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Assessment of Heavy Metal Contamination in Two Edible Fish Species and Water from North Patagonia Estuary

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Abstract: Estuaries worldwide have been severely degraded and become reservoirs for many types of pollutants, such as heavy metals. This study investigated the levels of Cd, Cu, Mn, Ni, Pb, and Zn in water and whole fish. We sampled 40 juvenile silversides *Odontesthes regia* and 41 juvenile puye *Galaxias maculatus* from the Valdivia River estuary, adjacent to the urban area in southern South America (Chile). Samples were analyzed using a flame atomic absorption spectrophotometer. In water samples, metals except Zn were mostly below the detection limits and all metals were below the maximum levels established by local guidelines in this estuary. In whole fish samples, concentrations of Cu, Zn, Pb, Mn, and Cd were significantly higher in puyes than in silversides. Additionally, Zn, Pb, and Mn were correlated to body length and weight in puyes, whereas Cd was correlated to body length in silversides. The mean concentration of heavy metals in silverside and puyes were higher than those reported in the literature. In silversides, all heavy metal levels were below the limits permitted by current legislation (FAO), whereas in puyes Pb and Cd levels were above the recommended maximum level established by international guidelines, therefore putting the human population at risk.

Keywords: bioaccumulation; metal toxicity; silverside; puye; *Odontesthes regia*; *Galaxias maculatus*; lead poisoning; cadmium poisoning

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1. Introduction

Estuaries are highly productive systems that support commercially important organisms and are a refuge for many species during spawning. However, estuaries worldwide have been severely degraded because they are semi-closed water bodies and are often close to urban areas, and therefore become reservoirs of many types of pollutants [1,2]. Heavy metals are one of the main groups of these pollutants and are present in the aquatic environment at elevated levels because of human activity [3]. Heavy metals can be dissolved or suspended in water and are then absorbed by aquatic organisms and accumulate in various organs. They are then transferred to food webs and create a risk to human health if concentrations reach high levels [4,5].

Fish provide minerals, proteins, nutrients, and metals to humans and are part of the daily diet of many people worldwide. Many fish species can bioaccumulate metals in their tissues, depending on size, age, feed type, and habitats [6]. The sea silverside (*Odontesthes regia*) is a marine fish endemic in South America, reported between Magellan Strait (53° S) and Antofagasta (23° S) in Chile [7]. Adults remain close to the estuary mouth and coast, whereas juveniles remain at the estuary bank among submerged vegetation [8]. Silversides are considered juvenile until they are approximately 14 cm long, and adults can be up to 22 cm long. The puye *Galaxias maculatus* has a sub-Antarctic distribution, founded in southern South America, South Africa, Australia, New Zealand, the Falkland Islands, the

Chatham Islands, and Tasmania. In Chile, it is often an abundant species in lakes, rivers, and estuaries, present between Aconcagua (30° S) and Tierra del Fuego Island (56° S) [9,10]. In estuarine populations, adults and juveniles are found in estuary banks, coexisting with *O. regia*. A puye is considered juvenile until it is approximately 5.8 cm long, whereas adults can be up to 14 cm long. *O. regia* and *G. maculatus* form part of the diet of birds, mammals, and fish and are also captured by artisanal fishermen, who make up a significant part of the fishing economy in southern Chile [11].

The Valdivia River estuary is one of the biggest estuaries in Chile, being an important nursery for silversides and puyes [12]. However, pollutants from upstream are transferred into this estuary as wastewater from the cellulose industry, sewage, and urban and agricultural effluents. Therefore, it is likely that the Valdivia River estuary has high levels of heavy metal contamination in the water itself and aquatic organisms. We aimed to determine heavy metal levels (Cd, Cu, Mn, Ni, Pb, and Zn) in water samples and *Odontesthes regia* and *Galaxias maculatus* from the Valdivia River estuary in southern South America. Obtaining knowledge of the pollutant load in this estuary affecting young fish will increase our understanding of the ecological effects associated with moderately disturbed estuaries and will contribute to the development of management programs of estuary ecosystems in South America.

