


RESEARCH ARTICLE

WILEY

# Non-indigenous fish in protected spaces: Trends in species distribution mediated by illegal stocking

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## Funding information

AMBER, Grant/Award Number: 689682; Juan de la Cierva

## Abstract

1. Many freshwater non-indigenous species (NISs) are stocked for recreational fishing, in some cases illegally in protected areas. In this study, fish communities were monitored using environmental DNA, electrofishing and anglers' catches as the sources of samples in a mountainous Biosphere Reserve in Asturias (northern Spain), where stocking is forbidden.
2. Three NISs have been introduced illegally in the protected area and have shown increasing populations in the last two decades. Two species used as fishing bait, *Squalius carolitertii* (chub) and *Phoxinus phoxinus* (minnow), are expanding in running waters. *Oncorhynchus mykiss* (rainbow trout) was also detected and is likely to have been introduced for angling or from fish farm escapes.
3. The results suggest that sustained illegal stocking contributed to the increase of the three NISs. In contrast, *Salmo trutta* (brown trout) of northern European lineages, identified from \*90 alleles at the *LDH-C1* locus, and formerly legally stocked for angling, is decreasing, most likely as a result of climate change. Climate change could also contribute to the expansion of the two non-indigenous cyprinids to colder upstream areas.
4. Through the application of a social survey, it was found that unlike other population groups, anglers in the region significantly preferred stocking over environmental improvement for the management of fish populations. The results obtained suggest that raising the awareness of anglers about the importance of safeguarding native fish species could help to prevent the spread of NISs in protected areas.

## KEYWORDS

brown trout lineages, citizen science, eDNA, monitoring, non-indigenous species, protected spaces

## 1 | INTRODUCTION

Species distribution in rivers depends on the characteristics of the system to which structural and functional elements of the communities are adapted (Vannote, Minshall, Cummins, Sedell, & Cushing, 1980). Changes in freshwater species occupancy are sometimes associated with human activities, however. Societies use rivers as a source of energy, water, and for other ecosystem services, and consequently many impacts are derived from human actions affecting rivers. Stocking, aquarium releases, and international shipping have been identified as the main activities contributing to the spread of non-indigenous freshwater species (Havel, Kovalenko, Thomaz, Amalfitano, & Kats, 2015). The construction of dams, canals, and reservoirs also creates new paths for species dispersal (Rahel & Olden, 2008). In some cases, reservoirs can act as shelters for non-indigenous species (NISs).

Fish are the most abundant group of NISs transported by humans in freshwater ecosystems (Gozlan, 2010). Fish development, spawning, and growth are heavily influenced by water temperature (Sharma, Jackson, Minns, & Shuter, 2007), and thus not all NISs can successfully establish self-sustaining populations in recipient ecosystems (Moyle & Marchetti, 2006), because of differences in water temperature compared with that of native ecosystems. The adaptation and spread of NISs are highly dependent on suitable thermal conditions, and global warming is likely to enhance some introduced species, such as Cyprinidae and Percidae (Lehtonen, 1996). Apart from warming temperatures, other consequences of climate change may favour the spread of NISs across freshwater ecosystems. Examples include the alteration of flow regimes that increase the probability of aquaculture escapes, and salinity decreases in estuaries caused by increased river flow that facilitate the introduction of NISs (Holopainen et al., 2016).

Many economic and ecological consequences of the introduction of NISs have been reported worldwide (Ardura, Zaiko, Borrell, Samuiloviene, & Garcia-Vazquez, 2017; Francisco, Blackburn, & Klinman, 2003; Gozlan, 2010; Simon & Townsend, 2003; Strayer, 2010); however, the real impacts of NISs are still unknown, as they should be considered at different levels over the long term, and these types of studies are scarce. In the Iberian Peninsula (southern Europe), 36% of NISs are fish species (Gozlan, 2010). One of the most important impacts of fish NISs on Iberian freshwater ecosystems is the alteration of native populations (Aparicio, Vargas, Olmo, & de Sostoa, 2000; Ayllon, Martinez, & García-Vázquez, 2006; Garcia-Marin, Sanz, & Pla, 1999; Horreo & Garcia-Vazquez, 2011; Rincon, Velasco, Gonzalez-Sanchez, & Pollo, 1990). As Iberian native fish are among the most threatened species in Europe (Miranda & Pino-del-Carpio, 2016), negative effects caused by NISs aggravate the condition of these vulnerable populations.

The creation of protected areas may help to reduce the spread of NISs because human impacts should be minimal in these areas, but in some cases the protection does not involve the prohibition of certain human activities, such as angling. The success of protected areas in preventing the introduction of NISs has been reported for marine protected areas (Ardura, Juanes, Planes, & Garcia-Vazquez, 2016;

Lubchenco, Palumbi, Gaines, & Andelman, 2003). However, the protection of terrestrial areas does not necessarily imply that the rivers within these areas are unaffected by human impacts (Juffe-Bignoli et al., 2016). For example, dams or water diversion for agriculture can occur outside the boundaries of protected areas and still have adverse consequences on freshwater habitats. An example of the mismanagement of freshwater ecosystems within protected spaces is the practice of stocking NISs for sport fishing (Saunders, Meeuwig, & Vincent, 2002). Although stocking has caused immense impacts on native wild fish populations (Gesundheit & Macías Garcia, 2018; Levin, Zabel, & Williams, 2001), it was and still is demanded by anglers in many regions worldwide. The Iberian Peninsula is no exception: stocking is currently driving the introgression of non-indigenous *Salmo trutta* (brown trout) in Spain (e.g. Horreo, Abad, Dopico, Oberlin, & Garcia-Vazquez, 2015).

The anglers themselves are an important part of the problem. Illegal stocking often occurs for the purpose of increasing recreational fishing through the release of fishing targets (Canonico, Arthington, Mccrary, & Thieme, 2005; Hickley & Chare, 2004; Johnson, Arlinghaus, & Martinez, 2009) or their prey (Elvira & Almodóvar, 2001; McPhee, Leadbitter, & Skilleter, 2002). Illegal stocking is suspected even in areas declared genetic refuges for native fish (Araguas et al., 2009). The awareness of anglers about the importance of preserving native biodiversity is thus essential for excluding stocking (Araguas et al., 2009, 2017), together with strict control and surveillance of the protected spaces.

