Universidad de Oviedo

Departamento de Ingeniería Química y Tecnología del Medio Ambiente

Programa Oficial de Doctorado en Ingeniería Química, Ambiental y Bioalimentaria

Incidencia de las invasiones piscícolas y sus impactos a nivel ecológico y sanitario sobre los recursos autóctonos en ríos de la región asturiana

Incidence of fish invasions and their impacts to ecological and health standards on indigenous resources in rivers of Asturias

Tesis Doctoral

Laura Clusa Cuesta

Directores: Eva García Vázquez y Eduardo Dopico Rodríguez

Oviedo 2018

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RESUMEN (en Inglés)

Rivers are one of the most important water resources on the planet. Fluvial fish species represent an important economic and ecological resource, such as the Atlantic salmon and the Brown trout. Many of their populations are threatened or in decline due to the degradation of their ecosystems and the introduction of exotic species.

In Spain there are a total of 45 freshwater exotic species, more than 60% are fish species, and 15% are non-arthropod invertebrates, mainly molluscs. In Spanish watersheds, most of the exotic species come from America or Asia but also some allochthonous introductions come from translocations between basins of different latitudes within the Iberian Peninsula. The early detection of these invasive species is an urgent need in order to develop measures to mitigate their impact. Sometimes this early detection is not easy, for this reason, alternatives methods have been developed, among which are techniques based on environmental DNA, and citizen involvement as a warning method.

The main objectives of this PhD Thesis consisted in developing specific markers for detecting invasive freshwater fish and mollusc species in Spain, their validation in water samples, assessing their performance in Europe, and evaluate the human factor in the introduction of exotic species, including knowledge of local population about them.

The results are reflected in seven scientific articles. For the design of specific markers, characteristic regions for each species were searched for in the cytochrome oxidase subunit I (COI) and 16SrDNA genes and specific markers were designed for thirteen invasive species in Europe: the catfish *Ameirus* sp (both for *A. melas* and *A. nebulosus*), the mosquito fish *Gambusia* sp (*G. holbrooki* and *G. affinis*), the black bass *Micropterus salmoides*, the pumpkinseed *Lepomis gibbosus*, the stone moroko *Pseudorasbora parva*, the New Zealand mudsnail *Potamopyrgus antipodarum*, the Asian clam *Corbicula* sp (*C. fluminea* and *C. fluminalis*), the red-rimmed melania *Melanoides tuberculata*, the Chinese pond mussel *Sinanodonta woodiana* and the Conrad's false mussel *Mytilopsis leucophaeata*.

The markers were validated in environmental DNA (eDNA) extracted from water samples obtained in several basins of the Iberian Peninsula: Ebro, Guadalquivir, Tajo and North. The same markers were successfully applied in the region of Lake Constance in Germany recording its effectiveness in other latitudes. Using next generation sequencing techniques (NGS) based on universal primers to amplify a region of the COI gene; it was possible to detect other invasive aquatic species. Comparing both methods, specific markers were more sensitive than NGS, highlighting their usefulness in the early detection of invasive species. Finally, a specific marker for salmonids was designed in the 16SrDNA gene, based on nested PCR and subsequent digestion of the fragment amplified with restriction enzymes. The marker was used to evaluate the salmonids present in the Nalón River.

The role of river barriers has been evaluated, using the Nalón River as a case study. Barriers negatively affected native species but also stopped the expansion upstream of exotic species. The ports, the areas between reservoirs and the areas of the rivers near big cities have been identified as the main hotspots of exotic species introduction that should be a priority objective of environmental monitoring and surveillance according to the results of this Thesis.

In the same way, Citizen science helped with the detection of exotic species in this work. The results of local citizens' surveys showed a limited knowledge of exotic species in Asturias. However, a positive attitude towards the eradication of these species and a high awareness to preserve the autochthonous species was detected.

Moreover the health, ecological and economic risks derived from the exotic species found in Asturias were described.

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FORMULARIO RESUMEN DE TESIS POR COMPENDIO

1.- Datos personales solicitante

Apellidos: Clusa Cuesta	Nombre: Laura

Curso de inicio de los estudios de doctorado	2014/2015

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Introducción justificativa de la unidad temática y objetivos	X	
Copia completa de los trabajos *	X	
Resultados/discusión y conclusiones	X	
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Artículos, Capítulos, Trabajos

Trabajo, Artículo 1

Titulo (o título abreviado)	An easy phylogenetically informative method to trace the globally invasive Potamopyrgus mud snail from river's eDNA
Fecha de publicación	5 de Octubre de 2016
Fecha de aceptación	30 de Agosto de 2016
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Si, revista PLoS ONE incluida en Science Citation Index
Factor de impacto	2,806

Coautor2 X Doctor ☐ No doctor . Indique nombre y apellidos	Dra. Alba Ardura
Coautor3 X Doctor ☐ No doctor . Indique nombre y apellidos	Dra. Fiona Gower
Coautor4 X Doctor ☐ No doctor . Indique nombre y apellidos	Dra. Laura Miralles
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Trabajo, Artículo 2

Titulo (o título abreviado)	An extremely sensitive nested PCR-RFLP mitochondrial marker for detection and identification of salmonids in eDNA from water samples
Fecha de publicación	28 de Febrero 2017
Fecha de aceptación	30 de Enero de 2017
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Si, revista PeerJ incluida en Science Citation Index
Factor de impacto	2,117

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Coautor4 X Doctor ☐ No doctor .	Indique nombre y apellidos	Dr. Agustín Roca
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Coautor6 ☐ Doctor ☐ No doctor .	Indique nombre y apellidos	
Coautor7 ☐ Doctor ☐ No doctor .	Indique nombre y apellidos	

Trabajo, Artículo 3

Titulo (o título abreviado)	eDNA for detection of five highly invasive molluscs. A case study in urban rivers from the Iberian Peninsula
Fecha de publicación	15 de Noviembre de 2017
Fecha de aceptación	1 de Noviembre de 2017
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Si, revista PLoS ONE incluida en Science Citation Index
Factor de impacto	2,806

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Trabajo, Artículo 4

Titulo (o título abreviado)	Public knowledge of alien species: a case study on aquatic biodiversity in North Iberian rivers
Fecha de publicación	13 de Febrero de 2018
Fecha de aceptación	9 de Enero de 2018
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Si, revista Journal for Nature Conservation incluida en Science Citation

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Trabajo, Artículo 5

	A simple, rapid method for detecting seven common invasive fish species in Europe from environmental DNA
Titulo (o título abreviado)	
Fecha de publicación	En producción
Fecha de aceptación	15 de Enero de 2018
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Sí, revista Aquatic Conservation: Marine and Freshwater ecosystems incluida en Science Citation Index
Factor de impacto	3,130

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Coautor6 □ Doctor □ No doctor . Indique nombre y apellidos	
Coautor7 □ Doctor □ No doctor . Indique nombre y apellidos	

Trabajo, Artículo 6

Titulo (o título abreviado)	
Fecha de publicación	
Fecha de aceptación	
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	
Factor de impacto	

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En caso de compendio de un número de artículos superior a seis, se incorporarán hojas suplementarias conforme a este modelo

15-Jan-2018

Dear Ms Clusa,

I am pleased to tell you that your manuscript entitled "A simple, rapid method for detecting seven common invasive fish species in Europe from environmental DNA" has been accepted for publication in Aquatic Conservation: Marine and Freshwater Ecosystems.

Article ID: AQC2890
Article DOI: 10.1002/aqc.2890
Internal Article ID: 15014510

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Professor Philip J Boon
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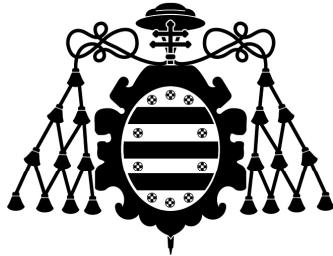
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Tesis doctoral

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Oviedo 2018
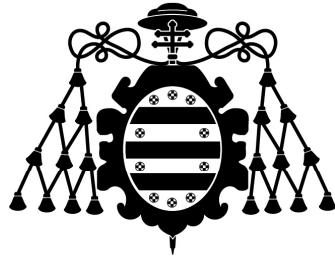
Universidad de Oviedo

Department of Chemical Engineering and Environmental Technology

PhD Programme in Chemical, Environmental and Bio-Food Engineering

Incidence of fish invasions and their impacts to ecological and health standards on indigenous resources in rivers of Asturias

PhD Thesis

Laura Clusa Cuesta

Oviedo 2018

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Como cada paso en la vida, cuando se cierra una etapa tan intensa hay muchas personas que merecen nuestra gratitud. A lo largo de estos 3 años y medio yo también he de reconocer el apoyo de un montón de personas y espero no olvidarme de ninguna:

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sonrisa y estar siempre dispuesta a ayudarme. Estoy segura de que me llevo una amiga para toda la vida.

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A ti, por creer en mí y permitirme soñar despierta,

*Mamá, papá, Isa, Miguel, Sven y Joana*

"La ciencia es un cementerio de ideas muertas, aunque de ellas puede salir vida"

Unamuno

"Yo soy de las que piensan que la ciencia tiene una gran belleza, un científico en su laboratorio no es sólo un técnico, también es un niño colocado ante fenómenos naturales que lo impresionan como un cuento de hadas"

Marie Curie

Índice

Índice

Resumen/Summary

Resumen

Los ríos son uno de los recursos hídricos más importantes del planeta; desde el comienzo de la humanidad, los primeros poblados se establecieron en torno a ríos y fuentes de agua. Todavía hoy las especies piscícolas fluviales representan un recurso económico y ecológico de prioridad para las zonas rurales. Particularmente, el salmón atlántico *Salmo sala*r y la trucha común europea *Salmo trutta* son especies bandera características del Arco Atlántico, donde muchas de sus poblaciones están amenazadas o en declive debido, entre otros factores, a la degradación de los ecosistemas y la introducción de especies exóticas.

En España hay un total de 45 especies exóticas de río, de las cuales el 60% son piscícolas, seguidas de un 15% de invertebrados no artrópodos, principalmente moluscos. Las especies introducidas compiten con las nativas por los recursos y el espacio, reducen la calidad del agua, desplazan a las especies nativas, transmiten enfermedades y, en general, son más resistentes a condiciones desfavorables. La mayoría de las especies exóticas de los ríos españoles son importadas de América o Asia, y algunas introducciones alóctonas provienen de translocaciones entre cuencas de distintas latitudes dentro la Península Ibérica. En todos los casos se producen pérdidas de la biodiversidad nativa local. La detección temprana de estas especies invasoras que puedan poner en peligro la integridad de los ecosistemas es por tanto una necesidad urgente para poder tomar a tiempo medidas conducentes a la mitigación de su impacto. A veces, esta detección temprana no es fácil *de visu*, especialmente si las especies exóticas están en muy baja densidad, son miméticas o tienen hábitos nocturnos. Se han propuesto métodos alternativos para su detección precoz, entre los que se encuentran las técnicas basadas en el ADN ambiental y la implicación ciudadana como método de alerta, al ser los usuarios de los ríos observadores cotidianos de los mismos. Por todo ello, los principales objetivos de esta Tesis Doctoral fueron el desarrollo de marcadores específicos para la detección de las especies invasoras de peces y moluscos de agua dulce en España y su validación en muestras de agua, además de su aplicabilidad en Europa, así como también la evaluación del factor humano en la introducción de especies exóticas, incluyendo las actuaciones en los cauces de los ríos y el nivel de conocimiento público acerca de las especies exóticas acuáticas.

Los resultados se reflejan en siete artículos científicos. Para el diseño de marcadores específicos, se buscaron regiones características para cada especie en los genes citocromo oxidasa subunidad I (COI) y 16SrDNA y se consiguieron diseñar marcadores específicos para las siguientes especies piscícolas: el pez gato *Ameirus* sp (tanto para *A. melas* como *A. nebulosus*), el pez mosquito *Gambusia* sp (*G. holbrooki* y *G. affinis*), la perca americana *Micropterus salmoides*, el pez sol *Lepomis gibbosus* y la rasbora *Pseudorasbora parva*. Y las siguientes especies de moluscos: el caracolillo del cieno neozelandés *Potamopyrgus antipodarum*, la almeja asiática *Corbicula* sp (*C. fluminea* y *C. fluminalis*), el caracol trompeta *Melanoides tuberculata*, la almeja china del cieno *Sinanodonta woodiana* y el falso mejillón de Conrad *Mytilopsis leucophaeata*. Todas ellas son invasoras en España y también en el resto de Europa. Los marcadores fueron validados en ADN ambiental extraído de muestras de varias cuencas de la Península Ibérica de diferentes dimensiones y características: Ebro, Guadalquivir, Tajo, ríos costeros de la cuenca Cantábrica. La diversidad de condiciones ecológicas confirma la versatilidad de los protocolos diseñados. Los mismos marcadores se aplicaron con éxito en la región del Lago Constanza y el río Rin en Alemania para seguir el patrón de invasión de *P. antipodarum* y *C. fluminea*, dejando constancia así de su eficacia en otras latitudes. Empleando técnicas de secuenciación masiva (NGS) basadas en cebadores universales para amplificar una región del gen COI y secuenciación en la plataforma Illumina, se consiguió detectar otras especies acuáticas invasoras, como la gamba asesina *Dikerogammarus villosus*, la medusa de

agua dulce *Craspedacusta sowerbyi* y el mejillón cebra *Dreissena polymorpha*. Comparando ambos métodos, los marcadores específicos fueron más sensibles que la NGS, resaltando su utilidad en la detección temprana de especies invasoras. También se diseñó un marcador específico de salmónidos en el gen 16SrDNA, basado en PCR (reacción en cadena de la polimerasa) anidada y posterior digestión del fragmento amplificado con enzimas de restricción. Este marcador fue validado en ADN ambiental, y empleado para evaluar los salmónidos presentes en el río Nalón.

Se han identificado los puertos, las zonas entre embalses y las áreas cercanas a grandes urbes como los principales focos de introducción de especies exóticas fluviales, que deberían ser objetivo prioritario de monitoreo y vigilancia medioambiental. En el río Nalón, como estudio de caso, se ha comprobado que las barreras fluviales afectan negativamente a las especies nativas, pero también frenan la expansión de especies exóticas aguas arriba. Los tramos situados entre embalses son los que contienen más especies alóctonas, y deberían considerarse objetivos prioritarios de restauración según los resultados de esta Tesis.

La ciencia ciudadana también ha ayudado a la detección y localización de especies exóticas en este trabajo. Los resultados de encuestas a ribereños mostraron un limitado conocimiento público de las especies fluviales exóticas locales en Asturias; sin embargo, se encontró una actitud positiva hacia la erradicación de estas especies y una alta concienciación pública para preservar la naturaleza autóctona. Estas actitudes respecto a las invasiones biológicas podrían ayudar a reducir o prevenir nuevas introducciones, y son una base para planificar futuros programas de ciencia ciudadana para su detección temprana.

El conjunto de esta Tesis Doctoral ha permitido desarrollar las herramientas genéticas necesarias para la detección temprana de trece especies invasoras en España, sentar las bases para futuros programas de ciencia ciudadana, identificar las zonas de riesgo de introducción de especies exóticas, e inferir los riesgos sanitarios, ecológicos y económicos derivados de las especies encontradas.

Summary

Rivers are one of the most important water resources on the planet, since the beginning of humanity the first settlements were established around rivers and water sources. In addition, fluvial fish species represent an important economic and ecological resource for rural areas. Particularly the Atlantic salmon *Salmo salar* and the Brown trout *Salmo trutta* are characteristic species of the Atlantic Arc. Many of their populations are threatened or in decline due to different factors among which are the degradation of their ecosystems and the introduction of exotic species.

In Spain, there are a total of 45 freshwater exotic species, more than 60% are fish species, followed by 15% of non-arthropod invertebrates, mainly molluscs. New introduced species compete with native species for resources and space, reduce water quality, displace native species, transmit diseases and, in general, are more resistant to degraded habitats. In Spanish watersheds, most of the exotic species come from America or Asia but also some allochthonous introductions come from translocations between basins of different latitudes within the Iberian Peninsula. In all cases losses of local native biodiversity is produced. The early detection of these invasive species that may affect the ecosystem is therefore an urgent need in order to develop measures to mitigate their impact. Sometimes this early detection is not easy, especially if the invasive species are in very low density, are mimetic or have nocturnal habits. For this reason, alternatives methods have been developed, among which are techniques based on environmental DNA, and citizen involvement as a warning method, since the rivers' users are daily observers of them. Therefore, the main objectives of this PhD Thesis consisted in developing specific markers for detecting invasive freshwater fish and mollusc species in Spain, their validation in water samples and assessing their performance in Europe. Evaluate the human factor in the introduction of exotic species, including actions in riverbeds and the exotic aquatic species knowledge of local population.

The results are reflected in seven scientific articles. For the design of specific markers, characteristic regions for each species were searched for in the genes cytochrome oxidase subunit I (COI) and 16SrDNA and specific markers were designed for the following fish species: the catfish *Ameirus* sp (both for *A. melas* and *A. nebulosus*), the mosquito fish *Gambusia* sp (*G. holbrooki* and *G. affinis*), the black bass *Micropterus salmoides*, the pumpkinseed *Lepomis gibbosus* and the stone moroko *Pseudorasbora parva*. And the following species of mollusks: the New Zealand mudsnail *Potamopyrgus antipodarum*, the Asian clam *Corbicula* sp (*C. fluminea* and *C. fluminalis*), the red-rimmed melania *Melanoides tuberculata*, the Chinese pond mussel *Sinanodonta woodiana* and the Conrad's false mussel *Mytilopsis leucophaeata*. All of them are invasive in Spain and also in the rest of the European territory. The markers were validated in environmental DNA (eDNA) extracted from water samples obtained in several basins of the Iberian Peninsula of different dimensions and characteristics: Ebro, Guadalquivir, Tajo, coastal rivers of the Cantabrian basin. The same markers were successfully applied in the region of Lake Constance and the Rhine River in Germany to follow the invasion pattern of *P. antipodarum* and *C. fluminea*, thus recording its effectiveness in other latitudes. Using next generation sequencing techniques (NGS) based on universal primers to amplify a region of the COI gene and sequencing on the Illumina platform, it was possible to detect other invasive aquatic species, such as the killer shrimp *Dikerogammarus villosus*, the freshwater jellyfish *Craspedacusta sowerbyi* or the zebra mussel *Dreissena polymorpha*. Comparing both methods, specific markers were more sensitive than NGS, highlighting their usefulness in the early detection of invasive species. Finally, a specific marker of salmonids was designed in the 16SrDNA gene, which by nested PCR (polymerase chain reaction) and

subsequent digestion of the fragment amplified with restriction enzymes allows. The marker was validated in eDNA and used to evaluate the salmonids present in the Nalón River.

In addition, the ports, the areas between reservoirs and the areas of the rivers near big cities have been identified as the main hotspots of exotic species introduction that should be a priority objective of environmental monitoring and surveillance. The role of river barriers has been evaluated, using the Nalón River as a case study. Barriers negatively affected native species but also stopped the expansion upstream of exotic species. The sections located between dams are those that contain more allochthonous species, and should be considered priority restoration objectives according to the results of this Thesis.

In the same way, Citizen science helped with the detection of exotic species in this work. The results of local citizens surveys showed a limited knowledge of exotic species in Asturias. However, a positive attitude towards the eradication of these species and a high awareness to preserve the autochthonous species was detected. These attitudes regarding biological invasions could help reduce or prevent new introductions, and are a basis for planning future citizen science programs for early detection.

This PhD Thesis allowed the develop of genetic tools for the early detection of thirteen invasive species in Spain, to explore the local knowledge about exotic species in the region, to lay the foundations for future citizen science programs, to identify the zones of risk of introduction of exotic species, as well as the health, ecological and economic risks derived from the species found.

Introducción
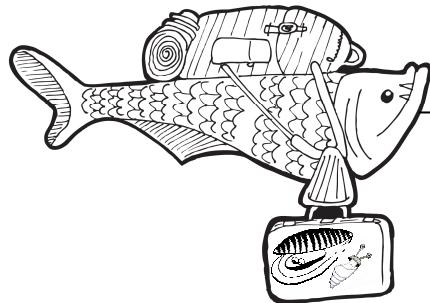
1. Los ríos y el transporte de especies

Los ríos son de vital importancia para la humanidad y uno de los recursos hídricos más importantes del planeta. Los primeros humanos establecieron sus asentamientos en torno a ellos. El establecimiento de rutas comerciales y el desarrollo industrial alteraron sus cauces y provocaron cambios en su flora, fauna y en las condiciones de explotación de los ríos como recurso. Progresivamente, esta globalización y la permanente actividad humana han provocado la aparición de especies no indígenas o no autóctonas (*Non-Indigenous Species*, NIS) en la mayoría de los ríos (Hulme 2009).

Las especies no autóctonas (también llamadas exóticas, *aliens* o especies no nativas) son especies que se encuentran fuera de su hábitat nativo y se transportan debido a actividades humanas. En algunos casos estas especies exóticas pueden proliferar, aumentar exponencialmente su población y dispersarse rápidamente en el nuevo ecosistema produciendo graves efectos en el nuevo hábitat y en la fauna residente, convirtiéndose en especies exóticas invasoras (EEI; Figura 1) (Occhipinti-Ambrogi y Galil 2004).
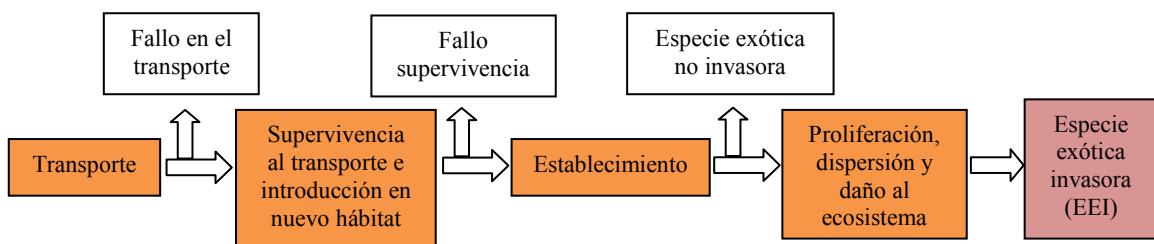
Figura 1. Etapas de la invasión. Procesos que deben superar las especies no autóctonas para convertirse en especies invasoras (señalados en naranja) basado en Kolar y Lodge (2001).

El transporte de estas especies es tan amplio que amenazan ecosistemas enteros alejados de su hábitat nativo. A nivel mundial, por ejemplo, en Australia la introducción de la gramínea africana (*Brachiura mutica*) altera toda la hidrología de los ríos (Bunn *et al.* 1998). Del mismo modo, en Australia y Nueva Zelanda la introducción de la trucha arco iris (*Oncorhynchus mykiss*) y de la trucha común (*Salmo trutta*) respectivamente están desplazando las poblaciones nativas de peces de la familia Galaxiidae (Cambray 2003; Townsend 2003). El mejillón cebra en el río Hudson en América consiguió alterar y transformar todo el ecosistema al tener una alta tasa de filtración y acumular grandes densidades (Strayer 2010). Más cerca, en Europa las especies más ampliamente distribuidas son dos especies piscícolas: el carpín (*Carassius auratus*) y la trucha arco iris (*O. mykiss*), teniendo poblaciones estables en 29 y 28 países europeos respectivamente (Savini *et al.* 2010). Además la introducción de otras especies piscívoras como son la perca americana (*Micropterus salmoides*), el lucio (*Esox lucius*) y el siluro (*Silurus glanis*) en la Península Ibérica está causando graves estragos en las poblaciones nativas de peces (García-Berthou *et al.* 2015).

2. Especies exóticas en España

España se divide en diez cuencas hidrográficas (Figura 2): la cuenca Norte que comprende la costa cantábrica, desde el río Bidasoa hasta el río Navia en Asturias; la cuenca de Galicia que va desde el río Eo hasta el río Limia; las cuencas del Duero, del Tajo, del Guadiana, del Guadalquivir y del Ebro; la cuenca del Sur desde el río Guadalete hasta el río Segura; la cuenca de Levante desde el río Vinalopó hasta el río Cenia; y finalmente la cuenca de Cataluña que comprende las de los ríos Ter y Llobregat (Doadrio *et al.* 1991).

Hay un total de 45 especies exóticas de agua dulce introducidas por toda la geografía española (Anexo I), siendo la cuenca del Ebro la más invadida con 34 especies exóticas, seguida de Cataluña y la cuenca del Tajo (Anexo I).

Figura 2. Hidrografía española. En la imagen se muestran las cuencas hidrográficas principales. Imagen modificada de Miranda y Pino del Carpio (2016).

Son las especies de peces y moluscos de agua dulce las que interesan en la presente Tesis Doctoral. De las 45 especies exóticas de río presentes en España, más de un 60% (28 especies) son especies piscícolas, seguidas de un 15% de invertebrados no artrópodos, principalmente moluscos (Figura 3).

La proporción de los diferentes taxones de especies exóticas también varía según las diferentes cuencas hidrográficas. Por ejemplo, más del 70% de especies introducidas en la cuenca del Guadiana son peces (14 especies de peces de 19 en total), mientras que en la cuenca de Galicia, con un menor número de especies exóticas, el 21% son especies de invertebrados no artrópodos (3 especies de 14 en total) (Figura 3) (Anexo I).
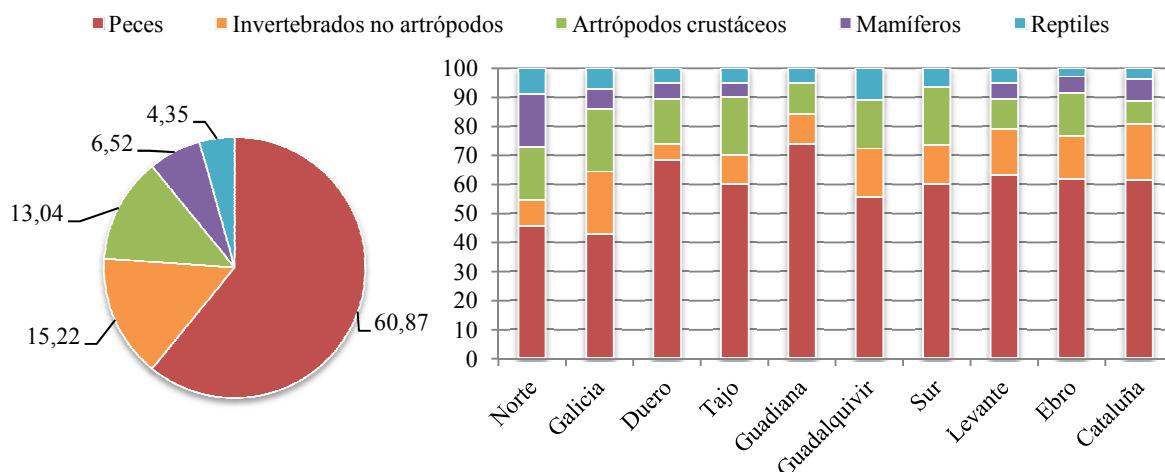
Figura 3. Especies exóticas de agua dulce presentes en España. En la figura de la izquierda se muestra el porcentaje de los distintos grupos taxonómicos de especies presentes en España a nivel global y a la derecha clasificados según las diferentes cuencas hidrográficas. Datos derivados del Anexo I.

No hay ninguna cuenca con menos del 40% de peces entre las especies introducidas (Figura 3). El número de especies piscícolas introducidas se ha triplicado en los últimos 60 años. Las cuencas con mayor riqueza de especies piscícolas (Ebro, Tajo y Júcar) también son las que mayor número de especies no nativas poseen (Miranda y Pino del Carpio 2016). Por ejemplo, en la cuenca Norte el número de peces exóticos ha pasado de 3 en 1952 a 4 en 1992 (Elvira 1995) y a 5 en la actualidad (Anexo I), mientras que en la cuenca del Ebro ha pasado de 5 en 1952 a 14 en 1992 (Elvira 1995) y a 21 en la actualidad (Anexo I). Ya en 2005 García-Berthou *et al.* confirmaron que los peces de agua dulce son las especies introducidas con mayor frecuencia en Europa y que sólo unas pocas son las responsables de la homogeneización de los ecosistemas: *Carassius auratus, Cyprinus carpio, Micropterus salmoides, Gambusia holbrooki, Oncorhynchus mykiss* (Toussaint *et al.* 2016). Muchas de ellas fueron introducidas en las últimas décadas en España (Elvira y Almodóvar 2001). Estas introducciones han hecho que el número de especies piscícolas nativas amenazadas en España sea de los más altos de Europa, con más del 80% de las especies clasificadas como vulnerables o en peligro (Clavero *et al.* 2004), además de ser un país donde más del 65% de especies piscícolas son endémicas (Miranda y Pino del Carpio 2016).

3. Normativa

Respecto a la legislación europea, el 22 de octubre de 2014 se creó el Reglamento UE Nº 1143/2014 del Parlamento Europeo y del Consejo de la Unión Europea sobre la prevención y la gestión de la introducción y propagación de especies exóticas invasoras. Esta normativa establece una lista de las especies exóticas invasores más dañinas y comunes a la región europea, además de las normas para su correcta identificación, manejo y detección (http://ec.europa.eu/environment/nature/invasivealien/index_en.htm). En concreto en su artículo 14, establece que cualquier Estado miembro debe mantener un sistema de vigilancia activa para detectar rápidamente la aparición de especies exóticas invasoras en sus ecosistemas. La Comisión Europea cuenta con una red de información de especies exóticas (European Alien Species Information Network, EASIN) puesta a disposición de los gobiernos y ciudadanos (https://easin.jrc.ec.europa.eu/).

