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Bioaccumulation of Microelements in Seaweeds by Scanning Electron Microscopy with an Energy Dispersive X-Ray Analytical System and Inductively Coupled Plasma-Optical Emission Spectrometer

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ABSTRACT

In the present paper, bioaccumulation characteristics of *Vaucheria sessilis* were presented. The ability of macroalga to bioaccumulate Co(II) and Mn(II) ions was investigated using scanning electron microscopy with an energy dispersive X-ray analytical system (SEM-EDX) and inductively coupled plasma optical emission spectroscopy (ICP-OES). After two weeks of the experiment, macroalga accumulated about: 71.3 mg/g of Mn(II) and 41.9 mg/g of Co(II) ions. Micrographs performed by SEM proved, that bioaccumulation occurred. Metal ions were bound on the surface and in the interior of biomass. Mappings of cations showed, that in the case of the surface of biomass (biosorption), the elements constituted aggregations. In turn, in the case of the cross section (bioaccumulation) they were evenly distributed. The ability to bioaccumulate microelement ions by *Vaucheria sessilis* creates opportunities for its wide application. This macroalga constitute a perspective raw material for agricultural applications, not only due to pronounced microelement accumulation capabilities but also due to the content of bioactive compounds.

INTRODUCTION

In the recent years, the interest in seaweeds in Poland has increased. More and more research is conducted in order to apply seaweeds (macroalgae) and their products in various areas, for example as functional food for human, animal feed or in agriculture (fertilizers, biostimulants) [1].

The advantage of macroalgae is the ability to accumulate metal ions from aqueous solutions. This property allows the multiple applications of algae. The most common is the use of algae as bioindicators, since they provide a time-integrated picture of the bioavailable pollutants [2]. The content of toxic metals in macroalgae is used to monitor the level of bioavailable metals in estuarine and sea shore areas. Also, the bioaccumulation of metal ions by aquatic organisms can be used in the metal removal from effluents in wastewater treatment. In this process, also nutrients can be removed from treated effluents (nitrates, phosphates, sulfates, organic and inorganic carbon compounds) [3]. This property gives the ability to the use the enriched with micro– and macroelements via bioaccumulation algae for agricultural purposes. However, bioaccumulating organisms should be selected among species which are resistant to high loads of pollutants and do not have mechanisms which protect from excessive accumulation inside the cell [4]. In the case of bioaccumulation, it is important to take into account the fact that excess concentrations of Zn, Ni, Cu, Fe, and Co can trigger an inherent defense mechanism that plants have developed that involves production of phytochelatins – polypeptides that bind metals. Phytochelatin production in response to high metal levels has been identified in land plants, vascular aquatic plants, fungi and marine and freshwater algae. This mechanism results in an accumulation of the excess metals within the plants with the final metal concentration often being significantly higher than found in water supplied to the plants [5].

In the literature it is reported, that algae accumulate metals by means of a two-stage process, consisting first of a rapid, reversible physico-chemical process of adsorption on the exterior surface (biosorption) and then of a slower metabolically regulated intracellular uptake. A crucial role play functional groups, which are present on the surface of the biomass (composition of the cell wall) [6,7].

In this paper, metal binding aspects of macralga were not examined for perspective utilization in environmental protection, but for the search of new applications for agricultural purposes. In the present study, alga *Vaucheria sessilis* was proposed because of two reasons: at first, it occurs commonly in Polish freshwaters *(V. sessilis* (Voucher) De Candolle, *V. geminata* (Voucher) De Candolle and *V. hamata* Walz.) and in the Baltic Sea (*V. thuretii* Woronin, *V. meduza* Christensen, *V. synandra* Woronin and secondly, it is easily cultivated under laboratory conditions [8,9]. Additionally, the cell wall of *Vaucheria* sp. is composed mainly of macromolecules, such as: monosaccharides (glucose, xylose, arabinose, ribose, N-acetylglucosamine, uronic acid), polysaccharides (cellulose, glucan, pectin, alginic acid) and proteins, which offer a host of functional groups that participate in bioaccumulation process [10-12].

