Sustainable applications of *Cladophora glomerata* hydrolysates: Boosting *Nostoc commune* biomass and utilizing biowaste for agricultural and environmental purposes

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Abstract

The green macroalga *Cladophora glomerata*, a species commonly infesting surface waters, was hydrolyzed by Liquid Hot Water treatment under acidic, neutral, and alkaline conditions. The liquid fraction obtained after hydrolysis was added to BG-11 medium to cultivate the valuable cyanobacterium *Nostoc commune* under mixotrophic conditions, while the insoluble fraction was tested as a biosorbent of metal ions. In addition, cyanobacteria were grown under autotrophic conditions also in the presence of ZnO nanoparticles, biosynthesized using aqueous algal *C. glomerata* extract. Mixotrophic conditions with the use of acidic and alkaline algal hydrolysates produced the largest amount of *N. commune*. The post-culture media after *N. commune* separation were tested as potential biostimulants of plant growth, while the alga itself and the solid residue after hydrolysis were tested for the biosorption capacity of Cr(III). The study presents an environmentally friendly method to transform the waste algal biomass into beneficial products for agriculture and the environment.

Keywords: *Cladophora glomerata*, thermal hydrolysis, *Nostoc commune*, germination tests, biosorption

1. Introduction

The biomass of the green macroalga *Cladophora glomerata* (Chlorophyta) is an environmental problem in both marine and freshwater reservoirs [1]. Municipalities often treat it as mixed waste, leading to landfill disposal. In this way, the opportunity to integrate this valuable algal biomass into the circular economy is lost, wasting large amounts of organic matter [2]. However, due to the unique chemical composition of C. glomerata, many valuable bioproducts can be obtained from this biomass, which are used in many sectors of the economy [1, 3, 4]. The key issue is the selection of an appropriate method for processing such biomass, adapted to potential future applications.

The Liquid Hot Water (LHW) technique, also known as autohydrolysis of biomass, is considered a green method for the hydrolytic cleavage of the polymers of biomass (e.g., polysaccharides, proteins, nucleic acids, etc.) and has many advantages such as utilization of hot pressurized water, as a solvent at elevated temperatures (160–240 °C) and pressurized conditions (5–20 bars) without additional chemicals [2, 5, 6]. The process carried out in optimal experimental conditions (temperature, time, pressure, solid:liquid ratio) is known to enhance sugar recovery from the biomass [6]. To the best of our knowledge, LHW technique has never been used for the hydrolysis of *C. glomerata* biomass. Applying this process, it is produced a liquid fraction containing soluble alga hydrolysates and a solid residue, which in accordance with the assumptions of a circular economy, must be managed.

In the present study, the liquid fraction coming from the LHW treatment was used as a source of sugars and minerals in the cultivation of *Nostoc commune* under mixotrophic conditions. This cyanobacterium is crucial for agriculture because it has atmospheric nitrogen fixation ability which is particularly important as it is a sustainable alternative to provide nitrogen as a fertilizer [7, 8, 9]. Moreover, the post-culture medium, after the separation of *N. commune*, also constituting waste, was tested as a potential plant growth biostimulant.

As for the valorization of insoluble *C. glomerata* residues, it is still a valuable source of active ingredients and nutrients, including polysaccharides, proteins, polyphenols, micro- and macroelements, which can be used in agriculture as soil additives. Moreover, due to its good sorption properties, the post-extraction residues can be used as a sorbent for the removal of heavy metal ions from wastewater. In a previous studies we have shown that the post-extraction residue after ultrasound-assisted extraction of *C. glomerata* biomass was able to bind 192 mg of Cr(III) ions/g of the biomass (for initial concentration of metal ions C_0 of 25–300 mg/L,

content of sorbent in the solution C_S of 10 g/L, and pH of 5) [3]. Thus, the *C. glomerata* residue from the LHW process was tested for sorption properties.

The aim of the research herein carried out was to exploit the application of the products obtained by the environmentally friendly LHW technique from the *C. glomerata* biomass – algal hydrolysates and post-hydrolysis solid residues.

2. Materials and Methods

The general research scheme is presented in Fig. 1.

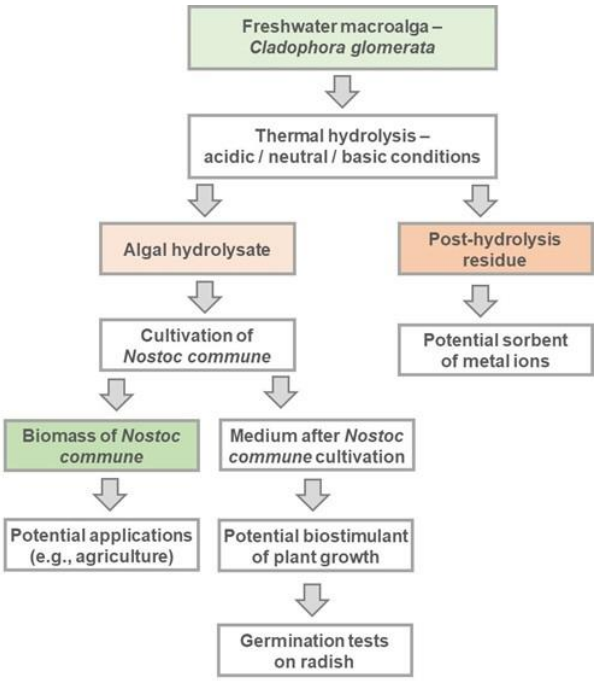

Fig. 1 General scheme of the performed experiments

The following research hypotheses were tested during the study:

1. Hydrolysates obtained from *Cladophora glomerata* using the Liquid Hot Water (LHW) technique will have a positive effect on the cultivation of *Nostoc commune*.
2. The extract residue after hydrolysis of *Cladophora glomerata* using the Liquid Hot Water (LHW) technique will have sorption capacity for metal ions from aqueous solutions (potential use as a biosorbent in wastewater treatment).

3. Post-culture media after *Nostoc commune* separation, applied during radish cultivation, do not cause phytotoxic effects (potential use as a plant growth biostimulant).

2.1. Alga – *Cladophora glomerata*

The biomass of freshwater macroalga *C. glomerata* was collected from the lake's surface in the village Tomaszówek (Łódź Province, Poland) in October 2016. The harvested macroalga was identified based on morphological characteristics according to the taxonomic literature for this area [10]. Then, the biomass was air-dried and milled using a grinding mill (Retsch GM300, Haan, Germany). During the sieve analysis (Retsch, Haan, Germany), the biomass with particle size lower than 400 µm was chosen for further experiments.

