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Obtaining phycobiliprotein-containing *Nostoc linckia* (Roth.) Born. et Flah biomass via bioconversation of waste water from reticulating aquaculture systems (RAS)

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Abstract

The paper studies the possibility of bioconversion of waste water from recirculating aquaculture systems (RAS). For this purpose, cyanobacteria *Nostoc linckia* (Roth.) Born. et Flah. was grown on waste water from RAS. In the process of cultivation, indicators of total mineralization, pH, and culture density were monitored. In the waste water, the content of various forms of nitrogen (NH_4^+, NO_3^-, NO_2^-), the content of P and S before and after the growth of cyanobacteria were determined It was noted that as a result of growing *N. linckia* in RAS waste water, a decrease in the content of various forms of nitrogen, sulfur and phosphorus is observed. Also, the content of phosphates decreased from 35×10^{-3} to 5×10^{-3} mg.L^{-1} and the content of sulfates from 95×10^{-3} to 25×10^{-3} mg.L^{-1}. Moreover, the resulting biomass of cyanobacteria contained 57 % of proteins, 20 % of lipids and 11% of carbohydrates. It was found that *N. linckia* biomass is a promising source of phycobiliprotein pigments: phycocyanin, allophycocyanin and phycoerythrin.

Introduction

In recent years, due to a significant increase in demand for aquatic food, land-based aquaculture is developing rapidly and intensively around the world. However, the continuous development of intensive aquaculture is tempered by certain problems, and the issue of wastewater disposal is especially acute (Mook *et al*. 2012; Chen *et al.* 2015). Wastewater discharged from fisheries is usually concentrated with nutrients (nitrogen, phosphorus) and mainly consists of fish waste products and feed residues.

Bioconversion makes it possible to dispose of various wastes in wastewater. This is the conversion of substances and energy under the influence of living organisms or in their cells. This process is inherent in any biological system, since the conversion of substances and energy occurs in metabolic reactions and in the processes of energy and substance exchange between living organisms and the environment. Under the influence of cells, metabolites turn into structurally related compounds. Various microorganisms were used to disinfect the contaminated environment, including bacteria, fungi, algae and plants (Vidali 2001; Leung 2004). In controlled conditions, organic waste degrades to harmless levels under the influence of microorganisms (Nayyef and Amal 2012; Lu *et al.* 2019). Growing cyanobacteria in the aquaculture wastewater ensures the removal of fish waste products and simultaneously, production of biomass that can be used to produce valuable

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products such as aquaculture feed, medicines, natural dyes, and biofuels (Michels *et al.* 2014).

The advantages of cultivating members of *Cyanobacteria* genus are higher yields, use of non-agricultural land, nutrients restoration from wastewater, and the efficient capture of carbon. Moreover, adaptation to a wide range of environmental conditions makes them a rich source of important and useful metabolites. These organisms have the potential to meet a wide range of global needs, but realization of this potential calls for lower production costs.