En España, el 2 de agosto de 2013, se promulgó el Real Decreto 630/2013, por el que se regula el Catálogo español de especies exóticas invasoras (https://www.boe.es/buscar/act.php?id=BOE-A-2013-8565). La última versión del catálogo (versión actualizada del 17 de junio de 2016) incluye un listado de todas las especies invasoras en el territorio español con la consiguiente prohibición de su venta, posesión, transporte, introducción, comercio de ejemplares vivos o muertos y adquisición en todo el territorio, en el cual se añadieron seis especies, entre ellas *Cyprinus carpio* y *Oncorhynchuss mykiss*, excluidas de la primera versión. Además, en el capítulo III, artículo 8, se establecen las medidas a cumplir por todas las Comunidades Autónomas sobre prevención y seguimiento de estas especies.

4. Modo de introducción de EEI

El transporte de especies acuáticas deriva principalmente de la acción humana (Havel *et al.* 2015). Los seres humanos transportan estas y otras especies de forma intencionada, accidental o mediante diversos vectores. La mayoría de las especies exóticas que se encuentran en Europa provienen de América o Asia y, una vez llegadas al continente, hay redes entre los distintos países (generalmente de usuarios, pescadores, acuicultores, comercios de acuarofilia, como se verá a continuación) que promueven su dispersión. Los países centroeuropeos, entre ellos

Alemania y Francia, han sido puertas de entrada y de posterior diseminación de muchas de estas especies. Por ejemplo, en España diez especies exóticas acuáticas que son originarias de América se introdujeron a través de Francia (García-Berthou *et al.* 2005). El transporte de la trucha arco iris (*O. mykiss*) a Austria, Bulgaria, Dinamarca, Polonia y más países del este de Europa se produjo por la translocación de esta especie desde piscifactorías alemanas (Stanković *et al.* 2015), del mismo modo la introducción del siluro (*Silurus glanis*) en 1974 en la cuenca del Ebro en la Península Ibérica, tuvo su origen en Alemania (Doadrio 2001; Carol 2007).

Las principales causas de introducción de especies de peces son la pesca deportiva, ya sea como objeto de pesca o como cebo, la acuicultura, la acuariofilia, para su uso ornamental u otros más específicos (Fuller 2015) (Anexo I). Por citar unos pocos ejemplos, la perca americana (*Micropterus salmoides*) y el pez gato (*Ameiurus melas*) (Figura 4) se introdujeron en Europa en muchos embalses y ríos para pesca deportiva (Copp *et al*. 2016; Savini *et al*. 2010). El pez mosquito (*Gambusia holbrooki*) (Figura 4) fue transportado desde América central a Europa para controlar el paludismo al alimentarse de mosquitos portadores del parásito que causa la malaria (Pyke 2008). Otra de las principales causas de dispersión de especies invasoras es la importación para acuariofilia, cuyos ejemplares muchas veces acaban liberándose a la naturaleza, como el pez sol (*Lepomis gibbosus*) (Maceda-Veiga *et al*. 2013) (Figura 4). Finalmente, otras especies pueden introducirse y pasar desapercibidas acompañando envíos de otros peces, como la rasbora (*Pseudorasbora parva*) que fue transportada junto con carpas chinas desde Asia a Rumania en 1960 (Gozlan *et al*. 2010; Simon *et al*. 2014).

Un caso curioso, por las paradojas ecológicas que implica, es el de los salmónidos, que han sido introducidos en muchas partes del mundo para pesca recreativa y acuicultura (Hasegawa y Maekawa 2006). Entre ellos, la trucha común europea (*Salmo trutta*) y la trucha arco iris (*Oncorhynchus mykiss*) que están incluidas en la lista de las 100 especies invasoras más perjudiciales, debido a sus efectos negativos en la fauna autóctona (Lowe *et al*. 2000). Como contrapartida muchos de estos salmónidos altamente invasores están en peligro en sus hábitats nativos, como es el caso del salmón atlántico (*Salmo salar*) en España (Horreo *et al*. 2011), cuyas poblaciones se han extinguido hace décadas en los ríos Duero, Tajo y Guadiana (Baillie y Groombridge 1996).

Figura 4. Imágenes de algunas de las especies de peces exóticos invasores más dañinos para el ecosistema. Arriba la primera a la izquierda y siguiendo las agujas del reloj: pez gato *Ameiurus melas*, lucio *Esox lucius*, pez mosquito *Gambusia holbrooki*, siluro *Silurus glanis*, perca americana *Micropterus salmoides* y pez sol *Lepomis gibbosus*. Fotografías realizas por Laura Clusa en el Acuario de Zaragoza.

No todas las especies exóticas provienen de otros países, pues a veces pueden producirse translocaciones de peces de una cuenca a otra. Ejemplos en la Península Ibérica son la colmilleja (*Cobitis paludica*), el piscardo (*Phoxinus bigerri*) o el gobio (*Gobio lozanoi*), que también producen grandes pérdidas de biodiversidad nativa (Ministerio de Medio Ambiente 2007). Esto se debe a que cada cuenca tiene una fauna acuática determinada, derivada del aislamiento geográfico ancestral. Por ejemplo, la cuenca asturiana está separada del resto de la península por una cadena montañosa, y para las especies piscícolas antes de la mediación humana sólo era accesible desde el mar, por lo que la mayoría de especies endémicas de la región son diádromas.

El ser humano es el causante de la mayoría de las translocaciones de peces, en muchos casos con el fin de favorecer la pesca deportiva, como la introducción del piscardo (*Phoxinus bigerri*) en el norte de España y el Duero, así como la colmilleja (*Cobitis paludica*) en las cuencas del Duero y del Nalón (Elvira y Almodóvar 2001; Doadrio *et al.* 2011). Recientemente, la política de trasvases de agua entre cuencas ha colaborado con la dispersión de especies. Por ejemplo, el trasvase Tajo-Segura ha ayudado a la introducción del calandino (*Squalius alburnoides*) así como de la boga de río (*Chondrostoma polylepsis*) en la cuenca del Júcar, además del carpín (*Carassius auratus*), el gobio (*Gobio gobio*) y la boga de río en la cuenca del Segura, y la bermejuela (*Chondrostoma arcasii*) en la cuenca del Guadiana (Elvira y Almodóvar 2001; Doadrio *et al.* 2011).

En cuanto a la translocación de invertebrados, esta se suele producir a partir de introducciones intencionadas para el control de plagas o para su cría como alimento. También son liberados accidentalmente a partir de instalaciones de acuicultura, transportados en agua de lastre, transferidos al establecer conexiones de agua mediante canales, en las suelas del calzado de excursionistas, en agua de acuarios, de forma accidental asociada a la pesca, mediante vectores animales, adheridos en plumas y patas de aves, etc. (Duggan 2010; Fuller 2015; Ricciardi 2015) (Anexo I). Por ejemplo, el caracol trompeta (*Melanoides tuberculata*) se introdujo en el Caribe para control de otros caracoles (Pointier y Jourdane 2000); la almeja china del cieno (*Sinanodona woodiana*) se importó en la Toscana (Italia) para la producción de perlas artificiales (Cianfanelli *et al.* 2007). Esta última especie también se introdujo de forma accidental en Europa junto con peces de origen asiático como la carpa plateada (*Hypophthalmichthys molitrix*) (Lajtner y Crnčan 2011). El agua de lastre fue el causante de la importación del falso mejillón de Conrad (*Mytilopsis leucophaeata*) en el río Guadalquivir en España (Escot *et al.* 2003), así como del transporte del caracol del cieno *Potamopyrgus antipodarum* desde Nueza Zelanda a Europa (Zaranko *et al.* 1997), y de la llegada de la almeja asiática (*Corbicula fluminea*) a Brasil (Beasly *et al.* 2003). La acuariofilia es la principal responsable de la introducción en España del caracol trompeta (*M. tuberculata*) (Jarillo y Salgado 2010). Finalmente, la construcción del canal Danubio-Meno-Rin ha permitido el transporte de innumerables especies invasoras entre dichos ríos (Leuven *et al.* 2009).

5. Barreras en los ríos: ¿Refugios para invasores?

Uno de los debates más candentes respecto a las especies exóticas y los recursos hídricos gira en torno a la presencia de presas y embalses. ¿Ayudan a la dispersión y proliferación de especies exóticas, o por el contrario frenan su avance y sirven de refugio para la fauna autóctona?

Las especies exóticas tienden a acumularse cerca de las grandes ciudades y puertos, en áreas de alta densidad humana donde se juntan un mayor número de vectores (Strayer 2010): agua de lastre, restos de acuarios, suelta de mascotas, etc. (Boltovskoy *et al.* 2006; Duggan 2010). Hay estudios que sugieren que los embalses y reservorios de agua son más susceptibles a

la invasión biológica porque el cambio de agua corriente a estancada afecta al flujo de agua, a la temperatura, a los sedimentos, y por tanto a la comunidad de especies (Johnson *et al.* 2008). Las especies invasoras se pueden adaptar fácilmente a estos ecosistemas alterados y dispersarse a otras áreas (Havel *et al.* 2015). En España el 50% de los sistemas fluviales están interrumpidos por la presencia de presas (Liermann *et al.* 2012). Clavero *et al.* (2004) encontraron que en la Península Ibérica las especies invasoras se establecían de forma más exitosa en embalses y reservorios de agua artificiales. La presencia de presas está relacionada con el número de especies introducidas y es uno de los factores más importantes que pone en peligro la conservación de especies nativas, especialmente los peces diádromos, al impedir su migración (*Salmonidae, Gobiidae*) (Han *et al.* 2008). Por ejemplo, Santos *et al.* (2017) en Portugal observaron una disminución de las poblaciones de peces nativos y un aumento de especies exóticas tras la construcción de dos embalses, además de perturbaciones tales como el crecimiento de algas, aumento de turbidez del agua y cambio en la comunidad de macroinvertebrados. La presencia de barreras en los ríos causa la pérdida de biodiversidad aguas arriba, y esto sucede en todos los continentes e islas. Otro ejemplo en ecosistemas muy diferentes a los europeos ocurre en el Caribe, donde Holmquist *et al.* (1998) observaron que la fauna piscícola nativa se había eliminado aguas arriba de barreras fluviales impasables.

Por otro lado, hay varios estudios que defienden la construcción de presas como un método para frenar el avance de especies exóticas y como refugio para especies autóctonas (Fausch *et al.* 2006; McLaughlin *et al.* 2007). En un río de montaña en Sierra Nevada (Andalucía), se consiguió frenar la expansión a tramos altos del río del cangrejo rojo americano (*Procambarus clarkii*) mediante la construcción de pequeñas presas, protegiendo de esta manera la población natural del cangrejo de río en peligro (*Austropotamobius pallipes*) (Dana *et al.* 2011). Además de ayudar a controlar especies no nativas previniendo su dispersión, mediante presas se previene también la de enfermedades asociadas a ellas (Rahel 2013), como es el caso de la lamprea marina (*Petromyzon marinus*) en la región de los grandes Lagos en América del Norte (McLaughlin *et al.* 2007).

Por todo lo comentado arriba, si bien está claro el efecto que produce la presencia de barreras fluviales sobre la biodiversidad acuática, no se conoce todavía el balance final de sus ventajas y desventajas ecológicas. Es muy posible que en algunas regiones donde las invasiones son un problema muy grave, las presas puedan considerarse medidas de contención; en cambio, en cuencas de regiones biogeográficas donde muchas especies nativas son diádromas la falta de conectividad será en sí misma un problema de primera magnitud, que impacta negativamente sobre la biodiversidad nativa. Es necesario por tanto abordar este tema con un enfoque regional. En el momento de comenzar esta Tesis no había ningún estudio publicado sobre el efecto de las presas presentes en la región asturiana en las especies fluviales exóticas y nativas.

6. Impactos producidos por las EEI en el ecosistema

Cuando una especie invasora coloniza un nuevo hábitat consigue transformar rápidamente todo el ecosistema (Havel *et al.* 2015). Un ejemplo devastador fue la introducción de la perca del Nilo (*Lates niloticus*) en el lago Victoria (este de África) en los años 50, la cual acabó con casi toda la diversidad acuática y llevó a la extinción a más de 200 especies endémicas de peces en cuestión de décadas (Witte *et al.* 1992; Goldschmidt *et al.* 1993).

Hay muchas razones para que se produzcan estas transformaciones a nivel de ecosistema. Numerosas especies introducidas compiten con las nativas por los recursos y el espacio (Ribeiro y Leunda 2012), reducen la calidad del agua, desplazan especies nativas,

transmiten enfermedades y muchas son más resistentes a condiciones desfavorables (Leunda 2010, Cambray 2003). Algunos ejemplos concretos se detallan en la Tabla 1.

Tabla 1. Impactos derivados de la introducción de especies acuáticas exóticas. Se detallan algunos ejemplos de los principales impactos ocasionados al introducir especies exóticas de peces y moluscos en un ecosistema.

	Impactos	Especie	Efecto	Referencias
Peces	Competencia por recursos y territorio	*Lepomis gibbosus*	Compite agresivamente por comida y territorio.	Almeida *et al.* 2014
		Gambusia holbrooki *Fundulus heteroclitus*	Compiten con el salinete (*Aphanius baeticus*), el fartet (*Aphanius iberus*) y el samaruc (*Valenica hispanica*).	Rincón *et al.* 2002 Elvira y Almodóvar 2001
		Oncorhynchus mykiss	Compite agresivamente con otros salmónidos.	Stanković *et al.* 2015
	Depredación de fauna autóctona	*Gambusia holbrooki*	Se alimenta de invertebrados, anfibios y huevos de otros peces.	Pyke 2008 Remon *et al.* 2016
		Micropterus salmoides	Depredación de peces nativos, anfibios, cangrejos e invertebrados.	Maezono y Miyashita 2002
		Esox lucius	Gran depredador piscívoro.	García-Berthou *et al.* 2015
		Salvelinus fontinalis	Depredación de anfibios en la Península Ibérica, *Rana iberica*.	Bosch *et al.* 2006
	Alteración del hábitat	*Ameiurus melas*	Aumento de turbidez en estudios de mesocosmos.	Braig y Johnson 2003
		Carassius auratus	Especie bentívora, se alimenta de nutrientes del fondo y provoca un aumento de la turbidez.	He *et al.* 2017 Richardson *et al.* 1995
	Introducción de parásitos	*L. gibbosus*	Introducción del parásito (*Onchocleidus sp*) en Noruega.	Sterud y Jørgensen 2006
		Pseudorasbora parva	Introducción de numerosos parásitos como *Sphaerothecum destruens*, capaces de trasmitirse a peces nativos.	Gozlan *et al.* 2010 Gozlan *et al.* 2005
		Carassius auratus *Cyprinus carpio*	Introducción del parásito *Lernaea cyprinacea*.	García-Berthou *et al.* 2007 Leunda 2010
	Hibridación	*Carassius auratus* *Cyprinus carpio*	Hibridación con otros ciprínidos.	Hänfling *et al.* 2005
		Alburnus alburnus	Hibridación con otros ciprínidos como el cacho *Squalius pyrenaicus* y el complejo *Squalius alburnoides.*	Almodóvar *et al.* 2012
	Alteraciones en la cadena trófica	*Lates niloticus*	Alteración de toda la cadena trófica en el Lago Victoria (África).	Witte *et al.* 1992 Goldschmidt *et al.* 1993
		Lepomis gibbosus *Micropterus salmoides* *Rutilus rutilus*	Extinción de *Gasterosteus acuelatus*, *Squalius laietanus* y *Barbus meridionalis* en el lago de Bañolas (España).	García-Berthou *et al.* 2015
		Esox lucius *Micropterus salmoides* *Silurus glanis*	Especies piscívoras que provocan cambios en la composición de especies y cadena trófica.	Leunda 2010 García-Berthou *et al.* 2015
Moluscos Su principal daño deriva de su alta tasa de reproducción y su tolerancia a condiciones ambientales extremas (Sousa *et al.* 2014).	Alteraciones en las partículas en suspensión	*Corbicula fluminea*	Reduce la comunidad de fitoplancton y altera el ciclo de nutrientes al ser un bivalvo con alta tasa de filtración.	Vaughn y Hakenkamp 2001
	Bloqueo de infraestructuras acuáticas	*Dreissena polymorpha*	Grandes masas que bloquean tuberías, canales y conductos acuáticos.	Connelly *et al.* 2007 Durán *et al.* 2010
		Mytilopsis leucophaeata	Área afectada por las descargas de agua de refrigeración de una planta de energía nuclear en el golfo de Finlandia.	Laine *et al.* 2006
	Introducción de parásitos	*Melanoides tuberculata*	Hospedador de un trematodo parásito capaz de infectar peces nativos.	Oscoz *et al.* 2010

Moluscos (Continuación)	Competición por recursos con fauna nativa	*Potamopyrgus antipodarum*	Alta tasa de reproducción, un adulto es capaz de producir 230 juveniles al año.	Zaranko *et al.* 1997
		M. tuberculata	Alcanza grandes poblaciones siendo una amenaza para los moluscos nativos como *Melanopsis etrusca* en Italia.	Cianfanelli *et al.* 2007
		Sinanodonta woodiana	Compite con otros moluscos por el uso de peces nativos como hospedador de sus larvas.	Douda *et al.* 2012
	Alteraciones en la cadena trófica	*C. fluminea* *D. polymorpha*	Reducción de biomasa y producción primaria.	Strayer 2010
		P. antipodarum	Consume hasta un 75% de la producción primaria alterando el ciclo de carbono y del nitrógeno.	Hall *et al.* 2003 Hall *et al.* 2006
			Perjudica distintas especies de salmónidos al no ser apto como alimento.	Vinson y Baker 2008 Sanderson *et al.* 2009
		Conchas vacías de bivalvos invasores	Al acumularse afectan a las poblaciones de anfípodos y al ecosistema.	Bódis *et al.* 2014

7. Métodos de detección de EEI

Son evidentes los serios problemas que las especies exóticas causan en el ecosistema (Chown *et al.* 2015), como se vio en el apartado anterior, siendo a veces capaces de causar la extinción de especies autóctonas (Clavero y García-Berthou 2005). La erradicación de estas especies podría favorecer la recuperación de la ictiofauna autóctona, como sucedió en Oshu (noreste de Japón), donde, después de la eliminación de la perca americana (*Micropterus salmoides*), se consiguió recuperar la fauna nativa (Tsunoda *et al.* 2010). En el noroeste de Gran Bretaña, las poblaciones nativas de *Rutilus rutilus* y *Abramis brama* aumentaron tras la eliminación de la invasora *Pseudorasbora parva* (Britton *et al.* 2009). Y más cerca, en la Laguna Grande del Parque Natural de Peñalara en la Comunidad de Madrid, se ha conseguido la erradicación del salvelino *Salvelinus fontinalis* y se han empezado a recuperar las poblaciones nativas de anfibios (*Rana iberica*) gravemente afectadas por este invasor (Martín-Beyer *et al.* 2011).

El momento de actuar contra las especies exóticas es decisivo, pues una vez que se convierten en invasoras y su población ha aumentado de forma descontrolada es muy difícil su eliminación, prácticamente imposible, además de llevar asociado un gran coste económico tanto para su erradicación como para paliar los impactos producidos por ellas. Por ello, los esfuerzos para luchar contra estas especies exóticas deben centrarse en las etapas previas a su establecimiento, o por lo menos antes del comienzo de la proliferación (ver Figura 1). La detección temprana, cuando aún están en muy baja densidad, es por tanto esencial para una respuesta eficaz y la prevención de una posible dispersión de estos invasores (Sousa *et al.* 2014; Havel *et al.* 2015).

Hasta ahora los únicos métodos disponibles de detección de peces e invertebrados consistían en métodos físicos, basados en electropesca, pesca con redes, trampas, con diferentes nasas, muestreos mediante buzos y otros, dañando en algunos casos la fauna nativa y siendo necesaria la participación de taxónomos expertos para clasificar las especies; incluso así, a veces se ha producido la identificación errónea de algunas especies crípticas (parecidas a las nativas en alguna de sus etapas vitales), que ha llegado a impedir la actuación temprana; el problema se ha evidenciado posteriormente a partir de estudios genéticos (Geller *et al.* 1997; Thomsen *et al.* 2012). El desarrollo en los últimos años de nuevas metodologías ha ayudado a la detección de especies invasoras en sistemas acuáticos (Figura 5).
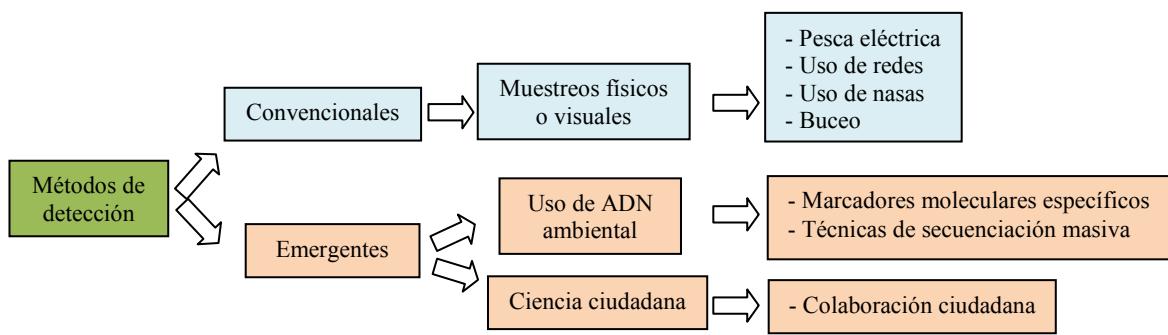
Figura 5. Esquema de los métodos de detección temprana de especies exóticas disponibles.

7.1 Uso de ADN ambiental

Un primer grupo de los nuevos métodos de detección de especies invasoras son las técnicas basadas en el ADN ambiental (Figura 5), ya que todos los seres vivos presentes en el agua liberan su ADN al medio a través de células epiteliales, secreción de moco, heces, branquias, etc. (Goldberg *et al*. 2014). Este ADN puede extraerse directamente a partir de una muestra de agua o sedimentos, y sirve para monitorizar la presencia de las especies presentes en el ecosistema. Una ventaja de esta metodología es que no depende de las condiciones climatológicas para el muestreo, requiere mucho menor esfuerzo, y además parece ser más sensible y eficiente que algunos métodos convencionales como la electropesca o la captura con redes (Blanchet 2012). Esto es especialmente importante para el monitoreo de especies elusivas o de hábitos nocturnos, y de las que están en baja densidad, lo que ocurre en los primeros estadios de una invasión (Jerde *et al*. 2011; Muñoz-Colmenero *et al.* 2017). La detección de especies individuales concretas se basa en el diseño de cebadores que anillan solamente en ADN perteneciente a la especie de interés. La señal positiva de amplificación en una PCR indica la presencia de esa especie objetivo en la muestra.

Desde el primer artículo que se valió del ADN ambiental para detectar la presencia de vertebrados en agua, en concreto del anfibio *Rana castebiana* (Ficetola *et al.* 2008), se han desarrollado con éxito numerosos marcadores moleculares específicos para especies de taxones muy diferentes, entre las que se incluyen moluscos (Ardura *et al*. 2015; Devloo-Delva *et al*. 2016; Clusa *et al*., 2016), peces (Furlan y Gleeson 2016; Takahara *et al*. 2013; Adrian-Kalchhauser y Burkhardt-Holm, 2016; Uchii *et al*. 2016), anfibios (Ficetola *et al*. 2008; Pilliod *et al*. 2014), reptiles (Piaggio *et al*. 2014; Davy *et al*. 2015), mamíferos (Foote *et al*. 2012; Ishige *et al*. 2017).

Además de los ensayos específicos, el avance en la metodología de secuenciación masiva mediante técnicas denominadas actualmente *Metabarcoding,* está llevando a otro nivel el uso del ADN ambiental, permitiendo la caracterización de la fauna acuática mediante una única PCR y ayudando a detectar especies exóticas presentes en el ecosistema (Rius *et al*. 2015; Deiner *et al*. 2016; Borrell *et al*. 2017). Estas técnicas, desarrolladas inicialmente para inventario de comunidades procariotas (Taberlet *et al*. 2012; Van Dijk *et al*. 2014), se basan en el uso de cebadores universales que hibridan sobre ADN de un gran número de taxones diferentes y amplifican un fragmento de ADN con señal filogenética fuerte, que permita diferenciar entre especies e identificarlas por comparación con las bases de datos. La mayoría de los proyectos de *Metabacoding* usan como *código de barras* genético (*Barcode*) parte del gen de la citocromo-oxidasa subunidad I y/o del gen del ARN ribosómico 18S (Leray *et al*. 2013; Capra *et al.* 2016), para especies eucariotas, y típicamente el gen del ARN ribosómico 16S para

especies procariotas (Shin *et al.* 2016). En esta Tesis se van a utilizar ambos tipos de técnicas, la especie-específica y la de *Metabarcoding*.

7.2 Implicación ciudadana

Otro gran grupo de métodos de detección de especies exóticas emergentes se recoge bajo el término de ciencia ciudadana (Figura 5). El ser humano es el causante de la mayoría de las introducciones de estas especies, por lo que tiene sentido involucrarlo en su detección. En los últimos años los proyectos, que incluyen ciencia ciudadana, han aumentado con el objetivo de implicar a los ciudadanos en investigaciones sobre cambio climático, conservación, restauración de la fauna, calidad de agua, detección de especies invasoras y otros temas (Silvertown 2009; Thomas *et al.* 2017). El advenimiento de la nueva era tecnológica ha facilitado el desarrollo e implementación de estos proyectos gracias a las aplicaciones disponibles para *smartphones* o a través de Internet (Newman *et al.* 2012).

En este tipo de proyectos los voluntarios pueden cubrir gran cantidad de terreno con sus observaciones, llegando a localizaciones y a un seguimiento temporal que pudieran no ser accesibles para los investigadores (Devictor *et al.* 2010). Por ejemplo, se ha conseguido mapear la invasión de los cangrejos *Carcinus maenas* y *Hemigrapsus sanguineus* a lo largo de toda la costa de Estados Unidos (Delaney *et al.* 2008). En Japón se ha conseguido eliminar 300.000 abejorros (*Bombus terrestris)* de la naturaleza (Kobori *et al.* 2016). En el norte de Italia y Suiza se ha podido localizar el insecto *Halymorpha halys*, además de desarrollar una guía de identificación para los ciudadanos que permita monitorizar la invasión de este insecto en otras regiones (Maistrello *et al.* 2016). En Alaska se empleó la ayuda de los ciudadanos para controlar las invasiones marinas (https://seagrant.uaf.edu/research/projects/summary.php?id=939). En el mar Mediterráneo se consiguió detectar el invasor *Abudefduf saxatilis* gracias a un programa de ciencia ciudadana que recoge datos de voluntarios en la página web: http://www.observadoresdelmar.es (Azzurro *et al.* 2013). También la implicación de comunidades de sectores concretos, como los pescadores, ha ayudado a concienciar a la población sobre la suelta intencionada de especies exóticas; en algunos estudios de conservación, su participación ha resultado ser muy útil (Granek *et al.* 2008), por ejemplo para obtener muestras de peces de forma no letal (Williams *et al.* 2015).

La utilidad de la colaboración ciudadana en la identificación de especies exóticas puede ser enorme, pero hay que ser consciente del conocimiento previo que se tiene de ellas. Estos programas pueden fracasar si los participantes carecen de la formación adecuada y de una educación ambiental fundamentada. El primer paso para diseñar cualquier programa de ciencia ciudadana es evaluar el conocimiento que tienen los ciudadanos de esa zona sobre las especies que se quieren monitorear. García-Llorente *et al.* (2008) estudiaron cómo se percibe el impacto causado por especies invasoras en la reserva natural de Doñana desde diferentes grupos de interés (turistas, conservacionistas, usuarios locales y otros) y su actitud frente a la erradicación de dichos invasores. Los resultados fueron en parte inesperados. Encontraron que existía poca correspondencia entre los datos oficiales, los reales y la percepción de los ciudadanos sobre los impactos causados por especies exóticas; sin embargo, el 97% de los encuestados apoyó la erradicación de estas especies independientemente del impacto percibido, especialmente de las que habían sido introducidas recientemente y aquellas que habían aparecido en los medios de comunicación. Se recomienda actualmente poner especial cuidado en la formación de los participantes en proyectos de ciencia ciudadana, que deben diseñarse a partir de su conocimiento y de su nivel de concienciación sobre el problema (Aceves-Bueno *et al.* 2017; Cunha *et al.* 2017; Garbarino y Mason 2016). En el ámbito geográfico principal de esta Tesis, el

Principado de Asturias, no existía a su comienzo ningún estudio ni prospección en este sentido en el campo de las especies invasoras acuáticas. Para cubrir este hueco se consideró necesario, por tanto, comenzar realizando una investigación de aproximación al conocimiento y percepción de los usuarios de los ríos y los ribereños sobre las especies invasoras piscícolas.

Objetivos
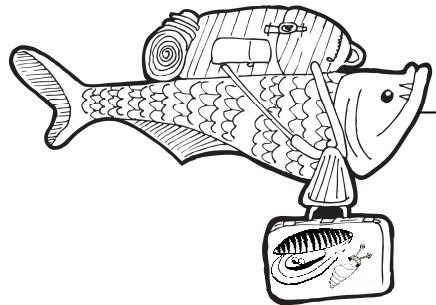
Objetivos

1) Desarrollar marcadores específicos para especies de peces y moluscos de agua dulce presentes en el listado de especies exóticas invasoras en España incluido en el Boletín Oficial del Estado 630/2013 del 2 de agosto de 2013, https://www.boe.es/buscar/doc.php?id=BOE-A-2013-8565, incluyendo su validación para aplicación en muestras de ADN ambiental.

2) Aplicar marcadores de ADN ambiental en Alemania, uno de los países europeos responsables de las introducciones de especies exóticas en el resto de Europa, para evaluar la influencia de las actuaciones antrópicas en la dispersión e introducción de especies exóticas en la región del Lago Constanza.

3) Estudiar el efecto de las barreras fluviales en la biodiversidad piscícola acuática en Asturias mediante técnicas multidisciplinares incluyendo ADN ambiental, usando el río Nalón como caso de estudio, para localizar los principales focos de especies invasoras.

