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Seasonal Differences in Water Pollution and Liver Histopathology of Iberian Barbel (*Luciobarbus bocagei*) and Douro Nase (*Pseudochondrostoma duriense*) in an Agricultural Watershed

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Abstract: Histopathology has been used as a very useful tool to provide information on the severity of tissue damage, injuries, and organ functionality. Thus, this work aimed to assess whether seasonal variations (summer and winter) in water quality had consequences on the liver histology of Iberian barbel (*Luciobarbus bocagei*) and Douro nase (*Pseudochondrostoma duriense*). The research was carried out in the Vilariça River, a tributary of the Sabor River in Portugal, which is used as spawning grounds by these endemic cyprinids. The liver histopathological changes, assessed through a semi-quantitative system, allowed the identification of 13 histopathological changes located in the hepatic parenchyma, bile duct, and blood vessels. The histopathological changes with a higher prevalence in both species were vacuolization of hepatocytes, endothelial rupture, necrosis, fibrosis, and degenerative vacuolization. The results showed that the severity degree of liver histological alterations ranged between moderate and severe, and the major severity degree was observed in *L. bocagei*, in the summer season, and at the sampling points located in the downstream and middle stream. The canonical analysis indicated that the exposure of fish to metals may increase the potential risk of liver damage. Thus, in the summer, the high concentrations of Fe, Cu, Zn, As, and Mn justified the prevalence of the biliary duct epithelial detachment, in both species, and the hyperplasia of biliary epithelium, in *L. bocagei*. In the winter, the high TSS and Cd, Ni, and Cr concentrations justified the prevalence of congestion of blood vessels and degenerative vacuolization in both species. The higher hepatosomatic index of fish caught in the winter was due to the high presence of degenerative vacuolization and hepatocyte vacuolization. The severity of liver histopathological changes reflected differences in the type of contaminants in different seasons and sampling periods, and was thus proven as a valuable indicator of water quality.

Keywords: water quality; metal concentration in river water; fish liver histopathology; hepatosomatic index; cyprinidae

1. Introduction

Pollution in aquatic environments has been reported to cause health problems in fish, such as reduced growth and fitness, low reproductive success [1], increased susceptibility to diseases and survival [2,3], and the occurrence of histopathological alterations [4]. These health problems have been attributed to the presence of a wide range of pollutants such as heavy metals [1,2], polycyclic aromatic hydrocarbons (PAHs) [3,4], pesticides (herbicides) [5], and endocrine-disrupting chemicals [6–8] in the aquatic environment. Pollution in aquatic environments is mainly due to discharges from industrial and municipal wastewater [9,10] and excessive agricultural fertilizers that reach the rivers [11]. Moreover, recurrent wildfires have serious consequences for soil erosion and nutrient exports, namely by deteriorating the water quality in the river basins [12–15].

In the assessment of the health status of fish, histology and histopathology have been used as very useful tools to provide information on the severity of tissue damage, injuries, and organ functionality [16]. Indeed, the analysis of histopathological changes in the organs of fish (liver, gill, kidney, etc.) has been used in environmental monitoring studies as excellent biomarkers to monitor aquatic contamination [17,18]. The liver has an important function in biotransformation, detoxification, and elimination of xenobiotics [16–18]. However, the consequent accumulation of contaminants in the liver can induce structural and functional alterations and reveal the failure of detoxification [16]. Histopathological changes such as hepatocyte vacuolization, melanomacrophage centers, necrosis, inflammation, fibrosis, and thickening of the bile duct wall are the most documented [17]. Many researchers who tried to link histopathological changes to contaminants in water interpreted them as nonspecific responses to the diversity of pollutants existent in the aquatic environment e.g., [2,17,19]. However, the relationship between histopathological changes and the accumulation of metals in the liver has been widely documented [2,20,21]. Heavy metals pose a serious threat because they are not biodegradable and, when accumulated in organisms, can reach toxic levels. Therefore, chronic exposure to high concentrations can constitute a threat to public health and aquatic organisms [2,20].

The Iberian barbel (*Luciobarbus bocagei*) (Steindachner, 1864) and Douro nase (*Pseudochondrostoma duriense*) (Coelho, 1985) are endemic fish species (Cyprinidae) of the Iberian Peninsula. According to the International Union for Conservation of Nature and Natural Resources (IUCN) red list of threatened species, the Douro nase is classified as vulnerable [22]. In turn, in the Red Book of Vertebrates of Portugal, both species are classified as least concerning [23]. Both species inhabit the Douro River and migrate to the Sabor and the Vilariça River to spawn. Unfortunately, the migration to the Sabor River was blocked due to the construction of the Baixo Sabor Hydroelectric Scheme (BSHS), composed of two dams without fishways [24,25]. Thus, the Vilariça River became an important river to complete the reproductive cycle of these endemic Cyprinidae [25,26]. However, the basin of this river is widely agricultural, leading to water quality problems due to excessive fertilization. Indeed, the water was previously classified as polluted and extremely polluted in the summer and winter, respectively. In summer, the pollution is due to less streamflow and excessive agricultural fertilizers. In the winter, the pollution is caused by a combination of factors, including high levels of precipitation, steep topography, a thin soil layer, a lack of vegetation cover, and wildfires, which cause the erosion of soil particles containing heavy metals [27]. For this reason, it is imperative to assess the consequences of agricultural practices and water quality on fish health in the Vilariça River.

According to a literature review, the present study is the first approach to the potential effect of water quality on the liver of Douro nase species using histopathology as an assessment tool. Accordingly, the main objectives of this study were (1) to assess whether metal concentrations recorded in water may cause histopathological changes in the liver of *L. bocagei* and *P. duriense* and their respective severity degrees; (2) to relate the seasonal differences in the liver histopathological changes of both species with to metal concentrations in river water; and (3) to assess the seasonal differences in hepatosomatic index of both species *L. bocagei* and *P. duriense*, and relate them with the histopathological changes observed in the liver.

2. Materials and Methods

2.1. Sampling Site

The study area was on the Vilariça River, located in the northeast of Portugal. This river is a tributary of the Sabor River, and both are tributaries of the Douro River (Figure 1).

The Vilariça River is a medium-sized, third-order stream [28] with a catchment area of 324 km^2 (http://www.dgterritorio.pt/, accessed on 12 July 2021). It is located in a mountain watershed with a low population density and little industrialization. However, it is exposed to intensive agricultural activity (42% of the basin area is agricultural land), and also to cumulative pollution from different upstream sources, such as wastewater, recurrent wildfires, and soil erosion [27]. The four water and fish sampling points were chosen according to the Water Framework Directive determinations (WFD-2000/60/EC) [29]: in rivers less than 30 m wide, the length of the sampling stretch was twenty times the width, whereas in rivers wider than 30 m, the length was ten times the width.

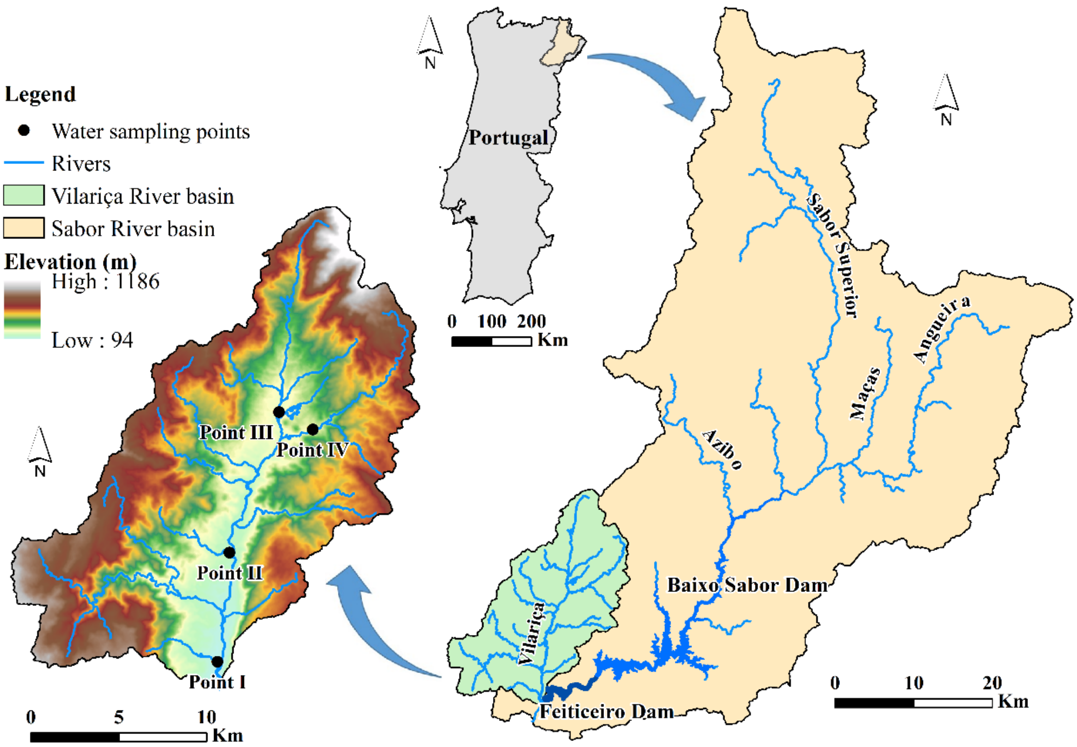

Figure 1. The Sabor and Vilariça Rivers basins are located in northern Portugal. In the Sabor River basin are represented the main rivers, the Feiticeiro Dam and the Baixo Sabor Dam of the Baixo Sabor hydroelectric scheme. The map of the Vilariça River basin shows the topography, drainage network, and sampling points.