Given the importance of preserving highly vulnerable native fish communities, it is important to avoid illegal stocking in protected spaces. Several factors that mediate the expansion of NISs should be considered. In this study, fish communities were assessed in the upper zone of a river basin, located within a biosphere reserve in Asturias (north-western Spain), where the introduction of non-indigenous fish and any type of stocking is strictly forbidden (Spanish Directive 162/2014, 29 December 2014). Biosphere reserves are designated with the aim of balancing biodiversity conservation and sustainable human development (Schliep & Stoll-Kleemann, 2010). Some biosphere reserves are designated to protect freshwater ecosystems with a whole-catchment approach (Saunders et al., 2002); however, as conservation measures are established by national legislation (UNESCO-MAB, 1996, p. 16), in some areas, activities that are potential sources for the introduction of NISs are permitted, such as aquaculture. In the area studied, the introduction of non-indigenous *Oncorhynchus mykiss* (rainbow trout), probably from aquaculture escapes, has been detected recently by environmental DNA (eDNA) (Fernandez et al., 2018).

The objective of this study was to monitor the trends in NISs and native fish distributions and to infer the origin of NISs from a combination of electrofishing, eDNA, and the voluntary donation of samples from anglers. The trends (expansion or contraction) of non-indigenous fish in recent decades were determined by comparison with previous inventories. The opinion of anglers and non-anglers in the region was elicited, as proposed by Araguas et al. (2009), to infer the local pressure(s) for (illegal) stocking that is generally assumed to be conducted by anglers (Johnson et al., 2009; McPhee et al., 2002). Our

expectations were: (i) following Lehtonen (1996), cyprinids would increase and cold-water non-indigenous salmonids would decrease; (ii) illegal stocking, detected in other protected areas in the region (Horreo & Garcia-Vazquez, 2011), would contribute to an increase in the two types of non-indigenous fish; and (iii) fish farm escapes (Fernandez et al., 2018) and reservoirs (Rahel, 2007) would contribute to enhancing the populations of non-indigenous salmonids.

## 2 | STUDY AREA

The Nalón River, located in the central part of the province of Asturias (northern Spain) (Figure 1a,b), is the largest freshwater system in Asturias and is one of the most important in the Iberian Bay of Biscay (140.8 km in length and an average discharge of  $55.18 \text{ m}^3 \text{ s}^{-1}$ ). This study took place in the upper zone of the river, a protected space within the United Nations Educational, Scientific, and Cultural Organization (UNESCO) biosphere reserve of Redes Natural Park. The area was officially designated a Natural Park in 1996, and in 2001 it was declared a biosphere reserve (Spanish law 8/1996, 27 December 1996). Streams inside this mountainous protected area are steep and run fast, with cold and well-oxygenated waters. They have been classified as being of 'good ecological status' under the European Water Framework Directive (WFD; Council of the European Communities, 2000) by the Government of the Principality of Asturias that is responsible for the assessment. These rivers contain a highly diverse fauna

(García-Ramos, Jiménez-Sánchez, Piñuela, Domínguez Cuesta, & López Fernández, 2006), where the dominant fish species is the brown trout, a species recognized in the region as having important economic and ecological value as the main target of recreational fishing.

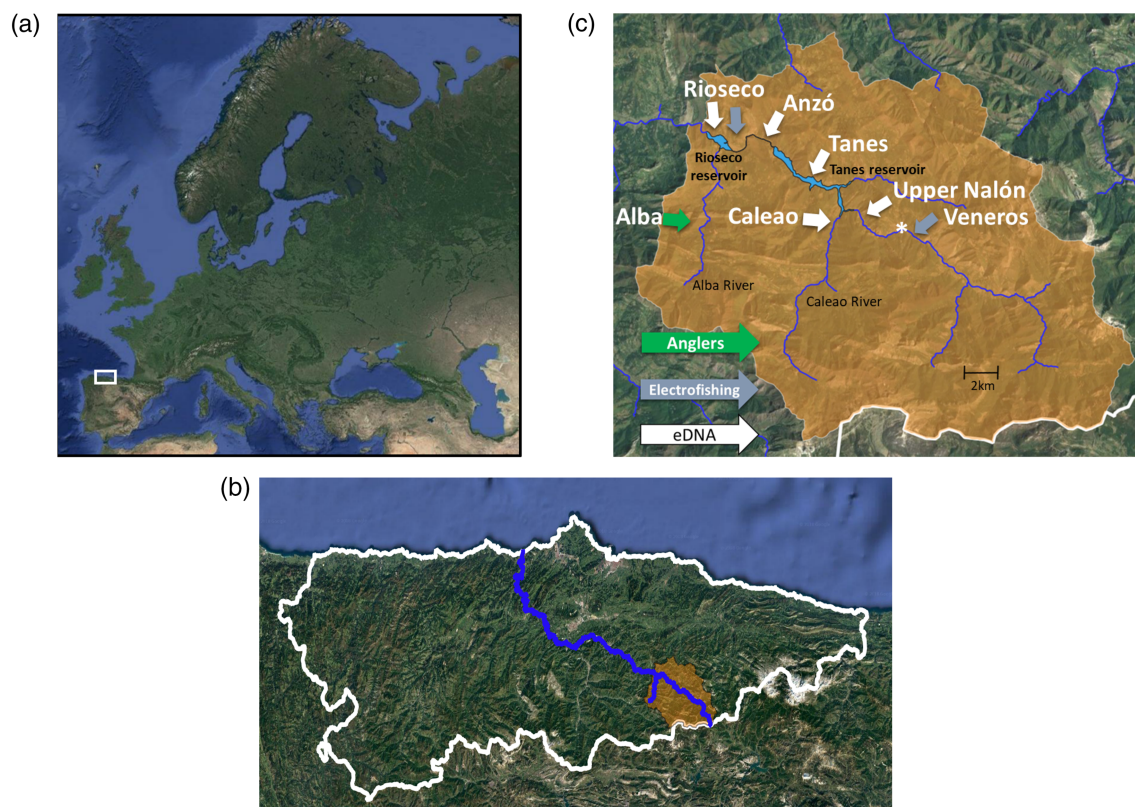
As in other temperate zones in the world, the climate is changing in this region, with an average increase of  $1.2^\circ\text{C}$  between 1996 and 2017 (data taken from the meteorological station 80150 in Oviedo:  $43^\circ 21' 00.0''\text{N}$ ,  $5^\circ 51' 36.0''\text{W}$ ).

