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 ORIGINAL PAPER

HEAVY METAL BIOACCUMULATION IN MARINE ORGANISMS FROM THE ROMANIAN BLACK SEA COAST

Magda NENCIU[1*], Andra OROS[1], Daniela ROŞIORU[1], Mădălina GALAŢCHI[1], Adrian FILIMON[1], George ŢIGANOV[1], Cristian DANILOV[1], Natalia ROŞOIU[2,3]

[1]National Institute for Marine Research and Development "Grigore Antipa", 300 Mamaia Blvd., Constanta, 900581, Romania
[2]"Ovidius" University of Constanta, Faculty of Medicine, Department of Biochemistry, Campus Building B, Constanta, Romania
[3]Academy of Romanian Scientists, 54 Splaiul Independentei, 050094, Bucharest, Romania
**Corresponding author, e-mail: mnenciu@alpha.rmri.ro, magdalena.nenciu@gmail.com*

Abstract. Similarly to other European seas, the Black Sea ecosystem has undergone severe changes in all its subsystems immediately after 1970, as a consequence of industrialization and intensive agriculture, resulting in eutrophication and other types of pollution: waste water discharges, oil spills, pesticide contamination, radioactive substances, heavy metal contamination etc. Heavy metals, one of the main pollutants reaching the Black Sea, are deposited in the various components of the aquatic environment (water, sediment and biota) and can be accumulated through the food chain in aquatic systems. Bioindicator species are ideal for monitoring and the most appropriate for the marine environment are considered mollusks, as filter-feeders. However, other marine species, such as algae, crustaceans and fish, have been used as indicators of contamination. Fish species living close to the seabed such as Syngnathids are more exposed to heavy metals than pelagic species, due to the fact that metals tend to accumulate in the substrate. This paper focuses on the bioaccumulation of heavy metals (Cu, Cd, Pb, Ni, Cr) in two species of Syngnathids from the Romanian shallow waters: the long-snouted seahorse (Hippocampus guttulatus, Cuvier 1829) and the greater pipefish (Syngnathus acus, Linnaeus 1758), compared to heavy metal concentrations recorded in the surrounding water and sediments, expressed by the Bioconcentration Factor (BCF) and the Biota-Sediment Bioaccumulation Factor (BSAF). For comparison reasons, the concentrations recorded by other fish and mollusk species from the same sampling areas are presented.

Key words: Syngnathidae, benthic species, heavy metals, biomonitoring, Bioconcentration Factor (BCF), Biota-Sediment Bioaccumulation Factor (BSAF**).**

Introduction

Given then capacity of marine organisms to accumulate via various pathways the heavy metals in the environment (in water, sediment or food), their use as bioindicators of marine pollution is supported by many examples. Mollusks have been considered the ideal species, due to their filter-feeding technique which allows them to interact with huge volumes of seawater, resulting in accumulation of pollutants. Consequently, extensive research has been

undertaken in this respect (Butler, 1971; Haug, 1974; Phillips, 1980; Sericano et al., 1990; Oros et al., 2003; Oros, 2009; Oros & Gomoiu, 2012; Roşioru et al., 2012; Roşioru et al., 2014).

Bivalve mollusks meet best the criteria set for bioindicator species of pollution (Haug, 1974; Phillips,1980; Schettino et al., 2012), namely:

- there must be a correlation between the bioaccumulation and the environmental background of contaminants;
- the species should tolerate high contaminant concentrations;
- the species should be sedentary and abundant in the study area;
- the species should be easy to collect and have a high survival capacity under unfavorable circumstances.

Apart from mollusks, other marine species have been investigated, such as algae, crustaceans (Rainbow et al., 1989; Powell & White, 1990; Moore, 1991; Barwick & Maher, 2003) and fish (ICES, 1989 & 1991; Misra, 1993; Van der Oost et al., 2003; Yarsan & Yipel, 2013).

Bioaccumulation refers to the accumulation of chemicals (heavy metals in this case) by all possible routes of exposure (surrounding water, sediment, food) (Kleinov et al., 2008). Bioconcentration and bioaccumulation are expressed by referencing the chemical concentration in tissue to that of water and sediment, respectively. Biomagnification, on the other hand, refers to a stepwise increase in the concentration of chemicals in higher trophic level organisms, resulting from the ingestion of contaminated lower trophic level organisms (ratio between the chemical bioaccumulation of a predator and its prey) (Nenciu et al., 2014a).

