treating the plants, each microalgae strain was centrifuged for 2 min. (4000 rpm) and then mixed with an appropriate amount of water to bring the cell number to 2.5×10^5 in 1 mL. A Fuchs-Rosenthal hemocytometer was used for cell counting. A volume of 20 mL of prepared monoculture was applied to each plant.

The effectiveness of the selected *Cyanobacteria* and *Chlorella* sp. strains cultured in laboratory conditions were compared to the environmental sample, which was a mixture of all algae found in a natural freshwater reservoir near Łódź (Poland, Zalew Sulejowski; Latitude 51.3642, Longitude 19.8886 51°21'51").

Bio-Algeen S90 (Schulze & Hermsen GmbH) was obtained commercially as part of an ecological biopreparation produced on the base of seaweed extract.

2.2. Treatments

The non-sonicated strains of *Cyanobacteria* and *Chlorella* sp. were applied three times to willow plants grown in the 5 liter pots, and in three-week intervals. The first application was made when the plants were 5 cm in height. The plants treated with water, Bio-Algeen S90 and environmental sample served as controls.

2.3. Assessments of Physiological Parameters of Biofertilized Plants

Growth and metabolic activity of willow were studied to explain the mechanism by which *Cyanobacteria* and algae stimulate development of plants and increase energy biomass yielding. Dynamics of shoot growth, their total length, amount and weight of fresh and dry biomass were measured to assess plant growth. The metabolic activity in plants was evaluated by determining the productivity of acid and alkaline phosphorylase, RNase, total dehydrogenase and nitrate reductase and also by index of chlorophyll content,

photosynthesis, transpiration, stomatal conductance, concentration of intercellular CO₂ and electrolyte leakage from leaves [25–27]. These parameters not only reflect the physiological activity of plants but are useful indicators of growth of plants and biomass yielding, as was found in our previous studies and the literature [24–27]. As our previous research [6,9,11] and commercial plantations show, willow cultivated with the conventional methods is an attractive source of sustainable energy. Since the influence of fertilization with *Cyanobacteria* and *Chlorella* sp. on the chemical composition and energy properties of these plants is unknown, the content of selected macroelements in leaves, calorific value of shoots, combustion heat and content of ash were examined.

To assess the plant growth kinetics, their height, sum of lengths of all shoots and their amount were evaluated in every 2–3 weeks during all vegetative periods. Fresh weight of the shoots and their dry biomass weight (dried at 130 °C for 72 h) was evaluated at the end of experiments [26,27].

Index of chlorophyll content in the plants was assessed with the chlorophyll meter SPAD-502 (Konica Minolta). Activity of gas exchange (net photosynthesis, stomatal conductance, concentration of intercellular CO₂, transpiration) were measured with the gas analyser TPS-2, (PP Systems International Inc., Amesbury, MA, USA) [28,29]. Activity of phosphorylases (acid and alkaline) and ribonuclease (RNase) in the leaves were assessed using the procedure presented by Knypl and Kabzinska [30]. Dehydrogenase activity was estimated by the method demonstrated by Gornik and Grzesik [31], using Spectrophotometer UVmini-1240 SHIMADZU. Nitrate reductase was evaluated with the technique developed by Jaworski [32]. Electrolyte leakage from tissues was examined after 2 and 4 h following placement of the segments of leaves in test tubes at 20 °C, which were filled with 3 mL of deionized water. Measurements were made with the CC-551 Elmetron conductivity meter [31]. Assessments of macroelemental content in plants and their energy properties were evaluated by certificated laboratories.

2.4. Proximate Analysis

Analysis to estimate the amount of ash in raw samples of *Salix viminalis* L. was performed using an electrical furnace. The research assumed that that no ash was lost during the torrefaction process. Thus, the ash content value was calculated for all solid residue from the overall mass yields. The proximate analysis of C, H, N, O and S contents were performed using Elemental Analyser Perkin/Elmer. All samples of *Salix viminalis* L. were assessed. All the measurements were conducted three times. The average value was calculated, and was corrected regarding water content (Table 2).

Table 2. Shoot number of willow plants fertilized foliarly with Bio-Algeen S90 and non-sonicated strains of *Cyanobacteria*, *Chlorella* sp. and environmental sample. The woody cuttings were planted to pots in the third week of April and the plants were grown without transplanting.

Applied Prepare, <i>Cyanobacteria</i> and Algae	Day and Month of Measurements				
	1 June	11 June	22 June	12 July	3 August
Control	1.2 a *	2.2 a	2.3 a	2.5 a	2.6 a
Bio-Algeen S90	1.4 b	2.3 b	2.7 b	2.9 b	2.9 b
<i>Microcystis aeruginosa</i> MKR 0105	1.5 b	2.5 d	3.1 cd	3.7 d	3.7 c
<i>Chlorella</i> sp.	1.5 b	2.4 c	3.0 c	3.4 c	3.6 c
<i>Anabaena</i> sp. PCC 7120	1.4 b	2.4 c	2.9 c	3.4 c	3.5 c
Environmental sample	1.3 a	2.3 b	2.7 b	2.9 b	2.9 b
LSD 0.05 **	0.1	0.08	0.1	0.2	0.2

* The means associated with the same letter are not significantly different according to Duncan multiple range test at the significance level of $p = 0.05$. ** The LSD were calculated at the significance level of $p = 0.05$.

2.5. Fuel Characteristics

2.5.1. Caloric Value

Two methods were used to measure the high heating and caloric values. One of the methods was based on using calorimetric bombs. The next method was based on performing calculations on the caloric value and knowing the amount of nitrogen, hydrogen and carbon. The first equation for caloric value estimation was used to find the High Heating Value, HHV, and was named the partial least squares regression (PLS) method (Equation (1)), which enabled calculation of a HHV base on a dry basis. High heating value was estimated using the formula below:

$$HHV(PLS) = 5.22C^2 - 319C - 1647H + 38.6CH + 133N + 21028 \quad (1)$$

where C = carbon, H = hydrogen, and N = nitrogen content defined on a dry mass percentage basis. C, H, N medium values are corrected by the dry ash content on a free basis.

In the presented research work, the methodology for analysis of the *Salix viminalis* L. carbonization process for biofuel production involved the following analytical techniques:

- Thermogravimetric analysis TG, DTA, TGA TG-FTIR, and TG-MS carbonization process for untreated and carbonized willow combustion;
- Proximate and technical analysis of the willow torrefaction process and final products;
- Gas analysis of torgas formed as a result of carbonization: FTIR TG-MS analysis;
- Fuel analysis (caloric value, ash content, moisture content) of willow carbonization process products;
- SEM-EDS ash analysis of carbonized *Salix viminalis* L. after combustion.

Research work on the willow thermo-chemical conversion was conducted by using a specifically designed biomass torrefaction installation with a batch reactor under inert atmosphere in nitrogen (Figure 1). Torrefaction process is a thermo-chemical conversion to upgrade substrates like biomass, wastes and other untreated materials, which takes place in a temperature limit between 200 °C to 350 °C to generate fuel with a higher energy density, mainly by the destruction of biomass polymers of lignin, hemicellulose and cellulose.

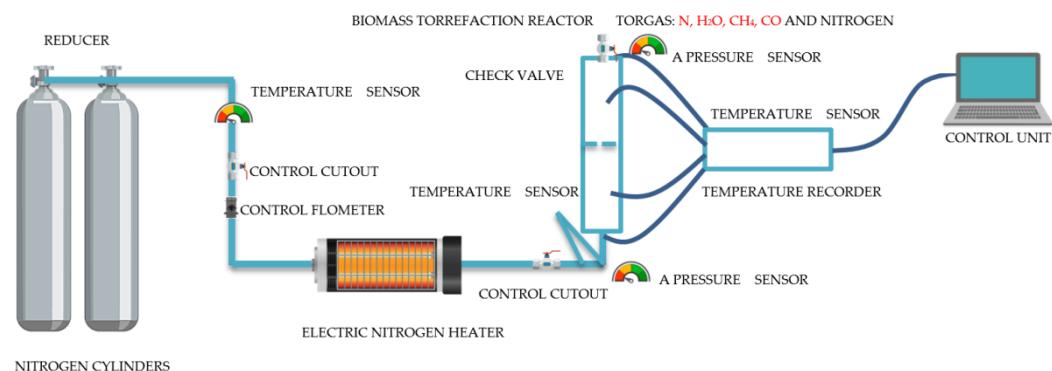


Figure 1. Batch reactor with electrical preheaters for the *Salix viminalis* L. thermo-chemical conversion in nitrogen.

