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ASSESSMENT OF CONSERVATION STATUS OF PETROLEUCISCUS BORYSTHENICUS CELENSIS FROM GURBAN RIVER, ROMANIA BY IDENTIFICATION OF PARASITES AND BACTERIA

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Abstract

The present research was conducted within the framework of a broader investigation with the objective of identifying freshwater parasites and bacteria on national territory, with the aim of gaining a deeper understanding of the relationships between parasites, hosts, and the environment, and to assess the potential detrimental effects of parasitic infestation on the conservation status of fish populations. Within this study, it was documented the first recordings of parasites Vorticella globularia and Epistylis sp. in the Romanian Petroleuciscus borysthenicus celensis fish species. A total of 42 specimens were collected from the Gurban River to study their infestation status, focusing on the skin, gills, and fins. From the samples analyzed, 16 specimens presented signs of infestation with five distinct parasite species (Ichthyophthirius multifiliis, Dactylogyrus vastator, Trichodina acuta, Vorticella globularia and Epistylis sp) and four bacterial strains (Aeromonas veronii, Shewanella putrefaciens, Aeromona hydrophila, and Citrobacter freundii). The results indicate that the skin is the organ most severely impacted by parasites and bacteria, followed by the gills. The fins, on the other hand, are the least susceptible to infection. Furthermore, the significant amount of parasitic infestation coupled with the high bacterial load of Aeromonas veronii indicates a plausible link between the two.

Keywords*: Petroleuciscus borysthenicus celensis; Fish parasites; Fish bacteria; Freshwater species; freshwater conservation*

Introduction

Petroleuciscus borysthenicus celensis (Bănărescu, 1954) (*pop.* Comana chub*)* [1], is a subspecies of *Petroleuciscus borysthenicus (Kessler, 1859)*, a small benthopelagic brackish and freshwater fish, 13cm in length, from the cyprinid family, which lives in the waters of the lower course of the tributary rivers of the Black Sea [2], the Sea of Azov [3], the Sea of Marmara [4] and the Aegean Sea [5]. Wrongly synonymized as *Leuciscus borysthenicus* [6], the species thrives in warm, shallow aquatic environments with temperatures up to 30–32°C [7], and prefers sandy or muddy substrates in areas characterized by gentle currents along the shorelines. It inhabits waters rich in vegetation such as *Alisma plantago-aquatica (Linnaeus, 1753)*, *Hydrocharis morsus-ranae (Linnaeus, 1753)*, *Potamogeton* sp., and *Vallisneria* sp. [8]. This

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species is subjected to parasite infestations such as *Diplozoidae* (common monogenean ectoparasites of cyprinid fish), *Paradiplozoon homoion* and *P. hemiculteri* [9], *Pseudoamphistomum truncatum, Metorchis xantosomus, M.bilis, Eustrongylides excisus* [10], *Ligula intestinalis, Schyzocotyle acheilognathi* [11] the widespread organisms representing an important issues studied in depth, both on wild and reared fish all over the world [12]. Such infestation in wild fish species that are living in natural or artificial lakes, coastal area of the seas, the lagoons, and rivers [13] represent not only a potential public health issue, but can also become a threat to the aquaculture sector [14]. Parasites can alter the behavior of the fish host [15] and prolonged infestations can lead to a weakened immune response in fish, making them more susceptible to other diseases and environmental stressors [16-18]. The determination of fish parasitic diseases and the associated pathogens in artificial or wild systems is crucial for sustainability [19], as wild fish in lakes and rivers are known to serve as reservoirs of symbionts, infecting fish in hatcheries supplied with water from such water courses [20]. Protozoan parasites undoubtedly comprise one of the most important groups of pathogens negatively influencing the welfare of both cultured and wild fish [21], parasitic ciliates being amongst the most pathogenic protozoa that infect fishes [22]. Such pathogens have not received much attention because of the technical difficulties inherent in their study, in comparison to the much larger helminth parasites [23].

The study stems from a concerning trend: a recent decline in the population of this fish species within Romania, which has sparked worries about their potential extinction; thus, our aim is to investigate the underlying causes behind this decline in order to implement protective measures. Notably, our research has uncovered the presence of two previously undocumented parasites, *Vorticella globularia* and *Epistylis sp* affecting these fish within Romania, along with the detection of bacterial pathogens.