In the available literature, bioaccumulation properties of *Vaucheria sessilis* are poorly examined. Marble et al. focused on bioaccumulation of metals (Mn, Fe, Ni, Cu, Co, Zn) by aquatic plants, algae (*Vaucheria*) and moss in Pinal Creek (Arizona State), that has been contaminated by acid-mining activities in the area [13]. These studies indicated that water speedwell, rabbitfoot grass and algae accumulated mainly Mn. In our previous paper, *Vaucheria sessilis* was used for the bioaccumulation of essential for plants micronutrients – Zn(II) and Cu(II) ions [14]. After two weeks of the experiment, macroalga accumulated 98.5 mg of Zn(II) ions in 1 g of dry biomass and 68.9 mg/g. It was suggested that the algal biomass with permanently bound microelements can find an application in many branches of the industry, for example as animal feed or component of natural fertilizers.

In the present paper, bioaccumulation of metal cations: Mn(II) and Co(II) by *Vaucheria* sessilis was investigated. Manganese is counted to essential micronutrients for higher and lower plants. This element is predominant in metabolism of organic acids, activates the reduction of nitrite and hydroxylamine to ammonia, plays role in important enzymes involved in respiration and enzyme synthesis, is activator of enzyme reactions such as oxidation/reduction, hydrolysis [15]. The second element – Co(II) is a beneficial micronutrients for higher and lower plants [15,16]. Cobalt is an essential component of several enzymes and co-enzymes. It affects growth and metabolism of plants in dependence on the concentration and status of cobalt in rhizosphere and soil [16].

The aim of the present paper was to evaluate the bioaccumulation properties of macroalga towards Mn(II) and Co(II) ions and to present how metal ions are accumulated on the surface and in the cross section of the biomass. Therefore, two methods were chosen: scanning electron microscopy with an energy dispersive X-ray analytical system (SEM-EDX) and inductively coupled plasma optical emission spectroscopy (ICP-OES). *Vaucheria sessilis* can constitute a perspective raw material for agricultural applications due to the high content of bioactive compounds and pronounced microelement accumulation capabilities. This innovative approach aims to produce natural, non-toxic algal products with cobalt and manganese which will influence the yield of the cultivated plants.

MATERIALS AND METHODS

Biomass of *Vaucheria sessilis*

The starting culture of macroalga *Vaucheria sessilis* was obtained from the Sammlung von Algenkulturen; Albrecht-von-Haller-Institute for Plant Science; University of Göttingen and was cultivated in the laboratory according to the procedure described by Sammlung von Algenkulturen Göttingen (SAG) (www.epsag.uni-goettingen.de/html/sag.html).

Bioaccumulation Process

Two solutions of algal medium (www.epsag.uni-goettingen.de/html/sag.html), which contained the following ions: Mn(II) and Co(II), were prepared in deionized water by dissolving appropriate amounts of $MnSO_4{\cdot}H_2O$ and $CoSO_4{\cdot}7H_2O$ (from POCh S.A. Gliwice, Poland, www.english.poch.com.pl/). The concentration of each metal ion in both medium solutions was 12.5 mg/l. pH of the solutions was adjusted to 7 with 0.1 mol/l standardized solution NaOH/HCl (from POCh S.A. Gliwice, Poland). pH measurements were conducted with pH-meter Mettler-Toledo - Seven Multi (Greifensee, Switzerland, www.mt.com) equipped with an electrode InLab413 with compensation of temperature. Into each solution about 2.5 g of wet biomass of *Vaucheria sessilis* was added (about 0.14 g/l of dry biomass). The bioaccumulation process lasted for two weeks in room temperature and daylight [17].

Analytical Methods

Scanning electron microscopy (SEM-EDX)

Natural and loaded with microelements biomass of *Vaucheria sessilis* was also examined by Scanning Electron Microscopy. The elemental analysis and mapping was performed at Wroclaw University of Environmental and Life Sciences (Electron Microscope Laboratory). Samples of macroalaga were fixed in 2.5% of glutaraldehyde (Sigma, www.sigmaaldrich.com). Then all the samples were dehydrated by ethanol (from 30% till 100% concentration). In the next step macrolaga was prepared in two planes for the observation of cross-section and its surface. Samples of the macroalga were mounted on appropriate stub and thereafter gold-sputtered (using ScanCoat six equipment - Oxford) and were observed and photographed with a Scanning Electron Microscope - Leo Zeiss 435 VP SEM (Oberkochen, Germany), operating at 20 kV. The microscope was equipped with a RONTEC energy dispersive X-ray system in order to obtain information on elemental composition of the surface of macroalgal cell wall. The X-ray spectrum of each macroalga loaded with a given microelement was obtained [14]. All papers must be written in the third person, e.g. 'it was found that', not 'we found'. You must not use 'this/our' paper/study/work, etc. when referring to your work; use 'the current' instead, throughout the whole paper. The Summary, Materials and Methods, and Results sections should also be in the past tense.