During decomposition of willow polymers, three areas were detected on the mass loss curves of *Salix viminalis* L. while the carbonization process was taking place in the batch reactor. The first curve corresponds to the hemicellulose which is the most reactive component. Depolymerization started at 220 °C and we could observe the end at 325 °C; after hemicellulose, a decomposed cellulose, whose depolymerization temperature range is between 300 °C to 375 °C, started depolymerization and the last component destroyed was lignin, which represents the wood's temperature range from 250 °C to 500 °C. The torrefaction process of lignocellulose willow was described by weight loss kinetics conducting different experimental devices. The above devices are mentioned: batch reactor, thermogravimetric analyzers (Netzsch 209 F1 Libra) and cylindrical furnaces. In the presented

research, a method using a thermogravimetric analyzer (TGA) was applied to obtain the weight loss kinetics during the willow carbonization process. Thermogravimetric analyses helped to obtain dynamic conditions in which the willow sample was stored at a certain heating ratio. In semi-scale installations and industrial scenarios we are working with much higher heating rates which are often much more dynamic than those in thermogravimetric electrical furnaces.

2.5.2. Ash Characteristics of the Torrefied *Salix viminalis* L. Biomass

The samples of willow biomass were analyzed by using scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) using an SEM FEI Quanta 200FEG microscope equipped with an EDX Oxford X-Max spectrometer. Experiments were conducted in at least 10 different spots for a specific sample by using the EDX technique on an average atom content and standard deviations were estimated for each of the specific elements.

2.6. Analyses of Willow Energy Properties and Torrefied Willow Characteristics

The willow particles were treated before the experiments. They were cleaned from contamination, separated from other substrates, chopped, cut into sections of 2 cm to 4 cm, ground and then sieved on an automatic bolter and finally dried in an electric oven at 110 °C for 4 h. Drying procedure was completed after the moisture content was 5%. Then, willow samples were tightly closed and intended for proximate and elemental analysis. C, H, N, O and S contents were estimated and the volatility matter, ash, moisture content, heat of combustion and calorific values were calculated. In a second step, proximate and technical analysis were also applied after the willow carbonization process took place in the nitrogen atmosphere. The mass of the samples was measured before and after the drying process and the results were used to evaluate the moisture content. In the carbonization process, the dried willow was split into three separate samples of 20 g and each of them was distributed on three screens of a batch reactor metal structure with different sizes of pores. The inert gas was pre-heated using electrical heaters for set-up isothermal conditions of temperature and pressure. We chose several selected samples for further analyses due to the fact that previous studies show a high mass loss of substrate—above 30% results in a high degree of the torrefaction. Too high of a carbon content provides uneconomical high energy loss above 10%. When a mass loss of willow is below 25%, willow is not fully carbonized (does not have the homogeneity and uniform physico-chemical characteristics and has a tendency to absorb water, resulting in decreasing calorific value).

3. Statistical Analysis

All experiments were conducted in three series and in three repetitions for separate treatment. Every repetition consisted of 10 plants cultivated individually in 5 L pots. The series and repetitions were deployed in a randomized block design. The calculated means from the series and repetitions were subject to analysis of variance using Program “STATISTICA version 10”. The means of selected measurements were gathered using the Duncan’s test at the significance level of 0.05.

4. Results and Discussion

4.1. Impact of Cyanobacteria and *Chlorella* sp. on Growth of Willow

Application of *Cyanobacteria* and *Chlorella* sp. to leaves significantly enhanced growth, metabolic activity and energy biomass yielding of the willow plants. The research demonstrated that the triple plant spraying with *Cyanobacteria*, *Chlorella* sp., Bio-Algeen S90 and with environmental samples resulted in significantly increased physiological properties and yield of willow biomass compared to plants which were treated with water only (Figures 2–4, Table 2). The spraying efficiency of the plants increased with the increase in the number of these treatments. Triple applications increased plant biomass yield of by over 25% compared to the control (Figures 2–4, Table 2). This was possible because the

triple foliar fertilization introduced the largest number of metabolites (contained in the applied microorganisms) into the plants, which resulted in a significant increase in the biomass yield as well as plant growth and health.

Biofertilization with two strains of *Cyanobacteria* and one of algae were more effective than other treatments. It increased to the greatest extent the dynamics of plant growth, number of shoots, their length, biomass dry and fresh weight, and also health status compared to the variants using water, Bio-Algeen S90 and the environmental sample. However, these enhancements were determined by the applied strains. *Microcystis aeruginosa* MKR 0105 increased shoot elongation slightly more than *Anabaena* sp. PCC 7120 or *Chlorella* sp. (Figures 2–4, Table 2). This might have been the effect of the structure and element content in these microorganisms, which has not been completely explained so far.

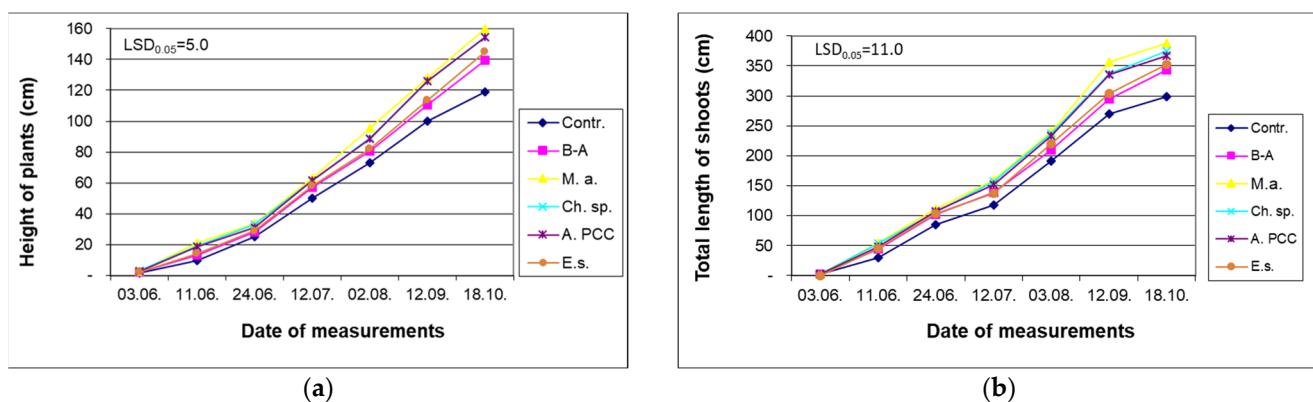


Figure 2. Dynamics of willow plant growth (a) and total length of shoots (b) fertilized foliarly with Bio-Algeen S90 (B-A) and non-sonicated strains of *Microcystis aeruginosa* MKR 0105 (M.a), *Chlorella* sp. (Ch. sp.), *Anabaena* sp. PCC 7120 (A. PCC) and environmental sample (E.s.). The woody cuttings were planted in pots in the third week of April and the plants were grown without transplanting. The LSD were calculated at the significance level of $p = 0.05$.

