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Holothuria tubulosa as a bioindicator to analyse metal pollution on the coast of Alicante (Spain)

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Abstract

Metal pollution is a major concern worldwide. The concentration of several metals in marine sediments from Alicante, Spain (Western Mediterranean): Cabo de la Huerta, Albufereta, San Gabriel and Cabo de Santa Pola has been studied, being areas with contrasted metal stress due to anthropogenic pressures, and their bioaccumulation in different tissues of *Holothuria tubulosa* (body wall, guts and intestine). The metals with more different levels among samples were Fe, Al, V, Mn, Pb, Ga, As, Cr, Zn and B. The body wall was the tissue that showed a significantly different signature of metal levels compared to the other body parts and the sediment. The guts, followed by the intestines, were the tissues with greatest bioaccumulation.

The standard guidelines for safety limits (US EPA) for As, Cr, Pb and Zn are in the range "non-polluted". In all the areas, the quality guidelines for Effect Level, Probable Effect Level, Effect Range Low and Effect Range Medium for As, Cr, Pb and Zn are much lower than those established, indicating no biologically adverse effects resulting from exposure to these metals. Regarding the potential ecological risk, for all metals it is <40 with low risk in all zones.

The metals studied present a negative Igeo (area not contaminated). Arsenic in Albufereta and strontium in all the areas studied are the only ones that present a level 2 (uncontaminated to moderately contaminated). The Enrichment Factor (EF), with Fe as the normalising element, had a level 1-3 (minor enrichment), with the exception of Pb, B, As and Sr.

Despite the concentrations in sediments being lower compared with other parts of the world, the Biota-Sediment Assimilation Factor from the body wall was higher at As (9.2) and B (7.3). It is necessary to highlight the high levels of As in the body wall (17 to 23 mg/kg of dry material), this is surprising, and it seems to be a general trend throughout the world.

Keywords

Heavy metals, Coast of Alicante, Sediment, *Holothuria tubulosa*, Sea cucumber

1. Introduction

The rise in marine pollution is directly related to the increased population in coastal areas and the development of anthropogenic activities. The increase in urban development on the coast of Alicante (Spain) as a result of tourism-related demands between 1987 and 2000 was very notable, thus making it the leading Spanish province as regards urban development [1]. This has led to the large-scale generation of urban sewage, which includes industrial and agricultural waste and rainwater containing contaminant substances [2], that are not completely eliminated in conventional treatment plants and which are discharged into the sea off the coast of Alicante by means of submarine pipelines.

Moreover, some change in the activities of the zone that has occurred in recent years has, along with climate change, resulted in an increase in runoff and flooding during episodes of intense rains, leading to the discharge of contaminant substances directly into the sea. The creation of a landscaped public space as a "floodable deposit" with which to mitigate flood problems and the use of this water for the vegetation in the "La Marjal" flood park (Alicante) is an excellent initiative, which has consequently decreased the discharge of contaminant substances directly onto the beach [3].

However, the port of Alicante involves numerous activities, including navigation in the area of merchant ship, fishing and recreational boats that contribute to a large emission of metals into the marine environment. In addition to this port, there is a recreational port in the Albufereta area and a harbour in S. Pola that have an influence on the metal's emissions.

Discharged metals tend to accumulate in sediments and in certain marine organisms, as a result of bioaccumulation of metals consumed directly from seawater or from the food chain. An example of this is the Mar Menor lagoon, which is located south of Alicante and has suffered from the dumping of mineral waste with high concentrations of metals bioaccumulated in various organisms in the area [4].

The *Holothuria tubulosa* species is abundant in the Mediterranean Sea (Figure 1). *Holothuria tubulosa* is a species that belongs to the Echinodermata phylum. It is relatively widespread and is the most predominant species in the benthic macrofauna in *Posidonia oceanica* meadows [5,6]. This species consumes surface sediments, since it feeds on detrital material and its associated microorganisms, as a result of which it plays a central role as regards recycling debris from the seabed. They are sedentary, can easily be collected and identified, and can be used as bioindicators [7-11]. The potential use of this echinoderm as a bioindicator of local pollution is being studied but few previous studies have, to date, paid attention to the characterisation of the distribution of metals differentiated by the body parts in *H. tubulosa*. One of them is the work carried out by Culha et al. [12] in the area of Turkey, an analysis was made of the metals in the aforementioned organism in the Marmara Sea and in the Dardanelles Strait.

Figure 1. Photograph of *Holothuria tubulosa* [Obtained from: https://animalesbiologia.com/invertebrados/equinodermos/ holothuria-tubulosa-cohombro-de-mar].

When we talk about contamination by metals, lead, zinc and chromium are normally included because they present serious environmental problems, along with other light toxic elements, such as aluminum, or some semi-metal, such as arsenic that can bioaccumulate. The concentration of the different metals in sediments is variable and in this work we are going to study whose concentration was above 1 mg of metal per kg of dry sample (aluminium, arsenic, boron, barium, chrome, iron, gallium, lithium, manganese, lead, strontium, vanadium and zinc), of four points on the coast of Alicante: Cabo de la Huerta (C. Huerta), Albufereta, San Gabriel (S. Gabriel) and Cabo de Santa Pola (S. Pola), and the bioaccumulation of each metal in three body parts of the *Holothuria tubulosa* (body wall, guts and intestine).

The study of various indices was, also, contemplated, such as: the Geo-accumulation index (Igeo), the Enrichment Factor (EF), the US EPA Standard guidelines for safety limits, the Sediment Quality Guidelines (SQGs), potential ecological risk (E_r^i) and the risk index (R_i). These will allow the information obtained from the *H. tubulosa* to draw conclusions regarding the quality of the study area and its influence on the marine ecosystem.

2. Materials and methods

2.1. Study areas

Four areas on the coast of Alicante were chosen for the realisation of this work: two areas situated at either end of the coast, to the north and to the south, corresponding to C. Huerta and S. Pola, respectively, and two central areas, one in the middle of the bay corresponding to S. Gabriel Beach, in an industrial area next to the Alicante docks, and the last on Albufereta Beach, which is more enclosed and where the water is renewed to a lesser extent (Figure 2).

Figure 2. Map of study areas and their respective geolocalised sectors. Yellow: C. Huerta, green: Albufereta, orange: S. Gabriel and blue: S. Pola. QGIS Development Team, 2020. QGIS Geographic Information System. Open Source Geospatial Foundation Project. https://qgis.org.

The sample area located further to the north, C. Huerta, contains the submarine pipelines of the San Juan and Mutxavista beaches, which are also two kilometres to the north and may influence the quality of their water. The most southern study area is that of S. Pola, signifying that waste from the municipality of Alicante and the anthropogenic activities in the areas of Urbanova, Arenales and Santa Pola could affect the environment of this zone. In both cases, pollution is caused as a result of coastal drift, creating currents that displace particles in a north-south direction [13].

