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Integrated environmental evaluation of heavy metals and metalloids bioaccumulation in invertebrates and seaweeds from different marine coastal areas of sardinia, mediterranean sea

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1   **Integrated environmental evaluation of heavy metals bioaccumulation in invertebrates and**

2   **seaweeds from different marine coastal areas of Sardinia, Mediterranean Sea.**

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16

17   **Running title:** Heavy metals accumulation studies in marine coastal areas of Sardinia,

18   Mediterranean Sea.

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26

27 **Abstract**

28 In this work, three gastropods *Patella vulgata*, *Osilinus turbinata,* and *Tahis clavigera*, one

29 echinoderm *Parancetrotus lividus*, one coelenterate *Anemonia sulcata*, and two seaweed *Padina*

30 *pavonica*, and *Cystoseira mediterranea* were collected from three different marine areas of Sardinia

31 in the Mediterranean sea and studied for heavy metals and metalloid content and accumulation

32 trends.

33 Inductively coupled plasma optical emission spectrometry (ICP-OES) was used for the

34 determination of Al, AS, B, Ba, Be, Cd, Co, Cr, Cu, Fe, Hg, Mn, Mo, Ni, Pb, Sb, Se, Sn, Sr, Te, Ti,

35 V and Zn in the selected samples. The results showed that *gastropods* were capable of accumulating

36 Al, Ba, Cu, Fe, Sr, and Zn; *seaweeds* can better concentrate Al, Fe, and Zn than all other species. At

37 the same time, *echinoderms and coelenterate* had limited ability to store specific metals, showing a

38 much more homogeneous distribution. PCA analysis allowed us to discriminate among the sites and

39 the species. Cala Zafferano was the area with the higher values of accumulation of all metals in all

40 species as expected, considering its proximity to industrial sites. The results of the analysis showed

41 clearly that heavy metal and metalloid accumulation was different for each species studied.

42 Therefore, for a correct environmental assessment of a given area, a comprehensive approach is

43 strongly recommended by exploiting the different properties of both accumulation and

44 concentration of the metals by different aquatic species.

45

46 Summary

47 Different species of aquatic living organisms have different heavy metals and metalloid

48 accumulation trends, even if they belong to the same family and the same water column or have

49 similar living habits.

50

51 **Keywords**: Heavy metals; accumulation; gastropods; *Paracentrotus, Anemonia*, seaweed, PCA

52

Introduction

Metals are usually found in the environment worldwide, their presence caused by natural events or because of anthropogenic activities, such as industrial sites and agricultural practice (Kamaruzzaman et al., 2011). The increase in environmental metal concentration in these areas can reach 100-1000 times the normal levels of Earth's crust (Carral et al., 1995). The hydrological cycle of water between the land, open water surface and sea can bring to significant pollution, exposing the aquatic ecosystems directly to a great variety of these metals causing severe contamination of marine organisms (Farombi et al., 2007).

Metals are mutually essential elements necessary to support biological activities and unneeded chemicals with an unidentified role (Roesijadi and Robinson, 1994). Heavy metals are problematic pollutants because, unlike most organic contaminants, they are non-biodegradable and can accumulate in living tissues and food, causing a significant threat to both human health and environmental safety. The most hazardous heavy metals and metalloids are Be, Cr, Hg, Cd, Pb, and As. These metal ions are low soluble in water, can cause toxicity, and have serious side effects on human health. After discharged in the water, they are usually accumulated by sediments and transferred to invertebrates and biomagnified in fish populations accumulating in the lipid tissue (Fergusson, 1990; Yi et al., 2011; Jakimska, 2011; Jaishankar et al., 2014; Jitar et al. 2015). An accurate assessment of the degree of metal contamination at a specific location requires an adequate estimation of these elements' natural levels in the biological communities and the natural environment. Mussels, as interdital filter feeders, accumulate pollutants coming from local contamination and distant areas influenced by sea current and thus can lead to inaccurate information on the real environmental situation of restricted territories. Therefore, environmental pollution monitoring studies relying on their ability to concentrate high levels of metal in the soft tissue used mussels, invertebrates, and marine vegetables widely (Philips, 1977; Ingston, 1982; Goldberg et al., 1983; Cossa, 1989; Bryan and Langston, 1992; Abisil et al., 1997; Hummel et al., 1997).

Commentato [IC1]: Ho messo la bibliografia dopo widely.

3

79   The use of sedentary herbivorous such as gastropods, and echinoderms, seaweeds holdfast, and

80   anemonia has several advantages. These living organisms are available all year, simple to collect,

81   resistant to transportation and conservation, and have enough tissues for the analysis.

82   In the last decades, metals bioaccumulation has been investigated in different biological

83   communities such as invertebrate gastropods (Vinogrjidov et al., 1953; Blackmore, 2000; Perez-

84   Lopez et al., 2003; Amin et al., 2006; Collado et al., 2006; Yüzereroğlu et al., 2009; Ramirez, 2013;

85   Kelepertzis, 2013; Boucetta et al., 2016; Duysak and Azdural, 2017; Aydin-Onen and Ozturk, 2017;

86   Gawad, 2018), anemone (Mitchelmore et al., 2003; Horwitz et al., 2014), or echinoderms (Salvo et

87   al., 2014, Chiarelli et al., 2019). Moreover, some authors investigated seaweed such as *Padina* spp

88   and *Cystoseira* spp (Aydin-Onen and Ozturk, 2017; Rodríguez-Figueroa et al., 2008; Benfares et

89   al., 2015; Ryabushko et al., 2017). However, most articles reported pollution from 4 to 15 metals in

90   one or two species.

91   The study aimed to compare metals accumulation performance of 7 living organisms (invertebrates

92   and vegetables) in three marine areas of Sardinia characterized by the different anthropic impact.