2. Materials and Methods

2.1. Study Area

The Valdivia River estuary is located in the Los Ríos Region of Chile (39°48′ S, 73°14′ W) (Figure 1). The area has a humid temperate climate with an average annual precipitation of 1600 mm and an average annual temperature is 12.9 °C. Most rainfall occurs between May, June, and July. The surface water level in the estuary fluctuates seasonally, increasing in the winter months because discharges are mostly caused by rain. The Valdivia River estuary has a semi-diurnal tidal regimen with ±1.48 m of mean tidal range and is classified as "positive microbial" [13] and tidal movement takes place 20 km inland of the river´s mouth. The Valdivia River estuary has a mean width of 700 m and a maximum depth of 18 m close to Valdivia city [8], which has a population approximately of 143,000 people. Two sampling sites were established within the estuary to evaluate water quality and fish samples, Site 1—Los Pelues, adjacent to the city area (39°50′ S–73°16′ W) and Site 2—Las Mulatas, downstream of the sewage treatment plant (39°48′ S–73°15′ W) (Figure 1).

2.2. Sample Collection

Field sampling was conducted in July 2010 (Austral winter). Surface water samples for heavy metal determination were collected at 0.2 m depth with 0.5 L polyethylene terephthalate bottles. Samples were taken in duplicate at each sampling station and high and low tide and transported in darkness at 4 °C to the laboratory.

We obtained 40 silverside juveniles and 41 puye juveniles in the study area. Fish were captured using a purse seine of 2 mm mesh, which measured 2 m × 6 m in the upper sub-littoral zone (0–1 m) at low tide. Captured specimens were fixed with 90% ethanol and transported in plastic bags to the laboratory where they were then weighed (using a plastic plate on an electronic analytical balance, 0.001 g precision) and measured (standard length using a plastic millimeter ruler, 0.1 mm precision). Whole fish were stored at −20 °C in pre-cleaned Teflon containers and labeled until chemical treatment. Plastic instruments were used to avoid contamination with metals.

2.3. Chemical Analysis

Water samples were analyzed in duplicate. A total of 500 mL of water was filtered using a 0.45 µm filter, then 1 mL of 70% nitric acid was added and pre-concentrated by evaporation on a Thermolyne Cimarec 2 heating plate under an extraction hood until it reached a sufficient volume to carry a capacity of 50 mL. The samples were packaged and stored until measured using an atomic absorption spectrophotometer (AAS) (Thermo Fisher

Scientific ICE 3000 Series, Cambridge, UK) Along with the samples, chemical treatment was performed on sample blanks, which were treated and stored as described above.

