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Disturbance in invasion? Idiopathic necrotizing hepatopancreatitis in the signal crayfish *Pacifastacus leniusculus* (Dana, 1852) in Croatia

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Abstract

As the most successful crayfish invader and possible vector for infectious agents, signal crayfish *Pacifastacus leniusculus* is among the major drivers of the native crayfish species decline in Europe. We describe histopathological manifestation and frequency of newly detected idiopathic necrotizing hepatopancreatitis along the invasion range of the signal crayfish in the Korana River in Croatia. Our results show extremely high prevalence of necrotizing hepatopancreatitis (97.3%), with 58.9% of individuals displaying mild and 31.5% moderate histopathological changes in the hepatopancreas, also reflected in the lower hepatosomatic index of analysed animals. Recorded histopathological changes were more frequent in the invasion core where population density is higher. Our preliminary screening of co-occurring native narrow-clawed crayfish *Pontastacus leptodactylus* showed lower incidence (33.3%) and only mild hepatopancreatic lesions, but potentially highlighted the susceptibility of native crayfish populations to this disease. Pilot analyses of dissolved trace and macro elements in water, sediment fractions and crayfish hepatopancreas do not highlight alarming or unusually high concentrations of analysed elements. Hepatopancreas microbiome analysis, using *16S* rRNA gene amplicon sequencing, identified taxonomic groups that should be further investigated, along with impacts of the disease on health and viability of both invasive and native crayfish populations.

KEYWORDS
crayfish, hepatopancreas, histopathology, invasive species

1 | INTRODUCTION

The number of introduced and established invasive alien species is continually increasing, and invaders alter and affect the structure and function of ecosystems worldwide (Ricciardi et al., 2017; Seebens et al., 2017; Simberloff et al., 2013). In freshwater ecosystems, invasive crayfish species have been recognized as one of the major threats to its biodiversity, structure and ecosystem services (Twardochleb et al., 2013). The signal crayfish *Pacifastacus leniusculus* (Dana, 1852) is the most widespread and the most successful crayfish invader in Europe with its presence reported in 29 EU countries (Kouba et al., 2014). According to the EU Regulation on invasive alien species No. 1143/2014, signal crayfish is listed on the list of species of EU Concern for which member states must restrict and regulate their use and take measures to early detect and rapidly eradicate establishing populations as well as to manage already

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established populations. In Croatia, signal crayfish is present in the continental part of the country, in the Mura, Drava and Korana Rivers and their tributaries and spreads rapidly through these watercourses (Dragičević et al., 2020; Hudina et al., 2009). In the Korana River, it was illegally introduced (Hudina et al., 2013) and represents a major threat to rich and diverse native crayfish fauna, since three out of five native European crayfish species (noble crayfish *Astacus astacus* (Linnaeus, 1758), narrow-clawed crayfish *Pontastacus leptodactylus* (Eschscholtz, 1823) and stone crayfish *Austropotamobius torrentium* (Schrank, 1803)) occur in the Korana River and its tributaries (Hudina et al., 2013; Lovrenčić et al., 2020; Maguire et al., 2011).

Due to the advantageous life history traits such as fast growth, early maturation, high fecundity and highly aggressive behaviour, signal crayfish may competitively exclude native crayfish (Gherardi, 2006; Pintor et al., 2008; Westman & Savolainen, 2002). However, pathogen introduction and transmission are the primary displacement mechanisms of native crayfish by invaders since signal crayfish (as well as other North American crayfish) is a latent carrier of crayfish plague disease agent, oomycete *Aphanomyces astaci* (Schikora, 1906). While North American crayfish invaders are relatively tolerant to crayfish plague, *A. astaci* infection frequently causes mass mortalities in native European crayfish and is considered responsible for decline and decimation of their numerous populations throughout Europe (Martín-Torrijos et al., 2019; Svoboda et al., 2016). In addition to transmission and spillover of novel pathogens, invaders may be susceptible to and transmit endemic pathogens of native species and may act as their reservoirs and multiply their negative impact on native species (Chinchio et al., 2020). Also, if environmental conditions are unfavourable and/or multiple infections occur, invaders may become susceptible to pathogens they are usually resistant to (Chinchio et al., 2020). These events may lead to population crashes of established populations of the invader, which has been recorded throughout Europe in the case of the signal crayfish (Aydin et al., 2014; Edsman et al., 2015). Thus, emerging diseases, which occur either due to movement of species and their pathogens or due to ecosystem alterations of either anthropogenic or natural origin, may modify the invasion success of the invader and mitigate its negative effects in the ecosystem (Chinchio et al., 2020; Fincham et al., 2019).

The hepatopancreas is the central metabolic organ of decapods, composed of blindly ending tubules and intertubular spaces filled with haemolymph, haemocytes, connective tissue and fixed phagocytes. It is also a major target organ for toxins and various pathogens from the environment, which can enter the body via the digestive tract, replicate within the hepatopancreatocytes and damage the organ (Vogt, 2020). Necrotizing hepatopancreatitis (Shields et al., 2012; Vincent & Lotz, 2007) or granulomatous hepatopancreatitis (Vogt & Rug, 1996) are unspecific diseases of hepatopancreas, which develop due to inflammatory response of crayfish to various aetiologies. Common pathological findings of infection agents in hepatopancreas are necrosis of tubular epithelial cells expressed as karyopyknosis and/or karyorrhexis (Jiravanichpaisal et al., 2009), followed by interstitial and luminal infiltration of haemocytes in order to sequestrate pathogens and necrotic tissue, leading to nodulation of tubule walls and melanized encapsulation (Longshaw, 2011; Shields et al., 2012). So far, bacteria recognized as causative agents of necrotizing hepatopancreatitis belong to genera *Staphylococcus*, *Vibrio*, *Escherichia*, *Aeromonas*, *Citrobacter* and *Pseudomonas* (Dragičević et al., 2021a). Additionally, necrotizing hepatopancreatitis bacterium (NHPB) was identified in shrimp farms as the causative agent of necrotizing hepatopancreatitis (Avila-Villa et al., 2012; Vincent & Lotz, 2007), and later renamed to *Hepatobacter penaei* (OIE, 2003), with the taxonomic position within the order Rickettsiales (Nunan et al., 2013). Viruses may also damage hepatopancreas of crayfish (Dragičević et al., 2021a; Edgerton et al., 2002), with *Pacifastacus leniusculus bacilliform virus* (PlBV) and *White spot syndrome virus* (WSSV) as two frequent candidates (Edgerton et al., 2002; Longshaw, 2011). However, many diseases (e.g., hemocytic enteritis), which can cause necrotic changes in crayfish hepatopancreas (Longshaw, 2011), are still classified as idiopathic. Except microorganisms, unfavourable environmental conditions such as long-term exposure to metals (i.e., lead, cadmium, zinc and copper) and their accumulation in hepatopancreas may also cause severe necrosis of tubules and their encapsulation and melanization (Kouba et al., 2010; Li et al., 2007; Longshaw et al., 2012; Tavabe et al., 2019; Wu et al., 2008).

In this study, we present the first evidence of an idiopathic necrotizing hepatopancreatitis in the signal crayfish from the Korana River in Croatia and a preliminary screening of its presence in the co-occurring native crayfish. We also compare the incidence and severity of idiopathic hepatopancreatitis along the invasion range of the signal crayfish in the Korana River, i.e., in populations from invasion core (long-established population with high crayfish abundance) and invasion front (recently established population at the very edge of the range with low crayfish abundance). Finally, we address the potential causative agents of the histologically manifested idiopathic hepatopancreatitis by (a) analysing the composition of bacterial communities in hepatopancreas using the amplicon sequencing approach based on the gene coding for *16S* rRNA and (b) performing the pilot analyses of trace and macro elements in surface water, river sediment and in native and invasive crayfish hepatopancreas.

2 | MATERIALS AND METHODS

2.1 | Study area

Korana River is a 134-km-long karstic river in central Croatia, which is characterized by mild-continental climate. It belongs to the Sava River basin, springs in the Plitvice Lakes National Park and flows northward (Roglić, 1974) to its confluence with the Kupa River in the town of Karlovac. Its annual temperature averages at 11.1°C (Gajić-Čapka & Zaninović, 2004), and it has an average discharge of approximately 29 m^3/s (Rebrina et al., 2015). The latest data on the signal crayfish distribution in the Korana River (Dragičević, Faller, et al., 2020) show that its invasive range covers over 30 km of the River's lower reach from whereon it spreads both upstream and downstream (Dragičević et al., 2020; Hudina et al., 2013). Furthermore, at both upstream and downstream end of its current distribution (invasion fronts), populations of native narrow-clawed crayfish are present and gradually displaced by the signal crayfish (Dragičević et al., 2020).