The S. Gabriel impact zone is the area closest to where ships leave Alicante docks where petrol spills and navigation could increase the levels of some of the metals studied in sediments and in the organisms of the area. This area is also surrounded by the “Barranco de las Ovejas” estuary and several industrial estates, and it previously had a considerable amount of industrial activity with serious effects on the marine ecosystem, leading to the deterioration of the *Posidonia oceanica*. The effects of the previous industrial activity can be seen in the marine sediments in the area, whose concentrations of B, Ba, Cr, Pb and Zn were analysed years after the industrial activities that had discharged waste directly into this area had ceased [14]. Aludium, an industry in the aluminium-processing sector, is currently located in this area, which is also affected by the large-scale dock activity and the waste from the water treatment plant that deals with the urban sewage from the south of Alicante (“Rincón de León”), along with the brine waste from the two seawater desalination plants that supply Alicante and which are located to the east of San Gabriel. Several red tides of *Gymnodinium impudicum* have occurred and these episodes were the result of specific climatological conditions, such as: lack of rain and periods of calm and high temperatures, along with an increase in the number of nutrients produced, possibly by the wastewater treatment plant of “Rincón de León”.

The area of Albufereta has been subject to many discharge episodes in the last 15 years. These were caused by a break in the pipeline, which principally contains faecal waste, and the fact that the area is highly urbanised (houses, a harbour and breakwaters from the extension to the harbour, among others), both of which have led to the beach being closed owing to unpleasant smells. The area has also been the victim of accidental diesel spillages from ships in the area, which have, on occasions, also led to the beach being closed to the public.

2.2. Sampling method

A total of 18 organisms of *H. tubulosa* were sampled, which are adequately organised by sectors and areas. Each of the areas considered was divided into two sectors separated by 200 metres (Figure 2). Three *H. tubulosa* individuals were collected randomly from each sector, and a surface sediment sample was simultaneously collected at the site in which each *H. tubulosa* was found. The samples were collected randomly in time in the different areas, during the period between March and May 2017, by means of diving. *H. tubulosa* samples were not found in the S. Gabriel area, and only the metals in sediment samples were analysed. Surface sediment was stored in polyethylene test tubes and *H. tubulosa* in polyethylene bags. Both types of samples were adequately conserved, kept cold and transported to the laboratory, where pre-treatment was carried out.

2.3. Pretreatment of *H. tubulosa* and sediment samples

The organisms were prewashed with tap water and then with distilled water, in order to avoid contamination. Three parts of each organism were considered in this study: the body wall, intestine and guts, and the samples were obtained by dissection using scissors. The degree of humidity was determined in each sample, heated at 105 ºC for 24 hours. Every sea cucumber sample was lyophilized after milling with an electric spice grinder and milled by rotational agate ball mill. Samples obtained placed in Teflon jars (0.5 g approximately) for digestion in microwave ovens, in the laboratory of the Research Support Services at the University of Alicante. To carry out the latter, 5 mL of HNO_3 and 2 mL of H_2O_2 (both from Merck p.a.) were added to the Teflon jars with the sample [15]. They were then placed in Milestone Start D equipment, in which the following sequence was employed: heated for 10 minutes from room temperature to 200 ºC, they were kept for 20 minutes, and were cooled to 30 ºC. Teflon jars were opened carefully and the extract obtained was filtered. Distilled water was added until a total volume of 25 mL.

With regard to the sediments, the samples correspond to the area and sector in which the *holothurias* had been found. The sediments were first dried to a constant weight in a heater at 105 ºC. Once dry, 0.5 g were placed in Teflon jars, using the same procedure as that employed with the *H. tubulosa*. Then an extract of the sediments and that of the body parts of the organisms were analysed together by employing ICP-MS to determine the metal composition.

2.4. Analysis of metals

The metals present in the filtered liquid extract, obtained after digestion of the *H. tubulosa* and sediments samples, were determined using the Inductively Connected Plasma Mass Spectrometry (ICP-MS) technique at the Research Support Services of the University of Alicante. This technique can quantitatively determine most of the elements of the periodic table at trace and ultratrace levels, starting from samples in aqueous solution. The sample, in liquid form, is transported by means of a peristaltic pump to the nebuliser system where it is transformed into an aerosol thanks to the action of argon gas. This aerosol is conducted to the ionisation zone in the form of a plasma generated by subjecting a flow of argon gas to the action of an oscillating magnetic field induced by a high-frequency current. Inside the plasma, temperatures of up to 8000 K can be reached. Under these conditions, the atoms present in the sample are ionised. The ions pass into the quadrupole filter through a growing vacuum interface, where they are separated according to their charge/mass ratio. Each of the tuned masses arrive at the detector where their abundance in the sample is evaluated.

The analysis was carried out with an Agilent model 8900 triple quadrupole or tandem inductively coupled plasma mass spectrometer. This system consists of two quadrupole mass transmission spectrometers in series with a non-selective octopole between them, only radiofrequency, which acts as a collision cell. Standard solutions used were the ICP multi-element standard solution IV (23 elements, Merck) and ICP multi-element standard (24 elements, Reagecon). The results were obtained with 10 standards, prepared with HNO_3, calibrated with regression coefficient near to 1 (r^2 = 0.9999 as minimum). The Standard Reference Material used to test the accuracy and precision of the device and the calibration curve were analysed in triplicate. In this study, the certified reference materials used were ERM®- CE278k (mussel tissue) for determining trace metals in *H. tubulosa* and CRM016 for the sediment samples (Table 1).

Table 1. Certified and measured values of trace metal concentrations in reference materials ERM®- CE278k (mussel tissue) and CRMO16 for sediment (mean ± SD)

Trace metals	Sediment CRM016 (mg/kg dry sample)	Measured (n=3) (mg/kg dry sample)	Mussel tissue ERM®- CE278k (mg/kg dry sample)	Measured (n=3) (mg/kg dry sample)
Al	8920 ± 660	9110 ± 150		
As	7.76 ± 0.44	7.58 ± 0.30	6.70 ± 0.40	6.20 ± 0.30
Ba	79.3 ± 2.8	78.5 ± 0.7		
Cr	14.5 ± 1.4	14.8 ± 0.1	0.73 ± 0.22	0.68 ± 0.10
Fe	16800 ± 515	16600 ± 325	161 ± 8	152 ± 5
Mn	180 ± 4	178 ± 2	4.88 ± 0.24	4.62 ± 0.22
Pb	14.1 ± 0.7	14.6 ± 0.1	2.18 ± 0.18	2.03 ± 0.02
V	22.5 ± 1.6	23.1 ± 0.5		
Zn	69.7 ± 2.1	71.0 ± 1.4	71 ± 4	69 ± 3

2.5. Statistical analysis of sediments and *H. tubulosa* from C. Huerta, Albufereta and S. Pola

An independent analysis of the contents of each body part of the echinoderm was carried out in order to discover the combinations of different metals. These body parts were: the sediment, along with the holothurian body wall, guts and intestine. This was done using a multivariate approach. These analyses employed a Euclidean similarity matrix based on the normalised (subtracting the mean and dividing by the standard deviation) data. First, a principal component analysis (PCA) was performed to visualise the ordination of the samples according to the metals contained in them and help to identify metals that showed similar distributions patterns for the sediment and the different body parts analyzed. Then, a cluster analyses was performed to classify samples and SIMPROF [16] was used as a complementary routine to find significant differences in the classification among samples. Additionally, a permutational analysis of variance (PERMANOVA) [17] was also performed, in which area (C. Huerta, Albufereta and S. Pola) and matrix (sediment, along with holothurian body wall, guts and intestine) were taken as fixed and factorial factors and site was considered to be a random factor nested in area. If the main test showed significant differences, then a pair-wise test was run to ascertain the interactions and/or the individual factors that showed significantly different metal content. In the case of the pair-wise test, the Monte Carlo test was also run for those cases in which the number of total permutations was low. The PERMANOVA was performed using the type III sums of squares (partial), assuming fixed effects adding up to zero for mixed terms and the permutation method used was the permutation of residuals in a reduced model. Prior to the PERMANOVA routine, a PERMDISP (Distance-based test for homogeneity of multivariate dispersions) analysis was used to measure the dispersion of the data for each independent factor, which is equivalent to an analysis of the homogeneity of variances in univariate analyses. After checking that the results of the PERMDISP indicated that the dispersion of the data was homogeneous, the PERMANOVA analysis was performed. Additionally, the SIMPER routine was run in order to discover which metals contributed the most to the differences among factors. All multivariate analyses were performed using the V.6 +PERMANOVA software package.

