Assessment of endangered freshwater pearl mussel populations in the Northern Iberian Plateau in relation to non–native species: xenodiversity as a threat

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Abstract

Assessment of endangered freshwater pearl mussel populations in the Northern Iberian Plateau in relation to non–native species: xenodiversity as a threat. In the last two decades, small populations of freshwater pearl mussels, *Margaritifera margaritifera,* have been recorded in Mediterranean rivers of the Iberian Northern Plateau. A survey was carried out in Castilla and León in 2018 to assess the development of populations of this species in all the rivers of known distribution and to update the threat classification. Thirty sections in the rivers Negro, Tera, Alberche and Águeda were positive for its presence, and another 50 stretches of seven rivers were negative. The species is currently distributed over about 22.5 km. Águeda and Tera populations have decreased dramatically in the last 14 years and are on the threshold of extinction. The Negro river supports the largest population, although the species has now disappeared in at least 61 % of the stretches that were inhabited in 2004. All populations showed very low densities and an ageing population structure, with no recruitment for decades. The presence of non-native invasive alien species (NIS) was higher than in a previous regional survey, with the signal crayfish representing the greatest threat. We observed changes in benthic microhabitats and direct predation of adults and glochidia conglutinates. In the Alberche River, in strict syntopy with *M. margaritifera* and two other mussel species, 10 NIS were detected. The current hydrological and ecological conditions in the Duero watershed support the settlement of exotic species to the disadvantage of native mollusks, which are more demanding in terms of microhabitats.

Key words: Freshwater invasion, Pearl mussels, Survey, Declining population, Signal crayfish, Duero watershed

Resumen

Evaluación de las poblaciones de náyades en peligro de extinción en la meseta norte de la península ibérica en relación con las especies alóctonas: la xenodiversidad como amenaza. Hace dos décadas que se conocen pequeñas poblaciones de *Margaritifera margaritifera* en ríos mediterráneos de la meseta norte de la península ibérica. En 2018 se realizó un muestreo en Castilla y León para conocer el estado de las poblaciones en todos los ríos en los que se sabe que se encuentra esta especie y actualizar el inventario de amenazas. Resultaron positivos 30 tramos de los ríos Negro, Tera, Alberche y Águeda, mientras que otros 50 tramos de siete ríos fueron negativos. La especie se distribuye actualmente a lo largo de unos 22,5 km. Las poblaciones del Águeda y el Tera se han reducido de forma drástica en los últimos 14 años y están al borde de la extinción. El río Negro alberga la población más numerosa, aunque ha perdido al menos el 61 % de los tramos ocupados en 2004. Todas las poblaciones presentaron una densidad muy baja y la estructura poblacional envejecida por la ausencia de reclutamiento desde hace décadas. La presencia de especies exóticas invasoras fue más elevada que en el anterior muestreo regional. El cangrejo señal resultó ser la especie exótica invasora más extendida y peligrosa para las náyades. Se observaron cambios en los microhábitats bentónicos y predación directa de adultos y conglutinados de gloquidios. En el río Alberche se detectaron 10 especies exóticas invasoras en sintopía con *M. margaritifera* y otras dos especies de náyades. Las actuales condiciones hidrológicas y ecológicas en la cuenca del Duero favorecen el asentamiento de especies exóticas, que son menos exigentes con los microhábitats que los moluscos nativos.

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Introduction

Freshwater pearl mussels (*Margaritifera margaritifera* Linnaeus, 1758) are a target species for the conservation of oligotrophic stream ecosystems throughout Europe (Geist, 2010). The species has been reported in the Spanish Duero River basin for two decades (Morales et al., 2004). During this period, while pearl mussels have been in decline, numerous aquatic species exotic to the Iberian Peninsula have become established in the same stretches of mountain rivers. According to Spanish legislation (Executive order 630/2013), many are considered invasive non–native species (NIS), and are included in the European Union strategy to combat NIS. Two exotic American crayfish (*Pacifastacus leniusculus* [Dana, 1852] and *Procambarus clarkii* [Girard, 1852]) are taxa of special concern (EU 1143/2014 and EU 1141/2016 Regulations) as reported by Vaeben and Hollert (2015). In the Iberian Peninsula *M. margaritifera* (Mm) has not been in contact with crayfish species and thus has no self–protective measures, either active or passive. In a review of 124 publications, Downing et al. (2010) showed that the presence of exotic species is the fourth most relevant cause of the decline of fresh water mussels.

These NIS have a negative impact on the conservation of this mussel, producing transformations in physical, chemical and/or ecological benthic microhabitats, typically through trophic networks (Dorn and Wojdak, 2004; Beisel and Lévêque, 2010) and interactions between species and synergy between pressures (fig. 1s in supplementary material). This negative effect is felt not only by native fauna communities through competition for food or substrate refuge, predation and territorial exclusion due to modifications in the food chain, but also pearl mussels, species with restricted mobility, and young brown trout (*Salmo trutta fario*) acting as hosts for glochidia larvae (Stenroth and Nyström, 2003). Some mollusks, such as the Asian clam *Corbicula fluminea* (O. F. Müller, 1774) or the mud snail *Potamopyrgus antipodarum* (Smith, 1889), can alter the oxygenated sand and gravel banks that juvenile mussels need for their growth, creating compacted and anoxic sediments (Sousa et al., 2008a, 2008b; Ferreira–Rodríguez et al., 2018). In addition, hypoxia increases their vulnerability to predators (Saloom and Duncan, 2005). This reduces the viability of mussels because of their high filtration rate, and effects them negatively due to the ammonium produced and excreted (Modesto et al., 2019). Furthermore, in sediments that are massively colonized by these exotic mollusks, the microbial communities –which control the biogeochemical cycling of nitrogen– undergo changes (Black et al., 2013), dominating communities as engineers (Sousa et al., 2009) by imposing their conditions on microhabitats, and reducing the recruitment of juvenile mussels that need highly specific epibenthic physicochemical conditions (Geist and Auerswald, 2007).