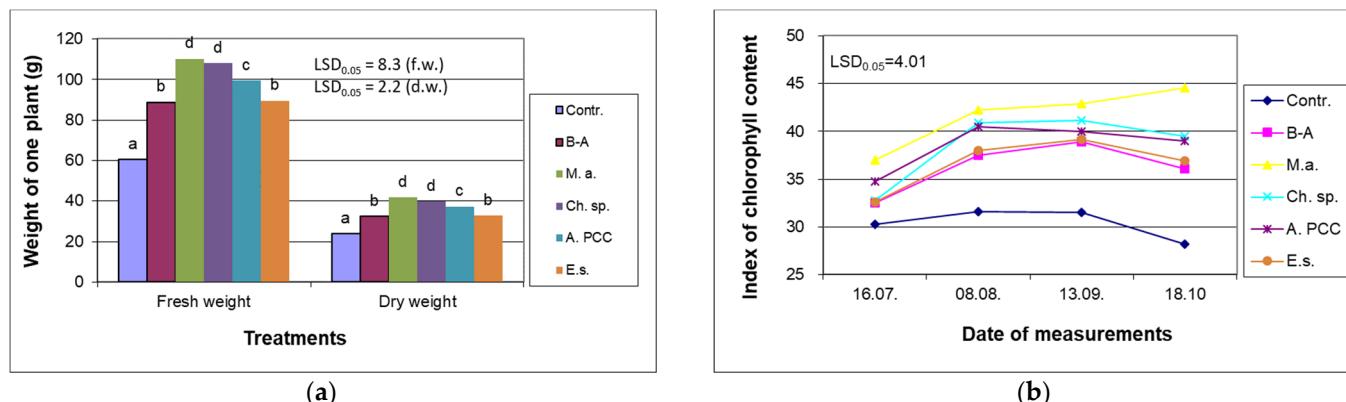


Figure 3. Dry and fresh weight of one willow plant (a) and index of chlorophyll content in willow leaves (b), fertilized foliarly with Bio-Algeen S90 (B-A) and non-sonicated strains of *Microcystis aeruginosa* MKR 0105 (M.a), *Chlorella* sp. (Ch. sp.), *Anabaena* sp. PCC 7120 (A. PCC) and environmental sample (E.s.). The data marked with the same letter (separately fresh and dry weight) are not significantly different according to Duncan's multiple range test at the significance level of $p = 0.05$. The LSD were calculated at the significance level of $p = 0.05$.

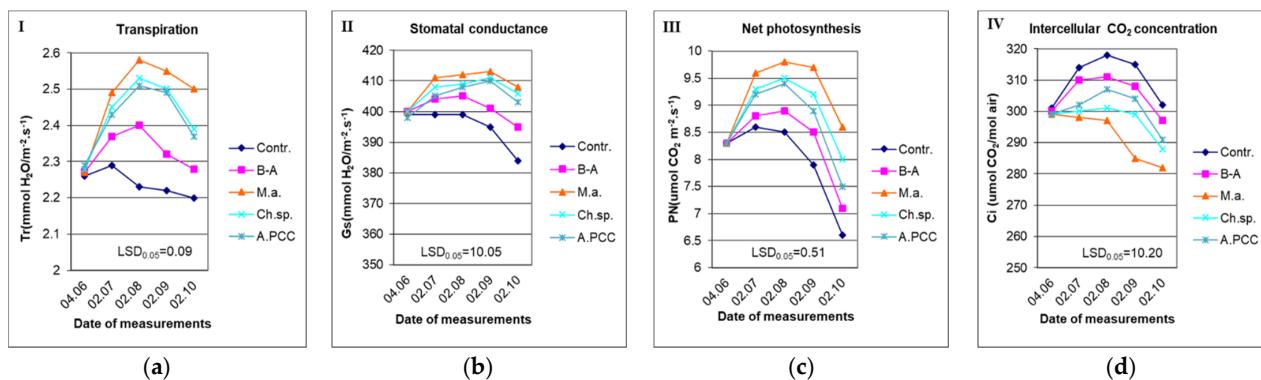


Figure 4. Transpiration (a), stomatal conductance (b), net photosynthesis (c), and intercellular CO_2 concentration (d) in willow plants fertilized foliarly with Bio-Algeen S90 (B-A) and non-sonicated strains of *Microcystis aeruginosa* MKR 0105 (M.a.), *Chlorella* sp. (Ch. sp.) and *Anabaena* sp. PCC 7120 (A. PCC). The LSD were calculated at the significance level of $p = 0.05$.

The results shown are similar to those reported by Falch et al. [19], Kreitow et al. [20], Burja et al. [21], Nain et al. [13], Rana et al. [33] and Kisielewska et al. [34] who demonstrated positive effects of *Cyanobacteria* on wheat crops. In grapevines, the application of a mixture containing seaweed extract and amino acids increased growth and development, leaf size and berry value. Increases in these parameters were ascribed to the hormones present in the seaweed extract, which increased the endogenous hormonal level. It could be related also with enzymes contained in this extract which usually improve the synthesis of sugars, acids and proteins [35]. Exogenous application of the seaweed extract caused 26.5% growth increase in “Thomson Seedless” grapes [36], while treatment with *Cyanobacteria* results in 10–25% enhancement [18]. Abdel-Mawgoud et al. [37] reported that treatments of watermelon with seaweed extract increased size of leaves. According to El Modafar et al. [38], bio-elicitors obtained from *U. lactuca* (green algae) can be useful for crop protection in view of the properties of natural defense. The obtained dates are also in agreement with our previous research, which demonstrated the stimulatory impact of cyanobacterial and algae strains on cutting, rooting, physiological activities and development of grapevines, on seed germination of maize or sunflower and growth of seedlings [9,17,39,40].

The enhanced growth of shoots, their number per plant and higher biomass yield caused by this biofertilization of willow (Table 2, Figures 2–4) could be the result of intensified physiological processes (mentioned below) (Tables 3 and 4, Figures 5–7) and of plant supplementation with auxins, cytokinins, gibberellins, amino acids and other important metabolites which are contained in *Cyanobacteria* and *Chlorella* sp. [4–10]. Hussain and Hasnain [41] reported that the regeneration of *Brassica oleracea* L. plants on MS medium containing the microbial origin hormones were similar after application of standard cytokinins and IAA. These natural substances absorbed and/or synthetized by *Cyanobacteria* and also increased the number of shoots in *Brassica oleracea*, similar to the presented research. Secretion of large IAA amounts by bacteria interacting with plants was also described by Glick et al. [42]. Similarly, Sergeeva et al. [43] stated that *Cyanobacteria*, and especially symbiotic isolates obtained from it, are also capable of accumulating and releasing IAA. They suggest that accumulation of IAA can be increased by exogenous tryptophan and may take place in the indole3-pyruvic acid pathway. These algae can respond developmentally to different signals of phytohormone when it is free-living or in plants. According to Johansson and Bergman [44], *Cyanobacteria* *Nostoc* sp. may stimulate the mitotic activity in cells which are near the place where this bacterium penetrates.

Table 3. Productivity of enzymes in willow leaves fertilized foliarly with Bio-Algeen S90 and non-sonicated strains of *Cyanobacteria*, *Chlorella* sp. and environmental sample.