Only two fish farms, both for rainbow trout, operate inside the reserve: one in Upper Nalón (near the Veneros sampling point) and the other in the River Alba (Figure 1c). In the region, local angling associations participate in the management of *Salmo salar* (Atlantic salmon) and brown trout populations through supportive breeding and assistance in stocking. Supportive breeding policies in the region are based solely on native stocks. The hatchery stocks have significantly improved in genetic quality since past imports of central European origin have been replaced by native breeders, with few exceptions (Horreo et al., 2015).

## 3 | METHODS

### 3.1 | Non-indigenous fish

Freshwater fish inventories from the rivers of Asturias were published in 1996, when the area was first designated as protected



**FIGURE 1** (a) Asturias (Northern Spain). (b) Nalón River and Redes Natural Park. (c) Sampling area within Redes Natural Park, showing the names of the sampling points and the techniques used. Rainbow trout (*Oncorhynchus mykiss*) fish farms are marked with an asterisk

(Reyes-Gavilan, Garrido, Nicieza, Toledo, & Braca, 1996). Information on the species in the biosphere reserve, including fish, was published later in 2006 (López Fernández, Suárez, López, & Ortega, 2006). In addition to these publications, the Regional Government conducts electrofishing surveys for fish species inventories that are updated sporadically. The last one was undertaken in 2014 (De La Hoz, 2014), using the same protocol as that deployed in the present study. Searches were conducted for accessible information in official reports, official depositories of the Regional Government of Asturias, scientific articles, and other media, including the internet and newspaper archives, about non-indigenous fish within the study area to compare them with the current inventory. Boolean operators for internet repositories were used with the following search terms: the names of the rivers and reservoirs inside the protected area (Caleao, Alba, and Nalón rivers; Tanes and Rioseco reservoirs); fish; and/or non-indigenous, introduced, native, inventory. The searches were conducted in Spanish and English.

Four non-indigenous fish species had been reported from the study area before the present survey (Table 1), including cyprinids such as *Squalius carolitertii* (the Iberian chub) and *Phoxinus phoxinus* (the minnow), which have been introduced to the region but are also present in other parts of the Iberian Peninsula; and salmonids such as the North American rainbow trout and a central European brown trout lineage imported from Germany (Morán et al., 1991). The two cyprinids are native to other Iberian regions but are naturally absent from the region studied and are used as bait. Other sources of information (e.g. fisher associations and media releases) may anticipate the report of some NISs.

Another source of non-indigenous fish is non-indigenous lineages of native species. Before the area was protected, there were

introductions of brown trout hatchery lineages from central Europe to enhance wild brown trout populations. The natural reproduction of non-indigenous lineages in the zone and hybridization with native brown trout have been detected from genetic analysis (Izquierdo, Castillo, Ayllon, De La Hoz, & Garcia-Vazquez, 2006; Morán et al., 1991).

## 3.2 | Sampling

Fish or their DNA were sampled from six locations within the protected area (Figure 1c) in 2016–2017, using three different techniques: electrofishing, a conventional and widely employed sampling method; water sampling for extracting eDNA; and citizen science, specifically with the collaboration of local anglers.

### 3.2.1 | Electrofishing surveys

Fish species were surveyed in November 2016 from accessible river areas with running water, including one location in the most upstream zone (Veneros) and another location just upstream of the impounded area of the Rioseco Reservoir, in the reservoir tail (Figure 1c, grey arrows). The official protocol for electrofishing (ML-R-FI-2015, NIPO: 280-15-122-6) was used, as set out by the Spanish Ministry of Agriculture, Fisheries, and Environment, with responsibility for implementing the WFD.

Electrofishing surveys consisted of a single pass, using a continuous pulsed DC between 500 and 850 V with an intensity between 34 and 20 A, calculated based on the conductivity of the transects selected. The protocol was essentially the same as that used in the 1990s when the previous inventories were performed, using a single

**TABLE 1** Non-indigenous fish reports. Records of non-indigenous fish in the Upper Nalón River inside the biosphere reserve. Geographic origin is the native origin of the species introduced in Redes Natural Park. Brown trout imported from Germany were domesticated in Spain and then stocked

| Non-indigenous fish species                                    |  |                                     |                                   |                     |  |
|--|--|-------------------------------------|-----------------------------------|---------------------|--|
| SPECIES  | Geographic origin                        | Year of first report                | Introduction purpose              | Reported habitat    | Source   |
| Non-indigenous lineages of brown trout ( <i>Salmo trutta</i> ) | Germany                                  | 1990                                | Recreational fishing              | Reservoir and river | (Morán, Pendás, Garcia-Vázquez, & Izquierdo, 1991)   |
| Iberian chub ( <i>Squalius carolitertii</i> )                  | Iberian Peninsula (Galicia and Portugal) | 2008 (absent in 2006)               | Bait                              | Rioseco Reservoir   | News in media ( <sup>a</sup> El Comercio Local Newspaper, March/2008); López Fernández et al. (2006); De La Hoz (2014) |
| Minnow ( <i>Phoxinus phoxinus</i> )                            | France                                   | 2006 (absent above Rioseco in 1996) | Bait                              | Reservoir and river | Reyes-Gavilan et al. (1996); López Fernández et al. (2006).  |
| Rainbow trout ( <i>Oncorhynchus mykiss</i> )                   | America                                  | 2006 (absent in 1996)               | Recreational fishing, aquaculture | Reservoir and river | Reyes-Gavilan et al. (1996), López Fernández et al. (2006), Fernandez et al. (2018)                                    |

<sup>a</sup><https://www.elcomercio.es/gijon/20081020/oviedo/miles-bogas-cangrejos-invaden-20081020.html>.



pass and controlled intensity. Individual fish were taxonomically identified to the species level. A small piece of adipose fin was excised from brown trout individuals and stored in ethanol for DNA analysis.