2.2. *Water Quality Sampling and Assessment*

The water samples were collected in the Summer of 2016 (27 July and 3 August), and in the winter of 2017 (7 March), at the sampling points I, II, III and IV of the Vilariça River (Figure 1). At the four sampling points, the following parameters were measured in situ: water temperature (T °C), dissolved oxygen (mg/L), pH, and conductivity (µS/cm), using multiparametric probes (HQ40d Multi, HACH; YSI Ecosense EC300, Loveland, Colorado, USA). To assess other physicochemical parameters, such as Nitrite (NO_2^-), Nitrate (NO_3^-), Phosphate (PO_4^{3-}), Total Suspended Solids (TSS), Calcium (Ca), Magnesium (Mg), Potassium (K), Sodium (Na), and Sulfates (SO_4^{2-}), the water samples were collected and transported to the laboratory in a cold environment. Water samples were also collected, acidified with 1% nitric acid and transported to the laboratory for Iron (Fe), Aluminum (Al), Arsenic (As), Cadmium (Cd), Lead (Pb), Cobalt (Co), Copper (Cu), Manganese (Mn), Zinc (Zn), Nickel (Ni), and Chromium (Cr) determination. These physicochemical parameters were determined in the Chemical Analysis Laboratory, at the University of Trás-os-Montes and Alto Douro, according to the procedures and the limits of detection specified in Table S1. Water samples were transported on ice and preserved at 4 °C until determination. The physicochemical parameters were measured between 24 and 48 h after collection.

The criteria for the water quality assessment followed the methodologies proposed for classifying the status of surface water bodies for multiple uses [30–32]. This classification was divided into five classes: not polluted, weakly polluted, polluted, very polluted, and extremely polluted, agreeing with the measured value in each parameter (Table S2). This methodology follows Decree-law No. 236/98 [33], and the Water Framework Directive (WFD-2000/60/EC) [29]. In Portugal, the WFD was transposed to the national legal framework with Law No. 58/2005 (Water law) [34] and the Decree-law No. 77/2006 [35]. Briefly, the WFD requires that the overall ecological status of a water body is determined by the results of the biological or physicochemical quality element with the worst class (i.e., the quality element worst affected by human activity). This methodology for water quality assessment is called the "one out-all out" principle. Accordingly, the water quality assessment of each sampling site monitored in the Vilariça River (points I, II, III and IV) results from the parameter with the worst classification.

2.3. Fishes Data Collection

The fishes were sampled during the same seasons and sampling points as the water samples, with the exception of point IV, where there were no fish in the summer of 2016, and fish were too small to be caught (<50 mm) in the winter of 2017. In points I, II and III, individuals of *L. bocagei* and *P. duriense* were captured using pulsed direct current backpack electrofishing equipment with a DC-500 V generator. After capturing, fish were anaesthetized by immersion in 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222), weighed, measured, and immediately euthanized by decapitation. For each fish, the liver was randomly sampled, weighed, and preserved in 4% buffered formaldehyde (Panreac, Castellar del Vallés, Spain) [27,36] for histopathological analysis. Fish sampling was conducted in accordance with the Ethical Guidelines of the European Union Council (European Directive 2010/63/EU) [37] and the Portuguese Agricultural Ministry [38] for the protection of animals used for experimental and other scientific purposes.

2.4. Histological Processing

The liver was fixed in neutral formaldehyde for 24 h, dehydrated through a series of graded ethanol (Fisher Scientific, Loughborough, UK) solutions (70–100%), cleared in xylene (Fisher Scientific, Loughborough, UK), and embedded in paraffin (Histosec® pastilles, Merck, Darmstadt, Germany) in a semi-enclosed tissue processor (Tissue-Tek II model 4634, Tokyo, Japan). Liver samples were then included in paraffin in a modular tissue embedding center (Leica EG 1160, Wetzlar, Germany) to be sectioned (3 µm thick) in a rotary microtome (Leica RM 2135, Wetzlar, Germany). The sampled sections, 3 replicates, were mounted on slides and stained with hematoxylin-eosin (H&E stain, Merck, Damstadt, Germany) before being coverslipped (Figure 2).

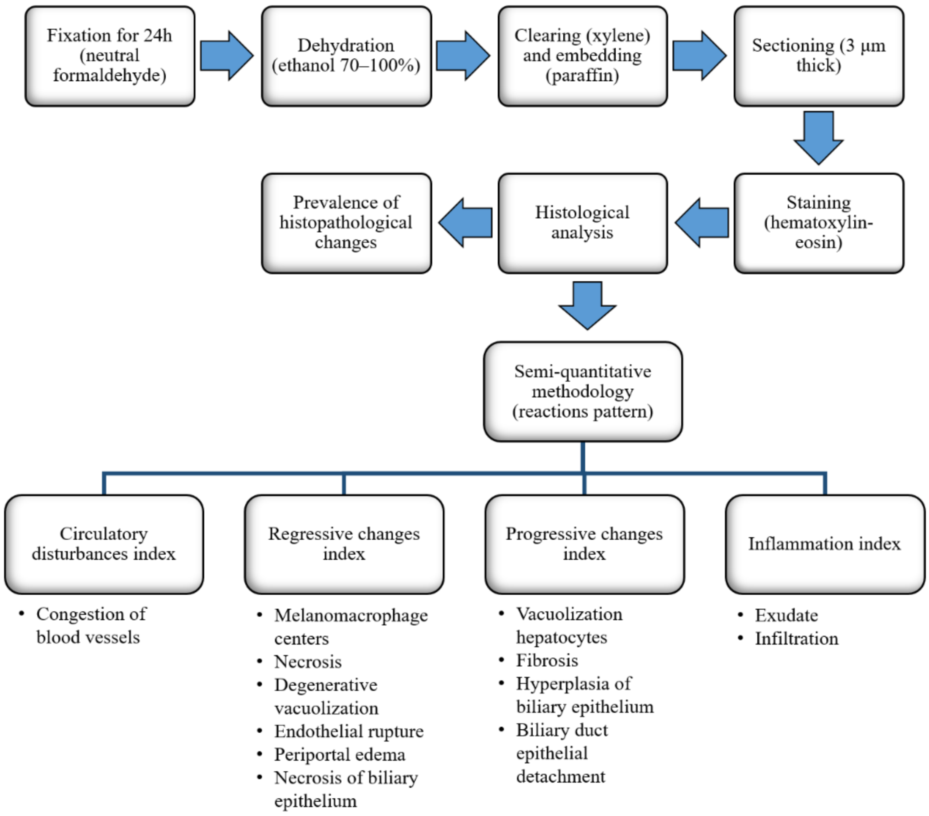

Figure 2. Diagram of liver histological processing and the semi-quantitative methodological analysis.

2.5. Histological Analysis of the Liver

Liver histological preparations were observed under a microscope (Olympus, IX 51, Tokyo, Japan) for the identification of histopathological alterations. A qualitative evaluation of the histopathological changes was first performed in each fish. The prevalence of each histopathological change, defined as the percentage of fish exhibiting that specific change [39], was then determined (Figure 2). The prevalence of each histopathological change (P) was expressed by dividing the number of fish in which the change was observed by the total number of fish analyzed:

$$P(\%) = \frac{n_{HChange}}{n_{total}} \times 100 \quad (1)$$

where $n_{HChange}$ is the number of fish with detected histopathological change and n_{total} is the total number of fish analyzed.

The semi-quantitative methodology, described by Bernett et al. [40], was adapted to assess the severity of the histological changes. This method classifies histopathological changes into five reaction patterns: circulatory disturbances, regressive changes, progressive changes, inflammation, and tumors. Each reaction pattern includes several histopathological alterations (Figure 2). The value attributed to each of these histopathological alterations results from the product of the score value by the importance factor. The score values depend on the extent of lesions and were defined as follows: 0 (unchanged), 2 (mild occurrence), 4 (moderate occurrence), and 6 (severe/diffuse occurrence). To each alteration, an importance factor was also assigned, namely, 1 (minimal pathological importance), 2 (moderate pathological importance), and 3 (marked pathological importance), indicating the relevance of the change and its pathological importance (Table S3). The sum of histopathological changes of each reaction pattern provides the index for the respective reaction pattern (Figure 2). In the present study, the histopathological changes of the tumors were not observed. Moreover, the sum of all reaction patterns yields the organ index.

The equation for the histopathological index of the organ is:

$$I_{org} = \sum_{rp}\sum_{alt}\left(a_{org\ rp\ alt} \times w_{org\ rp\ alt}\right) \quad (2)$$

where *org* represents the organ (i.e., liver), *rp* the reaction pattern, *alt* the alteration, *a* the score value, and *w* the importance factor.