2.2. Thermal hydrolysis of *Cladophora glomerata*

The dry powder of *C. glomerata* (10 g) was suspended in distilled water (300 mL) and let to rest overnight at room temperature. Then the suspension was centrifuged for 45 minutes at 3000 rpm and the solid was resuspended in 300 mL of water. This step was repeated a total of three times to wash the algae. After the third wash, the pH value of the washing water of *C. glomerata* algae was 7. Following that, the algal biomass was suspended in 200 mL of distilled water and subjected to thermal hydrolysis using the reactor (Highpreactor, BERGHOF), configured for 2 hours and 45 minutes at 170 °C, 32 bar and 200 rpm. The identical procedure was employed for thermal hydrolysis under acidic or basic conditions, with the exception that, prior to the reaction, the algae suspension was adjusted to pH 2 or 12.5 by the gradual addition of sulfuric acid (resulting in a final concentration of 2% v/v) or potassium hydroxide (resulting in a final concentration of 0.5% w/v), respectively. A variety of acids and alkalis act as external promoters in hydrothermal pretreatment techniques, which improve the biomass digestibility [5]. Following the reaction cycle, the suspension underwent cooling and centrifugation. The supernatants (algal hydrolysate) were collected and characterized for oxidizable sugar content and the concentration of micro- and macroelements. The solid residue was oven-dried overnight at 60 °C and the lyophilized post-hydrolysis residues were used for FTIR analysis. Additionally, the composition of the residue was analyzed in terms of elements content.

2.3. Cultivation of *N. commune* with *C. glomerata* hydrolysates

In all cases, the cells of *Nostoc commune* 1453-5 (Culture Collection of Algae at the University of Göttingen) were cultivated in 100 mL of BG-11 media, composed of (g/L):

$NaNO_3$ (1.5), HK_2PO_4 (0.0035), $MgSO_4$ (0.075), $CaCl_2$ (0.027), Citric acid (0.00655), Ferric ammonium citrate (0.006), $EDTANa_2Mg$ (0.001), Na_2CO_3 (0.02) and 1 mL of microelement stock solution (H_3BO_3 (2.86), $MnCl_2$ (1.81), $ZnSO_4$ (0.222), $NaMoO_4$ (0.39), $CuSO_4$ (0.079), $Co(NO_3)_2$ (0.0494)). Flasks were stirred at 120 rpm on a shaker (Thermo-Shaker, Eppendorf Innova 42 New Brunswick) at 25 °C under an illumination of 450 lx and a circadian cycle that provides 14 h of light and 10 h of darkness. Cell cultures were carried out in autotrophic (A) or mixotrophic (M) conditions (adding *C. glomerata* algal hydrolysates) using different media. Autotrophic cultivation included two variants: BG11 medium (A) and BG11 medium with zinc nanoparticles (ZnO NPs) at a dose of 0.005 mg/mL (A-ZnO NPs) (the concentration was chosen according to the study of Dziergowska et al. (2021) [3]). The biosynthesis and characteristics of used zinc oxide nanoparticles was described in the previous paper [3].

For mixotrophic conditions, the cultivation media were prepared by separately adding to the BG11 medium different volumes of acidic, neutral, basic hydrolysates from *C. glomerata*, which were determined based on the oxidizable sugars they contained (measured by the DNS method; section 2.6.1.). Each hydrolysate (acidic, neutral, basic) from *C. glomerata* provided the same amount of reducing sugars to the BG11 medium. Mixotrophic cultivation included the following variants:

1. Mixotrophy – BG11 medium containing 20% (v/v) *C. glomerata* hydrolysate produced by acidic hydrolysis process (M-AH);
2. Mixotrophy – BG11 medium containing 12% (v/v) *C. glomerata* hydrolysate produced by neutral hydrolysis process (M-NH);
3. Mixotrophy – BG11 medium containing 10% (v/v) *C. glomerata* hydrolysate produced by basic hydrolysis process (M-BH).

Ampicillin (50 μg/mL) was added to each flask to prevent contamination. The pH of all media was neutralized at a value of 7 with the addition of HCl or NaOH to verify the effect of the differently treated algal biomass on cell growth, as a different starting condition could falsify the result [11]. Samples of the cultures (3 mL) were taken at the starting point and at the end of the 3 weeks.

2.4. Germination tests on radish using post-culture media after *Nostoc commune* separation

After cultivation of *N. commune* in BG11 medium with *C. glomerata* hydrolysates and ZnO nanoparticles, the cyanobacterial biomass was separated, and the supernatants obtained,

constituting waste in this process, were tested for stimulation of the early phase of radish growth. Radish is a model plant, chosen for germination tests due to its rapid plant growth. After a week, it can be assessed whether the tested preparations have a phytotoxic effect on plants. Germination studies were carried out in 14 groups, which are listed in Table 1, along with the abbreviations of the names of these groups that were used when discussing the results.

Table 1

Variants for which germination tests were carried out on radish seeds

Group number	Treatment/Method	Group abbreviation
1	BG11 medium	BG11
2	Supernatant after separation of *N. commune* (Nc) cultivated in BG11	BG11-Nc
3	BG11 medium with the addition of acid hydrolysate (AH) from *C. glomerata*	BG11+AH
4	Supernatant after separation of *N. commune*, cultivated in BG11, with the addition of acid hydrolysate (AH) from *C. glomerata*	BG11+AH-Nc
5	BG11 medium with the addition of neutral hydrolysate (NH) from *C. glomerata*	BG11+NH
6	Supernatant after separation of *N. commune*, cultivated in BG11, with the addition of neutral hydrolysate (NH) from *C. glomerata*	BG11+NH-Nc
7	BG11 medium with the addition of basic hydrolysate (BH) from *C. glomerata*	BG11+BH
8	Supernatant after separation of *N. commune*, cultivated in BG11, with the addition of basic hydrolysate (BH) from *C. glomerata*	BG11+BH-Nc
9	Supernatant after separation of *N. commune*, cultivated in BG11 to which ZnO NPs were added before germination tests	BG11-Nc+NPs
10	Supernatant after separation of *N. commune*, cultivated in BG11, with the addition of acid hydrolysate (AH) from *C. glomerata* to which ZnO NPs were added before germination tests	BG11+AH-Nc+NPs
11	Supernatant after separation of *N. commune*, cultivated in BG11, with the addition of neutral hydrolysate (NH) from *C. glomerata* to which ZnO NPs were added before germination tests	BG11+NH-Nc+NPs

12	Supernatant after separation of *N. commune*, cultivated in BG11, with the addition of basic hydrolysate (BH) from *C. glomerata* to which ZnO NPs were added before germination tests	BG11+BH-Nc+NPs
13	Supernatant after separation of *N. commune*, cultivated in BG11 to which ZnO NPs were added during cultivation	BG11+NPs-Nc
14	Control group – distilled water (H_2O)	C

Germination tests were conducted on Petri dishes with a filter paper. Red radish seeds (*Raphanus sativus*) cv. Pharaoh from WerbAna Sp. z o.o. (Warsaw, Poland) were used as a model plant. The seeds were not sterilized before testing. Tests were carried out in triplicate for each experimental group, using 25 seeds per replication. Each Petri dish with seeds was watered with 3 mL of required solution (post-culture media after separation of *N. commune* grown in BG11 medium with modifications) or distilled water. The plants were kept in an incubator (POL-EKO, ST 5 C, Wodzisław Śląski, Poland) that maintains a constant temperature of 20 °C and humidity. The tests lasted for 10 days (12 h day/12 h night). After this time, the seedling parameters were evaluated: above-ground plant length measured with a ruler, dry above-ground plant weight, dry root weight and chlorophyll content in cotyledons (using SPAD 502 Plus Konica Minolta). Plant length and chlorophyll content was measured for all normal, fully developed seedlings. The mass of the aboveground part of plants and roots was determined for all normal, fully developed seedlings grown on a given Petri dish (each group performed in triplicate).