Bioconcentration is the result of the direct uptake of a chemical by an organism only from water and the result of such a process is measured by the Bioconcentration Factor (BCF), which represents the ratio of steady state concentration of the respective chemical in the biota (C_B) (mass of chemical per kg of organism/dry weight) and the corresponding freely dissolved chemical concentration in the surrounding water (C_W) (mass of chemical/l) (Geyer et al., 2000):

$$BCF = C_B/C_W.$$

Nevertheless, heavy metals do accumulate in marine biota from the sediments also, and this is expressed by the Biota-Sediment Bioaccumulation Factor (BSAF). BSAF is a parameter describing bioaccumulation of sediment-associated organic compounds or metals into tissues of ecological receptors (Kleinov et al., 2008).

The Biota-Sediment Bioaccumulation Factor (BSAF) is calculated using the following equation: $BSAF = C_B/C_S$, where C_B is the chemical concentration in the biota (mass of chemical per kg of biota/dry weight), while C_S is the concentration in the related sediment (mass of chemical per kg of sediment/dry weight) (Nenciu et al., 2014a).

Bioaccumulation is of great concern to authorities in the European Union and not only. The Regulation (EC) No. 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) stipulates that a substance fulfils the bioaccumulation criterion when the Bioconcentration Factor (BCF) is higher than 2000 (EC, 2006). The United States Environmental Protection Agency uses a Bioconcentration Factor (BCF) higher than 1000 as a concern trigger for potential bioaccumulation effects. A substance is considered "bioaccumulative" when it has a BCF ranging between 1000 and 5000 and "very bioaccumulative" if it has a BCF greater than 5000 (TSCA, 1976) (Table 1).

Table 1. Threshold values for the bioconcentration factor (BCF) ***(Nenciu et al., 2014a).***

Regulatory act	Threshold values
Regulation (EC) No. 1907/2006 (REACH)	≥ 2000 = bioaccumulative ≥ 5000 = very bioaccumulative
US EPA Toxic Substances Control Act (TSCA)	≥ 1000 = bioaccumulative ≥ 5000 = very bioaccumulative

Concerning the Biota-Sediment Bioaccumulation Factor (BSAF), there are no legal applicable threshold values, as BSAF values always depend on the physical-chemical properties of both the chemical and the sediment, as well as on the lipid content of the organism the chemical bioaccumulates into (Nenciu et al., 2014a). However, based on the calculated values, the different species of marine organisms can be classified into three groups, such as macroconcentrator (BSAF > 2), microconcentrator (1<BSAF<2) or deconcentrator (BSAF<1) (Dallinger, 1993).

Fish species living close to the seabed such as Syngnathids are more exposed to heavy metals than pelagic species, due to the fact that metals tend to accumulate in the substrate. This paper focuses on the bioaccumulation of five heavy metals (Cu, Cd, Pb, Ni, Cr) in two species of Syngnathids from the Romanian shallow waters: the long-snouted seahorse (*H. guttulatus*, Cuvier 1829) and the greater pipefish (*S. acus*, Linnaeus 1758), compared to heavy metal concentrations recorded in the surrounding water and sediments, expressed by the Bioconcentration Factor (BCF) and the Biota-Sediment Bioaccumulation Factor (BSAF). The two target species are coastal, they inhabit shallow areas, close to the seabed covered by macrophyte algae, thus very much exposed to all anthropogenic sources of contamination (Nenciu et al., 2013). As such, the bioconcentration and bioaccumulation of heavy metals in Syngnathid tissue can be used as bioindicators of the environmental quality along the Romanian Black Sea coast.

For comparison reasons, the concentrations recorded by other fish species from the same sampling areas are also presented (Nenciu et al., 2014b).

Material and method

In order to analyze the heavy metal concentrations in the two target species, the biological samples were collected from two shallow sampling stations (0-20 m): Cazino Constanta and Costinesti (areas where the species are abundant and easy to collect) (Fig. 1). Individuals belonging to the species *H. guttulatus* and *S. acus* were collected. The biological material was frozen and, subsequently, subjected to specific laboratory analyses.

