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Can Microplastics Influence the Accumulation of Pb in Tissues of Blue Crab?

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Abstract: The study of microplastics (MPs) and associated pollutants is essential for a better understanding of some of the factors that threaten marine ecosystems. The main objective of this study was thus to assess Pb distribution and accumulation in the tissues of blue crabs (*Callinectes sapidus*) exposed to MPs. Blue crabs were collected from the mouth of the river Segura (Guardamar, Spain) and fed on mussels from two Mediterranean areas with different levels of Pb contamination: Portmán Bay and San Pedro del Pinatar (Murcia, Spain). In addition, a batch of each group were exposed to MPs. After seven days of exposure, the crabs were euthanised, and tissues and faeces were analysed. The hepatopancreas was found to be the best tissue for measuring Pb concentrations after feeding; muscle tissue did not provide information on environmental quality. The meat (muscle) consumption of blue crabs from zones with high Pb content does not seem to constitute a risk for consumers, although the risk is not totally negated if all soft tissues are ingested. The presence of MPs in the water does not seem to increase the accumulation of Pb in these tissues of *C. sapidus*.

Keywords: blue crab; gills; hepatopancreas; lead; meat; microplastics

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1. Introduction

In the twenty-first century, marine pollution is now treated as a serious problem by scientists, governments, and society alike. The presence of pollutants in the sea poses a threat to wildlife, human beings, and ecosystems, even though its impact is very hard to quantify. Marine ecosystems have to face up to a cocktail of chemical pollutants. In addition to inherited contaminants such as heavy metals, there are a series of substances such as plastics [1–3] whose impact not only negatively affects marine species but could also be a threat to human health via the food chain.

Heavy metals represent one of the most serious pollution concerns in marine ecosystems [4,5] and, due to their toxic character, long-term persistence, and ability to enter and bioaccumulate in food webs, they constitute an important threat to human beings and aquatic life [6]. Lead is a non-essential, widely used toxic metal that has generated pollution and health problems worldwide [7]. It is regarded as a priority toxicant by the US Environmental Protection Agency [8] and Spanish legislation [9]. The presence of this metal in the environment continues to cause concern [10] and more guidelines are required to control which species of fish and seafood are consumed by humans, and how often, regarding the state of environmental pollution [11].

Waste plastic is a further concern because it is present in marine and freshwater ecosystems all over the world to such an extent that today plastic litter constitutes about 85% of all marine litter; its worldwide production has risen significantly and in 2018, 359 million tonnes of plastic were produced [12]. According to Jambeck et al. [13], by 2025, it is thought that 50–250 million tons of marine contamination will have originated from land-based sources. Larger plastic remnants break up into smaller pieces, giving rise to

'microplastics' (MPs), that is, fragments measuring <5 mm [14,15]. The MPs that have entered the sea remain there and are transported by winds and surface currents to different parts of the world in the form of water bodies [16,17]. This proliferation of MPs has led to the emergence of threats related to wildlife and human health. Interactions between heavy metals and MPs have been recorded, and MPs are thought to potentially act as vectors for heavy metals, thereby increasing the exposure of living organisms to these pollutants [18].

In light of the above, environmental monitoring plans in which aquatic organisms are used as 'sentinel' or 'bioindicators' are employed to evaluate the presence of plastics and heavy metals in marine ecosystems [19]. The blue crab (*Callinectes sapidus*) and the mussel (*Mytilus* spp.) are common species that are often used for this purpose [20–22]. The blue crab is a swimming decapod from the western coast of the Atlantic and the Caribbean, and it is widely distributed [23,24]. It is regarded as a euryhaline species because of its capacity to hyperosmoregulate in the salinities in which it lives [25]. Its distribution is influenced by the temperature and salinity of the water, being commonest in estuaries and seas with muddy bottoms [26–28]. In 2016, the Spanish Ministry of Fisheries included *C. sapidus* on its list of commercial fish species [29], and it is nutritionally well-accepted when described as 'Mediterranean blue crab meat' [30–32]. Studies of metal contamination in this species have multiple objectives, including its effects on human health and the fitness of crabs themselves [33], given that they reside in surface sediments and feed on benthic prey that normally live in contaminated areas. Furthermore, the crabs do not require authorisation for experimental procedures, and capture techniques do not involve any potential risks from an environmental point of view. Bivalves are by far the most widely used pollutant indicator organisms in environmental control studies in coastal areas [34]. Mussels are commonly used indicator species due to their wide distribution, abundance in the wild, and easy manipulation. Moreover, due to their well-documented usefulness and effectiveness, they are still extensively used for biomonitoring and experimental procedures (e.g., [35–39]). Their sessile condition and benthic and sedentary nature ensure that they persist in the area in which they are placed even if the environment is polluted [40]. Additionally, their capacity to filter [41] favours the entrance of contaminants into their systems, thereby allowing for the accumulation of chemical substances in their tissues whose analysis can provide key information about environmental pollution levels [42]. In this context, the aim of the present study was to assess how MPs affect lead concentrations in the tissues of *C. sapidus* fed on mussels taken from two Mediterranean environments with differing levels of Pb pollution.