2.5. Biosorption of Cr(III) ions by *C. glomerata* solid residue derived from LHW process

Solid residues after thermal hydrolysis of *C. glomerata* carried out under acidic, neutral and basic conditions were examined for their sorption properties. For this purpose, sorption experiments (kinetics and equilibrium) of Cr(III) ions by the above-mentioned biosorbents were carried out. Chromium ions were chosen as a model ion due to the simple method of determining their concentration in aqueous solutions – spectrophotometric method (Cr(III) ions form violet complexes with EDTA) [3].

The experiments on the kinetics of the biosorption process of Cr(III) ions were aimed at determining the time necessary to achieve the equilibrium state and to determine the biosorption capacity at equilibrium (q_t). The sorption capacity, q (mg/g), was calculated using the equation: $q = (C_0 - C_t) / Cs$

where: C_0 (mg/L) − the initial concentration of Cr(III) ions in the solution, C_t (mg/L) − the concentration of Cr(III) ions in given time t (min), and C_S (g/L) − the content of biosorbent in the solution.

The experimental conditions for sorption kinetics were as follows: initial pH of the solution equal to 5, C_0 of 300 mg of Cr/L and C_S of 1 g/L (determined in previous study [3]). The pH of the solution containing Cr(III) ions was measured and adjusted to the appropriate value by the addition of NaOH. During biosorption kinetics, to 200 mL of chromium solution (prepared using $Cr(NO_3)_3 \cdot 9H_2O$), 0.2 g of biosorbent was added. The mixture was stirred for 2 h at 200 rpm at the room temperature. After 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min samples were taken and filtered through a filter paper. The concentration of Cr(III) ions in the solution before and after the biosorption process was determined by spectrophotometric method. To describe the biosorption kinetics, the pseudo-second-order kinetic model was used [12, 13]:

$$\frac{t}{q_t} = \frac{1}{k_2 \cdot q_{eq\,2}^2} + \frac{t}{q_{eq\,2}}$$

where: q_t – amount of adsorbed metal ions on the sorbent (mg/g) at a given time t, q_{eq2} – sorption capacity at equilibrium (mg/g) and k_2 – the rate constant of pseudo-second order model of sorption (g/mg·min).

During experiments on biosorption equilibrium, nine solutions of Cr(III) ions at concentrations 25, 50, 75, 100, 125, 150, 200, 250, and 300 mg/L were prepared. To 40 mL of each solution, 0.04 g of biosorbent was added (C_S 1 g/L). The mixtures were stirred at 100 rpm, at room temperature. After 2 h of stirring (time determined from studies on the kinetics of the biosorption), each mixture was filtered through a filter paper and the concentration of Cr(III) ions in the solution after biosorption was determined analogously as in the case of studies on sorption kinetics. The values of the maximum sorption capacity (q_{max}, mg/g) for the tested sorbents were calculated from the Langmuir model [13]:

$$q_{eq} = \frac{q_{max} b C_{eq}}{1 + b C_{eq}}$$

where: q_{max} – maximum sorption capacity (mg/g), C_{eq} – concentration of Cr(III) ions in the solution at equilibrium (mg/L), b – constant related to the affinity of binding sites for metal ions (L/mg).

All biosorption experiments were performed in duplicate.

2.6. Analytical methods

2.6.1. DNS assay

Acidic, neutral and basic hydrolysates from *C. glomerata* were analyzed in terms of sugar content by the DNS assay as reported by Wood et al. (2012) [14].

2.6.2. Chlorophyll content in cultivated *Nostoc commune*

The biomass of *N. commune*, from each flask after cultivation was centrifuged and resuspended in 1.5 mL of methanol [15]. After being sonicated (Omni-Ruptor 250, Omni, St. Neots, United Kingdom) at 100 Watt for 5 min in an ice bath, the cells released the chlorophyll that was quantified through a spectrophotometer (Jasco V-630 bio, Jasco, Tokyo, Japan) measuring optical density (OD) at 680 nm.

2.6.3. FTIR analysis

This technique was used to characterize the biomass of *C. glomerata*, the solid residues after its thermal hydrolysis under acidic, neutral and basic conditions and corresponding hydrolysates (after freeze-drying). FTIR spectra were recorded using 1 mg of sample mixed with 250 mg of KBr to prepare pellets for FTIR analysis (Jasco, FT/IR-610). All samples were blank (only KBr) subtracted.

2.6.4. Multielement analysis

Hydrolysates from *C. glomerata* obtained by thermal hydrolysis, as well as post-extraction residues, were examined for their multi-element composition. The content of elements in examined samples was determined using the ICP-OES technique (Inductively Coupled Plasma – Optical Emission spectroscopy) on an ICP spectrometer (Agilent 5110) after prior mineralization of samples (0.5 g) in a Speedwave (Berghof) microwave mineralizer using 65% Suprapur nitric acid – 10 mL (Merck, Germany).

2.7. Statistical analysis

Results from the germination tests were statistically elaborated with *Statistica* 13 software (TIBCO Software Inc., Tulsa, OK, USA). The obtained data was analyzed with the Shapiro–Wilk normality test. If the data fit to a normal distribution, the homogeneity of variances was verified with the Brown-Forsythe test. A Tukey test was then used to compare any differences between the groups tested, and, more specifically, to compare all pairs of means following one-way ANOVA tests (results presented as a mean and standard deviation) [16]. In the case of a non-normal distribution, the Mann–Whitney test (to compare two groups) or the Kruskal–Wallis test (to compare more than two groups) were used (results presented as a median).

Results were considered significantly different when $p<0.05$. Principal Component Analysis (PCA) was performed using the Singular Value Decomposition (SVD) algorithm [17]. Raw data obtained from germination test analyses underwent several preprocessing steps, including normalization using the Manhattan norm, transformation through decay logarithm transformation, chromatogram mean centering, and scaling.

3. Results and Discussion

The raw material used in this research was the biomass of freshwater macroalga – *Cladophora glomerata*, which was characterized in terms of its chemical composition in the previous publications [3, 4].