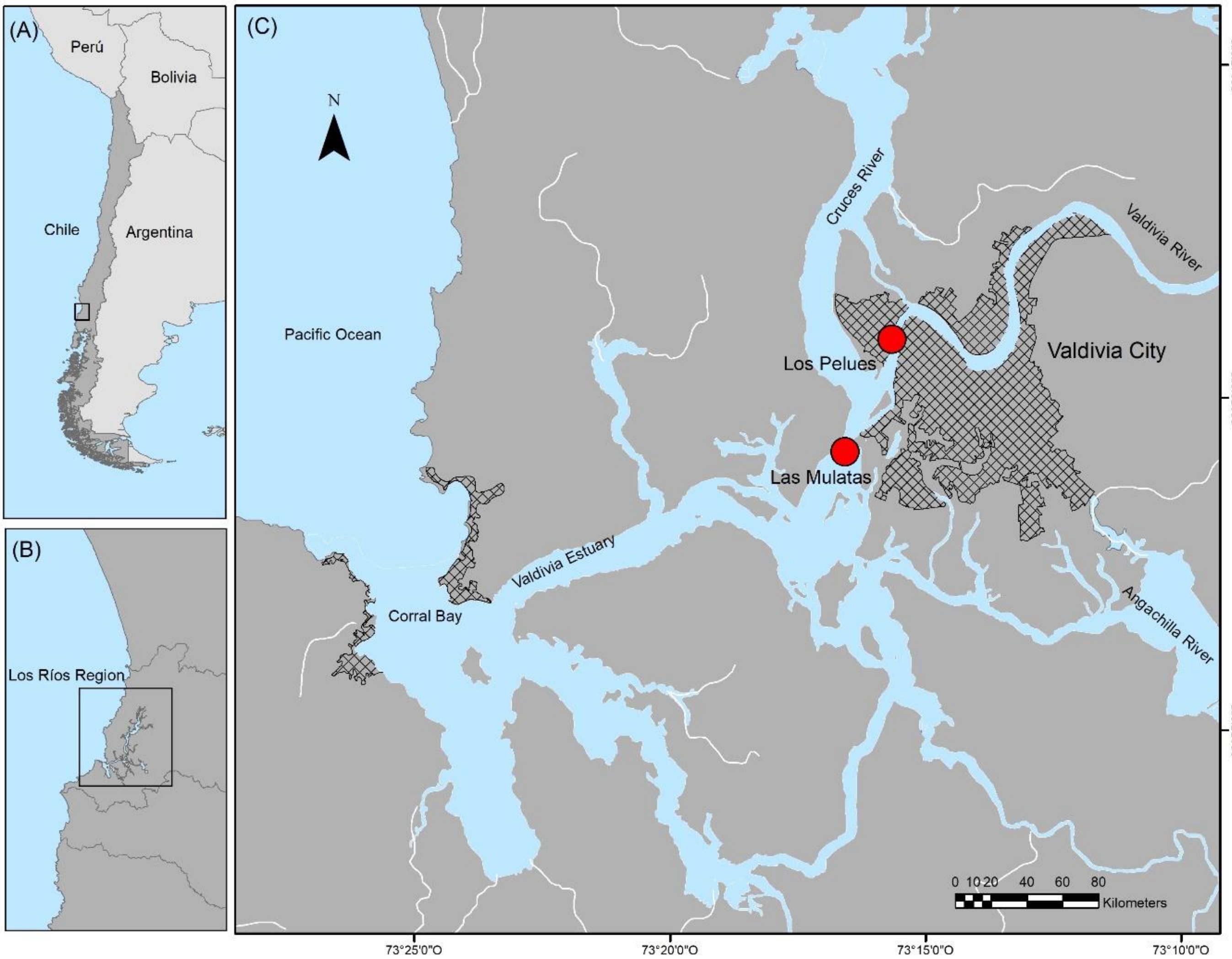

Figure 1. Map of the study area and the two sampling stations for *Odontesthes regia*, *Galaxias maculatus*, and water samples of the Valdivia River estuary in southern Chile. (**A**,**B**) Show the locality in South America and Chile. (**C**) Shows the Valdivia River estuary.

Tissue samples were lyophilized to a constant weight using a Freeze Dryer (Labconco, Kansas City, MI, USA), and then homogenized and stored in pretreated transparent plastic containers until they were analyzed. For digestion, 1.0 g of sample was weighed and 10 mL of nitric acid Suprapur was added (10 M acid concentration). Subsequently, the samples were almost dried under constant agitation under a hood and using a heating plate calibrated at 90 °C. The resulting solution was filtered and washed with double distilled water to a final volume of 50 mL in a pretreated volumetric flask. The analyses were carried out in parallel with their respective blank samples, and metal composition was measured by air-acetylene flame atomic absorption spectroscopy for Cu, Zn, Pb, Mn, Cd, and Ni (Spectrophotometer Thermo Fisher, iCE 3000). All the reagents used were of high purity (Suprapure, Merck, Darmstadt, Germany). The detection limits were Cu 4.5 µg/L, Zn 3.3 µg/L, Pb 13 µg/L, Mn 1.6 µg/L, Cd 2.8 µg/L, and Ni 8.0 µg/L.

