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HEAVY METAL CONTENT IN MACROALGAE FROM ROUMANIAN BLACK SEA

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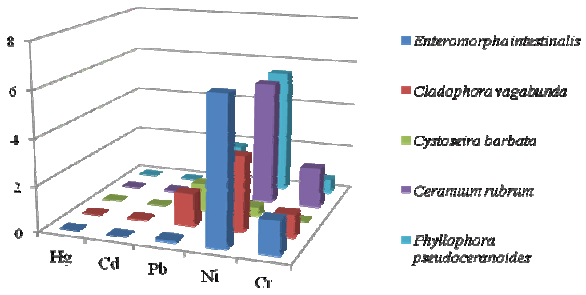
Levels of Hg, Cd, Pb, Ni, Cr, Mn, Zn, and Cu were determined in five macroalgae (*Enteromorpha intestinalis, Cladophora vagabunda, Cystoseira barbata, Ceramium rubrum* and *Phyllophora pseudoceranoides*) collected in June 2014 from the Roumanian Black Sea coastline. These heavy metals were quantified by inductively coupled plasma mass spectrometry (ICP-MS), after microwave digestion. A wide range of metal uptake ability among the different species was observed: *C. rubrum* showed a high capacity to accumulate Cu and Cr, *P. pseudoceranoides* - Mn and Pb, *C. vagabunda* – Hg, Zn and Cd, and *E. intestinalis* – Ni; hence, these species could be used as bioindicators for the respective metals. In our study, red algae displayed the highest capacity to concentrate heavy metals from surrounding water and sediment, followed by green and brown algae. The data presented could serve as a baseline for further investigations regarding the heavy metal contamination of the Romanian Black Sea coast.



INTRODUCTION

The Black Sea is one of the most isolated inland seas; the water exchange is low, the sea being connected in the south with the Mediterranean Sea by Bosphorus and Dardanelles Straits and with the Azov Sea by Kerch Strait in the north. Over the last decades, the Black Sea has undergone increasing environmental and ecological deterioration, due to contaminants from its 2 milion km^2 cachement area, that covers 22 countries in Europe and Asia.[1,2] This phenomenon is significant also in the Roumanian Black Sea coastline, as the ecosystem is affected by pollutants derived from anthropogenic sources: riverine outflow, industrial wastes, agricultural pesticides and fertilizers runoff, domestic solid wastes or sewage treatment plants.[3,4] Certain types of contaminants, such as heavy metals, have a significant impact on the marine environment and biota due to their high toxicity even in traces, bioaccumulation capacity and persistence in nature.[5]

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Macroalgae are the base of the aquatic trophic chain and possess the ability to accumulate high concentrations of heavy metals in their tissues, thus influencing the chemical content of higher trophic levels and becoming a potential threat to human health.[6] Over the past few decades, biological indicators have been used as monitoring tools for environmental pollution assessement. Water and sediment samples analysis provide information about the abundance of heavy metals and do not reflect their bioavailability in the marine ecosystem[7,8] Algae bind free metal ions and reflect better metals concentration and availability in the surrounding environment. Toxic metal ions are able to penetrate algae membranes *via* a molecular mimicry mechanism using ion carriers (*e.g.* Ca^{2+} channels) or amino acid transporters such as cysteine. Also, carboxyl, sulfate, hydroxyl and amino groups of algal cell-wall polysaccharides represent important complexation sites for metal cations.[9] Macroalgae are ubiquitous, sedentary, sensitive to environmental stressors, easily to collect and identify, thus possessing fundamental attributes as bioindicators of heavy metal contamination;[10] therefore, in order to assess ecosystem integrity and environmental deterioration, many field studies have used marine algae as monitors of metal contamination in coastal waters.[11-13]

As member of the European Union, Roumania considers the protection and preservation of Black Sea ecosystem as a national priority and cooperates with member states and also with The Black Sea Comission parteners in developing monitoring programmes for the ongoing assessment of marine environmental status.[14,15]