In the rivers studied, we found two exotic freshwater crayfish (NICs), the red swamp crayfish *P. clarkii*, and the signal crayfish *P. leniusculus*, both of which have become widely dispersed within the Spanish hydrographic network since the 1960s. Both produce cumulative effects in terms of the physical alteration of the riverbed (by excavation of galleries, removal of sediments or herbivory on submerged plants) and ecological changes in underwater communities (Crawford et al., 2006).

Vaeben and Hollert (2015) showed that invasive crayfish in European rivers not only decimate populations of other benthic invertebrates, but also submerged plants, and epilithon, which are primary resources for trophic networks. They also showed how these NICs displace fish from their shelters and even prey on juveniles (Stenroth and Nyström, 2003; Peay et al., 2009; Gladman et al., 2012). In this way, they reduce the viability of host populations and cause the medium– and long–term decline of pearl mussels by changing the structure of the community and trophic networks (Vaughn et al., 2008; Inoue et al., 2017). They also prey on mussels (Machida and Akiyama, 2013; Meira et al., 2019; Dobler and Geist, 2022) and on the other benthic macroinvertebrate communities (Stenroth and Nyström, 2003; Ercoli et al., 2015), destabilizing trophic networks at various levels (Dorn and Wojdak, 2004). The signal crayfish can occur in high densities and its greater activity than that of native crayfish can further exacerbate problems for native species (Wutz and Geist, 2013).

In all cases, its presence is associated with increased turbidity and the formation of cyanobacterial blooms (Yamamoto, 2010; Turley et al., 2017) that are detrimental to epibenthic filter–feeding communities. In addition, Liräs et al. (1998) observed how crayfish accumulate cyanotoxins during toxic algae blooms (HAB) (Vasconcelos et al., 2001), moving them further up the food chain. Given these effects, signal crayfish are considered one of the most dangerous invaders in European waters (Gherardi et al., 2011) for several species, including freshwater mussels, as evidenced by Meira et al. (2019) in other Iberian rivers.

The aim of this study was to update the distribution of *M. margaritifera* in Mediterranean rivers of the Iberian Northern Plateau, locate populations of other mollusks in syntopy, and identify conservation problems related to the proliferation of aquatic exotic species, including the abundance of the signal crayfish in the Negro River. We also sought to identify the basic descriptors of the population of this endangered species.

Material and methods

Study area

In summer 2018 we sampled eight rivers belonging to the Duero, Tajo and Miño–Sil watersheds in the northern plateau of the Iberian Peninsula. The sampling areas, approximately 80 km in length, were selected for survey within 15 UTM 10x10 km squares (cUTM) and are included in the management plan for species in the Autonomous Community of Castilla and León (Spain) in rivers of the Bibey, Tuela, Castro, Tera, Negro, Águeda, Duero and Alberche sub–basins (Morales et al., 2007ab), all of which are included in the European Natura2000 network. In complementary terms, in 2015–2017, surveys were carried out to search for the possible presence of shells on the banks of seven other nearby rivers: rivers Tormes, Eria, Órbigo, Cea, Esla, Aliste and Manzanas. All these rivers have Mediterranean pluvial hydrological dynamics during cool summers (continental with intense low water levels and hot summers, Csb, Köppen and Geiger classification). In addition, these rivers have a certain Atlantic influence, with high amounts of autumn and spring precipitation but low winter snowfall. Precipitation in the valley areas ranges between 800 and 1,000 mm/y, and up to 1,500 mm/y in the headwaters (Morales and Lizana, 2014). The riverbeds are affected by high–summer low water levels and high–winter floods. All these watercourses have a perennial flow, with the exception of the River Águeda in August and September (Morales, 2020).

All the rivers studied have acidic and cold waters, with low–mineralization. They are oligotrophic in nature (Morales et al., 2004) and have a typical pool–riffle sequence to lotic hydrological conditions. The dominant substrate is composed of blocks, coarse gravel, and sand, except for silt areas with of lenitic conditions (caused by old traditional watermills and dams for livestock). The riparian vegetation is dominated by alluvial forests containing alder (*Alnus glutinosa*) and ash (*Fraxinus excelsior* and *F. angustifolia*) (corresponding to 91E0(*) N2000 code habitat: *Alno–Padion*, *Alnion incanae*, *Salicion albae*), poplar (*Populus* spp.) and willow (*Salix* spp.) trees. All populations are considered within the Natura2000 Special Areas of Conservation, with rivers that also hold other endangered species such as *Macromia splendens, Oxygastra curtisii, Gomphus graslinii, Galemys pyrenaicus, Rana iberica* and *Cobitis calderoni*. Other conservation values in midstream watercourses are present in two habitats of interest: 3250/constantly flowing Mediterranean river with *Glaucium flavum* and 3260/floating vegetation of *Ranunculus* of plain, submountainous rivers.

Survey methods

The surveys were carried out during the summer–autumn season during the seasonal drought and when the rivers were at a low water phase. First, we searched for shells on the gravel banks of the riverbanks and the river channel. We then searched for adults in the wadeable areas using an opaque–walled bathyscope. In the 2018 survey, in each 10 x 10 km UTM grid (cUTM onwards), we sampled at least 1 km of watercourse in five stretches of varying length, following the recommendations of Leppänen (2018) for rivers with a low abundance of mollusks and the basic principles presented in the European CEN standard on monitoring of the fresh water pearl mussel (Boon et al., 2019). The riverbeds, in the wadeable areas, were thoroughly inspected (250–300 m in length) when the water level was at its lowest. We covered a total of 21.9 km in 81 georeferenced sections, using a sampling method that involved counting 50 m on the shoreline in 48 wadeable plots: totaling 17,200 m^2. Mussel density is expressed in specimens per 100 m^2. In the 2015–2017 shell survey, various distances (90–650 m) were covered in 37 transects of varying length on 29 cUTM in locations with environmental conditions for this species. The sampling locations and the minimum sampling area required were determined using the criteria established by experts.

We verified the presence of Mm juveniles in all stretches by sieving the sands and gravels inhabited by adults in sections using standard stainless steel sieves (1.0, 0.5, and 0.1–mm pore and 25 cm in diameter). In addition, when carrying out the underwater counts, we also quantified the presence of trout fry and other fish species, and signal crayfish. The abundance of crayfish (Pl) and brown trout (St) is expressed per sampled plot.