2.2 | Crayfish sampling

Crayfish sampling was performed in year 2018 (N = 48), 2019 (N = 25) and 2020 (N = 9) during September, when both crayfish species are reproductively active and both sexes are easily captured (Holdich, 2002; Hudina et al., 2013; Souty-Grosset et al., 2006; Table 1). Individuals were collected using baited LiNi traps (Westman et al., 1978) from four sites along signal crayfish invasion range: 1) upstream front (S1; Figure 1), upstream core (S2; Figure 1), downstream core (S3; Figure 1) and downstream front (S4; Figure 1; Table 2).

In order to examine whether such hepatopancreatic disease occurs in the native crayfish, we sampled three narrow-clawed crayfish at the downstream invasion front (Site S4; Tables 1 and 2). Finally, additional two narrow-clawed crayfish and two signal crayfish males were collected at site S4 in the Korana River and two narrow-clawed crayfish males at the site S5 in the Mrežnica River for the needs of analysis of total bioaccumulated trace and macro element concentrations in their hepatopancreas (described in chapter 2.5. and in the Supporting Information S1). Thus, for the needs of this study a total of 82 crayfish were collected, out of which 75 were invasive signal crayfish individuals and seven native narrow-clawed crayfish (Table 1). Since the narrow-clawed crayfish has been shown to exert competitive pressure on the expanding signal crayfish populations, which affects its dispersal rates (lower in downstream than upstream direction; Dragičević et al., 2020), we did not want to remove higher number of native crayfish from the Korana River. Hence, only seven native crayfish individuals in total were sampled: three for histopathology and four for metal analyses, aimed at obtaining preliminary information.

2.3 | Calculation of organosomatic condition indices and tissue sampling

In the laboratory, we first determined the sex of each individual (Table 2) and measured their total body length (TL; from the tip of the rostrum to the end of the telson, excluding setae, abdomen fully stretched, in mm; Streissl & Hödl, 2002), and weight (W_t, in grams). Each individual was carefully dissected and its hepatopancreas was isolated and weighted (W_{hep}, in grams). Hepatopancreas was always promptly and precisely isolated from the body by single cut at atrium line between stomach and hepatopancreas. Immediately before dissection, each individuals' nerve cord was rapidly cut ventrally from the thorax to the end of abdomen, according to available international guidelines for humane killing of crayfish (Conte et al., 2021); since no institutional or national ethical guidelines exist for crayfish. Recorded body length and weight data and hepatopancreas weight were used to calculate condition indices: (a) body condition index (Fulton condition factor; FCF = W_t/TL^3) and (b) hepatosomatic index (HSI = W_{hep} * 100/W_t), which are frequently used to assess the condition and overall health of the animal by determining their nutritional status and energy reserves (Carmona-Osalde et al., 2004; Lucić et al., 2012; Streissl & Hödl, 2002). Unexpectedly, during pilot dissections and hepatopancreas sampling for a wider study on microbiome of

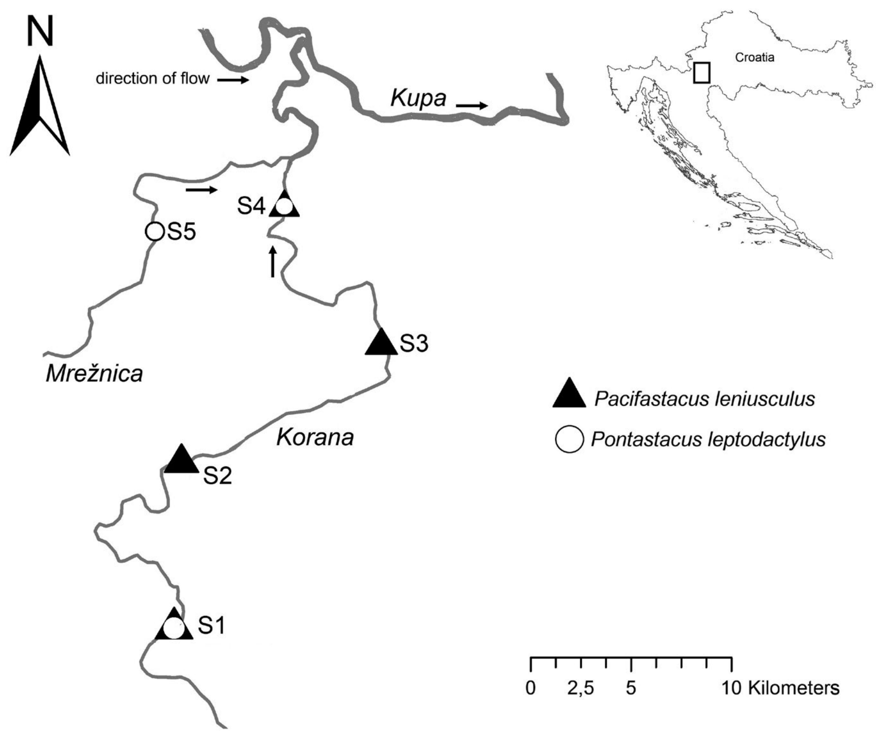

FIGURE 1 Sampling locations along the invasion range of the signal crayfish *Pacifastacus leniusculus* in the Korana River in Croatia (black triangles). Sites S1 and S4 represent upstream and downstream invasion fronts, while sites S2 and S3 upstream and downstream invasion cores. Sites S4 and S5 are sampling locations of the native narrow-clawed crayfish *Pontastacus leptodactylus* (white circles)

TABLE 1 The number (*N*) of crayfish individuals collected for different types of analyses performed in this study

Species	River	Histological examination of hepatopancreas	Total bioaccumulated trace and macro elements	Bioinformatic analysis
Pacifastacus leniusculus	Korana	73[a]	2	15
Pontastacus leptodactylus	Korana	3	2	0
	Mrežnica	0	2	0

[a]Hepatopancreas of 15 randomly selected individuals out of 73 sampled for histological assessment were also used for bioinformatic analysis.

TABLE 2 The number (*N*) and size of captured invasive signal crayfish *Pacifastacus leniusculus* and native narrow-clawed crayfish *Pontastacus leptodactylus* individuals used for calculation of organosomatic condition indices and histological assessment

Species	*Pacifastacus leniusculus*				*Pontastacus leptodactylus*
	Core		Front		Front
Location	Upstream (S2)	Downstream (S3)	Upstream (S1)	Downstream (S4)	Downstream (S4)
Males (*N*)	5	12	4	12	3
Females (*N*)	8	15	5	12	–
TL (± *SD*)	113.05 (± 7.65)	109.11 (± 7.92)	125.20 (± 10.88)	101.07 (± 11.89)	105.98 (± 9.92)
W_t (± *SD*)	45.43 (± 7.50)	44.51 (± 11.20)	69.97 (± 16.25)	35.68 (± 13.75)	37.17 (± 11.66)

Note: TL—average total body length in millimetres. W_t—average total body weight in grams. S1–S4 correspond to site codes on the Figure 1.

signal crayfish (Dragičević et al., 2021b), we discovered previously unrecorded gross morphological changes on hepatopancreas of several individuals. Hence, we decided to further investigate this finding and used available left lobe of hepatopancreas preserved in fixative for histological examination.

For microbiome analysis, each right hepatopancreatic lobe was placed in a sterile petri dish and carefully chopped into small pieces using a sterile disposable scalpel. Non-disposable dissecting equipment was alcohol and flame sterilized between each individual sample. The samples for microbiome analysis were frozen at −20°C.

2.4 | Histological assessment of the signal crayfish and the narrow-clawed crayfish

Hepatopancreas tissue samples from 73 signal crayfish, captured along its entire invasion range, and 3 native narrow-clawed crayfish (Table 1) were fixed in Bouin's solution, embedded in Paraplast embedding media (Sherwood Medical, USA) and sectioned at 7–8 μm on a rotary microtome (Shandon Finesse 325, Thermo Fisher Scientific, USA). Sections were stained following standard protocols with Mayer's Haematoxylin and Eosin Y (HE; Biognost, Croatia) and analysed using Nikon Eclipse E600 light microscope equipped with digital camera AxioCam ERc5s and ZEN2 lite software (Carl Zeiss Microscopy GmbH, Germany). All histological assessments were performed blinded, without prior knowledge of the species identity, sex and location of capture. To analyse histopathological changes within hepatopancreas qualitatively and quantitatively, 10 randomly digitally captured test fields (at magnification of 100×) were chosen per hepatopancreas for each individual. Each test field was assigned a score from 1 to 4 according to Zodrow et al. (2004): 1—no histopathology in any field; 2—mild histopathology, present in <25% of the fields; 3—moderate histopathology, present in 25%–75% of the fields; and 4—severe histopathology, present in >75% of the fields. Scores were based on both qualitative (severity of lesions) and quantitative (frequency of lesions) factors, while for negative control we used descriptions of healthy hepatopancreas from the available literature (Chabera et al., 2021; Štrus et al., 2019).

