Improving Biorefinery Sustainability and Profitability by Cultivating Aquatic Plants on Ozonized Distillery Effluents

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Industrial production of biogas offers a way to manage distillery leachate. The waste is usually subjected to anaerobic digestion for producing biogas. However, the effluent from anaerobic processes has high chemical oxygen demand (COD) and is harmful to the environment. An effective method of lowering COD is ozonation. Effluent from biogas plants after ozonation has the potential for use in breeding grounds for plants of the Lemnaceae family. Thus, they can provide a valuable additional source of biomass for the production of bioethanol. *Lemna minor* L. and *Spirodela polyrhiza* cultures were grown in media with the addition of 2.5% PFE, which had been treated by ozonation for between 6 and 50 min. Using ozonated effluent was an effective cultivation technique in all variants. The analyzed parameters were plant growth, chlorophyll index, fresh plant weight and photosynthetic traits (net photosynthesis, stomatal conductance, transpiration and concentration of intercellular CO_2). The best growth of *Lemna minor* L. was observed in the media with PFE treated for 12 min. Similar effects were obtained for *S. polyrhiza*, with ozone treatment for 12 and 25 min. The results show the potential of using ozone-treated post-fermentation leachate as a supplement in culture media.

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INTRODUCTION

Biorefineries that use biomass to produce energy, fuels, and chemicals are seen as a promising alternative to petrochemical technologies based on non-renewable raw materials (Zaman 2015). Biomass, in comparison to petrochemical raw materials, contains more oxygen, less hydrogen, and less carbon. Therefore, a larger group of chemical products can be obtained from lignocellulose than from petrochemical raw materials. However, the processing of biomass into fuels and valuable chemical compounds requires the use of more complex technologies, including pretreatment processes necessary to

obtain sugars or their derivatives, and further processes for obtaining final products. At present, the majority of lignocellulosic biomass processing technologies are in the pre-commercial phase and are not implemented in industrial installations (Cherubini and Strømman 2011). Among the possible methods of processing biomass into chemical products, only two technologies can be considered as fully commercial technologies: the production of bioethanol from lignocellulosic biomass (sawdust, corn straw) (Nguyen *et al.* 2017) and the production of lactic acid from glucose obtained from starchy raw materials (rye, triticale, maize, potatoes, *etc*.) (Altaf *et al*. 2007). For example, DuPont launched a plant in 2013 that uses corn straw (stems, leaves, and cob devoid of seeds) as the raw material. This biorefinery is the world's largest producer of cellulosic ethanol. Its production capacity exceeds 113 million liters of bioethanol annually, with greenhouse gas emissions reduced by 90% compared to the conventional gasoline production process (Rosen 2015). It may also be observed that most installations produce first generation fuels: biodiesel or bioethanol from sugar plants (sugar beet, sugar cane), starchy materials (*e.g.*, cereals, manioc), or oil (*e.g.*, rape, soy) (Rabaçal *et al*. 2017). An example of a modern biorefinery is the Vivergo plant founded by the companies AB Sugar, BP, and DuPont. Built in Hull (UK), the biorefinery produces 420 million liters of bio-ethanol per year from grain (Nichols 2013). An example of biofuel production based on oilseeds is provided by the Brazilian company Petrobras Biofuels. In their biodiesel production plant, oils are produced from soybean, castor, sunflower, cotton, as well as animal fats. The biorefinery achieves annual productivity of 170 million liters of biodiesel (Zonin *et al*. 2014). However, it should be emphasized that, regardless of the technology used to produce them, biofuels are still more expensive than conventional petroleum fuels, as a result of the large amount of energy required to produce them.

To improve the energy efficiency of biorefineries, partnerships have been set up between distilleries and biogas plants, which use the post-fermentation wastewater, which is still rich in nutrients and useful to biogas production (Fig. 1A). Following anaerobic fermentation, the biogas is burnt in cogeneration installations. The heat is used for the needs of the plant, and the electrical current is sold to electricity suppliers. However, biogas plants generate large amounts of leachates with high organic load, which are harmful to the environment and the surrounding population. In the authors' previous work, the use of sugar beet pulp for the production of ethanol (Berlowska *et al*. 2017), biogas (Ziemiński and Kowalska-Wentel 2017), lactic acid (Joanna Berlowska *et al*. 2018), and biodegradable polymers (Tomaszewska *et al*. 2018) was investigated. However, the authors did not consider waste disposal methods in the proposed biological-chemical technologies, and their ecological use in plant production as fertilizers (Romanowska-Duda *et al*. 2018, 2019; Dębowski *et al.* 2018, 2020; Kisielewska *et al*. 2020; Szufa *et al*. 2020).

The novelty of the current study is cultivation of aquatic plants on ozonized effluents from biogas plants. This is the first study in which ozonized effluents were used to produce biomass from aquatic plants of the family Lemnaceae. The process provides phytoremediation, as well as a supplement to the biogas plant charge or a valuable feed for animals. In the solution proposed in this work, the effluents are ozonated to reduce their organic charge and then used as a medium for the aquatic plants *Lemna minor* L. and *Spirodela polyrhiza* (Fig. 1B). Lemnaceae are a family of simple, fast-growing, floating aquatic plants. They are a suitable choice for wastewater treatment because of their high nutrient-uptake capabilities and resilience to severe environmental conditions. The Lemnaceae family consists of five genera (*Landoltia*, *Lemn*a, *Spirodela*, *Wolfa*, and *Wolfella*), and 38 species have been classified to date (Cui and Cheng 2015; Xu *et al*. 2015).

The rapid growth rates of these aquatic plants, their high starch content, and low lignin content make them a popular feedstock for bioethanol production (Calicioglu *et al*. 2018). The biochemical conversion of duckweed starch and cellulose into simple sugars for fermentation into alcohols has been described at both the laboratory and pilot scale (Kazemi *et al.* 2020; Su *et al*. 2014).