In the Negro River, we used traps baited with decomposing fish to determine the abundance of signal crayfish, and we placed groups of four closed pots (40 cm diameter) 50 m from the banks for 48 h in areas occupied by pearl mussels. All amounts captured are expressed as individuals per hour and trap (Pl/h–t). The mussel shells and crayfishes were measured 'in situ' using calipers (total length with 0.1 mm precision) and collected and stored in the laboratory for inspection for crayfish claw marks. Biometric data were grouped into intervals (using Sturges' rule) to construct size pyramids, and the comparison between sets was carried out using Welch's bilateral *F*–test for unequal variances.

Fig. 1. Location of the UTM grid including the range distribution of *M. margaritifera* in the Iberian Peninsula, study area and the distribution of *P. leniusculus* in the Castilla and León (CyL) (according to Spanish Biodiversity Database: MITECO, 2019). Hydrographical network are shown: RAG, River Águeda; RNE, River Negro; RTE, River Tera; RCA, River Castro; RDU, River Duero; RTU, River Tuela; RAL, River Alberche; RBI, River Bibey.

Fig. 1. Localización de la cuadrícula UTM que incluye el rango de distribución de M. margaritifera *en la península ibérica, la zona de estudio y la distribución de* P. leniusculus *en Castilla y León (CyL) (según la Base de Datos de la Biodiversidad de España: MITECO, 2019). Se muestra la red hidrográfica: RAG, río Águeda; RNE, río Negro; RTE, río Tera; RCA, río Castro; RDU, río Duero; RTU, río Tuela; RAL, río Alberche; RBI, río Bibey.*

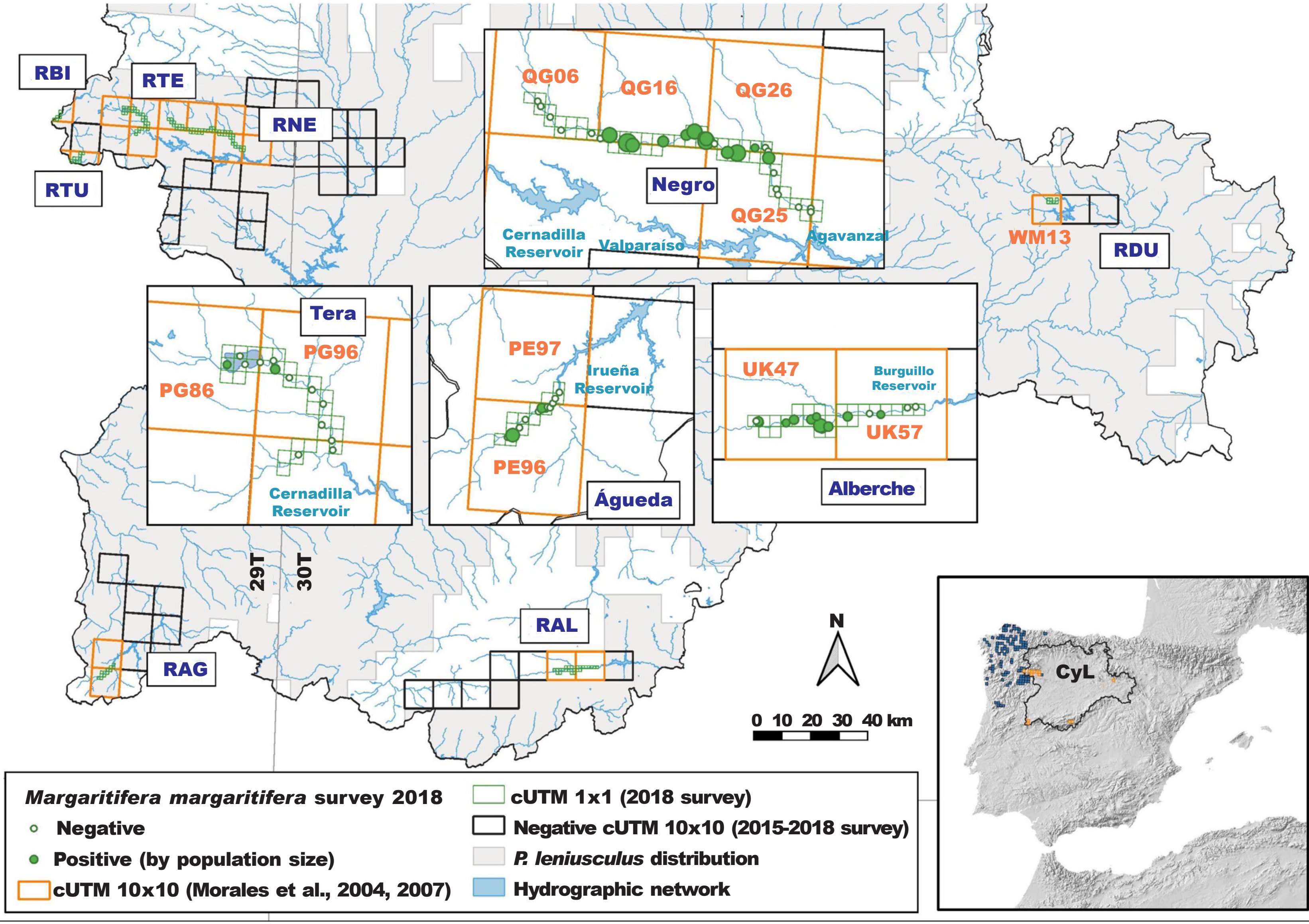
RBI
RTE
RNE
RTU
QG06
QG16
QG26
QG25
Negro
Cernadilla Reservoir
Valparaíso
Agavanzal
WM13
RDU
Tera
PG96
PG86
Cernadilla Reservoir
PE97
Irueña Reservoir
PE96
Águeda
UK47
Burguillo Reservoir
UK57
Alberche
29T
30T
RAL
RAG
N
0 10 20 30 40 km
CyL
Margaritifera margaritifera survey 2018
Negative
Positive (by population size)
cUTM 10x10 (Morales et al., 2004, 2007)
cUTM 1x1 (2018 survey)
Negative cUTM 10x10 (2015-2018 survey)
P. leniusculus distribution
Hydrographic network