Further on, hepatopancreas sections were stained for gram-positive and gram-negative bacteria with modified Brown and Brenn (BB) method (Churukian, 2009). We could not clearly distinguish gram negative bacteria from non-specific staining of connective tissue, cellular debris, mucus casts and other proteins present in the inflamed tissue; hence, only gram-positive bacteria were assessed for their presence (1) or absence (0). Since bacteria usually occur in the tubular lumen in the hepatopancreas, positive reactions from the lumen were not considered pathogenic and were excluded from this analysis.

2.5 | Sampling of crayfish, water and sediment for pilot multielement analysis

In addition to crayfish sampling, at sites S4 and S5, water and sediment samples were also collected for analysis of dissolved metals in the surface river waters of the Korana and Mrežnica, and for multielement analyses of sediment (fractions <2 mm). Site S4 (Figure 1) in the Korana River was chosen since it is potentially under the highest anthropogenic pressure compared to other sites in the Korana River—it is located in the town of Karlovac, near the confluence with the Mrežnica River. Site S5 (Figure 1) in the Mrežnica River was chosen as a comparative site, considered generally unaffected by anthropogenic activities (Dragun et al., unpublished data). At each river, the samples of surface water were collected in triplicate as described in the Supporting Information S1. Surface sediment samples were collected with a plastic shovel, sieved to 2 mm at field-moist conditions, air-dried and stored. Hepatopancreatic tissue from two invasive signal crayfish and two native narrow-clawed crayfish were sampled from site S4 in the Korana River. As with water and sediment samples, our aim was to compare the bioaccumulation in the signal crayfish from the Korana River with the same species from the pristine site (i.e., Mrežnica River). However, since in the Mrežnica River no signal crayfish have been recorded so far (Hudina et al., 2017), only native narrow-clawed crayfish individuals (two crayfish; Table 2) were used for the comparison of trace and macro element bioaccumulation between the two rivers. Crayfish were brought to the laboratory in individual containers, on ice and were dissected immediately. Hepatopancreas were isolated and challenged entirely for multielement analysis, without preforming histopathological assessment. Detailed description of all performed multielement analyses in water, sediment and hepatopancreas samples for this section is presented in the Supporting Information S1.

2.6 | DNA extraction, library preparation and bioinformatics analysis

In addition to histological assessment, in 15 randomly selected signal crayfish individuals during dissection (Table 1; 7 from invasion fronts, 8 from invasion cores) we sampled tissue from the right hepatopancreatic lobe. We extracted DNA using the NucleoSpin™ Microbial DNA kit (Macherey-Nagel, Germany) according to manufacturer's protocol for gram positive and gram negative bacteria with duration of sample lysis by agitation modified as in Pavić et al. (2020). Amplification and sequencing of the variable V3–V4 region of the *16S* rRNA gene were performed by Microsynth, Switzerland. Illumina library was prepared using *16S* Nextera two-step PCR using forward 341F (5′-CCTACGGGNGGCWGCAG-3′) and reverse 802R (5′-GACTACHVGGGTATCTAATCC-3′) primers and sequenced on an Illumina MiSeq using the MiSeq Reagent Kit v2 (2 × 250 bp paired-end). Bioinformatics analysis of raw sequences was performed in order to compare the difference in composition and abundance of bacterial communities between the individuals with histopathological status of hepatopancreas identified as mild histopathology (score 2) and those with moderate histopathology (score 3). Since mild and moderate histopathology were prevalent in subsampled individuals, comparison among all histopathological scores (no histopathology, mild, moderate and severe) could not be performed.

2.7 | Statistical analyses

Obtained histological assessment data were analysed using Statistica 13.3.0 (Statsoft Inc. 2017). Since both raw and transformed data violated the assumptions of parametric tests, the non-parametric analogues were used instead (McDonald, 2014). Specifically, we analysed correlation between measured parameters of histopathological changes of the hepatopancreas (histopathological scoring) and the organosomatic condition indices (FCF, HSI) of each animal (Spearman correlation), compared the differences in histopathological scoring between sexes, sampling years, position within the river (upstream vs. downstream) and between different positions within invasion range (invasion core and invasion front) using Mann–Whitney *U* test, as well as between all examined sites (Kruskal–Wallis ANOVA with Dunn's post hoc test). We also compared the frequency of gram-positive bacteria between sexes and between different positions within the invasion range (invasion core and invasion front) as well as between different histopathological scores (Fisher's exact test). In all analyses, if no statistically significant difference ($p > .05$) was observed between the sexes, sampling years or among sites along the invasion range (upstream and downstream invasion core and upstream and downstream invasion front), the data were pooled together. In bioinformatic analyses, differences between two sample subgroups (mild and moderate histopathology) were tested using the Benjamini–Hochberg corrected Kruskal–Wallis and PERMANOVA tests (Anderson, 2001) for alpha and beta diversity, respectively. Furthermore, ANCOM tests (Mandal et al., 2015) were used to identify amplicon sequence variants (ASVs) that are differentially abundant between locations using composition plugin within QIIME2. Detailed description of all performed analyses is presented in the Supporting Information S2.

3 | RESULTS

3.1 | Histological assessment of hepatopancreas of the signal crayfish

Pronounced gross lesions consisting of branched irregular black structures scattered throughout hepatopancreatic tissue were observed in five crayfish specimens, while irregular area of oedematous tissue lacking villi was also detected on native hepatopancreas samples and those fixed in Bouin's solution (Figure 2a,b). When only black rod-shaped irregular spiral or wavy brown to black areas were mechanically isolated from remaining soft tissue, different stages of melanized and granulomatous tubular casts were visible (Figure 3).

Detailed histopathological analysis of hepatopancreas from 73 individuals of the signal crayfish (Table 2) showed the high incidence of hepatopancreatitis (97.3%; $N = 71$) with only two individuals (2.7%) having hepatopancreas without histopathological lesions (histopathological score 1). The majority of individuals

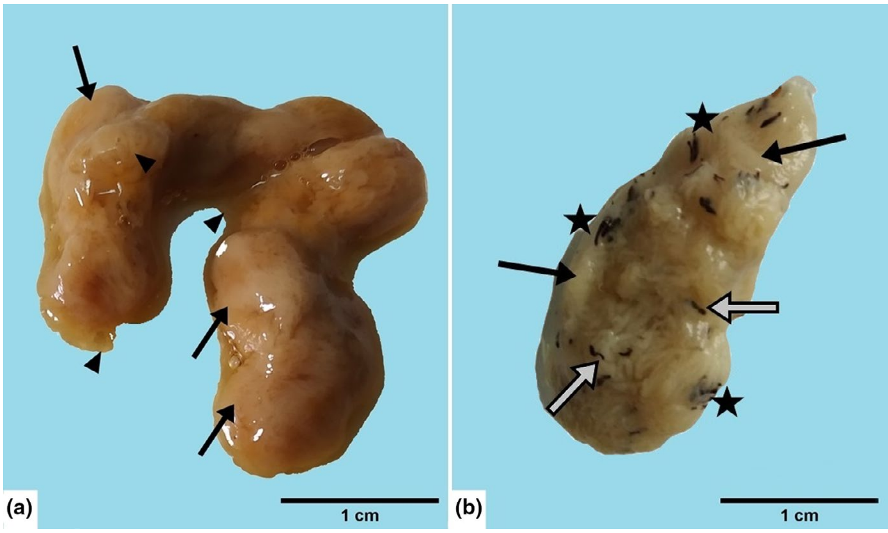

FIGURE 2 Representation of (a) native hepatopancreas of female signal crayfish *Pacifastacus leniusculus*, both lobes. Moderate hepatopancreatitis: arrows pointing irregular area of oedematous tissue lacking villous superficial appearance; arrowheads pointing normal superficial architecture of hepatopancreas. (b) Hepatopancreas of female signal crayfish fixed in Bouin's solution, left lobe. Severe multifocal to coalescing hepatopancreatitis: arrows pointing irregular areas of oedematous tissue lacking villous superficial appearance; grey arrows pointing single melanized tubule; asterisks pointing melanized hepatopancreatic branches. Macroscopic images, scale bars: 1 cm