3.1. Characteristics of *Cladopohora glomerata* hydrolysates

Thermal hydrolysis is widely recognized as an effective method for the valorization of waste biomass, enabling the recovery of valuable compounds such as reducing sugars and bioactive molecules, which can serve as substrates for downstream biotechnological applications (https://doi.org/10.1016/j.biortech.2021.125961). Thermal hydrolysis of *C. glomerata* under acidic, neutral and basic conditions resulted in algal hydrolysates with the pH values of 5.9, 7.2, and 10.1, respectively. The pH values prove that the acid or basic conditions were maintained until the end of the process. The appropriate amount of acid/base was added to the mixtures containing *C. glomerata*, which ensured that the set conditions were maintained throughout the process. Changes in the pH of the obtained hydrolysates may result from the autoionization of water, which produces hydronium ions that initiate the hydrolysis of biomass during hydrothermal treatment. The high concentration of hydronium ions in water, at high temperatures, serves as an acid catalyst [18]. Autohydrolysis of *C. glomerata* leads to the disruption of the biomass structure and the release of many active compounds contained in it. Due to the fact that the obtained *C. glomerata* hydrolysates were a component of the culture medium in the mixotrophic cultivation of *N. commune*, reducing sugars were determined in them. The highest concentration of reducing sugars was obtained in the *C. glomerata* hydrolysate obtained under alkaline, then neutral and finally acidic conditions, which was 256 µg/mL, 210 µg/mL, and 120 µg/mL, respectively. The different amount could be to a different solubilization of *C. glomerata* according to the pH value of the LHW process. As shown above, the pH change is typically more pronounced under acidic conditions, leading to a more neutral final pH. This suggests a potentially lower degree of hydrolysis compared to alkaline

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hydrolysis. Alkaline conditions may facilitate greater solubilization of carbohydrates and other sugars present in the biomass, leading to higher sugar concentrations in the resulting hydrolysate. Conversely, acidic conditions may not solubilize sugars as effectively, resulting in lower sugar concentrations in the acidic hydrolysate.

The obtained *C. glomerata* hydrolysates were examined also in terms of multi-element analysis and the concentration of micro- and macroelements in supernatants is presented in Table 2.

Table 2

Multi-element characteristics of hydrolysates obtained from *C. glomerata* (mg/L; mean±SD; N=2)

Element	Hydrolysates after *Cladophora glomerata* hydrolysis under		
	Acidic conditions	**Neutral conditions**	**Basic conditions**
As	<LOD	<LOD	<LOD
B	1.22±0.01	2.66±0.035	2.08±0.01
Ba	1.89±0.00	0.597±0.002	0.688±0.002
Ca	1094±10	501±3	513±0
Cd	<LOD	<LOD	<LOD
Co	<LOD	<LOD	<LOD
Cu	0.077±0.023	0.100±0.030	0.108±0.037
Fe	8.73±0.14	1.58±0.02	10.8±0
K	221±1	177±1	2205±23
Mg	41.6±0.3	28.6±0.1	33.5±0.2
Mn	3.44±0.02	0.840±0.009	1.79± 0.01
Na	6.88±0.01	5.81±0.07	9.56±0.32
P	6.22±0.05	1.85±0.14	15.2± 0.0
S	689±1	438±3	443±7
Zn	0.379±0.152	0.213±0.013	0.406±0.167

<LOD – below limit of detection

Liquid Hot Water hydrolysis of *C. glomerata* performed under acidic conditions facilitated extraction of light metal ions such as Ba (2.7 times more), Ca (2.1 times more) and Mg (24.2% more) in comparison with hydrolysis performed under basic conditions. These secondary

macronutrients (Ca and Mg) are important in the mineral nutrition of plants. The results for the sulphur and potassium concentration in the obtained hydrolysates are not compared due to the use of H_2SO_4 and KOH to adjust the pH of water during hydrolysis. The algal hydrolysate obtained under alkaline conditions was richer in microelements than the hydrolysate obtained under acidic conditions. The concentration of B in acidic hydrolysate was by 70.5% higher than in acidic hydrolysate, Cu by 40.3% higher, Fe by 23.7% higher, Zn by 7.12% higher. Hydrolysate obtained with KOH contained also more Na (by 38.9%) and P (2.4 times more) than hydrolysate obtained with H_2SO_4. Heavy metal ions in the tested hydrolysates were below the detection limit. These results also show that low pH of the solution favors the extraction of minerals (especially alkaline earth metals) from the algal biomass. Among the light metals tested, Ca, K, Mg and Na occur in the largest amounts in the freshwater biomass of *C. glomerata* – 168 829 mg/kg d.m., 22 212 mg/kg d.m., 1973 mg/kg d.m., and 518 mg/kg d.m., respectively [3]. Similar results regarding the extraction of individual micro- and macroelements (especially P, Mg, Na, Cu, Mn) were obtained by Neveux et al. (2020) for extracts obtained from the freshwater macroalga *Oedogonium intermedium* in acidic (0.05 M HCl) and basic (0.01 M KOH) conditions [19]. Also, Godlewska et al. (2017) indicated that with an increase in the pH of water (pH 3, 7, 10) used for the extraction of a mixture of Baltic macroalgae (*Polysiphonia* sp., *Ulva* sp., and *Cladophora* sp.), the concentration of Ca, Mg, Mn in the obtained extract decreased [20]. To obtain algal extract containing micro- and macroelements, extraction in an acidic environment is recommended. Hydrolysis of algal biomass in an alkaline environment does not result in a significant increase in the concentrations of the tested elements in the extract. The cations are more soluble in acidic than in alkaline conditions due to the formation of insoluble oxide or hydroxide.

Hydrolysis of *C. glomerata* carried out in neutral conditions allowed obtaining a hydrolysate that contained the highest concentrations of Ca (510±3 mg/L), S (438±3 mg/L), K (177±1 mg/L) and Mg (28.6±0.1 mg/L) [3]. The composition of this extract was compared with the extract obtained from the same alga but using ultrasound-assisted extraction. Also in this case, an extract was obtained containing the largest amounts of the following macroelements: K (872±10 mg/L), S (284±6 mg/L), Ca (198±1 mg/L) and Mg (31.4±0.1 mg/L). To sum up, *C. glomerata* extracted in an aqueous environment, regardless of the technique used, allows obtaining extracts containing mainly primary and secondary macronutrients.

3.2. FTIR analysis of *C. glomerata*, hydrolysates and solid residues after LHW process

The raw biomass of *C. glomerata*, hydrolysates from *C. glomerata*, obtained under acidic, neutral and alkaline conditions, as well as solid algal residues obtained after hydrolysis were also subjected to FTIR analysis to evaluate changes in the composition of examined samples (Fig. 2). Analyzes of *C. glomerata* biomass and the solid residue obtained after applying the LHW process to this biomass may be useful in identifying functional groups that may be responsible for binding metal ions from aqueous solutions (e.g., $-COO^-$ and $-NH_3^+$). The sorption properties of these two biosorbents were analyzed later in the article (section 3.6).