2. Materials and Methods

2.1. Sample Collection and Conditioning Period

2.1.1. Mussels

The mussels (*Mytilus galloprovincialius*) used in this study (4–5 cm in length) were obtained from an aquaculture farm located in the Ebro delta (Spain) in October 2018. Once their initial Pb concentrations had been analysed, mussels were relocated in two areas: (1) Portmán Bay, one of the areas with the highest levels of metal pollution in the whole Mediterranean basin [40,43,44], and (2) San Pedro del Pinatar, a non-polluted area [45]. For each location, three sampling stations were set up (coordinates: 689492X/4161139Y, 689559X/4161080Y, 689710X/4161065Y for Portmán Bay; and 704427X/4186597Y, 704387X/4186604Y, 704340X/4186601Y for San Pedro del Pinatar), and in each station, three bags, each of 30 mussels, were placed. After 113 days, the bags were retrieved: one half of the mussels from each bag was used to perform the analysis of the Pb concentrations and the other half to feed the crabs. Mussels were stored at −20° until the experiment began.

2.1.2. Blue Crabs

Thirty blue crabs were collected from the mouth of the river Segura (RSM) (Alicante province, coordinates 705558X-4220269Y) in October 2019 with a fyke net. The net was placed on the bottom of the river and retrieved four hours later. Crabs were brought back

to the laboratory in containers filled with water, and 24 were acclimatised for 10 days in tanks of artificial seawater (ASW) under laboratory conditions. The salinity of the ASW was similar to that of the RSM (osmolarity of 94.7 ± 2.3 mmol kg^{-1}). The other experimental conditions were as follows: pH = 8.1 ± 0.04, temperature = 18.0 ± 1.1 °C, continuous aeration, and a natural photoperiod. During their acclimatisation, crabs were fed on mussels from the Ebro delta (Spain) at a food equivalent of 4–5% of their weight and a Pb concentration of 0.070 ± 0.006 mg Kg^{-1} (wet weight). The biometric measures (geometric mean ± standard error) of crabs were 45.1 ± 2.8 g (weight), 4.3 ± 0.1 cm (carapace length between abdominal segment and rostrum) and 8.9 ± 0.3 cm (carapace width between lateral spines). The remaining crabs (n = 6, the 'Zero Hour', unexposed group) were euthanised immediately by hypothermia (30–40 min at −20 °C), sexed and their biometric parameters (weight, length and width) were recorded. Tissues from each specimen (hepatopancreas, muscle and gill) were carefully removed from the carapace with a surgical knife, transferred to 1.5-mL microtubes and stored at −20° until processed.

2.2. Experimental Procedure

Four groups were prepared based on feeding and MP exposure (Figure 1): (1) blue crabs fed on mussels with high Pb concentration from Portmán Bay with (1.1) or without (1.2) MPs in the water; and (2) blue crabs fed on mussels with low Pb concentrations from San Pedro del Pinatar with (2.1) or without (2.2) MPs in the water. The daily diet and consumption of each crab was registered and was equal to 5.5 ± 0.20% of their body weight (geometric mean ± standard error). For both groups, the MPs used were Aquatex−100, an oxidised polyethylene with a particle size of 80–100 µm and 0.99 g cc^{-1} density at 25 °C. The MP concentration was 25 µg L^{-1}, a realistic concentration that corresponds to the studies performed by Green et al. [46] and matches the prediction by Jambeck et al. [13] regarding the increase of global plastic waste by 2025. The experiment was carried out in three tanks per treatment (eight litres of ASW per tank, with the same experimental conditions of pH, osmolarity, temperature, aeration, and photoperiod, as described above) with two crabs in each tank (n = 6 crabs per treatment) for seven days. Once the experiment concluded, crabs were euthanised, and tissues were removed as described for crabs from the 'Zero Hour' group. As well, faeces were collected daily and placed in different tubes for each tank, then rinsed three times with bidistilled water, centrifuged, and dried at +60 °C until constant weight. Faeces from each group were pooled due to the low weight of the collected faeces. In addition, pools of several days were grouped: days 1–2, 3–4–5, and 6–7.