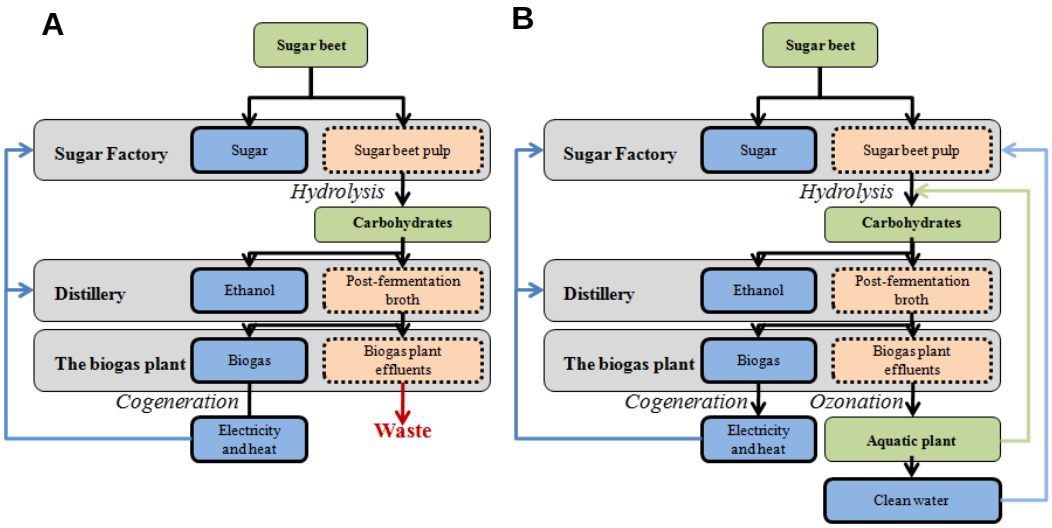

Fig. 1. A - Diagram of a bio-refinery producing ethanol from lignocellulosic raw materials from the sugar industry; B - Diagram of bio-refinery producing ethanol from lignocellulosic raw materials from the sugar industry supplemented with wastewater treatment processes and production of biomass from aquatic plants. Green squares show the raw materials used and blue squares are the final products.

The plants considered in this study are *Lemna minor* L. and *Spirodela polyrhiza*, which belong to the family Lemnaceae – the world's smallest angiosperms (Basiglini *et al*. 2018). Because of the ease with which they can be cultivated, their rapid growth rate, and the wide range of their possible uses, they are commonly used in biotechnological processes (Iatrou *et al.* 2015; Pena *et al*. 2017; Tang *et al*. 2017; Ma *et al.* 2018). Previous studies have explored their application in wastewater treatment (Zhao *et al*. 2015; Bokhari *et al.* 2016; Tang *et al.* 2017;); biomass generation (Gaur and Suthar 2017), which can be used in the production of biogas (Toyama *et al.* 2018), ethanol (Cui and Cheng 2015), and liquid fuel (Xu *et al*. 2018); or as a source of nutrients for humans and animals (Appenroth *et al.* 2017; Chakrabarti *et al*. 2018).

However, chemicals present in the culture media can significantly inhibit the growth of these plants (Bourioug *et al.* 2018). Therefore, to ensure effective and economically efficient cultivation of *Lemna minor* and *Spirodela polyrhiza* in media containing wastewater materials, it is essential to reduce the concentration of chemicals that are the growth inhibitors in the media. Anaerobic digestion of effluent from biogas plants is characterized by high chemical oxygen demand (COD) between 12,000 and 20,000 mg/L. One of the ways to lower the chemical load of sewage, as a preliminary stage in biotechnological processes, is ozonation (Muradov *et al*. 2014; Dziugan *et al*. 2016).

The use of ozone to purify leachate from biogas plants is one of the most effective methods of reducing the COD in sewage. Tests conducted at the Institute of Fermentation and Microbiology, Lodz University of Technology (Lodz, Poland), showed a strong decline in the COD of 200 mL of sewage during the initial ozonation period, from almost 14,000

mg/L to about 4000 mg/L. However, raising the ozone dose increases the COD again, to a value close to the initial level, as shown in Fig. 2. Ozone was also an effective disinfectant and fragrance corrector of sewage. Untreated wastewater had a strong and unpleasant odor, whereas the post-ozonated wastewater was odorless, after ozonation for 35 min, corresponding to an ozone dose of 0.42 g/1000 L. Therefore, if ozonation of sewage is to be conducted on an industrial scale, the oxidizer dose should be optimized, as a critical initial step.

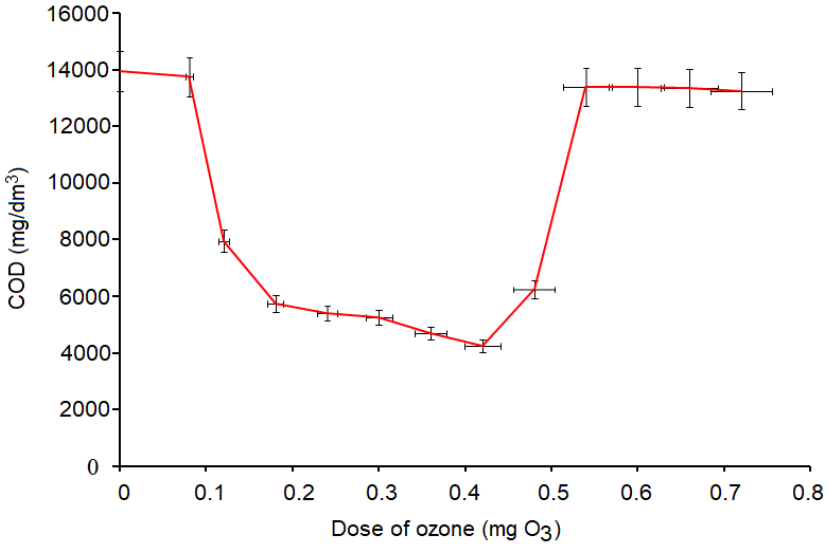

Fig. 2. COD changes depending on the ozone dose delivered to wastewater from a biogas plant

A **B** **C**

Fig. 3. Installation for the treatment of leachate digestates with ozone operating in a Polish distillery (A - general view; B - ozone generator; C - reactor for sewage ozonation)

The use of ozone in second-generation ethanol production technology from sugar beet can bring many technological and economic benefits. One advantage of this method is that ozone can be produced from oxygen *in situ*, thereby avoiding storage and transportation costs. Moreover, by generating their own heat and electricity, modern distilleries (or biorefineries) can considerably reduce the cost of producing bioethanol. In Poland, there is growing interest in the use of ozone as part of industrial biofuel production processes. For example, a treatment station for post-digestion leachate has been installed in one of the Polish distilleries (Fig. 3).