2.6. Calculation of indices

2.6.1. US EPA Standard guidelines of safety limits, Sediment Quality Guidelines (SQGs), potential ecological risk (E_r^i) and risk index (R_i)

The United States Environmental Protection Agency's (US EPA) standard guidelines of safety limits in marine sediment [18] are shown in Table 2. With regard to these metals, the sediment quality guidelines (SQGs), which were established by the National Oceanic and Atmospheric Administration (NOAA), are widely used to determine whether metals contained in sediments pose any threat to aquatic ecosystems or lead to an adverse biological impact. The SQGs establish several limit levels: Threshold Effect Level (TEL), below which adverse biological effects are not expected to occur; Probable Effect Level (PEL), above which adverse biological effects are expected to occur; Effect Range Low (ERL), which refers to the concentration at which a small percentage of biota is affected, and Effect Range Medium (ERM), which indicates a greater percentage of adverse effects resulting from metal exposure equal to or greater than this concentration level [18] (Table 2).

Table 2. Standard guidelines (US EPA) of safety limits applicable to metals in marine sediments and levels established by the National Oceanic and Atmospheric Administration (NOAA) for sediment quality guidelines (SQGs: TEL, PEL, ERL, ERM) [18].

Standard guidelines and SQGs (mg/kg)	As	Cr	Pb	Zn
Non-polluted	<3	<25	<40	<90
Moderately polluted	3-8	25-75	40-60	90-200
Heavily polluted	>8	>75	>60	>200
Effect Level (TEL)	7.2	52	30.2	124
Probable Effect Level (PEL)	41.6	160	112	271
Effect Range Low (ERL)	8.2	81	47	150
Effect Range Medium (ERM)	70	370	218	410

In the case of As, Cr, Mn, Pb, V and Zn, the potential ecological risk (E_r^i) and risk index (R_i) [19] were used to assess the comprehensive potential ecological risk of hazardous elements in the sediments:

$$E_r^i = T_r^i \frac{(C_i)_{sample}}{(C_i)_{background}} \quad \text{and} \quad R_i = \sum E_r^i$$

where T_r^i is Hakanson's (1980) coefficient for the toxicity of hazardous elements [20]. The corresponding coefficients, based on their toxicity, were As = 10, Pb = 5, Cr = V = 2 and Mn = Zn = 1, respectively. The levels of these indices are presented in Table 3.

Table 3. Relationship among the potential ecological risk E_r^i, risk index R_i and pollution levels [19].

E_r^i	Level
$E_r^i < 40$	Low
$40 < E_r^i < 80$	Moderate
$80 < E_r^i < 160$	Higher

R_i	Level
$R_i < 150$	Low
$150 < R_i < 300$	Moderate
$300 < R_i < 600$	Severe

$160 < E_r^i < 320$	High	$R_i > 600$	Serious
$E_r^i > 320$	Serious		

2.6.2. Geo-accumulation index (Igeo) and Enrichment Factor (EF)

To determine the degree of metal pollution in sediment, the Geo-accumulation index (Igeo) can be calculated as [18]:

$$I_{geo} = log_2\left(\frac{(C_i)_{sample}}{1.5(C_i)_{background}}\right)$$

where $(C_i)_{sample}$ is the concentration of metal i in the sample and $(C_i)_{background}$ is the concentration of metal i in the background. The factor 1.5 is a background matrix correction that was introduced in order to minimise the effect of possible variations in the background values as a result of lithogenic effects. The classification of this index is shown in Table 4.

Table 4. The degree of metal pollution according to Igeo-accumulation index (Igeo) level and according to Enrichment Factor (EF) level [18]:

Igeo	Level
<0	Unpolluted
0-1	Unpolluted to moderately polluted
1-2	Moderately polluted
2-3	Moderately to strongly polluted
3-4	Strongly polluted
4-5	Strongly to extremely polluted
>5	Extremely polluted

EF	Level
<1	No enrichment
1-3	Minor enrichment
3-5	Moderate enrichment
5-10	Moderately severe enrichment
10-25	Severe enrichment
25-50	Very severe enrichment
>50	Extremely severe enrichment

The Enrichment factor (EF) is commonly used to distinguish among metals originating from anthropogenic and natural sources [18]. The sediment sample is enriched with metals in comparison to the sample's background conditions for data normalisation purposes. With regard to determining EF values, iron (Fe) is commonly selected as the normalising element because it is a major sorbent phase for trace metals and is a quasi-conservative tracer of the natural metal-bearing phases in fluvial and coastal sediments. The EF can be calculated as the ratio of sample concentration metal (C_i) to the sample concentration of Fe (C_{Fe}) divided by the background ratio of metal concentration to Fe concentration. The equation is shown below:

$$EF = \frac{\left(\frac{C_i}{C_{Fe}}\right)_{sample}}{\left(\frac{C_i}{C_{Fe}}\right)_{background}}$$

The EF value of near unity denotes the elements that are naturally derived, while the EF values of several orders indicate elements of an anthropogenic origin. The classification of EF is shown in Table 4.

2.6.3. Biota-Sediment Assimilation Factor (BSAF)

The comparison between the results obtained for the body parts of *H. tubulosa* and those obtained for the sediments in order to discover which body part is the greatest bioaccumulator can be carried out using the Biota-Sediment Assimilation Factor (BSAF). This factor is defined as the relationship between the concentration of metal in the organism and that in the sediment [21]. This factor is calculated as BSAF = Corg / Csed, where Corg is the concentration of metals in the organism (mg / kg dry organism) and Csed is the concentration of metals in the sediment (mg / kg dry sample) [12].

3. Results and discussion

The results obtained for the metals analysed in the three different body parts considered (body wall, guts and intestine) are shown in the complementary material (Table CM1), along with those obtained for the sediment collected in each area and sector. A comparative study (Table 5) of the sediments described here was also carried out, with the results obtained for the S. Gabriel area in a previous study of the area by this research group 20 years ago [14]. In addition, it was compared with another nearby area of the Mediterranean, the Mar Menor [1], and those described in studies carried out in the Sea of Marmara and the Dardanelles Strait in Turkey [12,22] and in the Kavala, Strymonikos and Ierissos gulfs in Greece [23].