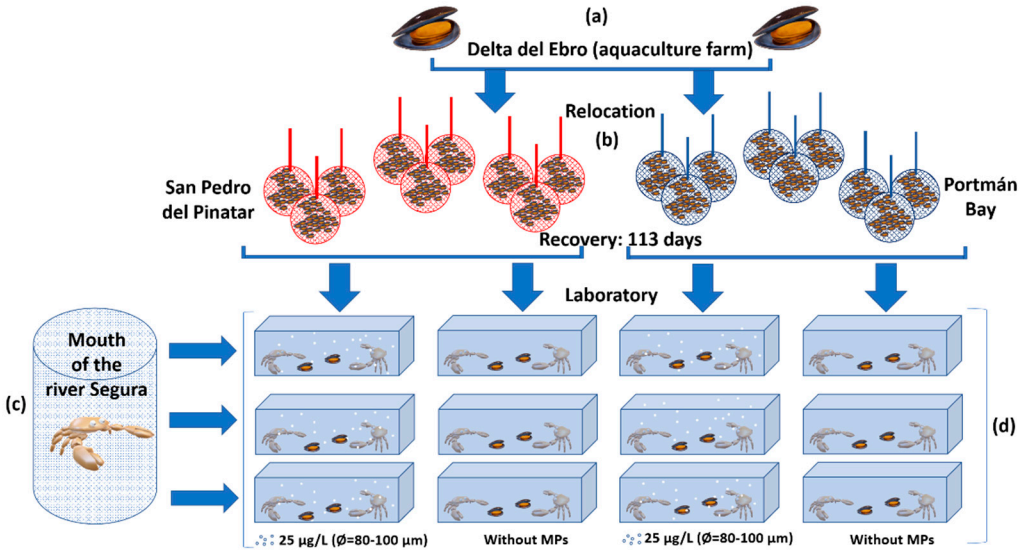

Figure 1. Phases of the study: (**a**) mussel acquisition (aquaculture farm, Delta del Ebro, Spain), (**b**) mussel relocation (San Pedro del Pinatar and Portmán Bay, Spain), (**c**) crab collection (mouth of the river Segura, Spain), and (**d**) experimental procedure (crabs fed on mussels from San Pedro del Pinatar or Portmán Bay, with or without microplastics (MPs) in the water).

2.3. Pb Analysis

To determine their Pb content, tissue and faeces samples, and ASW were analysed using inductively coupled plasma optical emission spectrometry (ICP-OES, ICAP 6500 Duo, Thermo Scientific, USA, with One Fast System, USA). Hepatopancreas, gill, and muscle tissues, as well as faeces, were treated with trace mineral grade nitric acid (69% Suprapure, Merck) and 33% H2O2 (Suprapure, Merck) in special Teflon reaction tubes. These tubes were heated at 220 °C for two minutes in a microwave digestion system (UltraClave-Microwave Milestone®, Italy) and later diluted to 10 mL with double deionised water (Milli-Q). The limit of detection was 0.001µg g^{-1}. Two replicates were analysed for every sample; the concentration values used in the analysis were the mean of two readings. For every 11 samples, one blank sample was analysed in the ICP-OES to check for possible metal contamination. Taking UNE-EN ISO 11885 as a reference for the determination of elements by ICP atomic emission spectroscopy, multi-element calibration standards (SCP Science, in 4% nitric acid) were prepared with specific Pb concentrations. Intermediate patterns were prepared for this element. The calibration device was established per batch, with a minimum of three points for every lot. Each run started with the calibration standards, continued with samples and intermediate patterns, and finished with the series with intermediate patterns (10% variation coefficient). The wavelength was 220.353 nm. The uncertainty and recovery percentages were 6.14 and 96.44, respectively, and the standard reference material was L577b (bovine liver). Lead concentrations were expressed in micrograms per gram in wet weight for tissues (µg g^{-1} ww) and dry wet for faeces (µg g^{-1} dw).