The present study aimed to investigate the possible usefulness of selected algae species as bioindicators of pollution degree in north-western Black Sea; in addition, these macroalgae species were compared regarding their heavy metal accumulation capacity. In an effort to gain some insight on heavy metal contamination along the Roumanian Black Sea coastline, the present study reports on Hg, Cd, Pb, Ni, Cr, Mn, Zn, and Cu levels accumulated by five macroalgae: *Enteromorpha intestinalis, Cladophora vagabunda, Cystoseira barbata, Ceramium rubrum* and *Phyllophora pseudoceranoides*. The species are abundant and representative for the studied area, but quantitative data on their heavy metal levels are scarce.[16,17] Inductively coupled plasma mass spectrometry (ICP-MS) was used for metal quantification in the investigated seaweeds; this analytical technique has been validated to determine trace and ultra-trace elements in algae, due to its high sensitivity and precision, with detection limits in the order of ng g^{-1}.[18]

RESULTS AND DISCUSSION

Macroalgae possess a high capacity to bind metals, being able to concentrate essential and non-essential metals from ambient water by several orders of magnitude.[19] The cellular wall polysaccharides possess functional groups (hydroxyl, sulfate, carboxyl) which act as ion-exchangers and bind metal cations.[9] Generally, metal content in macroalgae dependends on various biological (*e.g.* species phylogeny, thalus morphology, growth strategy, generation) and environmental factors (*e.g.* concentration and availability of elements in water, interactions between chemical elements, temperature, season, salinity, pH, light intensity, area geology) or on the interactions between both kinds of factors.[9,20,21]

Mean heavy metal concentrations (both essential and non-essential) in macroalgae sampled from the shoreline of the Black Sea varied from hundreds (Mn) to thousandths (Hg) scales of μg g^{-1} algae dry weight (table 1); metal concentrations decreased in the following order: Mn > Zn > Cu > Ni > Pb > Cr > Cd > Hg.

Table 1

Heavy metal content in Romanian Black Sea macroalgae (mean ± SD)

Species	Mn*	Zn*	Cu*	Hg**	Cd*	Pb*	Ni*	Cr*
Enteromorpha intestinalis	38.33±0.32	73.32±0.28	0.28±9.49	4.79±0.95	0	0.10±1.07	6.30±0.82	1.45±1.30
Cladophora vagabunda	79.8±0.5	119.02±0.95	<0.001	8.76±0.95	0.05±1.93	1.42±1.22	3.26±7.10	1.01±2.06
Cystoseira barbata	112.60±0.58	33.03±1.16	<0.001	2.60±0.19	0	1.22±0.69	0.41±0.65	0.003±4.68
Ceramium rubrum	22.94±1.13	104.66±0.68	13.85±0.91	3.65±3.37	0.05±3.11	1.81±1.17	5.39±0.95	1.77±1.28
Phyllophora pseudoceranoides	612.34±0.11	61.28±0.42	5.47±1.04	2.14±1.00	0	1.83±0.51	5.49±1.20	0.66±0.30

* μg g^{-1}; ** μg kg^{-1}

Among **essential elements**, the uptake order by the algal species was: Mn > Zn > Cu. The presence of high concentrations of Mn, Zn and Cu in macroalgae can be attributed to the fact that these elements are also important micronutrients for seaweed metabolic functions.[19]

Mn concentrations varied in a wide range, from 22.94 μg g^{-1} (*C. rubrum*) to 612.34 μg g^{-1} (*P. pseudoceranoides*). Studies regarding analogue seaweed species from Turkish and Bulgarian Black Sea coastline showed lower Mn levels (12.8-296.4 μg g^{-1}).[22,23] As a micronutrient, Mn is a component of metalloenzymes involved in photosynthetic oxygen evolution in chloroplasts, but high Mn content in plants has been associated with a reduction in photosynthetic rate.[24] Results from the present study showed lower Mn concentrations when compared to those of *Cladophora* sp. (712-1930 μg g^{-1}), collected from a polluted lagoon in south-eastern Baltic Sea.[12]

Minimal quantities of Cu were registered in *C. vagabunda* and *C. barbata*, meanwhile the highest concentration was measured in the red alga *C. rubrum* (13.85 μg g^{-1}). Cu levels were slightly above those reported for algae collected from a clean site, the sub-Antarctic ecoregion of Mangellanes (1.3-11.9 μg g^{-1}).[25] High levels of Cu (65.7 μg g^{-1}) were determined for algae collected from an area affected by major tourism activities in the Egyptian Mediterranean Sea.[26] Cu plays a dual role in the metabolism of macroalgae; it is an essential element for enzymes (amino oxydase, cytochrome *c* oxydase) and electron chain transport components involved in photosynthesis (*e.g.* plastocyanin). Generally, toxic levels of Cu have been related to inhibition of photosynthesis, with growth retardation and oxidative stress induced by generation of reactive oxygen species (ROS).[24,27]