Although several studies concerning the *H. tubulosa* have been carried out in other parts of the world. The data obtained from different research works has been gathered together in Table 6, which provides a comparison of the metals studied by various authors [12, 24-30] with the levels shown in this paper. It is necessary to highlight that the comparison with the data from other places was focused on analysing the concentrations found in the body wall of the *holothurias*, since this is the edible part of these marine species.

3.1. Individual study of metals according to areas and body parts of *Holothuria*

First, the study of the behaviour of each individual metal in each area and body part was done, by carrying out various representations in accordance with the concentration values obtained, which are shown in Figure 3. The concentrations found in the sediments from the different areas were, in general, similar to those in the intestines of *H. tubulosa*, some of whose values are even higher than those found in the sediments. This is logical since, this species feeds on surface sediments concentrating into non-assimilated compounds, such as metals.

The most abundant metals in the sediment and in the three body parts of the *H. tubulosa* were Fe, Al and, finally, Sr (Figure 3 a-d). Fe and Al are the most abundant element in the Earth's crust, therefore, it is usual to find high values of these two metals in marine sediments. The levels found in sediments of the coast of Alicante for Fe and Al were lower than those found in other parts of the world (Table 5) and the concentration of Fe in the body wall are similar or slightly higher (Table 6). The values obtained for Fe in Alicante are similar to those found in other Mediterranean regions, such as Calvi, Ischia and Marseille. The levels found in the Adriatic Sea are lower than those of the Mediterranean, while the area with the lowest concentration of this metal at a worldwide level is Sri Lanka. However, the highest concentrations are in Albufereta, the Saronikos Gulf and the Dardanelles Strait, together with Alaska.

Sr has the greatest concentrations in the animal's body wall (Figure 3 a), in comparison to the two aforementioned metals. It is found naturally in the soil in highly variable amounts [31]. The emissions produced by burning coal and oil increase the levels of this metal in the air, these particles end up back in the soil or at the bottom of lakes, rivers and seas, where their concentration can increase.

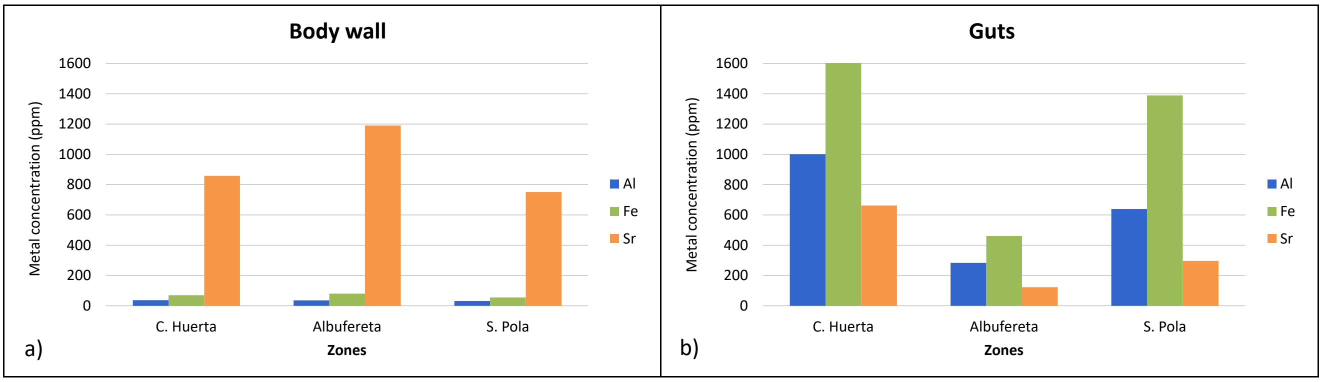
The other metals were, in general, found at a difference of 3 or 4 orders of magnitude. Figure 3 e-h shows the concentrations of As, B, Ba, Mn and Zn in the three body parts and in the sediments. With regard to the metals found in lower concentrations (Cr, Ga, Li, Pb and V), they are represented in Figure 3 i-l. In Figure 3 e, Fe and Al in the body wall are included to better comparation, and it will be noted that B has very similar values to Fe in all areas, and that the values of both are higher than those obtained for Al. The high concentration of B in body wall of the *H. tubulosa* in all areas stand out, since the values obtained for this metal are much higher than those found in the sediments. This metal that most stands out in guts in S. Pola. B presence in seawater may be natural (erosion of natural deposits of sandstone and limestone) or due to the discharge of B from wastewater (powdered laundry and liquid detergents, personal care and domestic or industrial cleaning products, manufacturing of building materials, paint and emissions due to traffic) is probably concentrated in this species due to its ability to cross membranes. This fact has also been observed in the reverse osmosis treatment used in seawater.

The next highest concentration in the body wall is to As. It is also possible to highlight the high concentrations of As present in the body wall and guts of the *H. tubulosa*, at around 20 mg/kg of dry material, which does not occur in the case of the intestines or in that of the sediments, in which the concentration of this metal is less than 7 mg/kg of dry material. It is fairly normal to find high contents of this metal in marine species [32]. The high levels in the body wall are similar to other areas for As (Table 6) this is surprising, and would appear to be a general tendency throughout the world, except in Guam Island, located in the Pacific Ocean, where they are lower. The presence of this metal in antifouling paints can have a great influence on these levels.

The principal metal found in the sediments and intestines of all the areas studied was Mn, which is logical as it is one of the most common elements in nature, together with Fe. With respect to the other places of the word the concentration Mn in sediments of this work are lower (Table 5). The values obtained for Mn in body wall of *Holothuria* was lower than those found in Alaska (Table 6).

The next metal with high concentration in the sediment is Ba, common elements in nature, which were found in similar concentrations in the intestines of *H. tubulosa* from different areas, although the values were significantly lower in the guts and body wall, and particularly in the latter

Regarding Zn (Figure 3 f), its values are the most prominent in guts, with similar concentrations in all study areas. With respect to the other places in the world compared (Table 6), the Zn values are very similar in *Holothuria*, obtaining higher values in Greece, Alaska, the Persian Gulf and the southern part of the Mar Menor in Murcia. In sediments, the values are lower than those found in other areas of the world (Table 5). It is one of the most common elements in the earth's crust, and it has numerous industrial applications, among which it is worth mentioning the antifouling paints of the boats in the port of Alicante, the galvanization processes and the emissions into the atmosphere derived from the combustion and abrasion process of tires.