Multielemental analysis by ICP-OES

The solutions before and after bioaccumulation process were analysed by Inductively Coupled Plasma-Optical Emission Spectrometer - Varian VISTA-MPX ICP-OES (Victoria, Australia) with ultrasonic nebulizer in the Chemical Laboratory of Multielemental Analyses at Wrocław University of Technology, accredited by ILAC-MRA and Polish Centre for Accreditation (No. AB 696) according to EN-ISO 17025. For the calibration of the apparatus, the multielemental standard (100 mg/L Astasol®, Czech Republic, www.analytika.net) was used. In order to prepare the calibration curve, the following working dilutions of the analytical standard were prepared: 1.0, 10, 50 mg/l. As a check standard", the standard solution - 10 mg/l was used. The acceptable result was assessed as 10%. The analytical process was controlled by the use of Certified Reference Material Hard Drinking Water (UK) - metals from LGC Standards (www.lgcstandards.com) (LGC6010) for analysis of solutions. Values of the measurements of the CRMs were within the certified range. The examined samples were measured in three repeats. The final result was an arithmetic mean, which differed less than 5% [14].

RESULTS AND DISCUSSION

Analysis of the biomass after bioaccumulation by Scanning Electron Microscopy. Scanning electron microscopy is a technique, which can be successfully used to investigate binding of metal ions to seaweeds. SEM allows to evaluate morphological changes in the surface (e.g., cell wall composition) after metal binding by functional groups. Changes in surface morphology are usually related to disruption of the cross-linking between the metal ion and negatively charged chemical groups, e.g., carboxyl groups in the cell wall polymers. Raw seaweeds usually contain high content of calcium and magnesium (naturally present from seawater) in the cell wall, which creates a net of cross-linking. When the seaweed is exposed to metal solution (e.g., with cadmium), the cadmium cations replace some of the calcium and magnesium ions thus changing the nature of the cross-linking on the surface and resulting in morphological changes [18].

In the present study, SEM-EDX pictures of living macroalga *Vaucheria sessilis* were performed after two weeks of bioaccumulation of Mn(II) and Co(II) ions. The aim of these experimenta was to prove that bioaccumulation occurred and metal ions were permanently bound in the interior of the biomass. In the case of bioaccumulation, when the living biomass is treated with metal ions, ions can be transported through the cell membrane of the biomass. The SEM micrographs revealed a significant changes in alga's morphology between control group (natural biomass) and the experimental groups (biomass with Mn(II) and Co(II) ions). Biomass enriched with Mn(II) and Co(II) ions was characterized by higher corrugation than natural biomass. On the **(Figure 1)**, the differences between natural biomass (a,a';b,b') and biomass after bioaccumulation of Mn(II) (c,c';d,d') and Co(II) (e,e';f,f') are presented. Observed differences between investigated groups of biomass enriched with Mn(II) and Co(II) were significant. *Vaucheria sessilis* enriched with Co(II) was characterized by increasing porosity and loose fiber structure (f). Moreover, scanning microscope revealed a loss of filaments thickness visible and deformation of the cell wall of both investigated groups. These observations are in agreement with previous studies. The dysfunction of typical biomass morphology was also observed in the case of *Vaucheria sessilis* enriched with Zn(II) and Cu(II) ions via bioaccumulation. Biomass enriched with Zn(II) ions exhibited medium disintegration of a meshwork structure. In the case of Cu(II) ions, a loss of filaments thickness and a high occurrence of reproductive structures was noticed [17]. Also Yang and Chen observed surface protuberance and microstructures in the raw seaweed of Sargassum sp. after biosorption of hexavalent chromium [19]. This phenomenon can result from calcium and other salt crystalloid deposition. As it was shown in EDX analysis, calcium was a major component of the seaweed surface.

SEM combined with EDX technique provides valuable input in determining the distribution of various elements over the seaweed surface [20]. In the present paper, the mapping of all elements on the surface and in the cross section was performed. The results confirmed that bioaccumulation of metal ions took place in the interior of the cell. Analyzing the mappings of Mn(II) ions can be noticed in the case of the surface (biosorption), the elements were very dense and irregularly arranged. In the cross section (c') of the biomass, a regularly arrangement on the whole area was noted. The distribution of Co(II) ions on the surface of the biomass was dispersed compared to the cross section (e').