4) Evaluar el conocimiento público acerca de las especies exóticas fluviales en Asturias mediante entrevistas a usuarios de los ríos, comparando ese resultado con datos oficiales de la región y muestreos convencionales *in situ*, para identificar prioridades en comunicación de la ciencia y educación ambiental respecto a las invasiones biológicas.

5) A partir de los resultados de los objetivos anteriores, determinar los principales riesgos ecológicos y sanitarios derivados de las especies exóticas fluviales encontradas en Asturias.

Metodología
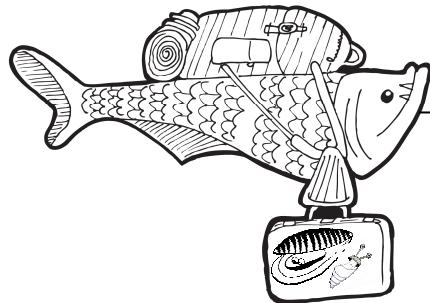
Metodología

En la figura 6 se resume un esquema de la metodología de ADN ambiental utilizada. Sus detalles y el resto, Materiales y Métodos, se encuentran en cada uno de los capítulos que componen la Tesis Doctoral.
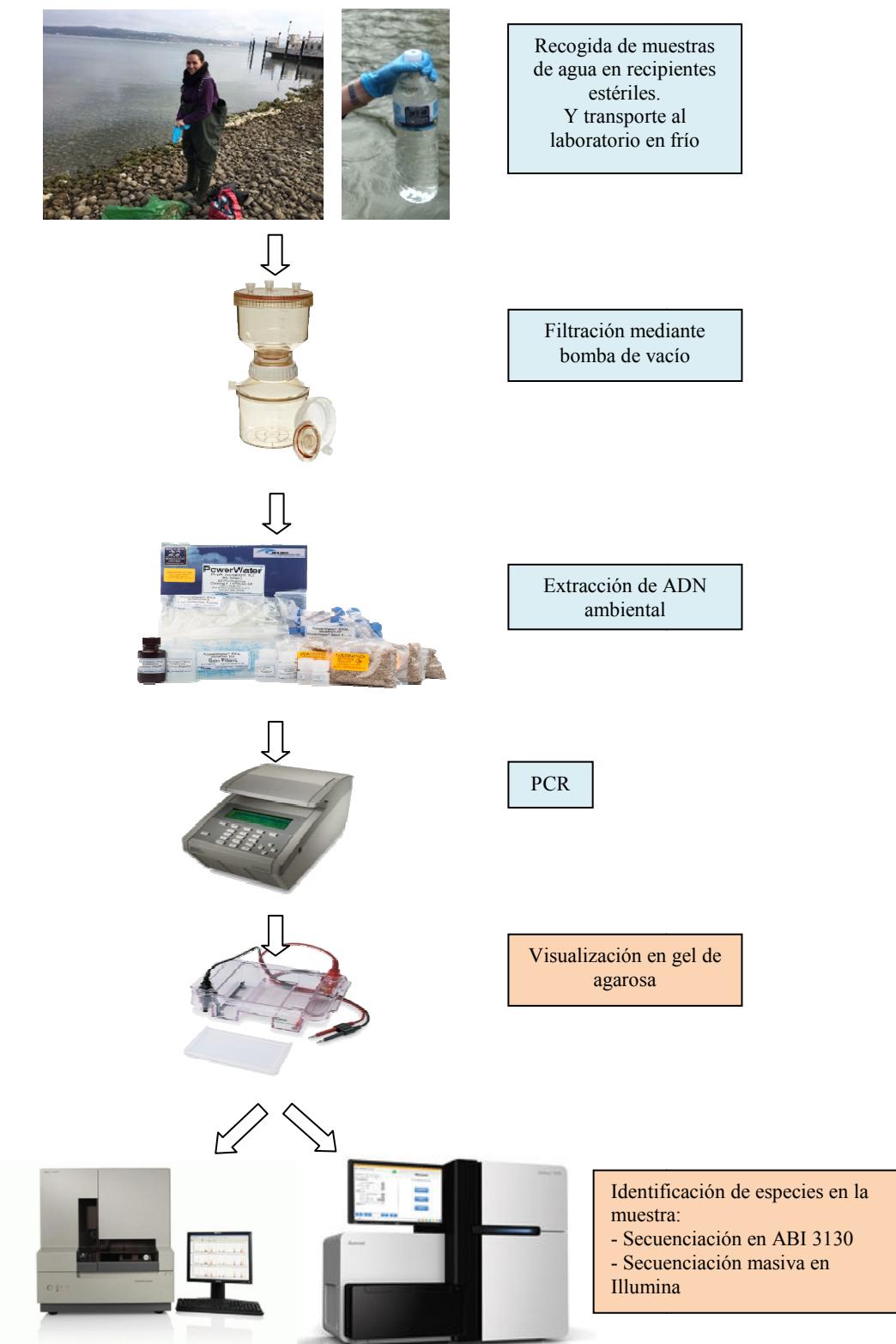
Figura 6. Esquema de la metodología de ADN ambiental utilizada. En azul se señalan los pasos pre-PCR realizados con un protocolo exhaustivo de control de contaminaciones, y en naranja los pasos post-PCR. Tienen lugar en laboratorios separados.

Resultados
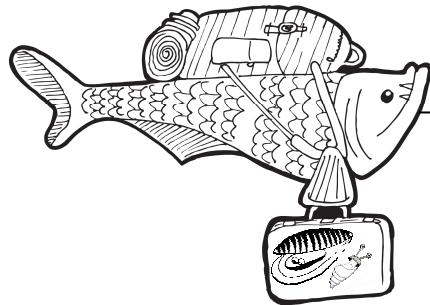
Resultados

Capítulo 1. Clusa L, Ardura A, Fernández S, Roca A and Garcia-Vazquez E. 2017. An extremely sensitive nested PCR-RFLP mitochondrial marker for detection and identification of Salmonids in eDNA from water samples. *Peer J* 5: e3045. DOI 10.7717/peerj.3045

Capítulo 2. Clusa L and Garcia-Vazquez E. 2018. A simple, rapid method for detecting seven common invasive fish species in Europe from environmental DNA. *Aquatic Conservation: Marine and Freshwater Ecosystems*. DOI: 10.1002/aqc.2890

Capítulo 3. Clusa L, Ardura A, Gower F, Miralles L, Tsartsianidou V, Zaiko A and 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

Capítulo 4. Clusa L, Miralles L, Basanta A, Escot C and Garcia-Vazquez E. 2017. eDNA for detection of five highly invasive molluscs. A case study in urban rivers from the Iberian Peninsula. *PLoS ONE* 12 (11): e0188126. DOI: 10.1371/journal.pone.0188126

Capítulo 5. Clusa L, García-Vázquez E and Machado-Schiaffino G. 2018. Assessing performance of species-specific primers for detecting aquatic invasive species in Lake Constance region using eDNA. Enviado: *Molecular Ecology*

Capítulo 6. Clusa L, Fernández S, Dopico E and García-Vázquez E. 2018. The role of barriers in aquatic fauna diversity: a case study in Nalón River, north of the Iberian Peninsula. En revisión: *Scientific reports*.

Capítulo 7. Clusa L, Miralles L, Fernández S, García-Vázquez E and Dopico E. 2018. Public knowledge of alien species: a case study on aquatic biodiversity in North Iberian rivers. En prensa, disponible online: *Journal for Nature Conservation*, https://doi.org/10.1016/j.jnc.2018.01.001.

Capítulo 1:

An extremely sensitive nested PCR-RFLP mitochondrial marker for detection and identification of Salmonids in eDNA from water samples

Clusa L, Ardura A, Fernández S, Roca A and García-Vázquez E

An extremely sensitive nested PCR-RFLP mitochondrial marker for detection and identification of salmonids in eDNA from water samples

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ABSTRACT

Background. Salmonids are native from the North Hemisphere but have been introduced for aquaculture and sport fishing in the South Hemisphere and inhabit most rivers and lakes in temperate and cold regions worldwide. Five species are included in the Global Invasive Species Database: rainbow trout *Oncorhynchus mykiss*, Atlantic salmon *Salmo salar*, brown trout *Salmo trutta*, brook trout *Salvelinus fontinalis*, and lake trout *Salvelinus namaycush*. In contrast, other salmonids are endangered in their native settings.

Methods. Here we have developed a method to identify salmonid species directly from water samples, focusing on the Iberian Peninsula as a case study. We have designed nested Salmonidae-specific primers within the 16S rDNA region. From these primers and a PCR-RFLP procedure the target species can be unequivocally identified from DNA extracted from water samples.

Results. The method was validated in aquarium experiments and in the field with water from watersheds with known salmonid populations. Finally, the method was applied to obtain a global view of the Salmonidae community in Nalón River (north coast of Spain).

Discussion. This new powerful, very sensitive (identifying the species down to 10 pg DNA/ml water) and economical tool can be applied for monitoring the presence of salmonids in a variety of situations, from checking upstream colonization after removal of river barriers to monitoring potential escapes from fish farms.

Subjects Aquaculture, Fisheries and Fish Science, Biodiversity, Environmental Sciences, Marine Biology, Molecular Biology
Keywords eDNA, Species-specific RFLP, Family-specific primers, Salmonids

INTRODUCTION

Salmonids are a fish group particularly interesting because, although native from the north Hemisphere, they are spread worldwide. Many species have been introduced into streams for recreational fishing (*Hasegawa & Maekawa, 2006*) and for aquaculture. Five species: *Oncorhynchus mykiss*, *Salmo salar*, *Salmo trutta*, *Salvelinus fontinalis* and *Salvelinus*

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namaycush are considered invasive and included in the Global Invasive Species Database (GISD) (http://www.issg.org/database) of the IUCN. Moreover, brown trout (*S. trutta*) and rainbow trout (*O. mykiss*) are within the One Hundred of the World's Worst Invasive Alien Species list, which includes species that cause serious negative impacts on biological diversity and/or human activities (*Lowe et al., 2000*).

The five salmonids listed above have been introduced worldwide. From its native range in Europe and North Africa, brown trout (*S. trutta*) has expanded to all continents except Antarctica (*MacCrimmon & Marshall, 1968*). Rainbow trout (*O. mykiss*) was one of the most widely exported salmonids in the beginning of the 20th century, for aquaculture from the USA to 28 out of 41 European countries (*Crawford & Muir, 2008*; *Savini et al., 2010*; *Stanković, Crivelli & Snoj, 2015*) including Spain (*Elvira & Almodóvar, 2001*). Atlantic salmon (*S. salar*), one of the most consumed fish in the world, can be found out of its North Atlantic native area as far as in Australia, New Zealand, Chile, West Coast of the US and Canada (*De Poorter, 2009*). Lake trout (*S. namaycush*) was also introduced in Europe from North America and Canada for recreational fishing. Some populations were established in deep, high-altitude lakes in the French Pyrenees and in alpine lakes in Switzerland (*Crossman, 1995*). Brook trout (*S. fontinalis*) is also established in some European countries: France, Austria, Germany, Switzerland etc. (*MacCrimmon & Campbell, 1969*). In Spain it can be found in Tagus and Ebro rivers and Cantabrian lakes (*Doadrio, 2001*).

The use of these exotic species for aquaculture and their accidental and intentional release or escape negatively impacts on native biodiversity and ecosystems (*Hewitt, Campbell & Gollasch, 2006*). To cite just a few examples, introduced salmonids have endangered native biota to the extinction or near-extinction of vulnerable species in Australia (*Morgan et al., 2004*), New Zealand (*Townsend, 1996*; *Townsend, 2003*), Argentina (*Consuegra et al., 2011*), Japan (*Kitano, 2004*) or Canada (*Dextrase & Mandrak, 2006*). Brook trout strongly impacted on the endemic Iberian frog *Rana iberica* (*Bosch et al., 2006*). Rainbow trout outcompeted other salmonid species for space and food (*Stanković, Crivelli & Snoj, 2015*), as brown trout (*Levin et al., 2002*) and brook trout (*Nakano et al., 1998*; *Blanchet et al., 2007*) also did in different regions of North America and Europe.

On the other hand, many salmonid populations are endangered in their native settings. A paradigmatic example is the decline of Atlantic salmon (*S. salar*) in their native rivers over the northern Hemisphere (*Klemetsen et al., 2003*; *Jelks et al., 2008*; *Hórreo et al., 2011*; *Chaput, 2012*). In Europe, 41% of native Salmonidae species are threatened (*Freyhof & Brooks, 2011*). The Atlantic salmon has undergone historical extirpation from rivers in Belgium, Czech Republic, Germany, Netherlands, Poland, Slovakia and Switzerland (*Freyhof, 2014*). Atlantic salmon populations from Duero, Tagus and Guadiana rivers are now extinct in Spain (*Baillie & Groombridge, 1996*), where only a few rivers in the north still support wild populations that are in continuous decline (*Hórreo et al., 2011*); their status is considered vulnerable (*Freyhof, 2014*). The reasons for this are principally habitat losses (damming, pollution), overfishing, and the introduction of invasive species (*Chown et al., 2015*). Infections, probably coming from fish farms, have also threatened European Atlantic salmon (*Krkošek et al., 2007*; *Whelan, 2010*), as well as other species such as the Arctic char (*Salvelinus alpinus*) and sea trout (*S. trutta*) in Norway (*Bjørn, Finstad &*

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Kristoffersen, 2001). During the 20th century, wild populations of brown trout decreased in Finland due to dams construction and overfishing (*Syrjänen & Valkeajärvi, 2010*). In other countries, like in Spain, some local sedentary populations of *S. trutta* (*Doadrio, 2001*) have been totally extirpated. This species is considered vulnerable in Spain (*Freyhof, 2011*).

In the last years, molecular tools such as barcoding and metabarcoding are becoming very useful for managing natural populations and communities (*Chown et al., 2015*). An emerging method to monitor and detect aquatic species is environmental DNA (eDNA) analysis. Metazoans can be detected from their DNA released into the environment through skin flaking and sloughed cells, mucus excretion and defecation in aquatic environments (*Goldberg, Strickler & Pilliod, 2015*). In many cases, eDNA amplification from PCR seems to be more sensitive and efficient than traditional surveillance approaches, like visual detection, and does not disturb the aquatic fauna (*Ficetola et al., 2008*; *Blanchet, 2012*; *Thomsen et al., 2012*). The use of specific primers on eDNA has been successfully demonstrated for a number of species. Examples are molluscs such as *Rangia cuneata* in the Baltic Sea (*Ardura et al., 2015*), *Xenostrobus securis* and *Potamopyrgus sp* in North Spain (*Devloo-Delva et al., 2016*; *Clusa et al., 2016*); fishes such as *Petromyzon marinus* (*Gustavson et al., 2015*), *Neogobius melanostomus* (*Adrian-Kalchhauser & Burkhardt-Holm, 2016*), *Cyprinus carpio* (*Uchii, Doi & Minamoto, 2016*). Salmonids with designed specific methodology based on eDNA include, amongst others, *Salvelinus namaycush* (*Lacoursière-Roussel et al. , 2015*), *Salmo trutta* (*Gustavson et al., 2015*; *Carim et al., 2016*), *Oncorhynchus mykiss* (*Wilcox et al., 2015*), *Salvelinus fontinalis* (*Wilcox et al., 2013*). These methods can be applied to detect, and in some cases roughly quantify, elusive or threatening species even at very low density. Methodologies based on eDNA may be particularly useful for inventorying salmonids from running or turbid waters, when traditional electrofishing or netting methods are not efficient (for example in reservoirs), and indeed when those sampling methods may disturb other vulnerable species cohabiting the same watersheds.

In this work, we developed a method based on PCR-RFLP (Polymerase chain reaction-restriction fragment length polymorphism) mitochondrial marker to detect the presence of salmonid species from water samples, in order to monitoring the presence of salmonid species focusing on North Iberia as a model region study. This region is interesting because it contains two native Salmoninae, *S. salar* and *S. trutta*, and three exotic species considered invasive by the International Union of Conservation of Nature (rainbow, brook and lake trout) that were introduced in rivers and lakes decades ago (*Doadrio, 2001*; *Elvira & Almodóvar, 2001*; *Crawford & Muir, 2008*). This was the second eDNA method validated for identifying Salmonidae from European water samples, after the *S. trutta* specific primer described by *Gustavson et al. (2015)*, and the first method to detect salmonid mixtures from a single PCR. The mixture of introduced and native species makes North Iberia a good case study for application of eDNA methodology to monitoring of feral populations. PCR-RFLP methodology has been successful for identification of different fish species (e.g., *Itoi et al., 2005*; *Reid & Wilson, 2006*), but has been employed on community DNA from aquatic samples only on protozoans (*Xiao et al., 2000*; *Galván et al., 2014*). Since it is relatively inexpensive, technically easy and fast, if successful from eDNA, it could be widely applied in ecology, conservation biology and management of aquatic resources in many

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zones of Europe, especially in the Atlantic Arc where the aquatic fauna is similar to North Spain's.

MATERIALS AND METHODS

Salmonidae specific primers

To obtain enough PCR product to perform the RFLP analysis, a nested PCR strategy was used. The method here described was based on the DNA fragment amplified with Salmonidae-specific primers described in *Zaiko et al. (2015)*. We designed a new primer pair to nest *Zaiko et al. (2015)* primers inside its amplification product. The 16S rRNA gene was chosen because, as a mitochondrial gene, it is more abundant than nuclear DNA in water samples (*Ficetola et al., 2008*), it is generally well conserved within species and exhibits higher variation between species (*Maretto et al., 2007*; *Zhang & Hanner, 2012*). All the 16S rRNA gene sequences available for the Salmonidae species *O. mykiss, S. trutta, S. salar, S. namaycush* and *S. fontinalis* were downloaded from the NCBI database of DNA sequences, either individual 16S DNA sequences or complete mitochondrial genomes. Polymorphisms were analyzed with the DNASP software V.5.10 (*Rozas et al., 2003*). The different haplotypes were visualized employing the BioEdit Sequence Alignment Editor software (*Hall, 1999*). Sequences were aligned with the ClustalW application included in BioEdit (*Thompson, Higgins & Gibson, 1994*). The Primer Blast application included in the NCBI webpage (*Ye et al., 2012*) was employed to design one forward 16S general primer, which amplified a fragment of 567 bp in the 16S rRNA gene using the reverse 16S-Br universal primer from *Palumbi et al. (2002)*.

The two Salmonidae-specific primers described by *Zaiko et al. (2015)* anneal within the 567 bp amplicon obtained from the new primers pair. These Salmonidae specific primers were tested *in silico* with the BLAST tool in the NCBI webpage (*Altschul et al., 1990*). The sequences retrieved with significant match (e-value of 0.046 for the forward primer and 0.18 for the reverse) were from the Order Salmoniformes. Both forward and reverse primers were checked, and the two BLAST results were contrasted to determine which species will probably amplify with both of them. One non-target species with significant match *in silico* was *Esox lucius*, which is an invasive species in the region and was included in the RFLP designed, to avoid any false positive after digestion.

To validate the new Salmonidae-specific primer *in vitro*, cross-amplification tests were performed. Samples from different species belonging to 15 fish families from the laboratory collection were used. They represent the 100% of the species inventoried in the study area, north coast of Spain (Table S1). DNA was extracted from muscle tissue with Chelex resine (*Estoup et al., 1996*). PCR amplifications were performed with the universal primers for the 16S gene (*Palumbi et al., 2002*), sequenced to confirm the species and used as DNA quality control. The primers pair was tested for PCR amplification on all the samples of Table S1 and *O. mykiss, S. salar, S. trutta, S. fontinalis* and *S. namaycush* as positive controls. The PCR conditions were as described in 'PCR conditions' but using 2 μl of template DNA extracted from tissue samples. We assayed the following annealing temperatures: 58 °C, 60 °C, 62 °C, 64 °C, 66 °C and 68 °C; and the following $MgCl_2$ concentrations: 2.5 mM, 2 mM, 1.5 mM, 1 mM. The best conditions were selected.

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PCR-RFLP method development

The RFLP (restriction fragment length polymorphism) protocol was designed within the DNA fragment amplified with the primers described by *Zaiko et al. (2015)*. To design species-specific RFLPs, all the haplotypes from the five species in study were aligned. Diagnostic single nucleotide sites (monomorphic within species and different between species) were identified for each species. The restriction enzymes recognizing those sites were selected and restriction pattern determined using the NEBcutter application (*Vincze, Posfai & Roberts, 2003*). For the species *S. namaycush* there were not enough sequences in the database. DNA extracted from 25 samples of this species kindly provided by the Université Laval of Québec were amplified with the universal primers for the 16S RNA gene (*Palumbi et al., 2002*) and sequenced. The new sequences were employed for RFLP design.

PCR conditions

In the first PCR, a fragment of 567 bp in the 16S rRNA gene was amplified with the forward 16S general new primer (see 'Salmonidae specific primers') and the reverse 16S-Br universal primer from *Palumbi et al. (2002)*. The amplification reaction was performed in a total volume of 20 µl, including Green GoTaq®Buffer 1X, 2.5 mM $MgCl_2$, 0.25 mM dNTPS, 1 µM of each primer, 4 µl of template DNA, 200 ng/µl of BSA (bovine serum albumin) and 0.65 U of DNA Taq polymerase (Promega). PCR conditions were the same as described by *Palumbi et al. (2002)*, but with 50 cycles instead of 35. Both negative control with only distilled water and positive control with *S. salar* DNA from tissue were included. This PCR confirmed the quality of DNA in the sample and discard false negatives due to excessive DNA degradation, and was used as template for the nested-PCR, amplifying a smaller fragment of the 16S rRNA gene with the Salmonidae specific primers.

   The nested PCR amplification with the pair of Salmonidae-specific primers described in *Zaiko et al. (2015)* was performed in a total volume of 20 µl, including Green GoTaq®Buffer 1X, $MgCl_2$, 0.25 mM dNTPS, 1 µM of each primer, 200 ng/µl of BSA and 0.5 µl of PCR product from the previous 16S amplification as template and 0.65 U of DNA Taq polymerase (Promega). The PCR conditions were the following: an initial denaturation step at 95 °C for 5 min, 35 cycles at 94 °C for 30 s, annealing at the temperature of choice for 30 s and elongation at 72 °C for 30 s. A final step of elongation was set at 72 °C for 10 min. In nested PCR two negative and two positive controls were included, one negative with only distilled water and another negative using as template the PCR product from the negative control in the first PCR and the same with the positive controls. PCR products were visualized in 2% agarose gels with 2.5 µl of SimplySafe™.

Restriction enzyme digestion validation

The PCR product amplified with the nested PCR described above was digested with FastDigest enzymes (Thermo Scientific). The digestion reaction was performed in a total volume of 15 µl, including 5 µl of PCR product (approximately 100 ng of DNA), 1.5 µl of Green Buffer 10X, 0.3 µl of Enzyme and 8.2 µl H_2O. The incubation time was 10 min at 37 °C for the *HindIII*, *SchI* and *VspI* enzymes and 10 min at 65 °C for *TaaI* and *Tru1I*. The

five species were digested with all the enzymes in order to validate the restriction pattern. The PCR-RFLP method was assayed using mixtures with different proportion of *Salvelinus namaycush* and *S. fontinalis* DNA as templates.

Sensitivity of the method

The detection limit of direct PCR with the two Salmonidae-specific primers alone (without nested PCR) was determined from serial dilutions of DNA of the five species (*S. salar, S.trutta, O. mykiss, S. fontinalis and S. namaycush*), starting from a known concentration (1 µg/ml).

The detection limit of the nested PCR (16S PCR followed of a PCR from the amplification fragment with the two Salmonidae-specific primers as described in 'PCR conditions') was done also from the same serial dilutions employed above. The dilution where no amplification was observed in agarose gel was considered the detection limit. DNA concentration was measured with a fluorometer Qubit®dsDNA BR Assay.

To test the sensitivity of the PCR and RFLP, several mixes of *S. namaycush* were tested. Mix 1 with 37.5 ng of DNA from *S. namaycush* and 12.5 ng of *S. fontinalis*, Mix 2 with 25 ng of each species, Mix 3 with 12.5 ng of *S. namaycush* and 37.5 ng of *S. fontinalis*, Mix 4 with 5 ng of *S. namaycush* and 45 of *S. fontinalis* and Mix 5 with 50 ng DNA of 6 Salmonidae species (*S. namaycush, S. fontinalis, S. alpinus, S. trutta, S. salar and O. mykiss*).

Method validation for eDNA

The method was validated in environmental DNA from aquarium samples as well as from field water samples obtained in locations with known salmonid populations.

Aquarium tests included two experimental situations: one of high density with six *Salmo trutta* juveniles (mean weight 1.714 ± 0.301 g) and another of low density with three *S. trutta* juveniles (two replicates: mean weights of 1.537 ± 0.405 g for Replica 1 and 1.400 ± 0.865 g for Replica 2). The brown trout juveniles were left swimming in aquariums of 15 L for 5 days. Everyday 10 L of water were replaced, after five days one sample of 1 L of water was taken from each aquarium for filtration and DNA extraction (see below) and nested PCR-RFLP was done.

For validation with field samples, one liter of water was collected from two positive and two negative control sites in the region of Asturias (north of Spain; Fig. 1). One positive control was Nora River (Asturias, north of Spain) at the coordinates 43.379283N, −5.788667W, with an average discharge of 20.98 m³/s. This tributary of Nalón River is isolated from the mainstream due to an impassable dam and contains a small resident population of *Salmo trutta*. No other salmonids or fish farms occur in the river. The other positive sample was the fishing reservoir "El Arenero" at the coordinates 43.346814N, −6.378065W. This one hectare surface pond contains *O. mykiss* released by the managers. The negative controls were the estuary of Aviles (coordinates 43.573223N, −5.922922W) and the Llanes Beach (coordinates 43.420461N, −4.752003W), where there are no salmonids.

Filtration and DNA extraction was done as explained in 'Measures for avoiding contamination in eDNA.' Nested PCR and RFLP were applied on the DNA extracted

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Figure 1 **Map from Asturias (Spain).** Water sampling sites: River Nora, River Nalón, fishing reservoir "El Arenero", Avilés and Llanes are shown.

from the water samples as described in 'PCR conditions' and 'Restriction enzyme digestion validation.' All samples were tested with the five restriction enzymes.

Measures for avoiding contamination in eDNA

Two separate areas were used for the whole process, one for pre-PCR and another one for post-PCR. Filtration of water samples was done in the pre-PCR room, where there were no positive DNA or tissue samples. Water samples were vacuum filtered using the Supor®-200 Membrane Filter (Pall Corporation) with 0.2 μm pore size and a reusable filter holder. The filter holder was dismantled, sprayed with 10% bleach, cleaned with detergent and 10% bleach, rinsed with distilled water and autoclaved between each sampling site. To ensure the cleaning process was correct, one sample with 1 L distilled water was filtrated between two problem samples and included in all eDNA analyses to confirm that contamination did not occur in the filtration or extraction process.

DNA was extracted with the PowerWater® DNA Isolation Kit (QIAGEN laboratories). The eDNA extraction was done in a separate laboratory unit inside a PCR laminar flow cabinet treated with ultraviolet light, where no salmonid tissue sample has never been used. The process was done using filter tips, to avoid contamination of the extraction kit and between samples.

The PCR reaction was prepared in the pre-PCR room inside a PCR cabinet treated with ultraviolet light. Once every sample was ready, closed and inside the PCR machine, the positive control was added in the post-PCR room and put into the machine, to avoid

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Figure 2 **Nalón River basin.** Dams along the river are shown; from downstream to upstream they are Valduno (D1), Priañes (D2), Furacón (D3), Rioseco (D4) and Tanes (D5). The fish farms are pointed as F1 to F7 and finally the sampling points are numbered in red from 1 to 16.

any contact between tubes with samples and with positive control. In every step, negative controls were added to ensure the samples were contamination free, as explained above.

Case study: Nalón River

The method was applied in Nalón River (Cantabrian corridor basin), of 140.8 km long and with an average discharge of 55.18 m³/s. There are five dams in its way long: Valduno, Priañes, Furacón, Rioseco and Tanes from downstream to upstream (D1–D5 respectively in Fig. 2). Seven fish farms are located along the river: one downstream in Pravia (Piscifactoría Barganeiro) where *O. mykiss* is farmed (F1); two in Cubia River, a tributary of Nalón River (Piscifactoría Alcubiella and Piscifactoría del Alba III), both with *O. mykiss* (F2 and F3); one in Somines (Piscifactoría Somines) with *O. mykiss* (F4); one in Laviana (Piscifactoría La Chalana) where *S. trutta* is reared (F5); and two upstream (Piscifactoría del Alba SA I in Soto de Agues and Piscifactoría del Nalón I in Veneros) with *O. mykiss* (F6, F7) (*Ministerio de Agricultura, Alimentación y Medio Ambiente, 2016*).

In February 2016, 16 different points along Nalón River were selected and sampled (1–16 in Fig. 2). Three liters of water were collected with sterile bottles from each point from upstream to downstream, putting the bottle as close to the bottom substrate as possible. They were put in ice and transported rapidly to the laboratory. Two samples of 1 L were filtered and extracted as described above, and one liter was stored frozen for confirmatory analysis if needed. Each replicate was extracted and analyzed separately in time. With the two eDNA samples, the PCR-RFLP method was performed twice, to discard false positive and false negative results due to technical failure. A minimum of two positive results from different extractions were considered valid to corroborate the presence of a species in

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the sample. In some cases where only one of the two eDNA replicates were positive, the PCR-RFLP method was performed three times to consider the result as positive. When the digestion results were not clear (too weak bands), the digestion was repeated using 10 µl of PCR template instead of 5 µl as described above. All the samples were tested with all the restriction enzymes.

Ethics statement

This project and the experimental procedure including aquarium stage of *Salmo trutta* was approved by the Committee of Ethics of the Government of the Principality of Asturias according to the Royal Decree 53/2013 of 1 February 2013 that regulates the use of experimental animals in Spain, with the permit code PROAE 25/2015.

