Environmental DNA for freshwater fish monitoring: insights for conservation within a protected area
Sara Fernandez, Miguel Sandin, Paul Beaulieu, Laura Clusa, José L. Martínez, Alba Ardura, Eva García-Vázquez

To cite this version:
Sara Fernandez, Miguel Sandin, Paul Beaulieu, Laura Clusa, José L. Martínez, et al.. Environmental DNA for freshwater fish monitoring: insights for conservation within a protected area. PeerJ, PeerJ, 2018, 6, pp.e4486. <10.7717/peerj.4486>. <hal-01757517>
Environmental DNA for freshwater fish monitoring: insights for conservation within a protected area

Sara Fernandez1, Miguel M. Sandin2, Paul G. Beaulieu3, Laura Clusa1, Jose L. Martinez4, Alba Ardura1 and Eva García-Vázquez1

1 Department of Functional Biology, University of Oviedo, Oviedo, Asturias, Spain
2 Sorbonne Université, Station Biologique de Roscoff, UMR7144, Roscoff, France
3 Tighe & Bond, Trout Unlimited, USA
4 Scientific-technical services, University of Oviedo, Oviedo, Asturias, Spain

ABSTRACT

Background. Many fish species have been introduced in wild ecosystems around the world to provide food or leisure, deliberately or from farm escapes. Some of those introductions have had large ecological effects. The north American native rainbow trout (Oncorhynchus mykiss Walbaum, 1792) is one of the most widely farmed fish species in the world. It was first introduced in Spain in the late 19th century for sport fishing (Elvira 1995) and nowadays is used there for both fishing and aquaculture. On the other hand, the European native brown trout (Salmo trutta L.) is catalogued as vulnerable in Spain. Detecting native and invasive fish populations in ecosystem monitoring is crucial, but it may be difficult from conventional sampling methods such as electrofishing. These techniques encompass some mortality, thus are not adequate for some ecosystems as the case of protected areas. Environmental DNA (eDNA) analysis is a sensitive and non-invasive method that can be especially useful for rare and low-density species detection and inventory in water bodies.

Methods. In this study we employed two eDNA based methods (qPCR and nested PCR-RFLP) to detect salmonid species from mountain streams within a protected area, The Biosphere Reserve and Natural Park of Redes (Upper Nalón Basin, Asturias, Northern Spain), where brown trout is the only native salmonid. We also measured some habitat variables to see how appropriate for salmonids the area is. The sampling area is located upstream impassable dams and contains one rainbow trout fish farm.

Results. Employing qPCR methodology, brown trout eDNA was detected in all the nine sampling sites surveyed, while nested PCR-RFLP method failed to detect it in two sampling points. Rainbow trout eDNA was detected with both techniques at all sites in the Nalón River’ (n1, n2 and n3). Salmonid habitat units and water quality were high from the area studied.

Discussion. In this study, a high quantity of rainbow trout eDNA was found upstream and downstream of a fish farm located inside a Biosphere Reserve. Unreported escapes from the fish farm are a likely explanation of these results. Since salmonid habitat is abundant and the water quality high, the establishment of rainbow trout populations would be favored should escapes occur. Environmental DNA has here proved to be a valuable tool for species detection in freshwater environments, and the probe-based qPCR highly sensitive technique for detection of scarce species. We would recommend this method for routine monitoring and early detection of introduced species within natural reserves.

How to cite this article Fernandez et al. (2018), Environmental DNA for freshwater fish monitoring: insights for conservation within a protected area. PeerJ 6:e4486; DOI 10.7717/peerj.4486
INTRODUCTION

Introduced fish species affect recipient ecosystems inducing changes in behaviour, distribution and abundance of native species, as well as affecting ecosystem functioning following the decrease of their favoured prey species (Strayer, 2010). An important source of fish introductions is inadvertent escapes from fish farms and aquaculture facilities (Naylor et al., 2005; Consuegra et al., 2011). Salmonids are native to the Northern Hemisphere but have been introduced and farmed worldwide causing disturbances to native species, especially in the Southern Hemisphere (Townsend, 2003; Valiente et al., 2007; Consuegra et al., 2011). Introduced salmonids interact with local fish in many ways: inducing behaviour changes (Cambray, 2003; Fausch, 2007; Landergren, 1999; Townsend, 2003; Wissinger et al., 2009); competing for food resources (Mooney & Cleland, 2001; Eby et al., 2006; Buria et al., 2010); causing changes in trophic webs (e.g., Diehl et al., 2000; McIntosh et al., 1996), and others.

Rainbow trout (Oncorhynchus mykiss Walbaum, 1792), a North American salmonid, is one of the most widely introduced fish species in the world and the most important freshwater fish exploited in aquaculture (Stanković et al., 2015). It is a well-known top predator in freshwater ecosystems (Oscoz et al., 2005; Fausch, 2007; Stanković et al., 2015). Rainbow and brown trout use similar resources and can thus compete for food or space (Oscoz et al., 2005). Introduced rainbow trout negatively impacts on European native brown trout (Salmo trutta L. 1758) populations, especially on those inhabiting small streams (Landergren, 1999). Rainbow trout is present in many European streams (Stanković et al., 2015). It was first introduced in Spanish waters in the late 19th century for sport fishing, and now is farmed there as well (Elvira, 1995). A few years ago, there was no evidence of self-sustaining rainbow trout populations in Spain (Doadrio, 2001), but it is expected that they will occupy river areas close to fish farms if escapes occur (Carss, 1990).

The native brown trout, although described as invasive in areas of the Southern Hemisphere, is catalogued as vulnerable in Spain because populations had been reduced by 20% at the end of the 20th century (Doadrio, 2001). The causes of its decrease are a combination of habitat losses, genetic introgression from introduced central European brown trout lineages, exotic species introductions and overfishing (Doadrio, 2001).