This paper set out to study the influence of post-fermentation effluents (PFEs) from a biogas plant treated with ozone on the growth of aquatic plants belonging to the Lemnaceae family. *Lemna minor* and *Spirodela polyrhiza* cultures were grown in water with PFE additives previously treated with ozone for a duration between 6 and 60 min. The analyzed parameters were as follows: plant growth, fresh plant weight, chlorophyll index, and photosynthesis traits (net photosynthesis, stomatal conductance, transpiration, and concentration of intercellular CO_2). The parameters were monitored to see whether the ozonated effluents had a positive effect on the growth of *Lemna minor* and *Spirodela polyrhiza*. Increasing the yields of aquatic plants by adding ozonated leachates from biogas plants could reduce the ecological footprint of the biorefinery, while at the same time increasing its profitability, because the additional biomass could itself be used for biofuel production.

EXPERIMENTAL

Post-fermentation Effluents

Effluent produced following anaerobic fermentation of a distillery post-fermentation medium was used to supplement cultivation media for *Lemna minor* L. and *Spirodela polyrhiza* (Fig. 4).

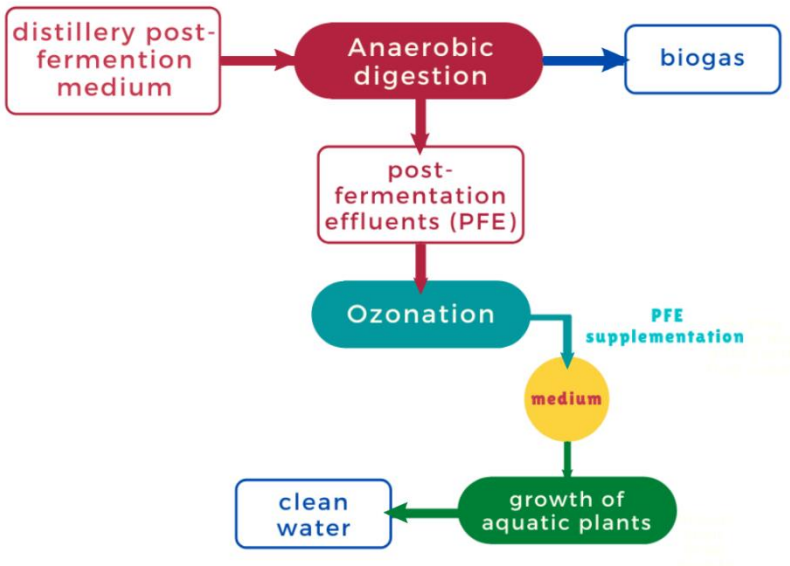

Fig. 4. Diagram of a utilization of PFEs

The PFEs were subjected to basic characterization in terms of COD, as well as total ammonium nitrogen (TAN), and phosphates, which were determined using a HACH-Lange DR6000 spectrophotometer (Hach Lange, Loveland, CO, USA) and HACH-Lange tests No. 8000, 8038, and 8048 (Loveland, CO, USA), respectively. The ozonation process was then performed. For this purpose, 20% (w/v) solution of the effluent in demineralized water was placed into a reactor, through which a mixture of oxygen and ozone was passed containing 80 g of ozone in 1000 L of gas. The flow of gaseous was 0.4 dm^3/min. Ozonation was performed for periods of 6, 12, 25, or 60 min. After ozonation, tests for COD, TAN, and phosphates were performed.

Plant Material

Two aquatic plants belonging to the Lemnaceae family were used in the study. *Spirodela polyrhiza* and *Lemna minor* (Fig. 5) were isolated from water reservoirs in the Lodz voivodeship (Poland). The plants were grown in *in vitro* cultures at the Laboratory of Plant Ecophysiology, Faculty of Biology and Environmental Protection, University of Lodz (Lodz, Poland).

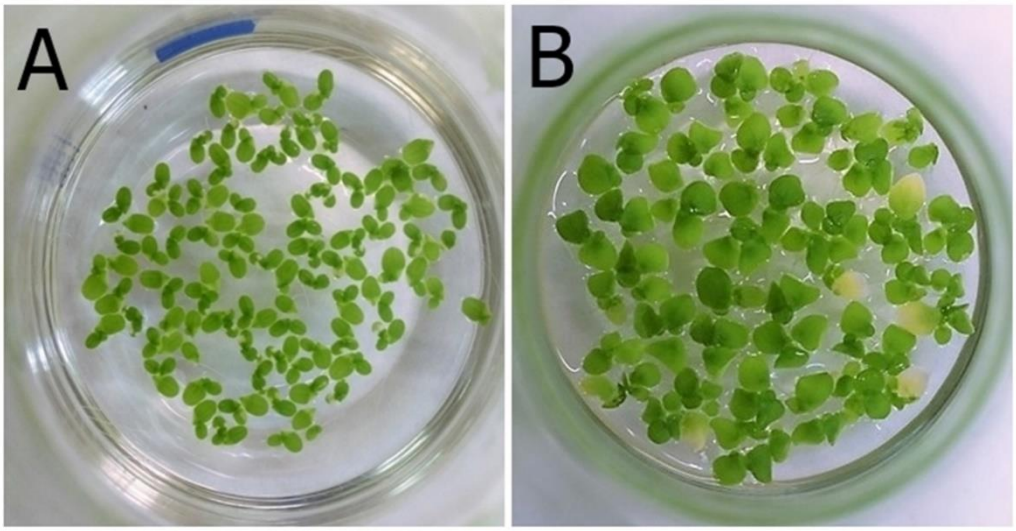

Fig. 5. Cultivation of (A) *Lemna minor* L. and (B) *Spirodela polyrhiza* plants used in the study

Culture Conditions

Spirodela polyrhiza and *L. minor* were cultivated in medium Z (Pádrová *et al*. 2015) (pH 6.4) as a control sample and the same medium was supplemented with different variants of ozonated PFEs: (**I**) Medium Z; (**II**) Medium Z + 2.5% non-ozonated PFE; (**III**) Medium Z + 2.5% PFE ozonated for 6 min; (**IV**) Medium Z + 2.5% PFE ozonated for 12 min; (**V**) Medium Z + 2.5% PFE ozonated for 25 min; (**VI**) Medium Z + 2.5% PFE ozonated for 60 min.