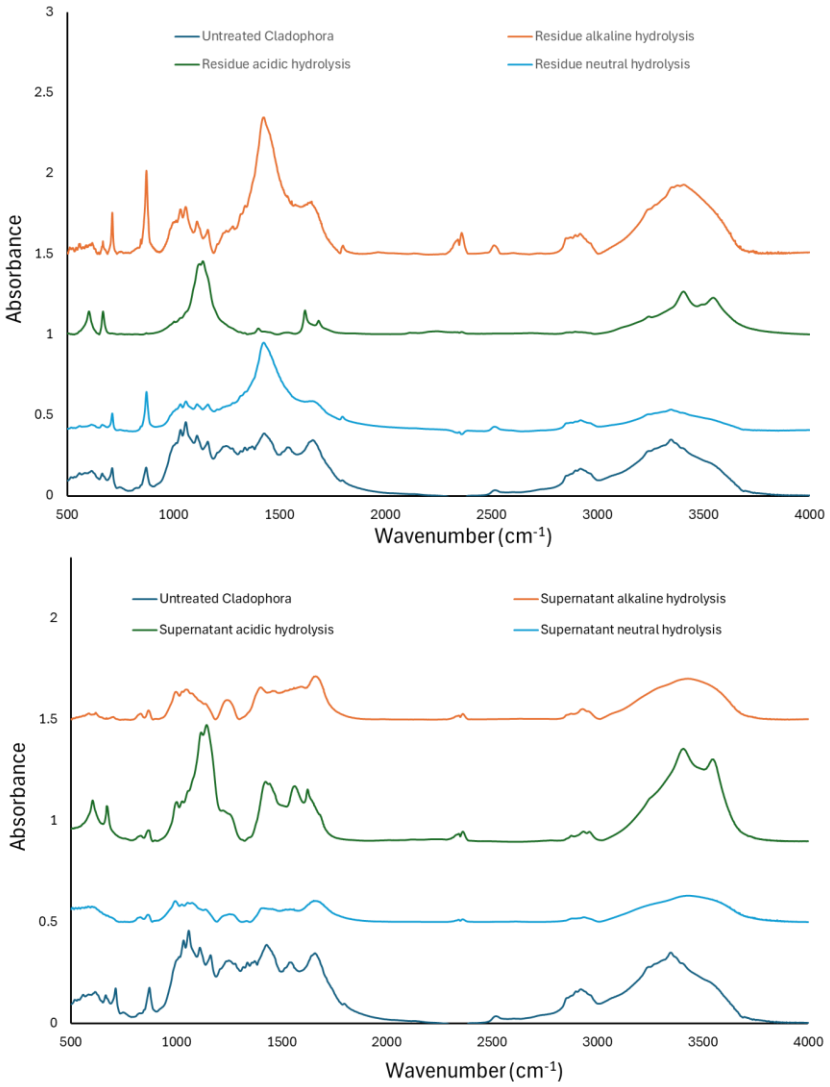

Fig. 2 The FTIR spectra of untreated and dried solid residue lyophilized (above) or lyophilized supernatant of *C. glomerata* after LHW treatment

Surayot et al. (2016) found that crude *C. glomerata* Kützing contains carbohydrates (62.8%), proteins (17.3%) and sulfate (19.9%) [21]. FTIR spectra of untreated powder of *C. glomerata* show the presence of peaks that can be assigned to -NH and -OH stretching vibration (3350 cm^{-1} and 3500 cm^{-1}), C=O stretching of amide bonds (1660 cm^{-1}), N-H bending of amide bonds (1540 cm^{-1}), which are typically assigned to proteins. The peak at around 1430 cm^{-1} can be assigned to C-O symmetric stretching of carboxylates groups ($-COO^-$) [22]. This latter assignment can be supported also by the analysis of the residue obtained in acidic conditions in which the same peak shows a marked intensity reduction because of the protonation of the carboxylic group (-COOH). Green algae are generally rich in sulfated polysaccharides, which are complex, heterogeneous, and bioactive anionic macromolecules. The presence of sulfate groups can be highlighted by the S=O stretching peaks at around 1160 cm^{-1} and 1440 cm^{-1}. However, this latter peak can be noted only in the residue obtained in acidic conditions, while in all the other residues it is hidden because of the more intense peak of the carboxylate groups. On the other hand, the very intense band at around 1130 cm^{-1} in the spectrum of the acidic residue can be assigned to the sulfate anions of the sulfuric acid employed for the hydrolytic process. In general, the same assignments can be done for both the dried solid residue and the lyophilized supernatant samples. However, these latter ones appear very similar to the *C. glomerata* untreated powder, indicating that the LHW allows the solubilization of the same organic fraction (e.g., polysaccharides and proteins) of the macroalgae in all conditions tested. According to the analysis of the FTIR spectra, residues coming from neutral and alkaline hydrolysis appear to be rich in carboxylate groups suitable for the removal of cations in biosorption applications (https://doi.org/10.1016/j.bej.2006.06.005).

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3.3. Cultivation of *Nostoc commune* with *Cladophora glomerata* hydrolysates

Hydrolysates from *C. glomerata*, obtained under acidic, neutral and alkaline conditions, were used as a component of BG11 medium, used in the cultivation of *N. commune* under mixotrophic conditions. In parallel, this microalga was grown in autotrophic conditions with or without the addition of ZnO nanoparticles. The results from the cultivation of *N. commune* under different experimental conditions are presented in Table 3.

Table 3

The influence of different culture conditions on the growth of *Nostoc commune*: A (autotrophy, BG11), A-ZnO NPs (autotrophy with ZnO nanoparticles), M-AH (mixotrophy with acidic *Cladophora* hydrolysate), M-NH (mixotrophy with neutral *Cladophora* hydrolysate), and M-BH (mixotrophy with alkaline *Cladophora* hydrolysate).

Condition	OD680 WEEK 0	OD680 WEEK 3	Dry weight (mg)
A	0.0887	0.282	42.9
A-ZnO NPs	0.109	0.125	25.5
M-AH	0.155	0.544	71.0
M-NH	0.193	0.0981	31.2
M-BH	0.135	0.422	73.4

The cultures showed significantly different growth across the various tested conditions. The results indicate that when basic or acid hydrolysates of *C. glomerata* were added to the culture medium (mixotrophy), cellular growth of *N. commune* was increased as compared to the autotrophy. Analyzing the dry mass results for the *N. commune*, it can be observed that its biomass almost doubled the amount produced in the photosynthesis process for M-AH and M-BH as compared to A (only BG11 medium). Among the tested hydrolysates from *C. glomerata*, the highest increase in microalgal biomass was in the case of the application of alkaline hydrolysate (M-BH), then acidic (M-AH). By looking at the dry weight results for *N. commune*, it can be observed that the biomass nearly doubled the amount when grown ~~produced just by photosynthesis~~ under mixotrophic conditions than under autotrophic conditions. When neutral conditions are used during the LHW process, only partial hydrolysis of *C. glomerata* biomass can occur, because neutral conditions are not sufficiently aggressive to completely break down the cell walls of the algal biomass, which limits the release of many active compounds. Hydrolysates from *C. glomerata* obtained under acidic, neutral and alkaline conditions provided the same amount of sugars during the cultivation of *N. commune*, but the concentration of other active compounds that may affect the growth of this microalga may have been lower in the case of the neutral hydrolysate. This is, for example, reflected in the elemental composition of the neutral hydrolysate from *C. glomerata*, which contained much lower concentrations of

macroelements – Ca, K, Mg, P, S and microelements – Fe, Mn, Zn compared to acidic and alkaline hydrolysates. The ability to grow under conditions of mixotrophy is common to many cyanobacteria, especially the filamentous ones [23]. Moreover, it has been demonstrated that the strain *Nostoc flagelliforme* can grow using many sugars, and in particular xylose as substrate [24]. The conducted research showed that the addition of Zn nanoparticles to the culture inhibited the growth of *N. commune*. The dose used may have caused stress in the cultured cyanobacteria ~~microalgae~~.