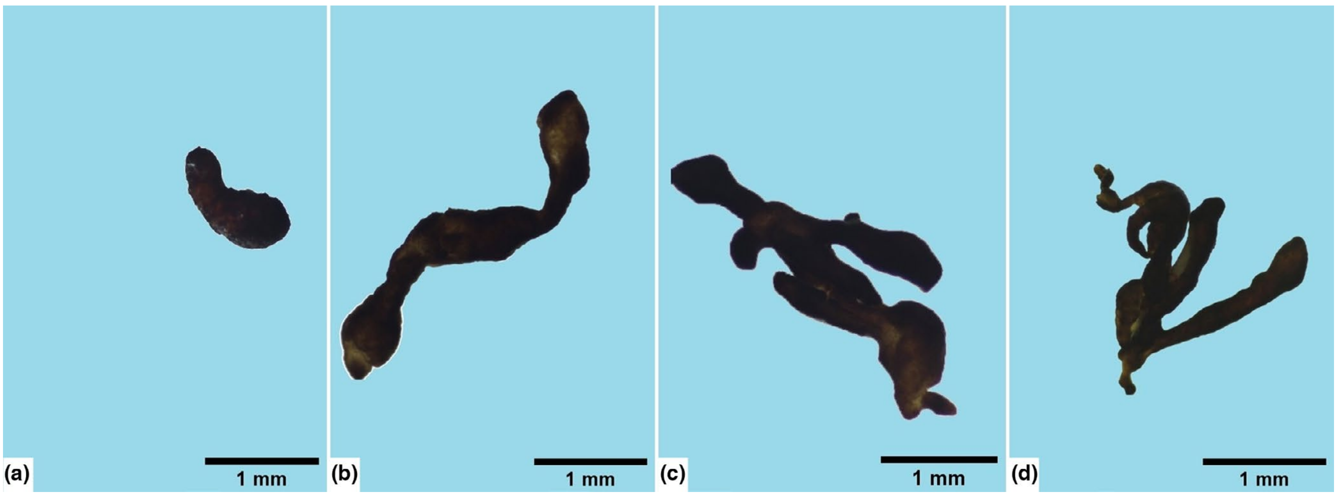

FIGURE 3 Different stages of melanized hepatopancreatic tubular casts, mechanically extracted from hepatopancreas of signal crayfish, *Pacifastacus leniusculus*, fixed in Bouin's solution. (a) Focal lesion, proximal zone tubule dilation and melanization, (b) Melanization of single tubule from distal to proximal zone, (c and d) two variants of hepatopancreatic branches melanization. Stereomicroscope images, scale bars: 1 mm

exhibited mild (histopathological score 2; 58.9%) and moderate histopathology (histopathological score 3; 31.5%), while 6.9% of crayfish exhibited severe histopathology (histopathological score 4; Figure 4).

Two analysed signal crayfish displayed normal structure of tubules and epithelial cells, with large central vacuoles in R cells and secretory vacuoles in B cells, and no lesions in hepatopancreas (histopathological score 1; Figure 5a). Specimens with identified lesions (histopathological score 2, 3, and 4) showed following histopathological signs: (a) epithelium atrophy with enlarged lumens of the tubules, (b) changes in cell composition (e.g. lower number of R cells with lipid droplets and lower number of B cells with large vacuoles), (c) floating cells detached from the basal lamina and disrupted integrity of the tubules (Figure 5b,c), (d) infiltration of granulocytes (Figure 5b), (e) focal necrosis (Figure 5c), (f) multifocal necrosis, (g) nodulation and encapsulation and (h) melanization of tubules (Figure 5d).

Focal necroses were quite frequent (>50% animals; Table 3; Figure 5c), while multifocal necroses, and nodulation and encapsulation, were observed in lesser extent (<50% animals; Table 3, Figure 5d). Focal necrosis, reported in 53.4% of animals, was visible as necrosis of an entire hepatopancreatic tubule, while surrounding tissues

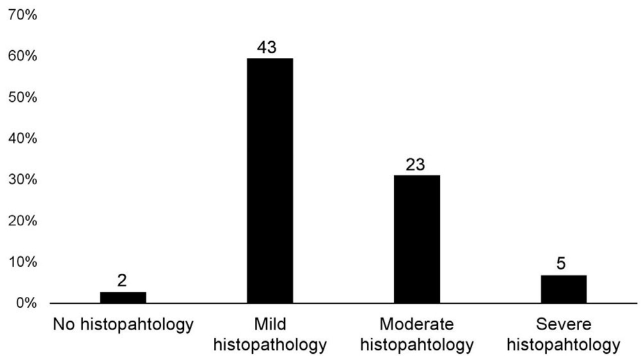

FIGURE 4 Histopathological scoring of hepatopancreas sampled from 73 signal crayfish *Pacifastacus leniusculus* individuals along its invasion range in the Korana River in Croatia. Scores are based according to Zodrow et al. (2004) and identified as: 1—no histopathology in any field; 2—mild histopathology, present in <25% of the fields; 3—moderate histopathology, present in 25%–75% of the fields; and 4—severe histopathology, present in >75% of the fields

occasionally showed signs of inflammation or differences in luminal and cellular architecture (Figure 5c). The final necrotic stage when melanized granulomas are formed was recorded in 50.7% of animals, when tubules were completely necrotic, with central debris, surrounding amorphous melanin capsule and outer cellular capsule. However, even when moderate histopathological lesions occupied more than 50% of tissue, we were able to distinguish intermediate zones (within the same histological sections) where some tubules displayed normal architecture (e.g., Figure 5c), regardless of whether sections were from a distal zone, B-cell zone or proximal zone. Finally, gram positive bacteria were observed in 32 animals (43.8%), mainly connected with necrotic tubules, and accompanied by inflammatory cells.

Histopathological scores were higher in crayfish with lower crayfish condition indices (FCF and HSI); however, only the correlation between histopathological score and hepatosomatic index (HSI) was statistically significant ($r_s = -.275$, $p < .05$). No significant differences were observed between sexes or sampling years in histopathological scores (Mann–Whitney *U* test, $p > .05$), and thus, sexes and sampling years were pooled together in subsequent analyses. Histopathological scores differed significantly between examined locations (Kruskal–Wallis ANOVA: $H_{(3,73)} = 11.88$, $p = .008$). In general, invasion fronts had higher histopathological scores compared to their respective fronts; however, only the differences between downstream front and upstream core were significant (Figure 6). Similarly, no significant differences in histopathological scores were observed between different sections of the river (upstream vs. downstream sites); however, different positions within the invasion range (core vs. front sites) differed significantly, with invasion core having significantly higher histopathological scores compared to invasion front ($U = 368.5$, $N1 = 40$, $N2 = 33$, $p = .001$). Gram-positive bacteria were significantly more frequent in individuals from invasion core than at invasion fronts ($p = .0001$) but exhibited no significant differences in their frequency between sexes or different histopathological scores.

3.2 | Pilot histological screening of native narrow-clawed crayfish

From three tested native narrow-clawed crayfish, one crayfish showed mild histopathological lesions, while two animals were without lesions in the hepatopancreas (Figure 7). Recorded lesions were visible as mild cell atrophy with enlarged lumens of the tubules, changes in cell composition (presence of smaller vacuoles within B cells) and infiltration of inflammatory cells in the connective tissue around tubules (Table 3).

3.3 | Pilot study: metal analyses of water, sediment and hepatopancreas samples

Preliminary analysis of dissolved metals in the surface river water and in the sediment fractions <2 mm is presented in the Supporting Information S1 (Tables S1 and S2), with metal levels in the water within limits recommended by national and international regulations (EPCEU, 2008; GRC 2019). The comparison of total bioaccumulated trace and macro elements in the hepatopancreas of native narrow-clawed crayfish from Korana and Mrežnica Rivers revealed comparable concentrations of the majority of analysed elements (15 elements had differences between sites below 50%, Supporting Information S1 (Table S3)). Higher concentrations in the hepatopancreas were still observed for a number of elements in the crayfish from the Korana River compared to Mrežnica River (As, Cr, Mn, Mo, Ni, Sb, U, V and Ca; 1.5–4.0 times), with the most pronounced differences being observed for Mn (Supporting Information S1, Table S2). In comparison, increased concentration in the hepatopancreas of only three elements (Ag, Cu and Pb) were found in the crayfish from the Mrežnica River (1.9–4.7 times higher compared to Korana River, with the most pronounced differences being observed for Cu). The comparison of hepatopancreatic bioaccumulation in native narrow-clawed crayfish and invasive signal crayfish, collected at the same site in the Korana River and thus living under the same conditions of metal exposure showed lower affinity for accumulation of extremely toxic metals, such as As, Bi, Cd and Ni, in the invasive signal crayfish (Supporting Information S1, Table S3).