Nostoc linckia Roth. Born. et Flah is a thallogen heterocyst cyanobacterium capable of nitrogen fixation (Fokina *et al.* 2011). Due to the sufficiently large size of thallome and a significant amount of mucus, it does not require complex approaches in the post-cultivation separation of biomass from the nutrient medium. Like most tallogens, *N. linckia* is responsive to changes in the nutrient medium and builds up biomass rapidly. These cyanobacteria are unpretentious to the composition of nutrient media (Gupta and Rastogi 2008) and are therefore considered an excellent choice for use in various biotechnological processes. Biomass of cyano-bacteria is characterized by a high content of amino acids and proteins (Rosales-Loaiza *et al.* 2017). The high content of basic nutrients allows using *N. linckia* as animal feed, specifically, in aquaculture. A variety of photo-synthetic pigments, which are used in the food, cosmetic and pharmaceutical industries, permits the use of this species as their producer. Recently, photosynthetic pigments such as phyco-biliproteins, which are usually present in cyano-bacteria, have attracted attention. C-phycocyanin is an active antioxidant able to suppress the proliferation of tumor cells, reduce the concentration of tumor necrosis factor in tissues, inhibit oxidative stress, prevent lipid peroxidation, DNA damage, destruction of cell membranes and cell death (Soundarapandian and Muruganandham 2008; Sinha *et al.* 2011). Phycocyanin allows to get the so-called anatomical portrait of a person (it selectively accumulates in atherosclerotic plaques and neoplasms, the localization of which is determined by ultrasound scanning) (Kardash *et al.* 2012). Most synthetic dyes are toxic, so interest in unconventional sources of raw materials for natural dyes has increased significantly. One of such promising food colors is protein phycoerythrin, an absolutely non-toxic red pigment with pronounced orange fluorescence, which is now widely used in the food, cosmetic and medical industries. In the United States, coloring materials based on this pigment are patented as fluorescent markers for immunodiagnostics. There is also evidence of the use of phycoerythrin and phycocyanin in oncology as radioprotectors. As a nutritional supplement, these pigments can improve the color of salmon flesh and also positively influence health and fertility in cattle (Valuta *et al.* 2015).

The aim of our work was to use recirculating aquaculture systems (RAS) waste water for growing cyanobacteria and obtain phycobiliprotein-containing biomass of *Nostoc linckia* (Roth.) Born. et Flah.

Experimental

Biological material and cultivation conditions

Studies were conducted using the monoculture of *N. linckia* (Roth.) Born et Flah, which is maintained in the collection of the Department of Biochemistry and Biotechnology, Yurii Fedkovych Chernivtsi National University. Museum *N. linckia* (Roth.) Born. et Flah. (HPDP-453) culture was obtained from the collection of the Institute of Hydrobiology of the NAS of Ukraine. *N. linckia* (Roth.) is a thallogen heterocyst cyanobacterium (Guiry and Guiry 2020), characterized by the ability to proliferate rapidly. This, as well as the ease of separation of biomass from the culture fluid makes this species a convenient object for biotechnological research.

Cyanobacteria were cultivated in RAS waste water and on the standard Fitzgerald's No. 11 medium in the modification of Zehnder and Gorhem as a comparison medium (Zolotareva *et al.* 2008). Water was taken from the mechanical filter of the RAS, aliquoted, sterilized in an autoclave at a temperature of 121 °C for 30 min and standardized at pH (7.5 – 8.0) and total salinity (495±5 ppm) (Khydyi *et al.* 2016).

Cultivation was carried out in 500-mL size Erlenmeyer flask under climate room conditions:

21±2 °C, illumination with fluorescent lamps of about 2,500 lux and a photoperiod light/darkness 16 : 8 h. The inoculation was carried out in the ratio of inoculum : nutrient medium – 1 : 10 in the sterile laminar box (Cheban *et al.* 2015). Cyanobacteria under these conditions were cultured for 50 days. Under these conditions, cyanobacteria were grown for 50 days.

In the process of cultivation, indicators of total mineralization, pH, and culture density were monitored. The content of various forms of nitrogen (NH_4^+, NO_3^-, NO_2^-), the content of P and S before and after the growth of cyanobacteria were determined in the waste water. The quantitative content of mineral elements in RAS waste water was determined according to generally accepted photocolorimetrically methods; nitrates were determined with phenol disulfonic acid, nitrites using Griss reagent, ammonia with Nessler's reagent, sulfates by the method of Ozerov and phosphates using a molybdenum mixture (Arsan *et al.* 2006).