### 3.2.2 | Water sampling for eDNA

Three litres of water were sampled using sterile bottles from five locations inside the protected area in March 2017: Rioseco Reservoir, Anzó (between the reservoirs), Tanes Reservoir, and the Caleao and Upper Nalón streams above the Tanes Reservoir (Figure 1c, white arrows). The protocol for water sampling followed that of Fernandez et al. (2018). A bottle containing distilled water was transported together with the sampling gear, opened in the field, and processed with the rest of the eDNA water samples as a sampling negative control to confirm that contamination did not occur during travel. To prevent contamination across sites, new sterile gloves were used to obtain the water samples at each site. Waders were carefully rinsed with 10% bleach before reaching and leaving each sampling point.

### 3.2.3 | Angler collaboration

Samples were also obtained from a collaboration with local anglers who contributed to this research by providing scales from their own catch and trophies; all angler samples were from the small, fast River Alba above Rioseco Reservoir (Figure 1c, green arrow). Local anglers were contacted in an open talk given in Rioseco's social centre during the 2016 World Fish Migration Day (<https://www.worldfishmigrationday.com/about>). They were trained to sample scales from the anterior dorsal part of the fish using tweezers to be stored in paper envelopes provided by the researchers. Anglers were asked to record the catch date and location (river zone) in writing on the envelopes. The council of Rioseco, a village in the target area, allowed anglers to deposit envelopes with scales on its premises, for collection by researchers after the 2016 angling season.

### 3.3 | Genetic analysis

Genomic DNA was extracted from scale and adipose fin samples following a Chelex resin protocol (Estoup, 1996). For species identification from scale samples, polymerase chain reaction (PCR) amplification of a cytochrome c oxidase subunit I (COI) gene fragment was performed using the primers designed by Kochzius et al. (2010). The final PCR volume was 20 µl and included full-strength Green GoTaq® Buffer, 2.5 mM MgCl<sub>2</sub>, 0.25 mM deoxynucleotide (dNTP), 10 pmol of each primer, 2 µl of template DNA, and 0.65 U of DNA Taq polymerase (Promega, Madison, WI). PCR conditions were 94°C for 4 min, followed by 40 cycles at 94°C for 50 s, 59°C for 50 s, and 72°C for 90 s, with a final elongation step of 72°C for 7 min. The amplicons generated were sequenced in the Sequencing Unit of the University of Oviedo's Scientific Technical Services. The resulting sequences were edited with SEQUENCE SCANNER 1.0 (Applied Biosystems, Sussex, UK). Then, each sequence was assigned to a species by comparison with public DNA databases using the BLAST tool

included on the National Center for Biotechnology Information (NCBI) webpage (Altschul, Gish, Miller, Myers, & Lipman, 1990), with the following settings: best match with minimum 99% identity and 99% coverage.

The PCR-restriction fragment length polymorphism (RFLP) technique described by McMeel, Hoey, and Ferguson (2001) based on the *LDH-C1\** locus analysis was applied to distinguish between non-indigenous and native individuals identified as brown trout, following the method of Izquierdo et al. (2006). Nucleotide sequences of the *LDH-C1\*90* and *\*100* alleles of brown trout differ at position 308 with A in the *\*100* allele and G in the *\*90* allele. Digestion with *Bs*II restriction enzyme of the DNA of *LDH-C1\*90/90* individuals generated two fragments, one with 360 nucleotides and another with 80 nucleotides. Digestion of DNA from *\*100/100* homozygotes generated a single uncut fragment of 440 bp, whereas the *\*90/100* heterozygotes generated all three bands: 440, 360, and 80 bp. Allele *\*100* is typical of autochthonous populations, whereas allele *\*90* is from introduced lineages (Izquierdo et al., 2006). To obtain the *LDH-C1\** gene fragment, the primers LDHxon3F and LDHxon4R were used for PCR amplification. PCR was carried out in a final volume of 20 µl, which included full-strength Green GoTaq® Buffer, 1.5 mM MgCl<sub>2</sub>, 0.25 mM dNTP, 10 pmol of each primer, 2 µl of template DNA, and 0.65 U of DNA Taq polymerase (Promega), on DNA extracted from scales and adipose fin clips. PCR conditions were as follows: 95°C for 5 min, followed by 30 cycles at 95°C for 1 min, 70°C for 1 min, and 72°C for 1 min, with a final elongation step at 72°C for 10 min. To obtain the RFLPs, a 10-µl aliquot of the PCR amplicon was digested with *Bs*II (New England BioLabs, MA, USA) in a total volume of 20 µl, according to the instructions provided by the enzyme supplier. The resultant fragments were separated by electrophoresis on a 2.5% agarose gel. The *LDH-C1\** locus was genotyped in brown trout individuals from the band pattern obtained from agarose gels (Figure S1).

The number of individuals of each *LDH-C1\** genotype previously found in the same area was taken from Izquierdo et al. (2006) and Morán et al. (1991).

### 3.4 | eDNA extraction and analysis

River water samples were vacuum filtered using a Supor® 200 Membrane Filter (Pall Corporation, Life Sciences, Ann Arbor, MI) with a 0.2-µm pore size using 1 L per filter and 3 L per sampling point (extraction replicates). One litre of distilled water was filtered at the end of the process to control for filtration contamination. Between each sample, the filtration area and apparatus were cleaned with 10% bleach to avoid cross-contamination.

Environmental DNA (eDNA) was extracted from filters with the PowerWater® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) under sterile conditions inside a laminar flow PCR cabinet, following the manufacturer's instructions. A negative control for extraction was added.