3.4 | Pilot study: differences in bacterial composition of hepatopancreas

Taxonomic-level determination revealed presence of 187 genera in the hepatopancreas of 15 signal crayfish individuals (Figure 8). Dominant genera included unknown genus of the order Rickettsiales (17.7%), unknown genus from the class Mollicutes (15.9%), and Candidatus Hepatoplasma (10.5%). The abundance of the category 'other' (i.e., genera with abundance less than 1%) comprised 12.2% of the microbial community in the hepatopancreas samples (Figure 8).

When we compared the abundance of genera between the groups showing mild histopathology and moderate histopathology, significantly different abundance was observed only for genus *Salmonella*,

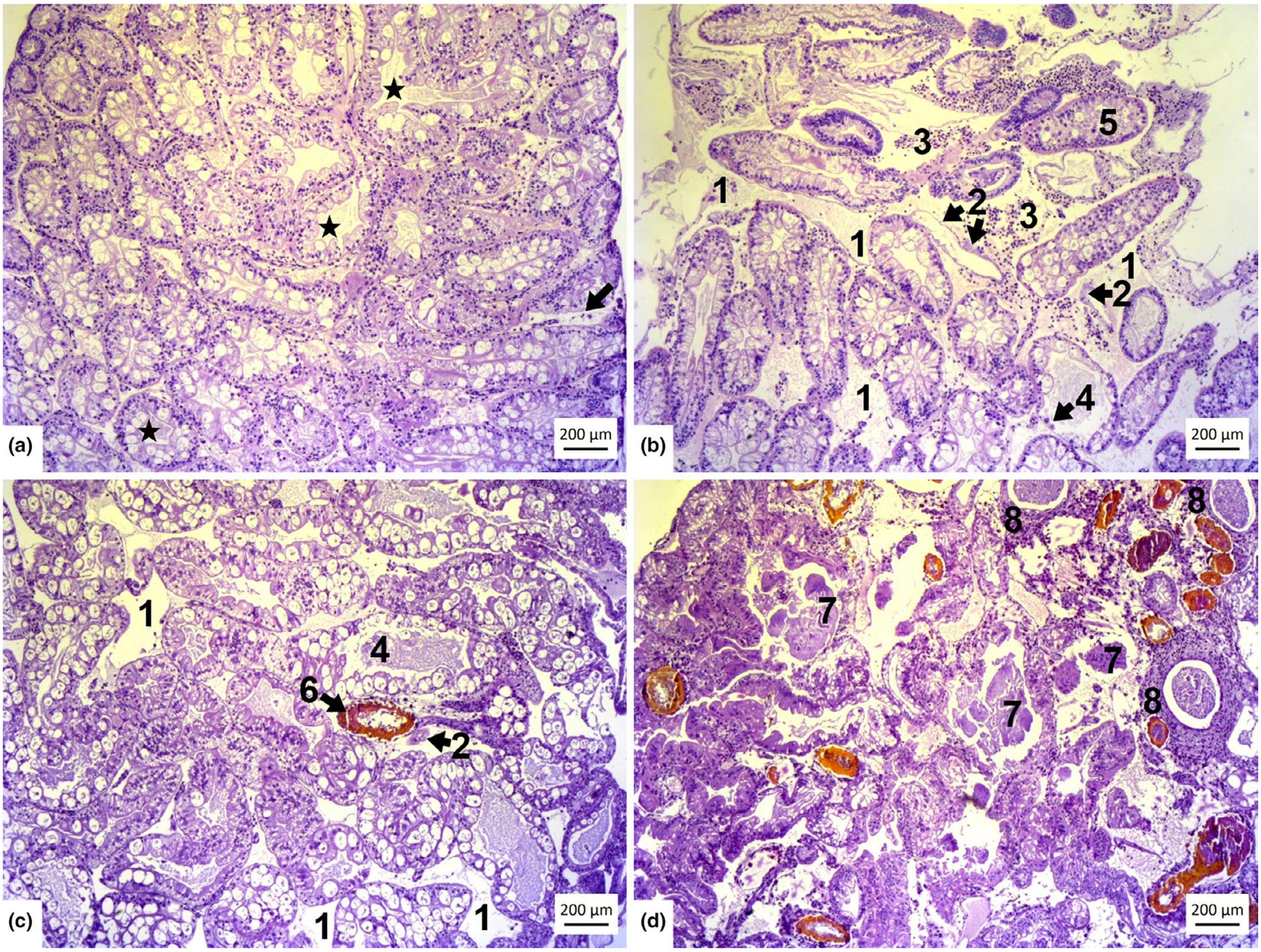

FIGURE 5 Histological presentation of hepatopancreas sampled from the left lobe of female specimens of signal crayfish *Pacifastacus leniusculus* in the Korana River (a) with no lesions: transversal and tangential sections of numerous tightly packed tubules of B-cell zone. Tubules are uniform containing proteinaceous luminal content (asterisks). Intertubular area are narrowed with arteries and intertubular sinuses discretely exposed. Only few perivascular inflammatory cells are present (arrow). R cells of B-cell zone massively vacuolated containing fat and protein droplets. Superficial epithelial cilia are occasionally pronounced. (b) Mild histopathological lesions: transversal and tangential sections of multiple tubules of B-cell zone widely extended by accumulation of haemolymph within intertubular space (1). Arteries and intertubular sinuses occasionally contain thick depositions (2) and multifocal aggregates of numerous inflammatory cells (3). Tubules are expressing occasional differences in luminal and cellular architecture (dilated lumen, segmental presence of naked basal membranes, floating cells and epithelium atrophy (4), or multifocal cellular slaughtering accompanied by discrete transepithelial migration of inflammatory cells (5). Majority of tubules expressed regular architecture. (c) Moderate histopathological lesions: transversal and tangential sections of multiple tubules of B cell zone with discrete multifocal expansions of intertubular spaces (1) and thickened arterial wall (2) close to terminally distracted tubule with intraluminal melanin deposition (6). Single tubule segment close to melanized area has dilated lumen, segmental area of naked basal membrane and epithelium atrophy (4). Only few inflammatory cells are visible within intertubular space surrounding necrotic tubules. Majority of tubules are expressing normal architecture containing fat and protein droplets within R cells of B cell zone. (d) Severe histopathological lesions: complete lack of normal architecture within transversal and tangential sections of tubules of B cell and distal zone. Within this section 75% of tubules are affected with one of previously described lesions, with more than 10 striking areas of melanization, multifocal to coalescing areas of tubular epithelial cells necrosis, floating cells and intraluminal accumulation of metaplastic epithelial cells (7) and proliferation of fibroblast like cells surrounding necrotic tubules (8, nodulation; Shields et al., 2012). Only a few intertubular spaces are expanded with inflammatory cells accumulation. HE, scale bars: 200 μm

which exhibited higher abundances in individuals with mild histopathology. Furthermore, no significant differences were recorded between two observed groups of crayfish hepatopancreas in alpha diversity. However, significant differences were recorded between the two groups in beta diversity (Bray–Curtis dissimilarity: $p = .007$, pseudo-$F = 2.04$; and Jaccard index: $p = .006$, pseudo-F: 1.44).