Erlenmeyer flasks were filled with 250 mL of different variants of the medium inoculated with 20 separate individuals (fronds) of *S. polyrhiza* or *L. minor*. The plants were cultivated in a phytotron at 24 °C, with continuous lighting supplied by 2 × 18 W/840 Philips Master TL-D lamps (Amsterdam, Netherlands). The culture was grown for 14 days. The fronds of the individual plants were counted on each day of the experiment. After 14 days of cultivation, the chlorophyll index and fresh weight of the plants as well as

parameters of the photosynthesis process (net photosynthesis, stomatal conductance, transpiration, and concentration of intercellular CO_2) were determined.

Chlorophyll Index

Index of chlorophyll content in the plants after 14 days of cultivation in the tested media was evaluated using a Konica Minolta SPAD-502 chlorophyll meter (Tokyo, Japan). The results were expressed in SPAD units (Grzesik and Romanowska-Duda 2014; Grzesik *et al*. 2017).

Fresh Weight of Plants

All fronds were transferred into pre-weighed polystyrene round bottom tubes with 1.0-mm holes. Next, the samples were centrifuged using an Eppendorf 5417R (Hamburg, Germany) at 3000 rpm for 10 min at 25 °C. The remaining supernatant was discarded and the tubes containing the tested plant biomass were weighed. Finally, the fresh weight was calculated by subtracting the weight of the tube from the weight of the tube with biomass (Oecd 2006).

Photosynthesis

Gas exchange parameters (net photosynthesis Pn [µmol CO_2 $m^{-2}s^{-1}$], transpiration Tr [mmol H_2O $m^{-2}s^{-10}$], stomatal conductance Gs [mol H_2O $m^{-2}s^{-1}$], and intercellular CO_2 concentration Ci [µmol mol^{-1}] for the tested *S. polyrhiza* and *L. minor* plants were measured using an Amesbury TPS-2 apparatus from PP Systems (Amesbury, MA, USA), in accordance with the methodology described above. All parameters were evaluated after 14 days of cultivation.

Statistics

Statistical differences in plant growth were compared using a one-way repeated measures analysis of variance (ANOVA; OriginPro 9.2.214, OriginLab Corp., Northampton, MA, USA). Statistical significance was set at the conventional level of 5% ($p < 0.05$). The profiles of the parameters for the plant cultures in different media were compared by hierarchical clustering using Clustvis (https://biit.cs.ut.ee/clustvis/), a web tool for visualizing clustering of multivariate data (Metsalu and Vilo 2015).

RESULTS AND DISCUSSION

Ozonation was investigated as a potential post-treatment step for post-fermentation effluent to enhance biodegradability and observe the influence of the initial organic matter concentration. At low concentrations, ozone mainly influences soluble COD compounds. Longer exposure time also affected particulate compounds, resulting in the solubilization of the COD fractions. Ozonation, despite its distinct technical advantages, may prove costly when applied to the entire wastewater volume. Ozonation efficiency was tested in terms of contact time. It seems that application of ozonation should be economically viable and not generate toxic intermediates affecting the process, which is consistent with the findings of Chiavola and co-workers (2021) and Mainardis and co-workers (2020).

In general, the PFEs ozonation time affected the number of plant fronds cultivated in supplemented media. The number of fronds of *L. minor* and *S. polyrhiza* after cultivation in different media are presented in Fig. 5. The largest numbers of fronds after 14 days of cultivation of *L. minor* and *S. polyrhiza* were observed in Medium 4 (PFEs with 12 min of ozonation), at 430 fronds and 279 fronds, respectively. Compared to the non-ozonated medium, these values were 99% higher for *L. minor* and 36% higher for *S. polyrhiza*. It is worth emphasizing that further ozonation led to smaller plant populations. In the medium supplemented with PFEs that had been ozonated for the longest time (60 min), the numbers of fronds of both plants were lower than for both the medium with PFEs without ozonation and the control medium (*L. minor*). Therefore, in terms of the increase in the number of individual fronds, the most favorable variant was Medium 4, supplemented with PFEs subjected to ozonation for 12 min.