For the reasons above, the evaluation of native and invasive fish populations is essential in monitoring the health of an ecosystem (Arlinghaus, 2006). This can be difficult, especially when their density is low, using conventional sampling methods such as electrofishing and netting (Clusa et al., 2017). Moreover, these types of sampling encompass some mortality (Snyder, 2004) and are not suitable for some ecosystems such as those located within protected areas. On the other hand, environmental DNA (eDNA), defined as the genetic material obtained directly from environmental samples such as soil, sediment, water, etc. (Thomsen & Willerslev, 2015), can enable the detection of species that can be elusive or
difficult to sample. This technique is commonly used today for species detection (Dejean et al., 2012; Ardura et al., 2015; Ardura et al., 2016; Clusa et al., 2016; Devloo-Delva et al., 2016) and biodiversity inventory (Zaiko et al., 2015; Civade et al., 2016). It is a non-invasive sampling technique that avoids distress to the fish allowing for compliance with the European Code of Conduct for Research Integrity (Drenth, 2011).

Amongst the methods of molecular analysis of eDNA, quantitative PCR (qPCR) has been shown to be highly sensitive, particularly when determining the presence of rare or low-density species (Laramie, Pilliod & Goldberg, 2015). An alternative method is nested PCR, sometimes coupled with RFLP (Restriction Fragment Length Polymorphism), for example that described in Clusa et al. (2017) as a sensitive tool for detecting several salmonid species in water samples. In this study, the objectives were two-fold. On the technical side we have compared the sensitivity of the two methodologies (qPCR and nested PCR + RFLP) to detect eDNA of salmonids from running waters. On the ecological side we have employed these methods to assess possible escapes of farmed rainbow trout in a mountainous protected area, the Biosphere Reserve and Natural Park of Redes (Upper Nalón Basin, Asturias, Northern Spain), where the only native salmonid present is brown trout.

**METHODS**

**Ethics statement**

This project and the sampling carried out in protected spaces was authorized by the entity legally entitled to do so in Spain: the Government of the Asturias Principality, with the permit reference 101/16. The authors adhered to the European Code of Conduct for Research Integrity (Drenth, 2011).

**Study area-upper Nalón Basin**

The Upper Nalón Basin is located in the central part of the region of Asturias (Bay of Biscay, Spain). As part of the UNESCO (United Nations Educational, Scientific and Cultural Organization) Biosphere Reserve and Natural Park of Redes, it has a high faunal diversity (García-Ramos et al., 2006). In the streams (river headwaters) brown trout (Salmo trutta) are the only native fish species, because two consecutive impassable dams (Fig. 1) impede the arrival of native migratory European eel (Anguilla anguilla) and Atlantic salmon (Salmo salar), which occur downstream (Juanes et al., 2011). There is a rainbow trout fish farm located in one of the headwater streams in a zone denominated Veneros (Fig. 1), but escapes have not been reported and rainbow trout individuals were not recorded within the studied streams so far.

**Field methods and macroinvertebrates sampling**

Sampling took place in November 2015. Targeted sampling areas were typical juvenile habitat: shallow, well oxygenated and with moderate current velocity. At the sampling time of the year, in the particular river zones sampled trout juveniles around one-year-old are expected to be by far the most abundant life stage (Alonso, Gortázar & García de Jalón, 2010). Two sampling points were chosen along the upstream Nalón River (points n1, n2) and six on one of its head tributaries, Caleao river (c1 to c6; Fig. 1). The two streams are
connected at the tail end of Tanes reservoir (Fig. 1). In each sampling point, the habitat was characterized based on the official protocol of the Spanish Ministry of Environment, Feeding and Agriculture (Alba et al., 2005), to check if it was appropriate for salmonids. A 20-meter transect was analysed per site. A profile of the substrate was drawn, characterized as percentage of blocks, boulders, gravel, sand and silt. The dominant vegetation type covering the river (river canopy) was identified as well as the shade percentage and continuity. Three measures of depth and width were taken within the 20-meter transect at each sampled site. From these data, the total amount of juvenile salmonid (‘rearing’) habitat units were estimated for each site based on the model described by Juanes et al. (2011). All profile variables were considered to assign a percentage of juvenile’s habitat to each sampling point. Then, it was standardized to 100 m² (estimated rearing units or ERU) (Table S1).

Physical-chemical water properties were recorded with a Horiba U-50 multimeter at three different points in each sampled site. To minimize the potential for biased data (i.e.: after a storm) abiotic parameters were replicated three times with a seven-day interval between each reading and the average of these three measurements was presented. Measured parameters included water temperature (°C), pH and TDS (Total Dissolved Solids) (Table 1).

In addition to abiotic measures, macroinvertebrates communities were studied to calculate water quality indices. Individuals were sampled following the kick-net sampling methodology described in the protocol employed (Alba et al., 2005). Collected specimens were identified down to Family level using an identification key (Oscoz, Galicia & Miranda, 2011), and family presence-absence was recorded. Ecological quality ratio (EQR) and
Table 1  **Sampling Stations (November 2015)**. Physical-chemical features (TEMP: Temperature. TDS: Total Dissolved Solids). ERU: Estimated Rearing Units for Brown Trout. EQR: Ecological Quality Ratio, based on macroinvertebrate families. The ecological state based on macroinvertebrates was classified following the official protocol of Environment, Feeding and Agriculture Ministry (NIPO: 770-11-308-X), and is highlighted in yellow for Moderate and green for Good (Alba et al., 2005). Detection (Nested PCR-RFLP) and quantification (qPCR) of Trout eDNA.