RESULTS

Primers designed

The new general forward primer was: 16S-new-F (5′-GCCTGCCCTGTGACTATGG-3′). Together with the universal 16S-Br reverse primer (*Palumbi et al., 2002*), they amplify a fragment of 567 nucleotides within the 16S rRNA gene, located between the sites 2046 and 2613 of the *Salmo salar* mitochondrion complete genome (GenBank: KF792729.1). The Salmonidae specific primers designed *in silico* from the analysis of databases and new sequences of salmonids were:

Forward primer: 16S-F-Salm (5′-AAGACCTGTATGAATGGCATC-3′)

Reverse primer: 16S-R-Salm (5′-TCGATAGGGACTCTGGGAGA-3′).

These primers amplify a fragment of 377 nucleotides within the 16S rRNA gene, located between the sites 2125 and 2502 of the same *S. salar* reference sequence used before. The assays of annealing temperatures for the PCR with Salmonidae specific primers showed that the best results were obtained at 68 °C with 2 mM MgCl$_2$. All the 16S rDNA sequences obtained and employed in this work are available in GenBank with the accession numbers stated in Table S1. In cross-amplification assays we have confirmed that the new primers only amplified from salmonids species. The sequence of the amplicons obtained for *O. mykiss*, *S. trutta*, *S. salar*, *S. fontinalis* and *S. namaycush* are available in GenBank with the accession numbers KU510521, KU510522, KU510523, KU510525 and KU510526 respectively.

The threshold of detection for direct PCR with the two Salmonidae-specific primers- and visualization in agarose gels was 0.1 ng/ml. We observed a band of the expected size in the dilution 1 to 10,000 from the five tested samples with an initial concentration of 1 µg/ml.

The detection limit for the nested PCR method (16S PCR with the new general primer designed and one universal Palumbi's primer followed by a PCR with the two Salmonidae-specific primers) was 10 pg/ml, since positive bands of the expected amplicon size were observed in agarose from the dilution 1 to 100,000 of the five samples with an initial concentration of 1 µg/ml.

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Table 1 Restriction patterns obtained with the enzymes considered for the five salmonid species. The bands in bold are diagnostic to identify each species.

Enzyme	FastDigest	Restriction site	Species	Bands	Rest of species
HindIII	FD0504	AAGCTT	*Salvelinus namaycush*	**231** and **146** bp	377 bp
VspI	FD0914	ATTAAT	*Salvelinus fontinalis*	**222** and **155** bp	377 bp
SchI	FD1374	GAGTC(N)$_5$	*Salmo salar*	**272** and **103** bp	374 y 3 bp
TaaI	FD1364	ACNGT	*Salmo trutta*	**205** and **172** bp	377 bp
Tru1I	FD0984	TTAA	*Oncorhynchus mykiss*	155, 156 and **66** bp	222, 150 and 5 bp

The species-specific PCR-RFLP

The restriction patterns within the fragment amplified with the new primers provided specific bands for all the considered species (Table 1). The diagnostic bands could be clearly differentiated in agarose gel (Fig. 3). Specific bands for *S. fontinalis* were 222 bp and 155 bp with the enzyme VspI, specific bands for *S. namaycush* 231 bp and 146 bp with HindIII, and so on. The non-target species *Esox lucius*, which has significant match with the primers, theoretically amplifies a fragment of 373 nt and was included in the design of the RFLP. None of the enzymes are supposed to digest the amplicon, except Tru1I which could give fragments of 140, 138, 75 and 21 bp. These fragments are clearly different from the diagnostic bands of the target species. Thus the RFLP patterns were well defined and allowed to differentiating each species.

Regarding the sensitivity of this method in agarose gels, in mixes of *S. namaycush* (lake trout) and *S. fontinalis* (brook trout) DNA (Table S2) it was possible to observe the clear diagnostic band of *S. namaycush* down to as little as 5ng of *S. namaycush* DNA. Moreover a light band could also be observed in Mix 5, where the DNA template was a mixture of 5 different species with only 7.5% of *S. namaycush* DNA. Thus this method was effective for recognizing a species also when there were different Salmonid species mixed in a site.

Detection of salmonids from water samples

The PCR-RFLP method was validated in both aquarium experiments and field water samples. The PCR was performed with the new general forward primer (16S-new-F) and the universal 16S-Br reverse primer (*Palumbi et al., 2002*). All the eDNA samples yielded amplification products of the expected size (Fig. 4A). Nested PCR provided a clear band of 370 nucleotides, the expected amplicon size, in both aquarium and positive field samples (Nora River and "El Arenero"); in the two other field samples no positive amplification was obtained from these primers, as expected since salmonids do not occur in Aviles estuary and Llanes beach (Fig. 4B).

The bands typical of S. *trutta* (205 and 172 bp) were obtained after digestion with the enzyme *TaaI* (Fig. 5) in aquarium samples, thus validating the use of this PCR-RFLP marker from water eDNA.

The water sample from Nora River, with a known population of *S. trutta*, and the water sample from "El Arenero," with a known population of *O. mykiss*, provided a clear band of 370 nucleotides with the Salmonid-specific primers (Fig. 4B). The PCR products obtained from River Nora and "El Arenero" water DNA were purified, sequenced and the sequence
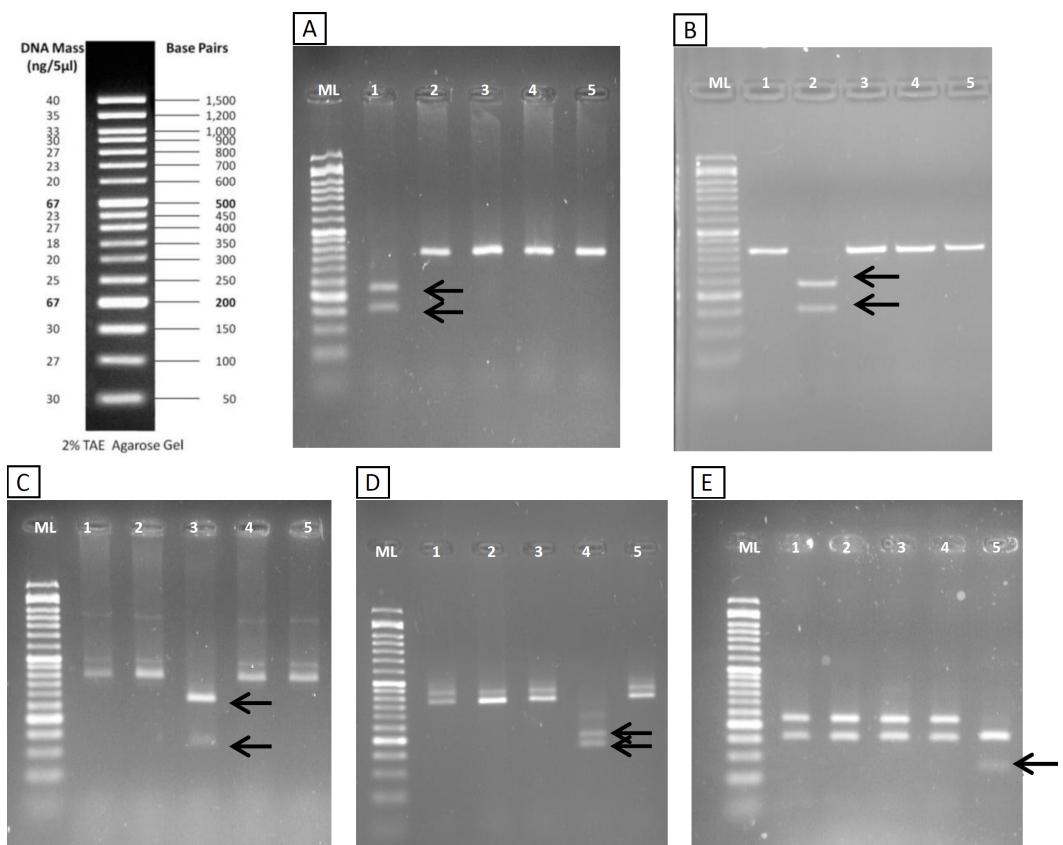
Figure 3 RFLP validation. Agarose gels (2%) showing results of digestion with VspI (A), HindIII (B), SchI (C), TaaI (D) and Tru1I (E). Lanes (from 1 to 5) in all gels are: Ladder (ML), *S. fontinalis*, *S. namaycush*, *S. salar*, *S. trutta*, *O. mykiss*. Diagnostic bands for each species are marked with arrows.
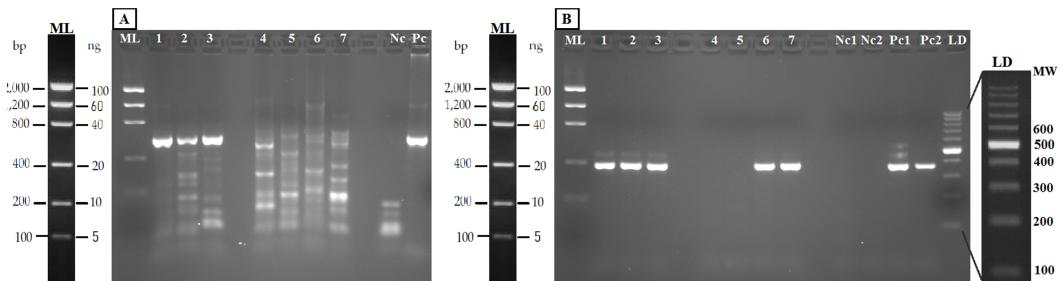
Figure 4 Agarose gels (2%) showing validation of the method with eDNA. Samples in both geles are aquarium samples: BThd (1), BT1 (2), BT2 (3), and field samples: Llanes (4), Avilés (5), Nora River (6) and fishing reservoir "El Arenero" (7), negative and positive controls are included (Nc and Pc respectively). (A) PCR product from 16S general PCR and (B) PCR product from Nested PCR with Salmonidae specific primers. Positive controls (Pc1) and (Pc2): nested and direct PCR, respectively, on *Salmo salar* DNA extracted from muscle. Negative controls are indicated as (Nc1 and Nc2) nested and direct PCR, respectively.

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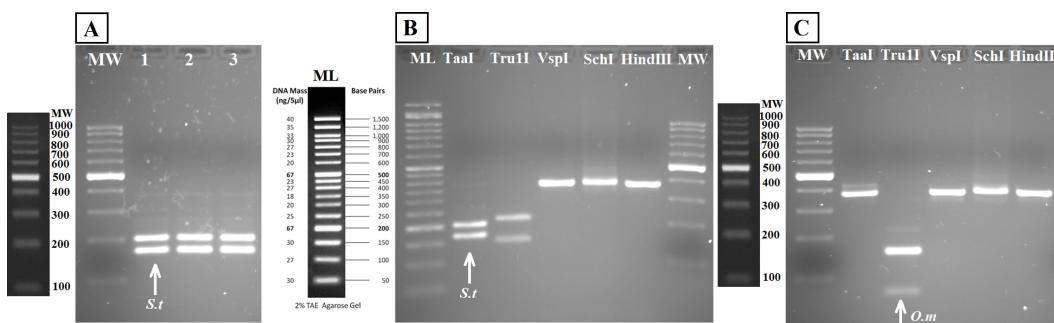
Figure 5 **Agarose gels showing restriction fragments obtained after digestion of amplicons from water DNA samples (Fig. 4B).** (A) Aquarium samples digested with TaaI, BThd (1), BT1 (2) and BT2 (3). (B) Restriction fragments of amplicons from nested PCR of Nora River water obtained with: TaaI, Tru1I, VspI, SchI, HindIII. (C) Restriction fragments of amplicons from nested PCR of ''El Arenero'' water, obtained with: TaaI, Tru1I, VspI, SchI, HindIII. Diagnostic bands of different species are marked with arrows. *Om* and *St* are *O. mykiss* and *S. trutta* respectively.

identified by BLAST as *S. trutta* and *O. mykiss* respectively. Both sequences are available in GenBank (Accession numbers KU510527 and KX904362).

RFLP digestions confirmed the species present in water samples in all cases (Figs. 5B and 5C). Typical bands of S. *trutta* (205 and 172 bp) were obtained after digestion with the enzyme *TaaI* in Nora River sample. In the sample from the fishing reservoir ''El Arenero,'' it is possible to identify *O. mykiss*; the bands expected of *O. mykiss* (66 bp) were obtained after digestion with *Tru1I*. This validates the method from field environmental samples with complex species mixtures, in both type of samples watercourse (Nora River) and ponds (''El Arenero'').

Case study: Nalón River

The results of Nalón River are shown in Table 2. The sampling points were separated from each other by an average distance of 6.74 ± 3.02 km. Multiple Salmonidae species were detected from the same sample and with the same PCR product. *Salmo salar* was found downstream in points 1 to 3. It should be noted that Narcea River, which is a tributary of Nalón River and a well known Atlantic salmon preserve, joins the mainstream in Pravia (upstream point 3).

S. trutta and *O. mykiss* were found along the whole river. In points 7 and 15 only *S. trutta* eDNA was found, while in point 9 *O. mykiss* was the only species found from eDNA. In point 10 none of these salmonids were detected with the new marker.

DISCUSSION

Here we described a robust marker for detection and identification of five species of salmonids from water samples based on RFLP from the product of a single PCR. Specific PCR primers are available for different Salmonidae such as *S. namaycush* (*Lacoursière-Roussel et al. , 2015*), *S. trutta* (*Gustavson et al., 2015*; *Carim et al., 2016*), *O. mykiss* (*Wilcox et al., 2015*), *S. fontinalis* (*Wilcox et al., 2013*), but for European waters only one has been

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Table 2 Náló River results. The positive identification of the species in each point is showed with an "X" and the negative identification with "−".

Sampling points	Coordinates	Distance between 1 point and the next (km)	S. trutta	O. mykiss	S. salar	S. fontinalis	S. namaycush
Sampling point 1 La Arena	43.548512N, −6.080661W	1.68	X	X	X	−	−
Sampling point 2 Soto del Barco	43.535637N, −6.080841W	10.49	X	X	X	−	−
Sampling point 3 Pravia	43.491283N, −6.103837W	7.90	X	X	X	−	−
Sampling point 4 San Román	43.448498N, −6.079927W	11.6	X	X	−	−	−
Sampling point 5 Bar Casa Aurina	43.403614N, −6.040419W	3.95	X	X	−	−	−
Sampling point 6 Valduno's dam	43.38987N, −6.0053W	8.76	X	X	−	−	−
Sampling point 7 Trubia	43.354393N, −5.963959W	6.39	X	−	−	−	−
Sampling point 8 Las Caldas	43.331509N, −5.930557W	8.50	X	X	−	−	−
Sampling point 9 Soto de Ribera	43.308495N, −5.870101W	6.99	−	X	−	−	−
Sampling point 10 Olloniego	43.315437N, −5.814595W	11.85	−	−	−	−	−
Sampling point 11 Lada	43.306736N, −5.697167W	9.87	X	X	−	−	−
Sampling point 12 San Martín del Rey Aurelio	43.273834N, −5.601774W	6.65	X	X	−	−	−
Sampling point 13 Laviana	43.237621N, −5.554755W	9.14	X	X	−	−	−
Sampling point 14 Rioseco's dam	43.223583N, −5.459807W	2.72	X	X	−	−	−
Sampling point 15 Anzó	43.225563N, −5.438601W	6.58	X	−	−	−	−
Sampling point 16 Tanes' dam	43.192474N, −5.382222W	−	X	X	−	−	−

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recently described for detection of brown trout (*Gustavson et al., 2015*). Our method enabled applications in a wider range of situations and species mixtures.

All previously described studies were based on qPCR, useful for knowing the density of one species. Our tool allows for a rapid overview of the Salmonidae community without the use of real-time PCR systems, and in the particular case of Spain it allowed to detect exotic and native salmonids at the same time. As it is, the method is ready to be used in Spanish waters, but it could be easily adapted for application in other region by checking for any cross-amplification with the local aquatic fauna.

Another advantage of the method was its technical accessible procedure that may allow to be routinely implemented in a laboratory, since it requires less special technical know-how or equipment than qPCR or NGS. RFLP-based methods are generally more economical and faster in comparison to metabarcoding (*Teletchea, 2009*; *Li et al., 2015*), and could be applied in routine sampling in a near future. The average cost of the method employed here was 13.4 euros per water sample including reagents for DNA extraction, PCR amplification and digestions with the complete set of enzymes (not the labor that may vary very much depending on salary wages and possible robotizing). The whole process would not take longer than one day, and it is possible to analyze several samples at the same time. It could also be robotized for genotyping in capillary electrophoresis using labeled primers, expectedly with better results because capillary electrophoresis has a better resolution than agarose gels. Compared with NGS metabarcoding, the digestion products can be directly interpreted and do not need bioinformatics analysis as NGS does (*Coissac, Riaz & Puillandre, 2012*; *Taberlet & Coissac, 2012*). DNA metabarcoding may be also limited by the difficulty to design universal primers (*Deagle et al., 2014*). Compared with other methodologies, such as SNPs (*Wenne et al., 2016*), it does not require high DNA quality; in fact in environmental samples the DNA is degraded and fragmented and despite it, it was possible to apply the method here described directly on water samples.

Since the Salmonidae-specific primers are highly sensitive, it is possible to use them directly on water samples for detecting salmonids, without the need of nested-PCR. This has been already proven from ballast water samples for confirming the presence of salmonid DNA detected from Next-Generation Sequencing (NGS) metabarcoding (*Zaiko et al., 2015*). Given its extreme sensitivity when using nested-PCR, our method could be applied in running waters. The method as it is could be applied to monitor the use of streams by Atlantic salmon, since it served to detect this species downstream the studied river. It could be used for a quick search of non-native populations of trout (e.g., Atlantic hatchery bred *S. trutta*) along classical DNA isolation from fin-clips. It could be easily adapted to coho salmon (*O. kisutch*) and sockeye salmon (*O. tshawytscha*) in Canada (*Irvine et al., 2005*), and other populations of Pacific salmon in the USA (*Gustafson et al., 2007*). It would be especially useful in protected spaces, enabling to detecting the presence of salmonids without disturbing wild populations with electrofishing.

On the other hand, it could be a useful tool to detect salmonids in places where these species are exotic and represent a danger to the local fauna. It could serve to detect escapes from aquaculture, a big problem for local wild populations (*Hewitt, Campbell & Gollasch, 2006*; *De Poorter, 2009*), and to detect populations of exotic salmonids—such as rainbow

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trout in Spain (*Elvira & Almodóvar, 2001*), or brown trout in New Zealand (*Townsend, 1996*; *Townsend, 2003*). In our case study (Nalón River) *S. trutta* and *O. mykiss* were detected from almost every sampling point, which we expected since there are *S. trutta* populations in Nalón River and some fish farms for *O. mykis.*

The biggest weakness of our method may be the mitochondrial sequences employed. Exotic salmonids can hybridize with native salmonids, since in this family genetically close species hybridize with each other (e.g., *Rubidge & Taylor, 2004*; *Hórreo et al., 2011*). Indeed, the marker here developed cannot detect hybrids because mitochondrial DNA has maternal inheritance. On the other hand it is based on DNA, a resistant molecule that can be amplified from dead animals, or from farm discharges. *Hänfling et al. (2016)* showed that eDNA from flowing streams may contaminate lake samples. *Deiner & Altermatt (2014)* demonstrated that eDNA from two invertebrates (*Daphnia longispina* and *Unio tumidus*) could be detected as far as nine to 12 km downstream from their populations were known to occur. Another contamination source could be avian feces, such as *Merkes et al. (2014)* showed. They found that the DNA of silver carp from avian excrement could be detected and the detection persisted for 28 days. Other authors have measured the degradation of eDNA in a ecosystem, such as *Strickler, Fremier & Goldberg (2015)*, who tested the effect of UV, temperature and pH in *Lithobates catesbeianus* eDNA obtaining positive detection from one to 54 days after species removal. On the other hand, *De Souza et al. (2016)* suggested that eDNA detection probability for the two species *Necturus alabamensis* and *Sternotherus depressus* was strongly affected by the season of sampling. In our particular case study of Nalón River *O. mykiss* was not detected from point 15 (Table 2 and Fig. 2) which is 10.85 km downstream the closest fish farm (Piscifactoría Nalón I in Veneros, F7 in Fig. 2), but was identified from point 9 which is 47.08 km downstream the closest farm (Piscifactoría del Alba SA I in Soto de Agues, F6 in Fig. 2). We could interpret that, at least in the second case, the presence of *O. mykiss* DNA was probably due to real individuals, coming from escapes of fish farms. To confirm positive results in the wild when a contamination source of eDNA is near, such as fish farms, it would be advisable to survey the place at different times, and to confirm the presence of individual escapes from conventional physical sampling.

CONCLUSION

This PCR-RFLP method is a sensitive tool able to detect the presence of five salmonid species by analyzing DNA extracted from water samples from a nested PCR and further simultaneous restriction digestions. This innovation may have various applications worldwide, either for detecting exotic salmonids or for monitoring native populations without disturbing them and the rest of aquatic fauna.

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ADDITIONAL INFORMATION AND DECLARATIONS

Competing Interests

The authors declare there are no competing interests.

Author Contributions

- Laura Clusa conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Alba Ardura, Sara Fernández and Agustín A. Roca contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Eva García-Vázquez conceived and designed the experiments, wrote the paper, reviewed drafts of the paper.

Animal Ethics

The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

This project and the experimental procedure including aquarium stage of *Salmo trutta* was approved by the Committee of Ethics of the Government of the Principality of Asturias according to the Royal Decree 53/2013 of 1 February 2013 that regulates the use of experimental animals in Spain, with the permit code PROAE 25/2015.

Data Availability

The following information was supplied regarding data availability:

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Genbank KU510485–KU510527 and KX904362

Clusa, Laura; Ardura, Alba; Fernández, Sara; Roca, Agustín; García-Vázquez, Eva (2017): An extremely sensitive nested PCR-RFLP mitochondrial marker for detection and identification of salmonids in eDNA from water samples. figshare. https://doi.org/10.6084/m9.figshare.4597888.v1.

Supplemental Information

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Table S1. Samples from different families of fishes from the laboratory collection employed for testing the Salmonidae-specific markers. All the 16S rDNA haplotypes from each species obtained and employed in this work are available in GenBank with the accession numbers stated in the table.

Phylum	Class	Order	Family	Species	Accession numbers
Chordata	Actinopterygii	Clupeiformes	Clupeidae	*Sardina pilchardus*	KU510505
Chordata	Actinopterygii	Clupeiformes	Engraulidae	*Engraulis encrasicolus*	KU510488
Chordata	Actinopterygii	Gadiformes	Gadidae	*Micromesistius poutassou*	KU510496
Chordata	Actinopterygii	Gadiformes	Gadidae	*Pollachius pollachius*	KU510500
Chordata	Actinopterygii	Gadiformes	Merlucciidae	*Merluccius merluccius*	KU510493-KU510494
Chordata	Actinopterygii	Lophiformes	Lophiidae	*Lophius budegassa*	KU510492
Chordata	Actinopterygii	Perciformes	Carangidae	*Trachurus*	KU510504
Chordata	Actinopterygii	Perciformes	Gobiidae	*Gobius*	KU510489
Chordata	Actinopterygii	Perciformes	Moronidae	*Dicentrarchus labrax*	KU510486
Chordata	Actinopterygii	Perciformes	Mullidae	*Mullus surmuletus*	KU510497
Chordata	Actinopterygii	Perciformes	Scombridae	*Scomber scombrus*	KU510503
Chordata	Actinopterygii	Perciformes	Sparidae	*Boops boops*	KU510485
Chordata	Actinopterygii	Perciformes	Sparidae	*Diplodus vulgaris*	KU510487
Chordata	Actinopterygii	Pleuronectiformes	Pleuronectidae	*Platichthys flesus*	KU510498-KU510499
Chordata	Actinopterygii	Pleuronectiformes	Scophthalmidae	*Lepidorhombus boscii*	KU510490
Chordata	Actinopterygii	Pleuronectiformes	Scophthalmidae	*Lepidorhombus whiffiagonis*	KU510491
Chordata	Actinopterygii	Pleuronectiformes	Scophthalmidae	*Psetta maxima*	KU510501
Chordata	Actinopterygii	Pleuronectiformes	Scophthalmidae	*Scophthalmus rhombus*	KU510502
Chordata	Actinopterygii	Pleuronectiformes	Soleidae	*Microchirus variegatus*	KU510495
Chordata	Actinopterygii	Salmoniformes	Salmonidae	*Oncorhynchus mykiss*	KU510508
Chordata	Actinopterygii	Salmoniformes	Salmonidae	*Salmo salar*	KU510514-KU510516
Chordata	Actinopterygii	Salmoniformes	Salmonidae	*Salmo trutta*	KU510509-KU510513
Chordata	Actinopterygii	Salmoniformes	Salmonidae	*Salvelinus alpinus*	KU510517
Chordata	Actinopterygii	Salmoniformes	Salmonidae	*Salvelinus fontinalis*	KU510518
Chordata	Actinopterygii	Salmoniformes	Salmonidae	*Salvelinus namaycush*	KU510519-KU510520
Chordata	Actinopterygii	Zeiformes	Zeidae	*Zeus faber*	KU510506-KU510507

Table S2. Sensitivity assays. Mix 1–5: Mixes of *S. namaycush* and *S. fontinalis* DNA used in the development of the PCR-RFLP method, indicating the percentage of *S. namaycush* and the amount of DNA of each species in the mix. In bold are the diagnostic fragments that can be seen in the agarose gel.

Nº	Enzymes	Incubation	Mixes	% *S. namaycush*	Expected Bands	Detection
Mix 1	HindIII	37°C-10'	*Salvelinus namaycush* 37.5 ng + *Salvelinus fontinalis* 12.5 ng	75	377, **231** and **146** bp	Positive
Mix 2	HindIII	37°C-10'	*Salvelinus namaycush* 25ng + *Salvelinus fontinalis* 25 ng	50	377, **231** and **146** bp	Positive
Mix 3	HindIII	37°C-10'	*Salvelinus namaycush* 12.5 ng + *Salvelinus fontinalis* 37.5 ng	25	377, **231** and **146** bp	Positive
Mix 4	HindIII	37°C-10'	*Salvelinus namaycush* 5 ng + *Salvelinus fontinalis* 45 ng	10	377, **231** and **146** bp	Positive
Mix 5	HindIII	37°C-10'	2 µl each: *Salvelinus namaycush, Salvelinus fontinalis, Salvelinus alpinus, Salmo trutta, Salmo salar and Oncorhynchus mykiss.*	7.15% (50 ng/700 ng DNA)	377, **231** and **146** bp	Positive

Capítulo 2:

A simple, rapid method for detecting seven common invasive fish species in Europe from environmental DNA

Clusa L and García-Vázquez E

Aquatic Conservation: Marine and Freshwater Ecosystems

A simple, rapid method for detecting seven common invasive fish species in Europe from environmental DNA

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ABSTRACT

1. Biological invasions are a global threat to biodiversity, and many come from deliberate introductions.
2. The American freshwater fish *Micropterus salmoides* and *Ameiurus* sp (*A. melas* and *A. nebulosus*) were introduced to Europe for recreational fishing; *Gambusia holbrooki* and *G. affinis* for mosquito population control and *Lepomis gibbosus* as an ornamental species. The Asiatic *Pseudorasbora parva* was acquired inadvertently as an accompanying species of fish consignments.
3. This paper presents a novel approach for detecting these species directly from water samples based on a panel of five taxon-specific primers within the 16S rDNA.
4. The primers were validated from tissue, in aquarium experiments, and from Ebro River water samples (Spain). With a simple PCR protocol followed by visualization in agarose gel or capillary electrophoresis it was possible to detect these species from environmental DNA concentrations as low as 0.89 to 100pg mL^{-1}.
5. This sensitive and economical tool can be useful to control the European invasions of these species and preserve the native biodiversity.

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Introduction

Biological invasions are an important threat to biodiversity (Chown et al., 2015), since they often result in local extinctions or extirpation of autochthonous species (Clavero & García-Berthou, 2005). Aquatic species are translocated worldwide for various purposes, from fishing to aesthetic pleasure (Havel, Kovalenko, Thomaz, Amalfitano, & Kats, 2015). In Europe, freshwater fish are the most frequently introduced aquatic species (García-Berthou et al., 2005). The north American largemouth black bass *Micropterus salmoides* and bullhead catfish *Ameiurus* spp. (*A. melas* and *A. nebulosus*) were introduced into many European waters for recreational fishing (Copp et al., 2016; Savini et al., 2010). The mosquitofish *Gambusia holbrooki* and *G. affinis* were widely introduced to Europe for mosquito control (Pyke, 2008), from their native range in North America (Sanz et al., 2013). Another cause of exotic fish spread is imports for aquarium trade that are often released into the wild. An example is the introduction of north American *Lepomis gibbosus* (pumpkinseed) to Europe as an ornamental species (Maceda-Veiga, Escribano-Alacid, de Sostoa, & García-Berthou, 2013). An unexpected impact of non-native fish farming in Europe was the inadvertent introduction of highly invasive accompanying species as contaminants in farm fish consignments.

For example the topmouth gudgeon (or stone moroko) *Pseudorasbora parva* was transported together with Chinese carp from Asia to Romania in 1960, and today it is present in almost every country in Europe (Gozlan et al., 2010; Simon, Gozlan, Britton, van Oosterhout, & Hänfling, 2014). The EU regulation No 1143/2014 of 22 October 2014 on Invasive Alien Species (http://ec.europa.eu/environment/nature/invasivealien/index_en.htm) states in its Article 14 that Member States should establish a surveillance system to detect rapidly the appearance of any invasive alien species in the environment of a Member State. Rapid detection is indeed important because biological invasions are better controlled in the initial invasion stages (e.g. Blackburn et al., 2011).