3.4. Germination tests on radish

Culture media after harvesting of *N. commune* may still contain many micro- and macroelements from the BG11 medium, as well as active compounds produced by microalga during its cultivation, and therefore may have a positive effect on plant growth. Using them as potential biostimulants of plant growth is consistent with the assumptions of the circular economy. The results regarding the influence of various post-culture media, after *N. commune* separation, on the biometric parameters of radish are presented in Table 4. In the previous section it was shown that the zinc oxide nanoparticles did not stimulate the growth of *N. commune*, but we wanted to check whether the nanoparticles added to the post-culture medium after collecting cyanobacterium would stimulate the growth of the plants. It was shown that the tested solutions had no phytotoxic effect on plant growth. The germination percentage of seeds for each group was above 90%.

Although the cultivation of microalgae under laboratory conditions is widespread, there is not much information in the literature about the use of the post-culture medium after the separation of microalgae in plant cultivation. One such study was conducted by Kholssi et al. (2019) reported that the post-culture medium of the microalga *Chlorella sorokiniana* had a biostimulant effect on wheat growth allowing a 30% increase in plant length when compared to the control (BG11 medium). Additionally, the total dry biomass of the aboveground and belowground parts improved by 22% and 51%, respectively [25]. These findings suggest that the nutrients and extracellular substances excreted by this microalga in the filtrate were relevant to the positive effects on plant growth.

Table 4

Parameters of radish growth treated with different liquid bioproducts

Group	Number	Whole plant length (cm)	Whole plant dry weight (g)	Root dry weight (g)	Chlorophyll content (SPAD)	Germination percentage (%)
		Mean±SD	Mean±SD	Mean±SD	Median	
BG11	1	5.15±1.25[a]	0.213±0.022	0.0525±0.0035	62.2	93
BG11-Nc	2	5.44±1.35[b,c]	0.180±0.011	0.0471±0.0042	57.9	92
BG11+AH	3	4.99±1.19	0.220±0.018	0.0503±0.0067	63.6	95
BG11+AH-Nc	4	5.57±1.52[d,e]	0.202±0.012	0.0563±0.0014	60.4	95
BG11+NH	5	4.75±1.33[f,g]	0.191±0.013	0.0524±0.0035	60.5	91
BG11+NH-Nc	6	5.05±1.14	0.183±0.019	0.0561±0.0048	56.8	95
BG11+BH	7	4.52±1.22[b,d,i,j,k]	0.174±0.015	0.0462±0.0051	59.6	92
BG11+BH-Nc	8	5.22±1.35[l]	0.196±0.008	0.0570±0.0045	59.8	96
BG11-Nc+NPs	9	5.26±1.26[m]	0.198±0.018	0.0559±0.0052	62.4	92
BG11+AH-Nc+NPs	10	5.21±1.43[n]	0.205±0.027	0.0544±0.0091	59.6	91
BG11+NH-Nc+NPs	11	5.66±1.44[f,i,o]	0.193±0.015	0.0564±0.0048	55.8	92
BG11+BH-Nc+NPs	12	5.47±1.59[j,p]	0.193±0.014	0.0488±0.0029	53.9	91
BG11+NPs-Nc	13	5.67±1.36[g,k,r]	0.202±0.028	0.0495±0.0104	61.8	97
C	14	4.34±1.00[a,c,e,l,m,n,o,p,r]	0.195±0.010	0.0436±0.0018	59.2	92

where:

- results with SD are presented as a mean; in other cases, the value in the table represents the median;
- a, b, c…statistically significant differences for $p<0.05$

The results obtained from tests on radish plants can be considered in several aspects:

1. Comparison of all experimental groups (1–13) to the control group (C) – it can be seen that both the BG11 medium alone (1) and the BG11 medium with the addition of hydrolysates from *C. glomerata* (AH – (3), NH – (5), BH – (7)), as well as the tested post-culture media after separation of *N. commune* (groups (2), (4), (6), (8), (9), (10), (11), (12), (13)) caused an increase in the above-ground length of plants, dry root mass. In the case of the dry weight of the above-ground part, lower values than in the control group were observed for BG11-Nc (2); BG11+NH (5); BG11+NH-Nc (8); BG11+BH (7); BG11+NH-Nc+NPs (11); BG11+BH-Nc+NPs (12), but these differences were small, in particular for BG11+NH (5); BG11+NH-Nc+NPs (11) and BG11+BH-Nc+NPs (12). In the case of chlorophyll, a lower value than in the control group was recorded for seedlings from the groups BG11-Nc (2); BG11+NH-Nc (8); BG11+NH-Nc+NPs (11) and BG11+BH-Nc+NPs (12). It can be noticed that both for the chlorophyll content in seedlings and for the dry weight of plants, the lowest values were obtained for practically the same groups. It can be assumed that the low chlorophyll content in seedlings (weaker photosynthesis) could be responsible for the lower plant weight;
2. Comparison of biometric parameters of plants grown in groups with BG11 medium after *N. commune* separation – BG11-Nc (2) and in groups with BG11 medium with the addition of algae hydrolysates after *N. commune* separation – BG11+AH-Nc (4), BG11+NH-Nc (6) and BG11+BH-Nc (8). Solutions based on BG11 medium with all hydrolysates (AH, NH, BH), had a positive effect on the increase in the dry weight of the above-ground part and root, chlorophyll (except group (6) BG11+NH-Nc) and plant length (except groups BG11+NH-Nc (6) and BG11+BH-Nc (8)). Post-culture medium with the addition of acid hydrolysate from *C. glomerata* had the greatest positive impact on the measured parameters among the tested hydrolysates;
3. Comparison of the influence of the culture medium containing algal hydrolysates (BG11+AH (3), BG11+NH (5), BG11+BH (7)) and the post-culture medium containing algae hydrolysates after collecting *N. commune* (BG11+AH-Nc (4), BG11+NH-Nc (6), BG11+BH-Nc (8) on the biometric parameters of radish. Post-culture medium, after removing *N. commune*, containing algal hydrolysate (BH) – (8) had a positive effect on all measured radish parameters compared to BG11 medium with BH (7), in which *N. commune* was not cultivated. In the case of groups BG11+AH-Nc (4) and BG11+NH-Nc (6), higher parameters than in the corresponding control groups (BG11+AH (3), BG11+NH (5)) were

obtained for the length of the above-ground and dry root mass. There was a decrease in the dry weight of the above-ground part and the chlorophyll content in seedlings in these experimental groups compared to the control group. But, of all the tested algal hydrolysates (AH, NH, BH), those produced in an acidic environment, added to the BG11 medium in which *N. commune* was grown and then separated, gave the highest measured parameters for radish, especially the above-ground part, dry plant weight and chlorophyll content in seedlings;