| Station | Local name | Water course | Coordinates | TEMP (°C) | pH | TDS (g/L) | ERU | EQR | Ecological state | Number of trout DNA molecules (qPCR) | Nested PCR-RFLP |
|---------|------------|--------------|-------------|----------|----|-----------|-----|-----|-----------------|-------------------------------------|-----------------|
|         |            |              |             |          |    |           |     |     |                 | Rainbow trout DNA molecules | Brown trout DNA molecules | Rainbow trout eDNA presence/ no presence | Brown trout eDNA presence/ no presence |
| c1      | Arrudos    | Caleao river | 43°08'46.8N 5°24'47.9W | 9.07     | 7.90 | 0.11      | 20.09 | 0.62 | Good            | 0                                    | 1,964.85         | 0                | X                |
| c2      | Caliao     | Caleao river | 43°09'11.2N 5°24'24.0W | 9.41     | 8.13 | 0.11      | 69.78 | 0.48 | Moderate        | 0                                    | 3,119.54         | 0                | X                |
| c3      | Encruceyada| Caleao river | 43°09'24.9N 5°23'39.4W | 9.60     | 8.27 | 0.12      | 17   | 0.48 | Moderate        | 0                                    | 1,125.65         | 0                | X                |
| c4      | Puentepiedra| Caleao river | 43°10'12.2N 5°23'32.3W | 10.48    | 8.36 | 0.13      | 20.11 | 0.48 | Moderate        | 0                                    | 4,394.81         | 0                | X                |
| c5      | NA         | Caleao river | 43°10'26.8N 5°23'29.2W | 10.73    | 8.44 | 0.13      | 27.39 | 0.48 | Moderate        | 0                                    | 2,231.15         | 0                | X                |
| c6      | NA         | Caleao river | 43°11'16.2N 5°23'01.3W | 10.92    | 8.39 | 0.13      | 53   | 0.48 | Moderate        | 0                                    | 1,459.83         | 0                | X                |
| n3      | Veneros    | Nalón river  | 43°10'35.2N 5°19'55.6W | –        | –    | –         | –    | –    | –               | 6,121.93                         | 1,775.02         | X                | X                |
| n2      | El Campu   | Nalón river  | 43°10'51.3N 5°20'27.7W | 10.89    | 8.35 | 0.12      | 86.17 | 0.66 | Good           | 5,456.07                         | 1,679.35         | X                | 0                |
| n1      | Cueva Deboyu| Nalón river  | 43°10'41.0N 5°21'36.3W | 10.96    | 8.38 | 0.12      | 75   | 0.55 | Good           | 94,342.76                        | 2,307.53         | X                | 0                |
the ecological state of each point were calculated based on macroinvertebrates family’s composition, giving a punctuation to each family based on their tolerance to contamination (Alba et al., 2005) (Table 1).

One extra point was selected in Nalón River to collect only water samples (n3). Taken upstream n2 (where a rainbow trout fish farm is located, see Fig. 1), this water sample (n3) was collected 20 m upstream fish farm discharges as a control to discard the possibility that rainbow trout DNA comes only from fish farm runoff.

**Water sample collection, filtration and eDNA extraction**

Water samples of 1.5 L each were collected in sterile plastic bottles from the sampling points. Water samples were vacuum-filtered using a Supor®-200 Membrane Filter (Pall Corporation, Life Sciences, Ann Arbor, MI, USA) with 0.2 µm pore size. The filtration room was free of external sources of contamination as it was separated from the molecular laboratory. The filtration system was cleaned up with 10% commercial chlorine based-bleach between samples to avoid contamination between sampling points. 1.5 L of Milli-Q water that was previously transported with the rest of the water samples from the field, was filtered at last following the same steps, and was considered as an extra sample to monitor possible field or filtering contaminations. Finally, filters were placed into 15mL tubes using sterile forceps and stored at −20 °C until DNA extraction.

DNA was extracted from filters with PowerWater® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) and preserved at −20 °C until further processing. The DNA extractions were conducted under sterile conditions inside a laminar flow PCR-cabinet, following the manufacturer’s instructions. A negative control was added at this step to monitor contaminations during the extraction process.

**Quantitative PCR procedures**

Quantitative PCR from eDNA using specific primers has been validated for brown (Gustavson et al., 2015) and rainbow trout (Wilcox et al., 2015) (Table 2). Details about qPCR protocols are included in this section, as recommended by Bustin et al. (2009):

Two specific TaqMan assays were selected as molecular markers: for brown trout, a 61 base pairs (bp) fragment of the mitochondrial cytochrome oxidase I gene (COI: Cytochrome Oxidase Subunit I) (Gustavson et al., 2015); and a 102 bp fragment of the NADH gene for rainbow trout (Wilcox et al., 2015). In silico analysis were done using the Primer Blast application included in the NCBI webpage (Ye et al., 2012) to check the specificity of the markers. No coincidences were found with related nor cohabiting species.

Pre-PCR analyses of eDNA samples were carried out in a room separated from the molecular laboratory where there is no DNA nor tissue samples, inside a flow PCR-cabinet. Negative controls from the field, filtration and extraction processes were included in PCR runs as well as a PCR negative control.

The qPCR (quantitative Polymerase Chain Reaction) runs were performed using 7,900 HT Fast Real-Time PCR System (Life Technologies, Inc., Applied Biosystems, Carlsbad, CA, USA). Amplification reaction mixtures for brown trout included: 10 µl of TaqMan Environmental Master Mix 2.0 (Life Technologies, Carlsbad, CA, USA), 0.4 µl of each
Table 2. qPCR molecular markers. TaqMan assays employed in the qPCR analysis for each targeted species. Primers’ and hydrolysis probes’ sequences.

| Species          | Source                  | Gene | Primer | Sequence (5′-3′)                        | Amplicon(bp) |
|------------------|-------------------------|------|--------|----------------------------------------|--------------|
| Brown trout      | Gustavson et al. (2015) | COI  | Forward| TTTTG TTTGGCCGTGTAGT                   | 61           |
|                  |                         |      | Reverse| TGGCTAAACAGGGAGGAGGT                   |              |
|                  |                         |      | Probe  | ACCGCCGTCTCT                         |              |
| Rainbow trout    | Wilcox et al. (2015)    | NADH | Forward| AGTCTCCTCCGTATATGC                  | 102          |
|                  |                         |      | Reverse| GATTTAGTTCATGAAGTGTCCGTAGTA            |              |
|                  |                         |      | Probe  | 6FAM-CCAACAACCTCTTAACCATC-MGBNFQ       |              |

Primers (final concentration of 0.2 µM), and 0.4 µl hydrolysis probe (final concentration of 0.2 µM), and DNA template (6 µl of eDNA extracted from water samples, or from 43 ng of tissue DNA), up to a final 20 µl volume. Amplification reaction mixtures for rainbow trout included: 10 µl of TaqMan Environmental Master Mix 2.0 (Life Technologies, Carlsbad, CA, USA), 0.6 µl of Forward primer (final concentration of 0.3 µM), 1.2 µl of Reverse Primer (final concentration of 0.6 µM) and 0.5 µl of hydrolysis probe (final concentration of 0.25 µM) and DNA template (6 µl and 43 ng of eDNA and tissue DNA respectively), also up to a final 20 µl reaction volume.