C. vagabunda accumulated the highest amount of Zn (119.02 μg g^{-1}), while *C. barbata* showed the lowest Zn uptake ability (33.03 μg g^{-1}). Ryan *et al.* (2012) reported mean Zn levels in the range described in our study for algae from clean areas of Ireland coast (12.2-137.6 μg g^{-1}).[28] These data are not consistent with the study of Schintu *et al.* (2010); this work on seaweeds collected from a lead-zinc smelter contaminated area (Sardinia, Italy) revealed considerably higher Zn levels, ranging from 1.3 to 780 μg g^{-1}.[29] Zn is the second most abundant transition metal after Fe; this essential micronutrient acts as a cofactor for numerous enzymes and redox proteins, such as DNA and RNA polymerase, carbonic anhydrase or alkaline phosphatase. Zn phytotoxic levels are characterized by retarded growth and development, due to decrease in photosynthesis and plant metabolism.[24]

With regard to **non-essential elements**, which are not considered mandatory for the algal metabolic activities, the mean concentration values varied in the following order: Ni > Pb > Cr > Cd > Hg. In the Black Sea, heavy metal pollution represents an environmental problem and it is mainly due to effluents from industrial processes and agricultural activities, wastewater discharges and shipping.[3] Such toxic elements affect normal metabolic functions of macroalgae, with an inhibition of photosynthesis and growth; also, their toxicity could be related to an increased ROS production, with a significant decrease of cellular antioxidant capacity.[9]

The mean concentrations of Hg ranged from 2.14 (*P. pseudoceranoides*) to 8.76 μg kg^{-1} dry weight (*C. vagabunda*). This range was similar to that reported earlier for edible algae (*Gelidium* and *Laminaria* sp.) collected from Spain (1-9 μg kg^{-1}; this range is below the limits required by food legislation regarding the maximum amount of metals in algae commercialised for human consumption).[30] Results from the present study showed significantly lower Hg concentrations when compared to macroalgae native to Izmir Gulf (26.1-336.3 μg kg^{-1}), a region characterized by a high degree of metal contamination due to industrial activities.[31] Hg is highly toxic in comparison with other heavy metals, as it is easily accumulated in terrestrial and aquatic plants. Phytotoxicity of Hg includes growth retardation, disturbance of cellular metabolism, photosynthesis inhibition and even death.[5,8]

Cd was detected only in *C. vagabunda* and *C. rubrum*, with values of 0.054 and 0.050 μg g^{-1}, respectively. Similar mean concentrations for Cd were determined in seaweeds collected from Thermaikos Gulf, an area with low anthropogenic impact (0.037-0.046 μg g^{-1}).[32] Nevertheless, Topcuoğlu *et al.* (2001) found higher Cd accumulating potential (concentration range: 0.35-2 μg g^{-1}) for several seaweed species collected from a polluted area near Istanbul, Turkey.[22] Cd is a major anthropogenic pollutant and its toxic effects are well documented; exposure of algae even at levels below 1 ppm causes alterations of membrane functionality, inhibition of chlorophyll biosynthesis and inhibition of enzymes involved in photosynthesis.[5,21]

The content of Pb in the analyzed macroalgae ranged between 0.10 μg g^{-1} in *E. intestinalis* and 1.83 μg g^{-1} in red alga *P. pseudoceranoides*. This range was found to be similar to that detected in *Ascophylum nodosum*, native to pristine areas of North Atlantic (0.11-2.11 μg g^{-1}),[33] although values above that range were determined in green, red and brown seaweeds collected from polluted sites from Gulf of Thessaloniki (up to 16.33 μg g^{-1}).[34] Excess of Pb causes inhibition of growth, mitosis, photosynthesis and water absorption, alters enzymes activity and disturbs mineral nutrition.[6]