Materials and Methods

Specimens Sourcing and Tissue Sampling

A total of 42 *Petroleuciscus borysthenicus celensis* wild specimens were captured from Gurban River, a tributary of Neajlov River which flows into Comana lake, Romania [24] of 320m (Fig. 1) and transferred in aquaculture water tanks under controlled conditions (19°C, 13.4mg/L O_2, pH = 7.8).

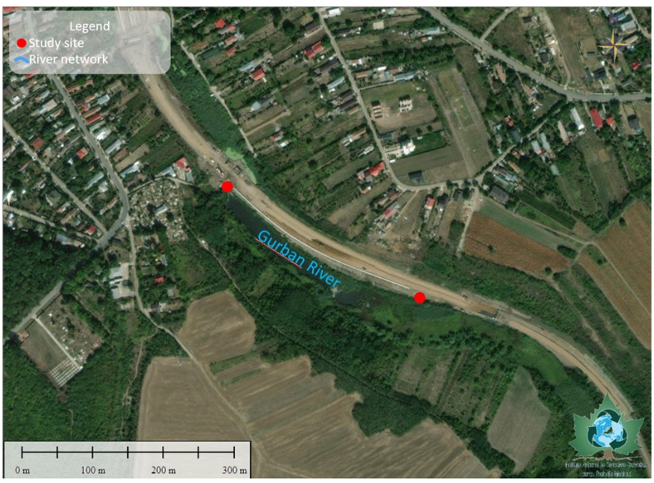

Fig 1. Electrofishing sampling area (two campaigns conducted in summer and fall season)

Two scientific fishing campaigns were conducted, one during the late fall season and another during the early summer. Biometric measurements for weight and length parameters were taken and descriptive statistics were obtained (minimum, maximum, average values, standard deviation and the coefficient of variation). After a macroscopic investigation for parasitic infestation, the 42 specimens were sacrificed [25] with the use of CO_2. Three tissue samples were collected from each specimen with the use of a scalpel from the skin and fins, the gills were positioned in Petri dishes with distilled water and placed on microscope slides followed by fixation in 70% alcohol. The microscopic investigation for parasites was performed with an Olympus BX43 light microscope (Olympus, Japan).

Table 1. Descriptive statistics for the entire group of 42 *P. borysthenicus celensis* specimens

	Min.	Avr.	Max.	SD	CV
Weight (g)	1.1	2.58	3.9	0.73	0.28
Lenght (cm)	5.2	6.22	7.4	0.67	0.11

Out of the 42 investigated specimens, 16 specimens were contaminated with parasites, leading to an infection rate of 38%.

Table 2. Descriptive statistics for the infected group of 16 *P. borysthenicus celensis* specimens

	Min.	Avr.	Max.	SD	CV
Weight (g)	1.2	2.95	3.8	0.91	0.31
Lenght (cm)	5.3	6.66	7.2	0.72	0.10

The sacrification of the specimens was performed within the premises and with the approval of National Institute for Research and Development in Environmental Protection Institute, in conformity with the Guide for the Use and Care of Laboratory Animals regarding reduction of animal suffering and number of animals sacrificed.

Parasites Staining and Examination

Following the primary investigation, staining was performed as follows: smears were air dried, fixed in 70% ethanol for 10min, and then stained with 5% Giemsa's solution in phosphate buffer (pH 7.3) for 30min [26]. Also, in order to study details of the ventral ciliature, air dried smears were saturated with a 2% aqueous solution of silver nitrate ($AgNO_3$) for 8 minutes and then rinsed thoroughly in distilled water following exposure to ultraviolet light for 20–25min [27]. The parasite species were identified on the basis of earlier studies, such as [28-31].

Bacteriological investigation

Out of the set of fish identified with parasite infections, three specimens were selected for bacteriological examination based on the exhibition of severely external lesions on the areas being analyzed, such as skin ulcerations, discolored spots, and tears in the fin tissue. These lesions were rinsed with distilled water and swabbed with a sterile swab (Copan Eswab, Italy). Each swab was used to sample an area of ≈ 2cm^2 and was afterwards inserted in 1.0mL Amies liquid transport media and rigorously shaken. From each, 20µL was inoculated in triplicate on Nutrient agar and Columbia agar supplemented with 5% sheep blood, prior to incubation at 25°C for 24 hours. The colonies that grew on media plates were counted and primary identification was performed using chromogen agar by seeding each individual colony.