2.4. Validation Methodology

The analytical method was validated using certified reference material DOLT-1 (dogfish liver) supplied by the NRC (National Research Council). Table 1 shows the results of the measurements of Cu, Zn, Pb, Mn, Cd, and Ni using the DOLT-1 certified reference material. Reference material had relative errors for tissue between −11.5% (Ni) and +8.82% (Pb).

Table 1. Cd, Cu, Mn, Ni, Pb, Zn concentrations ug/g (dry weight) in the DOLT-1 certified fish reference material (liver) from the National Research Council Canada.

	Certificate Concentration [a] μg/g	Observed Concentration [a] μg/g	Recovered Percentage
DOLT-1			
Cu	20.8 ± 1.2	21.2 ± 2.6	101.9
Zn	92.5 ± 2.3	94.8 ± 2.82	102.5
Pb	1.36 ± 0.29	1.48 ± 0.33	108.8
Mn	8.72 ± 0.53	8.13 ± 0.51	93.2
Cd	4.18 ± 0.28	3.96 ± 0.52	94.7
Ni	0.26 ± 0.06	0.23 ± 0.12	88.5

[a] Mean with the standard deviation of three independent measurements.

2.5. Statistical Analysis

Data were checked previously for normality, independence and homoscedasticity. The significance of differences between the heavy metal concentrations of fish was tested by Student's *t*-test. Pearson´s correlation analysis was performed between the weight and length of each fish species versus heavy metal concentration in the tissue. Box and whisker plots were constructed using RStudio statistical software [14].

3. Results

3.1. Heavy Metal Content in Water

A total of eight samples were analyzed by sampling site (two samples per tide, each one analyzed in duplicate). The results obtained for the estuary water samples at high and low tide from Los Peleus (site 1) and Las Mulatas (site 2) sites are presented in Table 2. Cu, Pb, Mn, Cd, and Ni values in water samples at the two sites and during both tides were below the detection limits. Zn concentration was higher downstream of the city (site 2) at both high and low tides.

Table 2. Heavy metal concentration in water from the Valdivia River estuary (μg/L) (winter, July 2010). WQRCh: Water quality regulations in Chile (maximum levels allowed in the Valdivia River estuary).

	Locality	Cu	Zn	Pb	Mn	Cd	Ni
High tide	Site I	<4.5	<3.3	<13.0	<1.6	<2.8	<8.0
	Site II	<4.5	4.8 ± 6.1	<13.0	<1.6	<2.8	<8.0
Low tide	Site I	<4.5	<3.3	<13.0	<1.6	<2.8	<8.0
	Site II	<4.5	4.1 ± 2.7	<13.0	<1.6	<2.8	<8.0
WQRCh		<20.0	<16.0	-	<40.0	-	-

3.2. Heavy Metal Content in Fish

Of the total 81 fish samples, the mean lengths of *O. regia* (N = 40) and *G. maculatus* (N = 41) were 6.3 cm (4.9–9.7 cm) and 4.8 cm (4–5.5 cm), respectively. The mean weights of *O. regia* and *G. maculatus* were 1.7 g (0.9–5.6 g) and 0.5 g (0.3–0.7 g), respectively. Mean concentrations in tissue of *O. regia* followed the order; Zn > Mn > Pb > Cu > Cd > Ni, while in *G. maculatus* tissue the order was Zn > Cd > Mn > Pb > Cu > Ni.