Metabarcoding analyses were performed at the Scientific Technical Services of the University of Oviedo (Spain) using universal primers for

the mitochondrial region of the *COI* gene, mCOLintF and jgHCO2198 (Leray et al., 2013), as barcode markers to detect fish species. Amplification was carried out in a total volume of 20  $\mu$ l and included full-strength Green GoTaq® Buffer, 2.5 mM  $MgCl_2$ , 0.25 mM dNTP, 20 pmol of each primer, 4  $\mu$ l of template DNA, 200 ng  $\mu$ l<sup>-1</sup> of bovine serum albumin (BSA), and 0.65 U of DNA Taq polymerase (Promega). PCR conditions in the Veriti Thermal Cycler (Applied Biosystems, Foster City, CA) were 95°C for 1 min, followed by 35 cycles of 95°C for 15 s, 46°C for 15 s, and 72°C for 10 s, with a final extension of 72°C for 3 min. Negative controls to monitor contamination and positive controls to check for inhibition were included in the amplification process. The success of the amplification was visually assessed on 2% agarose gel. PCR amplicons were purified from agarose gel using the Montage DNA Gel Extraction Kit (Millipore, Burlington, MA), quantified using the QuBit BR dsDNA kit (ThermoFisher Scientific, Carlsbad, CA), and double-checked in a Bioanalyser 2100 (Agilent Technologies, Santa Clara, CA) to confirm the fragment size, the absence of by-products, and to perform a more precise quantification. All samples were diluted down to 26 pmol to prepare an equimolar pool with all samples. The pool was processed by liquid emulsion PCR in the one-touch system using the Ion PGM™ OT2 Supplies Kit (Life Technologies, Carlsbad, CA) following the manufacturer's instructions. Then, the sample was loaded in the Ion '314' Chip (Life Technologies) and sequenced using the Ion Torrent Personal Genome Machine (Life Technologies), following the specifications in the Ion PGM™ Sequencing Kit. Low quality and polyclonal sequences were filtered automatically, and the PGM adaptor was trimmed within the PGM software.

QIIME 1.9.1 was used for bioinformatic analysis (Caporaso et al., 2011). To split the 'fastq' files into constituent 'fna' and 'qual' files the 'convert\_fastqual\_fastq.py' PYTHON script was used. To filter sequences by quality and size (minimum and maximum size of 250 and 400, respectively, and quality score of 25), the 'split\_libraries.py' PYTHON script was used. Then, primer trimming was performed with PRINSEQ 0.20.4 (Schmieder & Edwards, 2011). The *COI* gene database was constructed using the workflow developed by Baker (2017), and BLAST alignment was performed against the database of NCBI *COI* sequences (with a maximum *e*-value of  $10^{-50}$  and a minimum percentage identity of 97.0), employing the 'assign\_taxonomy.py' PYTHON script. Finally, operational taxonomic unit (OTU) tables, a list of OTUs obtained for each sample, and the number of sequences assigned to them were constructed with the algorithm 'fromTaxassignments2OtuMap.py'. This table was filtered to obtain fish read counts.

### 3.5 | Social survey

In-person surveys (*n* = 218, approximately 0.1% of the adult population of central Asturias) to determine the preferred management actions to improve river fish populations were conducted in different years. Anglers (*n* = 117) by the river were directly contacted while they were fishing (Horreo et al., 2015), and non-anglers (*n* = 101) in

neighbouring areas were contacted using snowball methodology, a method in which the participants provide contact details of other potential subjects to survey (Neis et al., 1999; Sadler, Lee, Lim, & Fullerton, 2010). After acceptance (by verbal consent) to participate in the survey, the subjects were informed that it was solely for research purposes and that their anonymity was guaranteed. An open question was posed: 'What would you propose for improving the state of fish populations in this river?' The answers were recorded in writing, and each participant checked that the notes accurately reflected her or his opinion.

### 3.6 | Statistical analysis

Contingent chi-square analysis was applied in the social survey for the frequency of each solution proposed (stocking versus habitat improvement) to test the differences between groups. It was also used to compare the frequency of brown trout *LDH-C1\** genotypes among years. Statistical significance was set at  $P < 0.05$ . When multiple groups were compared and the chi-square was significant, ad hoc pairwise a posteriori tests were performed, with the free software PAST 3 (Kot & Daniel, 2008).

## 4 | RESULTS

### 4.1 | Trends of non-indigenous fish inside the protected area

Before 1996, only minnow (Reyes-Gavilan et al., 1996) and non-indigenous brown trout lineages (Morán et al., 1991) were reported from the area now occupied by the biosphere reserve (Table 1). At that time, the minnow was restricted to altitudes lower than 500 m a.s.l. in this region, and its limit was below the Rioseco Reservoir (Reyes-Gavilan et al., 1996). Before the area was protected, non-indigenous brown trout from northern latitudes (German waters, central Europe) were legally stocked in the area to enhance native populations for the ultimate purpose of sport fishing (Morán et al., 1991), and reproduced naturally therein. Rainbow trout were reported from rivers and reservoirs of the biosphere reserve in 2006 (López Fernández et al., 2006), probably introduced for sport fishing, with additional inputs from aquaculture escapes (Fernandez et al., 2018). The Iberian chub was the most recent introduction, being reported from the Rioseco Reservoir in 2008 by regional newspapers (De La Hoz, 2014) (Table 1).