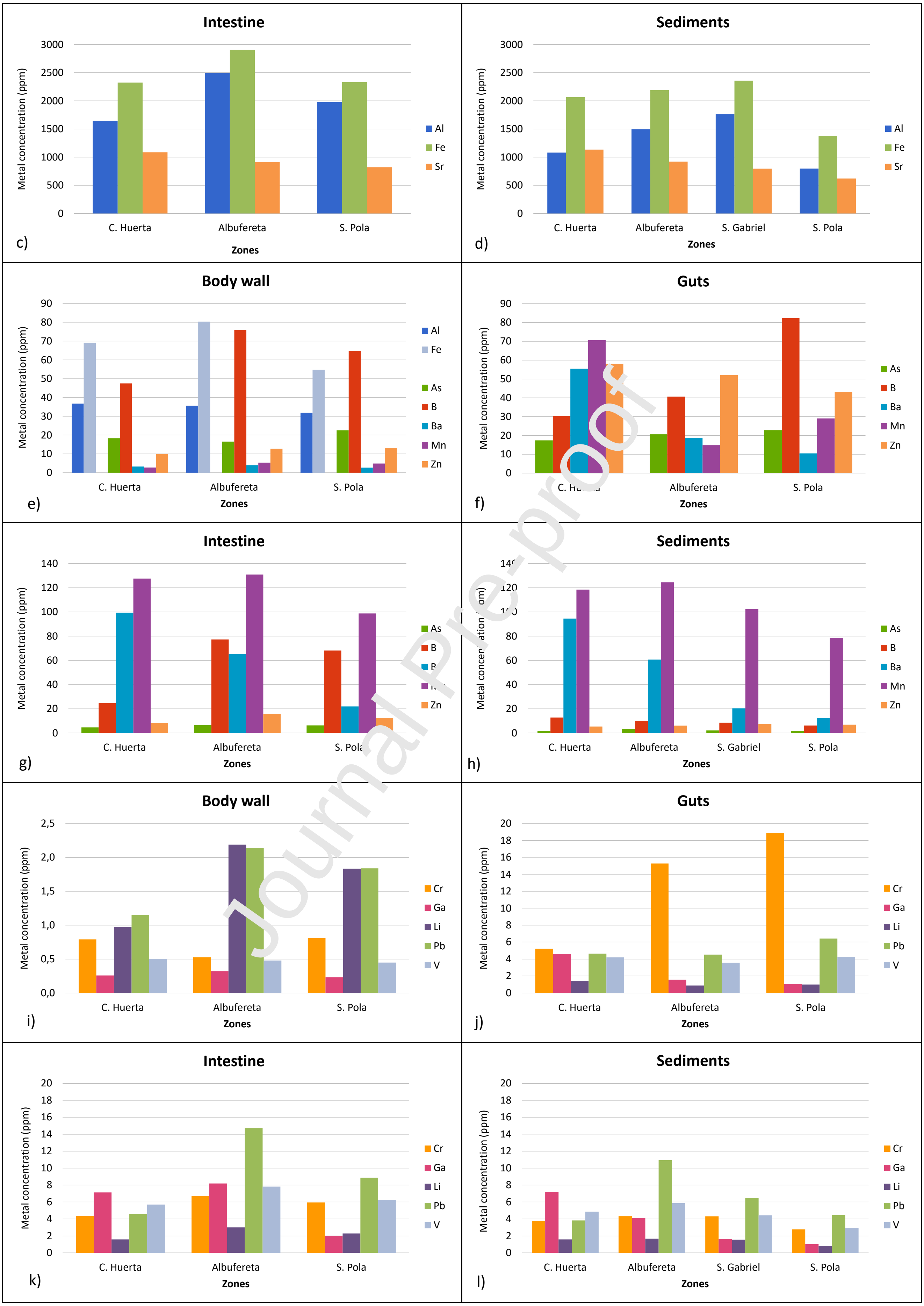


Figure 3. Mean concentration of metals (mg metal/kg dry sample) in body wall, guts and intestines of *H. tubulosa* and in the sediments from each area: C. Huerta, Albufereta, S. Gabriel and S. Pola (values from Table CM1).

Table 5. The concentrations obtained for the samples studied compared to the metal in other parts of the world and the potential ecological risk (E_r^i) and risk index (R_i) calculated for: As, Cr, Mn, Pb, V and Zn (mg/kg dry sample).

Zones	Al	As	B	Ba	Cr	Fe	Li	Mn	Pb	V	Zn	Reference
Mar Menor (Murcia)	3336	30.4			44.8	1931	31.6	408	769		1345	León et al. 2021
Dardanelles Strait (Turkey)						3780			14.6		54.5	Turk Culha et al. 2016
Şarköy. Marmara Sea (Turkey)					61.5	14896		373	22.7		43.6	Topcuoğlu et al. 2004
M. Ereğlisi. Marmara Sea (Turkey)					27.2	5956		274	31.9		34.1	Topcuoğlu et al. 2004
Menekşe. Marmara Sea (Turkey)					54.5	11875		384	21.6		50.9	Topcuoğlu et al. 2004
Kavala Gulf (Grecia)					80.8				52.8		140	Stamatis et al. 2019
Strymonikos Gulf (Grecia)					149				91.1		111	Stamatis et al. 2019
Ierissos Gulf (Grecia)					191				638		420	Stamatis et al. 2019
S. Gabriel (Alicante)			29.0	46.7	7.02				26.8		45.3	De la Muela 1997
C. Huerta (Alicante)	1081	1.79	12.8	94.5	3.79	2067	1.58	118	3.82	4.86	5.36	This work
Albufereta (Alicante)	1495	3.37	10.0	60.6	4.82	2191	1.67	125	10.9	5.86	6.07	This work
S. Pola (Alicante)	797	1.83	6.18	12.4	2.77	1378	0.82	78.7	4.46	2.92	6.85	This work
S. Gabriel (Alicante)	1763	2.09	8.52	20.4	4.31	2358	1.55	102	6.47	4.44	7.47	This work
E_r^i												**R_i**
E_r^i C. Huerta		10.5			0.06			0.17	1.29	0.10	0.08	12.2
E_r^i Albufereta		19.8			0.34			1.75	7.37	0.60	0.09	30.8
E_r^i S. Pola		10.8			0.22			1.10	3.01	0.30	1.05	16.4
E_r^i S. Gabriel		12.3			0.34			1.43	4.37	0.45	1.15	20.0

Table 5 shows concentration of As, Cr, Pb and Zn, with respect to the SQGs, these are in the range "non-polluted", it will be noted that the values for the metals are much lower than those established in all the areas, and it is, therefore, possible to conclude that there are no biologically adverse effects resulting from exposure to metals For these metals in other areas of the world, the values of SQGs would be in the range of "Heavily polluted". In all the areas, the quality guidelines for TEL, PEL, ERL and ERM for As, Cr, Pb and Zn are much lower than those established, indicating no biologically adverse effects resulting from exposure to these metals.

The potential ecological risk (E_r^i) and the risk index (R_i), calculated with the backgrounds from Wedepohl (1995) [33], are shown in Table 5. The highest values of E_r^i) for As can be highlighted in all zones (10-20). The Albufereta area reached the highest $R_i$value, around 30. The ecological risk to the total studied coastal zone is low as indicated by both indices.

Table 6. Comparison of the data obtained for the body wall of *H. tubulosa* in this study and those obtained in other parts of the world and for other species of *holothurias* (mg of metal / kg dry sample).