Ecological impacts of the above mentioned species have been demonstrated in Europe, where they compete with native species for habitat and food resources (Ribeiro & Leunda, 2012). *Lepomis gibbosus* exhibits aggressive behavior when competing for food and territory (Almeida, Merino-Aguirre, Vilizzi, & Copp, 2014). *Gambusia* species affect native fauna such as invertebrates and amphibians through predation (Pyke, 2008; Remon, Bower, Gaston, Clulow, & Mahony, 2016). They alter the plankton communities and subsequently the whole ecosystem (Hurlbert & Mulla, 1981; Hurlbert,

Zedler, & Fairbanks, 1972). Introduced black bass (*M. salmoides*), an aggressive predator, usually affects populations of small native fishes by predation, sometimes causing their decline or extinction (Maezono & Miyashita, 2002; Weyl & Lewis, 2016). *Ameiurus* sp may also have adverse ecosystem effects by increasing turbidity (Braig & Johnson, 2003). Moreover, these invasive species are hosts of many parasites. For example, *L. gibbosus* introduced new parasites (*Onchocleidus* sp) in Norway (Sterud & Jørgensen, 2006). *Pseudorasbora parva* carries many parasites, such as the rosette agent (*Sphaerothecum destruens)* (Gozlan et al., 2010; Pinder, Gozlan, & Britton, 2005), being capable of transmission to native fish species (Gozlan, St-Hilaire, Feist, Martin, & Kent, 2005).

Eradication of these invasive species, when possible, may allow the recovery of native fauna. This happened in 11 small ponds from Oshu city, north-eastern Japan, after eradication of *M. salmoides* (Tsunoda, Mitsuo, Ohira, Doi, & Senga, 2010). In one lake from the Lake District in the north-west of UK, populations of native *Rutilus rutilus* and *Abramis brama* increased after *P. parva* eradication (Britton, Davies, & Brazier, 2009). Early detection of non-native fish is crucial for a rapid and efficient response to prevent further establishment or spread.

In the last few years, environmental DNA (eDNA) survey methods have proved a promising tool for detecting and surveying invasive species in aquatic ecosystems. Metazoans can be detected from their DNA released into the environment through skin flaking and sloughed cells, mucus excretion and defecation (Goldberg, Strickler, & Pilliod, 2014). This method seems to be sensitive and efficient, and unlike most classic sampling methods (electrofishing, netting) it does not disturb the aquatic fauna (Blanchet, 2012; Ficetola, Miaud, Pompanon, & Taberlet, 2008; Thomsen et al., 2012). Specific PCR primers used on eDNA have been successful in detecting a number of species from water samples. Examples are molluscs (Ardura et al., 2015; Devloo-Delva et al., 2016; Clusa et al., 2016), fishes (Furlan & Gleeson, 2016; Gustavson et al., 2015; Takahara, Minamoto, & Doi, 2013; Adrian-Kalchhauser & Burkhardt-Holm, 2016; Uchii, Doi & Minamoto, 2016), amphibians (Ficetola et al., 2008; Pilliod, Goldberg, Arkle, & Waits, 2014), reptiles (Piaggio et al., 2014; Davy, Kidd, & Wilson, 2015) and mammals (Foote et al., 2012; Ushio et al., 2017).

The aim of this study was to check the potential of a simplified PCR-based method for early alert of seven common invasive fish species *A. melas, A. nebulosus, G. affinis, G. holbrooki, M. salmoides, L. gibbosus* and *P. parva* from water samples. If successful, the method could be applied by managers for river surveillance. For this purpose, new specific primers were developed and tested experimentally in vitro and in aquarium, as well as from field water samples. The seven species have been reported from many European countries, including Spain (Elvira & Almodóvar, 2001; Leppäkoski, Gollasch, & Olenin, 2002). They are in the Spanish official list of invasive alien species (Spanish Royal Decree 630/2013 of 2 August 2013, https://www.boe.es/buscar/doc.php?id=BOE-A-2013-8565). They all occur in Ebro River (north-east Spain), as reported in the webpage of the Regional Government of Aragón; thus, the new tool was field tested from Ebro River waters. The method could be also applied in other European waters for surveillance of these invasive alien species.

Materials and Methods

Species studied

For confirming the adequacy of the species choice an exhaustive search was performed in three databases for invasive species: EASIN (European Alien Species Information Network; http://easin.jrc.ec.europa.eu/ accessed in November 2016), DAISIE (Delivering Alien Invasive Species Inventories Europe; http://www.europe-aliens.org/ accessed in November 2016) and the GISD (Global Invasive Species Database; http://www.iucngisd.org/gisd/ accessed in November 2016) of the IUCN (International Union for Conservation of Nature). The criterion of choice used was species invasiveness to European countries. The species selected were those invasive to a higher number of European countries (the top five). The non-native species invasive to Europe were compiled (Table S1) and the five taxa of choice in this study (*Ameiurus* sp, *Gambusia* sp, *L. gibbosus*, *M. salmoides* and *P. parva*) are listed there amongst the commonest invasive non-salmonid fish to European countries. In Spain there are 61 species from 24 fish families officially listed as native to the Iberian Peninsula, and 36 non-native species from 15 families including the aforementioned ones (Table S2).

Design of species-specific primers

The method applied is based on conventional PCR. The 16S rRNA gene was chosen for the design of the primers based on reference nucleotide sequences from GenBank (www.ncbi.nlm.nih.gov/) plus the sequences obtained in this study. 16S rRNA is a mitochondrial gene, present in higher copy number than nuclear genes in eDNA samples (Thomsen, & Willerslev, 2015), it does not show great variation within species but it shows high variation between

Table 1: Taxon-specific primers designed in this study. Primer's sequence, annealing temperature, Mg^2 concentration, expected amplicon size (in base pairs); initial DNA concentration employed for testing sensitivity of the primer pairs (stock), maximum dilution (last dilution) and corresponding DNA concentration (detection limit) for which is possible to obtain a PCR product visible in agarose gel with the primer pairs in the conditions assayed.

Species	Primer	Sequence (5'-3')	Annealing Temperature	[Mg^{2+}]	Amplicon size	Stock	Last dilution	Detection limit
Gambusia sp	Ga-16S-F	GRAACCAACTGACCCCTGCTT	68°C	1mM	117pb	0.535 µg mL^{-1}	1/600 000	0.89 pg mL^{-1}
	Ga-16S-R	GTTTTGTGAGCTGCGGCTCTWTA						
Micropterus salmoides	MiSa-16S-F	WCATCCCRAAACAAAGGGCY	68°C	2mM	142pb	0.57 µg mL^{-1}	1/100 000	5.7 pg mL^{-1}
	MiSa-16S-R	AATTCTGTTCATTAGAGCGGAGG						
Ameiurus sp	Am-16S-F	CGTCAAGAACYCAGTTRAACT	65°C	1mM	134pb	0.7 µg mL^{-1}	1/5 000	140 pg mL^{-1}
	Am-16S-R	GWTTCTGYGACTTAGAGTTGTCA						
Pseudorasbora parva	PsPa-16S-F	GTTTAAYCATGTTAAACAACTTAT	58°C	2.5mM	192pb	0.5 µg mL^{-1}	1/5 000	100 pg mL^{-1}
	PsPa-16S-R	TTCGTTGATCGACTATGTGT						
Lepomis gibbosus	LeGi-16S-F	GGACACGGGGCTAAACCAAAT	68°C	1mM	113pb	0.535 µg mL^{-1}	1/600 000	0.89 pg mL^{-1}
	LeGi-16S-R	GGGCTCTTAGTTGTGGAATTGCA						

closely species, especially in fishes (Maretto, Reffo, Dalvit, Barcaccia, & Mantovani, 2007; Vences et al., 2016) and the number of sequences for fishes in databases is similar to other mitochondrial genes as COI or cytochrome b (Machida, Leray, Ho, & Knowlton, 2017). Sequences of this gene (either individual 16S DNA sequences or complete mitochondrial genomes) for the target fish and other species of a wide range of aquatic taxa were downloaded from GenBank and aligned with the ClustalW application included in BioEdit (Thompson, Higgins, & Gibson, 1994). Polymorphisms were analyzed with DNASP software (Rozas, Sánchez-DelBarrio, Messeguer, & Rozas, 2003). The different haplotypes were visualized employing BioEdit Sequence Alignment Editor (Hall, 1999). Within the 600 nucleotide amplicon obtained with the universal primers designed by Palumbi et al., (2002), regions conserved within each of the target species (identical in all sequences of that species) but different in the rest of reference species collected were located. These regions were used to design the set of specific primers (Table 1).

Fish tissue and water sampling in field and aquarium

Tissue samples were provided by Centro de Acuicultura Vegas del Guadiana. Genetic barcoding was done using cytochrome c oxidase subunit I (COI) (Geller, Meyer, Parker, & Hawk, 2013) and 16S rRNA genes (Palumbi et al., 2002) in order to confirm the species of each tissue sample.

To sample eDNA, 1L of water was collected in sterile plastic bottles from each sampling point, in both aquariums and river. Water samples were vacuum filtered using the Supor®-200 Membrane Filter (Pall Corporation) with 0.2 µm pore size (Turner et al., 2014) and a filter holder. The filtration apparatus was cleaned with 10% bleach, rinsed with distilled water and sterilized under UV light for 20 minutes between filtrations. Filters were put individually within 15mL tubes and stored at -20°C until DNA extraction.

DNA extraction and PCR conditions

DNA from tissue samples was extracted using Chelex resine as described by Estoup, Largiader, Perrot, & Chourrout, (1996). DNA from water samples was extracted directly from the filters with the PowerWater® DNA Isolation Kit (Mobio laboratories) following manufacturer's recommendations. The eDNA extractions were done under sterile conditions, in a laboratory unit where there were no other tissue samples, inside a PCR laminar flow cabinet treated with ultraviolet light to avoid any contamination of the environmental DNA. As a negative control for extraction (blank sample) 1L of distilled water was treated equally as the samples for all the processes and included in each analytical step to be sure that contamination did not occur, as described in Clusa, Ardura, Fernández, Roca & García-Vázquez (2017).

For positive control samples, DNA extracted from tissue of *A. melas, G. holbrooki, L. gibbosus, M. salmoides* and *P. parva* was PCR-amplified with the newly developed primers (Table 1). For confirming that cross-amplification negative results were not due to PCR failure, universal primers for the 16S rRNA gene (Palumbi et al., 2002) were employed for PCR amplification on the same samples.

The amplification reaction with the taxon-specific primers from tissue DNA was performed in a total volume of 20µL, including Green

GoTaq® Buffer 1X, MgCl₂, 0.25mM dNTPS, 1μM of each primer, 2μL of template DNA and 0.65 U of DNA Taq polymerase (Promega). The PCR conditions were the following: an initial denaturation step at 95ºC for 5min, 35 cycles at 94ºC for 30s, annealing at the temperature of choice for 30s and elongation at 72ºC for 30s. A final step of elongation was set at 72ºC for 10min. Different annealing temperatures and MgCl₂ concentrations for each pair of primers were assayed (Table 1). PCR products were visualized in 2% agarose gels with 2.5μL of SimplySafe™.

In the case of DNA extracted from water samples, the PCR conditions were the same as described above with some minor modifications. Fifty cycles were used instead of 35 and 6μL of DNA template. BSA (200ng mL^{-1}) was added in the PCR mix to avoid the effects of inhibitors in the sample (Jiang, Alderisio, Singh, & Xiao, 2005). In addition to the blank sample, negative controls containing only PCR reagents and distilled water were included in every PCR.

In silico and *in vitro* validation of designed primers

The new taxon-specific primers were tested first *in silico* with the BLAST tool in the NCBI webpage (Altschul, Gish, Miller, Myers, & Lipman, 1990) to confirm they aligned significantly only with the target species. To validate the marker *in vitro*, cross-amplification tests were performed using tissue DNA of different fish species occurring in Spanish waters. False positives may occur from native species of the same genus, perhaps of the same family. Three of the four families containing the species considered in this study (Centrarchidae, Ictaluridae and Poeciliidae) are non-native to Europe (Freyhof & Brooks, 2011), thus native species of such families do not occur from Spanish waters and false positives are not expected. However, there are Iberian native species from the Cyprinidae family, although not from the same genus considered in this study (*Pseudorasbora*),

and other exotic Cyprinidae genera as well (Table S2). Thus for cross-amplification two native cyprinids (*Phoxinus phoxinus*, *Squalius pyrenaicus*) and two non-native cyprinids (*Carassius auratus*, *Leuciscus idus*), and native species representative of three families common in Spanish waters: *Salmo trutta* (Salmonidae), *Platichthys flesus* (Pleuronectidae) and *Dicentrarchus labrax* (Moronidae) were tested.

The primers developed were tested for cross-amplification with the seven species above and the five target species of this study (*Ameiurus melas*, *Gambusia holbrooki*, *Lepomis gibosus*, *Micropterus salmoides*, *Pseudorasbora parva*).

The detection limit of PCR with taxon-specific primers, visualized in agarose gels, was determined from serial dilutions of a known DNA concentration for each species. The concentration previous to that where no amplification was observed in agarose gel was considered the detection limit. DNA concentration was measured with a fluorometer Qubit® dsDNA BR Assay.

In situ validation of designed primers

The method was also validated in environmental DNA from controlled aquarium water samples provided by Zaragoza's city Aquarium and Ebro's Delta Ecomuseum. Water samples (1L) from tanks containing individuals of each of the studied taxa and other fish were analyzed with the five newly designed primers (Table 2). The five sets of primers were used for each tank.

For validation with field environmental samples the method was applied in Ebro River as a case study. The seven species have been reported at several places in the Ebro basin (Ministerio de Medio Ambiente 2007). At 930 kilometers it is the second longest river in the Iberian Peninsula, and the second as well by flow rate, with an average discharge of 400 m^3 s^{-1} (Confederación Hidrográfica del Ebro, 2016).

Table 2: Aquarium experiments. Water volume in the aquarium (in L), number and size of individuals of the target species, other species present in each aquarium together with the target one (number of individuals in parenthesis).

Experiment	Volume (L)	Target species	Number of individuals (length in cm)	Other species (Number of individuals)
Aquarium 1	800	*Gambusia holbrooki*	10 adults (≤3cm)	*Salaria fluviatilis* (35)
Aquarium 2	6500	*Micropterus salmoides*	2 adults (45cm)	*Anguilla anguilla* (27), *Emys orbicularis* (3)
Aquarium 3	7640	*Ameiurus melas*	2 adults (23-25cm)	*Barbus graellsii (17)*, *Cyprinus carpio* (1)
Aquarium 4	2460	*Lepomis gibbosus*	4 adults (5-7cm)	*Barbus graessi* (8), *Gobio lozanoi* (10), *Parachondrostoma arcasii* (2)
Aquarium 5	200	*Pseudorasbora parva*	15 adults (8-12cm) and 7 juveniles (<8cm)	-
		Gambusia holbrooki	1 adult (2cm)	-
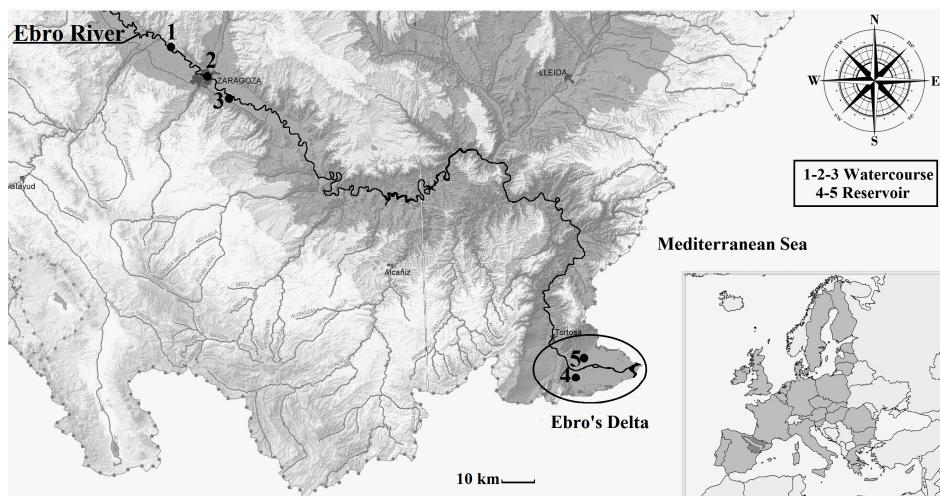
Fig. 1 Map of Ebro River and its location in Iberian Peninsula. The Ebro River is highlighted in black and the five sampling points are shown (downloaded from Confederación Hidrográfica del Ebro).

In December 2015, water samples were taken from five points along Ebro River. Three of them were sampled from running waters, far away from reservoirs, and two inside the river delta (Figure 1). The three samples from running waters were taken near the largest city crossed by the river (Zaragoza): one upstream from the city in Utebo (sampling point #1), another in the middle of Zaragoza city (sampling point #2) and the third one in Movera downstream (sampling point #3). The two samples collected in the Ebro River delta were point #4 and point #5 in two ponds, where Caiola and De Sostoa (2002) reported the occurrence of *P. parva*.

Two replicates of 1L water were collected with sterile bottles from each sampling point, putting the bottle as close to the bottom substrate as possible. They were immediately transported to the laboratory on ice and then frozen. At point #1, a survey was carried out along the riverside using a landing net. In total, 100 m were surveyed from the riverside. At the rest of the locations manual netting was not possible owing to very high river flow and rapid currents.

The primers were assayed twice to confirm the results: two replicate PCRs were done on each eDNA sample. All the positive bands found were purified, sequenced and the species confirmed by BLAST against GenBank.

Phylogenetic analysis of the DNA fragment amplified from genus-specific primers

In the case of the two genus-specific primers (one for the genus *Gambusia* and the other for the genus *Ameiurus*) additional phylogenetic analysis was done in order to check if the primers distinguish between the different species of a genus. Different reference sequences of *G. holbrooki*, *G. affinis*, *A. melas*, *A. nebulosus*, *A. bruneus*, *A. natalis* and *A. catus* were downloaded from GenBank. The

sequences obtained in this study as well as the additional reference sequences were aligned with the ClustalW application included in BioEdit (Thompson et al., 1994). A Neighbour-Joining tree (Saitou & Nei, 1987) was built using MEGA 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013), with 10000 bootstraps. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, Nei, & Kumar, 2004)

Results

Design of specific primers and experimental validation

Taxon-specific primers were designed for the analyzed taxa (Table 1). For *Gambusia* sp and *Ameiurus* sp the primers were genus-specific and amplified from the two species of each genus listed as invasive to Europe: *G. holbrooki* and *G. affinis*, *A. melas* and *A. nebulosus*.

From *in silico* BLAST assays, the new primers retrieved significant alignments only fwith the species for which they were designed. Consistently with these results, cross amplification was not found for the assayed species and for each pair of primers positive PCR amplification occurred only from DNA of the target species (Figure 2). A single clear band of the expected size was obtained with the primers designed for each target species, and the sequence obtained from the bands corresponded to the targeted species and gene (Table S3). Positive amplification of 16S rRNA gene with universal primers (Palumbi et al., 2002) was found for all the samples used in cross amplification tests (Figure 2A), confirming that DNA was of sufficient quality for successful PCR analysis. Sequences from genetic barcoding of COI and 16SrRNA genes of each tissue sample are

available in GenBank (accession numbers KU510486, KU510498, KU510509 and from KY231824 to KY231835).

The threshold of detection for PCR product visualization in agarose gels ranged from 100pg mL^{-1} for *P. parva* to 0.89pg mL^{-1} for *Gambusia* sp and *L. gibbosus* (Table 1).
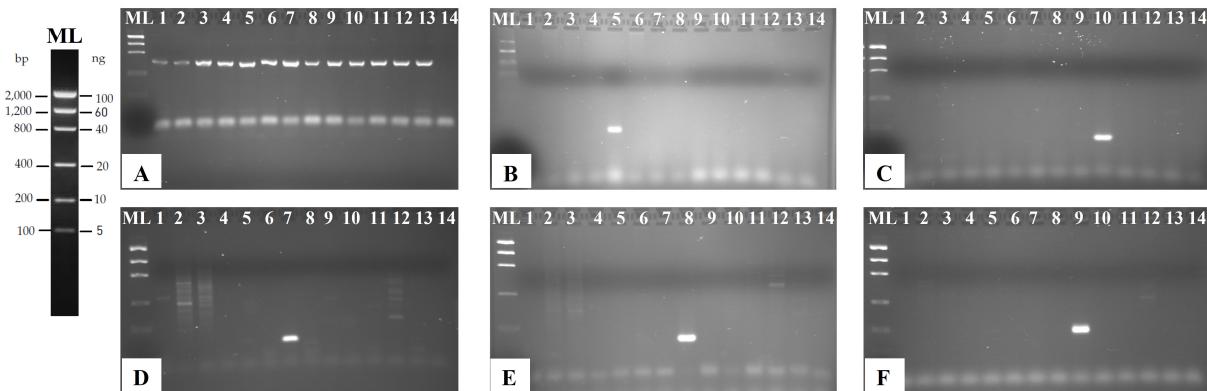
Fig. 2 Agarose gels (2%) showing the results of cross-amplification experiments for each specific marker. 16S rDNA PCR amplified with: A) universal primers (Palumbi et al., 2002); specific primers for *Ameiurus* sp. (B); *Gambusia* sp. (C); *Lepomis gibbosus* (D); *Micropterus salmoides* (E); *Pseudorasbora parva* (F). Lanes (from 1 to 14) in all gels are: Ladder (ML), 1-*Salmo trutta*, 2-*Dicentrarchus labrax*, 3-*Platichthys flesus*, 4-*Alburnus alburnus*, 5-*Ameiurus melas*, 6-*Carassius auratus*, 7-*Lepomis gibbosus*, 8-*Micropterus salmoides*, 9-*Pseudorasvora parva*, 10-*Gambusia holbrooki*, 11-*Phoxinus phoxinus*, 12- *Leuciscus idus*, 13-*Squalius pyrenaicus*, 14- Negative control.
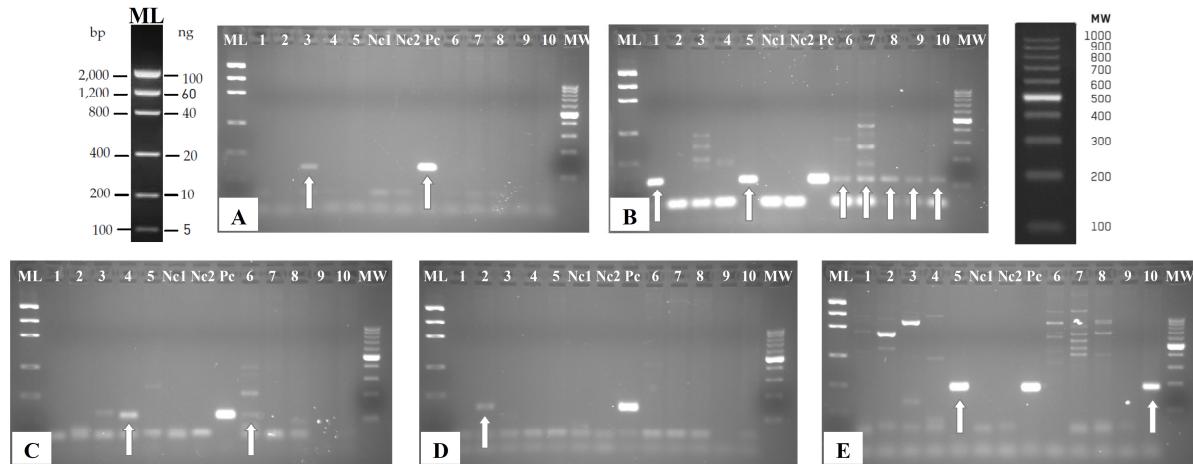
Fig. 3 Agarose gels (2%) showing PCR products from eDNA for each specific marker. A) *Ameiurus* sp; B) *Gambusia* sp; C) *Lepomis gibbosus*; D) *Micropterus salmoides*; E) *Pseudorasbora parva*. Lanes (from 1 to 15) in all gels are: Ladder (ML), aquarium 1 (1), aquarium 2 (2), aquarium 3 (3), aquarium 4 (4), aquarium 5 (5), Ebro River point 1 (6), Ebro River point 2 (7), Ebro River point 3 (8), Ebro River point 4 (9), Ebro River point 5 (10). Negative controls are indicated as (Nc1 and Nc2), negative control for extraction and negative control for PCR, respectively. Positive control with tissue DNA of each species (Pc).
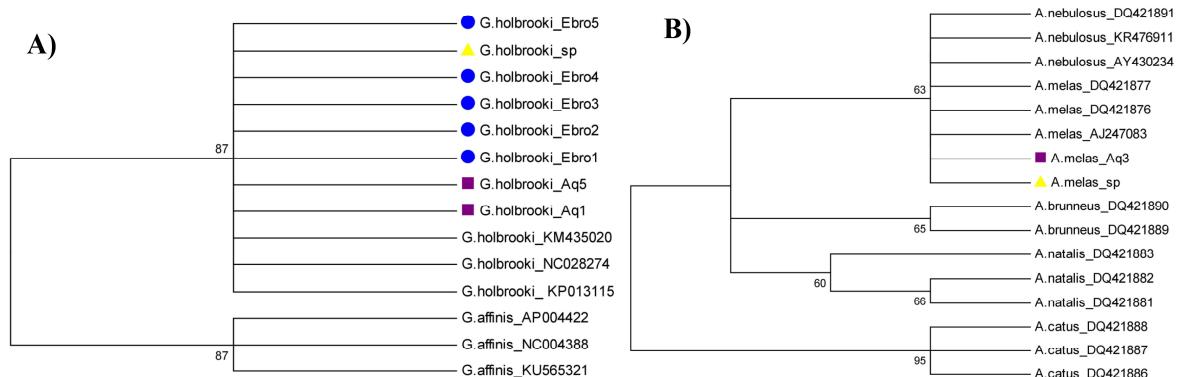
Fig. 4 Phylogenetic trees reconstructed from sequences obtained in this work and references from GenBank (the accession number is indicated). Tissue positive samples are indicated with a yellow triangle, aquarium samples are indicated with a purple square and Ebro River samples are indicated with a blue circle. A) *Gambusia* sp (76 nucleotides), B) *Ameiurus* sp (94 nucleotides).

For the experimental validation in the aquaria, each species was detected only in water from the tank where it was present. PCR from specific primers was successful even in the case of the tank containing only one small individual of *G. holbrooki* in Aquarium number 5 (Figure 3). All the bands marked with an arrow in Figure 3 were sequenced, and the species was confirmed by BLAST (Table S3 and DDBJ accession numbers LC198795- LC198812). The negative controls for extraction (Nc1 in Figure 3) were clean and contamination along the process could be discarded.

The phylogenetic analysis showed that the sequences obtained with the two genus-specific primer pairs could separate the two *Gambusia* species, but could not distinguish the two invasive species targeted within the genus *Ameiurus*. However, these two species (*A. melas* and *A. nebulosus*) clustered separately from the rest of *Ameiurus* species (*A. brunneus, A. natalis* and *A. catus*), that are exotic to Europe but not considered invasive (Figure 4A for *Gambusia* and 4B for *Ameiurus*).

Assays in field water samples: Ebro River

The results of Ebro River (Table 3, Figure 3) revealed DNA of three of the target taxa from the water samples analyzed: *Gambusia* sp*, L. gibbosus* and *P. parva*. Positive detection was obtained in the two replicates of eDNA samples taken. *Gambusia* sp were found from all the sampling points. *Lepomis gibbosus* was found from sampling point #1, and *P. parva* from point #5, in Ebro River delta where it had been reported by Caiola & De Sostoa (2002). For *Ameiurus* sp and *M. salmoides,* positive PCR amplification was not found from any Ebro River sample.

The positive bands were sequenced and are available in Table S3. The sequences amplified with *Gambusia*-specific primers from river water samples corresponded to the species *G. holbrooki*. The sequences clustered together with *G. holbrooki* reference sequences KM435020, NC028274, KP013115, supported by a robust bootstrap of 87 (Figure 4A).

Six *G. holbrooki* individuals were caught manually from sampling point #1, the only point where land nets could be used. Their physical occurrence confirmed the validity of eDNA analysis for detecting this species from running waters.

Table 3: Ebro River field eDNA results. Sampling points along the Ebro River and their coordinates, and PCR amplification results obtained with the taxon-specific primers designed in this study. Positive PCR amplification is marked with X. Negative PCR is indicated as "-".

Sampling points	Coordinates	*Ameiurus sp*	*Gambusia sp*	*Lepomis gibbosus*	*Micropterus salmoides*	*Pseudorasbora parva*
#1	41.736952N, -0.992233W	-	X	X	-	-
#2	41.658574N, -0.878066W	-	X	-	-	-
#3	41.632217N, -0.837865W	-	X	-	-	-
#4	40.64336N, 0.7104704E	-	X	-	-	-
#5	40.72397N, 0.721833E	-	X	-	-	X

Discussion

The set of specific primers designed and validated in this study has proved very sensitive for detection of seven of the commonest invasive species in Europe directly from water samples, and can be used for direct species detection from field water samples. Other specific primers for *P. parva* and *L. gibbosus* have been assayed experimentally in aquarium tanks (no running water), and in artificial ponds with known fish populations (Davison et al., 2016). In addition, other specific primers for *P. parva* designed by Keskin (2014) in the COI gene were successfully applied on river water samples. This study contains several innovations. This is the first case of primers validated for detecting *L. gibbosus* from running water samples; the first primers designed within the 16SrDNA gene for *P. parva*; and the first eDNA method at all, to our knowledge, for the other five species (*Gambusia* sp, *Ameiurus* sp and *M. salmoides*).