4 | DISCUSSION

This study reports on the newly detected necrotizing hepatopancreatitis in the population of the invasive signal crayfish in the Korana River and systematically analyses its histopathological features and changes along signal crayfish invasion range. Up

TABLE 3 Occurrence of histopathological lesions recorded in the hepatopancreas of invasive signal crayfish *Pacifastacus leniusculus* (*N* = 73) and native narrow-clawed crayfish *Pontastacus leptodactylus* (*N* = 3) sampled in the Korana River, Croatia

Histopathological lesions	No. and percentage of signal crayfish with observed lesions	No. and percentage of narrow-clawed crayfish with observed lesions
Epithelium atrophy with enlarged lumen of the tubule	71 (97.3%)	1 (33.3%)
Changes in cell composition	66 (90.4%)	1 (33.3%)
Floating cells	52 (71.2%)	
Infiltration of granulocytes	65 (89.0%)	1 (33.3%)
Focal necrosis	39 (53.4%)	
Multifocal necrosis	19 (26.0%)	
Nodulation and encapsulation	34 (46.6%)	
Melanization	37 (50.7%)	

to date, distinctive hepatopancreatic lesions have mainly been reported from farmed or wild populations in marine crustaceans (i.e. Hong et al., 2016; Shields et al., 2012), while after Vogt and Rug (1996) this study presents the second such report from a wild population of freshwater crayfish. Emerging wildlife diseases usually occur due to movement of pathogens or ecosystem alterations of either anthropogenic or natural origin, and may threaten local biodiversity (Cunningham et al., 2012; Daszak et al., 2000; Schmeller et al., 2020). In the case of successful invaders like the signal crayfish, such diseases may also affect invasion success and even alleviate its negative effects on the ecosystem (Fincham et al., 2019; Thomas et al., 2020). In this study, we recorded the extensive manifestation of histopathological lesions in hepatopancreas of the majority of examined signal crayfish individuals along its entire invasion range in the Korana River. Hepatopancreas is highly sensitive to physiological and environmental perturbations, critical in maintaining systematic metabolism, stability and balance of the digestive system and crucial in the regulation of the immune response (Vogt, 2020). Toxins and invading pathogens may impair its function directly or even indirectly since it is an important source of immune defence compounds and reservoir of lipids and glycogen, required for fighting diseases, egg production, moulting and growth (Vogt, 2020). Noteworthy, previous and ongoing research has shown that only 6% of the signal crayfish populations along entire invasion range were *A. astaci* positive (Pavić et al., 2020), while recorded *A. astaci* agent levels in both signal and narrow-clawed crayfish populations were very low (A0–A3 in the majority of analysed individuals of both species; Bielen et al., in preparation). This shows that both populations exhibit no signs of recent or ongoing crayfish plague outbreaks that could severely impact their immunological status and overall fitness. Since the aetiology causing this necrotizing hepatopancreatitis is still unknown, it is currently classified as idiopathic. In comparison with signal crayfish, mild histopathological lesions were detected in only one native narrow-clawed crayfish specimen, collected from interspecific populations at invasion front. Even though the number of analysed native narrow-clawed crayfish individuals was very low, we cannot exclude the option that idiopathic necrotizing hepatopancreatitis may not be species-specific and potentially affects both the native and invasive crayfish population.

Due to originally different purpose of the research and a smaller number of available intact unfixed organs, elaborate investigation of gross lesions was not performed. We were able to determine that all histological lesions except melanization were very discrete macroscopically and may be easily overlooked. While the majority (58.9%) of histologically screened individuals exhibited mild

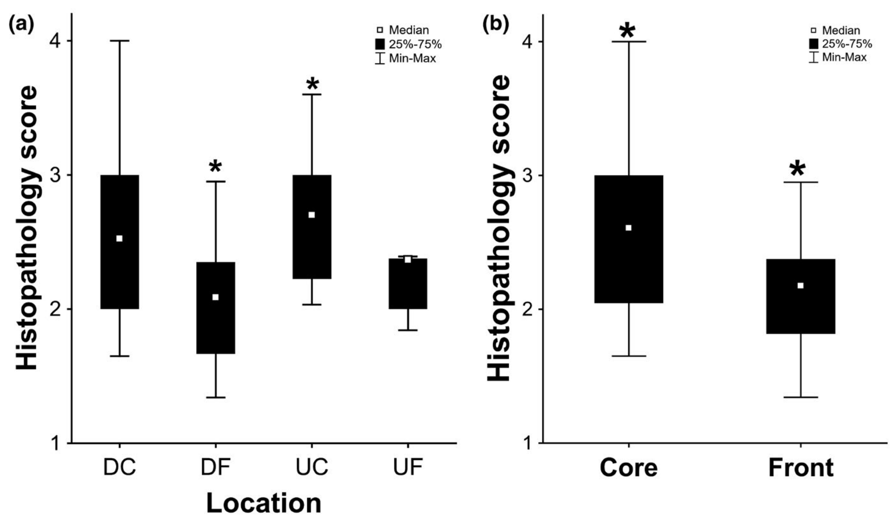

FIGURE 6 Comparison of recorded histopathological scores (1—no histopathological lesions; 2—mild histopathology; 3—moderate histopathology; 4—severe histopathology) in the hepatopancreas of the signal crayfish *Pacifastacus leniusculus* among (a) sites along the invasion range and (b) between different sections of the invasion range (invasion core versus. invasion front). Significant differences are marked with *

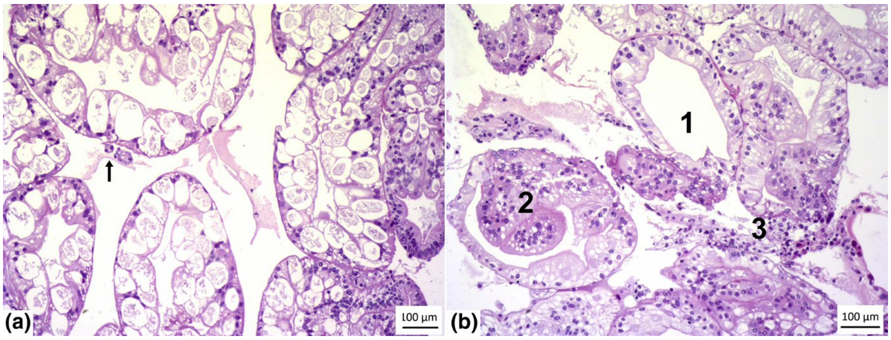

FIGURE 7 Histological presentation of hepatopancreatic tissue sampled from the left lobe of male specimens of the native narrow-clawed crayfish *Pontastacus leptodactylus* in the Korana River. (a) No histopathological lesions: transversal section of B cell zone of several tubules and tangential and transversal sections of distal zone. Presence of haemolymph and few inflammatory cells in the intertubular sinus (arrow). R cells of B-cell zone massively vacuolated containing fat and protein droplets. (b) Mild histopathological lesions: transversal section of B-cell zone of several tubules and tangential and transversal sections of distal zone. Expansion of luminal space, mild cell atrophy (1), presence of small fat droplets within R cells and intraluminal accumulation of metaplastic epithelial cells (2). Intertubular sinuses expanded with haemolymph and moderate number of inflammatory cells (3). HE, scale bars: 100 µm