Zones	Species	As	Cr	Fe	Li	Mn	Pb	Zn	Reference
C. Huerta (Alicante)	H. Tubulosa	18.3	0.79	69.2	0.97	2.76	1.15	9.84	
Albufereta(Alicante)	H. Tubulosa	16.6	0.53	80.4	2.19	5.40	2.14	12.8	
S. Pola (Alicante)	H. Tubulosa	22.6	0.81	54.8	1.83	4.92	1.84	13.0	
North Mar Menor (Murcia)	H. Polii	19.7					8.00	17.2	León et al. 2021
Central Mar Menor (Murcia)	H. Polii	22.9					9.00	14.4	León et al. 2021
South Mar Menor (Murcia)	H. Polii	25.8					54.9	44.1	León et al. 2021
Mediterránean Sea (Murcia)	H. Polii	22.9					5.90	17.1	León et al. 2021
Saronikos Gulf (Greece)	H. Tubulosa		0.80	74.0				36.0	Papadopoulou et al. 1976
South Adriatic Sea	H. Tubulosa	22.4	0.86	24.5			1.16	17.7	Sicuro et al. 2012
South Adriatic Sea	H. Polii	33.3	0.68	19.4			0.65	14.9	Sicuro et al. 2012
Calvi (Corsica)	H. Tubulosa			47.7			6.85	13.7	Warnau et al. 2006
Ischia (Italy)	H. Tubulosa			57.8			3.65	16.0	Warnau et al. 2006
Marseille (France)	H. Tubulosa			51.0			2.74	17.3	Warnau et al. 2006
Sri Lanka	H. Edulis		3.00	39.8			33.7	21.0	Jinadasa et al. 2014
Sri Lanka	H. Scabra		0.59	5.03			34.7	3.68	Jinadasa et al. 2014
Sri Lanka	H. Spinifera		1.31	20.1			205	8.77	Jinadasa et al. 2014
Sri Lanka	H. Atra		<0.04	11.7			97.5	24.4	Jinadasa et al. 2014
Agana Boat Basin (Guam island)	H. Atra	<0.01	<0.13				<0.36	12.6	Denton et al. 2006
Apra Harbour (Guam island)	H. Atra	18.4	0.13				<0.29	16.7	Denton et al. 2006
Agat Marina (Guam island)	H. Atra	<0.01	<0.19				<0.52	16.2	Denton et al. 2006
Merizo Pier (Guam island)	H. Atra	<0.01	<0.16				<0.43	21,2	Denton et al. 2006
Qeshm Island (Persian Gulf)	H. Leucospilota						21.39	43.03	Mohammadizadeh et al. 2016
Qeshm Island (Persian Gulf)	H. Scabra						1.92	23.29	Mohammadizadeh et al. 2016
Alaska	P. Californicus		6.70	184	6.60	43.6		40.4	Bechtel et al. 2013

The metals found in lower concentrations (Cr, Ga, Li, Pb and V) are shown in Figure 3 i-l and present a concentration in the body wall about ten times lower than in the other two body parts. The values of Pb and Li found in the body wall are the highest values found in all the areas studied, followed by Cr, and lower concentrations of Ga and V. It is worth highlighting the great similarity between the concentrations in the intestine and in the sediment, with the same distribution but with slightly lower values in the sediment

The value that stands out in the intestines is that of Pb found in the area of Albufereta, which coincides with the high concentrations of Pb found in that area. As mentioned previously, the individual assimilates the digestible organic part of the sediments and the most concentrated remains are left in the form of metals. The levels of Pb found in the Albufereta are related to the port that exists in the area, since this metal is used in antifouling paints of the boats, as well as in the weights used by anglers, since the area is rocky where the angling is habitual. The levels of Pb in *Holothuria* described herein are generally lower than those found in other parts of the world, with the exception of the island of Guam, where the levels of this metal are lower than the detection limit. Sri Lanka and the south of Mar Menor (Murcia, Spain) which have very high levels.

The values of Li (Table 5) obtained in sediments are lower than that obtained in Mar Menor (Murcia, Spain) and in *Holothuria* (Table 6) were lower than those found in Alaska, which was the only area in which values were found for this metal.

The high concentration of Cr found in the guts in the areas of Albufereta and S. Pola stand out, since they were much higher than those found in the area of C. Huerta, whose values were similar to those found in the intestines and sediment of all the study areas. This metal has various industrial applications, such as the chroming on parts of the leisure crafts in the areas studied. In the *Holothuria*, very similar values in the Saronikos gulf (Greece) and the Adriatic were found. The island of Guam, obtained the lowest values for this metal, while the maximum was registered in Alaska. It is surprising that Alaska is the region with the highest values for all the metals compared at a worldwide level.

If the results obtained for the S. Gabriel area are compared with those from the study carried out previously in the area by this research group 20 years ago [14], it will be noted that the levels of all the metals analysed in this area have decreased, and it is thus possible to state that the concentrations of metals discharged as the result of industrial activity have decreased over the years.

3.2. Analysis of results using PCA and PERMANOVA with sediments and *H. tubulosa* from C. Huerta, Albufereta and S. Pola

The statistical studies were carried out by substituting the outliers for the mean values, which were calculated using the remaining values. Figure 4 shows the PCA according to the content of the metals analysed in the sediment. This shows an ordination of the samples based on the body part (body wall, guts and intestine), while the area (C. Huerta, Albufereta and S. Pola) in which *H. tubulosa* have a less marked effect on the ordination of samples is also depicted. The levels of the metals analysed in the sediment and in the body wall of the holothurian samples were more homogeneous than in the intestine and guts of *H. tubulosa*. The position of the eigenvectors (correlations) indicated that the body wall of the holothurian had the lowest levels of metal content, and that the most important metals in this body part were As and Sr. The PC1 explained 44% of the variation, and divided the body wall and guts of the holothurian on the one side, and the sediment and intestine of *H. tubulosa* on the other. The values of the eigenvectors of the PC1 were mainly negative, indicating that the levels of metals were generally lower in the body wall of the holothurian, and also in its guts, than in other body parts. The metals that contributed most to the makeup of the PC1 were Fe, Al, V, Mn, Pb and Ga, with a correlation below -0.30, but As also contributed, and had the highest positive correlation (0.22). The PC2 explained 21% of the variation and isolated most of the samples of *H. tubulosa* guts and some samples of its intestine. For this axis, Cr, Zn, As and B had a correlation above 0.3, while Sr had the lowest negative correlation (-0.33) (Table CM2).

The cluster analysis of the samples according to the levels of metals showed an aggregation of the sediment samples from the rest (Figure CM1). The PERMANOVA showed significant differences as regards the interaction between the area and body parts (p=0.02 Monte Carlo) (Table 7). When comparing the sediment and each body part of the *H. tubulosa* from each area, the body wall was generally different from the other body parts and the sediment in all areas, and only the guts in Albufereta were not significant. Moreover, the guts and the intestines were significantly different in C. Huerta and S. Pola.

When comparing the levels of metals among areas, the area of Albufereta generally showed the highest levels in the sediments. This fact sides with the fact that this area had to be closed to the public on various occasions owing to incidents related to pollution (see section 2.1). The area of S. Pola which is located further to the south of the coast, showed in most cases the lowest level of metals in sediments.