93   Cala Zafferano (CZ) affected by heavy metal contamination, Capo Frasca (CF) and Capo

94   Spartivento (CS), representing potentially clean environments, exposed to NW, SW and SE

95   currents. Three gastropods *Patella vulgata*, *Osilinus turbinata,* and *Tahis clavigera*, one

96   echinoderm *Parancetrotus lividus*, one coelenterate *Anemonia sulcata*, and two seaweed *Padina*

97   *pavonica*, and *Cystoseira mediterranea,* were collected. All samples were subjected to

98   mineralization and analysis by ICP-OES to determine an exhaustive pattern of 23 metals and

99   metalloids and accumulation studies.

100 1. **Material and methods**

101 *2.1 Sample collection and processing*

102 Samples were handpicked from three sites of the Sardinia coast, Capo Spartivento (38° 52'41.90 "N;

103 8° 50'28.77 "E), Cala Zafferano Teulada (38° 53'43.26 "N; 8° 39'42.19 "E), and Capo Frasca (39°

104 42'54.98 "N; 8° 26'44.14 "E). Twenty samples of the gastropods *Patella vulgata*, *Osilinus turbinata*,

4

105 and *Thais haemastoma*, the echinoderm *Parancetritus lividus,* the coelenterate *Anemonia*

106 *sulcate,* and the seaweeds *Padina pavonica,* and *Cystoseira mediterranea*, were collected at a

107 maximum depth of 10 meters in August (Table 1). All species were present at all stations and

108 individually processed.

109 After collection, samples were immediately stored in ice cooler "drain plug open" to let the water

110 run out. This operation avoids deterioration during transport to the lab. Samples brought to the

111 laboratory were weighed, and gastropods soft tissue was separated from the shell. Sea urchin's

112 gonads were collected with a stainless-steel spatula from the shell opened with a stainless-steel

113 cutter for sea urchins. Anemone tentacles and seaweed aerial parts were selected using a stainless-

114 steel scissor. All samples, except gonads, were rinsed with deionized water to remove accidental

115 impurities, and finally stored at -20°C before analysis.

116 *Chemicals*

117 HNO$_3$ 67-69%, H$_2$O$_2$ solution 30%, and standards of Al, AS, B, Ba, Be, Cd, Co, Cr, Cu, Fe, Hg,

118 Mn, Mo, Ni, Pb, Sb, Se, Sn, Sr, Te, Ti, V, Zn were of ICP grade (Carlo Erba Reagents Milan, Italy),

119 HCl 34-37% super pure quality (Romil Spa Cambridge, England). Double-deionized water with a

120 conductivity less than 18.2 MΩ was obtained with a Milli-Q system (Millipore, Bedford, MA,

121 USA). Stock standard solutions were prepared at 1000 mg/L in HNO$_3$ 10%. Working standard

122 solutions were prepared daily by diluting the stock solution with MilliQ water.

123 *2.2 Moisture and ash*

124 Ten grams of homogenized samples were weighed and dried at 105°C in a thermostatic heater until

125 a constant weight was achieved (~ 24 h). After that, samples were stored in a desiccator to reach

126 ambient temperature and weighted for dried mater assessment. Dehydrated samples were then

127 carbonized at 450 C° in a porcelain crucible for five hours for total ash analysis. Moisture and ash

128 determinations were calculated as a percentage of the fresh product.

129

130 *2.3 Heavy metals and metalloids*

Commentato [IC2]: Weighed??

5

About 0.2 g of ashes obtained by carbonization were mineralized in a microwave CEM Mars6 system (CEM Corporation, Milano, Italy), by adding 10 mL of a mixture of HNO_3 and HCl (1:3), and 1 mL of H_2O_2. After the mineralization program, the solution was transferred into a 10 mL flask and brought to volume with MilliQ water with a conductivity less than 18.2 MΩ, filtered through 0.45 μm nitrocellulose membrane filter (Whatman, Milan, Italy) in another flask and finally subjected to analysis. Control solvent samples were simultaneously prepared to avoid false positives and contamination during the investigation.

Hg, As, and Se sample preparation were carried out using Agilent VGA-77 (Agilent, Milan, Italy) according to Agilent application notes (Agilent AN, 2014; Agilent AN, 2012). Analysis were carried out using a Varian 710ES ICP Optical Emission Spectrometer for the simultaneous analysis of 23 metals and metalloids. Operating conditions were as follows: radiofrequency (RF) generator power 1.2, frequency 40 MHz; argon (99.996% purity) was used both for plasma (15.5 L/min), nebulizer (200 Kpa) and optic supply (1.5 L/min). The sample uptake delay was 30 seconds, and the instrument stabilization delay 15 seconds. The spray chamber was a double-pass, glass cyclonic. Each measurement was made in triplicate. The power and pressure applied were 600 W and 100 PSI for 13 minutes. The limit of quantitation (LOQ) was calculated as ten times the standard deviation reading of the blank sample signal (Wenzl et al. 2016). Calibration curves were calculated on five points starting from the LOQ value and were considered acceptable for $R^2 \geq 0.999$.

2.4 Risk assessment

In this study, we used the target hazard quotient (THQ) for risk assessment. THQ represents the ratio of the estimated daily intake (EDI) / reference dose (RfD) for noncarcinogenic risk assessment methods indicating the risk level associated with xenobiotic exposure (Chien et al., 2002; Zhang et al., 2017). RfDs for oral intake were obtained from the Integrated Risk Information System (IRIS-EPA). When THQ is below 1 no adverse effect during a person's lifetime is expected. EDI was calculated as the concentration of heavy metals (mg/kg wet weight) × average daily consumption of

6

157 invertebrates in the local area (g/day bw) / body weight (Bw). To evaluate the risk of heavy metals

158 and metalloids from invertebrate consumption, we consider an average weight of 60 Kg. Total THQ

159 for each species was calculated as the sum of single metals THQ, while total THQ of each site was

160 calculated assuming the intake from all the selected species in the same day.

161 *2.5 Statistical Analysis*

162 Analysis of variance (ANOVA) was carried out with the software XLSTAT (Addinsolf LTD,

163 Version 19.4), Mean comparisons of the effects of treatments were calculated by the Fisher's least

164 significant difference test at $p \leq 0.05$.