### 4.2 | Fish inventory in 2016–2017

The four fish species previously reported inside the biosphere reserve were found in the 2016–2017 inventory using different sampling methods (Table 2). Summarizing the data obtained from each method, brown trout was detected from all the sampling sites and with all sampling methods (volunteer anglers, electrofishing, and eDNA; Table 2). Minnow was detected by both electrofishing and eDNA, and rainbow

**TABLE 2** Results of the 2016–2017 surveys. Fish (DNA or individuals) found within the protected area from electrofishing, scales provided by anglers, and next-generation DNA sequencing (NGS). \*Cannot differentiate between native and non-indigenous brown trout; \*\*could be escapes from a hatchery. Distribution: 1, native; 2, introduced from Germany; 3, translocated species that are native elsewhere on the Iberian Peninsula; 4, introduced from North America

| Sampling sites per technique                       |              | Electrofishing |         | eDNA    |             |        |       |      |         | Anglers | Trend of non-indigenous |
|--|--------------|----------------|---------|---------|-------------|--------|-------|------|---------|---------|-------------------------|
| Species  | Distribution | Veneros        | Rioseco | Veneros | Upper Nalón | Caleao | Tanes | Anzó | Rioseco | Alba    |                         |
| Indigenous brown trout ( <i>Salmo trutta</i> )     | 1            | X              |         | X*      | X*          | X*     | X*    | X*   | X*      | X       | –                       |
| Non-indigenous brown trout ( <i>Salmo trutta</i> ) | 2            |                | X       |         |             |        |       |      |         | X       | Regression              |
| Chub ( <i>Squalius carolitertii</i> )              | 3            |                |         |         |             |        |       |      |         | X       | Expansion               |
| Minnow ( <i>Phoxinus phoxinus</i> )                | 3            |                | X       |         |             |        | X     | X    |         |         | Expansion               |
| Rainbow trout ( <i>Oncorhynchus mykiss</i> )       | 4            |                |         | X       |             | X      | X**   |      |         |         | Expansion               |

trout was inferred from eDNA (Table 2). Iberian chub was detected only from scale samples provided by anglers.

The scale samples provided by anglers, obtained from fish caught in the River Alba upstream of the Rioseco Reservoir, were genetically identified as brown trout and Iberian chub. Their barcodes are available under GenBank accession numbers KY492314, KY49231425, KY492328–KY492330, KY492326, and KY492327. Two species were detected by electrofishing: *S. trutta* from the two sites surveyed (Veneros and Rioseco Reservoir tail) and *P. phoxinus* from the Rioseco Reservoir tail (Table 2).

For the eDNA inventory, raw sequences from next-generation sequencing (NGS) data were available in the NCBI sequence read archive (SRA accession: SRP128681) with BioSample number SAMN08295300. In total, 2 650 693 reads were recorded, and after filtering for quality 1 548 436 sequences were retained. Three fish species were identified in the dataset (Table S1): brown trout, minnow, and rainbow trout. Brown trout DNA was found at all sites. Minnow DNA was found from the two reservoirs (Rioseco and Tanes) and in the running water between them (Anzó). Rainbow trout DNA was found in the two rivers upstream and in the Tanes Reservoir (Table 3). Besides this DNA evidence, occasional observations of rainbow trout in the zone were mentioned by anglers in informal conversations.

For the non-indigenous brown trout lineage, four of the 55 brown trout samples analysed in this study (7.3%), all of which were caught by electrofishing from the Rioseco Reservoir tail (Figure 1c), possessed the *LDH-C1\*90* allele (Table 4). This allele is a marker specific to the non-indigenous lineage of central European origin introduced into the area (Izquierdo et al., 2006; Morán et al., 1991). The scales provided by anglers from the River Alba and the individuals analysed from other sampling points were all homozygotes *\*100/100* (Figure S1).

The *\*90* allele frequency (*q*) decreased over time and was clearly lower in 2016 than in previous years (Table 4). The difference in the distribution of genotypes among the three time points considered (1990, 1997–2003, and 2016) was highly significant (chi-square of 113.46, 4 df,  $P < 0.0001$ ). A posteriori testing revealed a highly significant difference between fish sampled in 1990 and fish sampled in 1997–2003 (chi-square of 44.9, 2 df,  $P < 0.0001$ ), and between that period and fish sampled in 2016 (chi-square of 20.9, 2 df,  $P < 0.0001$ ). For the trout sampled from the reservoir, a significant change was found only between 2003 and 2016 (chi-square of 87, 2 df,  $P < 0.0001$ ).

Regarding the trend (expansion or contraction) and the inference of illegal stocking of the non-indigenous fish detected in this study, the German non-indigenous brown trout is in regression in this biosphere reserve (Table 2), and there are no signs of illegal stocking. In

**TABLE 3** Detection of fish species using eDNA. Number of DNA sequences assigned to the detection of different fish species from metabarcoding analysis after next-generation sequencing (NGS) of eDNA samples, in the five sampling locations considered

| NGS_Reads counts<br>Species                  | Sampling points |             |      |         |       |        |
|--|-----------------|-------------|------|---------|-------|--------|
|  | Caleao          | Upper Nalón | Anzó | Rioseco | Tanes | Total  |
| Rainbow trout ( <i>Oncorhynchus mykiss</i> ) | 18 569          | 11 791      | 0    | 0       | 12    | 30 372 |
| Brown trout ( <i>Salmo trutta</i> )          | 172             | 105         | 36   | 22      | 118   | 453    |
| Minnow ( <i>Phoxinus phoxinus</i> )          | 0               | 0           | 82   | 20      | 91    | 193    |

**TABLE 4** *LDH-C1\** genotypes showing indigenous (\*100) and non-indigenous (\*90) alleles. Number of brown trout individuals of each genotype found in the current (2016) and previous studies, both in the Riosco Reservoir and in the rivers upstream: 1990, data taken from Morán et al. (1991); 1997–2003, data taken from Izquierdo et al. (2006).  $q(90^*)$ , frequency of the non-indigenous allele *LDH-C1\*90*;  $n$ , sample size