The final taxonomic identification was carried out using a Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) Mass Spectrometer Biotyper (MALDI-TOF Microflex LT/SH, BrukerDaltonik, Bremen, Germany). Isolated colonies were applied to MSP 96 target polished steel plates (Bruker Daltonics, Germany, Bremen). On each spot, α-Cyano-4-hydroxycinnamic acid (Bruker Daltonics, Bremen, Germany) matrix was added, along with the stock solution containing acetonitrile, trifluoroacetic acid, and distilled water.

For the identification process the SR Library Database (Database CD BT BTyp2.0-Sec-Library 1.0) in conjunction with MBT Compass (v.4.1) software were used.

Results and Discussions

Parasites

The five species of parasites were found on the gills, fins and skin of the fish studied. *Ichthyophthirius multifiliis (*Fouquet, 1876*),* commonly known as Ich, is a protozoan parasite that causes a disease known as *Ichthyophthiriasis* or white spot disease [32]. This parasite can have significant negative effects on fish health and can be particularly problematic in aquaculture and aquarium settings [33-35]. Regarding the morphological characteristics, *Ichthyophthirius multifiliis* is a large, oval to round and dark colored (due to the thick cilia covering the entire cell) mature trophont, that measures between 0.5-1mm. At this stage, a horseshoe or C-shaped macronucleus may be visible under 40x. magnification.

*Dactylogyrus vastator (*Nybelin, 1924*)*, that are usually found on the gills of cyprinid fishes, possess a haptor with a pair of anchors or hamuli, seven pairs of marginal hooks and four eyespots situated anteriorly to the pharynx [36-39]. *Dactylogyrus vastator* measures 0.8-1.15mm in length.

Trichodina acuta (Lom, 1961) are ciliated protozoans known for their mobility and their body enveloped by a slender membrane, encircled by an adoral ciliary spiral. Additionally, they feature a horseshoe shaped macronucleus and an adhesive disc equipped with a denticulate ring, containing small tooth-like structures [40]. *Trichodina acuta* have been isolated from gills ,fin and skin of various fish species [41-43].

Vorticella globularia (Müller, 1773) belongs to the family *Vorticellidae*, a morphologically distinct group within the subclass *Peritrichia* [44]. Infections by *Vorticella globularia* are common in many cultured fishes [45, 46]. Parasitic ciliates *Vorticella globularia* are common among wild and cultured tilapia, especially when the fishes are farmed at high stocking densities [47-49]. The presence of protozoan ciliates *Vorticella globularia* in cultured *Nile tilapia* in a number of farms in the central region of Saudi Arabia was reported by [26]. In *Cyprinus carpio* (Common carp), these parasites were only found in the gills and skin, with 80% of them being found on the skin. Also, on *Ctenopharyngodon idella* (Grass carp), the skin also had the most parasites (80%) compared to gills, whereas all other organs were parasite-free. Many *Vorticellids* on the skin of moribund fish prey on their tissues [50]. *A.M.K. Al-Musawi et al.* [51] reported this parasite on the skin of *Planiliza abu* in Euphrates River, Iraq, and on *Cyprinus carpio* and *C. idella* in West Bengal, India. *Vorticella globularia* is spherical in shape, usually between 161-163μm long and 151-156μm wide. The peristomial lip is narrow, and the stalk can reach up to 1002 μm in dimension.

Epistylis sp., a stationary, ciliated protozoan that forms colonies typically on the skin and occasionally on the gills of fish [31, 52-54].While it is not a genuine parasite, this organism acts as an epibiont, using fish as a surface for attachment, and induces tissue necrosis through the release of proteolytic enzymes [55]. It is an inverted bell-shaped body mounted upon branched non-contractile stalk. The peristome has a definite lip encircling the oral region and the peristomial ciliary rows wind only once around the peristome. The resulting biofouling and tissue harm usually leads to osmoregulatory stress and subsequent infiltration by opportunistic bacteria and water molds [54]. *Epistylis* sp. transmission occurs through the process of binary fission, where this organism reproduces. It is horizontally transmitted among fish through the transformation of the zooid (bell-shaped body) into a disc-shaped ciliated telotroch.

Given that three samples were collected from each fish, specifically from the skin, gills, and fins, and since all 16 fish exhibited the presence of parasites on their skin and gills, it can be concluded that the infection rate of these organs, in relation to the entire group of fish examined, is 38%. Regarding the fins, only 9 out of the 16 samples tested positive, indicating an infection rate of 21% when compared to the entire batch that was analyzed.