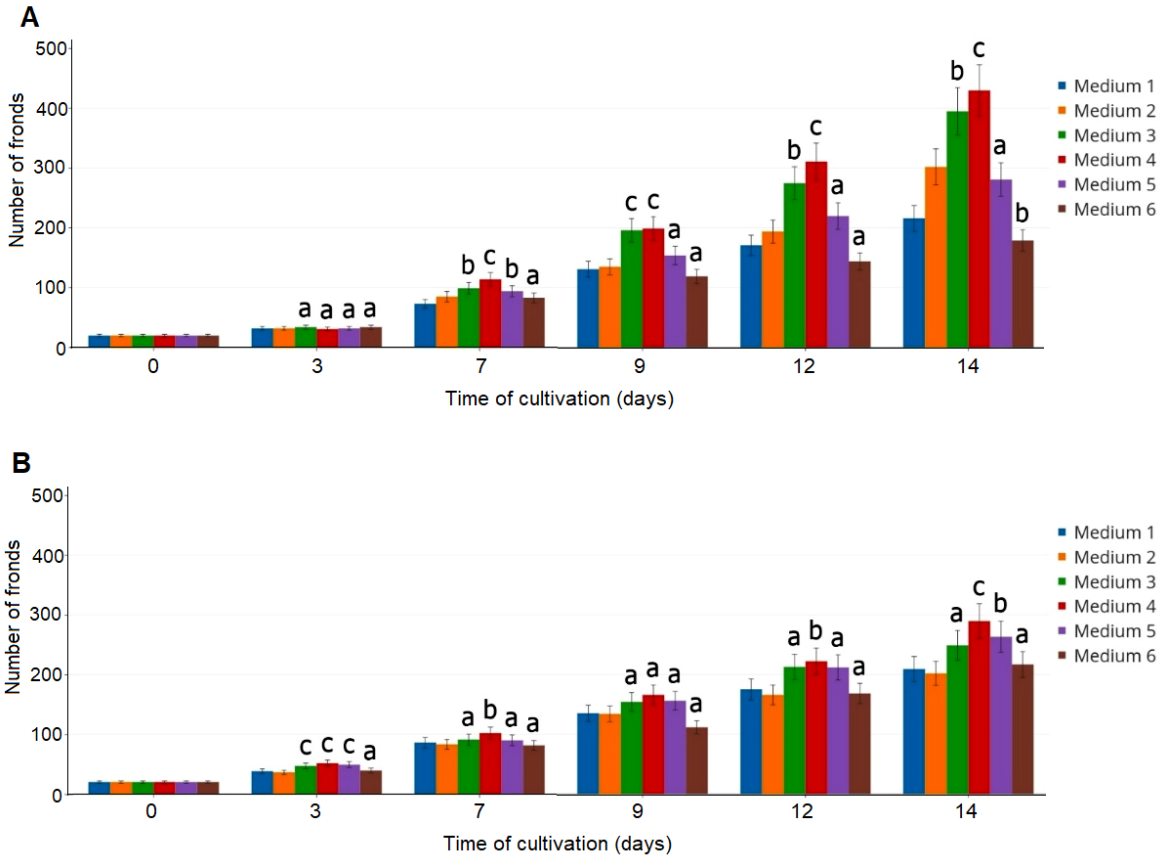

Fig. 6. Influence of the tested media on the number of fronds of *L. minor* L. (**A**); and *S. polyrhiza* (**B**). Results for media supplemented with effluent after ozonation over time 6, 12, 25, or 60 min (Media 3, 4, 5, and 6, respectively) are compared to the results for medium without ozone treatment (Medium 2) using one-way repeated measures ANOVA. Values with different letters are statistically different ($p < 0.05$): [a]— $p \geq 0.05$; [b]— $0.005 < p < 0.05$; [c]— $p < 0.005$.

The results of the growth parameters for the tested plants in media supplemented with PFEs after different times of ozonation are presented in Table 1. The results indicated that the best medium for the cultivation of *L. minor* was Medium 4, which showed the highest values for the analyzed parameters, photosynthesis (3.54 ± 0.11 µmol CO_2 $m^{-2}s^{-1}$), the chlorophyll index (25.31 ± 2.84 SPAD units), transpiration rate (3.32 ± 0.19 mmol H_2O

$m^{-2}s^{-1}$), and stomatal conductance (693 ± 16 mmol H_2O $m^{-2}s^{-1}$). At the same time, the intercellular CO_2 concentration (389 ± 40 µmol CO_2 mol^{-1}) was the lowest in this medium. Medium 4 was second only in terms of fresh weight (2.15 ± 0.13 mg), which was slightly lower than in the case of Medium 5 (2.40 ± 0.04 mg) (Fig. 7).

Table 1. Influence of Ozonation on Photosynthesis Parameters for *L. minor* and *S. polyrhiza*. The Highest Results for Each Feature for a Given Plant are Underlined and Denoted in Bold

***Lemna minor* L.**					
Tested Medium	**Photosynthesis (µmol(CO_2)$m^{-2}s^{-1}$)**	**Chlorophyll Index (SPAD units)**	**Transpiration (mmol(H_2O)$m^{-2}s^{-1}$)**	**Stomatal Conductance (mmol(H_2O)$m^{-2}s^{-1}$)**	**Intercellular CO_2 (µmol(CO_2)mol^{-1})**
Medium 1	3.10 ± 0.06	22.00 ± 1.30	3.10 ± 0.02	590 ± 8	435 ± 13
Medium 2	3.16 ± 0.03	24.00 ± 0.73	3.00 ± 0.08	612 ± 13	412 ± 7
Medium 3	3.35 ± .0.08^{c}	23.45± 1.92^{a}	3.12 ± 0.23^{a}	600 ± 35^{a}	402 ± 7^{b}
Medium 4	**3.54 ± 0.11^{c}**	**25.31 ± 2.84^{a}**	**3.32 ± 0.19^{c}**	**693 ± 16^{c}**	389 ± 40^{a}
Medium 5	3.00 ± 0.27^{a}	21.01± 3.40^{b}	2.94 ± 0.06^{a}	604 ± 43^{a}	438 ± 23^{b}
Medium 6	2.68 ± 0.06^{c}	23.26 ± 3.66^{a}	2.13 ± 0.04^{c}	578 ± 14^{c}	**467 ± 8^{c}**
Spirodela polyrhiza					
Tested Medium	**Photosynthesis (µmol(CO_2)$m^{-2}s^{-1}$)**	**Chlorophyll Index (SPAD units)**	**Transpiration (mmol(H_2O)$m^{-2}s^{-1}$)**	**Stomatal Conductance (mmol(H_2O)$m^{-2}s^{-1}$)**	**Intercellular CO_2 (µmol(CO_2)mol^{-1})**
Medium 1	2.23 ± 0.09	28.07 ± 1.91	2.89 ± 0.09	676 ± 12	450 ± 13
Medium 2	2.11 ± 0.08	24.13 ± 4.36	2.07 ± 0.03	597 ± 14	437 ± 10
Medium 3	2.39 ± 0.04^{c}	26.11 ± 3.17^{a}	1.98 ± 0.08^{b}	558 ± 7^{c}	427 ± 8^{b}
Medium 4	**3.14 ± 0.01^{c}**	26.12 ± 2.14^{a}	**4.05 ± 0.04^{c}**	879 ± 13^{c}	**468 ± 7^{c}**
Medium 5	2.82 ± 0.04^{c}	**29.37 ± 2.27^{b}**	3.87 ± 0.16^{c}	**981 ± 8^{c}**	457 ± 1^{c}
Medium 6	2.79 ± 0.02^{c}	26.17 ± 3.43^{a}	2.27 ± 0.01^{c}	786 ± 2^{c}	465 ± 8^{c}

(Medium 1) Medium Z; (Medium 2) Medium Z + 2.5% non-ozonated PFEs; (Medium 3) Medium Z + 2.5% PFEs ozonated for 6 min; (Medium 4) Medium Z + 2.5% PFEs ozonated for 12 min; (Medium 5) Medium Z + 2.5% PFEs ozonated for 25 min; and (Medium 6) Medium Z + 2.5% PFEs ozonated for 50 min. The results for Media 3, 4, 5, and 6 were compared to the results for the medium without ozone treatment (Medium 2) using one-way repeated measures ANOVA. Values with different letters are statistically different ($p < 0.05$): ^{a}— $p \geq 0.05$; ^{b}— $0.005 < p < 0.05$; ^{c}— $p < 0.005$.