The scoring scheme proposed by Zimmerli et al. [41] was used to classify the severity of the histological response based on the values of the organ index. Class I (index ≤ 10) represented normal organ structure with slight histological alterations; Class II (index 11–20) represented normal organ structure with moderate histological alterations; Class III (index 21–30) represented moderate modifications of normal structure; Class IV (index 31–40) represented pronounced histological alterations of the organ; Class V (index > 40) represented severe histological alterations of the organ.

2.6. Data Analysis

The Hepatosomatic Index (HSI) was used to evaluate the liver condition in sampled fish species, and also provided information about the impact of stressors on fish [15]. The HSI was calculated according to the following formula:

$$\mathrm{HSI} = \frac{LW}{TW} \times 100 \quad (3)$$

where *TW* represents the total weight (expressed in grams), and the *LW* represents the liver weight expressed in grams [15].

The HSI and the reaction patterns indices were evaluated through the Mann–Whitney rank sum test to detect seasonal statistical differences (between summer of 2016 and winter of 2017) for each species (*L. bocagei* and *P. duriense*). Statistical significance was accepted when $p \leq 0.05$ for the Mann–Whitney test. Moreover, Spearman's rank correlation coefficient was used to determine the correlations between HSI and the histopathological changes identified in both species. The correlation was considered statistically significant at the $p \leq 0.01$ level. The statistical analyses were executed using IBM SPSS statistics software, version 26, and with a significance level of 0.05 [42].

To provide an overview of the water quality by sampling points and seasons, the metal load was computed. This consisted of the sum of the concentrations of all metals, As + Al + Cr + Cd + Pb + Cu + Mn + Zn + Ni + Fe + Co (mg/L).

To investigate the statistical significance of the effects of water pollution on fish liver histopathology, canonical correspondence analysis (CCA) was applied. CCA is a method for multivariate analysis whose objective is to describe how species respond to particular sets of observed environmental variables. For this, CCA calculates a series of canonical functions that sum the relationship between a linear combination of dependent variables and a linear combination of independent variables. Therefore, two matrices were organized; one matrix contained the prevalence of each histopathological change, and the second matrix included the physicochemical parameters (conductivity, TSS, Zn, Cu, Fe, Mn, As, Cd, Cr, Pb, Al and Ni). The results consist of two types of scores: the points (represented by triangles) match liver response parameters, and the arrows represent the physicochemical parameters. Each arrow points in the expected direction of the increase in values of physicochemical parameters. The projections of points onto the line overlying the arrow can be used to approximate the optima of each liver histopathological change concerning values of those physicochemical parameters. The CCA was executed using CANOCO 5.0 software [43].

3. Results

3.1. Identification of the Liver Histopathology Changes

The liver histopathological assessment, in both *L. bocagei* and *P. duriense*, revealed the presence of changes in animals from all sampling points (I, II and III), and in both summer and winter. Several histopathological alterations were observed in both species, namely congestion of blood vessels (CBV), melanomacrophage centers (MMC), necrosis (N), vacuolization of hepatocytes (VH), degenerative vacuolization (DV), fibrosis (F), exudate (E) and infiltration (I), necrosis (NBE) and hyperplasia of the biliary epithelium (HBE), and biliary duct epithelial detachment (BDED), endothelial rupture (ER) and periportal edema (PE) (Figures 3 and 4). Melanomacrophage centers, also known as macrophage aggregates, characterized by the presence of brown pigment, occurred close to the blood vessels or bile ducts (Figure 3c and 3d, respectively) and scattered through the hepatic parenchyma (Figure 3c). In most sampled fishes, melanomacrophage centers were accompanied by inflammation (Figure 3c). Congestion of blood in vessels occurred in both portal veins and sinusoids (Figure 3e). The exudate, a fluid containing high protein levels and a large quantity of cellular debris exuded from blood vessels, was observed in the periportal region (Figure 3f). The periportal edema was characterized by a separation between the hepatic parenchyma and the bile duct, vessel, and artery (Figure 3f). In most sampled fish, endothelial rupture was found (Figure 4a). The necrosis occurred in the hepatic parenchyma, and surrounding the bile ducts and vessels (Figure 4b). In the bile ducts were observed necrosis (Figure 4c) hyperplasia of the biliary epithelium (Figure 4d), and biliary duct epithelial detachment (Figure 4d). The hyperplasia of the biliary epithelium was defined by an increased number of epithelial cell layers, whereas the biliary duct epithelial detachment was characterized by the separation of the bile epithelia from the surrounding connective tissue [40]. Some of the bile ducts were surrounded by lymphocyte infiltration. The vacuolization of hepatocytes, characterized by vacuoles of glycogen and lipids (Figure 4e), and the degenerative vacuolization, identified by the presence of large lipid vacuoles that cause the rupture of the cell membrane and nuclear changes, were also reported. In several fish, the enormous vacuole pushes the nuclei and cytoplasmatic material to the periphery of the cell, near sinusoidal vessels (Figure 4f).

3.2. Prevalence of the Liver Histopathological Changes

The histological assessment of the liver revealed that all fishes, from both species, exhibited several histopathological changes with varying prevalence values (Figure 5a). The more prevalent ones, in both species, were hepatocyte vacuolization (100%), endothelial rupture (>88%), necrosis (>77%), fibrosis (>72%), and degenerative vacuolization (>68%) (Figure 5a). The prevalence of the congestion of blood vessels and melanomacrophage centers showed the high differences between species. The melanomacrophage centers were more common in *L. bocagei* (68%) than *P. duriense* (9%), whereas the congestion of blood vessels was more common in *P. duriense* (59%) than *L. bocagei* (32%). Comparing the histopathological alterations between seasons, for *L. bocagei* (Figure 5b) and *P. duriense* (Figure 5c) individually, a marked seasonal difference was observed. In both species, the liver histopathological changes were more prevalent in summer than in winter. This was the case for melanomacrophage centers, endothelial rupture, fibrosis, hyperplasia of biliary epithelium, and infiltration. The periportal edema, necrosis of biliary epithelium, and biliary duct epithelial detachment were observed exclusively in summer and in *P. duriense*, whereas the biliary duct epithelial detachment was detected in *L. bocagei*. By comparison, the degenerative vacuolization and the congestion of blood vessels were more prevalent in winter, and degenerative vacuolization was observed in 100% of both species.