Finding positive amplification results from Ebro River running water was encouraging because it confirms the power of eDNA-based methodology. Despite high flow and rapid current in this river, it was possible to detect three different species directly from small volumes of running water. Turner et al. (2014) demonstrated that smaller pore filters (0.2μm) can recover eDNA quantities from small water volumes (similar to the ones used in this study) equivalent to those obtained from filtration of larger water volumes through larger pore filters. Other studies have employed from 250 mL up to 5 L of water samples (Goldberg et al., 2016). The *Gambusia* primers enabled detection of a *G. holbrooki* population in a zone (points #1-3) where the river is wide (133 ± 24m), and where classic sampling is very difficult. The occurrence of *P. parva* in the river delta, earlier reported by Caiola and De Sostoa (2002), was also confirmed using eDNA and revealed that the population is still there 15 years later.

Regarding the sensitivity of the five sets of primers, the detection limit was in the range of pg mL^{-1}, similar to that described by Davison et al. (2016) for *L. gibbosus* and *P. parva* specific primers. Therefore, the method would be useful for detecting these species in early invasion stages, when the population size is still low and might be overlooked from traditional sampling methods. Owing to its sensitivity, the method could be applied to detect the seven invasive species in other European streams where they are suspected. It could be especially useful in large streams, such as Rhine River, which is connected to nearly all the large rivers in south-western, southern, central and eastern Europe and could be the entrance of these invasive species (Leuven et al., 2009). For

Centrarchidae, Ictaluridae and Poeciliidae, which are non-native families of European rivers (Freyhof & Brooks, 2011), any positive result would indicate the occurrence of an exotic species. Sequencing the amplicon would confirm the identification of the non-native species and differentiate between congeneric species, except between the two *Ameiurus* species tested here.

The new tool developed here seems to be highly reliable from *in silico* and *in vitro* results, being sensitive and, theoretically (at least from the current status of reference databases) would not produce false positives from cross-amplification with other European fish species. However, more developments are recommended to completely prevent false positives. Although the BLAST assay only retrieved significant match with the target species, in theory it would be possible to get such cross-amplification with other species still not introduced in the databases. Expanding the current reference databases is necessary for adequate implementation of eDNA methodology for aquatic species detection (Goldberg et al., 2016). On the other hand, false positives may be caused by DNA from dead animals, avian feces, farm discharges or fishing bait (Merkes, McCalla, Jensen, Gaikowski, & Amberg, 2014; Hänfling et al., 2016; Clusa et al., 2017). eDNA may still be detected when the individuals are gone because it is persistent in cold waters (Ficetola et al., 2008). False positives may also be recorded because of contamination during fieldwork or in the laboratory (Thomsen, & Willerslev, 2015). Sampling replications, both temporal and from different places in a river, and the use of good laboratory and field practices, including the use of a blank control sample during fieldwork, will help to solve these problems (Ficetola et al., 2015; Goldberg et al., 2016).

Another important issue to consider when working with eDNA is the possibility of obtaining false negatives from field samples. False negatives may occur in the field for various reasons: when a species is scarce and its DNA has a low concentration (Ficetola et al., 2008); the presence of inhibitors in the sample (Goldberg et al., 2014); or when the activity of a species changes seasonally (De Souza, Godwin, Renshaw, & Larson, 2016). In the case described here, the absence of positive results for the *Ameiurus* sp and *M. salmoides* in the Ebro River could be example of false negatives. It is possible that the number of water sample replicates was insufficient, or that the populations were very scarce. It is also possible that they were not at the sampling points examined, since these species seems to have a preference for reservoirs (Doadrio, 2001), and three of the samples were taken far away from reservoirs.

Despite the problems discussed above, the success of eDNA for detecting populations was

confirmed from different studies. Doi et al., (2017) found a relationship between eDNA concentration and fish abundance in Saba river (Japan), where they detected *Plecoglossus altivelis* eDNA from all the places where visual detection was positive, but not when individuals were not found. Adrian-Kalchhauser, & Burkhardt-Holm (2016) successfully detected invasive gobies in Rhine River in Switzerland. These and other examples demonstrate that eDNA methods applied in rivers can cover equal or greater distances than traditional electrofishing (Evans, Shirey, Wieringa, Mahon, & Lamberti, 2017). Notwithstanding, the application of eDNA to monitoring river systems has some intrinsic limitations due to the nature of running waters. Goldberg et al. (2014) suggested that it is not possible to infer a spatial reference in lotic systems from eDNA, because suspended DNA may be transported far away from the population source. Deiner & Altermatt (2014) found eDNA from two target invertebrates 9-12 km downstream from established populations. Other studies have found DNA transport over shorter distances. Civade et al. (2016) showed downstream eDNA transport for only 2-3 km in low flow in the Tier River (France), and Jane et al. (2015) also found that eDNA travel was reduced at low flow. In any case, eDNA can at least give an overview of the biodiversity in a river system (Rius, Bourne, Hornsby, & Chapman, 2015; Deiner, Fronhofer, Mächler, Walser, & Altermatt, 2016). A positive PCR for any of the seven species in this study could be considered a signal of alert, and further investigation in the area, including conventional sampling, would be strongly recommended, because these species are non-native to all Europe (Leppäkoski et al., 2002). Amplicon sequencing to confirm the species would be necessary, as well as physical confirmation of the species occurrence (e.g. from conventional sampling or photographs), before attempting control and management.

Besides early detection, the tool developed here could be useful to monitor the spread of these invasive species (such as checking colonization of upstream dam areas as Yamanaka & Minamoto (2016) did on migratory fishes); for monitoring the efficacy of eradication programs (Davison, Copp, Créach, Vilizzi & Britton, 2017); or in protected areas to avoid disturbing wild populations (Civade et al., 2016). Methods based on eDNA may also be used for monitoring endangered species in their native range, similar to the studies of *Margaritifera margaritifera* (Stoeckle, Kuehn & Geist, 2016; Carlsson et al., 2017), *Lepisosteus oculatus* (Boothroyd, Mandrak, Fox & Wilson, 2016) and *Zearaja maugeana* (Weltz et al., 2017). A possible weakness of this non-quantitative method is that it determines only presence or absence of a species; however, it is easy to apply in routine surveys, since it does not require special technology. It is faster and more economical than metabarcoding (Comtet, Sandionigi, Viard, & Casiraghi, 2015; Taberlet, Coissac, Pompanon, Brochmann, & Willerslev, 2012) or qPCR (Darling & Blum, 2007), since the reagents needed for one sample cost about 12€ in 2017. Bioinformatics analysis is not necessary for interpreting the results, in contrast with Next Generation Sequencing methods such as metabarcoding (Coissac, Riaz, & Puillandre, 2012). The whole process can be completed in one or two days, and it is possible to analyze many samples at the same time.

As a result of the work described here, the set of taxon-specific primers developed is ready for detecting seven of the commonest invasive fish species in Europe directly from water samples, based on environmental DNA, even at very low densities. This powerful and economical method may be directly applied for early detection of all these species in European waters.

Acknowledgements

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. List of non-native fish species present in Europe and countries where they occur, from different databases.

Table S2. List of native and exotic freshwater fish species occurring in the Iberian Peninsula by family.

Table S3. Sequences of the amplicons obtained using species-specific primers from tissue and water samples.

Data Accessibility

Sequences of tissue for 16S rRNA and COI genes are available in GenBank with the accession numbers: KU510486, KU510498, KU510509, and KY231824- KY231850.

Sequences of the amplicons obtained using taxa-specific primers from tissue and water samples (both aquarium and Ebro River) are available in DDBJ (DNA Data Bank of Japan) with the accession numbers: LC198795- LC198812 and in supplementary Table S3.

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Supplementary Table S1. List of non-native fish species present in Europe and countries where they occur, from different databases. http://easin.jrc.ec.europa.eu/, http://www.europe-aliens.org/ and http://www.iucngisd.org/gisd/ accessed in November 2016.

Species	Albania	Armenia	Austria	Azerbaijan	Azores – Atlantic Ocean	Belarus	Belgium	Bosnia and Herzegovina	Bulgaria	Croatia	Cyprus	Czech Republic	Denmark	Estonia	Finland	Former Yugoslav Republic of Macedonia	France	Georgia	Germany	Greece	Hungary	Iceland	Ireland	Isle of Man	Italy	Latvia	Liechtenstein	Lithuania	Luxembourg	Montenegro	Netherlands	Norway	Poland	Portugal	Republic of Moldovia	Romania	Russia	Serbia	Slovakia	Slovenia	Spain	Sweden	Switzerland	Turkey	Ukraine	United Kingdom	Total number of different countries
Ameiurus melas			D				D			D		D					E, I		D				D		D						D					D					E, I		D			D	11
Ameiurus nebulosus			I			I, D	E			E		I	E, I, D		E, I		E, I	E	E, I		I		I		E, I, D						E, I	E, I, D	E, I, D			I	I	E	I	D	E, I	D	E	I	E, I, D		26
Aspius aspius							E										E								D						E						D						D	I			6
Carassius auratus auratus	I		I, D				I				I	I	I, D	D	D		E, I		E, I	E, I					E, I, D	I, D		I, D			E, I	E, I, D		I	I		I, D	D			E, I	E, D	D		E, I	I	30
Carassius carassius												D							D	E					D																E						3
Carassius gibelio						E			E				E	E			E		E	E	E										E	E	E			E		E			E	E	E	E	E	D	17
Coregonus albula						E								E	E		E		E							E					E	E	E				E					E, I	E		E, I, D	E, I	14
Ctenopharyngodon idella	I	I	I, D	I		I, D	I, D		I	I, D	E, I	I, D	I, D	I	I, D		E, I, D		E, I, D	E, I, D	I		E, I, D		I, D	I, D					E, I, D		E, I, D	E, I	I	I, D	D	E, D		I, D		E, I, D	D	I	E, I, D	E, I, D	33
Esox lucius					D																		I	I										E, I							E, I						6
Gambusia affinis	I	E	E						E	E, D	E, I						E, I, D		E	E, I, D	I				E, I									E, I		I, D	I, D			D	E, I			E, I, D	E, I		18
Gambusia holbrooki		I							I	E		I					E, I	E	E	E	I				E, I, D									E, I	I		I				E, I			E, I	E, D		14
Gymnocephalus cernuus						E				I		D	E				I		I				E									I											I			E, I	10
Hypophthalmichthys molitrix		I	I, D			I, D	D			D		D	I, D						E, I, D	E, I, D					I, D	I, D		D			I, D		I, D			I, D	D	E, D	I	I, D		I, D	D	E, I, D	E, I, D	I, D	27
Hypophthalmichthys nobilis	I	I	D			I, D			I	I, D		I	E, I	I, D			I		I	E, I, D					I, D	D		D			I, D		I, D		I	E, I, D	D	E, I	I	I, D	E	E, I, D	D	E, I, D	E, I, D	D	28
Lepomis cyanellus																		E	E, D																												2

Species	Total number of different countries	Albania	Armenia	Austria	Azerbaijan	Azores - Atlantic Ocean	Belarus	Belgium	Bosnia and Herzegovina	Bulgaria	Croatia	Cyprus	Czech Republic	Denmark	Estonia	Finland	Former Yugoslav Republic of Macedonia	France	Georgia	Germany	Greece	Hungary	Iceland	Ireland	Isle of Man	Italy	Latvia	Liechtenstein	Lithuania	Luxembourg	Montenegro	Netherlands	Norway	Poland	Portugal	Republic of Moldovia	Romania	Russia	Serbia	Slovakia	Slovenia	Spain	Sweden	Switzerland	Turkey	Ukraine	United Kingdom
Lepomis gibbosus	**21**			D				E, D			E, D		D				E	E		E, D	E, D	E				E, D						E, D	E, D	E, D	E		E, D		E, D		D	E		D		E, D	E, D
Leucaspius delineatus	7							E, D						E		D		E						D									D				E							E			E, D
Leuciscus idus	3																	I								E						I	E														E, I, D
Leuciscus leuciscus	4																				E			D		E							E					D							E		
Liza haematocheila	4																		E																			E							E	E, D	
Micropterus salmoides	**30**			I, D			I	I, D		E	D	I	I, D	I		I, D		E, I		E, I	E	I				E, I, D	I		I			I, D	D	I, D	E, I		D	I, D	E, D	I	D	E, I	D	D	E	I, D	E, E, D
Misgurnus anguillicaudatus	5						D					I														I, D	D		D								D	E			D	I		E		E, D	
Mylopharyngodon piceus	8						D			E	D		D													E						E		D	I		D	D	E, D				D			E, D	
Neogobius fluviatilis	10			E, D			D	E					D					E		E					E	E						E		E, D			E	D	E, D						E	E, I, D
Neogobius melanostomus	17			I			E, D	E, I	E, I	I			I	E, I	E, D	I		E, I		E, I	E, I			E, D	E	E, I	E	I	D			E, I	E, I	E, I	I		E	E, D	E, D		E	E, I	E, I	I		E	E, I
Oncorhynchus mykiss	**33**	I		I			E						D			D		E		E		I	E	E, D	E			I				D					I	I, D	E			E, I	E, I	I	I	E	E, I
Oncorhynchus tshawytscha	4												D			D																D															
Oreochromis mossambicus	6						E						I, D							E				E, D	E, I	E													E, D							E, D	I
Oreochromis niloticus	5							D			D							E		E	E					E												D								E, D	
Osmerus eperlanus	10						E	E					D	E				E		E						E							E	E				E, D	E			E	E			E	
Perca fluviatilis	9					D														E				E, D		E, I							E					D	E, D			E, I					
Perccottus glenii	12						E				E	I								E	E	E		D	E		D		E, D				E, D				E	E, D	E	E		E				E, D	E, I
Phoxinus phoxinus	5							E, D																		E, I																E		E	E		D
Pimephales promelas	3						E											E, I													E								D								D
Poecilia reticulata	9			D			E, D	E, I			E, D		E, D						E	E	E, D	E				E, D						E, D		E, D			D	D	E, D		D	E		E, D		E, D	D
Pseudorasbora parva	**26**	E		E, D			E, D	E, D		E	E, D		E, D	E, D				E		E, D	E, D	E				E, D				E	E	D	E, D		E, D			E, D	E, D, D	E, D		D	E		E, D	E	E, D	E, D

Species	Albania	Armenia	Austria	Azerbaijan	Azores - Atlantic Ocean	Belarus	Belgium	Bosnia and Herzegovina	Bulgaria	Croatia	Cyprus	Czech Republic	Denmark	Estonia	Finland	Former Yugoslav Republic of Macedonia	France	Georgia	Germany	Greece	Hungary	Iceland	Ireland	Isle of Man	Italy	Latvia	Liechtenstein	Lithuania	Luxembourg	Montenegro	Netherlands	Norway	Poland	Portugal	Republic of Moldovia	Romania	Russia	Serbia	Slovakia	Slovenia	Spain	Sweden	Switzerland	Turkey	Ukraine	United Kingdom	Total number of different countries
Rutilus rutilus					D						I									E, I, D			I, D	E	I, D									E, I													6
Salmo salar			D			E					I				E					E, I, D		E		E	E									E										I		E, D	11
Salvelinus fontinalis			I, E, D				I, D		I	D		I, D	E, I, D	I	I, D		E, I		E, I, D	E, I, D	I				E, I, D	I, D		I, D			E, I, D	E, I, D	E, I, D	I		I, D	I, D	E	I	I, D	E, I	E, I, D	I, D	I		E, I, D	30
Sander lucioperca						E	E		E				E				E								E, D						E, D						D				E			E	E	E, D	14
Scardinius erythrophthalmus																																					D				E, I						2
Silurus glanis													D							D					D																E					E, D	5
Squalius cephalus																																					I										1
Tilapia mariae																			E				E																								2
Tinca tinca																				D			E, I, D		I							I, D															4

E= EASIN database	
D= DAISIE database	
I= IUCN database	

Supplementary Table S2. List of native and exotic freshwater fish species occurring in the Iberian Peninsula, by family. Based on Doadrio (2001) and Maceda-Veiga (2013). Absence from the region: "-".

Native to the Iberian Peninsula		Non-indigenous species in the Iberian Peninsula	
Family	**Species**	**Family**	**Species**
Acipenseridae	*Acipenser sturio*	Acipenseridae	*Acipenser baerii*
			Acipenser naccarii
Anguillidae	*Anguilla anguilla*	Anguillidae	-
Atherinidae	*Atherina boyeri*	Atherinidae	-
Balitoridae	*Barbatula quignardi*	Balitoridae	-
Blenniidae	*Salaria fluviatilis*	Blenniidae	-
Centrarchidae	-	Centrarchidae	*Lepomis gibbosus*
			Micropterus salmoides
Cichlidae	-	Cichlidae	*Australoheros facetus*
			Astronotus ocellatus
Clupeidae	*Alosa alosa*	Clupeidae	-
	Alosa fallax		
Cobitidae	*Cobitis calderoni*	Cobitidae	*Cobitis bilineata*
	Cobitis paludica		
	Cobitis vettonica		
	Cottus aturi		*Misgurnus anguillicaudatus*
	Cottus hispaniolensis		
Cyprinidae	*Achondrostoma arcasii*	Cyprinidae	*Abramis brama*
	Achondrostoma occidentale		
	Achondrostoma oligolepis		
	Achondrostoma salmantinum		*Alburnoides bipunctatus*
	Anaecypris hispanica		
	Barbus guiraonis		
	Barbus haasi		*Alburnus alburnus*
	Barbus meridionalis		
	Gobio lozanoi		
	Iberochondrostoma almacai		*Barbonymus schwanenfeldii*
	Iberochondrostoma lemmingii		
	Iberochondrostoma lusitanicum		
	Iberochondrostoma olisiponensis		*Blicca bjoerkna*
	Iberochondrostoma oretanum		
	Luciobarbus bocagei		
	Luciobarbus comizo		*Carassius auratus*
	Luciobarbus graellsii		
	Luciobarbus microcephalus		
	Luciobarbus sclateri		*Ctenopharyngodon idella*
	Luciobarbus steindachneri		
	Parachondrostoma arrigonis		
	Parachondrostoma miegii		*Cyprinus carpio*
	Parachondrostoma turiense		
	Phoxinus bigerri		
	Phoxinus phoxinus		
	Phoxinus septimaniae		*Leuciscus idus*

Native to the Iberian Peninsula		Non-indigenous species in the Iberian Peninsula	
Family	**Species**	**Family**	**Species**
Cyprinidae	*Pseudochondrostoma duriense*	Cyprinidae	
	Pseudochondrostoma polylepis		
	Pseudochondrostoma willkommii		*Pseudorasbora parva*
	Squalius alburnoides complex		
	Squalius aradensis		
	Squalius carolitertii		
	Squalius castellanus		*Rutilus rutilus*
	Squalius laietanus		
	Squalius malacitanus		
	Squalius palaciosi complex		
	Squalius pyrenaicus		*Scardinius erythropthalmus*
	Squalius torgalensis		
	Squalius valentinus		
	Tinca tinca		
Cyprinodontidae	*Aphanius baeticus*	Cyprinodontidae	*Aphanius fasciatus*
	Aphanius Iberus		
Esocidae	-	Esocidae	*Esox lucius*
Fundulidae	-	Fundulidae	*Fundulus heteroclitus*
Gasterosteidae	*Gasterosteus aculeatus*	Gasterosteidae	-
Ictaluridae	-	Ictaluridae	*Ameiurus melas*
			Ictalurus punctatus
Latidae	-	Latidae	*Lates calcarifer*
Percidae	-	Percidae	*Sander lucioperca*
			Perca fluviatilis
Poeciliidae	-	Poeciliidae	*Gambusia holbrooki*
			Poecilia reticulata
Petromyzontidae	*Lampetra fluviatilis*	Petromyzontidae	-
	Lampetra planeri		
	Petromyzon marinus		
Salmonidae	*Salmo salar*	Salmonidae	*Hucho hucho*
			Oncorhynchus kisutch
	Salmo trutta		*Oncorhynchus mykiss*
			Salvelinus fontinalis
Serrasalmidae	-	Serrasalmidae	*Piaractus brachypomus*
Siluridae	-	Siluridae	*Silurus glanis*
Syngnathidae	*Syngnathus abaster*	Syngnathidae	-
Valenciidae	*Valencia hispanica*	Valenciidae	-

Supplementary Table S3. Sequences of the amplicons obtained using species-specific primers from tissue and water samples.

Sample	Species	Source	Forward primer name	Forward primer sequence (5'-3')	Reverse primer name	Reverse primer sequence (5'-3')	SEQUENCE (5'-3')
A.melas_sp	Ameiurus melas	Tissue positive control	Am-16S-F	CGTCAAGAACYCAGTTRAACT	Am-16S-R	GWTTCTGYGACTTAGAGTTGTCA	CCAATCTTCTGTTGGGGCGACCACGGGAGAAATAAAGCTCCCACGCGGACTGGGGCAACCCCTAAAACCAAGAGTGACAACTCTAAGTCGCAGAATC
A.melas_Aq3	Ameiurus melas	Aquarium 3	Am-16S-F	CGTCAAGAACYCAGTTRAACT	Am-16S-R	GWTTCTGYGACTTAGAGTTGTCA	CTTCTGTTGGGGCGACCACGGGAGAAATAAAGCTCCCACGCGGACTGGGGCAACCCCTAAAACCAAGAGTGACAACTCTAAGTCGCAGAATC
G.holbrooki_sp	Gambusia holbrooki	Tissue positive control	Ga-16S-F	GRAACCAACTGACCCCTGCTT	Ga-16S-R	GTTTTGTGAGCTGCGGCTCTWTA	GACCCCGGAGTAATAAAAACCCCGAGCGGACTGAAGACACCCTAGCTTCATAATATAGAGCGCCAGCTCACAAAAC
G.holbrooki_Aq1	Gambusia holbrooki	Aquarium 1	Ga-16S-F	GRAACCAACTGACCCCTGCTT	Ga-16S-R	GTTTTGTGAGCTGCGGCTCTWTA	GGCGACCCCGGAGTAATAAAAACCCCGAGCGGACTGAAGACACCCTAGCTTCATAATATAGAGCCGCAGCTCACAAAAC
G.holbrooki_Aq5	Gambusia holbrooki	Aquarium 5	Ga-16S-F	GRAACCAACTGACCCCTGCTT	Ga-16S-R	GTTTTGTGAGCTGCGGCTCTWTA	GGCGACCCCGGAGTAATAAAAACCCCGAGCGGACTGAAGACACCCTAGCTTCATAATATAGAGCCGCAGCTCACAAAAC
G.holbrooki_Ebro1	Gambusia holbrooki	Ebro river point 1	Ga-16S-F	GRAACCAACTGACCCCTGCTT	Ga-16S-R	GTTTTGTGAGCTGCGGCTCTWTA	TTGGGGCGACCCGGAGTAATAAAAACCCCGAGCGGACTGAAGACACCCTAGCTTCATTAGCTTCATAATATAGAGCCGCAGCTCACAAAAC
G.holbrooki_Ebro2	Gambusia holbrooki	Ebro river point 2	Ga-16S-F	GRAACCAACTGACCCCTGCTT	Ga-16S-R	GTTTTGTGAGCTGCGGCTCTWTA	CCCCGGAGTAATAAAAACCCCGAGCGGACTGAAGACACCCTAGCTTCATAATATAGAGCCGCAGCTCACAAAAC
G.holbrooki_Ebro3	Gambusia holbrooki	Ebro river point 3	Ga-16S-F	GRAACCAACTGACCCCTGCTT	Ga-16S-R	GTTTTGTGAGCTGCGGCTCTWTA	CCCCGGAGTAATAAAAACCCCGAGCGGACTGAAGACACCCTAGCTTCATAATATAGAGCCGCAGCTCACAAAAC
G.holbrooki_Ebro4	Gambusia holbrooki	Ebro river point 4	Ga-16S-F	GRAACCAACTGACCCCTGCTT	Ga-16S-R	GTTTTGTGAGCTGCGGCTCTWTA	ACCCCGGAGTAATAAAAACCCCGAGCGGACTGAAGACACCCTAGCTTCATAATATAGAGCCGCAGCTCACAAAAC
G.holbrooki_Ebro5	Gambusia holbrooki	Ebro river point 5	Ga-16S-F	GRAACCAACTGACCCCTGCTT	Ga-16S-R	GTTTTGTGAGCTGCGGCTCTWTA	ACCCCGGAGTAATAAAAACCCCGAGCGGACTGAAGACACCCTAGCTTCATAATATAGAGCCGCAGCTCACAAAAC
L.gibbosus_sp	Lepomis gibbosus	Tissue positive control	LeGi-16S-F	GGACACGGGGCTAAACCAAAT	LeGi-16S-R	GGGCTCTTAGTTGTGGAATTGCA	TGTCTTTGGTTGGGGCGACCGCGGGGAACCAAAAAACCCCACGTGGACTGGAATTATGCAATTCCACAACTAAGAGCCC
L.gibbosus_Aq4	Lepomis gibbosus	Aquarium 4	LeGi-16S-F	GGACACGGGGCTAAACCAAAT	LeGi-16S-R	GGGCTCTTAGTTGTGGAATTGCA	GGCGACCGCGGGGAACCAAAAAACCCCACGTGGACTGGAATTATGCAATTCCACAACTAAGAGCCC
L.gibbosus_Ebro1	Lepomis gibbosus	Ebro river point 1	LeGi-16S-F	GGACACGGGGCTAAACCAAAT	LeGi-16S-R	GGGCTCTTAGTTGTGGAATTGCA	GTCTTTGGTTGGGGCGACCGCGGGGAACCAAAAAACCCCACGTGGACTGGAATTATGCAATTCCACAACTAAGAGCCC
M.salmoides_sp	Micropterus salmoides	Tissue positive control	MiSa-16S-F	WCATCCCRAAAACAAAGGGCY	MiSa-16S-R	AATTCTGTTCATTAGAGCGGAGG	ATGTCTTTGGTTGGGGCGACCGCGGGGAAACACAAAACCCCATGTGGAATGGGACTACTCCCTCCTACAACTCAGAGCCTCCGCTCTAATGAACAGAATT
M.salmoides_Aq2	Micropterus salmoides	Aquarium 2	MiSa-16S-F	WCATCCCRAAAACAAAGGGCY	MiSa-16S-R	AATTCTGTTCATTAGAGCGGAGG	CTTTGGTTGGGGCGACCGCGGGGAAACACAAAACCCCATGTGGAATGGGACTACTCCCTCCTACAACTCAGAGCCTCCGCTCTAATGAACAGAATT
P.parva_sp	Pseudorasbora parva	Tissue positive control	PsPa-16S-F	GTTTAAYCATGTTAAACAACTTAT	PsPa-16S-R	TTCGTTGATCGACTATGTGT	ACTTATTAAAAAGCAAAAACTTAATGGAAAATAAACATTTACCTTCGGTTGGGGCGACCGCGGAGGAGAAAATCAGCCTCCGAGTGGAACGGGCTAAATACCTAAAACCAAGAGAGACAATCTCTAAGCCGCAGAAAATCTGACCAAAAATGATCCGACACATAGTCGATCAACGAA
P.parva_Aq5	Pseudorasbora parva	Aquarium 5	PsPa-16S-F	GTTTAAYCATGTTAAACAACTTAT	PsPa-16S-R	TTCGTTGATCGACTATGTGT	CTTATTAAAGCAAAAACTTAATGGAAAATAAACATTTACCTTCGGTTGGGGCGACCGCGGAGGAGAAAATCAGCCTCCGAGTGGAACGGGCTAAATACCTAAAACCAAGAGAGACAATCTCTAAGCCGCAGAAAATCTGACCAAAAATGATCCGACACATAGTCGATCAACGAA
P.parva_Ebro5	Pseudorasbora parva	Ebro river point 5	PsPa-16S-F	GTTTAAYCATGTTAAACAACTTAT	PsPa-16S-R	TTCGTTGATCGACTATGTGT	CTTATTAAAGAGCAAAAACTTAATGGAAAATAAACATTTACCTTCGGTTGGGGCGACCGCGGAGGAGAAAATCAGCCTCCGAGTGGAACGGGCTAAATACCTAAAACCAAGAGAGACAATCTCTAAGCCGCAGAAAATCTGACCAAAAATGATCCGACACATAGTCGATCAACGAA

Capítulo 3:

An easy phylogenetically informative method to trace the globally invasive *Potamopyrgus* mud snail from river's eDNA

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An Easy Phylogenetically Informative Method to Trace the Globally Invasive *Potamopyrgus* Mud Snail from River's eDNA

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Abstract

Potamopyrgus antipodarum (New Zealand mud snail) is a prosobranch mollusk native to New Zealand with a wide invasive distribution range. Its non-indigenous populations are reported from Australia, Asia, Europe and North America. Being an extremely tolerant species, *Potamopyrgus* is capable to survive in a great range of salinity and temperature conditions, which explains its high invasiveness and successful spread outside the native range. Here we report the first finding of *Potamopyrgus antipodarum* in a basin of the Cantabrian corridor in North Iberia (Bay of Biscay, Spain). Two haplotypes already described in Europe were found in different sectors of River Nora (Nalon basin), suggesting the secondary introductions from earlier established invasive populations. To enhance the surveillance of the species and tracking its further spread in the region, we developed a specific set of primers for the genus *Potamopyrgus* that amplify a fragment of 16S rDNA. The sequences obtained from PCR on DNA extracted from tissue and water samples (environmental DNA, eDNA) were identical in each location, suggesting clonal reproduction of the introduced individuals. Multiple introduction events from different source populations were inferred from our sequence data. The eDNA tool developed here can serve for tracing New Zealand mud snail populations outside its native range, and for inventorying mud snail population assemblages in the native settings if high throughput sequencing methodologies are employed.

Introduction

Human-mediated translocations of marine organisms have become a widely acknowledged global environmental issue nowadays [1, 2]. Maritime activities like merchant shipping or yachting aid the spread of many species out of their native distribution range, and global change may facilitate the success of exotic species in recipient ecosystems until they become

invasive with adverse effects on environment and economies [3, 4]. A successful invader must exhibit a set of differential features [5] allowing passing the different steps of the invasion process and involved barriers: transportation, establishment and spread [3, 6]. Such species usually become of a particular concern for environmental managers and interest for researchers studying patterns in biological invasions.