The results for *S. polyrhiza* were more varied. Medium 4 showed the highest values for fresh weight (2.86 ± 0.13 mg), net photosynthesis (3.14 ± 0.01 µmol CO_2 $m^{-2}s^{-1}$), and transpiration (4.05 ± 0.04 mmol H_2O $m^{-2}s^{-1}$), but also the highest intercellular CO_2 concentration (468 ± 7 µmol CO_2 mol^{-1}). However, the highest results for chlorophyll index (4.05 ± 0.04 SPAD units) and stomatal conductance (981 ± 8 mmol H_2O $m^{-2}s^{-1}$) were obtained with Medium 5. Despite differences in the results, both Medium 4 and Medium 5

stimulated the growth of *L. minor* and *S. polyrhiza*. The differences between Medium 2 (2.5 PFE without ozonation) and Mediums 4 and 5 were also statistically significant ($p < 0.05$). Thus, the process of ozonation positively affected the growth of *S. polyrhiza*. This observation was further confirmed by hierarchical clustering of the tested media in terms of the analyzed parameters (Fig. 8). For *L. minor*, Medium 4 was a separate clade, while for *S. polyrhiza* the separate clade included Medium 4 and Medium 5. The strongest determining factors were photosynthesis and stomatal conductance (Fig. 8A) as well as number of fronds after 7 days of incubation and fresh weight (Fig. 8B).

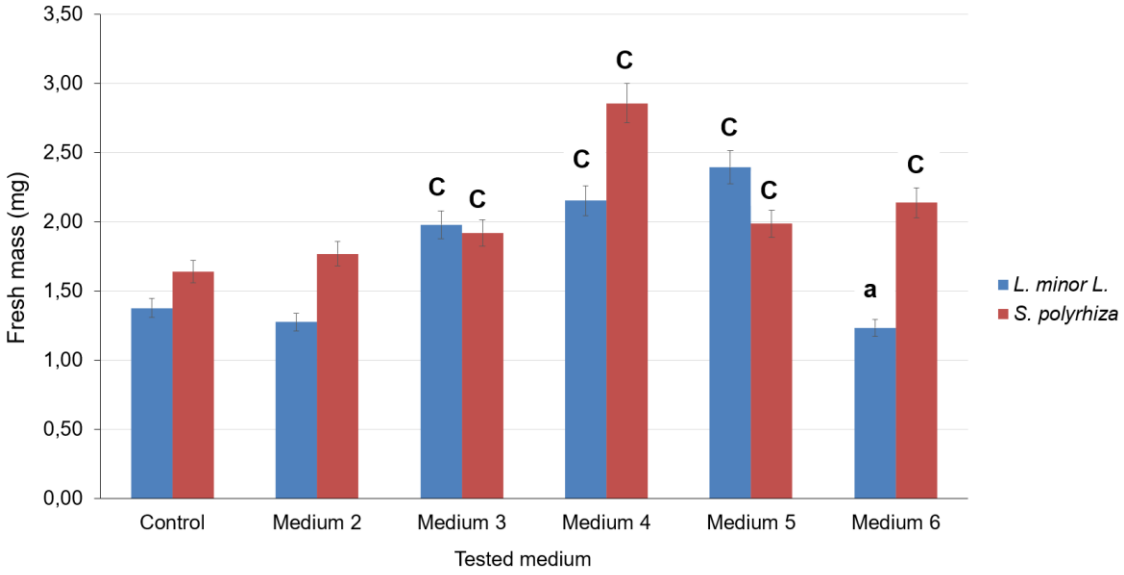

Fig. 7. Influence of the tested media on the fresh weight of *L. minor* L. (**A**); and *S. polyrhiza* (**B**). Results obtained for media supplemented with effluent after ozonation in proper time (Medium 3, 4, 5, 6) were compared to the results for medium without ozone treatment (Medium 2) using one-way repeated measures ANOVA. Values with different letters are statistically different ($p < 0.05$): [a]— $p \geq 0.05$; [b]— $0.005 < p < 0.05$; [c]— $p < 0.005$.

Ozone treatment has a wide range of possible applications across many industries. It has been used for the treatment of drinking water (Siddiqui *et al*. 1997), to remove organic matter (Zanacic *et al.* 2016), for inactivation of microorganisms in the food industry (Brodowska *et al*. 2017, 2018), and as an active agent for the removal of contaminants and pollutants from wastewater (Rosal *et al*. 2010; Schindler Wildhaber *et al.* 2015; Ashauer 2016; Bourgin *et al.* 2018). Ozonation has been employed in combination with specific organisms for the treatment of pulp and paper mill effluents (Bijan and Mohseni 2005; Remondino and Valdenassi 2018); in combination with biofilm for the removal of micro-contaminants from municipal influents (Gunnarsson *et al.* 2009); as well as in combination with anaerobic digestion in the treatment of wastewater containing pharmaceuticals (Carballa *et al.* 2007). In this study, the application of ozone (80 g in 1000 L) in the treatment of the PFE had a beneficial effect on the growth of *L. minor* and *S. polyrhiza*.

The results of basic chemical analysis of the medium after ozonation (Table 2) show that the process resulted in the increased availability of phosphate and nitrogen compounds. The concentration of phosphate increased from 2.73 ± 0.02 mg PO_4^{3-}/mL before ozonation to 3.39 ± 0.02 mg PO_4^{3-}/mL after ozonation. Similar trends were noted for ammonia

nitrogen and free nitrogen. Moreover, a significant decrease in the analyzed parameters was observed after cultivation of the plants. Slightly lower values were obtained for medium after the cultivation of *S. polyrhiza* compared to *L. minor*. Ozonation decreased the COD from 397 ± 1 mg/L (Medium 2) to 235 ± 1 (Medium 4). The COD values after the cultivation of *L. minor* and *S. polyrhiza* were 35 ± 1 and 45 ± 1 mg/L, respectively. The COD values after cultivation of the tested plants were lowest for Medium 4 and Medium 5. The results show that the use of ozonation alone is insufficient, as it only decreases COD slightly (41%). This is in line with results reported by Hadavifar and others (2016), who noted a 25% reduction of COD with ozonation of vinasse from alcohol distilleries (Gunnarsson *et al*. 2009).