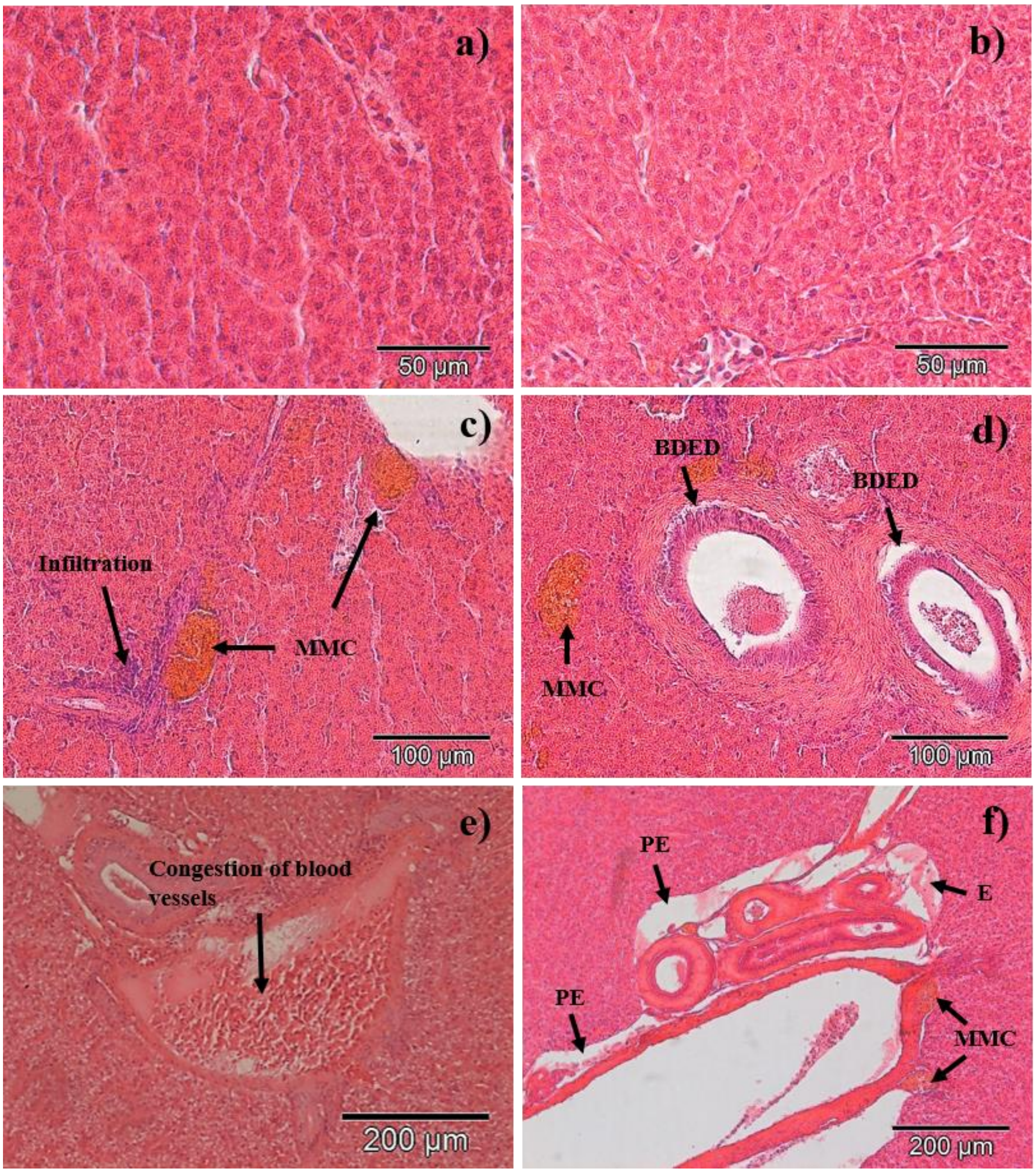

Figure 3. Normal histological architecture of the liver of *L. bocagei* (**a**) and *P. duriense* (**b**). Histopathological changes such as infiltration (**c**); MMC, melanomacrophage centers (**c**,**d**,**f**); BDED, biliary duct epithelial detachment (**d**); congestion of blood vessels (**e**); PE, periportal edema, and E, exudate (**f**) were observed in the liver of both species. Scale bars 50 µm (**a**,**b**), 100 µm (**c**,**d**), and 200 µm (**e**,**f**).

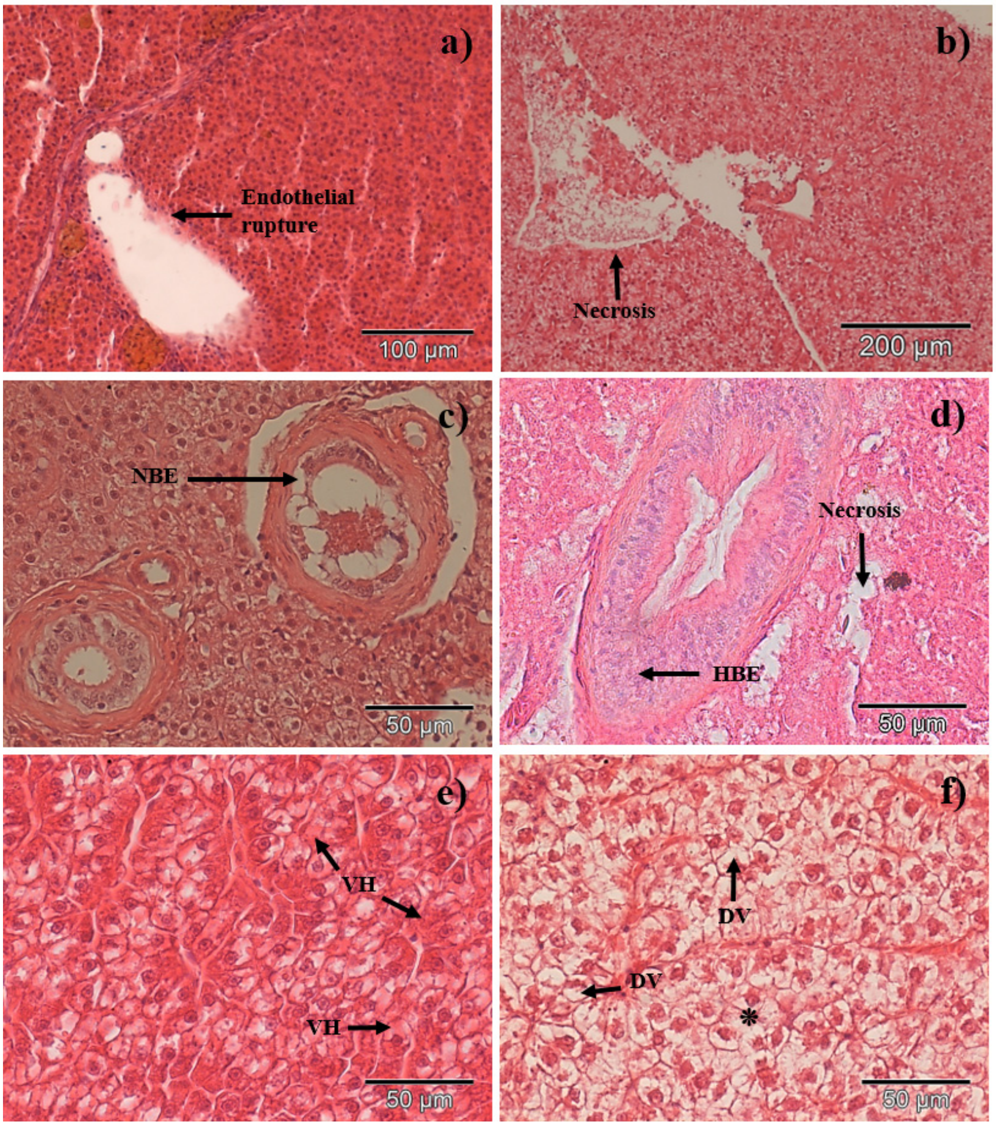

Figure 4. Histopathological changes as Endothelial rupture (**a**); necrosis (**b**,**d**); NBE, necrosis of biliary epithelium (**c**); HBE, hyperplasia of the biliary epithelium (**d**); VH, vacuolization of hepatocytes (**e**); DV, degenerative vacuolization and ❋, rupture of the cell membrane (**f**) were observed in the liver of both species. Scale bars 50 µm (**c**–**f**), 100 µm (**a**), and 200 µm (**b**).

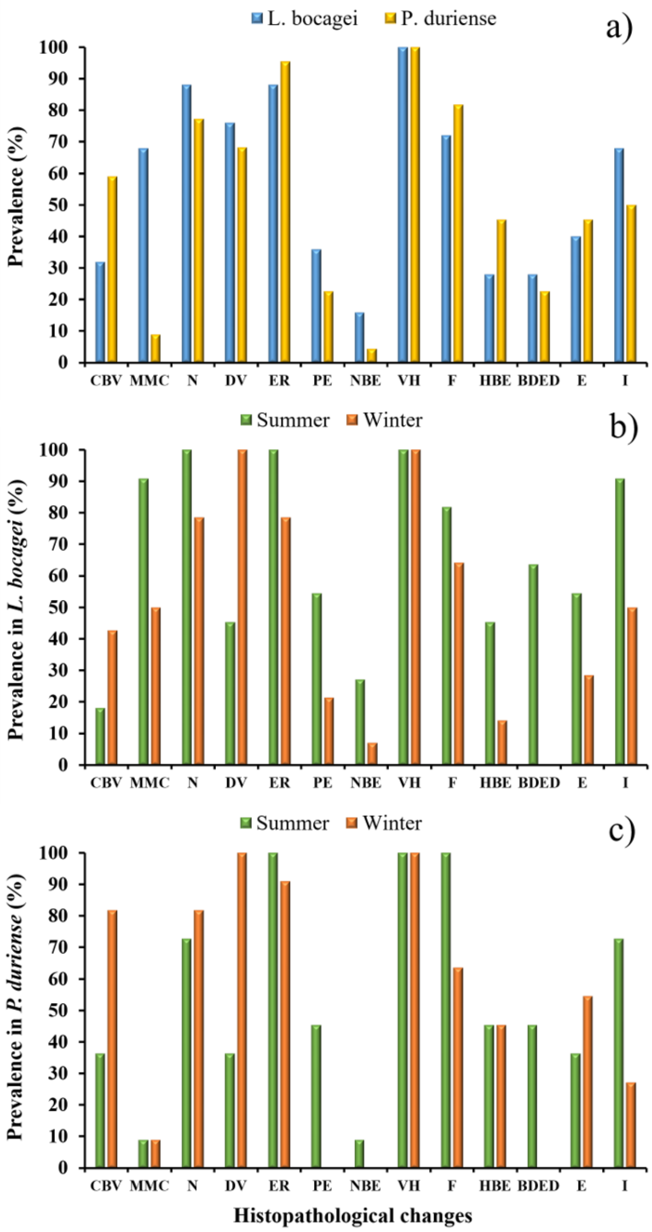

Figure 5. Prevalence (%) of liver histopathological changes observed in *L. bocagei* and *P. duriense* in both seasons (**a**), and in summer and winter for *L. bocagei* (**b**) and for *P. duriense* (**c**). The liver histopathological changes represent the observations in sampling sites (points I, II and III) of the Vilariça River. Abbreviations: CBV, congestion of blood vessels; MMC, melanomacrophage centers; N, necrosis; DV, degenerative vacuolization; ER, endothelial rupture; PE, periportal edema; NBE, necrosis of biliary epithelium; VH, vacuolization hepatocytes; F, fibrosis; HBE, hyperplasia of the biliary epithelium; BDED, biliary duct epithelial detachment; E, exudate; and I, infiltration.