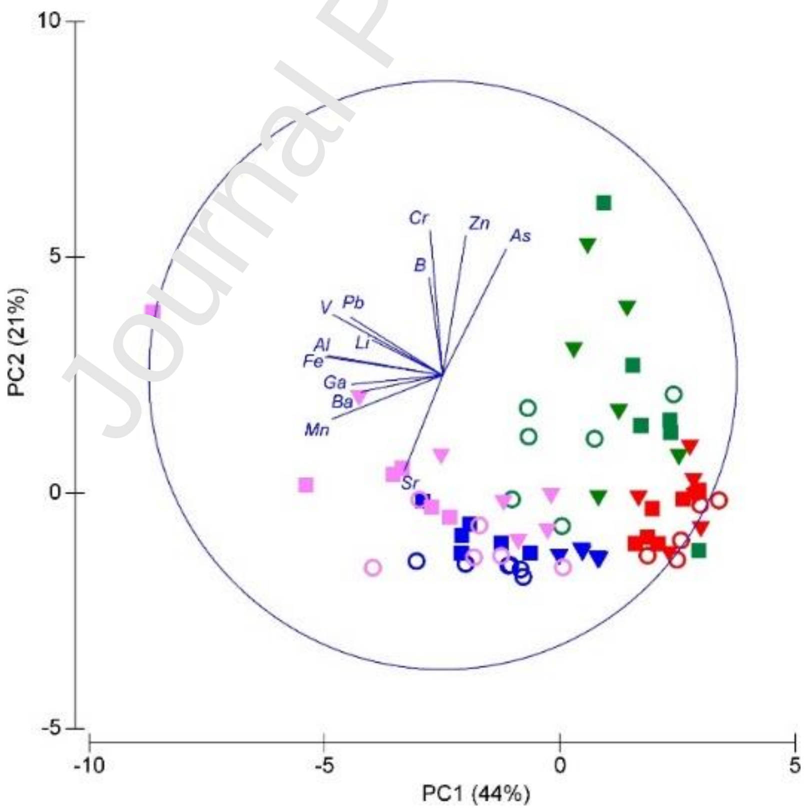


Figure 4. Principal component analysis (PCA) of the samples of the different body parts of *Holothuria tubulosa* [body wall (B, red), intestine (I, pink) and guts (G, green)] and sediment (blue), and areas [C. Huerta (○), Albufereta (□) and S. Pola (▽)] Two sites were sampled and replicated three times at each area.

Table 7. Summary of the effects of the area, site and the body parts on the levels of the metals analysed.

Source	df	SS	Pseudo-F	P (perm)	Unique perms	P (Monte Carlo)
Area	2	55 596	33 698	0.073	15	0.013
Body part	3	417.02	21 733	0.001	999	0.001
Si(Lo)	3	24 748	13 763	0.135	999	0.163
LoxCo	6	80.37	20 942	0.012	999	0.021
CoxSi(Lo)	9	57 566	10 672	0.362	998	0.389
Residual	48	287.7				

3.3. Assessment of metal pollution. Calculation of indices

3.3.1. Results of Geo-accumulation index (Igeo) and Enrichment Factor (EF)

In this subsection, a series of indicative indices are calculated in order to evaluate the level of pollution in the sediments in each of the areas studied. This was done by following the descriptions provided in Sharifuzzaman et al. (2015) [18]. In order to apply the Igeo and EF indices, the backgrounds used were provided from Wedepohl (1995) [33]. The numerical values calculated for these indices are shown in Table 8.

Table 8. Igeo and EF indices calculated with backgrounds from Wedepohl (1995) [33]

Index	Zones	Al	As	B	Ba	Cr	Fe	Ga	Li	Mn	Pb	Sr	V	Zn
Igeo	C. Huerta	-6.79	-0.511	-0.366	-3.21	-5.64	-4.97	-1.65	-4.10	-3.19	-2.54	1.18	-4.92	-4.19
	Albufereta	-6.32	0.402	0.722	-3.85	-5.45	-4.89	-2.45	-4.02	-3.10	-1.03	0.881	-4.65	-4.01
	S. Pola	-7.23	-0.479	-1.420	-6.14	-6.09	-5.56	-4.45	-5.04	-3.77	-2.32	0.312	-5.65	-3.83
	S. Gabriel	-6.08	-0.287	-0.954	-5.42	-5.45	-4.78	-3.78	-4.12	-3.40	-1.78	0.672	-5.05	-3.71
EF	C. Huerta	0.284	22.0	24.3	3.38	0.629	1.00	10.0	1.84	3.44	5.39	71.2	1.04	1.72
	Albufereta	0.370	39.1	17.9	2.05	0.676	1.00	5.40	1.83	3.44	14.5	54.5	1.18	1.84
	S. Pola	0.314	33.7	17.6	0.666	0.689	1.00	2.15	1.43	3.45	9.45	58.4	0.934	3.30
	S. Gabriel	0.406	22.5	14.2	0.640	0.627	1.00	2.00	1.58	2.61	8.01	43.8	0.830	2.11

The metals studied present a negative Igeo (Table 8, level 1), which indicates that these areas are not contaminated by the metals analyzed. Arsenic in Albufereta and strontium in all the areas studied are the only ones that present a level 2 (uncontaminated to moderately contaminated). The majority of the metals have a EF 1-3 (minor enrichment), with the exception of Pb who present a moderately severe enrichment, B severe enrichment, As very severe enrichment and Sr extremely severe enrichment. These high levels may be due to anthropogenic contamination processes, produced by the port of Alicante or wastewater discharges at different coastal points, among others. In the case of strontium, it could be attributed to natural processes since in this area the limestone rocks provide high levels of calcium carbonate, observing relationships between Ca and Sr [31].

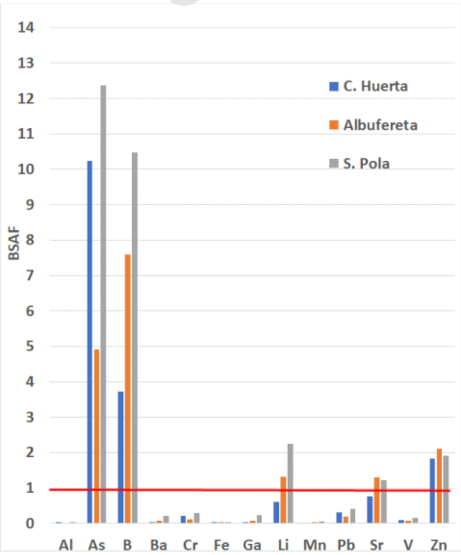
3.3.2. Results of the Biota-Sediment Assimilation Factor (BSAF)

The BSAF for each metal in each body part analysed of *H. tubulosa* per study area are shown in the complementary material (Table CM3). Figure 5 provides a graphic representation of them.

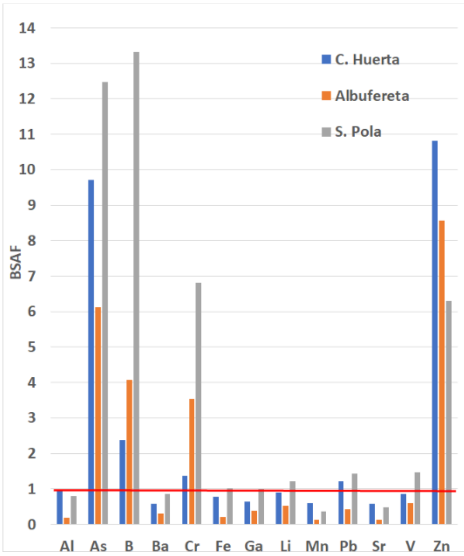
Figure 5 a show that the metals in the body wall that have undergone the greatest extent of bioaccumulation are As and B, while those that have undergone the lowest extent of bioaccumulation are Zn, Li and Sr. The latter have greater concentrations in S. Pola and Albufereta. The other metals were present in concentrations much lower than 1, which indicates that the organism itself metabolises these metals, thus decreasing the quantity present in the individual with respect to that which is found in the medium, or this echinoderm accumulates them in other body parts such as the guts or intestines.