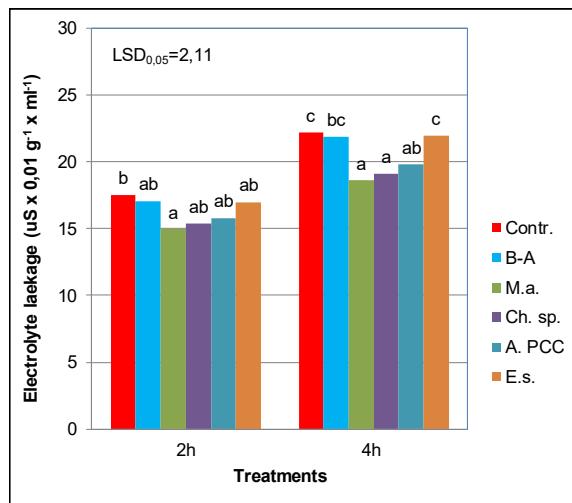
Applied Preparations, <i>Cyanobacteria</i> and Algae	Activity of Selected Enzymes				
	Phosphorylase Acid (mU g ⁻¹ f.w.)	Phosphorylase Alkaline (mU g ⁻¹ f.w.)	RNase (mU g ⁻¹ f.w.)	Dehydrogenases (mg Formazan · g leaf ⁻¹)	Nitrate Reductase (μmol NO ₂ g ⁻¹ f.w. h ⁻¹)
Control	0.54 a *	0.14 a	2.6 a	0.77 a	0.90 a
Bio-Algeen S90	0.76 b	0.25 b	3.75 b	1.22 b	1.32 b
<i>Microcystis aeruginosa</i>	0.94 c	0.38 c	4.81 d	1.62 d	1.72 d
<i>Chlorella</i> sp.	0.87 c	0.33 c	4.43 c	1.44 c	1.59 c
<i>Anabaena</i> PCC 7120	0.86 c	0.33 c	4.41 c	1.43 c	1.52 c
Environmental sample	0.67 b	0.25 b	3.76 b	1.23 b	1.35 b
LSD _{0.05} **	0.09	0.05	0.40	0.14	0.12

* The data associated with the same letter are not significantly different according to Duncan's multiple range test at the significance level of $p = 0.05$. ** The LSD were calculated at the significance level of $p = 0.05$.

Table 4. Increasing shoot length, chemical composition and energy properties of plants fertilized foliarly with Bio-Algeen S90 and non-sonicated strains of *Cyanobacteria*, *Chlorella* sp. and environmental sample.

Applied Preparations <i>Cyanobacteria</i> or Algae	Characteristics of Willow Plant Quality					
	Increase in the Length of Shoots %	Content of Macroelements in Plants	Calorific Value in the Operating State	Heat of Combustion in the Analytical State	The Ash Content in the Working State	
	N%	P mg kg d.w ⁻¹	K mg kg d.w ⁻¹	kJ kg ⁻¹	kJ kg ⁻¹	
Control	100.0 a *	3.40 a	2 166 a	31 910 a	15 247 a	18 722 a
Bio-Algeen S90	110.5 b	3.48 b	2 178 ab	31 920 a	15 140 a	18 799 a
<i>Microcystis aeruginosa</i>	125.1 d	3.64 c	2 210 c	31 980 b	15 223 a	18 769 a
<i>Chlorella</i> sp.	120.9 c	3.48 b	2 201 bc	31 962 b	15 224 a	18 699 a
<i>Anabaena</i> PCC 7120	118.2 c	3.58 c	2 199 bc	31 960 b	15 199 a	18 701 a
Environmental sample	113.7 b	3.47 ab	2 176 ab	31 922 a	15 187 a	18 745 a
LSD _{0.05} **	4.0	0.7	28.9	34.3	112.1	102.2

* The data associated with the same letter are not significantly different according to Duncan's multiple range test at the significance level of $p = 0.05$. ** The LSD were calculated at the significance level of $p = 0.05$.

**Figure 5.** Electrolyte leakage (after 2 to 4 h of soaking in water) from cells of willow leaves fertilized foliarly with Bio-Algeen S90 (B-A) and non-sonicated strains of *Microcystis aeruginosa* MKR 0105 (M.a.), *Chlorella* sp. (Ch. sp.), *Anabaena* sp. PCC 7120 (A. PCC) and environmental sample (E.s.). The data marked with the same letter are not significantly different according to Duncan's multiple range test at the significance level of $p = 0.05$. The LSD were calculated at the significance level of $p = 0.05$.

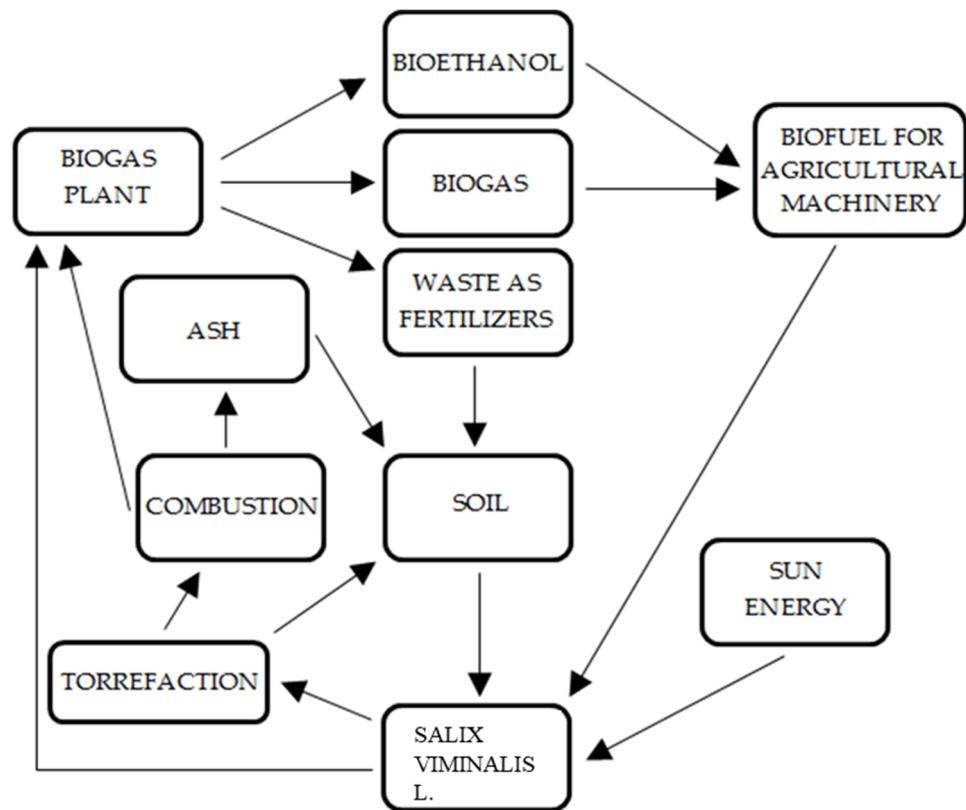


Figure 6. Circulation of by-products (nutrients) in the closed circular production of *Salix viminalis* L. as a source of energy fuel in four closed cycles.

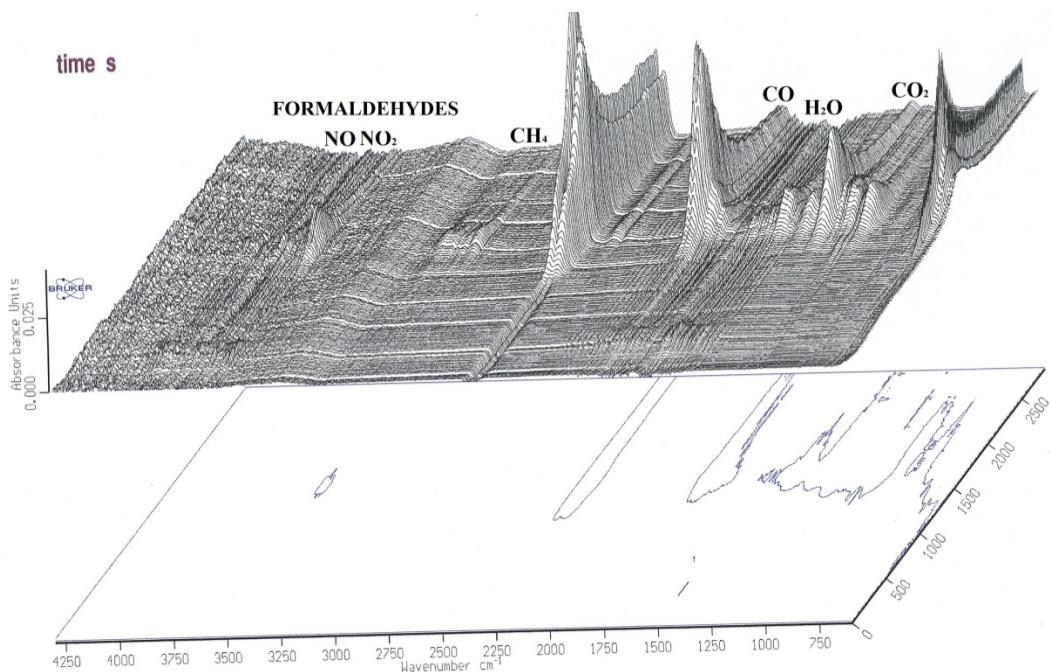


Figure 7. FTiR analysis of *Salix viminalis* L. torrefaction by-products: torgas during the willow torrefaction process under 245 °C.