The average concentrations of Cu recorded in the tissue samples were 5.4 µg/g (0.17–13.3 µg/g) in *O. regia*, and 10.4 µg/g (0.11–32.08 µg/g) in *G. maculatus* (Figure 2). Zn average concentrations were 104.7 µg/g (45.14–181.5 µg/g) in *O. regia*, and 155.2 µg/g (35.08–308.54 µg/g) in *G. maculatus* (Figure 2). Pb average concentrations were 8.5 µg/g (0.18-35.04 µg/g) in *O. regia*, and 22.7 µg/g (1.33–73.96 µg/g) in *G. maculatus* (Figure 2). Mn average concentrations were 10.8 µg/g (3.24–30.58 µg/g) in *O. regia*, and 22.9 µg/g (5.49–42.34 µg/g) in *G. maculatus* (Figure 2). Cd average concentrations were 4.1 µg/g (0.47–13.98 µg/g) in *O. regia*, and 28.4 µg/g (9.76–60.24 µg/g) in *G. maculatus* (Figure 2). Ni average concentrations were 3.3 µg/g (2.09–5.47 µg/g) in *O. regia*, and 6.4 µg/g (0.91–16.17 µg/g) in *G. maculatus* (Figure 2).

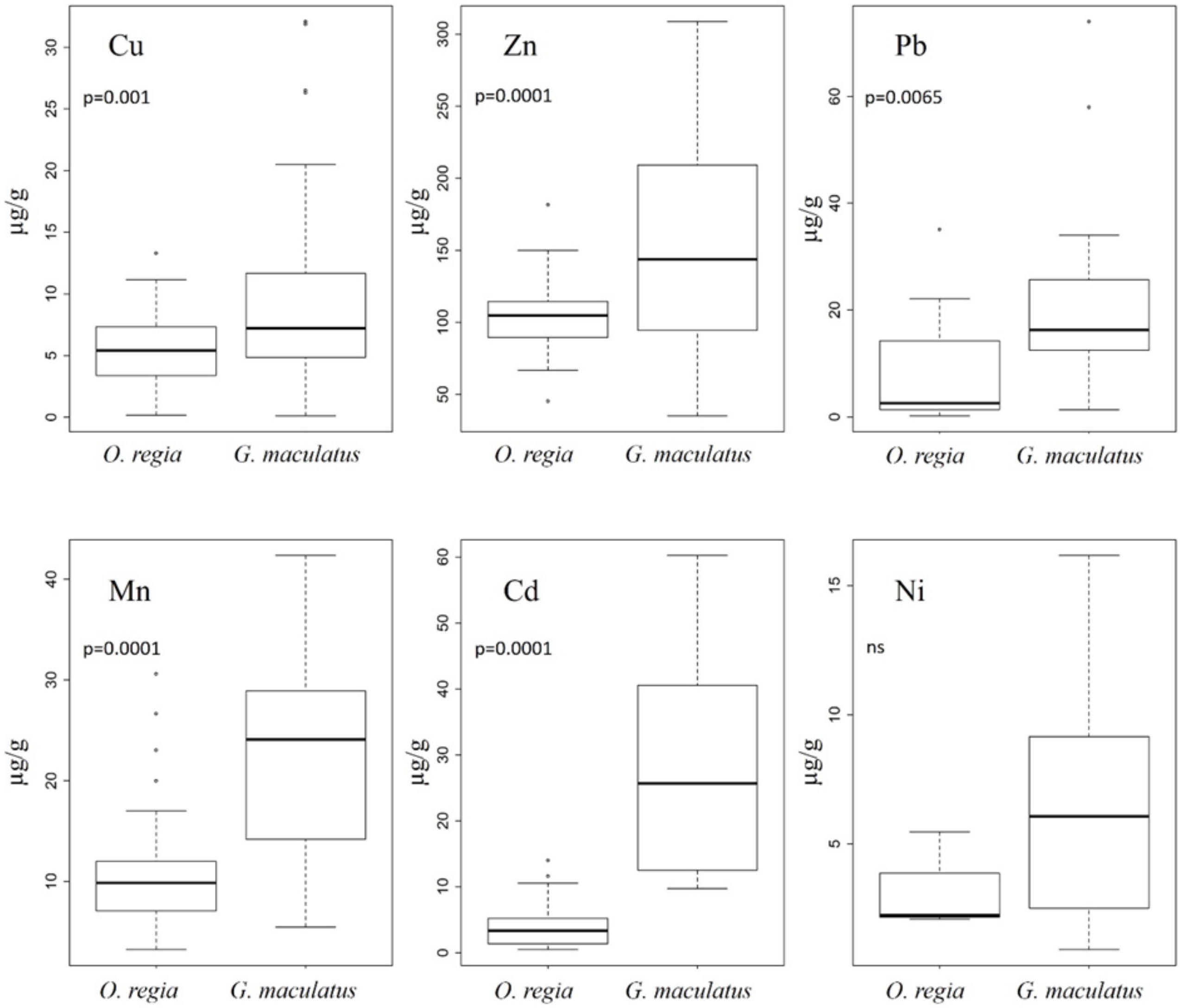

Figure 2. Boxplots of Cu, Zn, Pb, Mn, Cd, and Ni concentrations in samples of *Odontesthes regia* and *Galaxias maculatus* collected from the Valdivia estuary. Boxplots show the maximum and minimum as well as the interquartile ranges (25–75%) with solid lines representing median values.

Statistical analysis (Student's *t*-test, $p < 0.05$) showed significant differences in Cu, Zn, Pb, Mn, and Cd concentrations between fish species (Figure 2). Ni concentration (F = 6.50, $p > 0.05$) was not significantly different between fish.

3.3. Correlation between Heavy Metal and Fish

We found a significant negative correlation between Cd and the body length of silversides (*O. regia*) in the Valdivia River estuary (Table 3). In puyes (*G. maculatus*), we found significant negative correlations between Zn and Mn and body length, whereas Pb was significantly correlated with increased body weight (Table 3).

Table 3. Pearson correlation levels and significance between heavy metals from tissue, and body size variables (Length and weight) of *Odontesthes regia* and *Galaxias maculatus* individuals from the Valdivia River estuary. Statistically significant values for pairwise comparisons are denoted by * < 0.05, *** < 0.001.

	Body Length		Body Weight	