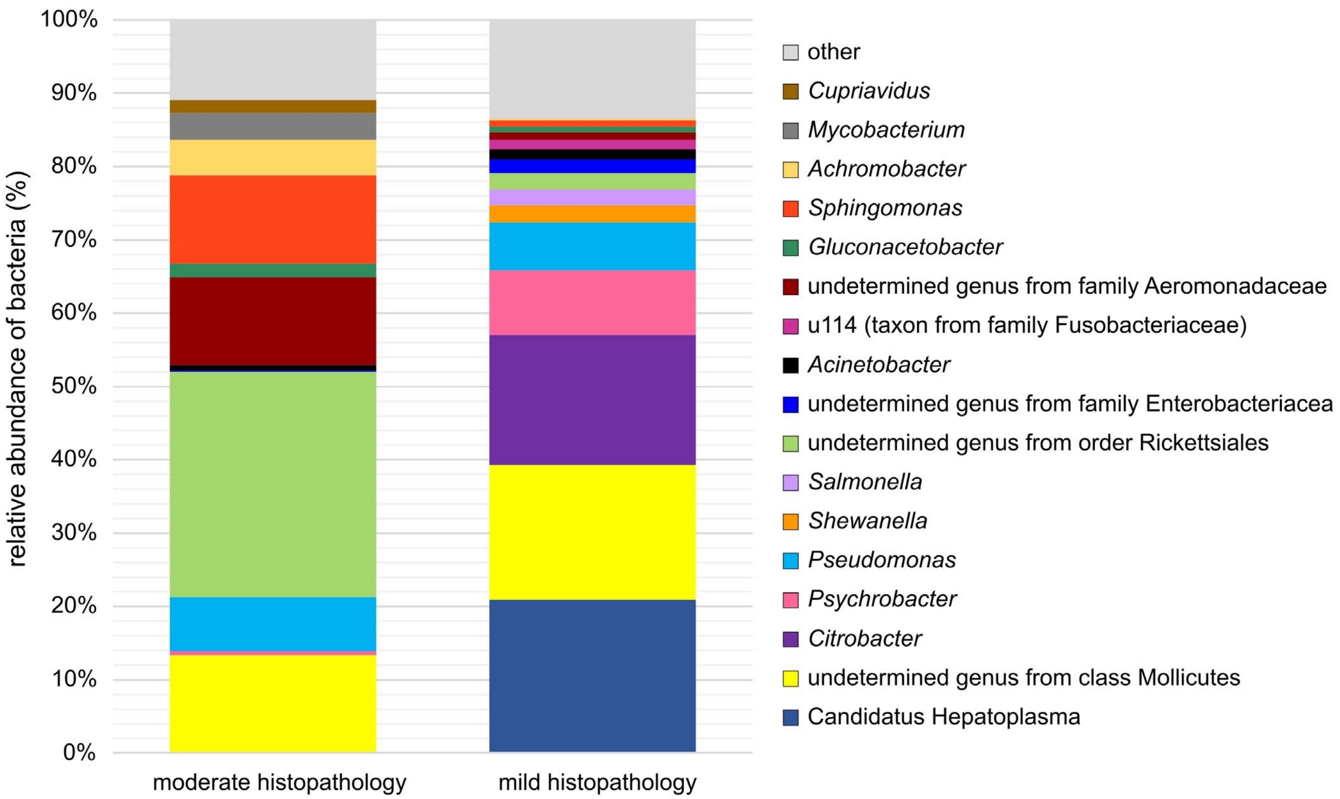

FIGURE 8 Relative abundance (%) of the overall most genera in hepatopancreas of signal crayfish *Pacifastacus leniusculus* individuals exhibiting moderate histopathology (left) and mild histopathology (right). Bacterial genera with an overall abundance >1% are shown, while the remaining genera were pooled and marked as 'other'

histopathological changes in hepatopancreas, we recorded only two signal crayfish with no hepatopancreatic lesions across the entire invasion range. Further on, even in animals with multifocal distribution of lesions and different degrees of tissue damage, there were parts of the hepatopancreas, which corresponded to the descriptions of normal architecture for freshwater crayfish (i.e. Chabera et al., 2021; Štrus et al., 2019). In the absence of crayfish from other locations, two signal crayfish without histopathological lesions, parts of unaffected tissue of specimens with lesions and available literature descriptions were consistent and altogether considered as a negative control.

Signs of atrophied hepatopancreas, characterized by the decrease of the tubule epithelial height and dilated lumens (Gibson & Barker, 1979) were observed in majority of individuals (Table 3), while other lesions such as changes in the composition of tubular epithelial cells, floating cells in the lumen and infiltration of inflammatory cells (Shields et al., 2012; Vincent & Lotz, 2007) occurred in high percentage of analysed animals (71.2%–90.4%). This is in accordance with other studies examining similar histopathological features in hepatopancreas of marine decapods (*Litopenaeus vannamei*; Vincent & Lotz, 2005) where more than 90% of hepatopancreatic tubules showed signs of inflammation. Focal and multifocal necrosis were observed in 53.4% and 26.0% of analysed signal crayfish, respectively, while in a similar research on clawed lobster *Homarus americanus* these types of changes were observed in only 9% and 6% of individuals (Shields et al., 2012). Nodulation and encapsulation (46.6%) and melanization (50.7%) were observed in almost half the collected animals which is similar to a study on lobsters (Shields et al., 2012). It should be noted that even when analysed specimens exhibited severe histopathological lesions, we roughly estimated that no more than 10% of melanized tubules were visible macroscopically. Melanin deposition around the damaged tissue and microorganisms, which physically prevents or retards pathogen growth (Cerenius & Söderhäll, 2004), is the most powerful immune response in decapods (Vogt, 2019). However, a rigid melanized capsule, which surrounds disintegrated hepatopancreatic tissue seems to remain in the organ until death of the animal (Vogt, 2020), without replacement with healthy tissue. Thus, future research should include experimental trials, necessary to determine the percentage of functional hepatopancreatic tissue required for maintenance of metabolic balance, before mortality occurs. Discovery of damaged arteries and sinuses (Figure 5b,c) potentially suggests systemic susceptibility and systemic cause of disease, and consequently may explain multifocal lesions and various degrees of lesions within the same organ.

Acute hepatopancreatic necrotic disease (AHPND) described in marine penaeid shrimp *Penaeus (Litopenaeus) vannamei* (Boone, 1931) is characterized by severe atrophy of the hepatopancreas and multifocal granulomatous lesions, resulting in anorexia, reduced growth, frequent secondary bacterial infections, lethargy and consequently high mortality rates in cultured shrimps (40%–100%; Hong, Lu, et al., 2016; Hong et al., 2016). Somewhat milder hepatopancreatic disease was described in the native noble crayfish *A. astacus* specimens (Vogt, 2020; Vogt & Rug, 1996) as granulomatous hepatopancreatitis, where through melanization and encapsulation of hepatopancreatic tubules infected with gram negative bacteria, intense dark brown and black nodules (granulomas) are formed. Similarly, in clawed lobster *H. americanus* idiopathic necrotizing hepatopancreatitis was represented by focal and coalescent lesions, necrotic tubules with nodulation of tubule wall and encapsulation of most likely bacteria-infected tubules, possibly coupled with environmental contaminants (Shields et al., 2012).

When AHPND described in penaeid shrimps was experimentally induced in the laboratory with pathogenic bacteria *Vibrio parahaemolyticus* carrying plasmids with toxic *pir*A and *pir*B genes (Aranguren Caro et al., 2020; Aranguren Caro et al., 2020; Tran et al., 2013), distinctive phases of disease were recognized: (a) acute phase characterized by tubule cell sloughing (floating cells), lower numbers of R cells and B cells, some haemocytic infiltration around tubules and multifocal necrosis while bacterial colonization is not observed; (b) terminal phase with necrosis, haemocytic infiltration both within and surrounding tubules and with massive secondary bacterial infections; and (c) chronic phase with granulomatous response in the tubules. We can speculate that sequence of events in our study may be similar: the acute phase of disease is visible as necrosis of hepatopancreatic tubules and intertubular inflammation, followed by encapsulation and nodulation of affected tubules, while presence of melanized granulomas in the tissue indicates the final immune reaction and suggests more subacute phase. Hence, recorded histopathological findings in the analysed crayfish could be defined as subacute to acute necrotizing hepatopancreatitis (Shields et al., 2012; Suong et al., 2017; Vincent & Lotz, 2007; Vogt, 2020). However, in the absence of known aetiology and experimental challenge trials we cannot determine with certainty the course of this disease and the gradation of lesions.

In this study, gram-positive bacteria were recorded in 43.8% of histological samples; however, we were not able to identify and quantify the gram negative bacteria, which are also among potential causative agents of this type of disease (Suong et al., 2017; Vincent & Lotz, 2007; Vogt & Rug, 1996) and their presence should be further investigated. Finally, while the examined signal crayfish appeared healthy, and no mortalities were reported from these populations during their regular monitoring (Dragičević et al., 2020; Hudina et al., 2013, 2017; Rebrina et al., 2015), we observed a significant negative correlation between the severity of histopathological changes and hepatosomatic index (HSI). While hepatosomatic index may be affected by a number of factors, including the season and crayfish density (Rebrina et al., 2015), the observed negative correlation indicates that it could be also affected by the intensity of histopathological change. In the latter case, the observed substantial damage to the hepatopancreas could potentially impair its function and exert negative effects on crayfish health, survival and population viability, which needs to be explored in more detail.

Along the invasion range, we observed significant differences between examined sites and between individuals at invasion cores and invasion fronts in histopathological scores, which were significantly higher at invasion core. In the Korana River, core and front populations differ substantially in crayfish density: crayfish abundance is 4–7 times higher at invasion cores compared to invasion fronts where signal crayfish co-occurs with the native and competitively inferior narrow-clawed crayfish (Dragičević et al., 2020; Hudina et al., 2016; Lele & Pârvulescu, 2017). High population density and subsequently higher competition intensity at invasion cores may increase the interaction rates between individuals (Pintor et al., 2009) and potential injuries, all of which may incur energetic costs and decrease the condition of individuals (Hudina et al., 2009). Impaired condition may in turn affect the immune status of individuals, and make them more susceptible to environmental stress, such as effects of pollution or pathogens (Aydin et al., 2014; Hong, Lu, et al., 2016; Hong, Xu, et al., 2016).