The sampling, preservation, preliminary processing and analysis methodologies of biota, water and sediment were consistent with the reference method recommended in the study of marine pollution (IAEA-MEL, 1999).

Before the determination of heavy metals, biological samples were freeze-dried, homogenized, weighed and subjected to digestion with 5 mL nitric acid (HNO_3 65%, Suprapur Merck) in sealed Teflon vessels (60 ml Savillex) on a hotplate at 120^0C. After completion of digestion, samples were brought to 100 ml volume with deionized water (18.2 MΩ cm, Millipore). In the solutions obtained after the digestion of samples, metals (copper, cadmium, lead, nickel, chrome) were analyzed using graphite furnace - atomic absorption spectrometers, type ATI-UNICAM 939Z and SOLAAR M6 Dual Thermo Electron-UNICAM. The accuracy and precision of the analytical method were checked with certified reference materials (CRM).

Fig. 1 Sampling locations for biota, water and sediment samples.

Water samples were also collected from the same locations. Water samples from the surface layer were collected with Nansen bottles and stored at refrigerator temperature until their subsequent analysis in the laboratory. Total metals have been determined in unfiltered seawater samples, acidified up to pH=2 with Ultrapur HNO_3. Nitric acid was used not only for preserving sample and partial solubilisation of particulate metals, but also as a matrix modifier, diminishing salts interferences. Metals were analyzed by graphite furnace - atomic absorption spectrometry (GF - AAS), using the equipment types ATI-UNICAM 939Z and SOLAAR M6 Dual Thermo Electron-UNICAM. Calibration was done with working standards prepared for each element, from stock solution of 1000 µg/l (Merck). The working domains are as follows: Cu 0-50 µgl; Cd 0-10 µg/l; Pb 0-20 µg/l; Ni 0-50 µg/l; Cr 0-50 µg/l.

Surface sediment samples were collected with a Van-Veen grab. The whole sediment was digested and analyzed, after the removal of the rough material (> 1 mm). Samples were freeze-dried and well homogenized before chemical analysis. For sediment decomposition, the acid microwave digestion method was applied, using Suprapur HNO_3 65%. After mineralization and cooling, samples were transferred into a 100 ml volumetric flask, with deionised water. In the solutions resulted after digestion, metals were analyzed by graphite furnace - atomic absorption spectrometry (GF - AAS), using the same equipment types ATI-UNICAM 939Z and SOLAAR M6 Dual Thermo Electron-UNICAM. Accuracy and precision were verified using certified reference materials (CRMs) with similar matrix as samples: BCSS-1, MESS-3 (marine sediments), which were analyzed together with each batch of samples.

After determining the heavy metal concentrations in the fish tissue, water and sediments, the BCF and BSAF were calculated, using the above mentioned equations.

Results and discussions

The results obtained revealed extremely high values recorded by copper in both species and both sampling stations.

In the Cazino Constanta Station, the highest values were recorded by copper both in *H. guttulatus* (14,000 µg/kg DW) and *S. acus* (7,890 µg/kg DW) tissue, followed by nickel in *H. guttulatus* (3,220 µg/kg DW) and *S. acus* (1,330 µg/kg DW) tissue. Lead recorded the maximum value in *S. acus* (1,220 µg/kg DW) tissue, as well as chrome (730 µg/kg DW) (Fig. 2).

The order of concentrations was as follows: *H. guttulatus* = Cu > Ni > Pb > Cd > Cr, *S. acus* = Cu > Ni > Pb > Cr > Cd.

In the Costinesti Station, copper also recorded the maximum values in both species: 17,270 µg/kg DW in *H. guttulatus* and 7,120 µg/kg DW in *S. acus*.

Chrome and nickel also recorded high values in *H. guttulatus* tissue: 11,790 µg/kg DW and 6,640 µg/kg DW, respectively (Fig. 3).

The order of concentrations was as follows: *H. guttulatus* = Cu > Cr > Ni > Pb > Cd, *S. acus* = Cu >Ni > Cr > Pb > Cd.