Metal	***O. regia***	***G. maculatus***	***O. regia***	***G. maculatus***
Cu	−0.09	−0.34	−0.05	−0.13
Zn	−0.24	−0.56 ***	−0.25	0.20
Pb	−0.26	−0.43	−0.20	0.59 *
Mn	0.02	−0.53 ***	0.02	−0.05
Cd	−0.43 *	−0.43	−0.32	0.58
Ni	−0.23	−0.43	−0.32	−0.30

4. Discussion

Our study evaluated the concentrations of Cu, Zn, Pb, Mn, Cd, and Ni in water and fish species in the Valdivia River estuary. The heavy metal loads reported were lower than other estuaries that are slightly polluted in South America [15]. The metal with the highest concentration was Zn; however, the levels were extremely low compared to polluted estuaries. For example, the Odiel Estuary in Spain and the La Plata estuary in Argentina has Zn concentrations between 83 to 223 µg/L [16] and 37 to 116 µg/L, respectively [17]. Our measurements show heavy metal concentrations in the Valdivia River basin are currently below the recommended values permitted by the current legislation (see Table 2), indicating a low risk of heavy metal contamination to the aquatic environment. Zn, Pb, and Cu occur naturally within the earth´s crust; however, here we recognized domestic wastewater as the predominant source of Zn and Cu, and sewage and urban effluents to be the main sources of Pb [18]. Zn concentrations in the water, both at low and high tide were higher downstream from the city; therefore, this could indicate the possible impact of the city on Zn levels in estuary water.

Zn was the metal with the highest levels in the water samples and in both fish species studied. High levels of Zn compared to other metals have already been reported in other aquatic organisms, including fish [6,19]. High Zn levels are naturally found in sediments of saline lagoons in Chile, which is attributed to naturally occurring phenomena [20]. Zn, Pb, Mn and Cd concentrations were highly correlated in both fish species, likely because they are important elements in animals that synthesize metalloenzymes and metallothionein [6]. Fish can regulate Zn and Cu concentrations in their tissue, including liver and muscle; however, excessive concentrations combined with other metals can be toxic to animals, resulting in reduced growth and respiratory distress [21,22].