As positive controls, DNA from tissue samples of each species were extracted with E.Z.N.A.® Tissue DNA Kit (Omega, Bio-Tek, Norcross, GA, USA) following the manufacturer’s instructions. The two molecular markers were tested first on control DNAs. A mixture of control DNAs from rainbow and brown trout at known concentrations were PCR amplified with the two specific markers to check for possible co-amplification or interference between them (Fig. S1). On each qPCR run, a positive control from tissue extractions of the targeted species was also added to monitor PCR inhibition.

PCR amplicons were generated with the two primer sets from tissue DNA in a total volume of 20 µl, including Green GoTaq® Buffer 1X, MgCl2, 0.25 mM dNTPS, 0.25 µM of each primer, 4 µl of template DNA and 0.65U of DNA Taq polymerase (Madison, WI, USA). PCR conditions were 95 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 57 °C for 30 s and 72 °C for 30 s, and a final step of elongation at 72 °C for 10 min.

The PCR amplicons obtained were quantified by fluorimetry using Qubit® dsDNA BR Assay Kit (Thermofisher Scientific, Carlsbad, CA, USA). The amount of DNA was transformed into molecules per µl, calculated from the known base composition of the amplicon sequence. A standard curve was constructed including a serial dilution (from 2.34 x 10⁹ to 2.34 x 10³ molecules/µL for rainbow trout and from 6.3 x 10⁹ to 6.3 x 10³ molecules/µL for brown trout) and used as reference for DNA molecules quantification in water samples. A dilution series from tissue DNA of each species was done to determine the lowest copy number of target DNA per litre of water detectable from each assay.

All the analyses of tissue and amplicon samples were conducted separately from environmental samples, keeping them away from any source of contamination.
**Table 3  PCR-RFLP Markers.** Primers’ sequences and amplicon’s length employed in the nested PCR assay for rainbow and brown trout eDNA detection.

| Nested PCR-RFLP | Gene   | Primer     | Source       | Sequence (5′–3′)                  | Amplicon (bp) |
|-----------------|--------|------------|--------------|----------------------------------|---------------|
| First-PCR       | 16S    | Forward    | *Clusa et al. (2017)* | GCCTGCCCTGTGACTATGG | 567           |
|                 |        | Reverse    | *Palumbi et al. (2002)* | CCGTCTGAACCTACATTG    |               |
|                 |        | Forward    | *Zaiko et al. (2015)* | AAGACCTGTATGAATGGCATC | 377           |
|                 |        | Reverse    | *Zaiko et al. (2015)* | TCGATAGGGACTCTGGGAGA  |               |

To confirm the correct target species detection, amplicons from some environmental samples were sequenced. Sequencing was carried out in the Sequencing Unit of University of Oviedo’s Scientific-Technical services.

**Species-specific PCR-RFLP**

The nested PCR-RFLP method was developed as described in *Clusa et al. (2017)* for detecting the two targeted species (brown and rainbow trout). Briefly: a first PCR was carried out to amplify a 567 bp fragment of the 16S rRNA gene, using as forward the 16S-new-F primer designed in the cited research (Table 3), and the reverse 16S-Br universal primer from *Palumbi et al. (2002)*. A nested PCR amplification was then performed with the pair of Salmonidae-specific primers described in *Zaiko et al. (2015)* (Table 3). The nested PCR product was digested with Taal and TruII FastDigest enzymes (Thermo Fisher Scientific Inc., Waltham, MA, USA) that produce diagnostic band patterns in agarose gel allowing to identify the two targeted species. With Taal enzyme, brown trout DNA gives two bands of 205 and 272 bp, and with TruII, rainbow trout DNA gives a band of 66 bp.

**RESULTS**

**Habitat quality measures**

The physical-chemical characteristics of the sampling points were within the optimum range for Salmonid juveniles (*Raleigh et al., 1984*; *Raleigh, Zuckerman & Nelson, 1986*). The numbers of juvenile salmonid habitat units (ERU) ranged between 17 in c4 and 86.17 in n2 (Table 1).

The ecological state measured from macroinvertebrate communities (EQR) was good at the points from the upper zone of Caleao River and in Nalon River (c6, n1 and n2), and moderate in the rest of the points. Detailed results of macroinvertebrate families along sampling stations are summarized in Table S2.

**qPCR assays**

Assays of control DNAs provided the same quantification cycle values in the samples with single species DNA as well as in the mixture DNA samples (17 cycles for rainbow trout marker and 15 cycles for brown trout marker; see Figs. S1A and S1B respectively). Thus, there were no interferences caused by the presence of DNA from the other species (Fig. S1).

Standard curves for brown and rainbow trout fitted the equations $y = -3.819x + 45.141$, $R^2 = 0.999$ and $y = -3.3128x + 39.2113$, $R^2 = 0.999$ respectively. The lowest detectable
number of copies was $7 \times 10^2$ and $2.6 \times 10^3$ molecules per liter for brown and rainbow trout assays respectively. From eDNA, negative controls were clean as expected i.e., no evidence of amplification. As positive amplification was obtained from all eDNA extraction samples for brown trout assay (see below), PCR inhibition testing was not performed.