3.3. Histological Reaction and Severity Degree Indices

Figure 6 shows the histopathological changes grouped into histological reaction indices, circulatory disturbance (CDI), regressive changes (RCI), progressive changes (PCI), and inflammation (II). To assess the differences between seasons, for *L. bocagei* and *P. duriense* individually, the histological reaction indices were compared through statistical analysis. In *L. bocagei*, the progressive changes and inflammation indices were higher in summer than in winter ($p < 0.05$).

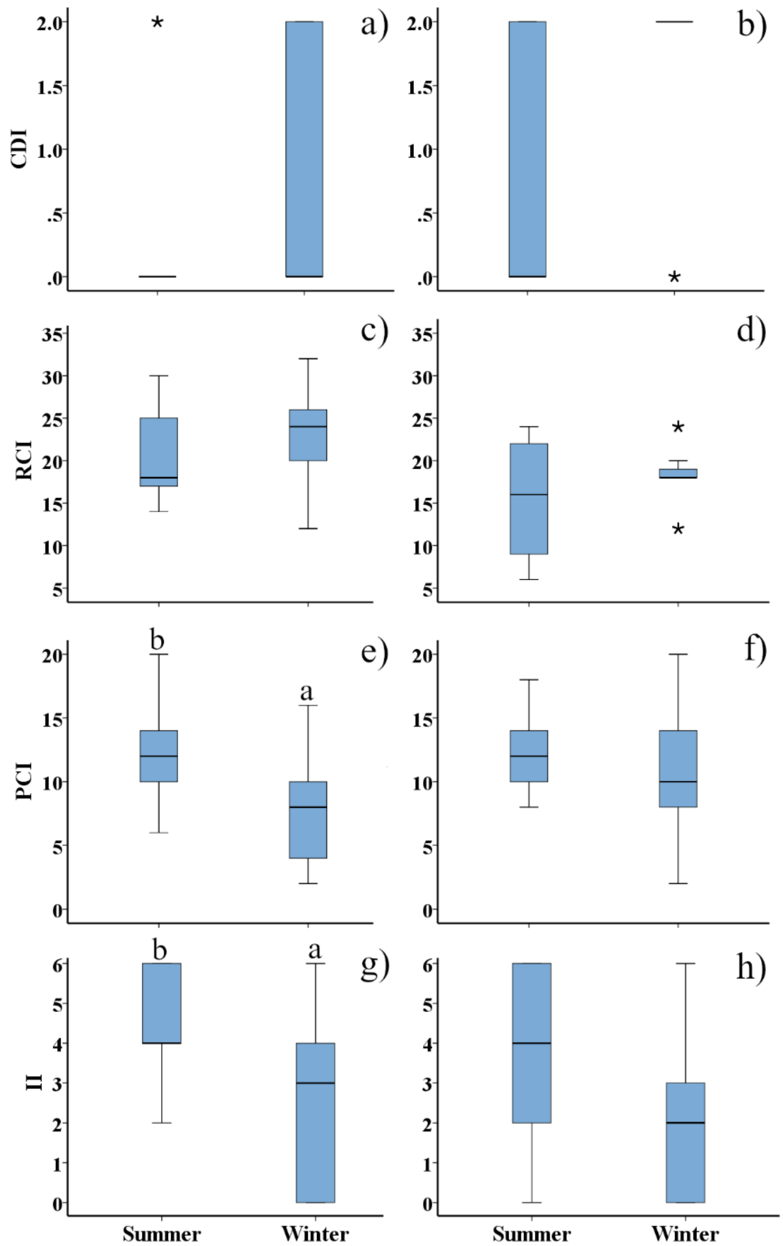

Figure 6. The indices of histological reaction patterns are CDI, circulatory disturbances index (**a**,**b**); RCI, regressive changes index (**c**,**d**); PCI, progressive changes index (**e**,**f**); and II, inflammation index (**g**,**h**), observed in the liver of *L. bocagei* (**a**,**c**,**e**,**g**) and *P. duriense* (**b**,**d**,**f**,**h**) in each season. The results are presented as box plots. The boundaries of the box plot indicate the 25th and 75th percentiles; the line within the box marks the median value; whiskers above and below the box indicate the 10th and 90th percentiles, whereas dots indicate outliers. Different letters indicate significant differences ($p < 0.05$) between seasons. Statistical analysis was performed using a Mann–Whitney rank sum test to detect differences within the indices of liver histopathological changes between seasons for each species.

The severity degree of histopathological changes (organ index) observed in the livers of both species, per season and sampling site, is represented in Table 1. The severity degrees ranged from moderate to severe, with the pronounced histological alterations being the most observed. In *L. bocagei*, the histological changes were classified with higher severity degrees (pronounced and severe) than in *P. duriense* (between moderate and severe). The fish caught in summer showed a higher severity degree (pronounced and severe) than fish caught in winter (moderate and pronounced). Moreover, the fish caught at the sampling points located downstream and middle stream of the river (I and II, respectively) showed higher severity degrees (pronounced and severe) than the fish caught at the sampling point located upstream of the river (III), (between moderate and severe).

Table 1. Organ index (severity degree) of histopathological changes observed in the liver of *L. bocagei* and *P. duriense* sampled in the summer of 2016 and the winter of 2017.

Sampling Point	*L. bocagei*		*P. duriense*	
	Summer 2016 (Mean and Range)	Winter 2017 (Mean and Range)	Summer 2016 (Mean and Range)	Winter 2017 (Mean and Range)
Point I	31.6 (24–36)	37.2 (34–40)	46 (46–46)	35.2 (32–40)
Point II	42 (36–52)	33.4 (20–50)	34.8 (18–46)	37.3 (24–44)
Point III	44 (44–44)	34 (26–42)	25.6 (18–30)	24.7 (22–28)

Class III (index 21–30): oderate modifications of normal tissue; class IV (index 31–40) pronounced histological alterations; and class V (index > 40) severe histological alterations of the organ [41].

3.4. Classification of Water Quality

The analysis of water quality revealed that 7 of the 24 physicochemical parameters exceeded the acceptable limits for good water quality for multiple uses (Table 2 and Table S2). In the summer of 2016, the water was classified as polluted and weakly polluted due to high temperature (>20 °C), low percentage of dissolved oxygen (<90%), and high Mn (>0.1 mg/L) and As (>0.01 mg/L) concentrations (Table 2). In the winter of 2017, the water classification varied from weakly polluted to extremely polluted due to low percentage of dissolved oxygen at one sampling point (<90%), and high TSS (>80 mg/L), NO_2^- (>0.02 mg/L), and Cd (>0.001 mg/L) concentrations (Table 2). Table 2 also shows that the parameters' concentrations differed between both seasons. In summer, the main elements found in surface water were Zn > Mn > NO_3^- > Fe > Pb > Cu > As. By comparison, in winter, the elements observed in higher concentrations in surface water were TSS > PO_4^{3-} > NO_2^- > Cd > Cr > Ni > Al. Moreover, the values of metal load per sampling site showed higher average concentrations in summer (0.8) than in winter (0.38), with the respective concentrations increasing from upstream to downstream in both seasons, from 0.66 to 0.9 in summer and from 0.41 to 0.47 in winter (Table 3).

3.5. Canonical Correspondence Analysis of Physicochemical Parameters and the Liver Histopathological Changes

Canonical correspondence analysis was applied to explore the relationship between physicochemical parameters and liver histopathological changes observed in both *L. bocagei* (Figure 7a) and *P. duriense* (Figure 7b). For *L. bocagei*, main vectors along the first two axes (eigenvalues 0.122 and 0.089, explaining respectively 29.6% and 17.1% of the variance $p < 0.01$), show that the high concentrations of Fe, Cu, and Zn explain the prevalence of biliary duct epithelial detachment in fishes. The high conductivity and As concentrations highlight the prevalence of hyperplasia of the biliary epithelium, and the high TSS and Cd, Ni and Cr concentrations explain the prevalence of congestion of blood in vessels and degenerative vacuolization (Figure 7a). For *P. duriense*, main vectors along the first two axes (eigenvalues 0.194 and 0.108, explaining respectively 38.1% and 24.4% of the variance $p < 0.01$), also show that the high concentrations of Zn, Cu, Fe, and Mn explain the prevalence of biliary duct epithelial detachment. Moreover, the high TSS and Cr and Cd concentrations explain the prevalence of congestion of blood in vessels and degenerative vacuolization (Figure 7b).

Table 2. Water physicochemical parameters in the sampling sites (points I, II, III and IV) of the Vilariça River.