Table 1. Results of the *M. margaritifera* survey and population status changes in the last fourteen years (2004–2018) in eight rivers of Castilla and León (for abbreviations of revers, see fig. 1): * the distribution of the species in this river is not natural, since specimens were rescued and transferred to Irueña reservoir and grouped in seven localities (Morales and Lizana, 2012); ** mussel (mu) population status according to Swedish standards (Degerman et al., 2009): 1, viable (> 20% < 5 cm and > 0% < 2 cm, > 500 mu); 2, viable? (> 20% < 5 cm or >10% < 5 cm and > 0% < 2 cm, > 500 mu); 3, non–viable (20% < 5 cm or > 20% < 5 cm and < 500 mu); 4, dying out (all > 5 cm, many individuals, > 500 mu); 5, almost extinct (all > 5 cm, few individuals, < 500 mu); 6, extinct (documented presence that has disappeared); relative abundance of freshwater mollusk species (++ abundant; + presence; – absence; = unchanged).

Tabla 1. Resultados del último muestreo de M. margaritifera *y los cambios demográficos en los últimos 14 años (2004–2018) en ocho ríos de Castilla y León (para las abreviaciones de los ríos, véase la fig. 1): * la distribución de la especie en este río no es natural, ya que los ejemplares fueron rescatados del embalse de Irueña y agrupados en siete localidades (Morales y Lizana, 2012); ** estatus de población de náyades (mu) según los tramos estandarizados en Suecia (Degerman* et al.*, 2009): 1, viable (> 20% < 5 cm ó >10% < 5 cm y > 0% < 2 cm, > 500 mu); 2, ¿viable?* (> 20% < 5 cm or > 10% < 5 cm and > 0% < 2 cm, > 500 mu); *3, no viable (< 20% < 5 cm ó > 20% < 5 cm, < 500 mu), 4, agonizante (todos > 5 cm, bastantes individuos, > 500 mu); 5, al borde de la extinción (todos > 5 cm, pocos ejemplares, < 500 mu); 6, extinta (presencia documentada de que ha desaparecido); abundancia relativa de especies de moluscos de agua dulce (++ abundante; + presencia; – ausencia; = sin cambios).*

	RAG	RCA	RNE	RTE	RTU	RDU	RAL	RBI
Stretches								
Number	12	1	28	7	5	5	15	4
Distance (km)	2.14	0.26	6.83	3.03	1.91	0.42	3.77	1.03
Positive survey								
Frequency (%)	25 (*)	0	46	29	0	0	73	0
Density in plots (mu/100 m^2)								
Median	1.60 (*)	0	5.04	1.43	0	0	0.66	0
Maximum	7.41 (*)	0	20.65	2.45	0	0	1.87	0
Population changes 2004–2018								
Estimated population decrease (%)	>95		40–54	81	0	100	=	
Estimated km occupied decrease (%)	93		61	90	0	100	=	
cUTM 1x1 km variation	–9	–1	–16	–5	0	–3	–1	–1
Population status (**)	5	6	4	5	–	6	5	6
Other aquatic mollusk species in 2018								
Unio delphinus	+	–	–	–	–	–	+	–
Anodonta anatina	–	–	+	–	–	–	++	–
Ancylus fluvitalis	–	+	++	++	+	–	+	+
Radix balthica	+	–	–	+	–	–	+	–

Results

Mussel surveys

We found live pearl mussels (Mm: *Margaritifera margaritifera*) in three of the 15 rivers surveyed in 2018, and confirmed their absence in two rivers where they had been known to inhabit. A total of 30/81 stretches (37.5%) along 22.5 km of the Negro, Tera, Alberche and Águeda rivers showed positive signs in the 2018 survey. This indicated that the species inhabited 29.9% of the potential area (fig. 1) initially established, but all samples in the upper Duero, Bibey, Tuela and Castro were negative (table 1). In addition, all epibenthic sediment points screening out for juvemiles (n = 30) were negative in 2018. The largest population (90.4%) was found in the Negro River (fig. 2), at an altitudinal range

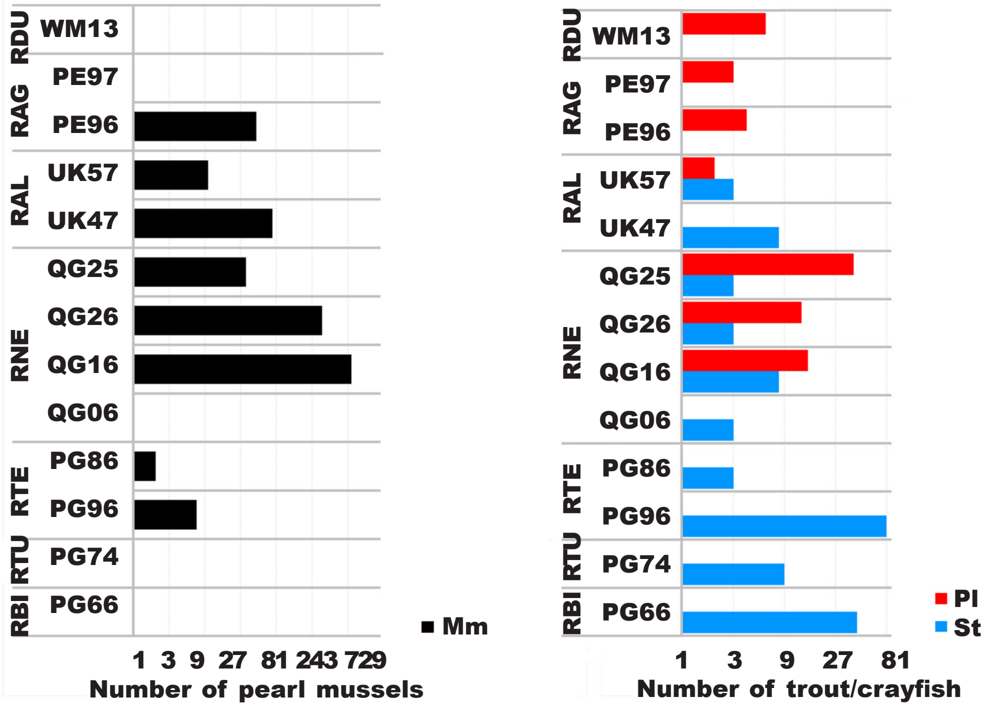

Fig. 2. Number of specimens for the studied species: A, pearl mussels (Mm); B, brown trout (St) and signal crayfish (Pl) found in the 2018 survey (cUTM's 10 x 10 km plots).