Values of quantification obtained from river water samples varied from $94.34 \times 10^2$ to $11.26 \times 10^2$ trout DNA molecules (Table 1). Brown trout eDNA was found from all nine sampling sites. Rainbow trout eDNA occurred from the three points located in Nalón River (Table 1), one 20 m upstream (Veneros) and two downstream from the rainbow trout fish farm located in that river. The amount of rainbow trout eDNA molecules was considerably higher than that of brown trout in the Nalón River sampling points (n1 to n3), especially in n1 where the highest amount ($94342.76$ eDNA molecules) was found. This point is located in the lowest zone of the sampling area (Fig. 1).

**PCR-RFLP**

The restriction patterns within the fragment amplified provided specific bands for brown trout (205 and 172 bp) after digestion with Taal enzyme in all sampling points except n1 and n2 (Fig. 2A), where only the specific band for rainbow trout (66 bp) was found after digestion with Tru1I enzyme (Fig. 2B). Rainbow trout typical RFLP pattern was also found in n3 after Tru1I digestion (Fig. 2C).

**DISCUSSION**

In this study, we have detected eDNA signals of rainbow trout on running waters that can be interpreted as fish farm escapes within a protected area. As in other studies, environmental DNA again proved its value as a tool for species detection in freshwater environments (Jerde et al., 2011). Moreover, the probe-based qPCR was confirmed to be a highly sensitive technique that has the potential to offer quantification and biomass estimations (Gustavson et al., 2015). Although the PCR-RFLP technique employed here had demonstrated its sensitivity for salmonids detection in water samples (Clusa et al., 2017), it was not able to detect brown trout DNA in all the sampling points of the current study (n1 and n2), where it was detected from qPCR. The high amount of rainbow trout eDNA in these two sampling points, clearly more abundant than brown trout DNA, was a likely cause of the false brown trout negative found using PCR-RFLP technique. That technique is based on initial PCR amplification of a salmonids-specific DNA fragment which is the template for enzymatic digestion; the initial PCR amplification of the much more abundant rainbow trout DNA probably led to very weak undetectable brown trout bands (at least on agarose gels). PCR-RFLP technique has also another limitation compared with qPCR, which is the probability of cross-contamination, more probable when using a gel-based system rather than an enclosed system as qPCR is.

Indeed, the results obtained using PCR-RFLP reinforced the conclusion of widespread rainbow trout occurrence in the upper Nalón River (n1, n2 and n3) found from qPCR. The two methods have detected rainbow trout DNA from running waters upstream of an impassable dam, where rainbow trout is not expected to occur naturally. Moreover, fish farm escapes have not been officially reported in this area, nor have established populations...
been documented. There are rainbow trout fish farms downstream of Tanes reservoir, but it is an impassable barrier, thus arrival of escapes from downstream fish farms is impossible. Unreported fish farm escapes within the Biosphere Reserve, or DNA runoff from the fish farm (not individuals) would be alternative explanations. It is also possible that eDNA comes from predator transfers via depositing of carcasses or defecation (Merkes et al., 2014), but much more improbable. The occurrence of rainbow trout individuals (not just trout DNA from fish farm water discharges or runoffs) was strongly suggested here from rainbow trout DNA obtained in the sample taken as a control upstream of the fish farm drainage (n3), because runoff goes with the river current and floating DNA cannot move upstream. Thus, unreported escapes were a likely explanation of these results, which show how important is the location of sampling points in eDNA studies from running waters (Deiner & Altermatt, 2014; Jane et al., 2015). In theory, if escapes occur the escapees may interact with native brown trout inside the protected area, where the introduction of exotic
species is strictly prohibited from Spanish legislation (BOPA Decree 48/2006, of 18 of May, implementing the Spanish Law 5/1991, of 5 of April, for Protection of Natural Spaces).

One of the problems of eDNA-based methodology is the possible occurrence of false positives (Beja-Pereira et al., 2009). However, this is likely not applicable to the present study. Negative controls during samples transport and processing were carried out to monitor possible contamination. Possible interferences of species-specific qPCR on DNA mixtures, that may happen in waters containing diverse communities, were also tested in vitro and discarded. We verified that there were neither interferences nor co-amplifications between the DNA from the two targeted species, which validates the use of the selected markers for eDNA detection and quantification where the two target species are cohabiting rivers. This is an innovation over previous studies based on eDNA where only one species was targeted (Gustavson et al., 2015; Wilcox et al., 2015). In the nested PCR-RFLP assays, cross-contamination was carefully prevented, as described in Clusa et al. (2017). All our negative sampling and extraction controls were clean; thus contamination can be reasonably ruled out in this study.

The results of the present study could be taken as an alert signal of the presence of rainbow trout in the ecosystem. Although the presence of other exotic species is documented along Nalón River, the information about rainbow trout occurrence in the ecosystem is clearly insufficient. It is generally believed that there are no self-sustaining rainbow trout populations in Spain, but there are no specific studies about its naturalization (Stanković et al., 2015). The methodology employed in this study could serve for a wide and systematic monitoring of this species from all the Iberian rivers basins. One of the benefits of eDNA is allowing to monitor more sites in a faster and cheaper manner (Deiner et al., 2016; Bista et al., 2017), screening environments for potential species from which ground truthing (i.e., electrofishing or netting in the present case) can be conducted later to verify. The river part studied here exhibits a good habitat for salmonids (Juanes et al., 2011). The sampling points where rainbow trout eDNA was found had 161.17 ERUs and the best water quality. Since salmonids habitat is abundant and the water quality high, if escapes of rainbow trout occurred the species establishment would be favoured by those good conditions. This would have both direct and indirect impacts at multiple ecological levels (Mack et al., 2009 Stanković et al., 2015), and would be an enormous threat for the protected ecosystem because the impacts of invasive fish propagate rapidly beyond the habitat initially occupied (Baxter et al., 2004). One of the possible consequences could be the fragmentation of native fish populations (Fausch, 2007). Brown trout populations would be especially affected in this case because River Nalón is already dammed, and upstream populations are forcedly isolated from downstream settings. On the other hand, the introduction of farmed salmonids in the wild (escapes or deliberate releases) is often followed by interspecific hybridization with wild individuals, due to altered behavior of farmed fish (e.g., Horreo et al., 2014; Horreo et al., 2018). Since the survival of brown trout x rainbow trout hybrids is extremely low (Scheerer & Thorgaard, 1983), if hybridization happened the reproductive potential of native brown trout would be diminished. Considering all the risks above together, and the fact that small and isolated brown trout populations are especially
vulnerable (Mooney & Cleland, 2001), their conservation within the protected area might be endangered if rainbow trout escapes are not carefully controlled.