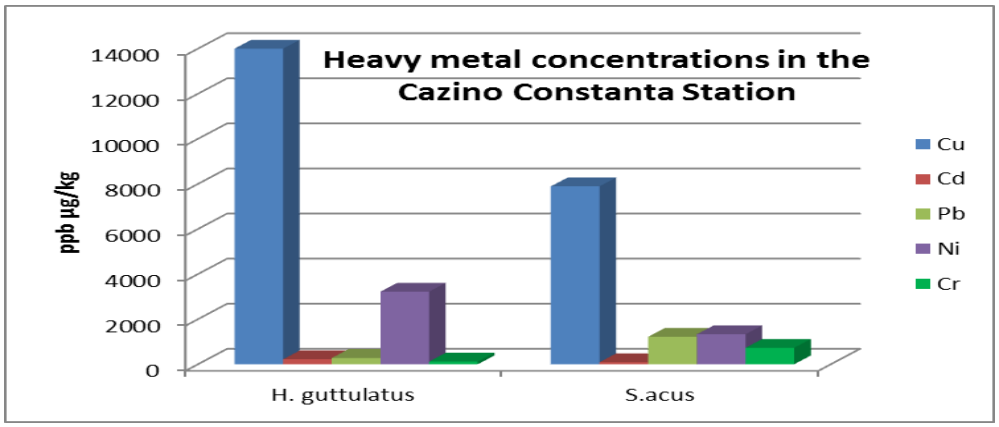

Fig. 2. Heavy metal concentrations in *H. guttulatus* and *S. acus* whole tissue - Cazino Constanta Station.

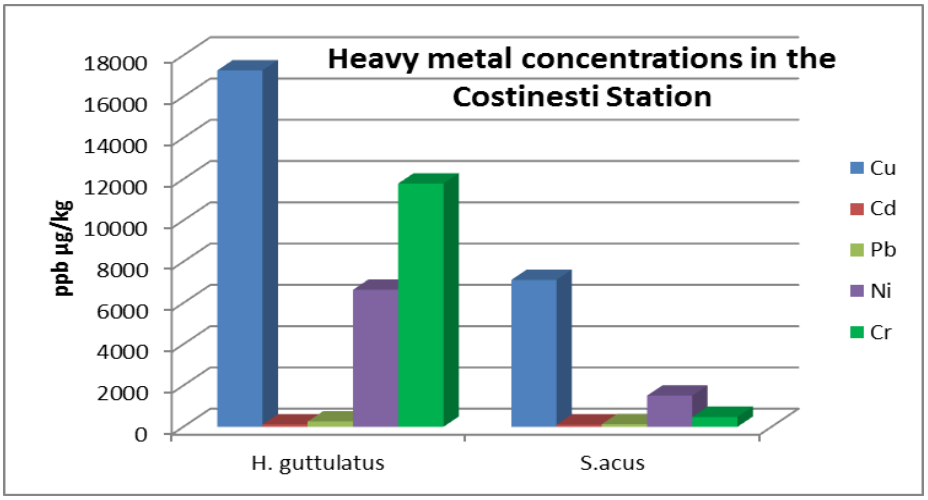

Fig. 3. Heavy metal concentrations in *H. guttulatus* and *S. acus* whole tissue - Costinesti Station.

Concerning the heavy metal concentrations in water, overall low values were recorded (Fig. 4). Higher values of lead were noticed in both stations (3.05 µg/l in Cazino Constanta and 2.95 µg/l in Costinesti). Chrome also recorded higher values in both locations (1.09 µg/l in Cazino Constanta and 1.81 µg/l in

Costinesti). Copper also recorded high values in Cazino Constanta (1.64 µg/l), while cadmium recorded the lowest values in both sampling stations: 0.84 µg/l in Cazino Constanta and 0.86 µg/l in Costinesti.

The order of concentrations was the following: Cazino Constanta - Pb > Cu > Ni > Cr > Cd; Costinesti - Pb > Cr > Ni > Cd > Cu.

With reference to heavy metal concentrations in sediments, high values were recorded by nickel at Cazino Constanta (73,090 µg/kg DW) and Costinesti (50,530 µg/kg DW). In addition, chrome also recorded high values, with a peak concentration in Costinesti (79,120 µg/kg DW), and 48,930 µg/kg DW in Cazino Constanta. The lowest values in sediments were recorded by cadmium: 210 µg/kg DW in Cazino Constanta and 170 µg/kg DW in Costinesti.