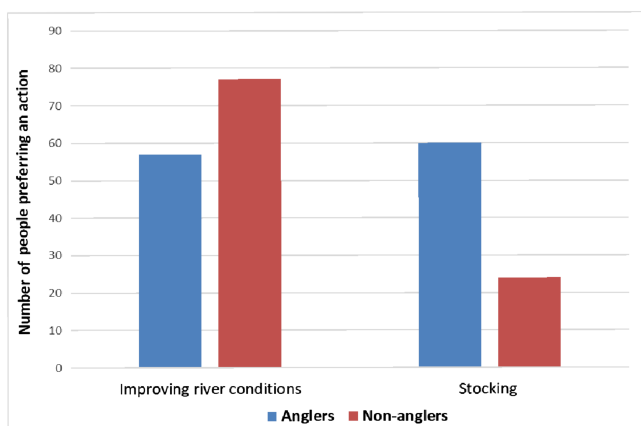
| Year<br><i>LDH-C1*</i> genotypes | 1990   |         |          | 1997–2003 |         |          | 2016   |         |          |
|----------------------------------|--------|---------|----------|-----------|---------|----------|--------|---------|----------|
|                                  | *90/90 | *90/100 | *100/100 | *90/90    | *90/100 | *100/100 | *90/90 | *90/100 | *100/100 |
| Rivers upstream                  | 0      | 22      | 55       | 0         | 15      | 264      | 0      | 0       | 28       |
| Riosco Reservoir                 | 100    | 0       | 0        | 50        | 0       | 0        | 0      | 4       | 23       |
| $n$                              | 177    |         |          | 329       |         |          | 55     |         |          |
| $q(90^*)$                        | 0.63   |         |          | 0.18      |         |          | 0.04   |         |          |

the past, these non-indigenous lineages of brown trout were found in rivers upstream and in the reservoirs, but now they are associated with the Riosco Reservoir alone, and the frequency of individuals carrying non-indigenous \*90 alleles has decreased significantly.

For rainbow trout, DNA was found from the Tanes Reservoir water and in the two streams upstream (Table 2). In 1996, this species was absent from the area, and the presence of its DNA in the Caleao Stream, where there are no rainbow trout farms, suggests natural expansion (from the reservoir) or illegal stocking. The Iberian chub, which was absent in 1996 and 2006, was found in running waters (the Alba River) for the first time; thus, it could be considered an expansion (Table 2) resulting from illegal stocking. The minnow was absent from waters at altitudes of >500 m a.s.l. in 1996. In the present survey, minnow DNA was found in the Tanes Reservoir, showing an expansive trend from its initial distribution in the area. As the Tanes dam is impassable, its presence upstream of the dam is solely the result of illegal stocking.

### 4.3 | Social survey

Survey results (Figure 2) showed significant differences among the groups of participants (anglers and non-anglers), with a contingency chi-square of 17.333, 1 df, and  $P = 0.0001$ . More than 50% of anglers chose 'stocking' as the preferred measure to improve fish populations,



**FIGURE 2** Results of the social survey. Numbers of individuals preferring stocking or improving river conditions for managing fish populations

whereas 'Improving river conditions' was the action preferred by the other group of participants interviewed in this study.

## 5 | DISCUSSION

The results of this study revealed a change in fish communities inside a temperate biosphere reserve in the last two decades. The number of species doubled from two (one indigenous and one non-indigenous) to four, and the two new species are non-indigenous. The population trends of the three NISs are expansive as they were found in more areas and also outside the reservoirs, whereas a non-indigenous lineage of German brown trout stocked in the area is strongly declining. These results highlight a problem in the management of protected areas, where the aquatic biota is sometimes neglected when applying conservation policies. Although this study has been developed in a particular geographical area, it is an issue also described in other ecosystems that should receive more attention from managers, as the recovery of aquatic biota is weaker than terrestrial communities in response to restoration actions (Pilotto et al., 2019). The lessons learned here could easily be applied in any other temperate or cold region of the world, where introduced non-native species seem to be already expanding to the detriment of native fish species, owing to a combination of uncontrolled releases and climate change. For example, in the UK, recreational anglers have been implicated in the spread of invasive fish (Anderson, White, Stebbing, Stentiford, & Dunn, 2014). In Wyoming, USA, fish illegally released by the public comprised as many as 46% of all the introduction events between 1961 and 2017 (Rahel & Smith, 2018). In fragile subarctic fresh waters the vulnerability of native fish communities to invasions is increasing under current climate change (Rolls, Hayden, & Kahilainen, 2017). These examples from different regions and situations illustrate the wide relevance of the present study.

In this fish survey, three techniques were used, allowing different information to be gathered that contributed differently to the fish inventories. The use of eDNA for species inventories has increased substantially in the last few years and is often applied as a sensitive complementary tool for conventional monitoring (Clusa, Miralles, Basanta, Escot, & García-Vázquez, 2017; Evans, Shirey, Wieringa, Mahon, & Lamberti, 2017; Kelly et al., 2017; Zaiko et al., 2015). eDNA allows a general description of the sampling area, larger than traditional sampling methods, because it includes the eDNA from



nearby areas (Deiner, Fronhofer, Mächler, Walser, & Altermatt, 2016). In this study, all species were detected except chub, which was recorded only from samples provided by anglers. The use of eDNA was able to detect one more species than electrofishing, obtaining comparable results and being even more sensitive. Electro-fishing, although invasive, is in some cases necessary to confirm presence and to obtain tissue samples for genetic analysis. The stress generated by this technique was avoided by the involvement of anglers obtaining tissue samples from their own catch. In addition to avoiding animal distress caused by electrofishing (Buckland-Nicks, Gillis, & Reimchen, 2012), angler contributions decreased the sampling effort and covered areas where electrofishing would not be effective. The introduction of a citizen science approach also addressed the logistical and financial limitations of monitoring. In addition, samples from anglers can be used for detecting rare or low-density species, such as the chub found in this study, that may otherwise go undetected. Although anglers prefer larger prey, larger prey is not representative of the population (Gledhill et al., 2015). In the present study, anglers caught chub, a species of little or no value as a trophy, to help with this research.