The density of the biomass was determined from the culture density indicators using an optical indicator at 750 nm with CaryWin UV 60 software (Agilent, USA). The transition from optical density units (D750) to the absolute dry biomass (ASB) value was carried out through the empirical coefficient *k*: ASB = *k* × D750 (Hevorhyz *et al.* 2008). The coefficient *k* (k = g.unit opt. density.L^{-1}) for the culture was determined experimentally in three independent reruns. Using the obtained values, a growth curve was drawn for the culture of *N. linckia.*

After the cultivation, the isolation of algae cells from the culture medium was carried out by centrifugation at 6,000 rpm within 15 min on "Herauses" Biofuga stratos. In the irrigated biomass, the content of proteins, lipids and carbohydrates, chlorophyll *a*, carotenoids and phycobiliproteins was determined.

Hydrophilic components of the biomass were extracted with 0.1 M phosphate buffer solution with a pH of 7.4 (Musienko *et al.* 2001). Extraction of hydrophobic pigments was performed with 100 % acetone. Optical density of the pigments was measured spectrophotometrically in the wavelength range of 400 – 800 nm with on a CaryWin UV 60 (Agilent, USA), and to obtain their concentration, a formulaic calculation has been put forward (Geffrey and Humphrey 1975). Determination of total protein content was carried out according to the method of Lowry (Lowry *et al.* 1951). Lipids were extracted using the Folch method and determined by the presence of sulfuric acid and phosphorus-vanillin reagent (Knight *et al.* 1972). The amount of total carbons was determined by the color reaction with the anthrone reagent (Olennikov and Tanhaeva 2006). The obtained indicators were calculated as a mass fraction (%) based on dry biomass.

Analyses of phycobiliprotein content

Phycobiliprotein complex was obtained with 0.2 M phosphate buffer, pH 7.0, at a biomass : extractant ration of 1 : 5. The concentration of phycobilin pigments was determined spectrophotometrically at analytical wavelengths of 450 – 700 nm using CaryWin UV 60 (Agilent, USA). The purity of the obtained fractions was calculated using standard formulas as the optical density ratio at D620 / D280 (Patil *et al.* 2006).

Data analysis

All data are presented as Mean ± SE. The significance of differences in the results was evaluated with one-way ANOVA. Statistical analysis was computed using MS Excel software and STATISTICA 6.0. Mean values were considered significantly different at $P \leq 0.05$ according to Student's criterion.

Results and Discussion

To ensure normal functioning of the cell culture of cyanobacteria, a sufficient content of essential nutrients in the culture medium is necessary. This has significant implications on the growth of microalgae and synthetic processes in them. Enriched with salts of nitrogen, phosphorus, and sulfur, RAS waste water has a qualitative composition of the main mineral elements similar to that of the Fitzgerald's medium (Cheban *et al.* 2015; Khydyi *et al.* 2016). Microalgae best absorb nitrogen in nitrate or ammonium form, however, when using the nitrate form of nitrogen, an intensive increase in biomass is observed, while

Table 1. Physical and chemical parameters of waste water from RAS (Mean ± SE, n = 4).

Characteristics of culture media	Waste water from RAS	Fitzgerald's medium
NO_3^- [mg.L^{-1}]	43.2±0.7	83.2±2.1
NO_2^- [mg.L^{-1}]	0.64±0.04	–
NH_4^+ [mg.L^{-1}]	0.51±0.02	–
PO_4^{3-} [mg.L^{-1}]	0.031±0.030	0.040±0.030
SO_4^{2-} [mg.L^{-1}]	0.094±0.020	0.031±0.010
pH	7.0 – 8.0	7.0 – 8.0
Total mineralization [ppm]	371.0 – 477.0	332.0 – 547.0
Conductivity [µS.mL^{-1}]	555.0 – 693.0	452.0 – 690.0
Redox potential [mV]	211.4 – 213.9	176.9 – 245.0

ammonium ions are able to suppress the development of the culture (Parshikova *et al.* 2010; Tejido-Nunez *et al.* 2019). With nitrogen deficiency, inhibition of algoculture growth is observed, which affects the content of basic nutrients in biomass.