The order of concentrations was the following: Cazino Constanta - Ni > Cr > Cu > Pb > Cd; Costinesti - Cr > Ni > Cu > Pb > Cd.

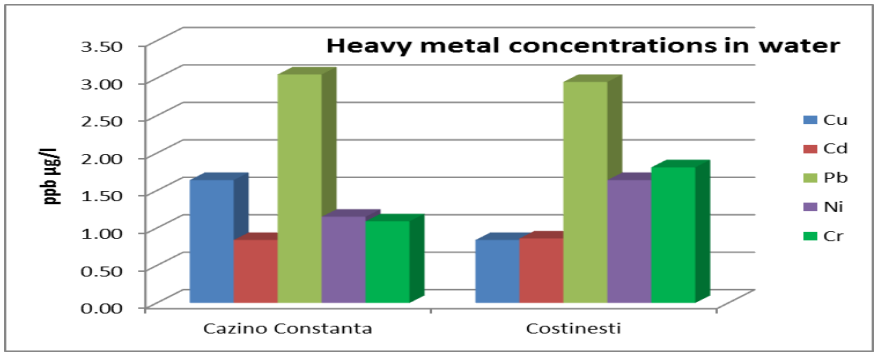

Fig. 4. Heavy metal concentrations in water (both stations).

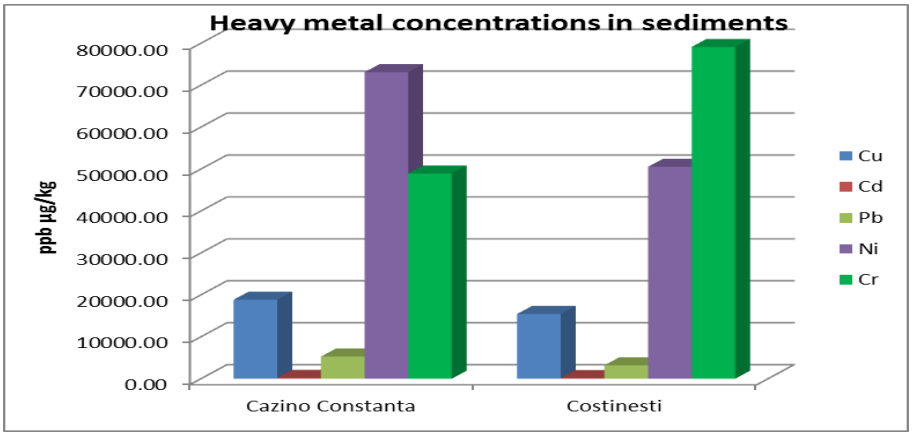

Fig. 5. Heavy metal concentrations in sediments (both stations).

Applying the above mentioned formulae, the following values were obtained for the Bioconcentration Factor (BCF) and the Biota-Sediment Accumulation Factor (BSAF) (in red exceedings of threshold values) (Table 2).

Table 2. BCF and BSAF values recorded for *H. guttulatus* and *S. acus*.

Cazino Constanta Station	**Cu**	**Cd**	**Pb**	**Ni**	**Cr**
BCF *H. guttulatus*	8536.58[c]	261.9	91.8	2800[a,b]	110.09
BSAF *H. guttulatus*	0.74	1.04[d]	0.05	0.04	0.002
BCF *S. acus*	4819.97[a,b]	107.14	400	1156.52[a]	669.72
BSAF *S. acus*	0.41	0.42	0.23	0.018	0.014
Costinesti Station	**Cu**	**Cd**	**Pb**	**Ni**	**Cr**
BCF *H. guttulatus*	20599.52[c]	139.53	88.13	4048.78[a,b]	6513.81[c]
BSAF *H. guttulatus*	1.11[d]	0.7	0.08	0.13	0.14
BCF *S. acus*	8476.19[c]	127.9	44.06	920.73	265.19
BSAF *S. acus*	0.46	0.64	0.04	0.02	0.006
[a] BCF ≥ 1000 (bioaccumulative) (REACH) [b] BCF ≥ 2000 (bioaccumulative) (TSCA) [c] BCF ≥ 5000 (very bioaccumulative) (REACH & TSCA)			[d] BSAF > 1 (microconcentrator) (Dallinger, 1993) [e] BSAF > 2 (macroconcentrator) (Dallinger, 1993) BSAF < 1 (deconcentrator) (Dallinger, 1993)		