Both juvenile fish species coexist and inhabit the littoral estuary among submerged vegetation. The principal component of the diets of small silversides and puyes are zooplankton and benthic prey (mainly invertebrates) [8,23]. Despite this similarity in their life habitats, puyes had higher levels of most heavy metals compared to silversides. Silversides may regulate metal concentration more efficiently than puyes because the principal way bioaccumulation of metals occurs in fish is by ingesting polluted prey. Our results suggest that puyes bioaccumulate more metals than silversides. Three of the six metals were significantly correlated to length and weight in puyes, indicating that large puye individuals do not efficiently assimilate metals (Table 3). Therefore, it seems that metabolic differences between the species cause the difference in heavy metal concentration rather than the food source; however, further research is needed to test this hypothesis.

Overexploitation of silversides and puyes for food purposes occurs in rural areas throughout Chile [11]. Juvenile stage puyes are consumed whole by humans, whereas only the muscle is edible in adult stages of silversides. In general, all the metals for the examined silversides from the Valdivia River estuary were below the Permissible Tolerable Daily Intake (PTDI) (see Table 4). Only copper concentration slightly exceeded the PTDI. On the other hand, in *G. maculatus*, Cu, Mn, and Cd levels were above the recommended values, indicating that consumption of puye could be a health risk to humans [24,25]. The estimated daily intake values presented in Table 4 were estimated assuming that a 70 kg human consumes 0.14 kg fish per week [26]. Even though copper and manganese are essential nutritional elements for humans, they can be toxic when ingested in high doses [27]. For instance, high levels of Cu be toxic and lead to an increased risk of neurodegenerative disease. Furthermore, Mn plays a fundamental role in neurodevelopment, brain function and is involved in oxidative stress response, and bioaccumulation Cd (a well-established carcinogen) can cause complications during pregnancy and even require abortion if exposure levels are high [27,28].

Table 4. A comparative account of the estimated daily intake of heavy metals from *O. regia* and *G. maculatus* (edible part) with the permissible tolerable daily intake (PTDI) (mg/day/70 kg body weight).

Metal	Mean Concentration (mg/kg-dry wt)		Estimated Daily Intake (mg/day/person)		PTDI (mg/day/person) *
	O. regia	*G. maculatus*	*O. regia*	*G. maculatus*	
Cu	5.4	10.4	0.11	0.21	0.07
Zn	104.7	155.2	2.09	3.1	35
Pb	8.5	22.7	0.17	0.45	9.8
Mn	10.8	22.9	0.22	0.46	0.35
Cd	4.1	28.4	0.08	0.57	0.25
Ni	3.3	6.4	0.07	0.13	70

* Reference from Türkmen and Dura, 2016 [32].

Pb in *G. maculatus* was the only heavy metal that was positively correlated with body weight. High levels of Pb can replace Calcium, accumulating in bones, the articular system and in the teeth of humans, eventually leading to encephalopathy, renal disease, colic disease, and anemia. Therefore, consuming excessive large puyes whole could also lead to possible negative effects on humans [29].

Heavy metal concentrations in whole fish of this study are higher than that reported in the muscle of estuarine fish, both from the same genus (*Odontesthes*) and other species (Table 5). Bioaccumulation of heavy metals in the same fish species can vary between studies due to several factors such as different metabolic rates, different metal concentrations in the environment, and extraction rates [30]. The high metal concentrations measured in this study could be due to performing the analysis with the whole animal, and not only with muscle tissue. This could explain our results because in juvenile silversides and puyes consumption of the whole individual implicates eating organs such as the liver, which is the main metabolic organ in fish and where the highest metal concentrations are found [31].

