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Seasonal differences in the content of phenols and pigments in thalli of freshwater *Cladophora glomerata* and its habitat

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ABSTRACT

Polyphenols are chemicals that primarily inhibit the growth of various autotrophic organisms. The presence of these metabolites greatly boosts the ecological dominance of eg. Cladophora, which creates large surface mats. The main goal of our work was to quantify the phenol and polyphenols (allelopathic substances) secreted by the macroalgae as a result of exposure to biotic stress caused by competition. The research was carried out on the *Cladophora glomerata* biomass collected from two freshwater ecosystems located in Wielkopolska Region (Poland, Fig. 1): Oporzynskie Lake (N52° 55′, E 17° 9′) and Nielba River (N5248′, E 17° 12′). Seasonal variability (May-October 2015) in the properties of *C. glomerata* mats in the river and lake ecosystem was also analyzed in relation to the physicochemical parameters of water. In addition, the content of pigments in the analyzed biomass was determined during the appearance of algae mats in water reservoirs. Biomass extraction was performed to determine the phenolic and carotenoid contents (chlorophyll and carotenoids) by using two extraction methods: microwave-assisted extraction (MAE) and supercritical fluid extraction (SFE). After isolation of the phenols from the thalli (mats) and the habitat, they were analyzed using the Folin-Ciocalteu method with some modifications, while the pigment content (chlorophyll and carotenoids) was evaluated by the spectrophotometric method Liechtenthaler (1987) with some variations. Analysis of the content of these components in algae extracts indicates that the tendency of changes in their contents was similar or the same. Growth and decrease of phenolic content (Cladophora T MAE and Cladophora T SFE) and pigments (MAE chlorophyll, SFE and MAE carotenoids) at the same time were independent of the insulation method used. The mats formed by *C. glomerata* on the surface of Lake Oporzyńskie were more stable and larger surface area than those on the Nielba River, which could explain differences in polyphenol concentrations in these two aquatic ecosystems. The results suggest a reduction in the secretion of phenolic compounds with an aging population of algae.

1. Introduction

Limited information about functioning astatic biocenoses forming in the littoral zone by filamentous green algae have prompted the authors to undertake detailed analysis of seasonal differences in the content of phenols and pigments in the thalli of freshwater *Cladophora glomerata* and also in its habitat. The available floristic data confirmed the presence of patches (mats) of filamentous green algae in the surface layer (free-floating thalli) and in the benthic zone (thalli attached to the bottom) in lakes and lowland rivers, which play an important role in aquatic ecosystems. However, competition between the mat-forming species may depend on (i) mutual replacement, (ii) exclusion of species or (iii) release of chemicals. Macroalgae from the group of chlorophytes that anchor to submerged vegetation can compete in the process of colonization of the water surface. The filamentous green algae, that tolerate a wide range of changes in habitat conditions (Pikosz et al., 2017), show the ability to produce an enhanced amount of polyphenols in response to biotic stress. It is supposed that secretion of polyphenols is a response to the competition from the Cladophora genus algae.

So far, only a few studies have been conducted regarding the

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contents of phenolic compounds and pigments composition in freshwater macroalgae. Such phenols as coumaric acid and galic acid (Kartal et al., 2009) have been found in freshwater *C. glomerata*. A high allelopathic potential of algae and water plants can be a result of high tissue concentrations of total phenolic compounds (6%–12% of dry matter), which are released into the surrounding medium (Hilt nee Körner et al., 2006). Some phenolic allelochemicals, such as tannic acid, exuded from submerged macrophytes (*Myriophyllum spicatum*) may inhibit development of other aquatic organisms, e.g.: phytoplankton or epiphyton (Gross et al., 1996). It has been proved that tannic acid might exhibit allelopathic effect on the green algae *Desmodesmus armatus* (Chodat) Hegewald and *Scenedesmus vacuolatus* Shihira and Krauss, as well as the diatom *Stephanodiscus minutulus* (Kützing) Cleve and Möller (Eigemann et al., 2013). The same polyphenol is able to inhibit the growth rates of green alga *Desmodesmus armatus* and at the same time may increase the growth rates of cyanobacteria *Microcystis aeruginosa* (Kützing) Kützing (Chang et al., 2012). Others compounds, such as: pyrogallic acid, gallic acid, ellagic acid and (+)-catechin have been also found as allelopathic polyphenols in *Myriophyllum spicatum* L. influencing the growth and photosynthetic activities of cyanobacteria *Microcystis aeruginosa* (Zhu et al., 2010). Polyphenols are able to penetrate cell membranes due to their amphiphilic or lipophilic structure (Leu et al., 2002).