The mean Ni content varied significantly among the investigated samples (0.41-6.30 μg g^{-1}). The range of Ni concentrations in algae from pristine areas of Antarctica coast was found to be between 1.8-8.8 μg g^{-1},[11] whereas for polluted sites of the Atlantic coasts of Ghana, Ni mean levels were above that range: 14.0-30.4 μg g^{-1}.[35] High Ni concentrations have been associated with plant necrosis, alteration of cell membrane permeability with impairment of nutrient balance, growth and development inhibition.[36]

Cr is a non-essential metal, being 100-1000 times more toxic to living cells in its Cr(VI) form than the Cr(III) common form.[9,21] In our work, Cr concentrations varied from 0.003 μg g^{-1} in brown alga *C. barbata* to 1.77 μg g^{-1} in *C. rubrum.* Similar results were reported for *Ascophylum nodosum,* a brown alga collected from clean areas of North Atlantic (0.05-1.07 μg g^{-1}).[33] Thus, higher amounts of Cr (1.8-7.0 μg g^{-1}) were detected in similar genera from Bulgarian Black Sea, collected from different regions affected by anthropogenic activities.[23]

According to species phylogeny, in this study red algae showed the highest capacity to concentrate metals from surrounding water and sediment, followed by green and brown algae. This is consistent with data from previous studies on macroalgae from Ireland South-East shoreline and similar species collected from the Bulgarian coast of the Black Sea.[23,28] However, a literature survey shows that there seems to be no consistent pattern for certain groups of algae to uptake and accumulate higher or lower amounts of metals.[13,37,38]

On a species basis, *C. rubrum* is a strong accumulator of Cu and Cr, *P. pseudoceranoides* of Mn and Pb, *C. vagabunda* of Hg, Zn and Cd, and *E. intestinalis* of Ni. Mean metal concentrations determined in the investigated algae indicate that minimum levels of Ni, Cr and Zn were found in *C. barbata*, Cu in *C. vagabunda*, Pb in *E. intestinalis*, Mn in *C. rubrum* and Hg in *P. pseudoceranoides.*

Interspecies variations in metal accumulation may be attributed to differences in algae thallus morphology and growth strategy;[34,39] significant differences were observed among the functional-form groups (Table 2). Generally, filamentous algae are characterized by increased photosynthetic and growth rates and possess a high surface for nutrient absorbtion.[40,41] In our study, filamentous seaweeds such as *C. rubrum* and *C. vagabunda* showed the highest metal contents for the majority of elements (Zn, Cu, Cr, Cd and Hg). These findings are supported by previous studies that reported on the capacity of *Ceramium* and *Cladophora* sp. to accumulate high metal concentrations.[12,34] Even though sheet-type *E. intestinalis* exhibits a high surface/volume ratio and presents a long history of use as a biondicator for metal pollution,[34,42] in our study the green alga did not provide a full account of metal contamination.

Table 2

Algae species, taxonomy, functional-form groups of the species and coordinates of the sampling sites

Species	**Taxonomic group**	**Functional-form group***	**Sampling site**	**Coordinates of the sampling site**
Enteromorpha intestinalis	*Chlorophyta*	Sheet	Constanța North	44°12'53"N 28°38'40"E
Cladophora vagabunda	*Chlorophyta*	Filamentous	Constanța North	44°12'53"N 28°38'40"E
Ceramium rubrum	*Rhodophyta*	Filamentous	2 Mai	43°47'22"N 28°34'21"E
Phyllophora pseudoceranoides	*Rhodophyta*	Thick-leathery	Constanța South	44°11'44"N 28°39'24"E
Cystoseira barbata	*Phaeophyta*	Thick-leathery	Mangalia	43°48'0"N 28°35'0"E

* 40,41

Thick-leathery algae (*C. barbata, P. pseudoceranoides*) showed lower mean concentrations of Zn, Cu, Cr, Hg and Ni than the filamentous macroalgae, which could be explained by lower rates of photosynthesis and growth, leading to decrease in assimilation of metals.[40,41] Nevertheless, *Phyllophora* and *Cystoseira* sp. showed high affinity for several elements such as Mn and Pb. This could be attributed to the fact that these two algae, in their natural habitat, grow in contact with sediment or coastal rocky bottom, thus accumulating heavy metals not only from surrounding water, but also from sediment.[43] In addition, the uptake of these elements could be related to the species biochemical composition: cellular wall polysaccharides such as alginate and carrageenan for *C. barbata* and for *P. pseudoceranoides*, respectively, have carboxyl groups with good ion chelating properties.[44]

EXPERIMENTAL

Analytical procedures

Total Hg, Cd, Pb, Ni, Cr, Mn, Zn, and Cu concentrations in macroalgae were determined with an Agilent 7700x ICP-MS (Agilent Technologies, Japan) with a third generation of Octopole Reaction System (ORS^3). Table 3 presents the ICP-MS operational conditions.