165 Analysis data sets were imported into SIMCA 14 (Umetrics AB, Umea, Sweden) for processing

166 using principal component analysis (PCA). Additionally, R2 (the coefficient of determination) and

167 Q2 (the cross-validated correlation coefficient) were used as measures for the robustness of a

168 pattern recognition model (Atherton et al., 2006).

169

170 **3. Results**

171 The selected sites had different anthropic conditions. Cala Zafferano (CZ) is in a military area,

172 downstream of an industrialized coastal area, and subjected to main sea NW currents. Capo

173 Spartivento (CS), located in the Gulf of Cagliari, is protected from the main current NW and

174 exposed to SW and SE, in contrast, Capo Frasca (CF) is located in the south of the Gulf of Oristano

175 but totally exposed to NW current. Both sites are far from industrial and agriculture areas (Figure

176 1). All organisms tested were present in each site and were selected with similar size and weight

177 among the species (Table 1). The analysis of water content and ashes showed similar values in the

178 three sites for gastropods, ranging in average from 74.44 ± 2.43% (*Osilinus*, CZ), to 79.30 ± 1.05%

179 (*Patella*, CZ). Ash content ranged on average from 3.75 ± 2.41% (Thais, CF), to 8.52 ± 25.75%

180 (*Osilinus*, CZ). However, the higher water levels were detected for *Anemonia*

181 *sulcata*, *Paracentrotus lividus* and *Padina pavonica* accounting in average for 90.85 ± 1.72%, 81.72

Commentato [IC7]: Perché maiuscolo?

Commentato [IC8]: Non deve andare in corsivo?

182 ± 2.77%, and 82.45 ± 1.82%, respectively, while the lower levels were for *Cystoseira*

183 *mediterranea* with an average value of 72.52 ± 7.12% (Table 2).

184 Seaweeds showed the higher mineral content with ash average values accounting for 15.35 ±

185 17.98% (g/100g ± RSD, FW) and 12.86 ± 19.98% (g/100g ± RSD, FW) in *Padina* CZ

186 and *Cystoseira* CS, respectively, almost three times higher than the analyzed invertebrates. Instead,

187 the lowest ash levels were detected in *Anemonia sulcate* CZ and *Paracentrotus lividus* CZ with

188 average values of 3.50 ± 7.89% and 2.10 ± 12.60% (g/100g ± RSD, FW), respectively.

189 Gastropods soft tissue, echinoderms gonads, and coelenterate anemone tentacles were used for the

190 analysis, while the juvenile areal parts were selected for seaweeds.

191 The ICP-OES analytical method allowed the analysis of 23 heavy metals and metalloids.

192 Calibration curves showed R^2 values ranging from 0.9967 (Pb) to 0.9997 (Co), and the limits of

193 quantification of the method were suitable to evaluate overall pollution and risk assessment (Table

194 3) (Wenzl et al., 2016).

195 Among the heavy metals and metalloids searched within the methods, eight were missing (Be, Co,

196 Hg, Mo, Sb, Se, Sn, Te), and only 6 (Al, Cu, Fe, Sr, V, Zn) were found in all samples from the three

197 sites. Gastropods and seaweeds showed in all sites the presence of 12 heavy metals and

198 metalloids, *Paracentrotus* 10, and *Anemonia* 9 (Table 4-6).

199 *3.1 Gastropods*

200 *Patella vulgata* showed higher values for seven heavy metals and metalloids (Al, Ba, Fe, Mn, Pb,

201 Sr, Ti) in the samples from CZ, while CS and CF showed even values. B, Cd, Cr, Cu, V, and Zn had

202 similar values in all three sites. Fe, Sr, and Al from CZ had the higher mean values accounting for

203 34.28 µg/g, 7.06 µg/g, and 6.44 µg/g, respectively, about four times higher than those from CF and

204 CS (Table 3). *Osilinus turbinatus* had the higher mean values in the samples from CZ for Al, Cr,

205 Cu, Mn, Fe, Ni, Pb, Sr, Ti, and Zn, among these, the highest concentrations were detected for Fe

206 40.22 µg/g, Sr 19.08 µg/g, Al 8.77 µg/g, and Ni 7.93 µg/g. B, Ba, Cd, and V had similar values in

207 the three sites. Also, *Thais* showed higher values in the samples of CZ. In particular, Al, Ba, Cu, Fe,

8

208   Pb, and V were the most concentrated, with Fe 36.89 μg/g, Cu 29.68 μg/g, Zn 21.91 μg/g, and Ba

209   10.04 μg/g with the higher values (Table 4).

210   Comparing the three gastropods in the three sites, Al, B, Cu, Fe, and Ti showed similar values in the

211   samples from CZ for *Patella* and *Osilinus*, Mn and Sr for *Patella* and *Thais*, while Cr had similar

212   values in all sites. In CZ, *Thais* showed higher mean values of Ba, Cd, Cu, and Zn, and lower mean

213   values for Al, B, and V.

214   In CS, B, Fe, and Ti had overlapping values in *Patella* and *Osilinus*, while Mn and Sr were similar

215   in *Osilinus* and *Thais*. Al, Ba, Cd, Pb, and Zn had different values in all sites.

216   CF showed more considerable variability among all metal's accumulations in the three species.

217   Considering all sites for the single species, the samples of CZ showed higher levels of all metals,

218   except for Zn, which showed overlapping values in the three sites for the three species.

219   *3.2 Seaweeds*

220   Heavy metal and metalloids distribution followed an entirely different and uneven distribution

221   among the sites and between the two seaweed species (Table 5). *Padina* and *Cystoseira* showed

222   both the higher values in the samples of CZ, Fe 225.46 μg/g, Al and Zn both at 104.4 μg/g, and Sr

223   77.46 μg/g in *Padina*, and Fe 184.03 μg/g, Al 41.85 μg/g, Sr 55.46 μg/g, Zn 41.84 μg/g, Ba 32.08

224   μg/g, B 12.60 μg/g, and Mn 6.58 μg/g in *Cystoseira*.