2.4. Data Analysis

The data are presented as geometric means ± standard errors of the means, and minimum and maximum concentrations. According to international guidelines [47], the middle-bound approach was taken (i.e., the values below the detection limit were equal to half this limit) to calculate mean concentrations. Lead concentrations were compared between exposure groups (by tissue and faeces) and between tissues (by exposure group). To check for data normality, a Shapiro-Wilk test was used. For the study of the groups, a log transformation of the data was carried out and parametric mean comparison tests were conducted (Student *t*-test and ANOVA with Tukey and Games Howell post-hoc test, and Levene's test for the equality of variances). Marginal differences between groups (p = 0.05–0.1) were confirmed by a non-parametric test (U de Mann–Whitney). Spearman's rank correlation coefficient test was applied (a) to Pb concentrations between tissues from each group, and (b) to biometric data and Pb concentrations from each tissue and exposure group. To compare the biometric measures, a Pearson test was carried out. In all cases, p-values of less than 0.05 were taken to be statistically significant. All statistical analyses were performed with IBM SPSS Statistics v.19.0 (IBM, New York, NY, USA) for Windows.

3. Results and Discussion

The Pb concentrations detected in mussels from the aquaculture farm in the Ebro delta were 0.076 mg kg^{-1}, while in mussels collected after the experimental period from San Pedro del Pinatar and Portmán Bay, concentrations were 0.220 ± 0.009 and 3.652 ± 0.188 mg kg^{-1}, respectively. These concentrations are realistic and are similar to others reported in previous studies [48]. The relationship of the Pb concentrations between the two ecosystems (unpolluted vs. polluted, 1:17) differed from those reported by other authors (1:2 [22]; 1:4 [49]). Based on the ingested food quantity and Pb concentrations, we estimated that the total Pb consumed by each crab (geometric mean ± standard error) was 23.5 ± 1.0 µg (for crabs fed on mussels from San Pedro del Pinatar) and 529.2 ± 20.4 µg (for crabs fed on mussels from Portmán Bay). Moreover, there was a close relationship ($r > 0.8$, $p < 0.001$) between the µg of ingested Pb and the biometric data (weight, carapace length, and carapace width) in both groups (crabs fed on mussels from San Pedro del Pinatar and from Portmán Bay), which guaranteed that the Pb ingested was proportional to the size of the crabs.

Descriptive data of Pb concentrations in the crab tissues are given in Table 1. San Pedro del Pinatar is considered by environmental studies to be a non-polluted area [45] and so, as expected, the crabs fed on mussels from this area had low Pb concentrations in their tissues. These concentrations were lower than those reported for the same species in other Mediterranean areas such as the Köyceğiz Lagoon (Turkey), Acquatina Lagoon (Italy), İskenderun Bay (Turkey) and Mersin Bay (Turkey) [20,31,50–52], and also lower than from countries such as USA, Brazil, and Venezuela [33,53–56]. The non-exposed crabs ('Zero Hour' group) had the same Pb concentration as those fed on mussels from San Pedro del Pinatar, which could be due to the fact that, after winning the European Riverprize Awards in 2016, the river Segura is now one of the least polluted rivers in Spain. On the other hand, Portmán Bay is one of the most polluted areas in the Mediterranean basin [40,43,44] and in the tissues of crabs fed on mussels from this latter area, Pb concentrations were higher than those reported by Genç and Yilmaz [20], but lower than those reported by a number of other authors [31,50–52]. In terms of countries such as USA, Brazil and Venezuela, the results were lower [33,54] or higher [53,55,56], depending on the tissue that was analysed.

Table 1. Descriptive statistics (geometric mean, standard error, minimum and maximum) of the Pb concentrations in tissues (μg g^{-1}, ww) and faeces (μg g^{-1}, dw) of *C. sapidus* after the 7-day treatment.

Exposure Group: Mussel Origin and MP Exposure	Muscle	Hepatopancreas	Gills	Faeces
San Pedro del Pinatar (without MPs)	0.004 ± 0.006^{AB} (*nd*−0.037)	$0.065 \pm 0.050^{ab\ A}$ (*nd*−0.322)	0.234 ± 0.072^{B} (0.117–0.583)	$30.493 \pm 4.939^{a,b}$ (18.234–45.046)
San Pedro del Pinatar + MPs	0.017 ± 0.013^{AB} (*nd*−0.076)	0.311 ± 0.060^{cdA} (0.144–0.588)	0.182 ± 0.390^{B} (*nd*−2.178)	$34.498 \pm 11.447^{c,d}$ (16.974–55.806)
Portmán Bay (without MPs)	0.003 ± 0.026^{AB} (*nd*−0.127)	1.178 ± 0.158^{aceAC} (0.876–1.884)	0.384 ± 0.199^{BC} (0.063–1.406)	$680.132 \pm 46.766^{a,c}$ (550.439–821.053)
Portmán Bay + MPs	0.008 ± 0.044^{AB} (*nd*−0.261)	1.565 ± 0.455^{bdfAC} (0.511–3.598)	0.519 ± 0.060^{BC} (0.374–0.758)	$649.338 \pm 56.101^{b,d}$ (503.079–772.040)
Segura River, 'Zero Hour'	0.006 ± 0.018^{AB} (*nd*−0.098)	$0.174 \pm 0.020^{ef\ A}$ (0.106–0.257)	0.095 ± 0.045^{B} (0.004–0.297)	n/a