Furthermore, infected crayfish may serve as a reservoir and spread the pathogen to other crayfish, possibly through cannibalism (Jiravanichpaisal et al., 2009), which may be more frequent in populations of higher density (Houghton et al., 2017). Previous research on signal crayfish condition along its invasion range in the Korana River has already identified density-dependent effects on measured body and organosomatic condition parameters, with individuals at invasion fronts being in better body condition and with females at invasion fronts exhibiting superior energetic status of hepatopancreas and gonads to those from invasion core (Rebrina et al., 2015). Thus, we assume that density may be one of the drivers of differences in histopathological changes between core and front populations observed in this study. Additionally, the lack of significant differences in histopathological scores between upstream and downstream river sections indicates that population characteristics (i.e., crayfish density) may be more prominent driver of the observed histopathological changes than the characteristics of the (abiotic) environment.

While the causative agent of this newly detected disease remains unknown, we have performed a pilot analysis of the most likely potential causative agents recorded in literature: pathogens and metal bioaccumulation (Avila-Villa et al., 2012; Dragičević et al., 2021a; Kouba et al., 2010; Li et al., 2007; Tavabe et al., 2019; Vincent & Lotz, 2007; Wu et al., 2008). Hepatopancreas has a central role in detoxification (Wu et al., 2008) and multiple studies have demonstrated high metal accumulation in hepatopancreas (Fikirdeşici-Ergen, 2020; Gherardi, 2006; Xiong et al., 2020) and related histopathological changes similar or identical to those identified within this study (Li et al., 2007; Tavabe et al., 2019; Wu et al., 2008). Pilot analyses of water and sediment metal load revealed that both the Korana River and the reference (unpolluted) site in the Mrežnica River appear as generally clean watercourses with only somewhat higher concentrations of dissolved Mn and Fe in the surface water of both rivers, and additionally higher concentrations of As and Ba (4.5 times and 1.5–2.5 times, respectively) in the Korana River, when compared with pristine karstic rivers in the region (Krka River source: Sertić Perić et al. (2018); Una River: Dautović (2006)). It must be taken into account that analyses were performed on a very low sample size and should be considered only as preliminary. Nevertheless, obtained data are in line with the results of surface waterbody monitoring according to Water Framework Directive (EPCEU, 2008) which assigned very good to good status at all 7 monitoring sites in the Korana River regarding general physical and chemical conditions and specific pollutants (Water Body Register, 2016). The recorded metal levels in the analysed sediments of the Mrežnica and Korana Rivers, with the exception of somewhat higher than expected Cd in the Mrežnica River, were comparable or even lower than expected values for the stream sediments in the area (Salminen et al., 2005), thus excluding the probability of anthropogenic effects. Total bioaccumulated trace and macro elements in the hepatopancreas of signal crayfish from the Korana and Mrežnica Rivers revealed comparable concentrations of the majority of analysed elements, while the native narrow-clawed crayfish and invasive signal crayfish from the same site in the Korana River differed in the affinity for accumulation of several metals, and lower affinity for extremely toxic metals, such as Bi and Cd. This potentially indicates better protective mechanisms against toxic metals of the invasive signal crayfish and is also in line with other studies demonstrating differences between native and invasive alien crayfish in their metal requirements and rates of metal uptake, accumulation, detoxification and excretion (Gherardi, 2006). Finally, comparison with the results reported in two other crayfish studies (on signal crayfish from the pristine Wieprza River (Poland), Nędzarek et al., 2020; and on red swamp crayfish *Procambarus clarkii* (Girard, 1852) from the mildly anthropogenically impacted Yangtze River ponds (China), Xiong et al., 2020) for the majority of elements indicated comparable hepatopancreatic concentrations, except for several trace elements (As, Cu, Se and Pb). Arsenic showed much higher concentrations in both the native and invasive crayfish in our study compared to literature data (up to 22 times, Supporting Information S1, Table S3), which is possibly associated with naturally higher As concentrations in the sediment fraction with grain size <2 mm in the Croatian rivers (3.4–4.8 mg/kg) compared to Wieprza River (0.79 mg/kg, Nędzarek et al., 2020). Since pollution and water quality can significantly affect the incidence and intensity of diseases observed in wild and cultured crayfish populations (Anderson et al., 2021), the possibility of arsenic impact on crayfish health should be taken in consideration in future research. Altogether, this pilot analysis merely indicates that no alarming or unusually high concentrations of metals were observed in either water, sediment or in crayfish hepatopancreas, and further in-depth analyses including different time points, higher number of individuals/samples and simultaneous assessment of histopathological condition and bioaccumulation in hepatopancreas are required.

In this study, potential bacterial causative agents were examined by comparing the bacterial composition of hepatopancreas between the individuals exhibiting mild and moderate histopathology. Multiple studies have recorded bacteria in hepatopancreas of crustaceans (Cheung et al., 2015; Dragičević et al., 2021b; Wu et al., 2021; Zhang et al., 2020). Additionally, some studies (Dragičević et al., 2021b; Zhang et al., 2020) found similarities between bacterial communities of hepatopancreas and other tissues, suggesting that bacteria are shared between tissues and that tissues of healthy crustaceans are not sterile. Due to high abundance of bacteria from order Rickettsiales and class Mollicutes which also includes highly abundant genus Candidatus Hepatoplasma, their role in health and immunity of crustaceans needs further investigation. Species belonging to genus Candidatus Hepatoplasma are reported as abundant in the digestive system and beneficial for crustacean health (Chen et al., 2015), while the members of the order Rickettsiales have been shown to cause a wide spectrum of histopathological changes in the hepatopancreas and other organs of crayfish, similar to those observed in this study (Edgerton & Prior, 1999; Jimenez & Romero, 1997; Powell, 2013). Here, significant differences in beta diversity were observed between individuals with mild and moderate histopathology in both bacterial abundance and composition, which is in line with the similar studies on shrimps (Xiong et al., 2017) and possibly connected with assumptions that secondary bacterial infections occur in terminal phases of disease when tubules are highly damaged (Aranguren et al., 2020).

The only significant differences in hepatopancreatic bacterial abundance in this study were recorded for genus *Salmonella*, with higher abundance in individuals with mild histopathology in comparison with moderate histopathology. Similar findings were reported by Dragičević et al. (2021b), who observed differences in abundance of genus *Salmonella* in hepatopancreas of crayfish from different locations within the same invasive range. Future microbiome studies should incorporate all stages of histopathological changes (from no histopathology to severe histopathology) by performing histopathological analysis prior to sample selection for DNA extraction, sequencing and subsequent analysis. This would provide a more in-depth insight into potential differences in bacterial composition in hepatopancreas with different incidence and severity of histopathological changes, especially between two extremes (no histopathology vs. severe histopathology), as in Chen et al. (2017), Cornejo-Granados et al. (2017) or Zheng et al. (2017). Additionally, our ongoing analysis of signal crayfish hepatopancreas virome (Bačnik et al., submitted) by high-throughput sequencing and metagenomic approach identified the presence of a putative signal crayfish associated reo-like virus, which exhibits similarity to *Cherax quadricarinatus* reovirus that causes necrosis and inflammation in hepatopancreatic tubules (Hayakijkosol & Owens, 2011). Hence, additional study is planned to explore the role of identified reo-like virus in this newly detected disease.

In conclusion, several factors such as: (a) the high prevalence of observed idiopathic necrotizing hepatopancreatitis in the signal crayfish along the entire invasion range, (b) relatively high percentage (31.5%) of individuals exhibiting moderate histopathology, (c) the crucial role of hepatopancreas in crayfish and (d) the reported high mortality rates of other decapod species (Avila-Villa et al., 2012; Chen et al., 2017; Vincent & Lotz, 2007; Xiong et al., 2015) with similar histopathological signs, highlight the importance of further research into recorded necrotizing hepatopancreatitis and its effects on signal crayfish health and its invasion success as well as effects on the native narrow-clawed crayfish. Further comparison of the severity of observed histopathological changes between the two co-occurring crayfish species is required since the native narrow-clawed crayfish seems to exhibit higher affinity for accumulation of some toxic metals which could also be contributing its displacement by the signal crayfish, observed in the Korana River.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors, upon request. The next-generation sequencing data that support the findings of this study are a part of the wider study by Dragičević et al. (2021b) and are openly available in EMBL Nucleotide Sequence Data Base (ENA) at https://www.ebi.ac.uk/ena/browser/home, reference number PRJEB43749.

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