Fig. 2. Número de ejemplares de las especies estudiadas: A, náyade (Mm); B, trucha (St) y cangrejo señal (Pl) encontrados en el muestreo de 2018 (parcelas cUTM 10 x 10 km).

of 802–913 m a.s.l. and consisting of about 2,000 mussels scattered over 15 km; total 23.5 km less than in the first survey. This was the only population for which we obtained counts of more than 100 animals per section. Moreover, this same location is where we found the most mussels in clumps, with up to 13 specimens in each cluster.

In the Alberche River, we found an estimated population of about 320 mussels in small clusters, scattered along 17.5 km of the riverbed. In the other two rivers that tested positive, the Tera and the Águeda, the number of mussels recorded barely reached 50 specimens in each river (fig. 2), representing a reduction of more than 90 % in a period of 14 and nine years, respectively. The average density in plots ranged between 0.66 Mm/100 m^2 (max: 1.9) in the Alberche River and 5.04 Mm/100 m^2 in the Negro (fig. 2). In the most favorable area of the Negro River, maximum density values of 20.6 Mm/100 m^2 were detected. In 2 plots, the density exceeded 20 Mm/100 m^2, in 34 plots it was less than 10, and in 14 plots it was less than 1 Mm/100 m^2.

We found four other species of freshwater mussels (table 1) in the stretches occupied by *M. margaritifera*. *Unio delphinus* and *Anodonta anatina* were abundant, especially in the Alberche River. No specimens or shells of *Potomida littoralis* were found.

NIS Species in pearl mussel rivers

The pearl mussels were found to be in strict syntopy with 12 aquatic species exotic to Iberian fauna (table 2), and NIS were found in 55 % of the plots, with a species richness of 1–4 species per plot. The Alberche River showed the highest xenodiversity, with 10 aquatic NIS identified.

In the Negro River, we observed the presence of signal crayfish (Pl: *Pacifastacus leniusculus)* in 20 out of the 28 plots surveyed, with a maximum abundance of 13 Pl/plot. In 3 out of 5 cUTM (fig. 1) containing pearl mussel populations, there were indications of interactions with signal crayfish. In addition, in 17 shells (28.3 % of the measurements), we observed gnawed edges, and some also showed the mantel was gnawed. We also found larvae and subadults of long–legged frogs (*Rana iberica*) and partially mutilated fish fry. In the Alberche and Águeda rivers, mussels shared benthic habitats in syntopy with the red swamp crayfish (*P. clarkii*), although we observed no evidence of predation. In the initial surveys (2004–2006) we did not detect this exotic species in any of the rivers studied.

A statistically significant negative relationship was found between the abundance of mussels and signal crayfishes in relation to the counts and plot density

Table 2. Presence of the non–native aquatic species in nine rivers of Castilla and León with past and recent populations of *M. margaritifera.* The year in which the presence of the species was discovered in the area is indicated in brackets: * not in strict syntopy. (For abbreviations of rivers, see fig. 1).

Tabla 2. Presencia de especies acuáticas exóticas invasoras en nueve ríos de Castilla y León con poblaciones históricas y recientes de M. margaritifera*. El año en que se descubrió la presencia de la especie en la zona se indica entre paréntesis: * no en estricta sintopía. (Para las abreviaturas de los ríos, véase la fig. 1).*

	Watersheds								
	Duero							Tajo	Miño–Sil
Rivers	RAG (2002)	RNE (2001)	RTE (2000)	RCA (2000)	Lake Sanabria–RTE (2014)	RDU (2012)	RTU (2000)	RAL (2006)	RBI (2007)
Natura 2000 ID code	ES4150032		ES4190067		ES4190105	ES4170083	ES4190131	ES4110078	ES1130007
Altitudinal range (m.a.s.l.)	796–772	965–789	1,000–960	925–910	1,004	1,097–1,084	791–735	835–834	1,002–993
Diatoms									
Didymosphenia geminata								•	
Ferns									
Azolla filiculoides	•							•	
Mollusks									
Corbicula fluminea		• (*)	• (*)						
Physella acuta	•		• (*)					•	
Potamopyrgus antipodarum								•	
Crustaceans									
Procambarus clarkii	•							•	
Pacifastacus leniusculus	•	•	• (*)			•		•	
Fishes									
Gambusia holbrooki			•		• (*)				
Lepomis gibbosus		•				• (*)		•	
Micropterus salmoides						• (*)			
Alburnus alburnus	• (*)					• (*)		•	
Mammals									
Neovison vison	•	•	•	•	• (*)	•	• (*)	•	•
Total xenodiversity = 12	5	4	4	1	2	5	1	10	1