In the **Table 1**, atomic concentration of the elements (%) on the surface and in the cross section of macroalga *Vaucheria sessilis* after bioaccumulation is presented. The changes in atomic concentration on the surface of the biomass after bioaccumulation concerned mainly the increase of carbon concentration, decrease of oxygen, calcium (except of MVs-Co), sodium. These results are in agreement with data obtained by ICP-OES. Decrease of the content of Ca(II) and Na(I) ions on the surface of biomass (increase in the solution) is due to ion exchange with metal ions from the solution – Mn(II) or Co(II) ions. These results agree with our previous observations on the bioaccumulation of Cu(II) and Zn(II) ions by *Vaucheria* sessilis [17].

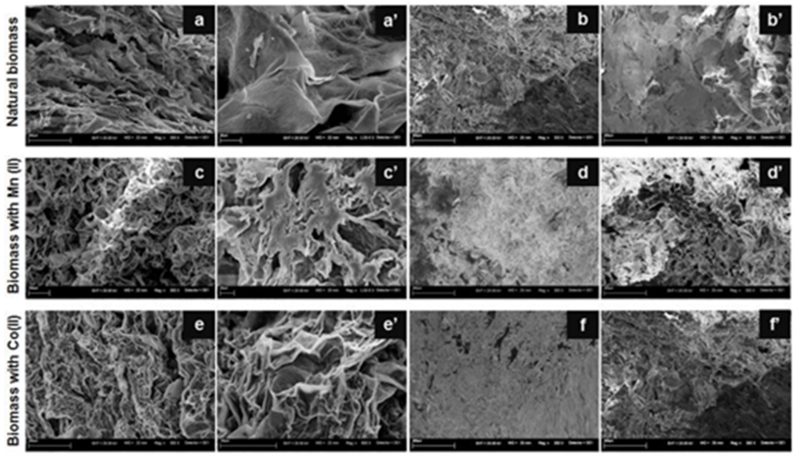

Figure 1. (a,a',b,b') Picture of the surface of natural biomass - *Vaucheria sessilis*; Picture of surface (b) and cross section (b'); (c,c',d,d') Picture of the surface of *Vaucheria sessilis* enriched with Mn (II) ions of surface (d) and cross section (d') of macroalga; (e,e',f,f') Picture of the surface of *Vaucheria sessilis* enriched with Co (II) ions; Picture of surface (f) and cross section (f') of macroalga.

Table 1. Atomic concentration of the elements (%) on the surface (I) and in the cross section (II) of macroalga *Vaucheria sessilis* after bioaccumulation. < LLD - below detection limit, Italics - below or above the detection limit Upper and lower limit of detection (%): Co (0.076-6.924); Mn (0.063-5.894) I-surface of macroalgae; II-cross section of macroalgae.

Atomic concentration of the elements (%)						
Element	I	II	I	II	I	II
	MVs – natural		MVs – Co		MVs – Mn	
C	48.2	37.5	55.4	53.7	52.7	50.5
O	48.2	55.2	32.1	41.1	43.3	42.0
Co	< LLD	< LLD	0	0	< LLD	< LLD
Mn	< LLD	< LLD	< LLD	< LLD	0	*0.04*
Ca	0.44	0.71	0.55	0.37	*0.27*	*0.33*
Na	0.57	0.67	0.23	0.35	0.54	1.04
Al	0.25	2.46	5.85	1.86	0.95	2.56

Atomic concentration of carbon in the cross section of MVs-Co and MVs-Mn was higher than in the natural biomass. Opposite observation concerned atomic concentration of oxygen. It was also noticed that atomic concentration of calcium decreased and sodium (except of MVs-Co) increased. The values of atomic concentration equal zero for Mn(II) (I) and Co(II) ions (I and II), does not mean that these elements were not accumulated. This means that their percentage share was much lower, than other elements (in this method it was assumed that the total concentration of all elements was 100 %).