As noted in Table 2, the highest BCF was recorded by copper in both sampling stations for both species. In *H. guttulatus*, copper was found to be *very bioaccumulative* in both stations (BCF > 5000), and in *S. acus* = *very bioaccumulative* in Costinesti (BCF >5000) and *bioaccumulative* (BCF >2000) in Cazino Constanta.

Another *bioaccumulative* metal was, according to the results obtained, nickel, which recorded exceedings in the Cazino Constanta Station both in *H. guttulatus* and in *S. acus* (BCF >2000).

In Costinesti, nickel was *bioaccumulative* (BCF >2000), but only in *H. guttulatus*. In Costinesti, a high BCF value was also recorded by chrome (BCF >5000), in seahorse tissue.

As *H. guttulatus* and *S. acus* are fish species living close to the substrate, clinging on macrophyte algae thalli (seahorses) or hiding in seagrass thickets (pipefish), which makes them prone to bioaccumulation from sediments, the BSAF values were also calculated (Table 2).

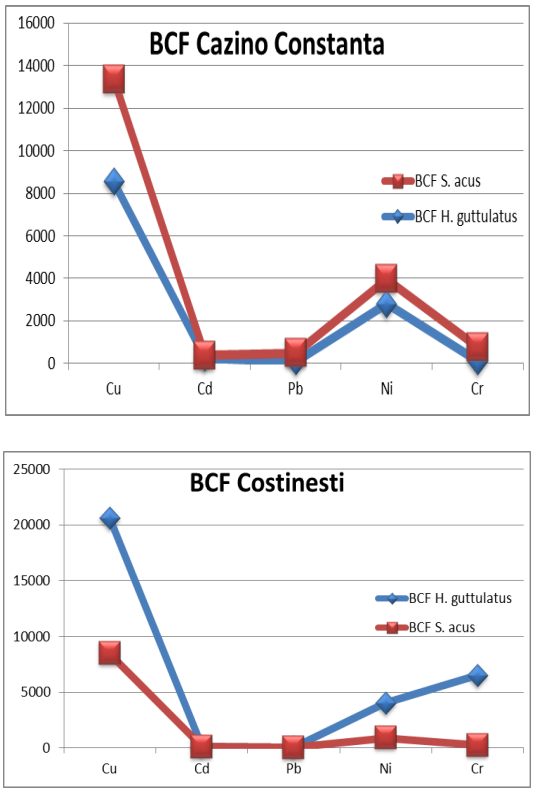

Fig. 6. BCF values in *H. gutt ulatus* and *S. acus* (both stations).

The Biota-Sediment Accumulation Factor (BSAF) values recorded in pipefish were low (BSAF<1), which rates thyem as *deconcentrators*. In seahorses, exceedings were recorded only for cadmium (Constanta) and copper (Costinesti) (BSAF>1), rating them as *microconcentrators*. The other trace elements recorded values which rate them as *deconcentrators* (BSAF < 1). Given the heavy metal bioaccumulation levels recorded by the two studied species, it was interesting to compare the results to values recorded by the five heavy metals in other fish species from the Romanian Black Sea coast (Fig. 8).

It can be noticed that, apart from copper and nickel, the concentrations of the other heavy metals (Pb, Cd and Cr) are comparable to those recorded in other fish species in shallow coastal waters. For copper, the similar concentration with the one recorded in turbot (*Psetta maxima maeotica*) tissue stands out, as turbot is exclusively benthic and very much exposed to bioaccumulation of trace metals directly from the substrate. Once again, the importance of other factors which act together with the bioavailability of heavy metals in the marine environment must be emphasized.