**Conclusion and implications for conservation**

From our results, and supporting Gustavson et al. (2015), qPCR is more effective for species detection when the eDNA abundance is low or if the number of eDNA molecules of two targeted species are very different. The use of qPCR for species detection in those cases would be recommended.

This is an example of the potential of eDNA for investigating the distribution of native and exotic fish in running waters. In this case, it served for surveying trout species inside a protected area, where sampling using conventional methodology (i.e., electrofishing or netting) should be disregarded as much as possible so as to not disturb vulnerable communities from upstream mountain landscapes. Therefore, we would recommend this strategy for routine monitoring and early detection of exotic species within natural reserves. Although the results support the occurrence of undeclared exotic rainbow trout, they should be confirmed by independent observations and conventional standard monitoring, to sample individuals and to check if rainbow trout presence is sporadic or on the contrary it reproduces in the river. We consider that a stricter control of the fish farm would be recommended, and efficient containment measures of farmed fish should be taken to prevent escapes.

**ACKNOWLEDGEMENTS**

The equipment and staff belonging to scientific-technical services of the University of Oviedo were crucial in the qPCR assays development for this study.

**ADDITIONAL INFORMATION AND DECLARATIONS**

**Funding**

Part of this research was supported by the Regional Government of Asturias, Grant for Excellence Groups GRUPIN-2014-093. Sara Fernández has received funding from the European Union’s Horizon 2020 Research and Innovation program under Grant Agreement No 689682 AMBER (Adaptive management of barriers in European rivers) project. Alba Ardura holds an incorporation-Juan de la Cierva fellowship. Laura Clusa holds a PCTI Grant from the Asturias Regional Government, referenced BP14-145. There was no additional external funding received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Grant Disclosures**

The following grant information was disclosed by the authors:
Regional Government of Asturias: GRUPIN-2014-093.
European Union’s Horizon 2020 Research and Innovation program: 689682.
Asturias Regional Government: BP14-145.
**Competing Interests**
Paul G. Beaulieu is an employee of Tighe & Bond, Trout Unlimited.

**Author Contributions**
- Sara Fernandez conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Miguel M. Sandin and Paul G. Beaulieu performed the experiments, analyzed the data, authored or reviewed drafts of the paper, approved the final draft, sampling.
- Laura Clusa conceived and designed the experiments, performed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Jose L. Martinez conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Alba Ardura conceived and designed the experiments, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Eva García-Vázquez conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.

**Field Study Permissions**
The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):

This project and the sampling carried out in protected spaces were authorized by the entity legally entitled to do so in Spain: The Government of the Asturias Principality, with the permit reference 101/16.

**Data Availability**
The following information was supplied regarding data availability:

The raw data are provided in the Supplemental Files.

**Supplemental Information**
Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.4486#supplemental-information.

**REFERENCES**
Alba J, Pardo I, Prat N, Pujante A. 2005. Metodología para el establecimiento el Estado Ecológico según la Directiva Marco del Agua. In: Protocolos de muestreo y análisis para invertebrados bentónicos. Zaragoza: Confederación Hidrográfica del Ebro-Ministerio de Medio Ambiente.
Alonso C, Gortázar J, García de Jalón D. 2010. Trucha común—Salmo trutta. In: Salvador A, Elvira B, eds. *Enciclopedia virtual de los vertebrados Españoles*. Madrid: Museo Nacional de Ciencias Naturales.

Ardura A, Zaiko A, Borrell YJ, Samuiloviene A, Garcia-Vazquez E. 2016. Novel tools for early detection of a global aquatic invasive, the zebra mussel Dreissena polymorpha. *Aquatic Conservation: Marine and Freshwater Ecosystems* 27(1):165–176 DOI 10.1002/aqc.2655.

Ardura A, Zaiko A, Martinez JL, Samulioviene A, Semenova A, Garcia-Vazquez E. 2015. eDNA and specific primers for early detection of invasive species—a case study on the bivalve Rangia cuneata, currently spreading in Europe. *Marine Environmental Research* 112:48–55 DOI 10.1016/j.marenvres.2015.09.013.

Arlinghaus R. 2006. Overcoming human obstacles to conservation of recreational fishery resources, with emphasis on central Europe. *Environmental Conservation* 33:46–59 DOI 10.1017/S0376892906002700.

Baxter CV, Fausch KD, Murakami M, Chapman PL. 2004. Fish invasions restructures stream and forest food webs by interrupting reciprocal prey subsides. *Ecology* 85:2656–2663 DOI 10.1890/04-138.

Beja-Pereira A, Oliveira R, Alves PC, Schwartz MK, Luikart G. 2009. Advancing ecological understandings through technological transformations in noninvasive genetics. *Molecular Ecology Resources* 9:1279–1301 DOI 10.1111/j.1755-0998.2009.02699.x.

Bista I, Carvalho GR, Walsh K, Seymour M, Hajibabaei M, Lallias D, Christmas M, Creer S. 2017. Annual time-series analysis of aqueous eDNA reveals ecologically relevant dynamics of lake ecosystem biodiversity. *Nature Communications* 8:14087 DOI 10.1038/ncomms14087.

Buria L, Albariño R, Díaz Villanueva V, Modenutti B, Balseiro E. 2010. Does predation by the introduced rainbow trout cascade down to detritus and algae in a forested small stream in Patagonia? *Hydrobiologia* 651(1):161–172 DOI 10.1007/s10750-010-0293-9.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry* 55:611–622 DOI 10.1373/clinchem.2008.112797.