Potamopyrgus antipodarum (New Zealand mud snail) is one of the extremely successful invaders in aquatic ecosystems worldwide. This ovoviviparous prosobranch is currently found in Australia [7], Asia [8–10], Europe [11, 12] and North America [13–15].

Being extremely tolerant, *P. antipodarum* is a good candidate to survive the transportation to a new region. The presumed vector of its initial transoceanic introduction to Europe and USA is ballast water [13]. Its further spread within the region could be aided by aquaculture (e.g. translocation of stock or equipment), fisheries (e.g. with boats or gear), recreational activities (e.g. with angling gear or pets) [16] or by natural vectors such as birds or fish [14, 17, 18].

Once it reaches the new region, it can colonize and adapt to a wide range of habitats: estuaries [14, 15], lakes [19], rivers [20], saltwater [21] and even open seas [22]. This mud snail competes with native invertebrates for resources in invaded habitats dominating the invertebrate communities [16, 23]. For example, it has caused the decrease of *Pyrgulopsis robusta* population in USA [24] and the decline of native benthos density and diversity in Poland [25]. They consume up to 75% of primary production, leading to altered nitrogen and carbon cycles in invaded ecosystems [26, 27]. It has been found to resists the impact of parasites [11], and also that of potential predators because it is a poor and often indigestible food for salmon and other fish species [17]. Moreover, Sanderson *et al*. [28] suggested that non-indigenous species like *P. antipodarum* are threatening the conservation of endangered salmon due to the alterations they cause in the trophic chain. Due to extremely fast population growth rate it can reach high densities in a short time after incursion, reducing the opportunities for control and mitigation measures. Therefore, early detection is in this case crucial for the efficient rapid response and prevention of the further invasion.

In the last few years, the use of environmental DNA has become a promising tool to detect and survey invasive species in aquatic ecosystems. This method seems to be more sensitive and efficient than traditional surveillance approaches, like visual detection, and does not disturb the aquatic fauna [29–31]. The use of specific primers on eDNA has been successfully demonstrated for a number of species. Examples are fish *Petromyzon marinus* and *Salmo trutta* [32], molluscs such as *Rangia cuneata* in the Baltic Sea [33] and *Xenostrobus securis* in North Spain [34], and others. *Potamopyrgus antipodarum* has also been detected previously directly from water samples [35], as presence-absence based on positive or negative PCR amplification of a fragment of the cytochrome b gene.

Städler *et al*. [36] suggested that the origin of European *Potamopyrgus antipodarum* is located in New Zealand. They found only two haplotypes of 16S rDNA across all Europe shared with snails from the North Island of New Zealand. The marked divergence among the two European haplotypes implies successful colonization by two distinct mitochondrial lineages.

The aim of this study was to demonstrate a cost-effective surveillance strategy for the species and to explore its invasion history in the North Iberian region. We developed specific primers for *Potamopyrgus* based on 16S rDNA sequences, for detecting this mud snail and inferring its lineage directly from water samples.

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Materials and Methods

The species studied

Potamopyrgus antipodarum is small in invaded regions (6–7 mm size in average), but can grow up to 12 mm in its native range (New Zealand). It has a solid operculum and an elongated shell [37]. It is capable to survive in a great range of environmental conditions: salinities 0–38 PSU [38–40], water temperatures 0–28°C [41], and can even resists short times of desiccation [3, 42]. Non-native populations are generally parthenogenetic, consisting almost exclusively of females [3]. One adult in a new habitat can produce an average of 230 juveniles per year [13]. This high reproductive capacity helps *Potamopyrgus* to establish and disperse quickly in a new area. Indeed this capacity is the main reason for the large ecological impact of *P. antipodarum*. Even a single individual can result in a massive invasion just in a few months.

Field sampling

River Nora (Asturias, north of Spain) is a tributary of the River Nalon basin, in the central Bay of Biscay region, of 67 km long and with an average discharge of 20.98 m^3/s. It is completely isolated from downstream by an impassable barrier and a reservoir for hydroelectric power supply (Priañes dam, 43°23′02″N 5°58′26″W) built in 1953. In February-March 2015, mud snails were sampled from three sites within the River Nora, separated from each other by three kilometers. From upstream to downstream, the sites were: Colloto (coordinates 43.379283, -5.788667); Lugones (coordinates 43.401321, -5.822816); and San Claudio (coordinates 43.382938, -5.931142). Ecological conditions were very similar in all sampling sites, with a bottom of stones and gravel, shallow depth, and resembling water flow.

The sampling protocol was the following: a 1m^2 quadrat was randomly selected, and all present *Potamopyrgus* individuals were manually collected from the stones (including the underneath sides). This was done simultaneously by three researchers from each site, thus three replicates of 1m^2 (approx.) were obtained per site. The average number of individuals per replica is a rough but comparable proxy of the density of the *Potamopyrgus* population present in each site. Additionally three liters of water were collected with sterile bottles from the same sampling locations before the search of *Potamopyrgus* individuals.

As negative field controls one liter of water was taken from Llanes beach (seawater), coordinates 43.420461, -4.752003 and mainstream River Nalon (freshwater), coordinates 43.180926, -5.341015. No *Potamopyrgus* individuals were found in these sites despite intensive exploration. No specific permissions were required for sampling in these locations. The River Nora is not within a national park or other protected area. It is of public access. The species *Potamopyrgus antipodarum* is not native from Spain. Moreover it is listed in the register of invasive species (Spanish Directive of 4 August 2013).

DNA extraction

From tissue samples DNA was extracted with mollusc DNA Kit (Omega Bio-Tek, USA) following the instructions provided by the manufacturer.

1 L of the water samples was filtered using the Supor®-200 Membrane Filter (Pall Corporation) with 0.2 μm pore size. The filtration apparatus was cleaned with 10% bleach, rinsed with distilled water and autoclaved between each sampling site. DNA was extracted with the Power-Water® DNA Isolation Kit (Mobio laboratories). The filtration process and eDNA extractions were done under sterile conditions, in a laboratory unit where there was no other tissue samples, to avoid any contamination of the environmental DNA. eDNA extractions also were done inside a PCR laminar flow cabinet prior to extractions treated with ultraviolet light. Blanks

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containing only water were used as controls in DNA extraction, to confirm that contamination did not occur in the process.

Design of specific primers

The 16S rRNA gene was chosen for the design of the primer, based on reference nucleotide sequences of 16S rDNA from GenBank plus the sequences obtained in the laboratory from *Potamopyrgus* samples of different origins. Sequences of this gene (either individual 16S DNA sequences or complete mitochondrial genomes), available for *Potamopyrgus* and other mollusk species were downloaded and aligned with the ClustalW application included in BioEdit [43]. Polymorphisms were analyzed with the DNASP software [44]. The different haplotypes were visualized employing the BioEdit Sequence Alignment Editor software [45]. The universal primers designed by Palumbi *et al.* [46] amplifying a 16S rDNA region of approximately 600 nucleotides were used for species barcoding. A region within these amplicons conserved in the genus *Potamopyrgus* but different in the rest of mollusk species was searched. This region was used to design a *Potamopyrgus* genus-specific reverse primer. As forward primer we used the universal 16SAr from Palumbi *et al.* [46].

Markers employed and PCR conditions

PCR amplification of 16S rDNA using the universal primers described by Palumbi [46] was done with the following protocol. The amplification reaction was performed in a total volume of 40 μl, including Green GoTaq®Buffer 1X, 2.5 mM MgCl$_2$, 0.25 mM dNTPs, 1μM of each primer, 0.65 U of DNA Taq polymerase (Promega) and 4 μl of template DNA. PCR conditions were the following: an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and a final extension step at 72° for 7 min. The PCR products were sequenced in the DNA sequencing service Macrogen Europe, and the species identifications were confirmed using the BLAST tool from the NCBI.

PCR amplification of partial 16S rDNA from tissue DNA using the new primers set (the newly designed reverse primer and the universal Palumbi's forward primer) was performed in a total volume of 20μl with the same conditions above, except for the annealing temperature. We assayed six different annealing temperatures: from 55°C to 60°C for selecting the best one (that provides clean and clear amplification products of the expected size with no extra bands). The assays of annealing temperatures showed that the best results were obtained at 60°C. All the PCR products were visualized in 2% agarose gels with 2.5 μl of SimplySafe™.

PCR amplification of a fragment of 16S rDNA from the bulk DNA extracted from water samples (eDNA) with the specific primer was performed in a total volume of 20 μl, including Green GoTaq®Buffer 1X, 2.5mM MgCl$_2$, 0.25mM dNTPS, 1μM of each primer, 6 μl of template DNA, 200ng/μl of BSA (bovine serum albumin) and 0.65 U of DNA Taq polymerase (Promega). The PCR conditions were the same as described above, at the best annealing temperature, but with 45 cycles instead of 35. Amplification products from water samples were purified with the Agarose-Out DNA purification kit (EUR®X) and sequenced by Macrogen service.

The cytochrome oxidase I (COI) gene was amplified from DNA extracted from tissue and water samples using the universal primers for invertebrates designed by Geller *et al.* [47] and following the protocol described therein. The difference between the protocols used for tissue and water DNA was the number of cycles in the PCR– 35 and 45 respectively. Negative controls containing only PCR reagents and distilled water were added in every PCR.

Marker validation

The new primer was first tested *in silico* by an alignment with the BLAST tool in the NCBI database [48].

Adult individuals of brackish and freshwater mollusks (five per species) were collected for testing *in vitro* possible cross-species amplification of the new primer (Table 1). PCR amplification with the universal primers of Palumbi *et al.* [46] was done.

The new set of specific primers was assayed on DNA extracted from eleven mollusk species described in Table 1.

The sensitivity of the specific primers was determined *in vitro* with serial dilutions of *Potamopyrgus antipodarum* DNA from a known concentration (43μg/ml). PCR amplification and visualization of the PCR product in a 2% agarose gel were performed for each concentration. DNA concentration was measured with a spectrophotometer (SimpliNano™ GEHealthcare).

From water eDNA, a fragment of the 16S rDNA was PCR-amplified with the new specific primers set using the protocol described in 2.4. As a positive control, the COI gene was amplified from each eDNA sample as described in 2.4, to test for the quality of the DNA and discard false negatives due to excessive DNA degradation, inhibitors or other reasons.

Validation of negative results

To confirm that the negative results of PCR with the specific primers performed on eDNA samples were true and not produced by any interference or inhibitor present in the template, the subsamples of the Llanes beach eDNA (6μl) were spiked with *Potamopyrgus antipodarum* DNA of two concentrations: 2μl of *P. antipodarum* stock DNA (43 μg/ml), and 2μl of the 1:50 000 dilution from the same stock. PCR amplifications were performed in the same conditions as explained before.

Phylogenetic analysis

Potamopyrgus individuals from River Nora and from different locations in New Zealand (as representatives of native populations), were collected and taxonomically classified *de visu* (Table 2). Three different sequences were obtained from these samples: COI gene [47], 16S rRNA gene [46] and partial 16S rDNA amplified with the specific primers set. Additional

Table 1. Adult mollusks sequenced in this study for 16S rRNA and cytochrome oxidase I genes.

Species	Habitat	Origin	Common name	Collection site
Potamopyrgus antipodarum	freshwater, brackish	non- native	New Zealand mudsnail	Nora River
Mytilus galloprovincialis	marine	Spanish native	Mediterranean mussel	Aviles estuary
Mytilus trossulus	marine	non-native	Foolish mussel	Baltic Sea
Ruditapes philippinarum	marine	non-native	Japanese carpet Shell	Aviles estuary
Xenostrobus securis	brackish	non-native	Axe-head mussel	Aviles estuary
Mya arenaria	marine	non-native	Soft-shell clam	Baltic Sea
Crassostrea gigas	marine	non-native	Giant oyster	Aviles estuary
Tylomelania kuli	freshwater	non-native	Sulawesi snail	pet shop
Tylomelania toradjarum	freshwater	non-native	Sulawesi snail	pet shop
Neritina canalis	brackish	non-native	Nerite	pet shop
Neritina punctulata	freshwater	non-native	Nerite	pet shop

Bivalves and gastropods (five individuals per species) employed for the evaluation of cross-amplification of the specific primers. The origin (native or non-native) is given in relation with Spanish waters.

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Table 2. *Potamopyrgus* samples, collected from Asturias and New Zealand, sequenced in this study for 16S rRNA and cytochrome oxidase I genes.

Sample	Place	Country	Species
Pa Ast1 01	Colloto- River Nora	Spain	*P. antipodarum*
Pa Ast2 01	Lugones- River Nora	Spain	*P. antipodarum*
Pa Ast2 02	Lugones- River Nora	Spain	*P. antipodarum*
Pa Ast2 02	Lugones- River Nora	Spain	*P. antipodarum*
Pa Ast3 01	San Claudio- River Nora	Spain	*P. antipodarum*
Pa NZ1 01	Collins River	New Zealand	*P. antipodarum*
Pa NZ2 01	Onomalutu River	New Zealand	*P. antipodarum*
Pe NZ3 01	Maitai River Site 1	New Zealand	*P. estuarinus*
Pe NZ3 02	Maitai River Site 1	New Zealand	*P. estuarinus*
Pe NZ3 03	Maitai River Site 1	New Zealand	*P. estuarinus*
Pe NZ4 01	Maitai River Site 2	New Zealand	*P. estuarinus*
Pe NZ4 02	Maitai River Site 2	New Zealand	*P. estuarinus*
Pe NZ4 03	Maitai River Site 2	New Zealand	*P. estuarinus*
Pe NZ5 01	Mangroves Matua Rangarawa	New Zealand	*P. estuarinus*
Pe NZ5 02	Mangroves Matua Rangarawa	New Zealand	*P. estuarinus*
Pe NZ5 03	Mangroves Matua Rangarawa	New Zealand	*P. estuarinus*

doi:10.1371/journal.pone.0162899.t002

sequences assigned to *Potamopyrgus* species were downloaded from GenBank. For each gene, the sequences were aligned with the ClustalW application included in BioEdit [43]. The alignment was converted to MEGA file and a phylogenetic neighbor-joining tree was built using MEGA 4.0 [49], with 10000 bootstrapping and the evolutionary distances were computed using the Tamura-Nei method [50].

Results

Specific primers

The new specific primer designed *in silico* within the 16S rDNA sequence was:

Reverse primer: 16SPA-R (5′ –TCAAAGATTTTGGATCATAGCT–3′).

Using the 16SAr described by Palumbi *et al.* [46]: 16SAr (5′ –CGCCTGTTTATCAAAAACAT–3′) as a forward primer and the new 16SPA-R as a reverse primer, a region of 380 nucleotides within the 16S rRNA gene was amplified. The region is located between sites 5350 and 5730 of the *Potamopyrgus antipodarum* mitochondrion complete genome with GenBank accession number GQ996421.1.

Marker validation

From BLAST assays *in silico*, the new primer retrieved significant alignments, with 100% identity, 100% coverage, 0.018 E-value and score of 44.1, with *Potamopyrgus antipodarum*, *P. estuarinus*, *P. doci*, *P. opidanus*, *P. troglodytes* sequences of 16S rRNA gene. The same values were also obtained with 16S rDNA sequences of *Caldicochlea globosa*, an Australian endemic aquatic snail, and several species of the genus *Sororipyrgus* that are Hydrobiidae snails endemic in New Zealand. All these species except for *Potamopyrgus antipodarum* are currently absent from European aquatic ecosystems.

PCR reactions for assessing primers' specificity discarded cross-amplification with other mollusks assayed in this study (Table 1A). Consistently with *in silico* results, *in vitro* tests showed that the specific primers give positive PCR amplification (amplicons visible in

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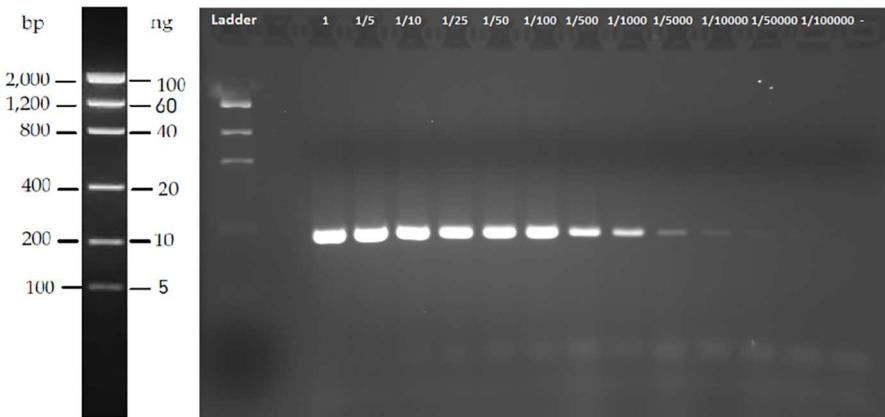
Fig 1. Agarose gel showing PCR amplification products obtained with the species primers' set for 16S rRNA gene from serial dilutions of *Potamopyrgus antipodarum* DNA (43µg/ml): 1 (no dilution), 1:5, 1:10, 1:25, 1:50, 1:100, 1:500, 1:1000, 1:5000, 1:10000, 1:50000, 1:100000 and a negative control.

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agarose gels; data not shown) on the species listed in Table 1A only from DNA samples of *Potamopyrgus*.

The threshold of detection for PCR-visualization in agarose gels was 0.86µg/l, because we obtained a weak but visible band of the amplicon size in the dilution 1 to 1:50 000 from a sample with a concentration of 43µg/ml (Fig 1).

Potamopyrgus antipodarum population in Asturias

In River Nora the *Potamopyrgus antipodarum* population was not identical in the three sampling sites. From the sampling results, the estimated population density was higher in the midstream location of Lugones (Table 3), with 63 individuals/m²; meanwhile downstream San Claudio has quite low density of 6 individuals/m². None of the individuals was >7mm. In the downstream site the relative abundance of juveniles (<3mm) was clearly lower than in upstream areas (Table 3).

In all eDNA samples obtained from water, PCR with the universal COI primers [47] yielded amplification products of the expected size around 650 nucleotides (Fig 2). The water samples from River Nora provided positive PCR amplification with the taxon-specific primers designed herein (Fig 3A). In the other two control sites, Llanes beach and River Nalón; no amplification was obtained with these primers as expected since *Potamopyrgus* mollusks are not present there. The positive bands observed in agarose gel for River Nora water samples were purified, sequenced and the sequences unequivocally identified as *Potamopyrgus antipodarum*, GenBank accession numbers KU933000-KU933002. The PCR products gave clear chromatograms directly readable, without any trace of nucleotide mixture in any site.

Table 3. *Potamopyrgus antipodarum* specimens collected from different sites within Nora River, classed by size, and total density. The same sampling protocol from three replicates of 1m² was employed in all sites.

Site	Density (individuals/m²)	Individuals $\geq$ 3mm	Individuals < 3 mm
Colloto (upstream)	18	33.3%	66.7%
Lugones (midstream)	63	39.7%	60.3%
San Claudio (downstream)	6	83.3%	16.7%

doi:10.1371/journal.pone.0162899.t003

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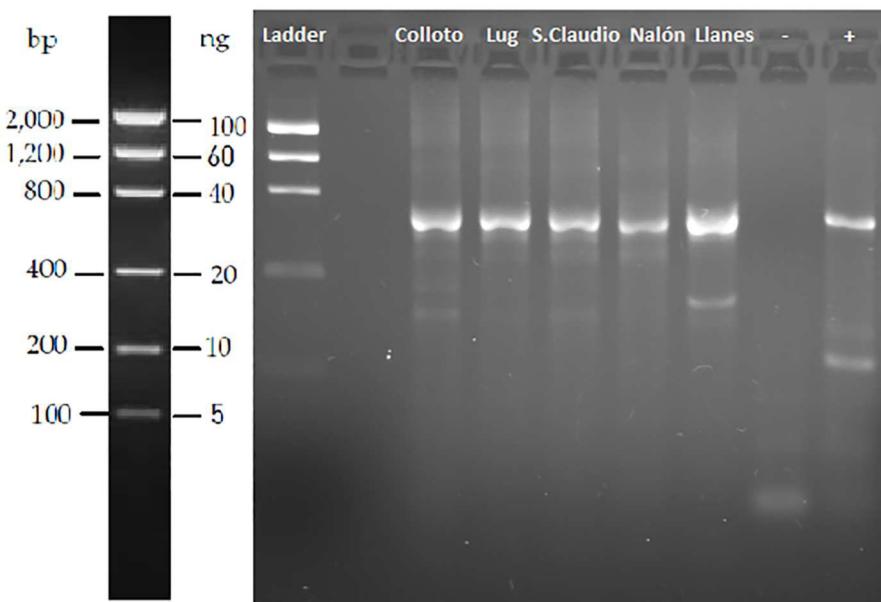
Fig 2. Amplification products of the cytochrome oxidase I gene obtained from PCR with universal primers on water samples. Sampling sites: River Nora: Colloto, Lug (Lugones) and San Claudio, River Nalón and Llanes beach;–and + are negative and positive controls respectively.

doi:10.1371/journal.pone.0162899.g002

In the agarose gel it can be seen that the band for Lugones is bigger and brighter than for San Claudio location (Fig 3A), concordantly with different population densities. The method can be considered quite sensitive because PCR product was detectable in agarose gel even for San Claudio sample where the observed density was only 6 individuals/m^2 (Fig 3A).

On the other hand, the negative results obtained from field water samples were confirmed by the additional validation test. Positive PCR amplification from Llanes beach water sample was obtained when *Potamopyrgus* DNA was added (Fig 3B). A clear band was seen in the two mixtures, one of high concentration with an amount of approximately 86 ng of *P. antipodarum*
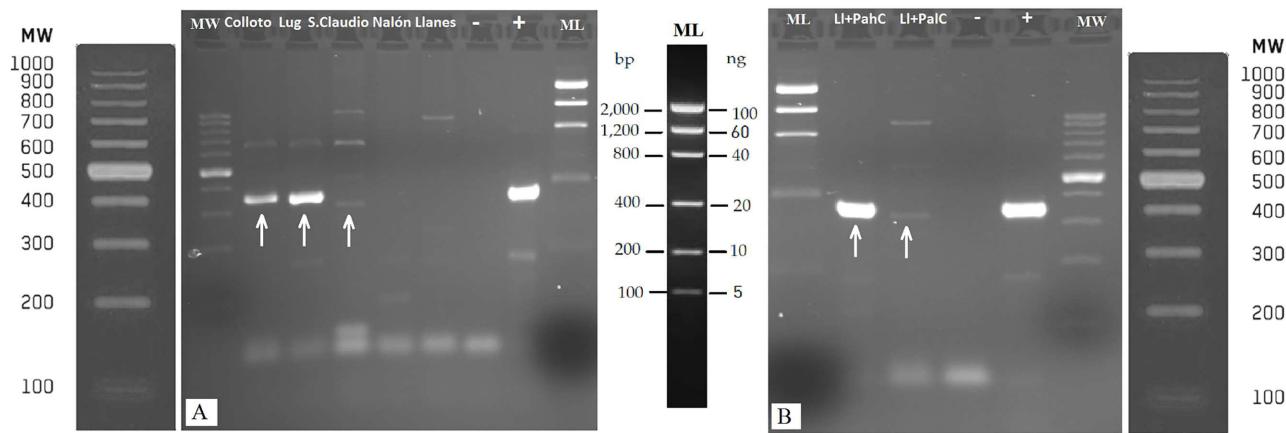
Fig 3. A) PCR products of the partial 16S rRNA gene obtained with the taxon-specific primers, on DNA extracted from water samples of River Nora (Colloto, Lugones and San Claudio sites), River Nalón and Llanes beach. Positive amplifications are marked with an arrow. B) Validation of negative results: amplification products of the same gene obtained from Llanes beach water DNA spiked with *Potamopyrgus antipodarum* DNA. Ll+PahC and Ll+PalC are high and low concentration of *Potamopyrgus antipodarum* respectively (43µg/ml and dilution 1:50000 respectively).–and +, negative and positive controls respectively.

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DNA and the other of low concentration with approximately 1.72pg of DNA. The positive results obtained in this last PCR indicate that there were no inhibitors in the environmental samples. This confirms that the negative results obtained from environmental DNA were not due to the presence of inhibitors in the water sample but to the absence of *Potamopyrgus* DNA in the samples. Therefore false negatives were discarded.

Phylogenetic inferences

From the individuals analyzed in this study a total of 26 haplotypes were found: 11 (two from Asturias *P. antipodarum* individuals), 8 (two from Asturias individuals) and 7 (also two from Asturias) for COI gene, long, and short 16S rDNA fragments respectively. The haplotypes obtained in this study are available in NCBI GenBank database with the accession numbers KU932989-KU932999 (COI gene), KU933003-KU933010 (16S rDNA large fragment). The shorter 16S rDNA amplicon obtained from taxon-specific primers corresponds to the sequence comprised between site 01 and site 325 on KU933003- KU933010.

The tree reconstructed from the COI gene (Fig 4A) and 16S rDNA (Fig 4B) haplotypes obtained in our mud snail samples with universal primers separated consistently the samples from San Claudio (downstream) from those collected mid- and upstream. Downstream samples clustered with New Zealand samples (Onomalutu River) while the rest of River Nora samples, all with the same haplotype, clustered with River Collins samples, also from New Zealand. The *Potamopyrgus estuarinus* samples of Maitai River and the Matua Rangarawa Mangroves (New Zealand) clustered, as expected, in an independent branch for the two genes. They were separated by locations (Maitai River in one branch and Matua Rangarawa Mangroves in another) for 16S rRNA gene (Fig 4B), with apparent geographical differentiation.

For the shorter 16S rDNA fragment amplified with the primers designed herein, the two haplotypes of *P. antipodarum* found in Asturias (from both water samples and mud snail individuals) were also separated in different clusters (Fig 5). Samples from Lugones and Colloto (purple diamond in Fig 5) formed a monophyletic group with one haplotype (JQ346709) found in Germany, France, Hungary, Poland, Lithuania and United Kingdom; with the haplotype AY955377 found in Australia (Tasmania), and the New Zealand haplotype AY955376. The haplotype found downstream River Nora (PaAst3-03, San Claudio location) was in a separate clade supported by a bootstrap value of 65, containing the haplotype JN639014 found in Estonia and Wales; the haplotype JN639014 found in Hammond Harbor in Oregon and Devils Lake in Wisconsin; and the New Zealand haplotype AY955393 (North Island). The haplotypes of New Zealand South Island were also separated in this tree, the haplotype from River Collins being monophyletic with the upstream and midstream Asturian samples and other European references, and the Onomalutu River haplotype exhibiting an intermediate and less clear position in the middle of the two branches (Fig 5). Indeed the haplotypes obtained from Asturias water samples (blue circles in Fig 5, GenBank accession numbers KU933000-KU933002) matched perfectly with the haplotypes of the individuals found from the same place.

The *Potamopyrgus estuarinus* samples from New Zealand analyzed in this work formed a clearly differentiated clade with a *P. estuarinus* reference sequence from GenBank (AY634082), supported by a bootstrap value of 99. Since the two species are closely related, this confirms the phylogenetic value of this relatively short marker.

Discussion

This is the first record of *Potamopyrgus antipodarum* from the central basin of the Bay of Biscay (Asturias, North of Spain). In the Iberian Peninsula the species has been detected in Atlantic and Mediterranean basins [16, 20], but not in the Bay of Biscay façade.

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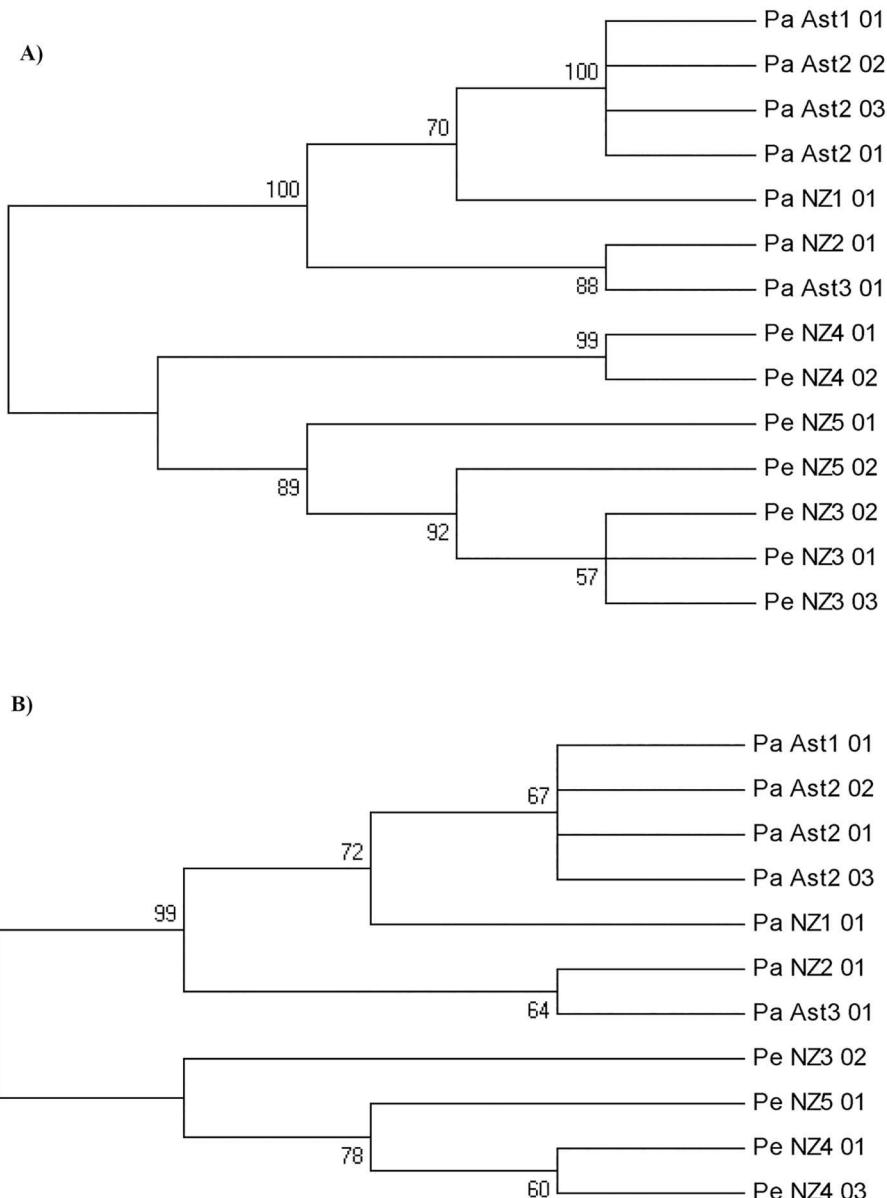
Fig 4. Phylogenetic tree reconstructed from: A) cytochrome oxidase I gene (621 nucleotides), and B) 16S rDNA haplotypes (496 nucleotides), obtained with universal primers [46, 47] from the individuals analyzed in this study. Pa and Pe are *Potamopyrgus antipodarum* and *P. estuarinus* respectively.