The expansion of NISs in a zone always begins with (legal or illegal and deliberate or inadvertent) introductions (Chown et al., 2015; García-Berthou et al., 2005; Maceda-Veiga, Escribano-Alacid, de Sostoa, & García-Berthou, 2013; Ribeiro & Leunda, 2012). In most studies where illegal stocking is reported, it is presented as a suspicion but not as confirmed fact (Araguas et al., 2017; Johnson et al., 2009). In the present case, however, the isolated condition of the water bodies, restricted to a mountainous area by impassable dams, ensures that the introduction of new species or their expansion above dams cannot occur naturally, as in other cases where illegal stocking is only suspected to be the cause (Araguas et al., 2017; Horreo & García-Vázquez, 2011). Although the physical and environmental conditions in reservoirs may favour the establishment of NISs (Clavero, Blanco-Garrido, & Prenda, 2004; Han, Fukushima, Kameyama, Fukushima, & Matsushita, 2008; Havel et al., 2015), dams have blocked the expansion of NISs to upper zones of the Nalón River (Clusa, Miralles, Fernández, García-Vázquez, & Dopico, 2018). Indeed, natural expansion upstream of the dams may occur for the species that are already in the reservoirs. From the results of the present study, where new species have appeared upstream of impassable dams, illegal stocking seems to be the main cause of the increase of non-indigenous fish in the reserve, providing further support that this is a factor in other studies too (Araguas et al., 2009; Johnson et al., 2009). In this study, fish farm escapes are only a secondary factor in the introduction of NISs (Fernandez et al., 2018).

Angler pressure for stocking in the region was deduced from the social survey and is reflected in the reality that they have released, illegally, both bait and target catch species (cyprinids and salmonids, respectively) in the protected area. The presence of Iberian chub and minnow in the Tanes Reservoir where it never existed previously strongly suggests that it was stocked by anglers. These results support the trend previously reported that anglers release NISs as unused bait (Banha, Diniz, & Anastácio, 2017). For that

reason, it is crucial to increase the cooperation of anglers in the management of protected areas, as anglers are the main drivers of introductions (Hickley & Chare, 2004). This should involve raising awareness of the adverse effects of NISs. In addition, fines should be levied to penalise illegal introductions (Johnson et al., 2009). This is a government responsibility that is not carried out here for several reasons: for example, because of the lack of vigilance regarding illegal fishing practices, or to avoid creating social conflicts in which anglers might view the protected area as a threat to their activity.

Shifts in the distribution and abundance of aquatic species are also occurring as a result of climate change (Hughes, 2000), which is modifying aquatic ecosystems through the warming of the water and through alterations in stream flow patterns (Rahel & Olden, 2008). This type of biological change has been documented, mostly by depth and latitude, for marine ecosystems (Perry, Low, Ellis, & Reynolds, 2005). In inland waters, shifts in community composition are also expected. For fish communities, Lehtonen (1996) noted that global warming would result in an increase in cyprinid and percid dominance and in a decline in salmonids and other cold-water fish. This global warming paradigm, where the spread of southern species is being facilitated (Walther et al., 2009), may also be supported by the results on non-native brown trout from the present study. Although there are no physical data on these stocks, the frequency of carriers of the non-indigenous specific allele *LDH-C1\*90* has steadily decreased in the reserve since 1990, reflecting the decline in the status of this species. As the foreign stock of brown trout introduced in the region was of northern European origin (Morán et al., 1991), its reduction under current climate warming conditions would be expected. In the case of the southern Iberian chub, climate warming would have enabled its expansion into natural running waters in the River Alba. The exception would be rainbow trout, which also seems to be expanding in the system, against the predictions of Lehtonen. Rainbow trout is a cold-water species, and it would be expected that it would recede. An explanation for its presence in the Caleao Stream might be that it is colder than the reservoirs because it is higher up in the mountains, so it is possible that rainbow trout enters the stream in search of a colder environment. In mountain streams, cold-water fish may move to colder waters, expanding their distribution in the upper river reaches. Alternatively, or concomitantly with natural movement, illegal stocking could also explain the presence of rainbow trout in Caleao Stream.

The presence of NISs in rivers is likely to affect native populations. Native brown trout are listed as Vulnerable in Spain because natural populations have been reduced by 20%, owing to habitat loss, introductions of non-indigenous lineages, such as are found in the present zone (Izquierdo et al., 2006), and overfishing (Doadrio, 2001). The presence of non-indigenous fish species cohabiting in rivers with brown trout may result in a threat to their native populations, affecting their behaviour, genetic background, and distribution (Cucherousset & Olden, 2011); thus, in this basin, the threat to the native brown trout population needs to be highlighted to avoid its decline.

Anglers have contributed historically to river management in this (Horreo et al., 2015) and other regions. On the one hand, their activity is the main reason for the introduction of NISs, but on the other hand they contribute to river conservation through their involvement in research programmes (Couvet, Jiguet, Julliard, Levrel, & Teyssedre, 2008; Gledhill et al., 2015; Granek et al., 2008; Williams, Holmes, & Pepperell, 2015). In this study, the anglers surveyed preferred to invest money in supporting breeding than in improving the river environment, contrary to the other group surveyed. The intentional introductions observed in this study were carried out mainly for fishing activities, as has occurred in other regions worldwide (Arlinghaus, 2006; Crawford & Muir, 2008; Fausch, 2007; Wissinger, McIntosh, & Greig, 2006). It is important, therefore, to promote the role of anglers in conservation, encouraging them to stop illegal introductions. Considering that 42% of all aquatic introductions are deliberate (Gozlan, 2010), this is crucial to avoid the spread of NISs.

## ACKNOWLEDGEMENTS

This study has received funding from the European Union's Horizon 2020 Research and Innovation programme under grant agreement no. 689682 (project AMBER). AA holds an incorporation-Juan de la Cierva fellowship. The equipment and staff belonging to the scientific technical services of The University of Oviedo have been involved in this study.

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Zaiko, A., Martinez, J. L., Ardura, A., Clusa, L., Borrell, Y. J., Samuiloviene, A., ... Garcia-Vazquez, E. (2015). Detecting nuisance species using NGST: Methodology shortcomings and possible application in ballast water monitoring. *Marine Environmental Research*, 112, 64–72. <https://doi.org/10.1016/j.marenvres.2015.07.002>

## SUPPORTING INFORMATION

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**How to cite this article:** Fernández S, Arbolea E, Dopico E, Ardura A, García-Vázquez E. Non-indigenous fish in protected spaces: Trends in species distribution mediated by illegal stocking. *Aquatic Conserv: Mar Freshw Ecosyst*. 2019;29:2240–2252. <https://doi.org/10.1002/aqc.3238>