RAS waste water is a suitable medium for cyanobacteria cultivation since it has a high content of nitrogen compounds (ammonium, nitrite and nitrate), phosphates and dissolved organic carbon (Lananan *et al.* 2014) coming from fish waste products and food residues (Crab *et al.* 2007). This composition of discharged water promotes the growth of various microorganisms, including microalgae and cyanobacteria. The quality composition of RAS waste water and the Fitzgerald's medium was found to be similar in terms of basic mineral elements. In both cases, there is a sufficient amount of nitrogen, phosphorus, carbon.

Studies shown that the physical and chemical parameters of RAS waste water are fully comparable with Fitzgerald's medium (Table 1). Therefore, for our research, RAS waste water was used as a nutrient medium. It was noted that *N. linckia* grows exponentially during the first 30 days with subsequent stabilization (Fig. 1).

From the day 40 of cultivation, there was a decrease in the amount of the biomass was observed, which is explained by a decrease in the available nutrients in the medium. This is confirmed by the decrease in the total mineralization index. The growth pattern of the culture was the same both in waste water and in the control medium. The amount of biomass obtained, taking into account the simultaneous use of media, did not significantly differ.

The main advantage of using cyanobacteria for waste water treatment is their availability, autotrophy, high reproduction rate and high ratio of body surface area to cell number. These bacteria are photosynthetic organisms that assimilate N and P during growth. Microalgae-based technologies are a promising solution for aquaculture waste water treatment (Gao *et al.* 2016; Guldhe *et al.* 2017; Egloff *et al.* 2018).

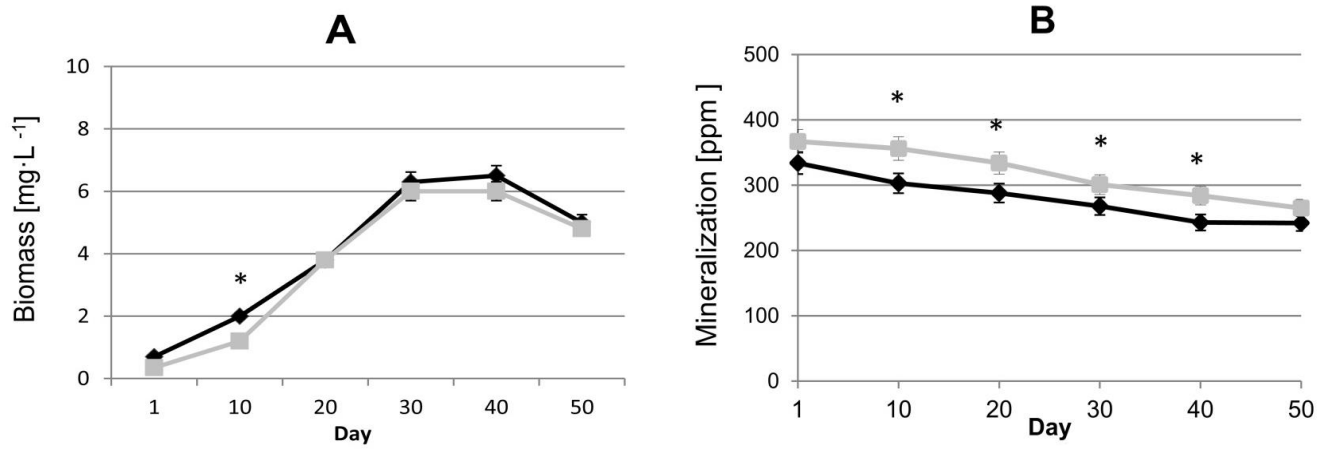

Fig. 1. *N. linckia* biomass (**A**) and total mineralization (**B**) cultured at waste water from RAS: Fitzgerald medium (diamonds), waste water from RAS (squares). Note: * – significant difference relative to control values ($P \leq 0.05$).

Table 2. Biochemical composition of *N. linckia* biomass under various cultivation conditions (Mean ± SE, $n = 4$).