225   Al, Fe, and Zn in *Padina* showed higher concentration in CZ compared to CS and CF, and

226   to *Cystoseira* samples in all sites. *Cystoseira* levels of Al, B, Ba, Cu, Fe, Mn, Sr and Zn were higher

227   in CZ samples, while Cr, Pb, Ti and V were higher in CF.

228   *3.3 Echinoderms and coelenterate*

229   *Parancetrotus lividus* accumulated only a few metals at moderate concentrations, Al, Sr, and Zn

230   were highly concentrate in CZ. At the same time, the other trace elements showed values in an even

231   range in the three sites. The higher levels were detected for Zn 9.24 mg/g, Al and Sr 5.64 μg/g, and

232   Fe 4.25 μg/g in CZ. The levels of Fe were similar in CZ and CF, and lower in CS (Table 6).

9

233 *Anemonia sulcata* showed the presence in the tentacles of some minerals at low levels, among the

234 heavy metals detected, Fe 4.07 μg/g, and Al 2.21 mg/g were the most abundant in CZ. Also, Sr and

235 Zn showed values slightly higher in CZ than in CS and CF (Table 5).

236 Accumulation results from all samples showed an overall content of metals higher in Cala

237 Zafferano, especially for Fe, Al, and Zn in seaweeds. These findings confirmed an increase in the

238 environmental pollution in this area which suffered the anthropic impact of the industrial area due

239 to the exposition to NW current (Figure 1).

240 Among the heavy metals and metalloids considered most dangerous, Hg was never detected at a

241 value above the LOQ of the method (0.05 μg/g), which was ten times below the MRL set for fish

242 (CR-EC No 1881/2006). Arsenic was below the LOQ in all samples except in seaweed, and

243 *Cystoseira* showed higher values in all sites. Pb was not present in *Paracentrotus* and *Anemonia*,

244 and the samples of *Osilinus* from CS and CF. In the other samples, its values were all below the

245 MRL (0.3 μg/g), except in the samples of CZ accounting for 0.50 μg/g in *Patella* and 0.46 μg/g

246 in *Thais*. Cd was not detected in *Anemonia*, while showed values below the MRL (0.05 mg/g)

247 in *Paracentrotus* and *Padina* from CS and CF. All other samples showed values above the MRL,

248 with *Patella* showing a value four times higher of the MRL, and *Thais* 20 times higher. No

249 differences were detected among each species between CS and CF sites.

250 *3.4 Multivariate analysis*

251 Multivariate analysis statistics could help in better discriminate among the different species, due to

252 the high number of samples and variables from the analysis. In this paper, we have used the

253 principal component analysis (PCA) approach do describe if the various species studied had

254 different heavy metals accumulation capacity and which were the most discerning heavy metals.

255 PCA biplot of score and loadings for CZ and CS showed a separate group of datasets for each

256 species, allowing to identify 12 clustering groups and to discriminate metals along PC1 and PC2

257 axis (Figure 3).

258 PCA emphasize the differences in accumulation of the seaweed *Cystoseira* and *Padina* in CZ
259 samples to the other species studied in the same area and to all the other samples collected in CS.
260 Among the gastropod, *Thais* was the most discriminating species. CZ and CS samples were
261 separated from the other gastropod's samples, invertebrate and seaweed, moreover, the samples
262 from CZ were split from CS along the PCA1 axis stressing a more influence of the residues of the
263 most discriminating metals for this axis. The samples of *Patella*, *Osilinus* and *Paracentrotus* fall in
264 a restricted zone of the PCA plot, despite this they were well separated between CZ and CS along
265 the PC1 axis. The metals Pb, Ba, Fe, Mn, Al, Cr, and B were the most discriminating along the PC1
266 axis, while Cu, Zn, Mo, and Cd were the most discriminating in PC2 axis (Figure 3).
267 Risk assessment showed total THQ for each species below 1, indicating no adverse effects from the
268 consumption of the selected organisms. In all sites, *Thais* showed higher values, while *Anemonia*
269 showed lower values in all sites (Table 7). Moreover, total THQ for the site obtained from the sum
270 of total THQ of the various species were below 1, confirming that no adverse effects were reached
271 also consuming the different species in the same day.
272 The values of total THQ for the site confirmed that CZ had higher harmful metals concentration
273 than CS and CF, and the amounts consumed should be taken into consideration (Table 7).

274 **4. Discussion**

275 Biomonitoring studies have the scope to assess the environmental occurrence of pollutants
276 potentially toxic for human and other living organisms. Different approaches can be used, such as
277 bioconcentration, that studies the direct partition form the environment to target organisms, and
278 biomagnification that is strictly related to the diet leading to increase xenobiotic concentration in the
279 feeder than in the diet. In contrast, bioaccumulation describes the accumulation and enrichment of
280 contaminants in a selected living organism. This process is species-dependent since different
281 organisms have different feeding habits, mechanisms of detoxification and metabolism (Jasminska
282 et al. 2011). Metals and metalloids are commonly present in the environment and can be essential

Commentato [IC11]: Ripetizione. Lo hai già messo a inizio frase.

283　elements supporting biological activities or have adverse effects decreasing aquatic species or being

284　dangerous for human being trough the food chain.

285　Gastropods, echinoderm, and anemonia species are usually eaten in Sardinia. THQ showed that the

286　intake of single metals from the selected areas trough the diet did not create an alarm for the

287　population if food quantities remains in the local typical FDI (food daily intake).

288　Metals do not degrade and along the trophic pyramid tend to increase, and anthropic activity or

289　geochemical conditions can influence their concentration, the natural levels can grow significantly,

290　becoming harmful to human safety. Literature data reported xenobiotic accumulation on suitable

291　species used as indicators. Although most studies have addressed local issues, their impact is

292　important to assess the different bioaccumulation trends of the organisms used.

293　Perez-Lopez et al. (2003) studied the contamination of *Patella vulgata* from Zn, Cd, Pb and Cu with

294　levels ranging in the soft tissue from 91 to 429 µg/g for Zn, 5.6 µg/g for Cd, 1-3 µg/g for Pb, and 2-

295　10 µg/g for Cu.