High copper values in some marine organisms can be explained by the presence of haemocyanin, a copper-based protein molecule (unlike the iron-based haemoglobin) which carries oxygen through the body (Rainbow et al., 1989). The best-known example of an animal with copper-based blood is the horseshoe crab (*Limulus polyphemus*), but a number of other arthropods and various mollusks also have copper-based blood. The protein in copper-based blood, called haemocyanin, functions better than iron-based haemoglobin would in carrying oxygen through the mollusks' bodies in the cold, oxygen-poor depths of the sea. However, it is not the case of Syngnathids and the other Black Sea fish species, which, as vertebrates, have iron-based blood (Rainbow et al., 1989). Consequently, the source of copper must be external and probably of anthropogenic origin.

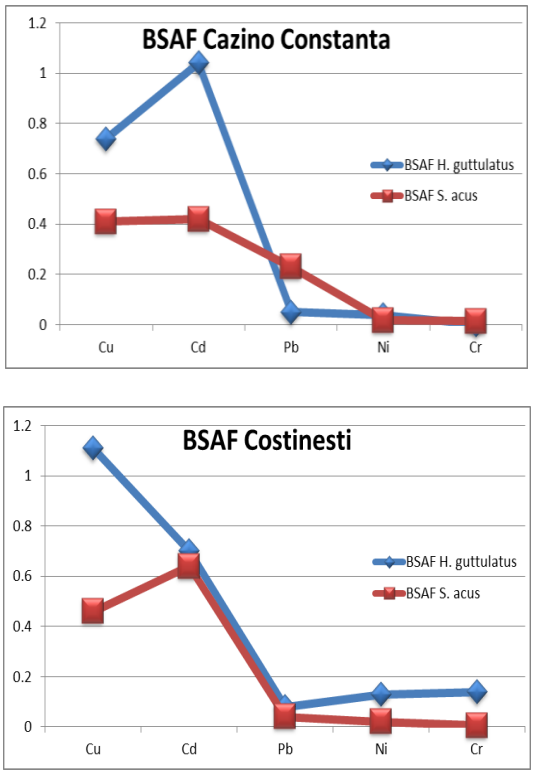

Fig. 7. BSAF values in *H. guttulatus* and *S. acus* (both stations).