For each tissue and faeces: exposure groups with the same lowercase had significant statistical differences. For each exposure group: tissues (muscle, hepatopancreas, and gills) with the same capital letter had significant statistical differences. MPs=microplastics; n/a = not analysed; *nd* = not detected.

The order of Pb concentrations was the following (the same superscript indicates significant statistical differences between tissues): hepatopancreas^{1} > gills^{2} > muscle^{1,2} in crabs from the control group ('Zero Hour') and crabs fed on mussels from San Pedro del Pinatar; and hepatopancreas^{1,2} > gills^{1,3} > muscle^{2,3} in crabs fed on mussels from Portmán Bay. According to Duruibe et al. [57], the accumulation of heavy metals is higher in chest tissues than in appendages, and muscles only accumulate a small amount of metal [58], which agrees with our results. In this sense, a low percentage of crabs with Pb above DL in muscles was recorded (56.7%), which coincides with the figures reported by Sivaperumal et al. [59], while most of the hepatopancreas and gill samples had Pb concentrations above the DL (96.7%).

Both these tissues (hepatopancreas and gill) commonly have the highest amounts of metal due to their nature and the role they play in the organism. The hepatopancreas is the principal tissue for storing toxicants, while the gills are a large absorptive organ system and one of the main points in which crabs are in contact with the noxious substances [24]. Regarding metals, the hepatopancreas (digestive gland) has been described as the main organ for detoxification and accumulates more toxicants than the gills [20,60]. However, no significant differences were reported between these two tissues in the crabs from the 'Zero Hour' group and the crabs fed on mussels from San Pedro del Pinatar (Table 1). It is known that metals can enter the organism in two ways [54,61,62]: (1) directly via the water that enters the gills or (2) indirectly via diet, which could explain the differences found between

the hepatopancreas and the gills in the crabs fed on mussels from Portmán Bay given that the metal concentrations in the water in the tanks were low (0.007 ± 0.008 mg L^{-1}).

Interestingly, when these organs reach saturation, it is thought metal excess is transferred to other tissues via hemolymph [24,49]. Larger crabs were fed on greater amounts of food to ensure that they ingested more total Pb ($r > 0.8$, $p < 0.05$). However, there was no correlation between the biometric data and Pb concentrations in tissues. On the other hand, the concentration of Pb in faeces was almost 20 times higher in individuals fed on mussels from Portmán Bay than in individuals fed on mussels from San Pedro del Pinatar (Table 1). Although faeces were collected daily, we cannot confirm whether the crabs from both exposure groups excreted Pb on a daily basis or whether Pb was concentrated in the most recent excretions. However, this efficiency in Pb excretion through faeces could help avoid a generalised spread throughout the animal and so explain why no correlation was detected between the biometric data and Pb concentrations in tissues. Metal uptake and its regulation in invertebrates have been explained by theoretical models. Rainbow [63] has stated that crustaceans could assimilate non-essential metals (1) without excreting them and store them in a detoxified way inside the organism (mostly bound to metallothioneins), and (2) by excreting them but with no changes in concentrations, since excretion and metal uptake rates tended to balance out. According to this author, the excretion of non-essential metals occurs when there is an excess of the detoxified metal in a body compartment. Despite the fact that both the hepatopancreas and gills are directly involved in metal uptake, storage, and excretion due to their great involvement in metallothionein synthesis [64], other authors have reported significantly higher metallothionein concentrations in hepatopancreas than in gills [65], which could explain why higher Pb concentrations were found in the hepatopancreas of crabs fed on mussels from Portmán Bay. Thus, the hepatopancreas of crabs from this group could act as temporary Pb deposits, at least in the short term.