The hastened willow plant growth and the higher yield of biomass might be affected by bioactive compounds synthesized by *Cyanobacteria* and *Chlorella* sp. [10], which was also observed in studies conducted on grapes, gillyflower, rice and wheat [4,9,14,16,17,40,45].

According to Sahu et al. [18] *Cyanobacteria* have the ability to transform complex substances into simple nutrients available for plants and to increase plant yield by 10–25%. In addition, these microorganisms have a positive impact on soil structure and production of glue substances. They can not only excrete substances increasing growth but also have capacity for water holding due to their absorbing structure. Research of Saadatnia and Riahi [46] indicate that *Cyanobacteria* biomass increased decomposition of soil, thus limiting its pollution and preventing growth of weeds. Additionally, Wilson [47] described that treatment with *Cyanobacteria* increases phosphate content in soil by excretion of biological acids. The higher amount of produced biomass can absorb about 20% more CO₂, which is particularly important in mitigating this gas in the atmosphere.

The presented studies also show that the impact of the applied monocultures of *Cyanobacteria* and *Chlorella* sp. was more beneficial than plant spraying with the environmental sample obtained from the reservoir of fresh water. This suggests that the environmental sample may contain less productive and possibly toxic algae that affects plant growth to a lesser extent [40,48,49]. Therefore, monocultures of *Cyanobacteria* and algae must be carefully selected in terms of profitable plant fertilization.

4.2. Effect of *Cyanobacteria* and *Chlorella* sp. on Permeability of Cytomembranes and Physiological Activity in Willow Plants

Foliar biofertilization increased numerous physiological processes which accelerated plant growth, giving a greater yield of willow biomass (Tables 2–4, Figures 2–5). This shows that the used *Cyanobacteria* and *Chlorella* sp. strains contained compounds which initiated physiological processes modifying plant development.

Biofertilization with *Cyanobacteria* and *Chlorella* sp. increased the index of chlorophyll content in plants and intensified the processes of photosynthesis, transpiration and stomatal conductance, connected with a reduced intercellular concentration of CO₂ (Figures 5 and 6), which was also found in maize [40]. These discoveries are also similar to Khan et al.'s [35] research, who demonstrated that the plant spraying with mixture of seaweed extract and amino acids improved chlorophyll contents in grapevine leaves by 19% compared to the untreated ones. This increase in chlorophyll content may be due to the ability of the treated plants to take up higher levels of amino acids from microalgae [50,51]. The seaweed extract applied to apple leaves increased the chlorophyll content by 12% as well as photosynthesis and respiration rates [52]. Increasing the vigor and chlorophyll content in the leaves, and thus increasing photosynthetic activity, along with enriching the willow tissue with active secondary metabolites synthesized by the strains used, may be of key importance for the acceleration of willow plant growth and its energy properties.

The research shows that the foliar fertilization using strains of *Cyanobacteria* and *Chlorella* sp. limited cytomembrane permeability in willow plants. From leaf samples treated with *Cyanobacteria* and *Chlorella* sp., less electrolytes flowed into the water in which they were immersed, which proves greater stability and lower permeability of cytomembranes in the cells. The reduced electrolyte leakage from the cells of leaves (Figure 7) might result from deposition of periplasmic enzymes (phosphates) on cytoplasmic membranes or between them and the cell wall [53]. As a result of the treatments, the activity of acid and alkaline phosphatases in the willow increased (Table 3).

Application of *Cyanobacteria* and algae to willow plants increased the total activity of dehydrogenases (Table 3), which are important enzymes of the respiratory cycle in cells. This confirms the studies of De-Mule et al. [54] and De-Caire et al. [55] which indicated a significantly positive impact of algae addition to compost. This treatment improved dehydrogenase activity in compost compared to the untreated one. The applied *Cyanobacteria* also increased the productivity of dehydrogenases in Virginia mallow plants [56].

Application of *Cyanobacteria* and algae also enhanced the activity of acid and alkaline phosphatases in the willow plants (Table 3). Phosphatases plays a major role in mineralization of organic phosphorus to inorganic forms which are accessible to plants, and thus they show a key part in increasing energy biomass [57]. The phosphatases are also a good indicator of metabolite activity which are secreted by *Cyanobacteria* and algae. They exhibit

the potential of the organic phosphorus mineralization [58]. The phosphate uptake by algae and *Cyanobacteria* is determined by pH. The highest uptake of these ions takes place at pH 6–7, similarly in plants. *Cyanobacteria* usually contain acid and alkaline phosphatases. Alkaline phosphatases are intensively synthesized in the case of phosphorus deficiency. Their synthesis and activity are reduced when plants contain a large amount of phosphorus.

Cyanobacteria and algae applications increased ribonuclease (RNase) activity in the willow leaves (Table 3). RNase is responsible for enzymatic degradation of different fractions of ribonucleic acid. The germination of seeds, apoptosis, plant aging, attack of pathogens and deficiency of phosphorus cause the increased activity of this enzyme [59–61]. According to Booker's research [62], ribonuclease can modify the activity of individual genes by degrading mRNA transcripts and can cause changes in the concentration of these compounds' molecules. RNase activity stimulation can shape defense mechanisms in plants. In the case of willow, increased RNase activity was associated with improved plant health, which is important in energy biomass production on an industry scale.

4.3. Effect of Cyanobacteria and Algae on Composition and Energy Value of Willow Biomass

The used monocultures of *Cyanobacteria* and *Chlorella* sp. enlarged the number of microelements (N, P, K) in the willow tissues (Table 4). It could be the consequence of atmospheric nitrogen assimilation by undamaged cells of the used monocultures, which could additionally influence not only plant development but also the chlorophyll content in leaves and thus increase energy biomass yield [12,14,15]. The increased quantity of important macroelements was also noted by Abd El Monien and Abd-Allach [3] in grapes fertilized foliarly with monocultures of *Chlorella vulgaris*. Grapevines sprayed several times with amino acids and seaweed extract contained more macroelements in leaves important for growth than after other treatments [35]. Mohammadi et al. [63] showed that applied biofertilizers significantly increased the nutrient uptake by wheat.

The used biofertilization of willow did not greatly affect the calorific value, combustion heat and content of ash in willow biomass. This shows that growth stimulation by the applied strains does not affect the energy value of particular units of the plant biomass, but it increases its yield and thus the higher energy amount per area. It does not change the ash content either, but it can enrich its fertilizer value by increasing N,P,K content. Therefore, these plants fertilized with algae can be used for energy production as biofuel. The literature on this field is hard to find.