The observed parasites belong to four species of Protozoa (*Ichthyophthirius multifiliis, Trichodina acuta, Epistylis* sp, *Vorticella globularia*) and one species of Monogenea

(*Dactylogyrus vastator*). Parasitic and bacteriologic infestation was clearly visible on the investigated *Petroleuciscus borysthenicus celensis* (Figure 2a). *Ichthyophthirius multifiliis* caused noticeable damage to the skin and necrosis of the caudal fins as previously reported [42], (such are indicated by the red circle in Figure 2b). Additionally, it led to the removal of scales from the skin, particularly in the tail area below the lateral line (Figure 2b). *Ichthyophthirius* attaches to the skin and gill epithelium, causing mechanical damage during the feeding and attachment process, thus resulting in the formation of ulcers and lesions on the skin. Severe *Ichthyophthirius* infections can lead to fish mortality, especially if the parasites are not treated promptly [32]. These parasites are characterized by a characteristic horseshoe shaped nucleus, as they frequently appear in a squash preparation or wet smear, generally about 10 µm wide and 30–40µm long [56].

Similarly, *Trichodina acuta* causes damage to the skin and necrosis of the anal fins [33, 57]. This parasite attaches to the skin and gill epithelium using structures like cilia and adhesive disks, and stimulates an increased production of mucus by the fish as a response to the irritation [58]. *Trichodina acuta* body shape varies from cylindrical to discoidal [45], the aboral disc is typically suctorial with hook-like elements possessing, both centrifugal processes and centripetal rays.

The monogenea *Dactylogyrus vastator* infected the gills and skin, possibly also creating entry points for secondary infections by bacteria, that further compromise the health of the fish [59]. *Dactylogyrus vastator* can cause damage to the skin of fish as they attach themselves using specialized structures like hooks and suckers [60], and also often target the gills of fish, where they attach and feed on host tissues, thus resulting in damage to the delicate gill filaments [61]. The characteristics of *Dactylogyrus* sp. include the appearance of four eye-spots, 14 marginal hooks (7 pairs), one to two connective bars and two needle-like structures and spindle-shaped *Dactylogyrus vastator* -type seminal vesicles [62]. *Dactylogyru*s *vastator* has an adult structure that is up to 2mm in length [63].

The infestation with the ciliated protozoan *Epistylis* sp. on the body (indicated by the black arrow in Figure 2c) is generally considered opportunistic and often not lethal on their own, although severe infestations, or those in combination with other stressors, can contribute to fish mortality [55]. *Vorticella* sp. was also present, specifically on the pelvic fins (indicated by the red arrow in Figure 2d), the parasite is characterized by having a retractile stalk and ribbon-shaped macronucleus. *Epistylis* and *Vorticella* these organisms primarily live independently, however, when fish experience unfavorable environmental conditions, they can adapt to become an ectoparasite [64]. Following staining, the mentioned species have been microscopically investigated (Figure 3 A–J).

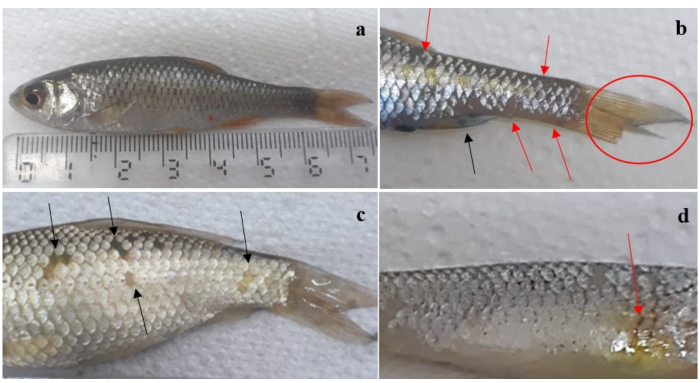

Fig. 2. a) *Petroleuciscus borysthenicus celensis* specimen; b) Signs of parasites on the fish body (the red circle shows the extent of erosion of the caudal fin, the black arrow shows the effect on the pelvic fins, the red arrows show the removal of scales from the skin); c, d) Appeareance of *Epistylis* sp on the anatomical region of the body (black arrows) and that of *Vorticella* sp on the pelvic fin (red arrow).