Table 2. Influence of Medium Ozonation and Plant Cultivation on Free Phosphate and Nitrogen

Tested Medium	Chemical Oxygen Demand (mg/L)	Phosphate ($mgPO_4^{3-}/L$)	Phosphorus (mgP/L)	Ammonia Nitrogen ($mgNH4^{+}/L$)	Nitrogen (mgN/L)
Medium 2	**397 ± 1**	2.73 ± 0.02	0.89 ± 0.02	6.51 ± 0.05	5.38 ± 0.02
Medium 4	235 ± 1[a]	**3.39 ± 0.02[a]**	**1.08 ± 0.03[a]**	**9.60 ± 0.10[a]**	**7.88 ± 0.05[a]**
Medium 4 after *L. minor* L.	35 ± 1[a]	1.94 ± 0.01[a]	0.60 ± 0.02[a]	4.21 ± 0.01[a]	3.47 ± 0.02[a]
Medium 4 after *S. polyrhiza*	45 ± 1[a]	1.29 ± 0.04[a]	0.41 ± 0.03[a]	3.80 ± 0.04[a]	3.13 ± 0.03[a]

The results Medium 2 (without ozone treatment) were compared with results for Medium 4 (12 min ozonation) using one-way repeated measures ANOVA. The results after cultivation of *L. minor* and *S. polyrhiza* were compared to Medium 4. The compared values are statistically different ($p < 0.05$): [a]— $p < 0.005$.

In general, the mechanism for removing contamination by ozonation is based on direct reaction with ozone or hydroxyl radicals formed during the decomposition process (Schindler Wildhaber *et al.* 2015). While electron-rich moieties (olefins, tertiary amines, thioethers, and activated aromatics) react easily and quickly with ozone, the hydroxyl groups that form during the decomposition process react with alkanes and amides (Bourgin *et al*. 2018). As a result of the oxidative transformation of organic matter, the content of assimilable organic carbon or biodegradable organic carbon also increases. Consequently, the resulting compounds can be utilized by organisms introduced in subsequent biological stages of wastewater treatment (Zimmermann *et al*. 2011; Schindler Wildhaber *et al.* 2015). In the context of the present study, in which the content of easily digestible forms of nitrogen and phosphorus increased, this can explain the improved growth of the tested plants in media supplemented with 2.5% PFEs ozonated for 12 min.

The effect of nitrogen and phosphate on the growth of Lemnaceae plants has been studied in the literature (Ge *et al.* 2012; Zhao *et al*. 2014; Iatrou *et al*. 2015). In studies conducted by Paolacci and co-workers (2016), the highest number of fronds of *L. minor*. and the highest biomass were noted for 0.03 g/L of nitrate. The lowest biomass was associated with the medium without nitrate. The optimum concentration of nitrogen for *L. minor* appears to lie in the range from 2.8 mg/L to 350 mg/L, depending on the tested plant. For good plant growth, the presence of phosphate in the water medium is also necessary. The best results of growth of *L. minor* were obtained when the control medium contained 93 mg/L of P.