The presented results and the cited literature point out that application of non-toxic monocultures of algae and *Cyanobacteria* can be beneficial for development and vigor of willow plants as ecological biofuel after its upgrading in the torrefaction process. Direct application of dense fresh cyanobacterial cultures into the plants may reduce the cost of their production and improve cell viability [64]. Majority of algae have the ability to create symbiotic relations with a wide number of different plant species. *Cyanobacteria* are also the key element of different ecosystems and take a crucial part in cycling of nitrogen and carbon. Swarnalakshmi et al. [65] discovered the interrelationships between nitrogen fixation and enlarged uptake of phosphorus by plants. They used *Cyanobacteria* as a matrix for Azotobacter and Pseudomonas in wheat crop. The presented research shows that the studied *Cyanobacteria* and *Chlorella* sp. can be used in organic willow production for energy needs as an alternative to the expensive and toxic artificial fertilizers and pesticides which pollute the environment. It is in line with Hegazi et al.'s [66] research on common beans which indicated that the recommended dose of nitrogen contained in artificial fertilizers can be lowered by 1/4 to 1/2 after using nitrogen fixing *Cyanobacteria*.

4.4. Circulation of By-Products (Nutrients) in the Closed Circular Production of *Salix viminalis* L. as a Source of Energy Fuel in Four Closed Cycles

The presented research and our previous work [26,67–71] allow us to construct a nutrient circulation scheme in the circular production of *Salix viminalis* L. as a source of energy fuel in four closed cycles (Figure 6).

As part of the first cycle, the nutrient contained in waste by-product generated from salix digestion for methane production (energy fuel) are applied to the soil as natural fertilizers, which are then taken up by the *Salix viminalis* L. plants and used as fuel in burning or reused as raw material for biogas and alcohol (fuel). The waste by-product obtained may be reused again as natural fertilizers applied to the soil. The second closed circulation cycle of nutrients consists of moving them from the plant biomass to the ash in the combustion process of torrefied biomass. In ash, they are moved to the soil as natural fertilizers and again to the plants. The third closed cycle concerns energy which is produced by a biogas plant in the form of biogas and alcohol which is used to power agricultural machinery necessary in *Salix viminalis* L. cultivation. This energy, and also that of the sun, is accumulated in biomass which is used then in digestion to methane, production of alcohol and biogas or it is released in the combustion process or used for other purposes. The fourth cycle is the use of *Salix viminalis* L. produced in previous cycles as a raw material for the production of torrefied biomass, which can be used for energy purposes and also as the biofertilizer added to soil. The presented studies demonstrate that the application of *Cyanobacteria* and *Chlorella* sp. to *Salix viminalis* L. plants in various conditions of a changing climate may have a large impact on reducing the recommended amounts of artificial fertilizers that pollute the environment. Moreover, the application of algae as fertilizer can be much safer for the environment and living organisms than chemical fertilizers [71,72].

Research results on the *Salix viminalis* L. torrefaction process using batch reactor presented (Tables 5 and 6, Figures 7–12) show that in the willow torrefaction process temperature is the most important factor in designing semi-scale continuous reactors for commercial production of carbonized solid biofuels. This result shows that production of carbonized solid biofuels from willow to obtain the best process conditions of torrefaction should finish with mass loss on a 30% level and energy loss of the willow on a level of 10%.

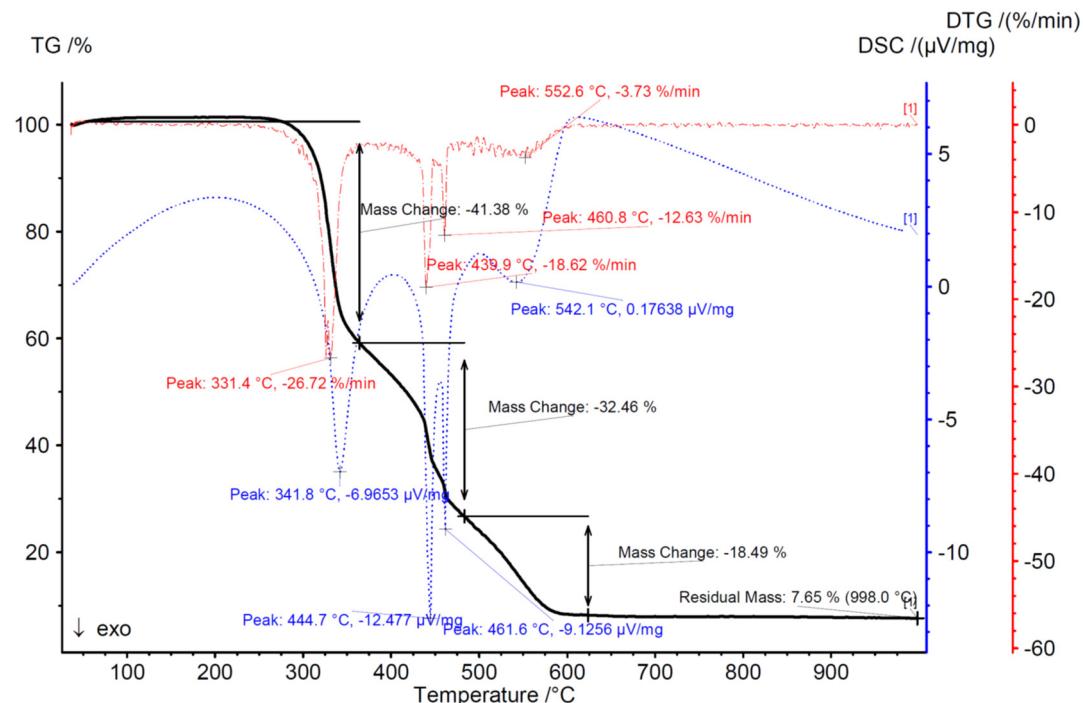
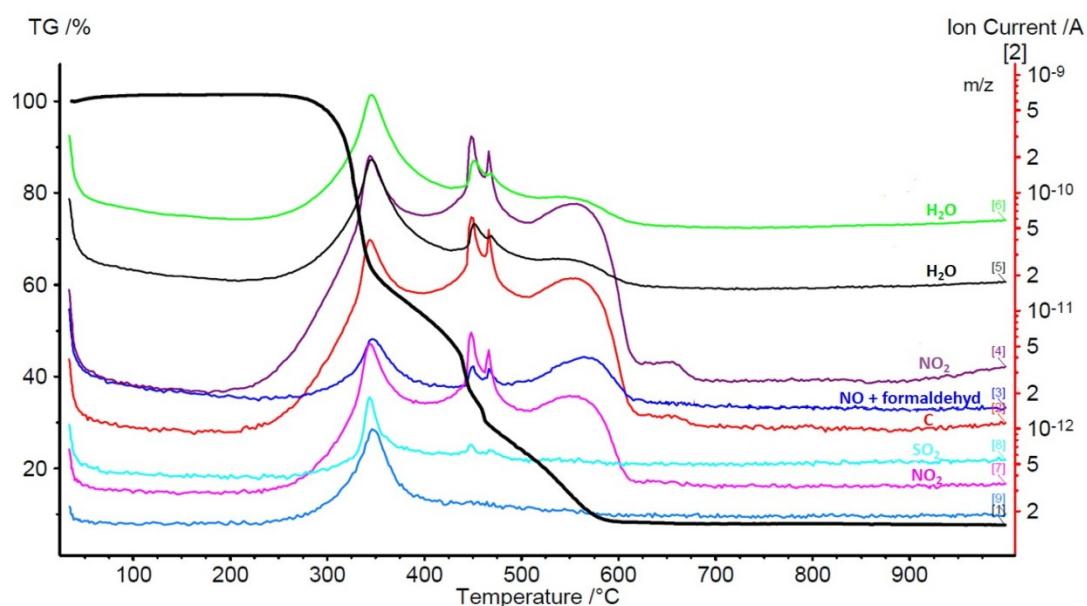
Table 5. Mass reduction of the *Salix viminalis* L. carbonization process in nitrogen using a batch reactor.