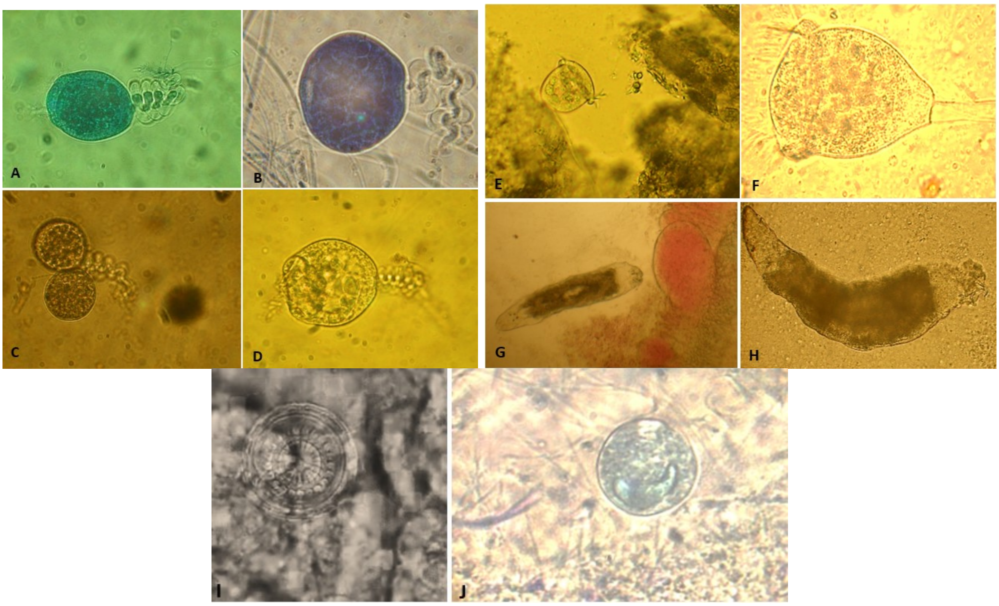

Fig. 3. *Vorticella globularia.* isolated from skin: (A and B stained Giemsa; C and D stained with sliver nitrate). *Epistylis sp.* isolated from the skin (E and F, stained with sliver nitrate). *Dactylogyrus vastator s.* isolated from *s*kin *(G)* and from gills *(H), Trichodina acuta.* isolated from the skin (I, stained with sliver nitrate) and *Ichthyophthirius multifiliis* isolated from the skin (J, stained with Giemsa)

Figure 4 presents the classification based on the total amount of injuries found on the body of *P. borysthenicus celensis,* ranging from the most damaging parasite (*Ichthyophthirius multifiliis*) to the least harmful parasite (*Dactylogyrus vastator*). Furthermore, it has been established that *Ichthyophthirius multifiliis* is the most damaging parasite among the five taxa analyzed, causing the greatest number of injuries across various infection sites, followed by *Trichodina acuta*, *Vorticella globularia* and *Epistylis*. On the other hand, the group *Dactylogyrus vastator* is found to be the least harmful.

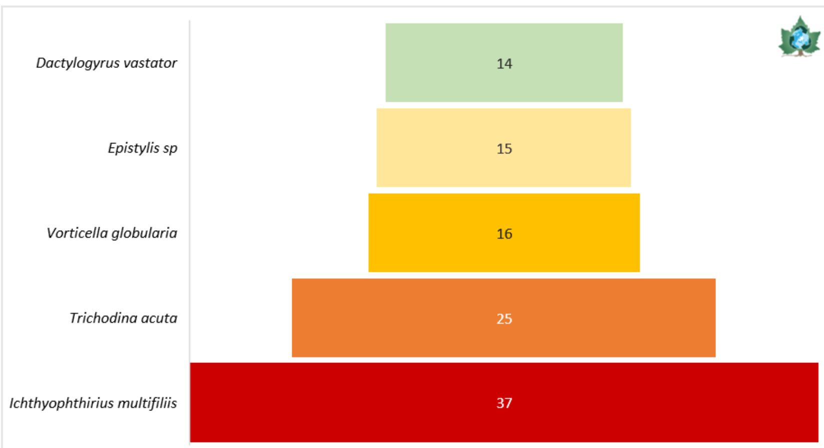

Fig. 4. Classification of parasite taxa based on the number of injuries identified on *Petroleuciscus borysthenicus celensis*

Figure 5 illustrates the specific predilection of each of the five discovered parasites for particular infection sites on the fish's body (skin, fin and gills). The skin is the organ most susceptible to parasitic infestation, followed by the gills. The results also show that the fin is the area least vulnerable to parasitic infestation.