296　Kelepertzis (2013), studying the heavy metal concentration in *Patella*, postulated that many

297　mineralized areas give rise to higher levels of Pb (96 vs 8 µg/g), Zn (196 vs 48 µg/g), Mn (36 vs 10

298　µg/g), and Cu (28 vs 6 µg/g) bioconcentration. In contrast, Ni (9 µg/g), and Cr (8 µg/g) were even,

299　the levels of metals decreased in the line Zn > Pb > Mn> Cu.

300　Duysak and Azdural (2017) studied the accumulation from 8 sites in the different tissues

301　of *Patella* of Fe, Zn, Cd, Cu, Co, Ni, Mn, Pb, Cr and Al, with the higher concentration in all sites

302　for Fe, Zn, Cd, Co, Pb, and Al.

303　Collado et al. (2006) found that mean total concentrations of Cd, Cu, Pb, and Zn in *P. rustica* were

304　0.37 ± 0.05, 1.77 ± 0.09, 1.27 ± 0.07 and 8.84 ± 0.71 µg/g DW (mean $\pm$ S.E.) respectively; whereas

305　in *P. candei crenata* were 0.71 ± 0.10, 2.94 ± 0.11, 0.09 ± 0.01 and 33.74 ± 1.15 µg/g DW (mean $\pm$

306　S.E.).

Commentato [IC12]: Through?

12

307 Gadaw (2018) studied the accumulation in the gastropod *Lanistes carinatus* of Mn, Fe, Co, Ni, Cu,

308 Zn, Cd, and Pb. With values ranging from 240 to 567 µg/g dw for Fe, 5.12 – 32.8 µg/g dw for Mn,

309 Zn 5.35-28.15 µg/g dw, 0.69-3.25 µg/g dw for Cu, 4.42-13.87 µg/g dw for Ni, 0-9.24 µg/g dw for

310 Co, 0.03-0.04 µg/g dw for Cd, and 0.24-0.40 µg/g dw for Pb.

311 Jakimska et al. (2011) reported the levels of Zn and Fe in gastropods. In these molluscs these two

312 metals have essential biochemical functions, Zn is a constituent of haemocyanin, Fe is a constituent

313 of goethite (α-FeOOH) responsible for the proper functioning of the radula. This fact can explain

314 the high levels of Zn and Fe in gastropods. Moreover, the levels of Zn are related to Cu levels

315 through the metabolic processes and homeostasis, metallothionein mediated.

316 Amin et al. (2006) studied the accumulation of Cu, Zn, Fe, Pb, Cd in the soft tissues of *Thais*

317 *aculeata* with values of 150.3 ± 58.0 µg/g DW, 158.4 ± 27.9 µg/g DW, 316.7 ± 125.3 µg/g DW,

318 11.3 ± 5.9 µg/g DW and 0.6 ± 0.3 µg/g DW, respectively.

319 Ramirez et al. (2013), studied the levels of Cd (0.69-15.81 µg/g), Cu (10.82-55.68 µg/g), Pb (0.21-

320 1.36 µg/g), and Zn (15.78-32.09 µg/g) in *Osilinus*. Later Boucetta et al. (2016) reported the

321 determination in *Osilinus* of Cd, Cr, Cu, Ni, Pb, Zn in two stations, recording different values in the

322 two stations, accounting for 318.10±38.20 µg/g and 6.51±1.47 µg/g for Zn and Pb in the first

323 station. And 3.63±1.14 µg/g for Cd, 32.70±2.90 µg/g for Cu, and 24.80±20 µg/g for Ni in the

324 station 2.

325 Salvo et al. (2014) studied the accumulation of Hg, As, Cr, Ni, Cu, V, Cd and Pb in P*aracentrotus*

326 *lividus*, their findings suggested the use of *Paracentrotus* as a bioindicator for pollution in defined

327 areas. Similar findings were presented by Angioni et al. (2012, 2014) analyzing polycyclic aromatic

328 hydrocarbon (PAH) pollution in different Sardinian coastal areas.

329 Horovits et al. (2014) analyzed symbiotic algae and the tentacles of *Anemonia* for the

330 bioaccumulation of 12 trace elements. *Anemonia viridis* regulates its internal trace element

331 concentrations by compartmentalization and excretion owing a high resilience to environmental

332 contamination from high levels of metals. Pb, Fe, and Cu were mostly associated with symbiotic

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algae, while Cd, As, and Zn were associated with the *Anemonia*. 85% of Fe was in the alga fraction, Pb and Cu were evenly distributed, while Zn, As, and Cd were mostly distributed in the *Anemonia*. Mitchelmore et al. (2003) studying the accumulation of heavy metals in anemone pointed out that symbiotic anemones accumulate Cd, Ni e Zn higher than aposymbiotic anemones, and the levels were depleted when recovered in seawater.

Data on the contamination of *Cystoseria* have been reported by Ryabushko et al. (2017) on Pb (35-50 μg/g), Cd (2-3.5 μg/g), Zn (40-65 μg/g), and Cu (13-24 μg/g), and by Benfares et al. (2015) on Cd, Pb (0-5.1 μg/g), Cr (0.1-1.9 μg/g), and Hg.

The same specimen with different size can give rise to different levels of xenobiotics; therefore, to avoid individual variation, we selected specimens from the same species with similar size in each site and among sites.

Literature most studied metals were Cd, Cr, Cu, Pb, and Zn, by using one or two organisms, mainly comparing polluted vs clean areas. The merged data from Sardinia obtained in the present study showed values for all living organisms in the lower range compare to literature data for Cd, Cr and Cu, while Pb and Zinc levels in gastropods were higher than the levels reported in the Canary Islands (Table 8).

Mercury detected in mussels collected along the coastline of Sardinia from two sampling campaigns showed values ranging from 0.035 to 0.11 μg/g and 0.048 to 0.83 μg/g (Ipolyi et al. 2004). Moreover, data reported on carnivorous and herbivorous or omnivorous fish showed that the seconds accumulate till 10 times less the concentration of the former (Levitan et al., 1974; Liu et al., 2014). Therefore, values of Hg below the LOQ in the samples of the present study were not surprising since most of the living organisms studied are mostly herbivorous or seaweed (Table 1).