Our study has some limitations that must be considered. The low number of sampling sites could limit the representativeness of our results to other estuaries of Chile. Additionally, the degree of the heavy metal concentration varies between estuaries, according to size, tidal range, and proximity to urban areas, making our results specific to the Valdivia River estuary. Therefore, to evaluate if ingesting juvenile silversides and puyes constitutes a risk to human health in Chile, new sampling efforts in other estuaries that are more or less polluted must be considered in future studies.

Table 5. The concentration (µg/g) levels of heavy metals in whole samples of *Odontesthes regia* and *Galaxias maculatus* from this study, and concentration of heavy metals in the muscle of other estuarine fish.

Species	Location	Date	Cu	Zn	Pb	Mn	Cd	Ni	Reference
O. regia	Valdivia Estuary—Chile	2010	5.4	104.7	8.5	10.8	4.1	3.3	This paper
G. maculatus	Valdivia Estuary—Chile	2010	10.4	155.2	22.7	22.9	28.4	6.4	This paper
O. bonariensis	De La Plata Estuary—Argentina	2011	-	12	0.2	0.3	-	0.1	Avigliano et al., 2015 [33]
O. bonariensis	Adela Lagoon—Argentina	2011	-	14	0.1	0.4	-	0.1	Avigliano et al., 2015 [33]
O. bonariensis	Barrancas Lagoon—Argentina	2011	-	12.6	0.1	0.4	-	0.1	Avigliano et al., 2015 [33]
Odontesthes sp.	De La Plata Estuary—Uruguay	1998	1.1	32	-	-	-	-	Viana et al., 2005 [34]
M. furnieri	De La Plata Estuary—Uruguay	1998	1	17	-	-	-	-	Viana et al., 2005 [34]
M. manni	Budi Lagoon—Chile	2004	-	7.3	0.3	1.8	0.1	-	Tapia et al., 2009 [35]
M. cephalus	Black Sea region—Turkey	2014	1.5	-	0.1	0.4	0.1	0.7	Soner Engin, 2015 [36]
M. cephalus	Maule Estuary—Chile	2005	5.9	-	0.4	-	-	-	Tapia et al., 2012 [37]
M. cephalus	Mataquito Estuary—Chile	2005	6.2	-	0.4	-	-	-	Tapia et al., 2012 [37]
E. maclovinus	Maule Estuary—Chile	2005	3.6	-	1.9	-	-	-	Tapia et al., 2012 [37]
E. maclovinus	Mataquito Estuary—Chile	2005	3.8	-	1.6	-	-	-	Tapia et al., 2012 [37]

5. Conclusions

The water samples collected from the Valdivia River estuary did not exceed the environmental limits for the heavy metals analyzed and were below the recommended values permitted by the current legislation. Zn concentration was different between high and low tides, as well as among sampling stations, suggesting an input of metals into the estuary within the urban area. Comparison of heavy metals between fish species showed significant differences between the concentration of copper, zinc, lead, manganese, and cadmium, with higher concentrations in puyes than silversides. All heavy metal concentrations analyzed in both species were compared to other estuarine fish. The Cu concentration in *O. regia* and the Cu, Mn, and Cd concentrations in *G. maculatus* were over the permissible tolerable daily intake for human consumption, meaning that consumption may pose a risk to human health. We observed significant correlations between body length and Zn, Mn, and Cd concentrations in both species, and between body weight and Pb concentration to puyes. At present, the Valdivia River estuary is processing a guideline limit for heavy metal concentrations, and other physic-chemical variables. Therefore, our results provide empirical information that can be used for the management of estuaries in Chile. Further analyses must be carried out to evaluate the source of contamination in the estuary, as well as evaluate the effect of contamination in specific kinds of large estuary fish, in which muscle tissue is predominantly consumed by humans.

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Institutional Review Board Statement: The experiment/samples were performed in accordance with the guidelines for the use of laboratory animals established by the Comisión Nacional de Ciencia y Tecnología de Chile (CONICYT) and the Universidad Austral de Chile.

Informed Consent Statement: This study is not applicable due to it is not involving humans.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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