It is important to point out that muscle tissue had the lowest Pb concentrations and was the only tissue that showed significant differences from the other tissues for all treatments. This result confirms our predictions and supports the idea that muscles have a low accumulative potential since they are not metabolically active tissues. Comparable findings have been reported by Bordon et al. [49], who used similar Pb concentrations in *Callinectes* spp., and for all treatments (contaminated food, water, and combined), these authors obtained the lowest amounts of Pb in muscles. Hence, it seems that muscles are not a target tissue for this metal and so Pb accumulation may only begin when an excess reaches muscles via the hemolymph from gills and the hepatopancreas. Despite this typical pattern of low heavy metal accumulation in muscles, there are exceptions in which higher levels are found in muscles than in the hepatopancreas and gills, as has been noted by Mohamed and Osman [66] for *Oreochromis nilotius*. On the other hand, muscle meat from appendages and abdomens are important tissues for pollution control due to their capacity to transfer metals through the food web chain [67] and highlight potential risks to human health. *Callinectes sapidus* is widely consumed due to its protein content (14–19%) and body size, which has led scientists to declare it to be an interesting source of protein for human exploitation [32,68]. Metal concentrations were lower than the maximum permissible levels established by the EU Commission Regulation 2015/1005 [69] for muscle meat in crustaceans (0.5 mg kg^{-1}). In this study, the highest Pb concentrations in muscles were obtained from a crab fed on mussels from Portmán Bay (0.261 mg kg^{-1}) and exposed to MPs in the water, and so a priori, there would seem to be no reason not to consume meat from those specimens.

No statistical differences in Pb tissue concentrations were found between crabs exposed and not exposed to MPs (Table 1), although the highest Pb concentrations were found in tissues of crabs fed on mussels from Portmán Bay that were exposed to MPs. Based on our results, it seems that an increase in Pb accumulation could occur when MPs are present in the water. When MPs occur in their oxidised form, they can adsorb and act as vectors of contaminants such as heavy metals that are present in the water, thereby increasing the

exposure of organisms to metal pollutants [70–72]. In the present study, the MPs (AQUATEX 100) were an oxidised polyethylene that could have adsorbed and concentrated the Pb present in the water, which could have facilitated the entrance of Pb bound to MPs in crabs in two ways: (1) in the hepatopancreas while feeding and (2) through the gills in the water. Nevertheless, and as mentioned above, the Pb concentrations in the water were very low, and the exposure time was only seven days. Differences in metal adsorption by virgin and beached polyethylene pellets have been recorded in experiments under estuarine and marine conditions by Holmes et al. [73,74]. According to these authors, the metal uptake by plastics pellets was greater in plastics that had spent more time in seawater ('aged plastics') than in others that had not been in contact with water. This difference in metal adsorption is caused presumably by the changes in the surface properties that occur when plastic pellets are in contact with the marine environment. In addition, the adsorption of metal ions can be increased through processes such as photo-oxidative weathering, the accumulation of biofilms and chemical hydrogenous precipitates [73,75,76]. In this sense, Brennecke et al. [77] have reported an increase in heavy metals in MPs after 14 days. In our study, the exposure to MPs was only for seven days and so further studies are necessary to test whether or not this tendency becomes significant as the exposure time increases. In addition, there is concern about the ingestion of MPs. Redondo-Hasselerharm et al. [78] report the presence of MPs in the body and faeces of *Gammarus pulex*, while other studies have concluded that sediments can constitute a source for ingestion of MPs [79]. However, a recent study has provided evidence that, owing to their scavenging and digestive activity, benthic crustaceans could facilitate the fragmentation of the MPs that have accumulated in sediments and thus give rise to a new kind of 'secondary' MPs [80]. According to Renzi et al. [81], this process could affect aquatic organisms and contribute to the bioaccumulation of metals and chemical substances released from ingested MPs. Thus, more studies on the interaction of crustaceans with MPs are required to clarify these ideas.

4. Conclusions

In conclusion, *Callinectes sapidus* could be used as a bioindicator for studying the occurrence of this non-essential metal (lead) in polluted areas as it does not play any role in crustaceans' life cycles and its presence permits us to determine how polluted an ecosystem is. The hepatopancreas is the best tissue for measuring Pb concentrations after dietary exposure. The gills may be of interest in highly contaminated zones. Muscles, on the other hand, provide little information relating to environmental conditions and the presence of Pb. The presence of MPs in the water could increase the accumulation of Pb in the tissues of *C. sapidus*. However, to confirm this hypothesis, longer-term studies are still required. Finally, the human consumption of muscle tissue from *C. sapidus* from zones with high Pb content does not seem to constitute a risk for consumers, although there could be some risk if all soft tissues are ingested.

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