Physicochemical Parameter	Summer 2016		Winter 2017	
	Mean (Min–Max)	Classification	Mean (Min–Max)	Classification
Temperature (°C)	22.7 (21.2–26.2)	polluted	12.2 (10.1–14.5)	not polluted
Total Suspended Solids (mg/L)	4.25 (0–9)	not polluted	(110–180)	extremely polluted
Dissolved oxigen (lab O_2 %)	73.6 (50.23–89.45)	polluted	112.42 (88.5–128.7)	weakly polluted
pH	7.2 (6.79–7.91)	not polluted	7.7 (6.79–8.28)	not polluted
Conductivity (uS/cm)	264.3 (167.6–337.6)	not polluted	277.7 (161.1–332.2)	not polluted
Nitrate (mg NO_3^-/L)	2.92 (1.31–3.8)	not polluted	0.66 (0–1.38)	not polluted
Magnesium (mg Mg/L)	10.7 (6.9–13.7)	not polluted	8.4 (4.6–10.8)	not polluted
Potassium (mg K/L)	2.18 (1.49–2.95)	not polluted	2.08 (1.52–2.61)	not polluted
Phosphate (mg PO_4^{3-}/L)	0.0125 (0–0.05)	not polluted	0.125 (0.01–0.23)	not polluted
Nitrite (mg NO_2^-/L)	0.0025 (0–0.01)	not polluted	0.0178 (0–0.035)	polluted
Calcium (mg Ca/L)	4.57 (2.16–6.6)	not polluted	6.85 (3.48–8.4)	not polluted
Zinc (mg Zn/L)	0.0796 (0.045–0.13)	not polluted	0.0135 (0.0071–0.0310)	not polluted
Manganese (mg Mn/L)	0.1032 (0.0497–0.1984)	weakly polluted	0.0218 (0.0086–0.0401)	not polluted
Iron (mg Fe/L)	0.41 (0.29–0.49)	not polluted	0.1000 (0.03–0.16)	not polluted
Lead (mg Pb/L)	0.002 (0–0.0048)	not polluted	0.0005 (0.0004–0.0006)	not polluted
Copper (mg Cu/L)	0.0028 (0.0015–0.0053)	not polluted	0.0008 (0.0002–0.0012)	not polluted
Arsenic (mg As/L)	0.0124 (0.00121–0.01895)	weakly polluted	0.0052 (0.0022–0.0069)	not polluted
Cobalt (mg Co/L)	0.0002 (0–0.0009)	not polluted	0.0000 (0–0)	not polluted
Sodium (mg Na/L)	35.7 (24.9–44.5)	not polluted	41.03 (35.9–45.6)	not polluted
Sulfates (mg SO_4^{2-}/L)	-	-	21.13 (12.51–26.65)	-
Cadmium (mg Cd/L)	0.0003 (0.0001–0.0006)	not polluted	0.0013 (0.0011–0.0017)	polluted
Chromium (mg Cr/L)	0.0032 (0.0024–0.0042)	not polluted	0.0049 (0.0044–0.0055)	not polluted
Nickel (mg Ni/L)	0.0026 (0.0012–0.0041)	not polluted	0.0037 (0.0015–0.0057)	not polluted
Aluminum (mg Al/L)	0.2023 (0.14142–0.2445)	not polluted	0.2401 (0.1889–0.3176)	not polluted
Class of water quality for multiple uses	Polluted		Extremely polluted	

Table 3. Metal (As + Al + Cr + Cd + Pb + Cu + Mn + Zn + Ni + Fe + Co mg/L) concentrations measured in the summer of 2016 and winter of 2017 in sampling sites (points I, II, and III) of the Vilariça River basin.

Sampling Sites	Summer 2016	Winter 2017
Point I (downstream)	0.9	0.47
Point II (middle stream)	0.83	0.26
Point III (upstream)	0.66	0.41
Average	0.8	0.38

3.6. Hepatosomatic Index and Total Weight

The statistical analysis of the hepatosomatic index of both species caught in summer and winter at the sampling points (I, II, and III) of the Vilariça River is presented in Table 4. The results show significant differences between seasons, with higher HSI observed in animals caught in winter, for both species. The HSI of *L. bocagei* was 2.1 ± 0.88 and 0.84 ± 0.37, and of *P. duriense* 1.74 ± 0.53, and 1.07 ± 0.45 in winter and summer, respectively. In winter, the spatial distribution of the HSI showed an increase from downstream (point I) to upstream (point III) for both species. In *L. bocagei*, the HSI increased from 1.55 to 2.58, and in *P. duriense*, from 1.62 to 1.91, from downstream to upstream, respectively (Figure 8a and Table 4). In summer, the HSI increased from downstream to middle stream (point II) and then decreased to upstream, for both species. Accordingly, the higher HSI was observed in the middle steam, with 1.14 and 1.45 in *L. bocagei* and *P. duriense*, respectively. Contrary to the HSI, the fishes' total weight in winter increased from downstream to the middle stream and then decreased to upstream, for both species. The highest total weight was observed in the middle stream, of 77 and 29 g for *L. bocagei* and *P. duriense*, respectively (Figure 8b and Table 4). In summer, the weight of fishes showed a decrease from downstream (332 g) to upstream (46 g) in *L. bocagei*, and in *P. duriense*, it declined from 45 to 32 g, respectively.

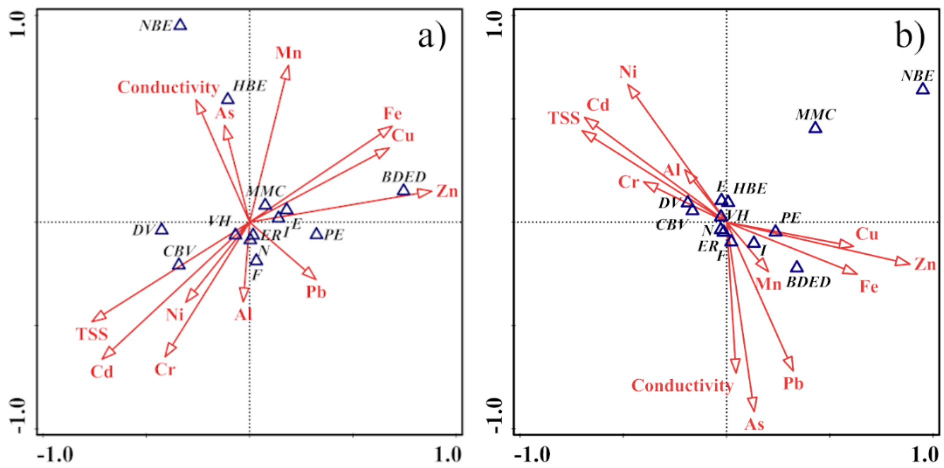

Figure 7. Canonical correspondence analysis (CCA) biplot of physicochemical parameters and liver histopathological changes observed in *L. bocagei* (**a**) and *P. duriense* (**b**). The data were provided from samplings (points I, II, and III) taken in the Vilariça River performed during the summer of 2016 and the winter of 2017.

Table 4. The hepatosomatic index of *L. bocagei* and *P. duriense* captured in the summer of 2016 and the winter of 2017 at the sampling points (points I, II, and III) of the Vilariça River.

Specie	Season	Sampling Point	Sample Size (n)	Hepatosomatic Index (HSI)
L. bocagei	Summer	Point I	5	0.52 ± 0.28
		Point II	5	1.14 ± 0.15
		Point III	1	0.93
	Total Summer		11	0.84 ± 0.37 a
	Winter	Point I	5	1.55 ± 0.31
		Point II	7	2.35 ± 0.62
		Point III	2	2.58 ± 2.24
	Total Winter		14	2.1 ± 0.88 b
P. duriense	Summer	Point I	1	1.25
		Point II	5	1.45 ± 0.32
		Point III	5	0.65 ± 0.06
	Total Summer		11	1.07 ± 0.45 a
	Winter	Point I	5	1.62 ± 0.47
		Point II	3	1.79 ± 0.55
		Point III	3	1.91 ± 0.74
	Total Winter		11	1.74 ± 0.53 b

Values are depicted as the mean ± standard deviation. Within each species, significant differences ($p < 0.05$) between seasons are indicated in bold by different letters. Statistical analysis was performed using a Mann–Whitney rank sum test to detect differences in biometric parameters between seasons for each species.

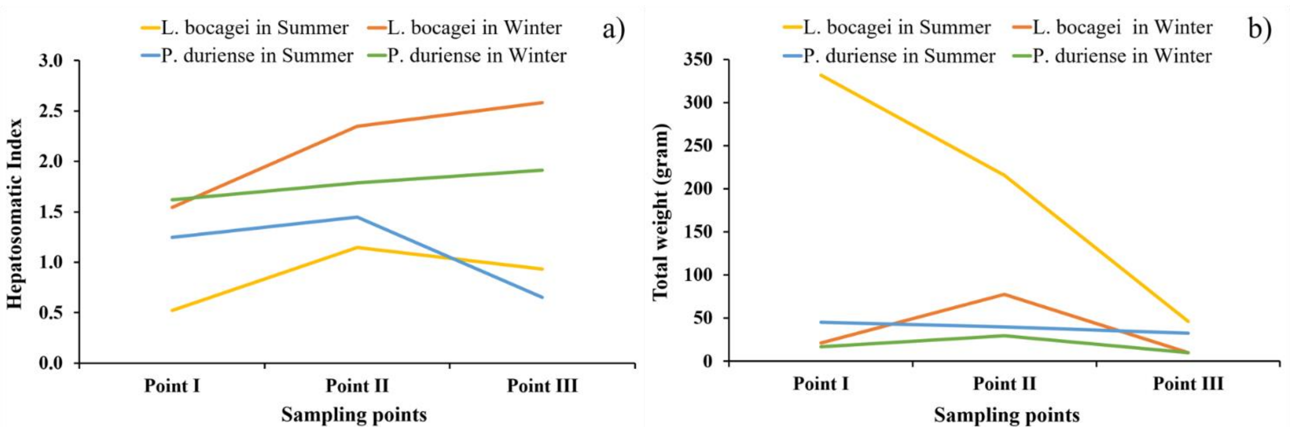

Figure 8. Hepatosomatic index (average) (**a**) and total weight (average) (**b**) of *L. bocagei* and *P. duriense* captured in the summer of 2016 and the winter of 2017 at the sampling points (points I, II, and III) of the Vilariça River.