The results from the studies examined showed that local pollution cannot be used for global discussions but take pictures of a scenario in restricted areas. The Mediterranean basin, which is an enclosed sea, has different contamination characteristics related to the anthropic effects on the coasts and on the sea of the single surrounding countries.

> **Commentato [IC13]:** Forse bisogna mettere Hg?

359 Therefore, the real value of these studies is related to the global information of the accumulation

360 trends of the different living organisms.

361 The present study elucidated that the species of gastropods, *Paracentrotus*, *Anemonia* and seaweed

362 living in the same water column (upper littoral zone) had different uptake capability on metals.

363 These data confirm that accumulation is species and site-dependent, being related to pollution

364 magnitude, feeding habitude and geochemical morphology of the site.

365 MVA analysis emphasizes the different accumulation capacity of the seven species collected at the

366 different upper littoral areas of Sardinia, allowing to distinguish areas with different pollution

367 contaminations in a similar contest.

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369 **5. Conclusions**

370 The data reported showed different capabilities of the species involved in accumulating different

371 metals. Gastropods are more capable of accumulating Al, Ba, Cu, Fe, Sr, and Zn, but in different

372 amount. *Thais* levels of Cu and Zn are much higher than *Patella* and *Osilinus*, seaweeds can

373 concentrate Al, Fe, and Zn (*Padina* more than *Cystoseira*) in large extent in respect to all other

374 species. At the same time, *Paracentrotus* and *Anemonia* have limited ability to concentrate on

375 specific metals but have a much more homogeneous distribution. THQ showed that Thais

376 contributed for almost 50% to total THQ of the site and that among the heavy metals Cd was the

377 most impacting on the overall health concerns for invertebrate eating.

378 All these species have been referred to be model organisms for biomonitoring environmental

379 quality and pollution.

380 Therefore, for a correct environmental assessment of a given area, a diversified approach is strongly

381 recommended by exploiting the different properties of both accumulation and concentration of the

382 metals by different aquatic species.

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Table 1. Humidity and ash content of the edible part of gastropods, Paracentrotus, anemonia, and of the seaweed sampled.

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	Humidity g/100g ± RSD FW			Ash g/100g ± RSD FW		
	C. Zafferano (CZ)	C. Spartivento (CS)	C. Frasca (CF)	C. Zafferano (CZ)	C. Spartivento (CS)	C. Frasca (CF)
Patella vulgata	79.46 a(a)	78.91 a(a)	78.01 a(a)	4.61 a(a)	4.29 a(a)	5.04 ab(a)
Osilinus turbinatus	74.44 a(a)	77.54 a(a)	75.62 a(a)	8.91 b(a)	5.10 a(b)	6.59 a(ab)
Thais haemastoma	76.53 a(a)	74.90 a(a)	76.20 a(a)	5.18 a(a)	3.75 ab(b)	4.24 b(ab)
Paracentrotus lividus	84.06 b(a)	79.54 a(a)	81.56 b(a)	2.10 c(a)	2.75 b(a)	3.30 b(a)
Anemonia sulcata	89.06 b(a)	91.44 b(a)	91.96 c(a)	3.50 c(a)	3.85 ab(a)	3.71 b(a)
Padina pavonica	81.67 b(a)	81.50 c(a)	84.18 b(a)	15.35 d(a)	13.13 c(a)	6.30 a(b)
Cystoseira mediterranea	70.21 c(a)	68.91 d(a)	78.44 b(b)	10.85 e(a)	12.86 c(a)	10.48 c(a)

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ᵃ **Values among species (without brackets) or among sites (in brackets) followed by unlike letters differ significantly by Fisher's least significant difference (LSD) procedure, P ≤ 0.05. Letters without brackets relate to comparisons of the influence of species within each site. Letters in brackets relate to comparisons of the influence of the sites among each species for each parameter.**

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Table 2. Heavy metals method parameters.

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Element	λ	MRL µg/g	LOQ	Linear regression equation	R²
Al	237.3		0.10	y=670.89x-45.48	0.9978
AS	188.98		0.10	y=79.38x-10.14	0.9991
B	249.77		0.50	y=5151.33x+789.8	0.9995
Ba	493.40		0.05	y=8035x-289.9	0.9993
Be	313.04		0.005	y=259128x-6848	0.9985
Cd	226.50	0.05	0.005	y=4804x-3.55	0.9995
Co	228.61		0.025	y=6622.7x+58.6	0.9997
Cr	267.71		0.01	y=9908.7x+97.7	0.9994
Cu	324.75		0.01	y=17117.2x-95.93	0.9996
Fe	259.94		0.10	y=3059.9x+80.8	0.9992
Hg	194.16	0.5	0.05	y=392.2x+11	0.9994
Mn	257.61		0.01	y=70780.6x-8029	0.9990
Mo	204.59		0.05	y=604.4x-5.5	0.9988
Ni	216.55		0.05	y=1642.2x-22.3	0.9986
Pb	220.35	0.3	0.05	y=297.3x-19.4	0.9967
Sb	217.58		0.10	y=49.7x-5.8	0.9981
Se	196.02		0.025	y=157.7x-11.5	0.9992
Sn	189.92		0.05	y=74.3x-4.9	0.9986
Sr	407.77		0.10	y=829945.3x+17943	0.9984
Te	214.28		0.10	y=106.6x-10.1	0.9972

Ti	336.12		0.01	y=14275x+521.8	0.9976
V	292.40		0.005	y=13753.2x+231.2	0.9996
Zn	213.85		0.05	y=4785.9x-316	0.9986

Table 3. Heavy metals (μg/g ± RSD%) in gastropods from the three sites investigated.