Multielemental analysis of the solution before and after bioaccumulation process by ICP-OES. In the **Table 2**, the composition of the solution before and after bioaccumulation of Mn(II) and Co(II) ions by *Vaucheria sessilis* is presented. Bioaccumulation capacity was determined from the mass balance and by the direct analysis. After two weeks of the experiment, biomass of macroalga accumulated about: 71.3 mg/g of dry biomass of Mn(II) ions and 41.9 mg/g of Co(II) ions. During bioaccumulation, alkali and alkaline earth metals were released by the biomass of *Vaucheria sessilis*. The order for each macroalga was as follows (where MVs – means macroalga *Vaucheria sessilis*): MVs-Mn: Ca (30.7 mg/g dry mass) > K (26.6 mg/g) > Mg (13.1 mg/g) > Na (6.24 mg/g) and MVs-Co: K (46.8 mg/g dry mass) > Ca (37.6 mg/g) > Mg (18.2 mg/g) > Na (6.70 mg/g). This proves, that during the first step of bioaccumulation – biosorption – alkali and alkaline earth metals were replaced by metal ions from the solution. It can be assumed, that in the case of *Vaucheria* sessils K(I) and Ca(II) cations played a main role in cation exchange in biosorption process.

Table 2. The concentration of elements in the solution before (I) and after (II) bioaccumulation of Mn (II) and Co(II) ions by *Vaucheria sessilis* < LLD - below low limit of detection (Al <0.0234 mg/L) I-before; II-after.

Concentration (mg/L) of elements in solution:				
Element	I	II	I	II
	Mn(II) ions		Co(II) ions	
	bioaccumulation by *Vaucheria sessilis*			
Co	0.02 ± 0.005	0.019 ± 0.005	14.4 ± 2.2	8.53 ± 1.28
Mn	13.2 ± 1.98	3.22 ± 0.48	0.04 ± 0.01	0.16 ± 0.02
Ca	2.69 ± 0.40	6.99 ± 1.05	2.66 ± 0.40	7.93 ± 1.19
Na	1.45 ± 0.22	2.33 ± 0.35	1.13 ± 0.17	2.07 ± 0.31
K	94.2 ± 14.1	97.9 ± 14.7	91.9 ± 13.8	98.5 ± 14.8
Al	0.09 ± 0.02	0.08 ± 0.02	0.03 ± 0.006	< LLD
Mg	2.38 ± 0.36	4.22 ± 0.63	2.21 ± 0.33	4.75 ± 0.71

The application of bioaccumulation process by macroalga allowed to obtain biomass with permanently bound microelements. This valuable, natural and non-toxic material can be used in different areas – for example in agriculture. As it was shown it the literature, seaweeds (fresh, dry) and their products (extracts, composts, soil conditioners) are used in agriculture to enhance plant growth and productivity [1,21]. Algal-based products are known to stimulate seed germination, assist in nutrient uptake, help to protect plants from insects, disease and frost and support root development [1,22].

Natural resources of seaweeds, which are rich in minerals, can constitute the alternative or supplementation to mineral fertilizers. The majority of nutrition supplements comprise trace elements as inorganic salts but the bioavailability of inorganic forms is not so pronounced [23]. The solution can be natural components of fertilizers obtained by biosorption/bioaccumulation process [24]. Molnár et al. suggested that metal enriched algae can be considered as highly efficient tools of microelement supply, as it involves considerable amounts of metals in bioavailable forms (organic bonds) [23]. In the work of Tuhy et al. it was shown that biological fertilizer components with zinc based on algal biomass were characterized by higher bioavailability of Zn(II) ions when compared to traditional fertilizers: zinc sulfate and Zn-EDTA. Moreover, it was found that the use of enriched biomass led to the biofortification of plants with this nutrient [24]. Additionally, plants in the experimental group were higher than in the control and had more intense green colour. Another approach involves the use of macroalgae as a valuable raw material for the extraction of biologically active compounds for agricultural use (e.g., plant biostimulants, bioregulators of plant growth) [17]. As can be seen, the prospects for algal products for agricultural purposes are promising because of increased public awareness.

Vaucheria sessilis is a widespread alga, but has not been thoroughly studied yet. The performed experiments showed that it can act as a good bioaccumulator of metal ions. Macroalga showed higher bioaccumulation capacity towards Mn(II) than for Co(II) ions. Scanning electron microscopy micrographs have been employed to explain the interaction of metal ions with biomass. In the case of the surface (biosorption), the elements constituted aggregations and in the case of the cross section (bioaccumulation) they were evenly distributed. The SEM micrographs revealed also significant changes in morphology of alga. The application of two methods: ICP-OES and SEM-EDX allowed characterization of bioaccumulation process of metal ions by *Vaucheria sessilis*.

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