The Spearman correlation coefficient shows that HSI is positively correlated with degenerative vacuolization ($R^2 = 0.69$, N = 47, *p*-value < 0.01) and vacuolization hepatocytes ($R^2 = 0.38$, N = 47, *p*-value < 0.01), and negatively correlated with the biliary duct epithelial detachment ($R^2 = 0.55$, N = 47, *p*-value < 0.01) (Figure 9).

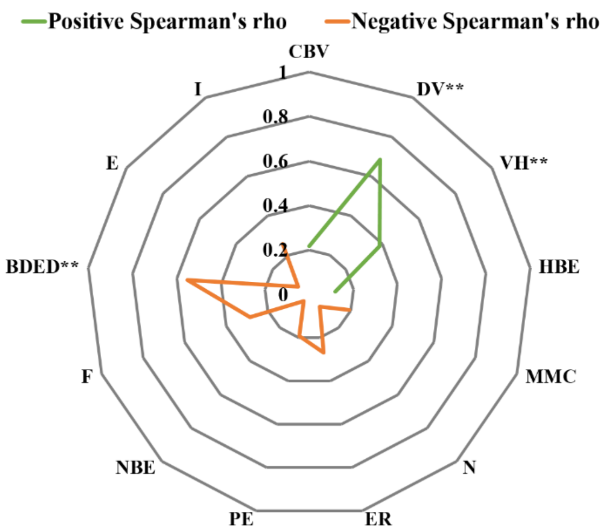

Figure 9. Spearman's rank correlation coefficient between the hepatosomatic index and the value of each histopathological change observed in fishes caught in the summer of 2016 and the winter of 2017 in the Vilariça River basin. The radar chart contains the positive or negative correlation coefficient of each histopathological change. Abbreviations: CBV, congestion of blood vessels; MMC, melanomacrophage centers; N, necrosis; DV, degenerative vacuolization; ER, endothelial rupture; PE, periportal edema; NBE, necrosis of biliary epithelium; VH, vacuolization hepatocytes; F, fibrosis; HBE, hyperplasia of the biliary epithelium; BDED, biliary duct epithelial detachment; E, exudate; and I, infiltration. Correlations is significant at the 0.01 level (**).

4. Discussion

4.1. Comparative Analysis of the Liver Histopathology between L. bocagei and P. duriense

The vacuolization of hepatocytes, endothelial rupture, necrosis, fibrosis, and degenerative vacuolization were the liver histopathological changes most observed in both species, with the exception of the melanomacrophage centers that were more common in *L. bocagei* and the congestion of blood vessels in *P. duriense*. The melanomacrophage centers were identified in 68% of *L. bocagei*, whereas in *P. duriense* they were only observed in 9%

(7.6 fewer times). Macrophages and macrophage aggregates are normal components of the liver in some fish species, acting (among other functions) as an antigen-trapping defensive system for lymphoid cells and in the sequestration of products of cellular turnover and potentially toxic substances, such as xenobiotics [44]. In the presence of environmentally stressful conditions, it has been documented that macrophages increase in size and frequency, suggesting they are reliable biomarkers for water quality in terms of deoxygenation and chemical pollution [44]. The presence of macrophage aggregates has also been documented in *L. bocagei*, at the Vizela River [45,46]. Coimbra et al. [46] observed greater hepatic changes in fish caught in polluted sites, showing that the textile industry effluents interfere with the health of fish. Carrola [45] found uncommon hepatic macrophagic foamy-cell nodules in fish collected in years of poorer water quality classification. In addition, Raldúa et al. [8] argue that *Barbus graellsii* collected downstream of industrial effluent discharges in the Vero river (Ebro basin, NE Spain) was affected by water pollution. In the same study, the histological analysis of the liver and kidney of *L. bocagei* evidenced an increase in the number and size of macrophage aggregates in most individuals collected downstream of industrial effluent.

The congestion of blood vessels in *P. duriense* was identified in 59% of fish, whereas in *L. bocagei* was only observed in 32%. However, many studies have documented the prevalence of this alteration in several fish species exposed to both natural and anthropogenic sources of pollution, e.g., [17,19]. Kostić et al. [19] in the Duboko locality on the Sava River (Belgrade region) showed that circulatory alterations, such as congestion of sinusoids and the presence of stasis inside the blood vessels, in addition to leukocyte infiltration, were the alterations most common in three benthivorous cyprinids. Kaptaner et al. [17] also observed the congestion of blood vessels in the cyprinid *Chalcalburnus tarichi* from the Lake Van basin (Eastern Turkey).

Although both species share the same habitat conditions and, therefore, the same water quality, the results showed that the liver metabolism of both species was not the same. This shows that different species may have different responses to similar environmental conditions. This observation supports the argument that the variety in the lesion types may be due to the species sensitivity to the contaminants, in addition to individual life history and contaminant exposure history e.g., [8,17].

4.2. Assessment of the Liver Histopathology and Physicochemical Parameters in the Summer

The liver histology assessment of both species showed that the histopathological changes were more prevalent in summer. In addition, for *L. bocagei*, the progressive changes and inflammation indices were statistically higher in summer than in winter. Moreover, the canonical analysis highlighted that the conductivity and As (the metal that exceeds the acceptable limits for good water quality) influenced the prevalence of hyperplasia of the biliary epithelium in *L. bocagei*. In fact, the As concentration and the prevalence of the hyperplasia of biliary epithelium in *L. bocagei* were higher in summer than in winter, in addition to the metals Zn, Mn, Fe, Pb, Cu, and As, and the melanomacrophage centers and infiltration. Similar results were obtained by Peixoto et al. [20] with *L. bocagei* captured in a polluted stream (the Vizela River). The authors concluded that the presence of metals such as Al, Cu, Zn, and Cr accumulated in liver tissue may be associated with the presence of melanomacrophage centers and lymphocyte foci.

According to Mohamed [47], the stasis of blood may be responsible for cellular degeneration and necrosis in the liver. In addition, Kostić et al. [19] argued that the sinusoidal congestion blocks blood from the hepatic artery and the interbiliary portal vein, which has to pass through the sinusoids on its way to the central vein. To reach the central vein, the blood needs to be pumped harder, and this can explain the presence of fibrosis in the periportal and portal areas e.g., [48]. This histopathological change, frequently observed in field studies, has been suggested to be a chronic response to chemical injury [17]. Moreover, the presence of leukocytes is part of the non-specific cellular defense, such as phagocytosis and phagocyte killing. Thus, the presence of leukocytes indicates an inflammatory reaction, reflecting an immunological response to environmental contaminants, e.g., [19]. Inflammation can be associated with edema [40], hepatocellular degeneration processes, and later, necrosis [2]. In the study area, lymphocyte infiltration appears in most fish, associated with melanomacrophage centers, and both were observed scattered in hepatic parenchyma, surrounding biliary ducts, and in the presence of changes such as periportal edema and endothelial rupture. Moreover, the necrosis was observed scattered in the hepatic parenchyma and surrounding biliary ducts. The same observations were reported by other researchers, suggesting that the presence of hepatic necrosis is an indicator of toxic injury by contaminants [5,17,19]. Necrosis is strongly associated with oxidative stress and free radical generation, which causes enzyme inhibition, cell membrane damage, and inhibition of protein synthesis, resulting in increased cell death.

In the bile duct, alterations such as necrosis, and hyperplasia of biliary epithelium were also observed by other authors. Kaptaner et al. [17] observed necrosis of biliary epithelium in the liver of *Chalcalburnus tarichi* exposed to contaminants, such as heavy metals and endocrine-disrupting chemicals. Agamy [49] also observed necrosis in the biliary epithelium of *Siganus canaliculatus*, in an experiment to investigate the effects of acute exposure to light Arabian crude oil. Wester and Canton [50] observed epithelial hypertrophy and hyperplasia in the bile duct of *Poecilia reticulata* exposed to methyl mercury chloride. The biliary duct epithelial detachment, characterized by the split of epithelium cells from connective tissue, only occurred in summer, with a prevalence of 64% in *L. bocagei* and 45% in *P. duriense*. The statistical analysis of both species (Figure 7) revealed that the metals with the highest concentration in summer (Zn, Cu, and Fe) can explain the presence of biliary duct epithelial detachment.