Investigated indicators	Fitzgerald's medium	Waste water from RAS
Total proteins [%]	56.6±2.6	55.1±2.3
Total lipids [%]	19.8±0.8	20.1±0.8
Carbohydrates [%]	10.7±0.4	11.3±0.5
Chlorophyll *a* [mg.g^{-1}]	17.4±0.8	16.2±0.8
Carotenoids [mg.g^{-1}]	11.3±0.5	9.5±0.4

Note: * – significant difference relative to control values ($P \leq 0.05$).

One of the most important properties of the species *N. linckia* is its high protein content, which makes this genus a candidate for the continuous production of fertilizers and protein supplements in animal feed (Habib *et al.* 2008). During the cultivation of the cyanobacteria *N. linckia*, a biomass was obtained with the following content of basic nutrients and pigments (Table 2).

When cultivating a culture of *N. linckia* on RAS waste water, a significant decrease in the content of various forms of nitrogen, sulfur, and phosphorus is observed (Fig. 2). As it turns out, in the process of growing biomass there is a complete absorption of the ammonium form, and the amount of nitrate form decreases by 8-times. Also, as a result of nitrification, ammonium could be classified as nitrite. It should be noted that ammonium and nitrate nitrogen under certain conditions are equivalent sources of nitrogen for cyanobacteria. The predominant absorption of ammonium nitrogen occurs when NH_4^+ is the only source of nitrogen (Shilova *et al.* 2020). In cyanobacteria, ammonium, either extracted from the external environment or formed intracellularly, is incorporated into carbon skeletons mainly through the glutamine synthetase/glutamate synthase cycle (Herrero *et al.* 2001).

The content of phosphates and sulfates decrease by 7- and 4-times, respectively. Therefore, growing cyanobacteria on RAS waste water not only allows obtaining protein-rich biomass, but also significantly purifies waste water during bioconversion.

Cyanobacteria, unlike higher plants and bacteria, contain, in addition to chlorophyll and carotenoids, water-soluble pigments – phycobiliproteins – chromoproteins, the non-protein part of which includes phycobilin chromophores. They are the main light-absorbing pigments in cyanobacteria (Valuta *et al.* 2015). Since *N. linckia* is a promising source of phycobilins, we developed a scheme for the preparation of phycobilin protein complex and ways to improve it. This scheme includes stages of the steps of isolating a pigment preparation, spectroscopy and purity testing, and determining the amount of pigment. It is known that the extraction efficiency of any cell components depends on the extractant used.

The phycobiliprotein complex of cyanobacteria dissolves well in phosphate buffer or alcohol solutions is well soluble in phosphate buffer or in alcohol solution, the latter also allow increasing the shelf life of the obtained preparation. Typically, these compounds are extracted at room