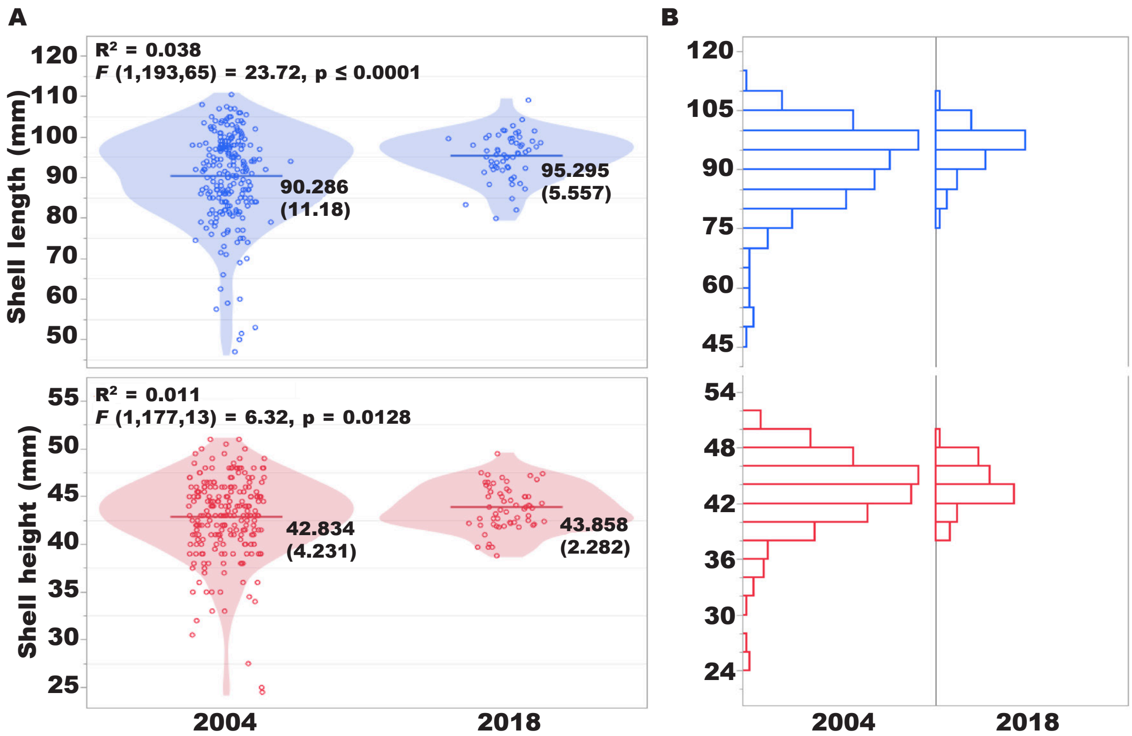

Fig. 3. Biometric analysis (A, scatter and violin plot; B, histogram) of shell–size in the two surveys in Negro River separated by a 14–year period (average and standard deviation indicated in brackets).

Fig. 3. Análisis biométrico (A, diagrama de dispersión y violín; B, histograma) de tamaños de la concha en los dos muestreos del río Negro, realizados con 14 años de diferencia (media y desviación estándar indicadas entre paréntesis).

(Pl = 1.1883 exp [0.7263 x Mm]; r = 0.335, p = 0.02 / Pl = 1.7638 exp [0.0021 x Mm]; r = 0.296, p = 0.007 respectively), but not with the abundance of brown trout (St: *Salmo trutta* var *fario*) during the pearl mussel spawning season (St = 2.3012 exp [–0.021 x Mm]; r = 0.1364, p = 0.37].

Signal crayfish in the Negro River

Adult signal crayfish were caught in 5 out of 6 trapping sessions, over a course of 89 hours of surveying, with results of between three and 48 crayfish per session: the signal crayfish (n = 98) abundance fluctuated between 1.021 and 0.047 Pl/h–t. Some differences were found between the abundance of crayfish in the lower reaches of the river and upstream. The average size of females was 5 mm larger than that of males, which were less abundant in all stretches surveyed. The sex ratio was close to 1 male for every 2 females, with a maximum of 4:9. During crayfish trapping, 156 cyprinids and 0 trout were captured passively.

Mussel populations trend

In 2018, the population sizes found were smaller in all cases, with reductions of more than 80% in the Tera and Águeda rivers (table 1), and less in the other rivers. This reduction equated to a loss of 71.7 % of the area occupied 14 years earlier, and currently only four rivers remain inhabited. The largest population was again found in the Negro River, and in 2018 a cluster of 617 pearl mussels, a higher count than in the previous survey, was located, despite an estimated 40 % reduction in the population and with an 8–km stretch of the riverbed being less inhabited.

Figure 3 compares the change in size (TL) of mussels found in the riverbed during both surveys of the Negro River that had died due to natural causes. The sizes considered medium were larger in 2018 (TL_{Me2004} = 92.0 vs TL_{Me2018} = 95.5 mm) and were mainly in the range of 90–105 mm (83,3 % in 2018 and 55,9 % in 2004). Comparing the size pyramids, we observed a statistically significant shift of 5 cm in the modal length frequencies, which could correspond to demographic changes over the 14–year period (F = 23.7, p < 0.001 for length (TL) and F = 6.3, p = 0.01 for shell height). The decades–long absence of juvenile recruitment –already detected in 2004– was confirmed, as was a reduction in the presence animals with an LT < 85 mm (table 3). This indicates a significant reduction in the number of younger animals and an increase of 27.4 % in older individuals.

Table 3. Size distribution (by ranks) of *M. margaritifera* in the 2004 (229 shells) and 2018 (60 shells) in Negro River (RNE) surveys: TL, interval shell lenght (in mm); n, sample size; p, number positive plots.

Tabla 3. Distribución de la talla (por rangos) de las conchas de M. margaritifera *en los muestreos del río Negro (RNE) de 2004 (229 conchas) y en 2018 (60 conchas): TL, longitud del caparazón; n, tamaño de muestra; p, número de parcelas positivas.*

	RNE	
	2004 survey	2018 survey
TL (mm)	(p = 67)	(p = 28)
< 70	4.34% (n = 10)	0.0%
70–80	9.61% (n = 22)	1.67% (n = 1)
80–90	30.13% (n = 69)	15.00% (n = 9)
90–100	39.30% (n = 90)	66.67% (n = 40)
100–110	16.59% (n = 38)	16.67% (n = 10)

Discussion

Here we studied populations of *M. margaritifera* in the Northern Iberian Plateau. Small, non–functional groups of spatially isolated metapopulations had been discovered in these rivers over the last two decades (see review at Araujo et al., 2009; Lopes–Lima et al., 2017) but the numbers of this species have decreased, the distribution area has shrunk, and there is a total absence of recruitment (Morales et al., 2004). This species is typical of very shallow waters in turbulent rivers with very clear water and as the animals do not bury themselves in the summer they can be studied without using snorkeling or diving techniques, as is recommended for species that live in deeper waters (Degerman et al., 2009; Boon et al., 2019). In the Iberian Peninsula, these populations are the most threatened since according to the classification of the population status (table 1) in Sweden (Degerman et al., 2009), they are below the minimum viable population size, contrasting with Iberian populations in Galicia or Portugal (Lois et al., 2014; Sousa et al., 2015) and others in central Europe (Geist and Auerswald, 2007; Stoeckle et al., 2020).