4.3. Assessment of the Liver Histopathology and Physicochemical Parameters in the Winter

In both species, the liver histology assessment showed that alterations such as congestion of blood vessels and degenerative vacuolization were more prevalent in winter. In addition, the canonical analysis revealed that the higher TSS and Cd, Ni, and Cr concentrations in winter can explain the prevalence of these histopathological changes in both species. However, the vacuolization of hepatocytes occurred in all fish of both species and seasons. The work developed by Coimbra et al. [46] also observed, in *L. bocagei*, the presence of liver vacuolization in fish caught in the polluted Vizela River. Its presence in the liver is normal and represents somatic energy storage. However, the increase of hepatic lipid accumulation has been documented as a pathology, and is very common in fish exposed to contaminants [2,17,51]. The relationship between Cd exposure and the increase in vacuolization of hepatocytes and blood congestion of veins and sinusoids has already been documented. The work developed by Liu et al. [18] with *Acrossocheilus fasciatus*, in a laboratory, reported an increase in the hepatocyte vacuolization after Cd exposure. In addition, the blood vessels and sinusoids become blocked by red blood cells, especially in the central vein. Moreover, Van Dyk et al. [1] observed a drastic increase in the vacuolization of hepatocytes, in the liver of *Oreochromis mossambicus*, after exposure to Cd and Zn. The authors attributed the presence of fat vacuoles to a general failure of lipid metabolism that arises from a decrease in the utilization of energy reserves or pathological synthesis, i.e., this condition is not a result of the uptake of abundant lipid precursors but is a problem of removing the fat from the hepatocytes. Degenerative vacuolization has also been reported in *Barbus barbus* [48] and other fish species living in contaminated areas [17,47,52].

4.4. Hepatosomatic Index Assessment

Statistical analysis showed that the HSI was higher in winter than summer for both species (Table 4). In addition, the correlation between the HSI and the value of each histopathological chance showed that the higher HSI in winter was due to greater vacuolization of hepatocytes, that is, the increase in glycogen and lipids stored in the liver, although the lower presence of vacuolization of hepatocytes in summer, immediately after the spawning season of both species (http://esa.ipb.pt/) (accessed on 12 July 2021), can be due to the mobilization of lipids for the development of the gonads. Indeed, in the fish reproduction period, the lipids are mobilized from the liver and muscle to the gonad for its development [53–55]. The work developed by Encina and Granado-Lorencio [56] with *Barbus sclateri*, in the Guadalete River (Spain), showed that the highest somatic energy content was found in March–April and then decreased until the end of summer. Depletion of somatic energy seems to be associated with high metabolic demands in adult fish with spawning-related activity. Moreover, Kandemir and Polat [54] found that the cycles of lipid storage and mobilization are directly connected with food abundance. In addition, Camargo and Martinez [57] argue that the tendency of the animals to show less glycogen in summer may be related to the higher metabolic rate caused by the higher temperatures. However, the possibility that the lower HSI observed in summer may be due to the effect of water pollution cannot be ruled out. The lowest HSI in summer (0.84 for *L. bocagei* and 1.07 for *P. duriense*) is in agreement with the higher prevalence of histological changes, observed in both species and with the high concentrations of heavy metals measured in the water. Indeed, the work developed by Carrola et al. [45], also with *L. bocagei*, evidenced a lower CF in polluted sites (0.89 and 0.84) than in control conditions (1.14). In contrast, the work developed by Gonino et al. [15], in burned areas, and van Dyk et al. [58], in polluted rivers, points in the opposite direction. The work developed with *L. bocagei* in the burnt catchment, in central Portugal, showed a significant increase in the HSI of fish from sites with a high concentration of ash [15]. In addition, the study by van Dyk et al. [58] with *Clarias gariepinus* in South Africa and Botswana showed that the HSI was significantly higher in animals from polluted sites than from reference sites, with a higher incidence of damage such as hepatocellular vacuolation and steatosis.

In winter, the higher HSI and the fish with less total weight were observed upstream in both species. In summer, also, the total weight of the fish was lower upstream; nonetheless, the HSI was higher in the middle stream. The higher HSI after spawning months (in summer) can be due to the start of the storage of hepatic glycogen, revealing that the middle stream is where there can be greater availability of food. The presence of smaller fish upstream may be due to lower current velocity, higher proximity to refuge areas, and a lower probability of the presence of large predators.

4.5. The Relationship between Metal Load and the Severity Degree of Histopathological Changes

The comparison of the severity degree of histopathological changes between species showed that *L. bocagei* presented more histological alterations than *P. duriense* (Table 1). In addition, these histopathological changes were higher in fish caught in summer than in winter for both species. Moreover, the fish captured at sampling points located downstream and in the middle stream presented more histological alterations than the fish captured upstream. The fact that *L. bocagei* has a greater number of anomalies than *P. duriense* may be due to the type of feeding and the own metabolism of the species. Furthermore, the severity degree of histopathological changes observed in both species followed the same spatial pattern as metals concentration (Tables 1 and 3). Thus, it was observed that there was an increase in metals concentration from upstream to downstream of the river. The metals concentration was also higher in summer than in winter. Thus, the high number of changes observed in the liver fish caught in the summer can be explained by the higher metals concentration in the river.

The comparison of physicochemical parameters between seasons showed that the major concentrations of NO_3^-, Mg, K, Zn, Mn, Fe, Pb, Cu, and As occurred in summer. These increases are probably due to agricultural fertilization applied in the olive groves and the vineyards during the growing and ripening phases, which vary between April and October for the olive groves and between March and August for the vineyards [27]. In summer, the presence of these chemical compounds in the river water may be influenced by the irrigation regime and by lower river water due to the low intensity and frequency of precipitation. This can justify the higher severity degree of histopathological changes observed in individuals caught in summer. Moreover, the study developed by Kostić [19] with three benthivorous cyprinids concluded that the liver histopathological index showed the highest values in summer. The authors argue that the increase in the liver histopathological index can be attributed to chemical and infectious agents, but also to the increased metabolic rate of the fish liver during the warm season.

By comparison, in winter, the physicochemical parameters in higher concentrations were TSS, PO_4^{3-}, NO_2^-, Cd, Cr, Ni, and Al. The high concentration of the TSS in the river water is mainly due to erosion, which is explained by climatic, geologic, topographic, land cover, and anthropic factors [27]. The Vilariça River basin is characterized by precipitation concentrated in the winter and spring seasons, steep topography, thin soil layers (mainly lithosols), low vegetation cover (scrub and herbaceous vegetation, open spaces, and burnt areas), and wildfires. These factors are highlighted as the main causes of soil erosion in the Vilariça River basin. Moreover, the current management of olive groves, vineyards, and fruit trees, with regular tillage, herbicide application, and the absence of vegetative cover among the trees, has also contributed to soil loss. The higher concentration of PO_4^{3-}, Cr, Cd, and Al in the river water in winter is probably due to their accumulation in the soil, reaching the water bodies in events of intensive precipitation.

5. Conclusions

This study showed that the heavy metals present in the water of the Vilariça River are in concentrations that can cause damage to the liver of fish. The injuries affected the hepatic parenchyma, blood vessels, and bile ducts in both cyprinids, *L. bocagei* and *P. duriense*. The liver histopathological changes, such as hepatocyte vacuolization, endothelial rupture, necrosis, fibrosis, infiltration, and degenerative vacuolization, were the most observed in both species. These histopathological changes were more common in summer, and in *L. bocagei* it was possible to observe significant increases in progressive and inflammation disorders. Moreover, the statistical analysis showed that the prevalence of hyperplasia of biliary epithelium in *L. bocagei* may be due to the conductivity and the concentrations of As. In addition, in both species, the concentrations of Zn, Cu, and Fe explain the prevalence of biliary duct epithelial detachment in fishes.

In winter, the congestion of blood vessels and degenerative vacuolization were more prevalent, and were explained by high TSS and Cd, Ni, and Cr concentrations. In addition, the greatest presence of degenerative vacuolization and hepatocyte vacuolization was responsible for the higher hepatosomatic index in individuals caught in the winter, in both species. The comparison of the severity degree of histopathological changes showed that *L. bocagei* caught in the summer, from the sampling points located downstream and in the middle stream, presented more histological alterations. This study demonstrated that understanding the harmful effects of metals in fish and their safe, allowable concentrations in the aquatic environment is critical for fish conservation. In addition, the Iberian barbel had a greater number of anomalies, making it a better biomarker for monitoring studies on the water quality and ecological status of northern Portuguese rivers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14030444/s1, Table S1: The analytical method, detection limit, and equipment used to determine the physicochemical parameters at the sampling points in the Vilariça River; Table S2: The five classifications of surface water bodies for multiple uses according to Pereira [32], Rodrigues et al. [33], and SNIRH [34]; Table S3: Liver histopathological changes observed in *L. bocagei* and *P. duriense* were grouped into five reaction patterns. An importance factor, ranging from 1 to 3, was attributed to each change.

Author Contributions: R.M.B.S. write the manuscript, methodology, software, identification of liver injuries of the fishes, data collection (fish and water quality) in the field. S.M.V.M., review and editing the manuscript, methodology and supervise the identification of liver injuries of the fishes and data collection (fish and water quality) in the field. F.A.L.P., review and editing the manuscript, methodology, supervision and project administration and funding acquisition. R.M.V.C., project

administration and funding acquisition. L.F.S.F., supervision, project administration and funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Fish sampling was conducted in accordance with the Ethical Guidelines of the European Union Council (European Directive 2010/63/EU, 22/09/2010) and the Portuguese Agricultural Ministry (Portaria no 1005/92, 23/10/1992) for the protection of animals used for experimental and other scientific purposes.

Informed Consent Statement: Not applicable.

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