4. Comparison of the addition of ZnO nanoparticles to the post-culture medium after separation of *N. commune*, containing BG11 medium with the addition of appropriate hydrolysates – BG11+AH-Nc+NPs (10), BG11+NH-Nc+NPs (11), BG11+BH-Nc+NPs (12), versus the appropriate post-culture media, but without the addition of NPs – BG11+AH-Nc (4), BG11+NH-Nc (6), BG11+BH-Nc (8). ZnO nanoparticles were added to solutions applied to Petri dishes with radish seeds before germination tests began. The results obtained were ambiguous, but for all experimental groups using the medium with the addition of ZnO nanoparticles, a decrease in the chlorophyll content in radish seedlings was observed compared to the mentioned control groups. Among the groups with ZnO nanoparticles, the highest radish measured parameters compared to the control group were obtained for BG11+NH-Nc+NPs (11) vs. BG11+NH-Nc (6) – the plant length was higher by 11%, plant dry weight by 5% and root dry weight only by 0.5%. In general, the addition of ZnO nanoparticles to the tested media just before application to the seeds did not have a significantly positive effect on the value of the measured parameters;
5. However, the addition of ZnO NPs to the BG11 medium after cultivation and separation of *N. commune* (BG11-Nc+NPs (9)) had a positive effect on the weight of the whole plants (increase by 9.1%), root dry weight (increase by 15.7%), and chlorophyll content (increase by 7.2%) compared to the analogous group, but without ZnO NPs, added to the solution before application to radish seeds (BG11-Nc (2)).

The results of germination tests indicated that the various *N. commune* post-culture media used can be applied as preparations stimulating plant growth in the initial phase. In most cases, for the measured radish growth parameters, the values obtained were higher than in the control group. Among all experimental groups, the highest results for the measured radish parameters were obtained for the groups (4) BG11+AH-Nc and (13) BG11+NPs-Nc in comparison to the

control group (14). For example, the whole plant length in group (4) was 22.1% higher than in the control group (14), and root dry weight by 22.6%.

3.5. Analysis of the results from germination tests on radish by Principal Component Analysis

Principal Component Analysis (PCA) was conducted to investigate the relationship among variables contributing to the variance of the observed data. The first two principal components (PC1 and PC2) collectively explained 79.4% of the total variance and were represented in a two-dimensional space (Fig. 3). PC1, aligned along the horizontal axis, accounted for most of the variance (42.5%), while PC2, positioned on the vertical axis, contributed to 36.9% of the overall variance (Table 5).

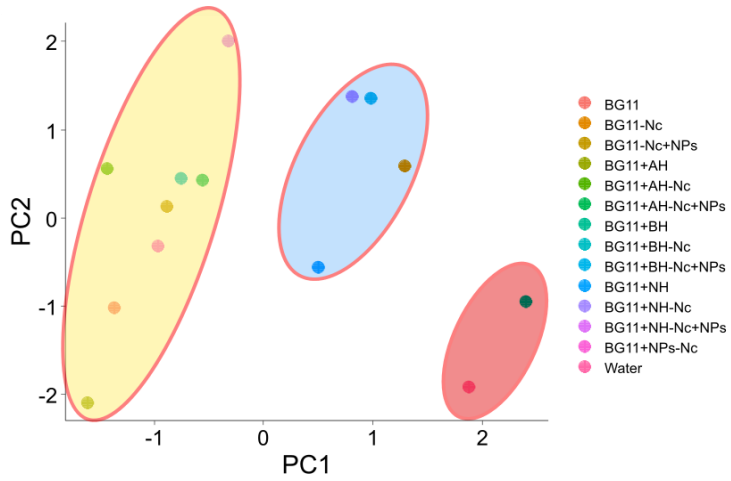

Fig. 3 Scatter plot of the studied variables on the first two principal components (PC1 and PC2) of different growth conditions to improve radish germination

Graphical visualization of the data through PCA plot provides an immediate overview of the structure and relationships within the data, facilitating the interpretation of analysis results. From this analysis, three main areas were identified where samples located closely in the principal component space may share similarities in their composition or response to measured variables. Specifically, the following conditions were identified for each area:

1. Yellow ellipse: BG11, BG11+AH, BG11+NPs-Nc, BG11-Nc+NPs, BG11+AH-Nc, BG11+AH-Nc+NPs, BG11+BH-Nc, and BG11+NH-Nc+NPs;

2. Blue ellipse: BG11+NH, BG11+Nc, BG11+BH+Nc+NPs, and BG11+Nc+NH;
3. Red elipse: H2O and BG11+BH.

The positive and negative values of PC1 and PC2 signify the relative orientation of samples concerning the principal variations observed in the data. Specifically, positive values on PC1 denote a stronger positive correlation with the sample characteristics associated with that component, while negative values indicate a negative correlation. Similarly, for PC2, positive values indicate a stronger positive correlation with the sample characteristics associated with this component, while negative values denote a negative correlation. These observations highlight the presence of correlations among the tested conditions, with groups of samples clustering based on similar characteristics within the principal component space. This analysis provides valuable insights into the structure and relationships within the data, underscoring the importance of PCA in exploring and understanding the complexity of multivariate data.

Table 5

Eigen analysis of the correlation matrix

Principal Component Analysis	**PC1**	**PC2**
Eigenvalues	1.69	1.47
Total variance (%)	42.5	36.9
Cumulative eigenvalues	1.69	3.17
Cumulative variance (%)	42.5	79.4
Matrix of transformed data		
Variable	PC1	PC2
BG11	-1.36	-1.02
BG11-Nc	1.29	0.59
BG11+AH	-1.61	-2.09
BG11+AH-Nc	-1.43	0.55
BG11+NH	0.50	-0.56
BG11+NH-Nc	0.81	1.37
BG11+BH	2.40	-0.94
BG11+BH-Nc	-0.75	0.44
BG11-Nc+NPs	-0.88	0.12
BG11+AH-Nc+NPs	-0.55	0.42

BG11+NH-Nc+NPs	-0.32	2.00
BG11+BH-Nc+NPs	0.98	1.35
BG11+NPs-Nc	-0.96	-0.32
Water	1.88	-1.91

3.5. Biosorption properties of solid residues after thermal hydrolysis of *Cladophora glomerata*

Biosorption properties of solid residues obtained after thermal hydrolysis of *C. glomerata* under acid, basic and neutral conditions were examined towards Cr(III) ions. In the conducted research, it was crucial to propose a method of valorization of each waste generated. Kinetics of biosorption of Cr(III) ions by *C. glomerata* residues is presented in Fig. 4, whereas the equilibrium of biosorption in Fig. 5. Studies on the kinetics of the sorption process showed that equilibrium was established after approximately 90 minutes.