In the Alberche River, the presence of aquatic NIS is higher than in the other rivers in the study, but there are better conditions in the sediment and more abundant water flow in summer since more trout and fewer animals were found here than in the Negro River. Of particular concern is the presence of signal crayfish in the stretches observed in this study; they were found to live in strict syntopy with mussel species and are an increasing pressure (Morales et al., 2007ab). Added pressures include the lack of summer flow, warmer waters, and the dumping of untreated urban organic discharge into rivers and runoff that occurs during events linked to extreme climatic events (Diez et al., 2012; Morales and Lizana, 2014). NIS are greatly associated with the negative impact that influences the conservation of *M. margaritifera*, and act negatively on host species, water quality and alder conservation (more details in fig. 1s in supplementary material).

The conservation problems and pressures on the habitat responsible for this decline are not only local in nature within each stretch but also exist on a larger (sub–basin, regional, etc.) and more global scale, with multiple synergies and both spatial and temporal dimensions (fig. 1s in supplementary material). The problems derive mainly from a reduction in the quality of epibenthic microhabitats due to lack of water flow (reduction of precipitation, especially snow), the predominance of waters that are warmer than what is normally expected in trout spawning areas, and the extremely low water levels during extreme periodic droughts (Morales and Lizana, 2014; Garrido Nogueira et al., 2019) (more details are given in figures 1s and 2s in supplementary material).

Local anthropic pressures and climatic events are shared by all populations and affect the physicochemical conditions of the water through nutrient enrichment and warming, scarce longitudinal connectivity due to the succession of dams, and the frequent clouding produced by summer storms, which fall with great erosive power on areas without forest cover or on burned areas (Morales et al., 2007ab; more details in fig. 1s in supplementary material). As a result, sinkholes are lost due to siltation of gravels,clays and silts (Morales et al., 2004; Morales and Lizana, 2014), thereby increasing glochidia mortality (Ziuganov et al., 1994). Given the precarious demographic situation of this species, at or below the minimum viable level in all populations (table 1), each individual is important. During the breeding season, pearl mussels expel a mass of whitish–colored, dense–looking mucilage, the conglutinate. This mass attracts the fry that will then be infected by the glochidia when they are handled at the time of trying to eat them. We have found that crayfish consume the embryos and remove the adults by extracting them from the sandy substrate. While the adults are in the horizontal position they are vulnerable to the current dragging them along and are unable to find a vertical position in the riverbed. The resulting effect is unknown as they may eventually be flushed out during floods (Morales and Lizana, 2014).

The most negative effect on these almost sessile mollusks is direct predation, with crayfish being the only predator in the rivers studied, as occurs in other rivers (Meira et al., 2019; Sousa et al., 2019; Dobler and Geist, 2022). Colonization of *P. leniusculus* is positively related to winter temperature and positive connectivity of gravel beds (Nyström et al., 2006; Olsson, 2008); they also prefer this type of habit together with *M. margaritifera*. Degerman et al. (2009) report a similar scenario to the Negro River; they support the eradication of crayfish and activities to restore habitats in order to ensure the conservation of mussels.

The presence of *P. leniusculus* has been documented for no more than 10 years in the Negro and Alberche rivers, and the highest densities are found in the stretches where most mussels live. Nonetheless, during this period of expansion no control actions have been carried out to date. In both cases, the maximum trout density coincides with the absence of mussels. Vaeben and Hollert (2015) showed that the most negative effects of crayfish are related to their increasing density in the riverbed, indicating the need to initiate their immediate removal. Both *U. delphinus* and *A. anatina* have undergone an equally intense decline in the Negro River; while both species are frequent in the Alberche River, coinciding with a lower presence of crayfish in the mussel beds. In the first survey, no such NIS species were present in the stretches occupied by freshwater mussels (Morales et al., 2004, 2007b).

No conclusive relationship has been found between xenodiversity and the presence/absence or abundance of *M. margaritifera*, but there is a lower density of trout in both the mussel survey and crayfish plots, and this could be the first early symptom of a serious problem. Moorhouse and Macdonald (2011) have shown that it is possible to control crayfish in rivers through early and rigorous action, thus preventing their expansion along the riverbed and slowing down the simplification of communities and the disruption of trophic networks (Dorn and Wojdak, 2004; Machida and Akiyama, 2013; Meira et al., 2019) with the synergistic presence of NIS species.

Conclusion

This study shows urgent management measures are needed to eliminate or control the presence of exotic species and reduce their negative effects on the most Mediterranean populations of pearl mussels. There is a need to develop a specific conservation plan adapted to their special characteristics: low density, isolation, ageing and lack of recruitment, declining trout host populations, siltation of the beds and hydrological uncertainty accentuated by climate change. These effects are likely to be exacerbated in the very near future by the invasion of alien species.

Effective conservation requires prioritizing research and actions to identify extrinsic factors (environmental variables and threats) and mitigate current and potential direct impact on conservation status in a short timeframe. This can only be achieved by improving the environmental conditions (hydrological and biological) that mussels, trout and the rest of the native benthic community need. This is a priority for the conservation of these species at risk of extinction, especially considering the increasing hydrological pressure from the consequences of climate change (O'Briain, 2019) and the critical threat from the signal crayfish, as demonstrated in experimental and field studies in Iberian (Meira et al., 2019; Sousa et al., 2019) and European rivers (Dobler and Geist, 2022). Over the past decades, the loss of integrity in the biological communities of these rivers and the consequent absence of trout fry when glochidia are present in the water has exacerbated the difficulty for *M. margaritifera* to complete its life cycle.

The presence of xenobiota endangers the survival of aging mussel adults, the only source of genetic material for implementing conservation plans in situ and ex situ in the immediate future. In conclusion, the current river conditions favor the settlement of exotic species of wide ecological valence and induce the decline of native mollusks that are more demanding in their naturally occurring microhabitats.