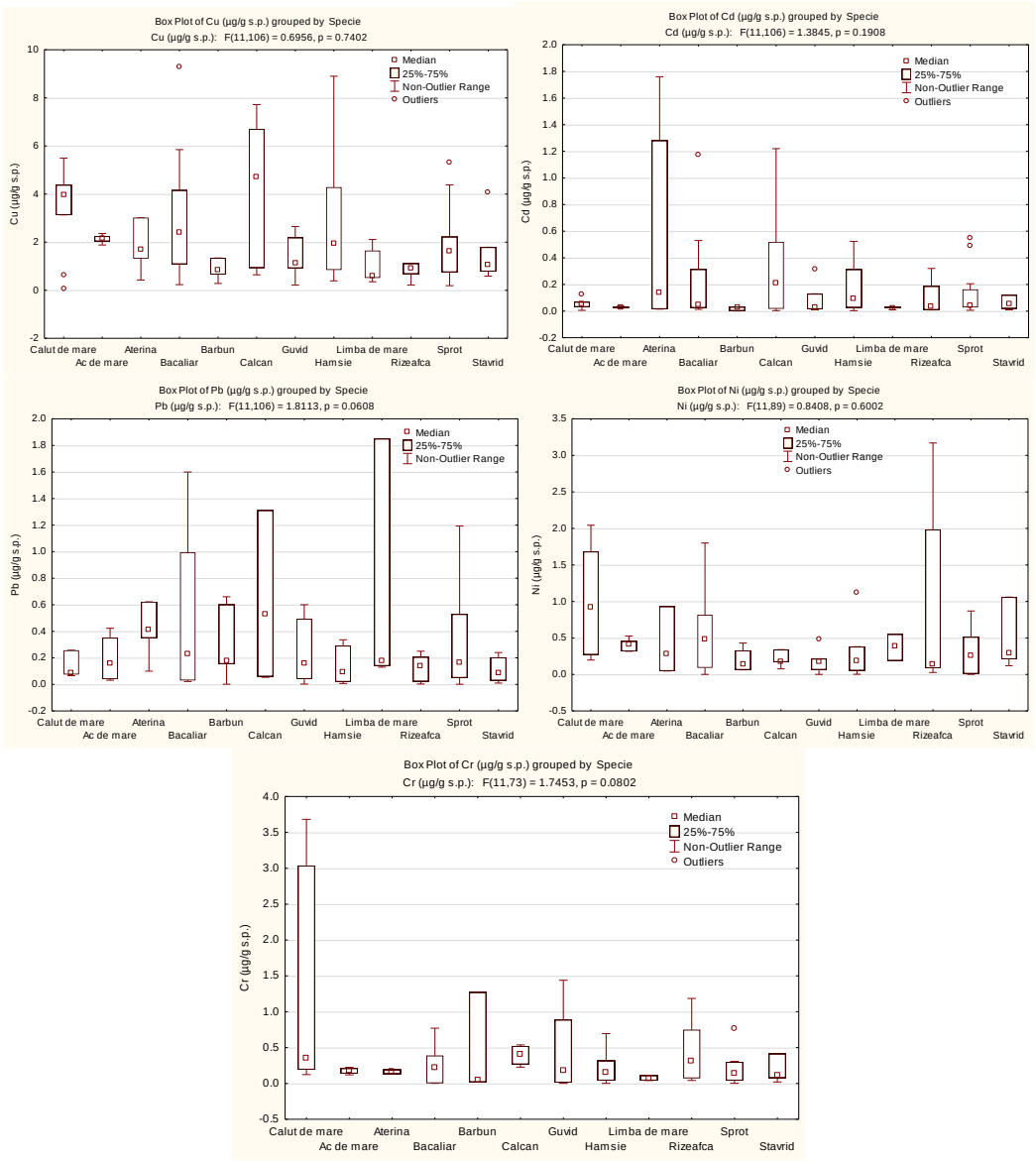

Fig. 8. Heavy metal contamination of Syngnathids compared to other fish species in Romanian Black Sea waters.

Conclusions

The results obtained showed that seahorses and pipefish from shallow Romanian coastal waters bioaccumulate heavy metals at different rates, with some differences between sampling stations as well (statistically significant, $p < 0.05$). For instance, copper recorded the highest values in both species: a mean value of 17.27 ±1.96 μg/g DW in seahorses (Costinesti) and 7.89 ± 0.36 μg/g DW in pipefish tissue (Cazino). High values were also recorded in seahorse tissue by nickel (6.64 ±0.66 μg/g DW) and chrome (11.79 ±1.13 μg/g DW) in Costinesti, and in pipefish tissue by lead (1.22 ± 0.27 μg/g DW) in Constanta. The Bioconcentration Factor (BCF) recorded high values (>5000, *very bioaccumulative*) for copper in the Constanta station and for nickel (>2000, *bioaccumulative*) in both species. In Costinesti, the results were similar for copper (>5000, *very bioaccumulative*) in both species, while nickel and chrome recorded high values (>5000, *very bioaccumulative*) only for seahorses. The Biota-Sediment Bioaccumulation Factor (BSAF) values in pipefish were low (<1), while for seahorses high values were recorded only for cadmium (Constanta) and chrome (Costinesti). These results indicate that *H. guttulatus* and *S. acus* uptake heavy metals from the surrounding environment, being bioaccumulative and bioconcentrators. Concerning the interspecific differences, seahorses, due to their lower mobility and smaller habitat ranges as compared to pipefish, tend to accumulate more heavy metals. Moreover, comparable levels were found between heavy metals accumulated in Syngnathids and in benthic fishes (turbot) from shallow Romanian Black Sea waters.

The high copper values in some marine organisms can be explained by the presence of haemocyanin, a copper-binding protein. However, it is not the case of Syngnathids and the other Black Sea fish species, which, as vertebrates, have iron-based blood (Rainbow et al., 1989). This leads to the conclusion that the source of copper must be external and probably of anthropogenic origin (it may be caused by vessel traffic, as copper is used as antifouling agent on hulls) (Nenciu et al., 2014b).

Nevertheless, based solely on the data available so far and the calculated factors, one cannot draw the conclusion that one sampling station is more polluted than the other. The influence of diet on the heavy metal uptake (biomagnification), as well as of other physiological factors (age, sexual maturity, diseases etc.) are also to be taken into account for future research.

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