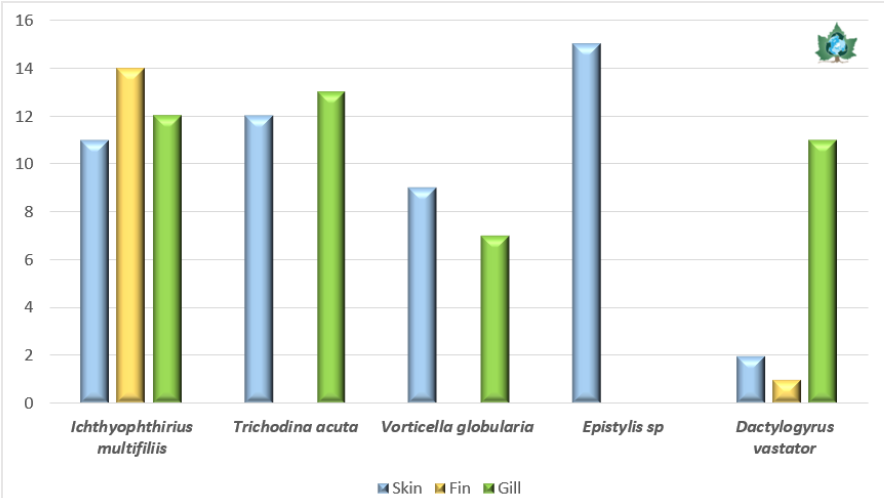

Fig. 5. Specific predillection of each parasite species for particular infection sites on the fish's body

It is important to consider the parasites' preference for infection sites, as follows: *Ichthyophthirius multifiliis* mostly affects the fin and to a lesser extent the skin; *Trichodina acuta* on the other hand, prefer the gills and were not found on the fins; Vorticella *globularia*. likewise has its own specific location of infestation, the skin, while no signs of this parasite were observed on the fins; *Epistylis* sp only affected the skin, while *Dactylogyrus vastator* predominantly attacked the gills and fins.

Pathogenic Bacteria

On Columbia agar supplemented with 5% sheep blood, a large number of white-greyish, small and smooth colonies grew (Table 4), identified as *Aeromonas veronii* (Hickman-Brenner et al., 1987*)*, followed by a modest growth on Nutrient agar of *Shewanella putrefaciens* (Lee et al. 1981), two colonies of *Aeromona hydrophila* (Chester, 1901) and one colony of *Citrobacter freundii* (Braak 1928).

Table 4. Pathogens identified per specimen, (n = 3)

Speciment number	Species identified	CFU/mL
Specimen 1	*Aeromonas veronii*	2300
	Shewanella utrefaciens	200
	Citrobacter freundii	50
Specimen 2	*Aeromonas veronii*	10000
	Shewanella utrefaciens	150
	Citrobacter freundii	50
Specimen 3	*Aeromonas veronii*	10050
	Aeromona hydrophila	100

* CFU = colony forming units. CFU/mL presented as averaged and calculated as 20μL, 50×.

Taking into account the bacterial load, *Aeromonas veronii* prevails in all three specimens studied, thus it can be identified in all the 16 specimens with a probability of 81%, *Shewanella* *putrefaciens* and *Citrobacter freundii* having a probability of infestation of 54% and *Aeromona hydrophila* with a probability of 27%.

The identified pathogens can be considered common, however there is a large discrepancy between the bacterial load of *A. veronii* in regard to the other species detected. For instance, *Shewanella putrefaciens* is known as an important spoilage bacterium of seafood and infects several aquatic animals such as the European sea bass [65]. *Citrobacter freundii* stands out as a significant opportunistic pathogen associated with nosocomial infections, leading to sepsis and inducing diverse and histopathological lesions in various internal organs of both humans and animals (this impact is particularly notable in dogs and fish [66, 67]). *Aeromonas hydrophila* is a motile, Gram-negative, rod-shaped bacterium with a preference for warm climates. This heterotrophic organism is commonly present in fresh or brackish water and demonstrates adaptability to both aerobic and anaerobic environments. Generally regarded as a secondary invader [68, 69] (it depends on the existence of primary infection to infect), upon entering a host's body, it travels through the bloodstream to reach the first available organ. *Aeromonas hydrophila, just like Aeromonas caviae and Aeromonas veronii* biotype sobria, can cause gastroenterites in humans [70] and motile *Aeromonas* septicaemia in fish [71, 72] characterized by reddened fins, inflammation of the anus, diffuse hemorrhages on the skin, exophthalmia and abdominal swelling [72].