The behaviour of the guts (Figure 5 b) is similar to that of the body wall, in that the most bioaccumulated metals were As and B. In guts, Zn had accumulated in values similar to those of the two previous metals, and Cr had also accumulated in this area of the echinoderm, but to a lesser extent. As will be noted, the other metals obtained values that were closer to 1 than the values found for the body wall, which are much lower, thus corroborating the hypothesis that they are metabolised or accumulated in the guts.

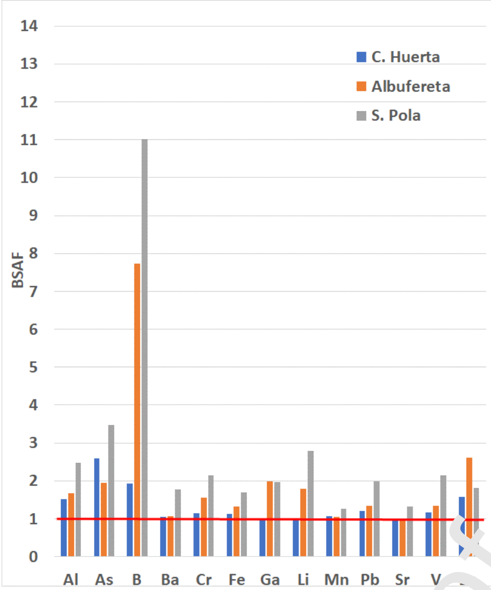
It is clear that the part of the *H. tubulosa* that obtained values very close to 1 for all the metals studied was the intestine (Figure 5 c), which coincides with the previous finding that the concentrations present in this part of the *H. tubulosa* are the most similar to those found in the sediments. Only B obtained values much higher than 1, particularly in the areas of S. Pola and Albufereta. The next metal that had most bioaccumulated in all areas was As, followed by Al (Al 0.183 in sediment and 1.25 in intestines), Zn and Li, but with much lower levels than those found for B.



a)



b)



c)

Figure 5. Concentration of metals in body wall (a), guts (b) and intestine (c) of *H. tubulosa*, normalised with respect to the concentration of metal in the sediment in the C. Huerta, Albufereta and S. Pola areas (mg metal/kg dry sample).

Our results show that each body part of the holothurian has a different trend as regards the accumulation of metals. Upon observing the mean attained for each body part of the *H. tubulosa* studied, it will be noted that the guts show the greatest bioaccumulation (BSAF = 2.67) of the three body parts studied, followed by the intestines (BSAF = 2.04) and finally the body wall (BSAF = 1.68).

In some of these works, the BASF is calculated. The values of this study for Fe (0.03-1.69), Pb (0.20-1.99) and Zn (1.57-10.82) are higher than those found by Culha (Fe: 0.01-0.04, Pb: 0.09-0.34 and Zn: 0.1-0.75). Usually, the As (1.94-12.47) and Zn (1.82-10.82) values are similar in this work to those found by Leon et al. (As: 3.94-16.01 and Zn: 1.52-8.94), while in the case of Pb (0.3-1.99) it is much lower than the values found by these authors in the Mar Menor lagoon (0.42-31.1) with the influence of a mining area, and located in the south of the Alicante coast.

4. Conclusions

This study is focused on those metals whose concentration in sediments was greater than 1 ppm (aluminium, arsenic, boron, barium, chrome, iron, gallium, lithium, magnesium, lead, strontium, vanadium and zinc). It is worth highlighting the great similarity between the concentrations of metals in the intestines and sediment, with the same distribution, but with higher values for the intestines.

The standard guidelines for safety limits for As, Cr, Pb and Zn are in the range “non-polluted” and the quality guidelines for TEL, PEL, ERL and ERM are much lower than those established, with no biologically adverse effects resulting from exposure to these metals. The potential ecological risk (E_r^i<40) and the risk index (R_i<105) is low in all zones. The highest values of E_r^i are for As in all zones (10-20) and of R_i(30.8) in Albufereta.

The levels in the sediments found on the coast of Alicante are lower than those found in the rest of the world. With regard to the body wall, the high levels of As stand out, and this appears to be a general trend throughout the world. The levels of Fe in Alicante are similar to those found in other

Mediterranean regions (Calvi, Ischia and Marseille), but are higher than those of the Adriatic Sea. In the case of Cr, the values for these areas are very similar to those in other parts of the world, and this is also the case of Zn. The levels of Pb are generally lower than those registered in other areas, and this is also true of Mn and Li when compared to those found in Alaska.

Regarding PCA, the metals that showed the largest difference on their levels among samples were Fe, Al, V, Mn, Pb, Ga, As, Cr, Zn and B. The body wall was the tissue that was significantly different to the other body parts and the sediment according to levels of the analysed metal. If the levels of the metals are compared solely by areas, the highest levels in the sediments tend to be in the area of Albufereta and the lowest values were generally found in S. Pola, which is located further to the south of the coast.

The metals studied present a negative Igeo, which indicates that these areas are "unpolluted" by the metals analyzed. Arsenic in Albufereta and strontium in all the areas studied are the only ones that present a level of "unpolluted to moderately polluted". The majority of the metals have EF level of "minor enrichment", with the exception of Pb with "moderately severe enrichment", B "severe enrichment", As "very severe enrichment" and Sr "extremely severe enrichment". These high levels may be due to anthropogenic contamination processes, produced by the port of Alicante or wastewater discharges at different coastal points, among others or by natural processes, in the case of strontium.

The guts, followed by the intestines, were the species compartments with greatest bioaccumulation of the three body parts studied. The most abundant metals in the sediments are Al, Fe and Sr, and this is also the case of the guts and intestines of the *H. tubulosa*. With regard to the body wall, the concentrations of Sr (750 to 1200 mg/kg of dry material) and As (17 to 23 mg/kg of dry material) stand out, but the BSAF for Sr is around 1 or less, while that for As oscillates between 5 and 12, which indicates a high accumulation of this metal in the body wall. There was a high concentration of B in all the body parts of the *H. tubulosa*, which was a much higher value than that found for the sediments, with a BSAF of between 2 and 13. The BSAF for Mn and Zn were generally close to 1 in all the body parts, with the exception of the guts, for which the values were between 6 and 11. The sequence of metals found in lower concentrations is: Pb, Li > Cr > Ga and V. The high concentrations of Cr found in the guts in the areas of Albufereta (BSAF 3.5) and S. Pola (BSAF 6.8) stand out. With regard to the intestines, the values obtained for Pb in Albufereta also stand out, although in this case the BSAF is 1.4.

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Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Highlights

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Holothuria tubulosa is studied as metals pollution bioindicator in Alicante (Spain).

Al, As, B, Ba, Cr, Fe, Ga, Li, Mn, Pb, Sr, V and Zn were analized in marine sediment.

Concentrations are above of 1 mg/kg of dry sediment but different in body parts.

Biomagnification occurs to As, with high levels in the body wall from the sediment.

The PERMANOVA showed differences between the zones, Santa Pola the lowest levels.