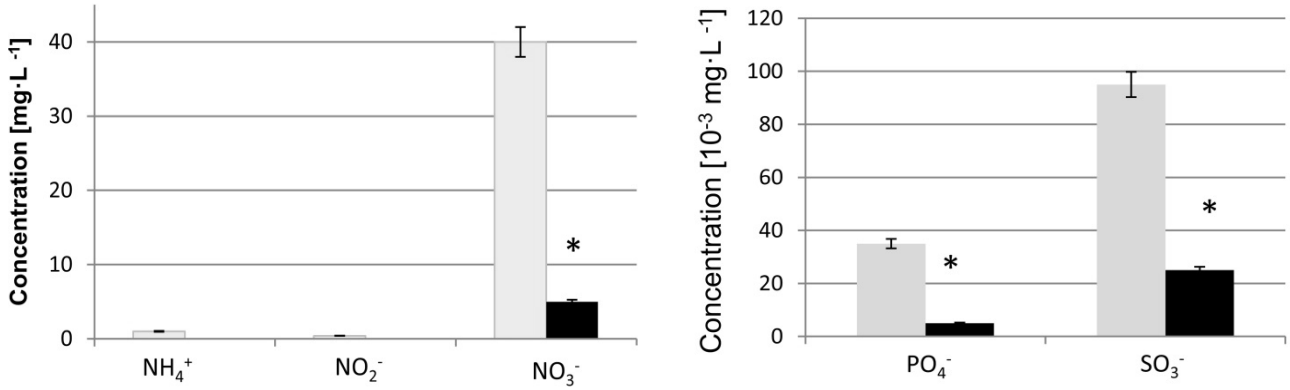

Fig. 2. The content of various forms of nitrogen, phosphorus and sulfur in waste water from RAS before (gray columns) and after cultivation of *N. linckia* (black columns). Note: * – significant difference relative to control values in waste water from RAS before cultivation ($P \leq 0.05$).

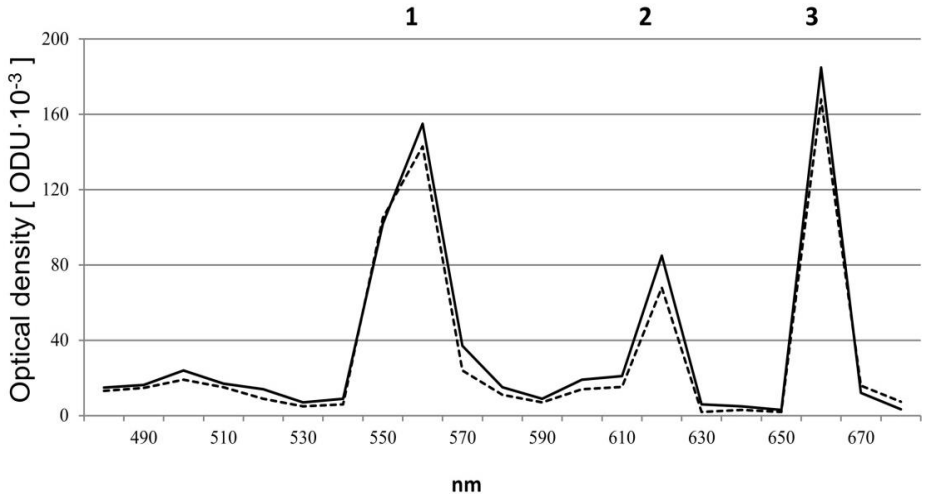

Fig. 3. Absorption spectra of phycobiliproteins from the biomass of *N. linckia*, where: Fitzgerald's medium (filled line), waste water from RAS (dashed line); 1 – phycoerythrin, 2 – C-phyco-cyanin, 3 – alophykocyanin.

temperature, but with chilled extractants. So as an extractant we used 0.2 M phosphate buffer with a pH of 7.0. To ensure complete extraction of phycobiliproteins, this operation was performed three times. Phycobiliproteins are the main components of cyanobacteria cells: it is known that in some species they can make up to 40 % of the cellular protein.

Three compounds were found in our phycobiliprotein preparation, the absorption maxima of which correspond to phycoerythrin (560 nm), (620 nm) and alophycocyanin (650 nm) (Fig. 3). It should be noted that phycoerythrin is not always present in the cells of cyanobacteria, it is characteristic mainly for filamentous forms, *N. linckia*, in particular.

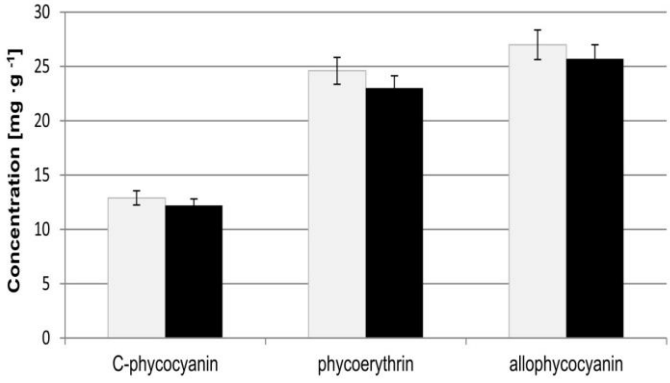

Fig. 4. The content of phycobiliproteins in *N. linckia* cells when cultured on waste water from RAS: Fitzgerald's medium (gray columns), waste water from RAS (black columns). Note: * – significant difference relative to control Fizgerald medium values ($P \leq 0.05$).