Cambray JA. 2003. Impact on indigenous species biodiversity caused by the globalisation of alien recreational freshwater fisheries. *Hydrobiologia* 500:217–230.

Carss DN. 1990. Concentrations of wild and escaped fishes immediately adjacent to fish farm cages. *Aquaculture* 90:29–40 DOI 10.1016/0044-8486(90)90280-Z.

Civade R, Dejean T, Valentini A, Roset N. 2016. Spatial representativeness of environmental DNA metabarcoding signal for fish biodiversity assessment in a natural freshwater system. *PLOS ONE* 11(6):e0157366 DOI 10.1371/journal.pone.0157366.

Clusa L, Ardura A, Fernández S, Roca AA, García-Vázquez E. 2017. An extremely sensitive nested PCR-RFLP mitochondrial marker for detection and identification of salmonids in eDNA from water samples. *PeerJ* 5:e3045 DOI 10.7717/peerj.3045.
Clusa L, Ardura A, Gower F, Miralles L, Tsartsianidou V, Zaiko A, Garcia-Vazquez E. 2016. An easy phylogenetically informative method to trace the globally invasive potamopyrgus mud snail from river’s eDNA. *PLOS ONE* **11**(10):e0162899 DOI 10.1371/journal.pone.0162899.

Consuegra S, Phillips N, Gajardo G, De Leaniz CG. 2011. Winning the invasion roulette: escapes from fish farms increase admixture and facilitate establishment of non-native rainbow trout. *Evolutionary Applications* **4**:660–671 DOI 10.1111/j.1752-4571.2011.00189.x.

Deiner K, Altermatt F. 2014. Transport distance of invertebrate environmental DNA in a natural river. *PLOS ONE* **9**(2):e88786 DOI 10.1371/journal.pone.0088786.

Deiner K, Fronhofer EA, Mächler E, Walser J-C, Altermatt F. 2016. Environmental DNA reveals that rivers are conveyer belts of biodiversity information. *Nature Communications* **7**:12544 DOI 10.1038/ncomms12544.

Dejean T, Valentini A, Miquel C, Taberlet P, Bellemain E, Miaud C. 2012. Improved detection of an alien invasive species through environmental DNA barcoding: the example of the American bullfrog *Lithobates catesbeianus*. *Journal of Applied Ecology* **49**:953–959 DOI 10.1111/j.1365-2664.2012.02171.x.

Devloo-Delva F, Miralles L, Ardura A, Borrell YJ, Pejovic I, Tsartsianidou V, Garcia-Vazquez E. 2016. Detection and characterisation of the biopollutant *Xenostrobus securis* (Lamarck 1819) Asturian population from DNA Barcoding and eBarcoding. *Marine Pollution Bulletin* **105**:23–29 DOI 10.1016/j.marpolbul.2016.03.008.

Diehl S, Cooper SD, Kratz KW, Nisbet RM, Roll SK, Wiseman SW, Jenkins Jr TM. 2000. Effects of multiple, predator-induced behaviors on short-term producer-grazer dynamics in open systems. *The American Naturalist* **156**:293–313 DOI 10.1086/303390.

Doadrio I. 2001. Atlas y Libro Rojo de los Peces Continentales de España. Madrid: Dirección General de Conservación de la Naturaleza, Museo Nacional de Ciencias Naturales.

Drenth PJ. 2011. A European code of conduct for research integrity. *Available at http://www.allea.org/wp-content/uploads/2015/07/A-European-Code-of-Conduct-for-Research-Integrity_final.10.10.pdf*.

Eby LA, Roach WJ, Crowder LB, Stanford JA. 2006. Effects of stocking-up freshwater food webs. *Trends in Ecology & Evolution* **21**:576–584 DOI 10.1016/j.tree.2006.06.016.

Elvira B. 1995. Freshwater fishes introduced in Spain and relationships with autochthonous species. In: Philipp DP, Epifanía JM, Marsden JE, Claussen JE, eds. *Protection of aquatic biodiversity, Proc. of the world fisheries congress, Theme 3*. New Delhi: Oxford & IBH Publishing, 262–265.

Fausch KD. 2007. Introduction, establishment and effects of non-native salmonids: considering the risk of rainbow trout invasion in the United Kingdom. *Journal of Fish Biology* **71**:1–32 DOI 10.1111/j.1095-8649.2007.01682.x.

Garcia-Ramos JC, Jiménez-Sánchez M, Piñuela L, Domínguez Cuesta MJ, López Fernández C. 2006. *Patrimonio geológico en Asturias: la cuenca alta del río Nalón y la Cost a de los Dinosaurios*. Oviedo: Servitec.
Gustavson MS, Collins PC, Finarelli JA, Egan D, Conchuir R, Wightman GD, King JJ, Gauthier DT, Whelan K, Carlsson JEL, Carlsson J. 2015. An eDNA assay for Irish Petromyzon marinus and Salmo trutta and field validation in running water. *Journal of Fish Biology* 87:1254–1262 DOI 10.1111/jfb.12781.

Horreo J, Luis O, Turrero P, Perez J, García-Vázquez E. 2014. Long-term species balance in sympatric populations: implications for Atlantic salmon and brown trout. *Frontiers of Biogeography* 6:111–118.

Horreo JL, Valiente AG, Ardura A, Blanco A, Garcia-Gonzalez C, Garcia-Vázquez E. 2018. Nature versus nurture? Consequences of short captivity in early stages. *Ecology and Evolution* 8:521–529 DOI 10.1002/ece3.3555.

Jane SF, Wilcox TM, Mckelvey KS, Young MK, Schwartz MK, Lowe WH, Letcher BH, Whiteley AR. 2015. Distance, flow and PCR inhibition: EDNA dynamics in two headwater streams. *Molecular Ecology Resources* 15:216–227 DOI 10.1111/1755-0998.12285.

Jerde CL, Mahon AR, Chadderton WL, Lodge DM. 2011. “Sight-unseen” detection of rare aquatic species using environmental DNA. *Conservation Letters* 4:150–157 DOI 10.1111/j.1755-263X.2010.00158.x.