Sample nr.	Mass Reduction, g	Mass Loss, %	Residential Time, min.	Torrefaction Temp., °C
1	20/13.17	34.15	15	243.89
2	20/13.92	30.40	14	244.67
3	20/13.70	31.50	16	238.99
4	20/13.97	30.15	14	245.00
5	20/12.69	36.55	17	241.99
6	20/12.36	38.20	20	230.02
7	20/12.03	39.85	18	230.47

The use of biogas solid residues increased the heat of combustion in the analytical state and calorific value in the working state and slightly decreased the ash content in the plants. Table 5 presents the final results of the experiment using a batch reactor for making a *Salix viminalis* L. carbonization process in a nitrogen atmosphere. The specific research objectives have proved that under temperatures of 245 °C and a carbonization in reactor time of 14 min, the willow mass reduction (the most important factor in industrial scale production which has the biggest influence on costs of biofuel production) was the closest one in all obtained experimental results to 30% (30.15%).

Table 6. Proximate analysis and technical analysis of *Salix viminalis* L. treated and untreated willow in carbonization process.

Energy Crop	Moisture (%)	C ^{ad} , (%)	N ^{ad} , (%)	H ^{ad} , (%)	S ^{ad} , (%)	Cl, (%)	Volatile ^{ad} (%)	Ash (%)	High Heating Value, (MJ/kg)
<i>Salix viminalis</i> L.	7.12	45.69	0.52	6.37	0.05	0.115	91.29	7.12	19.79
Torrefied <i>Salix viminalis</i> L:									
(244.67 °C, 14 min)	4.05	52.51	0.19	5.85	0.05	0.014	73.37	3.01	23.48
(243.89 °C, 15 min)	3.93	53.20	0.19	5.85	0.05	0.014	72.81	3.2	24.02
(245.0 °C, 14 min)	3.78	54.53	0.19	5.72	0.05	0.014	72.27	3.46	25.10

^{ad} Add dry basis.**Figure 8.** TGA analysis of torrefied *Salix viminalis* L. combustion process.**Figure 9.** TGA-MS analysis of the torrefied *Salix viminalis* L. combustion process.

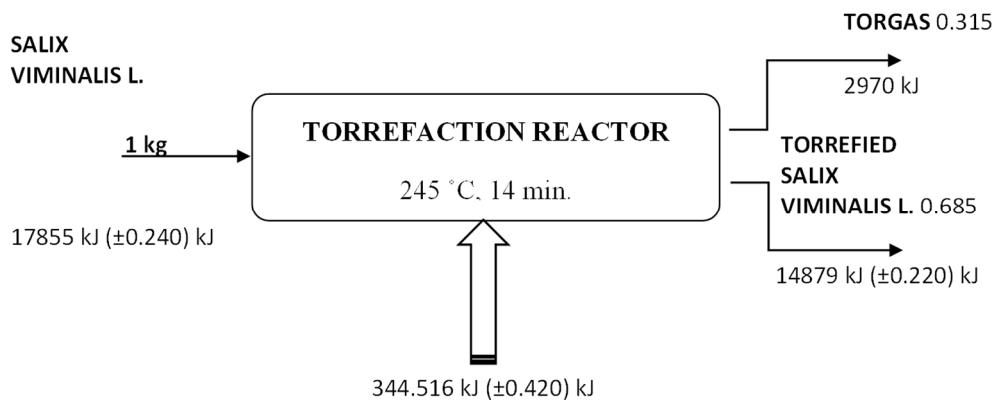


Figure 10. Mass and energy balance of the *Salix viminalis* L. torrefaction process ($t = 245\text{ }^{\circ}\text{C}$, 14 min).

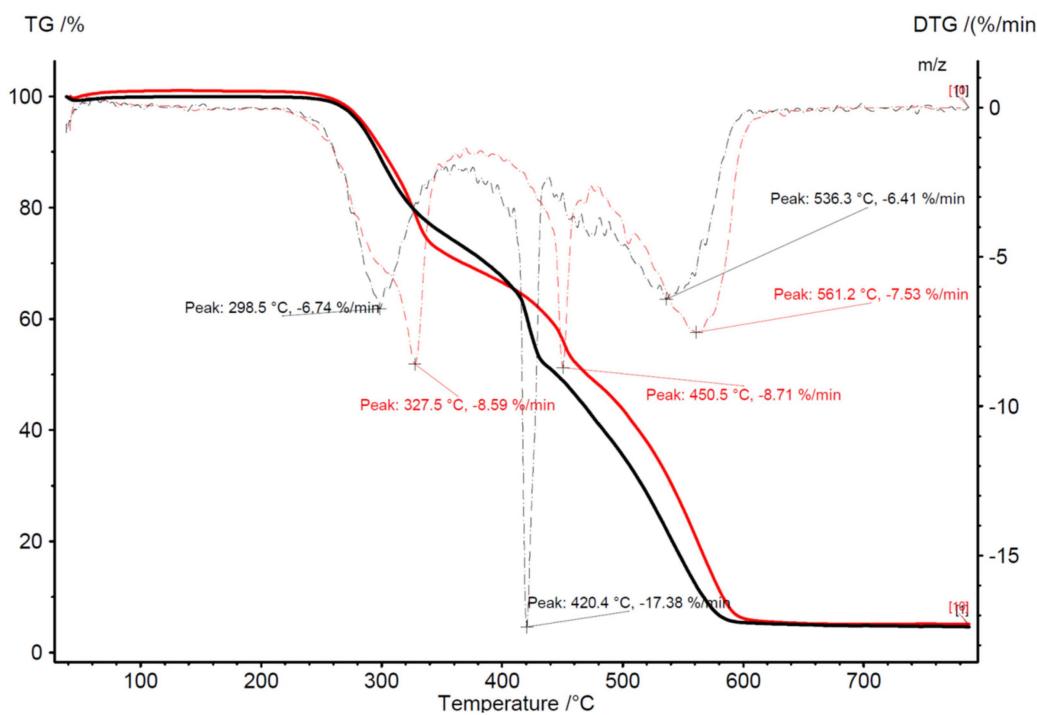


Figure 11. TGA analysis of the torrefied *Salix viminalis* L. combustion process.

Table 6 presents a proximate analysis of *Salix viminalis* L. treated and untreated in the torrefaction process in a batch reactor. It shows that the C% (weight) in the torrefied willow rises together with an increase in the *Salix viminalis* L. temperature of the carbonization process. It was opposite to the weight percentages of C_aH and O, and it presents a lowering trend.

By conducting FTiR analysis in the same moment with TGA analysis of the *Salix viminalis* L. carbonization process in isothermal conditions of 245 °C to obtain carbonized solid biofuel, volatile matter composition was produced, called “torgas” with a composition that can be seen in Figure 7.

Figures 8 and 9 present the TGA analysis of the combustion process of roasted *Salix viminalis* L. and TG-MS analysis. The colored lines are related to volatile components which are generated during the combustion process of torrefied willow: water, carbon dioxide, nitric oxide, sulphur dioxide, nitrogen dioxide, formaldehyde and carbon.