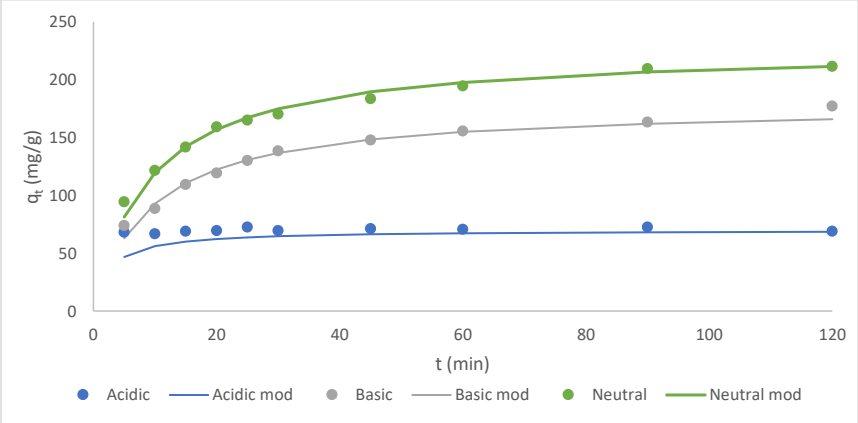

Fig. 4 Kinetics of biosorption of Cr(III) ions by *Cladophora glomerata* residues (C_0 300 mg/L, 25 °C, C_S 1.0 g/L, pH 5)

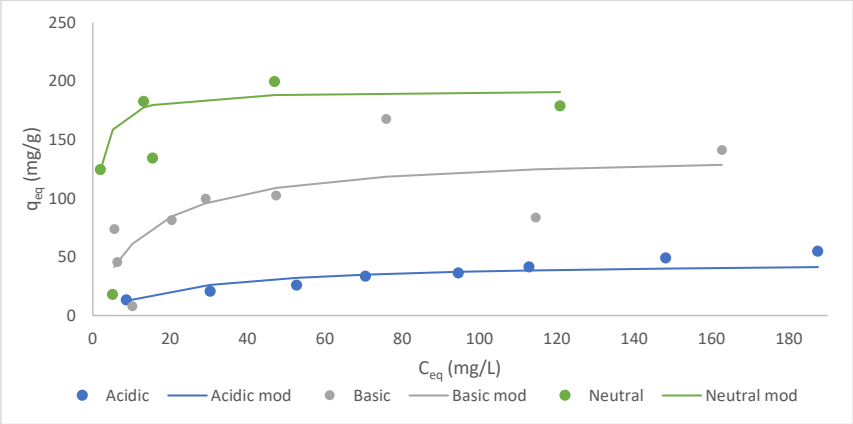

Fig. 5 Equilibrium of biosorption of Cr(III) ions by *Cladophora glomerata* residues (C_0 25 – 300 mg/L, 25 °C, Cs 1.0 g/L, pH 5)

The highest equilibrium biosorption capacity (calculated using the pseudo-second-order kinetic model) and maximum biosorption capacity (calculated using Langmuir equation [26]) were obtained for the algal solid residue prepared under neutral conditions (227 and 192 mg/g, respectively). The second highest values of equilibrium and maximum biosorption capacity were received for the residue obtained under basic conditions (179 and 139 mg/g, respectively), whereas the lowest for the residue obtained under acidic conditions (69.9 and 69.0 mg/g, respectively). The presented study results proved that all bioproducts, especially the ones received under neutral and basic conditions, can be used as sorbents of Cr(III) ions from aqueous solutions. Usually, biosorption capacity of different metal ions is lower for algal post-extraction residues (received during extraction of different compounds, e.g., polysaccharides, agar, alginate or oil) than for the algae itself. The equilibrium sorption values are usually under 100 mg/g [27]. The equilibrium biosorption values in the presented study were lower than the one calculated for *C. glomerata* algae in our previous study (345 mg/g). However, the results for maximum biosorption capacity (for the neutral residue) were higher than the one obtained for *C. glomerata* itself (169 mg/g) [3].

In this study, a higher maximum biosorption capacity of Cr(III) ions was determined for the *C. glomerata* residue from alkaline hydrolysis compared to the residue from acidic hydrolysis. This is consistent with the research results presented by (Bhatti et al., 2007), who investigated the removal of Zn(II) ions from aqueous solution using horseradish tree (*Moringa oleifera*) pretreated with sulfuric acid and sodium hydroxide. *Moringa oleifera* pre-treated with sodium

hydroxide showed a higher sorption capacity for Zn(II) ions than that treated with sulfuric acid [28].

4. Conclusions

This study proposes a comprehensive method for managing *Cladophora glomerata* biomass. Using the LHW technique, hydrolysates were obtained from macroalga under acidic, basic and neutral conditions. These hydrolysates were used as a source of sugars in the mixotrophic cultivation of the cyanobacterium *Nostoc commune*, with each hydrolyzate providing the same dose of reducing sugars to the BG11 medium. The highest concentration of reducing sugars (256 µg/mL) was contained in the hydrolysate obtained under alkaline conditions. The highest increase in *N. commune* cell mass was observed in the culture in which alkaline hydrolyzate from *C. glomerata* was used, which allows us to conclude that other active compounds/micro- and macroelements contained in this hydrolysate had a positive effect on their growth. In the conducted research, an attempt was also made to manage the post-culture medium after the separation of *N. commune*. These solutions can serve as potential biostimulants of plant growth (e.g., radish), increasing the length of plants, the mass of the above-ground part and root, and the chlorophyll content. Another waste for which a valorization method was proposed was the solid residue from the *C. glomerata* autohydrolysis. This biomass was used as a biosorbent, removing metal ions from wastewater. The residue after hydrolysis of *C. glomerata* in alkaline conditions had better sorption properties (139 mg of Cr(III) ions/g of the biosorbent) than the residue obtained in acidic conditions (69 mg/g). The research hypotheses formulated at the beginning of the study were therefore confirmed. This research confirms the possibility of using *C. glomerata* biomass to obtain products with a wide range of applications (e.g., in agriculture) and additionally fit into the assumptions of a circular economy.

CRediT authorship contribution statement

All authors contributed to the study conception and design; Data curation: IM, FS; Formal analysis; EL, KŁ, MD, NN, GO, FS, IM; Funding acquisition: IM, FS; Investigation: EL, KŁ, MD, NN, GO, FS, IM; Methodology: EL, KŁ, MD, NN, GO, FS, IM; Project administration: IM, FS; Resources: IM, FS; Supervision: IM, FS, GO; Visualization: EL, KŁ, FS, IM; Roles/Writing - original draft: EL, KŁ, MD, NN, GO, FS, IM; and Writing – review & editing: GO, FS, IM. All authors read and approved the submitted manuscript.

Declaration of Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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