Juanes F, Gephard S, De J, Hoz L, Moran P, Dopico E, Horreo JL, Garcia-Vazquez E. 2011. Restoration of native Atlantic salmon runs in northern Spain: do costs outweigh benefits? *Knowledge and Management of Aquatic Ecosystems* 402:22 DOI 10.1051/kmae/2011078.

Landegren P. 1999. Spawning of anadromous rainbow trout, Oncorhynchus mykiss (Walbaum): a threat to sea trout, Salmo trutta L., populations? *Fisheries Research* 40:55–63 DOI 10.1016/s0165-7836(98)00215-x.

Laramie MB, Pilliod DS, Goldberg CS. 2015. Characterizing the distribution of an endangered salmonid using environmental DNA analysis. *Biological Conservation* 183:29–37 DOI 10.1016/j.biocon.2014.11.025.

Mack RN, Simberloff D, Lonsdale WM, Evans H, Clout M, Bazzaz FA. 2009. Biotic invasions: causes, epidemiology, global consequences, and control. *Ecological Applications* 10:689–710.

McIntosh AR, Townsend CR, Mcintosh AR, Townsend CR. 1996. Interactions between fish, grazing invertebrates and algae in a New Zealand stream: a trophic cascade mediated by fish-induced changes to grazer behaviour? *Oecologia* 108(1):174–181.

Meres CM, McCalla SG, Jensen NR, Gaikowski MP, Amberg JJ. 2014. Persistence of DNA in carcasses, slime and avian feces may affect interpretation of environmental DNA data. *PLOS ONE* 9:e113346 DOI 10.1371/journal.pone.0113346.

Mooney HA, Cleland EE. 2001. The evolutionary impact of invasive species. *Proceedings of the National Academy of Sciences of the United States of America* 98:5446–5451 DOI 10.1073/pnas.091093398.

Naylor R, Hindar K, Fleming IA, Goldburg R, Williams S, Volpe J, Whoriskey F, Eagle J, Kelso D, Mangel M. 2005. Fugitive salmon: assessing the risks of escaped fish from net-pen aquaculture. *BioScience* 55:427–437 DOI 10.1641/0006-3568(2005)055[0427:fsatro]2.0.co;2.
Oscoz J, Galicia D, Miranda R. 2011. Clave dicotómica para la identificación de macroinvertebrados de la Cuenca del Ebro. Journal of Chemical Information and Modeling 53:66 DOI 10.1017/CBO9781107415324.004.

Oscoz J, Leunda PM, Campos F, Escala MC, García-Fresca C, Miranda R. 2005. Spring diet composition of Rainbow Trout, Oncorhynchus mykiss (Walbaum, 1792) in the Urederra River (Spain). Annales de Limnologie–International Journal of Limnology 41:27–34 DOI 10.1051/limn/2005003.

Palumbi SR, Martin AP, Romano SL, McMillan WO, Stice L, Grabowski G. 2002. The simple fool's guide to PCR. Honolulu: Dept. of Zoology, University of Hawaii.

Raleigh RF, Hickman T, Solomon RC, Nelson PC. 1984. Habitat suitability information: rainbow trout. Washington, D.C.: US Fish and Wildlife Service.

Raleigh RF, Zuckerman LD, Nelson PC. 1986. Habitat suitability index models and instream flow suitability curves: brown trout. Federal Government Series FWSOBS 82/10.71. Washington, D.C.: US Fish and Wildlife Service.

Scheerer PD, Thorgaard GH. 1983. Increased survival in salmonid hybrids by induced triploidy. Canadian Journal of Fisheries and Aquatic Sciences 40:2040–2044 DOI 10.1139/f83-235.

Snyder DE. 2004. Invited overview: conclusions from a review of electrofishing and its harmful effects on fish. Reviews in Fish Biology and Fisheries 13:445–453 DOI 10.1007/s11160-004-1095-9.

Stanković D, Crivelli AJ, Snoj A, Stankovi D, Snoj AS. 2015. Rainbow trout in Europe: introduction, naturalization, and impacts. Reviews in Fisheries Science & Aquaculture 23:39–71 DOI 10.1080/23308249.2015.1024825.

Strayer DL. 2010. Alien species in fresh waters: ecological effects, interactions with other stressors, and prospects for the future. Freshwater Biology 55:152–174 DOI 10.1111/j.1365-2427.2009.02380.x.

Thomsen PF, Willerslev E. 2015. Environmental DNA—an emerging tool in conservation for monitoring past and present biodiversity. Biological Conservation 183:4–18 DOI 10.1016/j.biocon.2014.11.019.

Townsend CR. 2003. Individual, population, community, and ecosystem consequences of a fish invader in new zealand streams townsend multilevel effects of a fish invader 39. Conservation Biology 17:38–47 DOI 10.1046/j.1523-1739.2003.02017.x.

Valiente AG, Juanes F, Nuñez P, García-Vazquez E. 2007. Is genetic variability so important? Non-native salmonids in South America. Journal of Fish Biology 71:136–147 DOI 10.1111/j.1095-8649.2007.01674.x.

Wilcox TM, Carim KJ, Mckelvey KS, Young MK, Schwartz MK. 2015. The dual challenges of generality and specificity when developing environmental DNA markers for species and subspecies of Oncorhynchus. PLOS ONE 10:1–13 DOI 10.1371/journal.pone.0142008.

Wissinger SA, McIntosh AR, Ami H, Reig SSG. 2009. Impacts of introduced brown and rainbow trout on benthic invertebrate communities in shallow New Zealand lakes. Freshwater Biology 51:2009–2028 DOI 10.1111/j.1365-247200601629x.
Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics* **13**:134 DOI 10.1186/1471-2105-13-134.

Zaiko A, Martinez JL, Ardura A, Clusa I, Borrell YJ, Samuiloviene A, Roca 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 DOI 10.1016/j.marenvres.2015.07.002.