Table 3

Instrumental parameters
for heavy metals determination using ICP-MS

Instrumental parameter	
RF incident power, W	1550
Plasma argon flow rate, L min^{-1}	15
Carrier Gas flow rate, L min^{-1}	1.01
Sample Depth, mm	8
Nebulizer pump, rps	0.1
Nebulizer temperature,ºC	2
Scanning mode	NO GAS
AMU gain	127
Dwell time, µs	100
Sampling period, s	3
Number of readings per replicate	3

The external calibration technique was followed for the quantitative analysis of the samples, using ICP multi element standard solution Merck IV CertiPur and SPS-M-321 (Holger Teknologi, Norway). The calibration curves were built on 10 different concentrations (0.001, 0.005, 0.01, 0.026, 0.05, 0.1, 0.26, 0.5, 1.5, 5 µg mL^{-1}) so that the concentrations of all analytes in the samples were within linear range of calibration curves. Measurements were carried out using the full quantitative analysis mode. Re was used as internal standard.[43] All reagents were of analytical grade (Merck, Darmstadt, Germany). The accuracy of the method was verified by analysis of certified reference material METRANAL 8 – Green algae (metals) (Analytika, Prague, Czech Republic); results were in agreement with indicative values.

Sample collection and digestion

Macroalgae samples were collected from the Black Sea coastal areas of Roumania in June 2014. The species, their taxonomic group and the sampling area are shown in Table 2. Fresh seaweed material was washed thoroughly first with tap water; all epiphytes, organic and mineral particles were manually removed. All samples were then rinsed in distilled water and ultrapure water; the air dried samples (40 ºC) were powdered and stored at -4 ºC until analysis. The species were identified by Assoc. Prof. Daciana Savu ("Ovidius" University, Constanța, Roumania) and voucher specimens were deposited in the Laboratory of Pharmacognosy, Faculty of Pharmacy, "Grigore T. Popa" University of Medicine and Pharmacy – Iași, Roumania.

For digestion 0.25-0.5 g of each sample were directly weighed into polytetrafluoroethylene digestion vessels. Then 7.0 mL of concentrated HNO_3 (65%) and 2.0 mL of H_2O_2 (30%, catalyst) were added and digested using laboratory microwave unit MARS 6 (CEM Corporation North Carolina, USA). The combustion procedure was as follow: (1) 1,000 W at 80 ºC for 5 min, (2) 1,000 W at 50 ºC for 5 min, (3) 1,000 W at 190 ºC for 20 min and (4) 0 W for 30 min for cooling. Digested samples were transferred into polypropylene volumetric tubes and made up to 50 mL with ultrapure deionised water. Three replicates of each sample were analyzed. Blank samples were also carried out.[43]

CONCLUSIONS

The present study provides data on the heavy metal contamination of marine macroalgae along the Roumanian Black Sea coastline. In our study, heavy metal concentrations in algae were within the range reported in the scientific literature for other algae collected from non-polluted areas; metal concentrations decreased in the following order Mn > Zn > Cu > Ni > Pb > Cr > Cd > Hg. These findings provide valuable information on the relatively low degree of heavy metal contamination of macroalgae from Roumanian Black Sea coastline. Furthermore, these data could serve as a baseline for further investigations regarding the heavy metal contamination of the Roumanian Black Sea coast.

The accumulation of heavy metals is related to phyllogeny, as red algae possess a higher capacity to concentrate heavy metals than green and brown algae. Also, the metal uptake is highly dependent on thallus morphology and growth strategy: filamentous seaweeds show a higher metal uptake than sheet and thick-leathery algae. *C. rubrum* is a strong accumulator of Cu and Cr, *P. pseudoceranoides* of Mn and Pb, *C. vagabunda* of Hg, Zn and Cd, and *E. intestinalis* of Ni; these species are abundant and representative for the studied area and could be used as bioindicators for assessment of heavy metal contamination along the Roumanian Black Sea coastline.

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