metal	*Patella vulgata*			*Osilinus turbinatus*			*Thais*		
	C. Zafferano (CZ)	C. Spartivento (CS)	C. Frasca (CF)	C. Zafferano (CZ)	C. Spartivento (CS)	C. Frasca (CF)	C. Zafferano (CZ)	C. Spartivento (CS)	C. Frasca (CF)
Al	6.44a(a)	1,41b(b)	1.35b(b)	8,77 a(a)	2,94 b(c)	2.80 b(c)	0,37B a(d)	0,18 b(e)	0.15 b(e)
AS	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
B	1,63a(a)	1,44a(a)	1.37a(a)	1,54 a(a)	1,35 a(a)	1.40 a(a)	1,14 a(b)	0,90 a(b)	0.92 a(b)
Ba	1,21a(a)	0,52b(b)	0.48b(b)	1,98 a(c)	1,63 a(c)	1.57 a(c)	10,04 a(d)	7,07 b(e)	6.58 b(e)
Be	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Cd	0,19a(a)	0,23a(a)	0.20a(a)	0,08 a(b)	0,10 a(b)	0.09 a(b)	0,90 a(c)	1,14 a(c)	1.01 a(c)
Co	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Cr	0,03a(a)	0,03a(a)	0.03a(a)	0,11 a(a)	0,03 b(a)	0.03 b(a)	0,04 a(a)	0,03 a(a)	0.03 a(a)
Cu	0,28a(a)	0,26a(a)	0.27a(a)	2,29 a(a)	1,64 b(a)	1.58 b(a)	29,68 a(a)	8,71 b(a)	7.58 b(a)
Fe	34,28a(a)	7,37b(b)	6.58b(b)	40,22 a(a)	15,36 b(c)	14.91 b(c)	36,89 a(a)	10,73 b(d)	11.51 b(d)
Hg	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Mn	0,33a(a)	0,07b(b)	0.05b(b)	0,69 a(c)	0,23 b(a)	0.21 b(a)	0,30 a(a)	0,36 a(a)	0.32 a(a)

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Mo	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ni	< LOQ	0,13a(a)	0.11a(a)	7,93 a(b)	0,26 b(c)	0.25 b(c)	< LOQ	0,08 a(a)	0.05 a(a)
Pb	0,50a(a)	0,07b(b)	0.08b(b)	0,18 a(c)	< LOQ	< LOQ	0,46 a(a)	0,16 b(c)	0.14 b(c)
Sb	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Se	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Sn	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Sr	7,06a(a)	1,57b(b)	1.65b(b)	19,08 a(c)	8,04 b(a)	7.58 b(a)	6,01 a(a)	6,10 a(a)	6.05 a(a)
Te	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ti	0,13a(a)	0,07b(b)	0.06b(b)	0,18 a(a)	0,09 b(b)	0.10 b(b)	< LOQ	< LOQ	< LOQ
V	0,28a(a)	0,30a(a)	0.31a(a)	0,60 a(b)	0,59 a(b)	0.54 a(b)	0,06 a(c)	0,51 b(b)	0.48 b(b)
Zn	1,69a(a)	1,88a(a)	1.75a(a)	0,62 a(b)	2,16 b(c)	1.98 b(c)	21,91 a(d)	20,31 a(d)	21.03 a(d)

[a] **Values among species (without brackets) or among sites (in brackets) followed by unlike letters differ significantly by Fisher's least significant difference (LSD) procedure, P ≤ 0.05. Letters without brackets relate to comparisons of the influence of species within each site. Letters in brackets relate to comparisons of the influence of the sites among each metal.**

Table 4. Heavy metals seaweeds.

Element	Padina pavonica			Cystoseira mediterranea		
	C. Zafferano (CZ)	C. Spartivento (CS)	C. Frasca (CF)	C. Zafferano (CZ)	C. Spartivento (CS)	C. Frasca (CF)
Al	104,40a(a)	9,13b(b)	7,30b(b)	41,85a(c)	3,85b(d)	15,09c(e)
AS	0,73a(a)	0,25b(b)	0,26b(b)	1,06a(c)	2,56a(c)	1,05a(c)
B	< LOQ	0,74a(a)	< LOQ	12,60a(b)	5,10b(c)	< LOQ
Ba	9,96a(a)	3,52b(b)	0,68c(c)	32,08a(d)	17,77b(e)	4,46c(b)
Be	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Cd	0,07a(a)	0,01b(b)	0,02b(b)	0,08a(a)	0,02b(b)	0,08a(a)
Co	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Cr	0,18a(a)	0,02b(b)	0,06b(b)	0,15a(a)	0,03b(b)	0,24c(ac)
Cu	0,42a(a)	0,10b(b)	0,15b(b)	0,79a(c)	0,18b(b)	0,15b(b)
Fe	225,46a(a)	11,41b(b)	15,79b(b)	184,03a(a)	3,94b(c)	3,56b(c)

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Hg	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Mn	2,88a(a)	0,84b(b)	< LOQ	6,58a(c)	1,40b(a)	0,73c(b)
Mo	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ni	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Pb	0,71a(a)	0,11b(b)	0,25c(c)	0,58a(a)	0,13b(b)	0,92c(d)
Sb	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Se	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Sn	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Sr	77,46a(a)	5,39b(a)	5.65b (b)	55,46a(a)	1,53b(c)	1,15b(c)
Te	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ti	2,60a(a)	0,20b(b)	0,92c(c)	1,18a(c)	0,03b(d)	4,41c(e)
V	0.72a(a)	0,21b(b)	0,25b(b)	1.10a(a)	0,52b(c)	1,50c(d)
Zn	104,40a(a)	0,23b(b)	11,42c(c)	41,85a(d)	1,50b(e)	2,97c(f)

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[a] **Values among species (without brackets) or among sites (in brackets) followed by unlike letters differ significantly by Fisher's least significant difference (LSD) procedure, P ≤ 0.05. Letters without brackets relate to comparisons of the influence of species within each site. Letters in brackets relate to comparisons of the influence of the sites among each metal.**

Table 5. Heavy metals in echinoderms, and coelenterate.