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Supplementary material

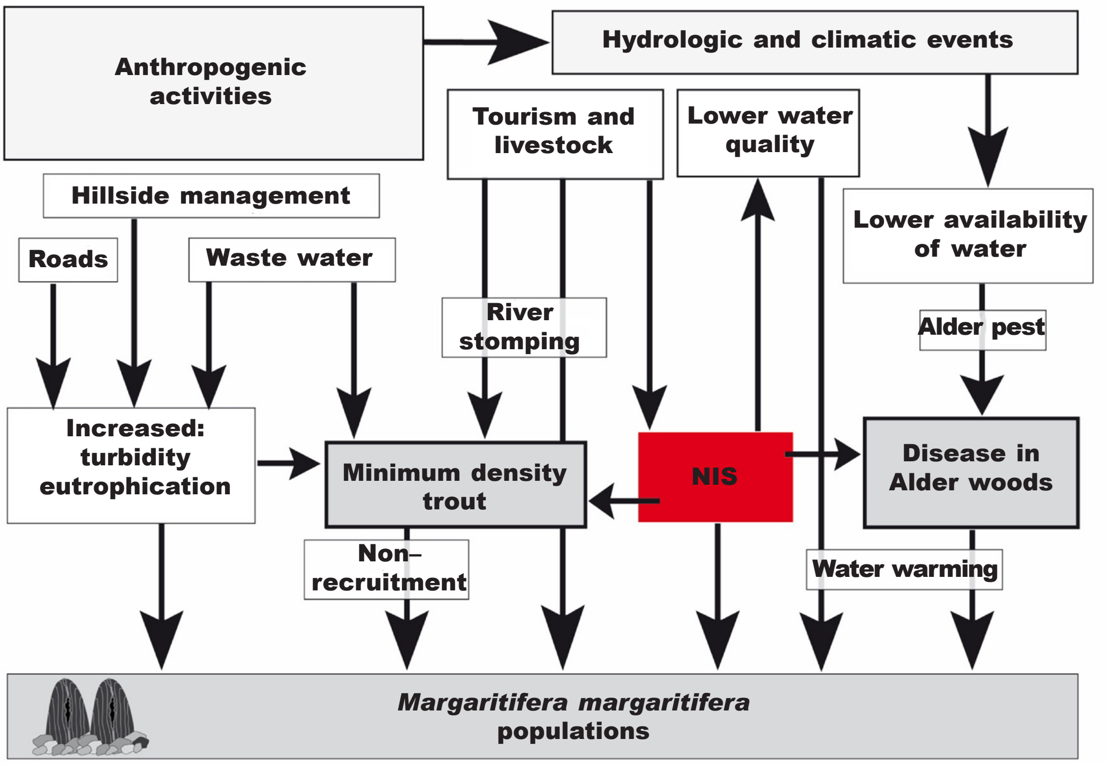
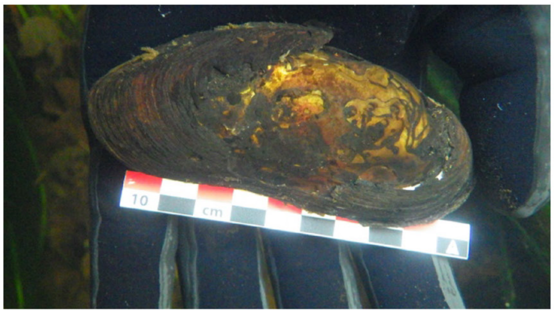


Fig. 1s. Schematic diagram of the complex network of direct and indirect stressors on *M. margaritifera* populations in Castilla and León. These non–native species (NIS) have a negative impact on conservation by changing fluvial ecosystems physically, chemically and/or ecologically, through trophic networks, interactions between species and the synergistic effect between pressures. They impact on native benthic communities through competition for food or substrate, and by predation on larval or adult phases. (Hillside management includes cropland and forest management of pinewood firebreaks; roads include culverts and ditches; tourism includes sport fishing and bathing).

Fig. 1s. Diagrama esquemático de la compleja red de factores de estrés directos e indirectos que inciden en las poblaciones de M. margaritifera *de Castilla y León. Las especies exóticas invasoras (NIS) tienen un impacto negativo en la conservación, ya que modifican física, química y/o ecológicamente los ecosistemas fluviales, a través de las redes tróficas, las interacciones entre especies y el efecto sinérgico entre presiones. Afectan a las comunidades bentónicas nativas a través de la competencia por el alimento o el sustrato, y por la depredación de las fases larvarias o adultas. (La gestión de las laderas incluye las tierras de cultivo y la gestión forestal de los cortafuegos de los pinares; las carreteras incluyen los drenajes y las cunetas, y el turismo incluye la pesca deportiva y el baño).*

Non–recruitment of juveniles in decades and very old aged adults

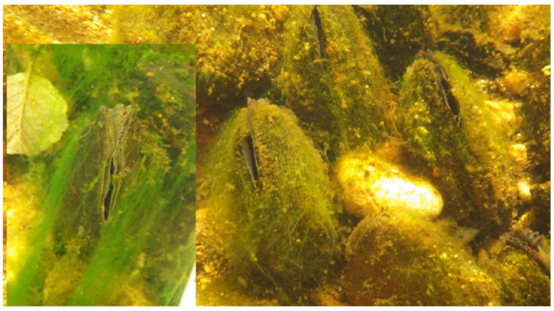
The youngest mussel (Alberche)



The most aged mussel (Negro)

Lower population density and eutrophicated mussels beds

Summer eutrophication of rivers (Águeda)



Filamentous algae colonization (Negro)

Biological invasion and disease

Signal crayfish invasion (Negro)

Alder disease and severe drought (Águeda)

Anthropic impacts on the riverbeds

Cattle stomping on the riverbed (Tera)

Shell of the trampled mussel (Negro)

Fig. 2s. Graphic key to the conservation problems of freshwater mussel populations in 2018, in relation to direct anthropic pressures, impacts related to climate change and the spreading of non–native species.

Fig. 2s. Clave gráfica de los problemas de conservación de las náyades en 2018, en relación con los factores de estrés de origen antrópico, efectos relacionados con el cambio climático y la expansión de las especies exóticas.