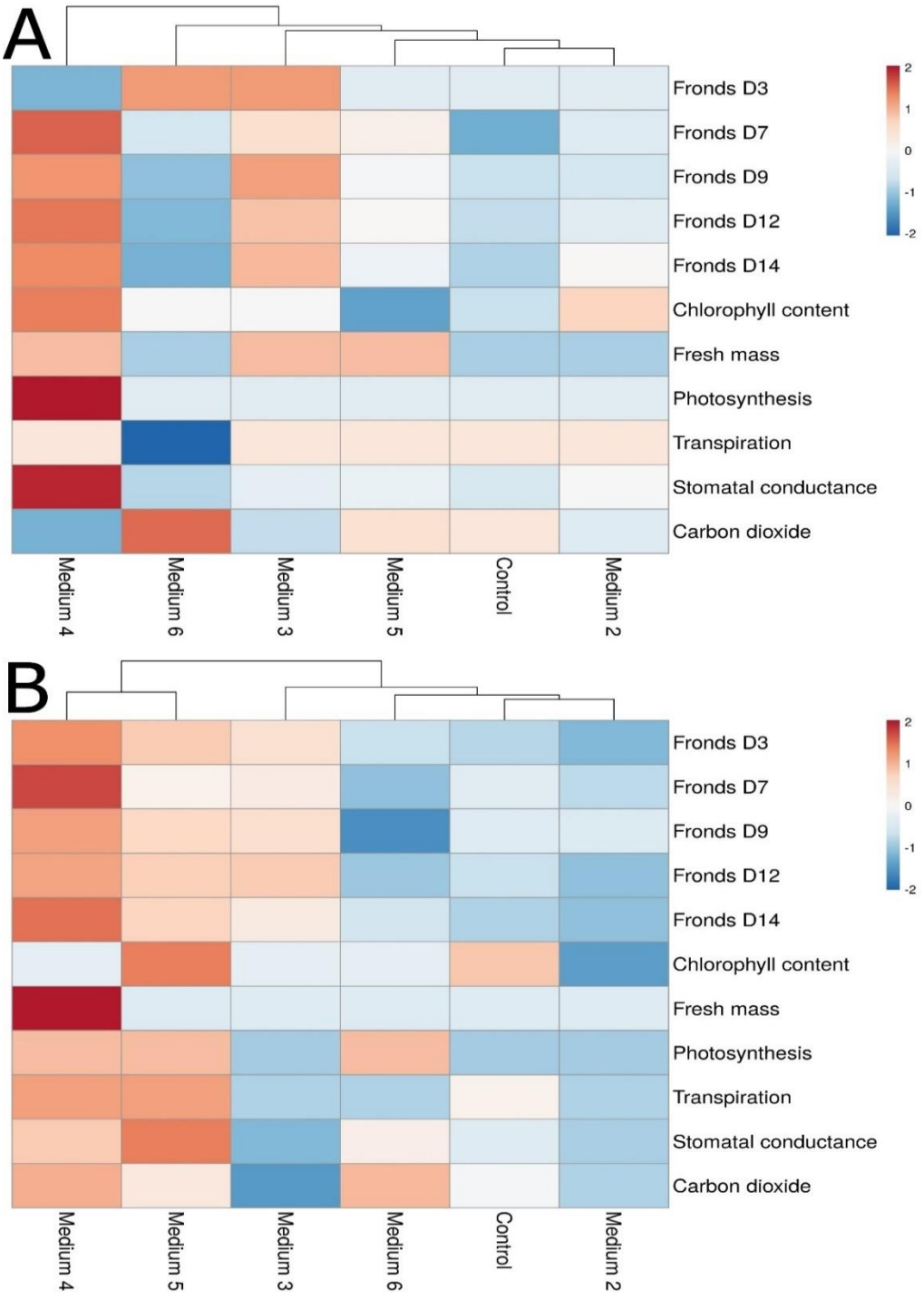

Fig. 8. Hierarchical clustering of analyzed parameters for *L. minor* (**A**); and *S. polyrhiza* (**B**) according to the tested media. Manhattan distance was used hierarchical clustering.

Another study found that number of *L. minor* fronds increased with higher phosphate concentrations and increases in temperature (up to 25 °C) (Appenroth 2002). In

this study, the maximum growth rate of *L. minor* and *S. polyrhiza* was achieved in the medium with 1.08 mg/mL of P and 7.88 mg/mL of N. It should be emphasized that the variation in these results may stem from the different nutritional requirements and metabolic activity of the plants used in this study, as well as from the other compounds present in the tested media (Njambuya *et al*. 2011; Appenroth and Adamec 2014).

As well as the effects of nutrient components on plant population and fresh weight, the growth environment has a major impact on parameters such as chlorophyll content. It is widely known that chlorophyll plays a major part in the energetic metabolism of green plants, and therefore any significant change in its levels will have a marked effect on the entire metabolism. *Lemna minor* has been used as a test organism for ecotoxicological assessment of industrial effluents by Radić *et al.* (2010). They found that the tested wastewaters inhibited growth rates, based on frond numbers and biomass, and also decreased chlorophyll content. The same authors concluded in a separate study that *L. minor* can be used as a tool for testing the toxicity of surface waters, and that one of the parameters that change under the influence of water parameters is the concentration of chlorophyll (Radić *et al*. 2011). In a study conducted by Chaudhuri *et al.* (2014), cadmium at concentrations of 3 mg/L reduced the chlorophyll content of *L. minor* to 0.7 mg/g, while for *S. polyrhiza* the value was 0.13 mg/g. These authors concluded that *Spirodela* plants were more susceptible to cadmium than *Lemna* plants (Chaudhuri *et al*. 2014). The effect of copper exposure on photosynthetic pigments in *L. minor* and *S. polyrhiza* was evaluated in a study by Kanoun-Boulé *et al.* (2009). They found that the content of a-type chlorophyll and carotenoids decreased as the concentration of copper rose. At the same time, b-type chlorophyll seemed less affected by exposure to the toxic agent. It should be mentioned that the studies discussed here varied in terms of temperature, pH, type of wastewater, and the composition of the resulting medium, as well as in terms of the tested plant species. Therefore, it is difficult to compare their results with the present work. Moreover, most research has focused on determining the impact of certain factors on the number of fronds, fresh weight, and chlorophyll content. In this study, additional photosynthesis parameters were examined (transpiration, stomatal conductance, and intracellular CO_2 content). The authors' results indicate that ozonation of PFEs before addition to a culture medium can have a beneficial effect on biomass yields.

Wastewater containing ozonated PFEs from a biogas plant can be considered for use in the cultivation of aquatic plants to improve the profitability and sustainability of biorefineries (Fig. 1B). Polish law allows the water discharged into watercourses to have parameters not exceeding 0.25 mg/L for phosphorous and 5 mg/L for nitrogen. Ozonated effluents with *Lemna minor* and *Spirodela polyrhiza* did not exceed these values for nitrogen and only second microorganisms led to normative results for phosphorus. The use of reclaimed wastewater as a source of process water reduces the ecological footprint of such installations and cost of energy used for fuel production. Lowering the costs of bioethanol production can be achieved by recycling the biomass of the aquatic plants through the fermentation process. After partial chemical, physical, or biological degradation, the biomass obtained from the process of wastewater treatment is a good fermentation medium, which is rich in fermentable carbohydrates. It may be used successfully as an additional substratum for bio-ethanol production. The amount of Lemnaceae and duckweed biomass that can be produced on an area of water rich in nutrients is 39.2 to 44.0 t/ha·year. This is comparable to or even higher than the yield of biomass that can be obtained from bioenergy grass such as miscanthus (5.0 to 44.0 t/ha·year) (Miranda *et al*. 2016). Because of their high growth rates, these water plants have

already been used as a feedstock for bioethanol production (Cheng and Stomp 2009; Xu *et al*. 2011). To increase the yield of bioethanol from lignocellulosic biomass, it is widely recommended to perform simultaneous saccharification and fermentation (Olofsson *et al*. 2008; Berłowska *et al*. 2016). Further enhancement of ethanol fermentation efficiency in media derived from aquatic plants can be achieved by using the ozonization method to stabilize the broth (Berłowska *et al*. 2018). Further research is necessary to increase the scale of cultivation of *L. minor* and *S. polyrhiza* in waters enriched by ozonized biogas plant effluent. The collected biomass will be used as a fermentation medium in ethanol production processes coupled with biogas production processes.

CONCLUSIONS

1. Post-fermentation effluents can be used as a raw material to supplement conventional culture media for *L. minor* and *S. polyrhiza*. Prior ozonation of the PFEs significantly improved the growth efficiently of these plants.
2. The most preferred variant was the medium supplemented with 2.5% of PFEs subjected to ozonation for 12 min. The plant growth parameters for this variant were the highest of the supplemented media, but also significantly higher than the results obtained for conventional medium (Medium Z). Thus, ozonation prior to basic biomass cultivation of aquatic plants leads not only to more effective wastewater purification but also to higher yields of biomass.

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