In our previous paper we compared the efficiency of different extraction methods, and then chose the most effective technique to isolate pigments as bioactive substances from freshwater green algae, such as: *Cladophora glomerata* (L.) Kütz. *Cladophora rivularis* (L.) Hoek and *Ulva flexuosa* Wulfen, in the same conditions (temperature, time) (Fabrowska et al., 2017). However, in fact, little is known about the interactions between the mat-forming species and the response of organisms to biotic stress caused by competition. Moreover, a quantitative determination of phenols and polyphenols (allelopathic substances) secreted by macroalgae to the habitat as a result of their exposure to biotic stress caused by competition is also an objective of our study. According to literature data, polyphenols are the main chemicals inhibiting the growth of different autotrophic organisms (Macias et al., 2008; Nakai et al., 2000, 2001, D'Ambrosca et al., 2006). The role of phenols and polyphenols as allelopathic substances has been evaluated. The presence of phenols and polyphenols can significantly stimulate the ecological dominance of e.g. Cladophora, that forms large surface mats. Our another objective was to determine the total amount of polyphenols synthesized in response to the effects of stress factors and the physiological conditions in the resulting mat by examining the composition and concentration of photosynthetically active pigments (chlorophylls and carotenoids). Seasonal variability in the properties of *C. glomerata* mats in the river and lake ecosystem was also analyzed in relation to the physicochemical parameters of water.

2. Materials and methods

2.1. Algae material and water samples

Samples of *C. glomerata* were collected manually from two freshwater ecosystems located in Greater Poland region (Poland, Fig. 1): Lake Oporzynskie (N52°55′; E17°9′), and the Nielba River (N52°48′; E17°12′). The biomass of algae was collected week by week, starting from the first week of May 2015 till the end of October 2015. All samples were randomly selected from the mat at about 10 cm from the water surface. The filamentous green algae samples were collected into plastic containers and transported in a refrigerated container (at 4 °C) to a laboratory. After harvesting, the filaments were rinsed repeatedly with distilled water. Next, fresh algal biomass was weighed and dried in a drying chamber FD with forced air (Binder) under the temperature of 40 °C until a dry matter of the water content < 15% was obtained. Afterwards, dry algae were milled using a laboratory mill with a grinding tank. Dried algal biomass was stored in plastic containers protected from light at room temperature. Each time to check the taxonomic purity of the material, morphometric measurements of the length and width of cells, pyrenoids (stained with Lugol's iodine) and nuclei (stained with 1% acetocarmine) were observed. To assess the physicochemical preferences of macroalga in mats we collected water samples from 0.1 m, using a customized sampler. Samples for analyses of chlorophyll a and other chemical analyses were collected using a sucking pump equipped with hoses (ø 10 mm) adapted to bring up water from a particular sampling depth into a calibrated vessel.

During the weekly sampling some physicochemical parameters of water were measured, such as: temperature (°C), pH, oxygen saturation (OS %), dissolved oxygen (DO mg O_2 L^{-1}), electrolytic conductivity (EC μScm^{-1}) and total dissolved substance (TDS mg L^{-1}). All these variables were measured in the field using a YSI multi-parameter probe. For a detailed analysis of the chemical parameters, water samples (500 mL) were taken, conserved with chloroform ($CHCl_3$) and stored at -10 °C for further analyses. Water samples (0.5 L) for pigment analysis were filtered through Whatman GF/F filters according to the ISO 10260 standard method. The concentrations of orthophosphates, nitrates, ammonium nitrogen and sulfates were determined using a spectrophotometer HACH DR 2800. The phytoplankton chlorophyll *a* (chl *a* $\mu g\ g^{-1}$) concentration was determined in laboratory on a spectrophotometer using ethanol as extraction solvent. At the same time water samples for analysis of polyphenols were collected from both sampling sites. Water was sampled both from the middle of algal mats and from the area outside the mats. About 0.5 L of water was collected from the lake and the river week by week into plastic containers. Water samples were ultra-filtered to remove any microorganism, stored in a laboratory freezer at −20 °C and protected from light until analysis.

2.2. Chemicals and reagents

The chemicals used in this study included: ethanol (specially pure, 99,8%, POCH, Gliwice, Poland), carbon dioxide (CO_2) for SFE with a purity 4.5 (99.995%) was purchased from Messer (Chorzów, Poland), Lugol's solution (I_2/KI) was obtained from Sigma-Aldrich, Folin-Ciocalteu reagent (Merc), sodium carbonate anhydrous (pure, POCH. Gliwice, Poland), fucoxanthin (Sigma-Aldrich). Deionized water was filtered through qualitative filter paper (413, size: 42.5 mm, VWR) and then used to prepare solutions for experiments.

2.3. Extraction

Algae were subjected to two types of extraction: MAE (*microwave assisted extraction)* and SFE (*supercritical fluid extraction)*. MAE process was performed using the microwave oven (Mars Xpress) and SFE was carried out in the Extractor MV-10 (Waters MV-10 ASFE System) with a fraction collector. Every time 2 g of dry weight of algae were used for the extraction. For both processes 40 °C was the extraction temperature and 2 h was the extraction time. For MAE, 50 mL of ethanol was used as a solvent, whereas for SFE, CO_2 was applied as the main solvent and ethanol as a co-solvent. The other parameters specified for MAE were: power - 800 W and yield - 100%. For SFE the following parameters were set: CO_2 flow – 10 mL min^{-1} (58 min – dynamic mode, 4 min – static mode, 58 min – dynamic mode), EtOH flow – 1.14 mL min^{-1} and