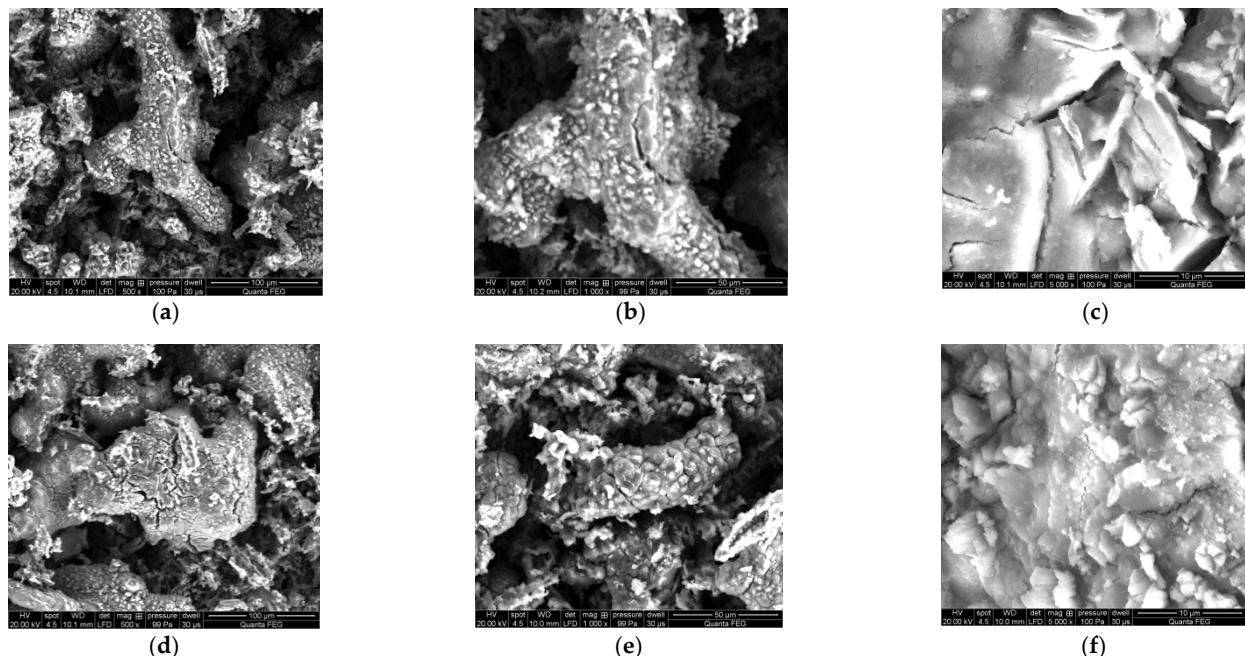


Figure 12. SEM-EDS microscopic images (a) 100 μm , (b) 50 μm , (c) 10 μm of ashes from torrefied *Salix viminalis* L. after combustion and (d) 100 μm , (e) 50 μm , (f) 10 μm of ashes from untreated *Salix viminalis* L. after combustion.

An elemental analysis shows that during the TG-MS analysis of the carbonized process of *Salix viminalis* L. under 245 $^{\circ}\text{C}$ in volatile components, we could find CH₄: 0.1%, C₂: 0.06%, CO₂: 81.24%, CO: 18.94% (Figure 9). In Figure 10, we find the mass and energy balance of the *Salix viminalis* L. carbonization process in nitrogen using a batch reactor. By conducting energy and mass balance, it was calculated that 344.516 (± 0.420) kJ is the energy which is necessary for the torrefaction process to generate carbonized solid biofuel from *Salix viminalis* L. The willow plants cultivated on low quality soils and biofertilized with algae can serve as a feedstock for the carbonization process. To improve its productivity, the elaboration of optimal parameters of the carbonization processes and carbonized biofuel production are needed, as well as performing the physical and chemical analysis of carbonized solid biofuels with uses to carry C and to produce fertilizers (Table 7) [73–79].

Torrefaction, which is a promising thermo-chemical conversion of willow into solid biofuel with higher caloric value, unfortunately cannot be properly used to reasonably reduce concentrations of volatile components such as K, Cl, and S in the willow, and this fact may mitigate their adverse impact on the heat transfer and corrosion rates in the boiler if it is used as a solid biofuel.

The surfaces of torrefied willow ash samples were covered by numerous K, Cl and S-rich amorphous deposits. In contrast, fewer deposits were observed on the surfaces of untreated willow ash samples. Nevertheless, SEM-EDS analysis of torrefied willow has proved that during the torrefaction process, small percentages of K, Cl, and S can volatize out of willow and can be concentrated due to condensation on the outside surfaces in the form of amorphous or crystallized clusters of salts KCl and K₂SO₄ with a size of 1–100 μm . Therefore, a new question arises regarding if there will be the salt deposits in the batch reactor after torrefaction—however, this was not observed during operation with batch reactor for a period of six months. SEM-EDS analysis of the ash composition after burning at 700 $^{\circ}\text{C}$ of torrefied willow showed a very favorable composition of mineral substances which can be reused as additives to organic fertilizers or as carriers of elements K (22.08%), P (3.05%) and C (23.25%). SEM-EDS was performed on fly ash of willow of sintered at 700 $^{\circ}\text{C}$ and leaching toxicity of heavy metals analyzed by horizontal vibration extraction procedure (HVEP).

The research shows that a temperature of 245 °C during carbonization of *Salix viminalis* L. biomass for solid biofuel conversion under isothermal conditions is low in comparison to other energy crops [80–82].

Table 7. Content of elements in the ash from untreated *Salix viminalis* L. and torrefied *Salix viminalis* L. burnt biomass.

Assessed Material	C	O	K	Ca	Mg	Fe	Si	P	S	Cl	Dry Mass
	[Atomic, %]										[%]
Ash composition from torrefied <i>Salix viminalis</i> L. (average values)	23.75	45.82	22.01	2.60	0.84	0.02	0.29	3.07	0.17	1.43	100.00
Standard deviation, σ	2.99	2.19	2.85	0.49	0.30	0.00	0.10	0.60	0.05	0.40	
Ash composition from untreated <i>Salix viminalis</i> L. (average values)	33.50	33.08	21.09	2.22	0.98	0.11	0.46	1.16	0.19	3.13	100.00
Standard deviation, σ	3.28	4.04	3.82	0.78	0.30	0.04	0.42	0.62	0.05	1.19	

5. Conclusions

The presented research shows that the studied, non-toxic *Cyanobacteria* and *Chlorella* sp. can be used in organic willow production for energy needs as alternative to the expensive artificial fertilizers and pesticides which pollute the environment. The triple application to leaves of *Cyanobacteria* and *Chlorella* sp. Bio-Algeen S90 and with environmental samples significantly accelerated growth, metabolic activity and increased biomass yield of the willow plants, as to compare to plants that were treated with water only. The treatments with *Cyanobacteria* and *Chlorella* sp. were most conducive to increasing the willow biomass, which was suitable for torrefaction.

The obtained results indicate that the temperature at which 30% weight loss and 10% energy loss occurs during the carbonization of *Salix viminalis* L. biomass fertilized with *Cyanobacteria* and *Chlorella* sp. is 245 °C. The research shows an increase in the calorific value as a result of the *Salix viminalis* L. torrefaction process from 19.79 to 25.10 MJ kg^{−1}. The amount of ash after the torrefaction of willow was still at a low level compared to biomass not subjected to this process. Ash content in torrefied willow biomass was lower than 3%, while in Polish hard coal it was over 15%.

The presented methodology of *Salix viminalis* L. production is ecological and the energy properties of the obtained large amounts of biomass can be increased in the carbonization process. The *Salix viminalis* L. cultivated using the elaborated environmentally friendly biofertilization with algae can be define by low cultivation costs. The unit of caloric value of the elaborated and carbonized solid biofuel requires about 25% less costs than when applying traditional chemical fertilizers. In addition, energy crops produced under the use of algae fertilization can be directly used for energy production and can be converted to carbonized solid biofuel with high caloric value [83–85].

The obtained results regarding the usefulness of algae in the fertilization of willow can be summarized by the following conclusions:

1. The triple foliar biofertilization with *Cyanobacteria* (*Microcystis aeruginosa* MKR 0105, *Anabaena* sp. PCC 7120) and green algae (*Chlorella* sp.) significantly increase the willow plant development, and several metabolic events have a key influence on plant growth and biomass yielding, of which energy value can be improved in the torrefaction process.
2. The use of non-toxic *Cyanobacteria* and *Chlorella* sp. is cost-efficient and environmentally friendly and increases productivity of willow crops when used as biofuel for energy production.

3. New physiological possibilities concerning enhancement of natural plant defense is considered one of the most promising strategies for ecological and integrated protection of energy crops cultivated on large areas.
4. The developed technology for the production of willow biomass by means of fertilization with algae and increasing its energy properties in the thermo-chemical process enables the production of environmentally friendly energy as an alternative to that obtained from fossil fuels.

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