First described by *F.W. Hickman-Brenner et al.* [73], *A. veronii* manifests through ulcers, fin and/or tail rot, abdominal swelling, exophthalmia, and hemorrhaging [74, 75]. These symptoms vary among infected fish, with distinct pathogenicity observed based on specific bacterial isolates or strains [76]. In recent years there has been a rising incidence of extensive outbreaks caused by *A. veronii* [77, 78] in fish, as well as in humans [79]. *A. veronii* strains have been increasingly isolated from diseased fish and have also been linked with the immune-related gene expression [80]. Due to its characteristic trait of forming ulcerations, it is safe to assume that either *A. veronii* opens a gateway for parasitic infestation or enhances the parasitic damage.

Furthermore, it is important to acknowledge the fact that climate change can induce modifications [81-85] in fish pathogenic bacterial communities and their distribution. For instance, a 2018 governmental report recorded *A. veronii* infecting fish in the UK for the first time [86], its presence being attributed to the high ambient water temperatures experienced during the spring.

Conclusions

The results of this study demonstrate the influence of parasites on the overall health of the *Petroleuciscus borysthenicus celensis* community. Furthermore, the infestation of parasites such as *Ichthyophthirius multifiliis*, *Trichodina acuta*, *Vorticella globularia*, *Dactylogyrus vastator* and *Epistylis* sp. creates favourable conditions for the proliferation of secondary infections caused by different species of bacteria. These bacteria primarily exploit the ulcerations caused by parasites as entry points or vice versa, further studies are required to establish the relationship between them. The identification of the two parasitic species *Vorticella globularia*. and *Epistylis* sp. in *Petroleuciscus borysthenicus celensis* is hereby reported for the first time and can be attributed to the presence of anthropogenic impact such as livestock/aquaculture farms upstream of the studied area, as well as the influence of climate change, however, additional investigations are needed to confirm this hypothesis.

The findings indicate that the overall parasite infection rate for the entire set of specimens investigated is 38%. This parasite infection rate is consistent for both the skin and gills, with all samples testing positive and for the fins is lower at 56.25%, since only 9 out of the 16 samples tested positive.

Based on the results of this study, it can be established that *Ichthyophthirius multifiliis* is the most damaging parasite among the five taxa analyzed, causing the greatest number of injuries across various infection sites. On the other hand, *Dactylogyrus vastator* is found to be the least harmful.

It is important to consider the parasites' preference for infection sites, as it follows: *Ichthyophthirius multifiliis* mostly affects the fin and to a lesser extent the skin; *Trichodina acuta*, on the other hand, prefer the gills and were not found on the fins; *Vorticella globularia*. likewise has its own specific location of infestation, the skin, while no signs of this parasite were observed on the fins; *Epistylis* sp. only affected the skin, while *Dactylogyrus vastator* predominantly attacked the gills and fins.

In addition to this, the bacteriological examination reveals the presence of four bacterial strains, as follows: *Aeromonas veronii, Shewanella putrefaciens, Aeromona hydrophila*, and *Citrobacter freundii*. *Aeromonas veronii* occured in all investigated specimens, with an infestation probability of 81%, *Shewanella putrefaciens* and *Citrobacter freundii* having a probability of infestation of 54% and *Aeromona hydrophila* with a probability of 27%.

It is essential to have a thorough comprehension of the life cycle of each parasite and the specific susceptibilities of different fish species, as evidenced by this research and many others. This is because parasites susceptibility may vary among species, as bacteria and parasites are opportunist, infecting tissue that are already affected. Given that *Aeromonas veronii* exhibited the highest number of colony-forming units per mL, it can be deduced that there could be a link between this species and parasitic infestation. However, further investigations are necessary to clarify such connection in order to gain a better understanding of the extent of the interconnections that exist between bacteria and parasites, in the context of climate change which causes modifications to occur in the communities of ichthyofauna and pathogens, as well as how this consequently impacts the aquatic ecosystem which already exposed to anthropogenic pollution.

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