There is a link between the amount of pigments and the conditions of nitrogen supply, which is possibly due to the chemical structure and functions of the main components of the photosynthetic system.

So, when cultivating *N. linckia* in RAS waste water, its biomass revealed C-phycocyanin – 12.2 $mg.g^{-1}$ (1.2 %), phycoerythrin – 23 $mg.g^{-1}$ (2.3 %), alophycocyanin – 25.7 $mg.g^{-1}$ (≈ 2.6 %) (Fig. 4).

The amount of analyzed compounds in *N. linckia* biomass did not differ significantly with the nutrient medium. It is obvious that the same growing conditions, pH of the medium and the amount of basic nutrients in both applied nutrient media make it possible to obtain the amount of phycobiliproteins in the biomass, almost identical in both cases.

Table 3. Purity index by optical density of the phycobylprotein preparation derived from biomass *N. linckia* (Mean ± SE, $n = 4$).

Investigated indicators	Fitzgerald's medium	Waste water from RAS
Phycoerytryn [ODU]	1.42	1.35
C-Phycocianin [ODU]	0.78	0.64
Alophycocianin [ODU]	1.62	1.54

Note: * – significant difference relative to control values ($P \leq 0.05$).

We also calculated the purity of individual fractions of phycobiliprotein pigments according to standard formulas (Table 3). Degree of purification is determined by an indicator measured by the ratio of optical densities at two wavelengths: D_{620}/D_{280}. In the aqueous extract, this value should be at least 0.3 – 0.4 (Kardash *et al.* 2012).

Thus, the use of RAS waste water as a nutrient medium can reduce the costs of biomass cyanobacteria production, as well as cleanse RAS waste water from excess nutrients and use it as a reverse.The use of the obtained biomass as a source of the phycobiliprotein complex allows receiving a cheaper product with the components of high purity. So, the waste water from RAS can be used as an alternative medium for the cultivation of *N. linckia*. The resulting biomass in terms of total protein, lipids, carbohydrates, and basic photosynthetic pigments does not significantly differ from that grown on Fitzgerald's control medium.

The performed operations have shown that cleaning RAS waste water by growing cyanobacteria *N. linckia* on it is possible. Such bioremediation measures allow pursuing several goals simultaneously: to purify water from chemical agents and obtain valuable cyanobacterial biomass. These approaches are now widely used both for microalgae and cyanobacteria (Ansari *et al.* 2017; Tejido-Nunez *et al.* 2019). Understandably, to complete the task, additional examinations for the presence of bacterial contamination and the safety of the obtained biomass are required (Halfhide *et al.* 2014; Fulbright *et al.* 2018). However, even at this stage, it can be concluded that the cultivation of cyanobacteria in RAS waste water is a promising trend in the field of aquaculture.

Conclusion

The chemical composition of RAS waste water is comparable to the Fitzgerald's medium in terms of the amount of various forms of nitrogen, phosphorus and sulfur. This makes it possible to use RAS waste water as an alternative medium for growing the cyanobacteria *N. linckia*. This technology eliminates chemical pollution of water, significantly reduces the content of nitrites and nitrates as well as the level of sulfur and phosphorus in water.

The biomass grown in waste water has a similar content of proteins, lipids and carbohydrates as in the control medium. From this biomass, a highly purified preparation of phycobilin protein pigments can be obtained, containing C-phycocyanin (12.2 $mg.g^{-1}$), phycoerythrin (23 $mg.g^{-1}$) and allophycocyanin (25.7 $mg.g^{-1}$).

Conflict of Interest

The authors declare that they have no conflict of interest.

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