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Element	*Paracentrotus lividus*			*Anemonia sulcata*		
	C. Zafferano (CZ)	C. Spartivento (CS)	C. Frasca (CF)	C. Zafferano (CZ)	C. Spartivento (CS)	C. Frasca (CF)
Al	5.64a(a)	0,27a(a)	1,74a(a)	2,21a(a)	1.95a(a)	2.05a(a)
AS	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
B	1.25a(a)	0,53a(a)	1,27a(a)	0,63a(a)	0.59a(a)	0.60a(a)
Ba	0.98a(a)	0.25a(a)	0,40a(a)	0,13a(a)	0.12a(a)	0.10a(a)
Be	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Cd	0.06a(a)	0,04a(a)	0,03a(a)	< LOQ	< LOQ	< LOQ
Co	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Cr	0.02a(a)	0,04a(a)	0,02a(a)	< LOQ	< LOQ	< LOQ
Cu	0.27a(a)	0,14a(a)	0,17a(a)	0,20a(a)	0.12a(a)	0.16a(a)
Fe	4.25a(a)	1,85a(a)	4,38a(a)	4,07a(a)	3.81a(a)	2.95a(a)
Hg	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Mn	< LOQ	< LOQ	< LOQ	0,22a(a)	0.35a(a)	0.27a(a)
Mo	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ni	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Pb	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Sb	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Se	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Sn	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Sr	5.64a(a)	0,79a(a)	2,89a(a)	0,64a(a)	0.29a(a)	0.31a(a)
Te	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Ti	0.09a(a)	0,02a(a)	0,08a(a)	< LOQ	< LOQ	< LOQ
V	0.12a(a)	0,08a(a)	0,10a(a)	0,02a(a)	0.03a(a)	0.02a(a)
Zn	9.24a(a)	4,87a(a)	2,47a(a)	0,62a(a)	0.22a(a)	0.13a(a)

[a] **Values among species (without brackets) or among sites (in brackets) followed by unlike letters differ significantly by Fisher's least significant difference (LSD) procedure, $P \leq 0.05$. Letters without brackets relate to comparisons of the influence of species within each site. Letters in brackets relate to comparisons of the influence of the sites among each metal.**

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681   **Figure 1**. Localization of the three sites in Sardinia, main current ↑NW, and ● industrial area.
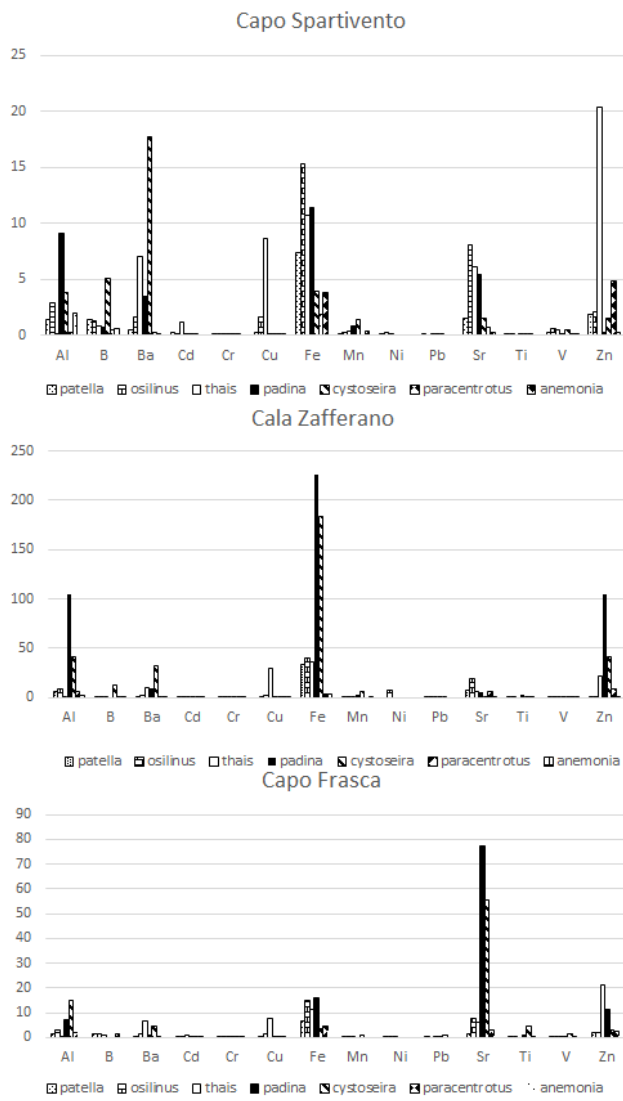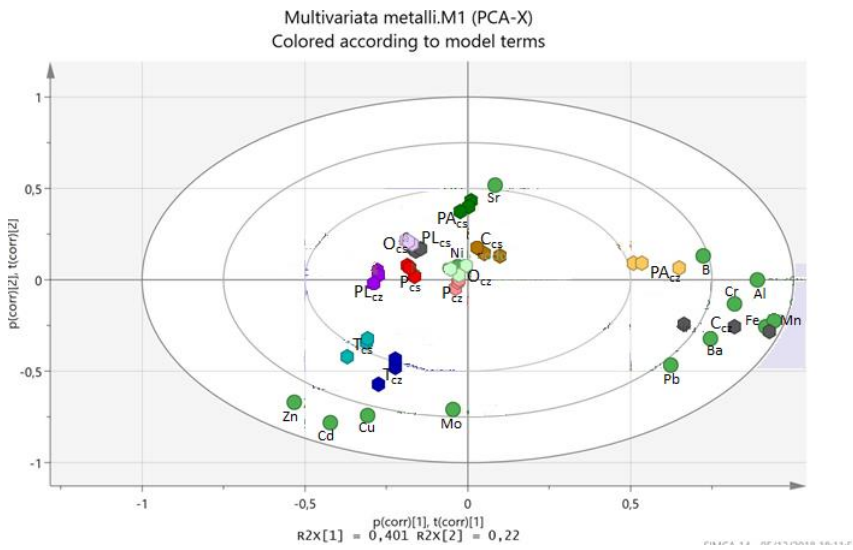
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Figure 2. Histograms of the distribution of the metals among the species in the three locations.

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Figure 3. PCA biplot of the score and loading in CZ and CS sites.