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The results obtained in this study are surprising in several ways. First, in a small river (River Nora) and at short distance among sampling locations we have found two different haplotypes. These haplotypes correspond to the haplotypes *t* and *z* described for European *Potamopyrgus antipodarum* by Städler *et al.* [36]. These authors found the two haplotypes together only in two locations: Loch of Stennes (Orkney, Scotland) and Slack estuary (Nord-Pas de Calais, France). In the rest of sites studied across Europe only one haplotype was present in each location. The two haplotypes do not seem however be admixed in the same place. The sequences obtained from water samples did not exhibit any sign of overlapped chromatogram peaks in
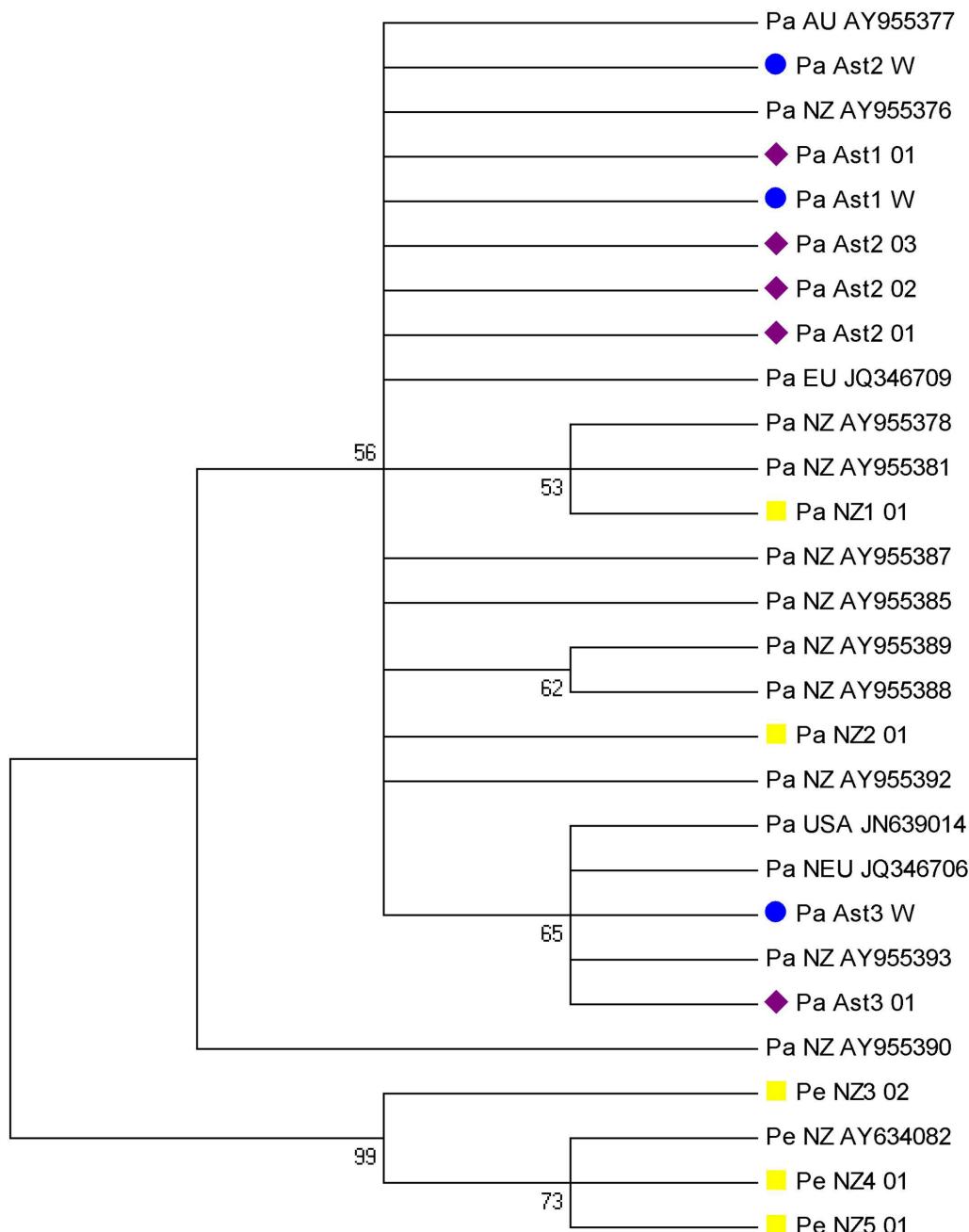
Fig 5. Phylogenetic tree of partial 16S rDNA sequence (325 nucleotides) with the taxon-specific primer reconstructed from the *Potamopyrgus* haplotypes (Pa, *P. antipodarum*; Pe, *P. estuarinus*) obtained in this work and references obtained from GenBank (the accession number is indicated). The geographic origin of the voucher *P. antipodarum* specimens are: Ast1; Ast2; Ast3; USA; NZ; NZ1; NZ2; NEU and EU are: Colloto, Lugones, S. Claudio (Asturias 1, 2, and 3), Wisconsin, New Zealand; Collins River, Onomalutu River (New Zealand, South island); Estonia, France (European samples). Sequences obtained from the water samples, Asturias individuals and New Zealand individuals (south island) sampled in this study are indicated with a blue circle, a purple diamond and a yellow square respectively.

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the polymorphic sites described by Städler *et al*. [36]. Low densities and scarce juveniles found downstream suggest that a second and recent introduction occurred in San Claudio site.

Another interesting result of this study was high sensitivity of the taxon-specific primers developed for detecting *Potamopyrgus* DNA in water samples. Goldberg *et al*. [35] designed a marker in the cytochrome b gene region that was able to detect *Potamopyrgus* individuals at densities as low as 11 individual/m^2, filtering 4-L water samples. In our study successful amplification of the 16S rDNA based marker, with amplicons visible on agarose gel, was obtained from 1-L water samples and almost half density (6 individual/m^2). This viviparous mud snail does not have a planktonic stage, so the DNA detected from water samples is most likely free-floating DNA.

These results are really encouraging because, since imply usefulness of this for early detection of the species when the population density is still low at the initial stage of invasion or on the edge of the range expansion area. This PCR method is economical (the estimated average cost was 10 euros per water sample) and faster in comparison to Metabarcoding [51], and also to qPCR [52] and could be easily added into routine surveillance programs.

The method has a shortcoming, however. Simple positive amplification and visualization in agarose gel (or by capillary electrophoresis), that can serve for detecting the species in Europe and North America because it is unique in its genus there, are not enough for population monitoring in its native settings. *In silico*, and proved *in vitro* for *P. estuarinus*, the primers can anneal with other species of the genus *Potamopyrgus* that are present in Australia and New Zealand. The DNA region employed here as a marker has the phylogenetic power to discriminate between closely related species of this genus (Fig 4). The same primers could be used in native settings using high throughput methodologies [53], or simply cloning-sequencing to separate the different amplicons. Since the region amplifies well from water samples, after further development it could be employed as an additional method for surveys of native *Potamopyrgus* species assemblages.

The origin of the *Potamopyrgus antipodarum* found in Asturias seems to be the same as for the rest of Europe, since the two haplotypes described by Städler *et al*. [36] were found. The particular introduction pathway to the region, however, is still unclear. Ballast water, one of the inferred vectors of this invader [13], can be reasonably discarded in our case because the invaded habitats are not accessible from the sea (isolated by an impassable dam). Upstream River Nalón we found no *P. antipodarum* individuals neither traces of its DNA in the water (negative controls). Aquaculture can also be disregarded because there are no aquaculture facilities in River Nora valley. Short-distance transport by fishermen as suggested by Alonso and Castro-Díez [16] is plausible. Casual hikers may contribute to short-distance transport as well. The bird-mediated transport suggested by Lassen [54] is also plausible, since the region is in the middle of the 600-km corridor of northern Spain that is an important and rich wintering ground for many birds [55, 56]. Another possibility, still unexplored, is that they could come from aquarium releases as accompanying fauna of fish pets, as already described for other species [57].

Loo *et al*. [58] predicted extremely fast spread of this species, forecasting a total invasion of North America freshwater ecosystem in a relatively short time if actions are not taken to prevent its expansion. It seems that in the Cantabrian range region, or at least in the river where it was detected for the first time, the population density is still not too high, especially downstream. Rapid application of containment measures and eradication efforts, as well as a close surveillance of the present populations could be strongly recommended.

Conclusion

We developed a specific set of primers to detect *Potamopyrgus* species directly from the water samples (environmental DNA). With this molecular tool it is possible to establish the species

identity and the phylogenetic characteristics of the invasion, sequencing PCR amplicons obtained from environmental samples. This powerful (and economical if limited to visualization on gel) tool can be useful for early detection of New Zealand mud snail in its expanded range of invasion.

Acknowledgments

Author Contributions

Conceived and designed the experiments: EGV AZ.

Performed the experiments: LC AA VT.

Analyzed the data: LC LM AA VT.

Contributed reagents/materials/analysis tools: LC LM AA VT.

Wrote the paper: LC EGV AZ LM AA FG VT.

Sampling: LC LM AA VT FG AZ EGV.

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Capítulo 4:

eDNA for detection of five highly invasive molluscs. A case study in urban rivers from the Iberian Peninsula

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eDNA for detection of five highly invasive molluscs. A case study in urban rivers from the Iberian Peninsula

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Abstract

Biological invasions are an important threat to biodiversity especially in aquatic ecosystems, and their frequency is generally higher near urban areas. Potentially invasive non-indigenous molluscs were deliberately introduced into European waters for food (*Corbicula fluminea*) and biocontrol (*Melanoides tuberculata*), and unintentionally introduced by ballast water (*Mytilopsis leucophaeata*, *Corbicula fluminea*), stock contamination (*Sinanodonta woodiana)*, accidental escapes from aquaculture (*Sinanodonta woodiana*), aquarium trade releases (*Melanoides tuberculata*) and even attached to aquatic birds (*Corbicula fluminea*). Three rivers from the Iberian Peninsula were monitored near the three most populated inland cities to evaluate the presence of these invasive molluscs through PCR amplification using taxon-specific primers from eDNA. New primers were designed within 16S rRNA and cytochrome oxidase subunit I genes, tested *in silico* from BLAST methodology and experimentally *in vitro* before application in the field. *C. fluminea* was found in Ebro River (near Zaragoza); *M. leucophaeata* in Guadalquivir River (near Sevilla). *M. tuberculata* and *S. woodiana* were found from enclosed areas (lake and reservoir respectively) upstream, respectively, Zaragoza and Madrid. The new tools are ready to be used in other regions where these species are also invasive.

Introduction

Biological invasions are one of the most important threats to biodiversity. Particularly in aquatic ecosystems the number of invasive species has increased in the last decades, due to globalization and closely related to human activities [1, 2]. Human-mediated transport together with global warming could promote the rapid and uncontrolled dispersion of invasive freshwater species [3]. An example is the rapid spread of *Mytilopsis leucophaeata* (native to the Gulf of Mexico) and the zebra mussel *Dreissena polymorpha* (native to the Caspian Sea) in the Baltic Sea [4].

The ways of introduction of aquatic species are numerous. Invertebrates are deliberately introduced for food and biocontrol, and may be also unintentionally released from accidental aquaculture escapes, ballast water, water connections, hitchhikers, stock contamination, pet

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and aquarium trade [5, 6]. For example, *M. leucophaeata* was introduced in Baltic Sea as well as in Guadalquivir River in Spain by ballast water transport [7, 8]. Multiple introductions of the same species have also been reported. *Melanoides tuberculata*, native from eastern Africa and the Middle East was deliberately introduced in 1980 for snail control in the Caribbean [9], and also inadvertently from aquarium trade [10]. *Sinanodonta woodiana* whose native range is Eastern Asia, was introduced in Tuscany (Italy) for production of artificial pearls [11], but in Poland it arrived probably with fish consignments as a parasite [12], since it has an obligatory parasitic stage [13]. *Corbicula fluminea*, also native to Asia, was transported inadvertently in ballast water to Brazil [14], where it was also introduced from aquarium releases and attached to feet or feathers of aquatic birds, and deliberately released as a food resource and fish bait [15].

The effects caused by the molluscs cited above are enormous and mainly derived from their high reproductive rates and their environmental tolerance. They can alter the suspended particles and sediments, introduce new parasites and diseases and compete with native species [16]. The parthenogenetic *M. tuberculata* is a threat for the Italian endemic *Melanopsis etrusca* due to its high population density [11]. Moreover *M.tuberculata* hosts a trematode parasite that infests local fish [17]. *C. fluminea* reduces the local phytoplankton community due to high filtration rates, altering the nutrients cycling [18], as it happened in the Potomac River in Maryland, USA [19]. In addition, this species tolerates higher concentrations of mercury than native molluscs, surviving better in polluted areas [20]. *S. woodiana* became the dominant species in Poland and dispersing quickly to the rest of Europe [21], partly due to its high ability to parasitize native fish species outcompeting native molluscs [22]. *M. leucophaeata* is even able to survive in the cooling tanks of nuclear plants and spread from there [23]. Its ability to tolerate high temperatures and chlorine concentrations makes *M. leucophaeata* a huge biofouling problem once established [24, 25] as it happened with *D. polymorpha* [26]. Even the empty shells of these molluscs can cause serious damage to the ecosystem. In the Danube empty shells of *C. fluminea* and *S. woodiana* accumulated, sheltering amphipods and isopods and attracting predator populations [27]. The examples above illustrate the impact of these invasive molluscs in European and other freshwaters. The human population density is one of the best predictors of biological invasions [28–31]. As other invasive species, exotic molluscs tend to accumulate near big cities and ports, in highly anthropogenic areas where the invasion vectors accumulate [32]: ballast water, hull fouling, aquarium wastes, pet releases e.g [33, 34]. Thus water bodies nearby the cities should be logically main monitoring targets, in order to control potential entries of new undesired species; especially when those species, as the molluscs cited above, have been already reported as biological invasions in the same and/or other regions.

Sousa *et al.* [16] suggested using novel tools such as environmental DNA (eDNA) for the early detection of exotic species. These methodologies are based on extracting DNA directly from environmental samples (water, sediments) and identify the species present there from DNA traces. The techniques are becoming cheaper, are non-invasive, highly sensitive, independent of weather conditions for sampling, and may help to control target species [35]. There are several examples of molluscs' detection using eDNA from European waters, both invasive species such as *Rangia cuneata* [36], *Dreissena polymorpha* [37], *Xenostrobus securis* [38], *Potamopyrgus antipodarum* [39] and locally endangered natives like *Margaritifera margaritifera* [40].

The main objectives of this study were two-fold. First to design an easy and fast method to detect presence-absence of four freshwater molluscs invasive to Europe from eDNA: *Corbicula fluminea*, *Melanoides tuberculata*, *Mytilopsis leucophaeata* and *Sinanodonta woodiana* based on simple PCR. Second, to validate the new primers in situ testing the hypothesis of positive eDNA results in the areas where either conventional sampling and/or official records were

obtained. For this, samples upstream, within and downstream the three most populated non coastal Spanish cities were employed. Expectations were sampling points within and downstream cities provided more positives, given reported association between human population density and invasive species [28–31].

Materials and methods

Study region and target species

The mollusc species analysed in this study (*Corbicula fluminea*, *Melanoides tuberculata*, *Mytilopsis leucophaeata*, *Sinanodonta woodiana*) are considered invasive in the Iberian Peninsula. They are included in the current official list of invasive species in Spain (Spanish Directive of 4 August 2013 RD 630/2013).

Three main rivers near to the most populated cities inland the Iberian Peninsula were selected to evaluate the potential association of these invasive species with urban areas. Manzanares River crosses Madrid (3 165 000 inhabitants) and is a tributary of Tajo River (the longest river basin of the Iberian Peninsula). Guadalquivir River crosses Sevilla (690 000 inhabitants), and Ebro River crosses Zaragoza (661 000 inhabitants). Water samples were collected upstream (Point 1) (coordinates: Madrid 40.404968N, 3.722536W, Sevilla 37.404152N, 5.998669W and Zaragoza 41.736952N, 0.992233W), within (Point 2) (coordinates: Madrid 40.400108N, 3.718048W, Sevilla 37.404307N, 5.998946W and Zaragoza 41.658574N, 0.878066W) and downstream (Point 3) (coordinates: Madrid 40.326673N, 3.654334W, Sevilla 37.403653N, 6.006897W and Zaragoza 41.632217N, 0.837865W) each considered city. Two additional points were sampled from upstream enclosed areas with reported occurrence of one of these species: Santillana reservoir (River Manzanares coordinates 40.719003N, 3.855379W) and the lake of Alhama de Aragón (Jalón River, Ebro's tributary, coordinates 41.294383N, 1.898593W), which have respectively *Sinanodonta woodiana* (Madrid Community Official Bulletin Decreto 102/2014 of 8 September 2014) and *Melanoides tuberculata* [17].

Sampling included: taking water samples (see "Water sample collection and eDNA extraction" section below), and *a posteriori* intensive search of the invasive species found in a site from water eDNA, if any. For comparable sampling effort, the bottom (stones, pebbles, sediments) were carefully inspected from an area of 5m² in each revisited sampling point until the species was found, or during one hour.

No specific permissions were required for sampling in these locations; all of them are of public access and the species: *C. fluminea*, *M. leucophaeata*, *M. tuberculata* and *S. woodiana* are not native from Spain.

Specific primers design

The design and validation of taxon-specific primers was based on the methodology described by Clusa *et al.* [39] and Ardura *et al.* [37]. 16S rRNA and COI (cytochrome oxidase subunit I) genes were selected to design the specific marker, since they are mitochondrial and expected to be in abundance in eDNA [41]. All the 16S and COI sequences from the target species, related species and other aquatic species of a wide range of taxa were downloaded from the NCBI database. All different haplotypes were visualized with BioEdit [42] and the sequences were aligned with the ClustalW application included therein [43]. A region conserved in all the haplotypes of the target species but different in the rest of species was searched and used to design two specific primers per species. Both primers were tested with the Oligo Analyzer 3.1 tool included in the Integrated DNA Technologies webpage (http://eu.idtdna.com/calc/analyzer) in order to obtain similar annealing temperature and to check the primers not to form hairpins or dimers.

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Primers validation

The first validation step was to check the primers *in silico* by an alignment with the BLAST tool of the NCBI webpage [44]. For *in vitro* validation, tissue from muscle from different molluscs and fishes from the laboratory were used to check for possible cross-species amplification of the designed primers. The four genera are absent in the Iberian Peninsula [45, 46]. Therefore species that belong to different families including molluscs which are common in the Iberian Peninsula (S1 Table) were selected for the *in vitro* assays. To discard the not very likely but possible cross-reactivity with fish species we included four fishes from different families that are abundant in Spanish waters (S1 Table). DNA was extracted from tissue with Chelex resine [47] in the case of fish samples and with the mollusc DNA Extraction Kit (Omega Bio-Tek, USA) following the instructions provided by the manufacturer in the case of mollusc samples. To confirm good DNA quality in each sample COI gene was amplified with universal primers following the protocol described by Geller *et al.* [48]. Thus absence of PCR amplification with specific primers cannot be attributed to lack of good DNA in a sample but to the absence of the target species. All the markers were tested on all the eDNA samples. The sensitivity of the specific primers was determined *in vitro* with serial dilutions of the target species DNA from a known concentration quantified with Qubit 2.0 fluorimeter (Invitrogen, ThermoFisher Scientific) following the same protocol described by Clusa *et al.* [39]. Stocks concentrations were in the range of 1–5 µg/ml. Dilutions made were: 1:5; 1:10; 1:25; 1:50; 1:100; 1:500; 1:1000; 1:5000; 1:10000; 1:50000 and 1:100000. The previous concentration to the one where no amplification was observed in agarose gel was considered the detection limit.

Water sample collection and eDNA extraction

From January to April 2016, two replicates of 1L water were collected with sterile bottles from each sampling point, putting the bottle as close to the bottom substrate as possible. The different sites were sampled in different days and always from upstream to downstream. All the material was cleaned with bleach between samplings following Goldberg *et al.* [49], to ensure decontamination of the equipment. Samples were immediately frozen and transported to the laboratory. Water samples were vacuum filtered using the Supor®-200 Membrane Filter (Pall Corporation) with 0.2 µm pore size and a filter holder. The filter holder was dismantled, sprayed with 10% bleach, cleaned with detergent and 10% bleach, rinsed with distilled water and sterilized by 30 minutes under UV light between samples. Filters were stored individually within 15ml tubes at -20˚C until DNA extraction. DNA was extracted with the PowerWater® DNA Isolation Kit (Mobio laboratories) following manufacturer's recommendations. The two replicates of each sampling point were extracted separately in time. The eDNA extractions were done under sterile conditions, in a laboratory unit where there were no other tissue samples, inside a PCR laminar flow cabinet treated with ultraviolet light to avoid any contamination of the environmental DNA. To ensure the cleaning process was correct, one sample with 1L milliQ water was filtrated between two problem samples and included in all eDNA analyses to confirm that contamination did not occur during the filtration or DNA extraction process.

PCR conditions

The amplification reaction with the species-specific primers from tissue DNA was performed in a total volume of 20µl, including Green GoTaq® Buffer 1X, MgCl2, 0.25mM dNTPS, 1µM of each primer, 2µl of template DNA and 0.65 U of DNA Taq polymerase (Promega). The PCR conditions were the following: an initial denaturation step at 95ºC for 5min, 35 cycles at 94ºC for 30s, annealing at the temperature of choice for 30s and elongation at 72ºC for 30s. A final step of elongation was set at 72ºC for 10min. We assayed different annealing temperatures

and MgCl$_2$ concentrations for each pair of primers. In every PCR a positive control using tissue DNA of the target species (from voucher specimens kindly provided by the Museo Nacional de Ciencias Naturales, Madrid) and a negative control containing PCR reagents and distilled water (to discard contamination during preparation of PCR) were included. PCR products were visualized in 2% agarose gels with 2.5μl of SimplySafe™.

The individuals sampled in situ were Barcoded for the COI gene using Geller et al. [48] primers jgLCO1490 and jgHCO2198, for species confirmation, with the PCR conditions indicated by the authors and using muscle tissue as DNA source. DNA extraction from tissue was as reported above. The new designed primers were used to amplify their DNA as well.

In the case of DNA extracted from water samples, the PCR conditions were the same as described above with some minor modifications. Fifty cycles were used instead of 35; 6μl of DNA template and BSA (200ng/ml) was added in the PCR mix. In addition to the positive and negative controls for PCR, a negative control for extraction was included. All the PCRs from eDNA were done in a PCR cabinet where no tissue sample was handled, treated with ultraviolet light before preparing the mix in order to avoid contamination of the samples and using pipette filter tips. The positive control was added outside the cabinet and separately from water samples.

All the positive bands obtained from eDNA were purified following instructions either with the Exo-BAP (EURx) or cutting the band with the agarose out DNA purification kit (EURx) in case of multiple bands and sequenced to confirm the species. In each eDNA replica two PCRs were done for each species. A minimum of two positive amplifications from one extraction or one positive result from two different extractions were required to consider a species was present in a sample.

Ethics statement

DNA from fish species were from the laboratory collection. The study was approved by the Ethics Committee from the Principality of Asturias with the permit of reference number 99/16 (for the project MINECO-13-CGL2013-42415-R) and with the permit of reference number 101/16 (for the project EU RIA 689682 –AMBER).

Results

Specific markers

The specific primers designed for the target species are shown in Table 1. From the BLAST test, the combination of the two new primers for each species retrieved significant alignments only

Table 1. Taxon-specific primers designed in this study.

Species detected	Primer	Sequence (5'-3')	Annealing Temperature	[Mg^{2+}]	Amplicon size	Detection limit
Corbicula sp	CoFl-16S-F	GAATAACTTAAATGTAGGT	55°C	2 mM	165 bp	0.375 ng/ml
	CoFl-16S-R	AGCAAACTTCTTCTTAAATAT				
Melanoides tuberculata	MeTu-16S-F	GGTCTRACGAAAGCAATACT	58°C	2 mM	230 bp	3 ng/ml
	MeTu-16S-R	GCTTTGCTKGATCTAAAYYT				
Mytilopsis leucophaeata	MyLe-COI-F	GGTTGTAACAACGCACGGTTTAG	66°C	1 mM	193 bp	0.76 ng/ml
	MyLe-COI-R	CACCTTCTCTGAAAGCCGAGC				
Sinanodonta woodiana	SiWo-COI-F	GGGTCAGCCMGGRAGGCTTTTA	68°C	1 mM	258 bp	0.202 ng/ml
	SiWo-COI-R	TGTTCACCCTGTACCAACRCCC				

Primer's sequence, annealing temperature, Mg^2 concentration, expected amplicon size (in base pairs) and minimum DNA concentration (detection limit) for which is possible to obtain a PCR product visible in agarose gel with the primer pairs in the conditions assayed.

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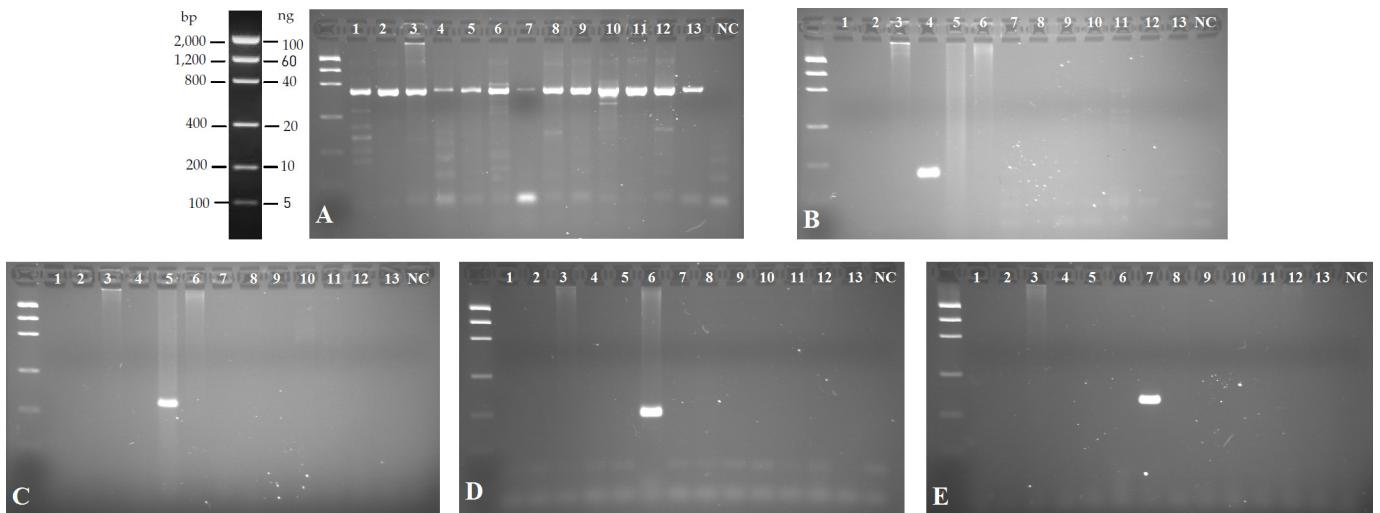
Fig 1. Agarose gels (2%) showing the results of cross-amplification experiments for each specific marker. PCR amplified with: A) universal primers [48]; and specific primers for *Corbicula sp.* (B), *Melanoides tuberculata* (C), *Mytilopsis leucophaeata* (D); *Sinanodonta woodiana* (E). Lanes (from 1 to 13) in all gels are: Ladder, 1-*Mya arenaria*, 2-*Rangia cuneata*, 3-*Dreissena polymorpha*, 4-*Corbicula fluminea*, 5-*Melanoides tuberculata*, 6-*Mytilopsis leucophaeata*, 7-*Sinanodonta woodiana*, 8-*Potamopyrgus antipodarum*, 9- *Bithynia tentaculata*, 10-*Salmo trutta*, 11-*Phoxinus phoxinus*, 12- *Carassius auratus*, 13-*Micropterus salmoides*, NC- Negative control.

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with the target species, except in the case of *Corbicula fluminea*. For this last species it was not possible to find a specific marker nor in 16S or COI. A *Corbicula* genus-specific marker was designed within the 16SrDNA gene that anneals, from BLAST, on *C. leana* (a synonym of *C. fluminea* [50]) and *C. largillierti*, which is native to Asia and invasive in South America [51, 52].

In the cross-amplification test, the set of primers were tested against the collection of molluscs and fish species described above (S1 Table). All samples employed provided positive amplification with universal primers [48], confirming the presence of good quality DNA (Fig 1A). For the newly designed taxon-specific primers no amplification was found from any of the aquatic species assayed except from DNA of the target species of each primer (Fig 1B, 1C, 1D and 1E). The detection limit for each marker (Table 1) was close to 1 ng/ml for all the species.

Field results

The four designed markers provided positive amplification from real eDNA samples (Fig 2), in the three rivers. All eDNA samples were positively PCR-amplified with universal primers [48], thus eDNA was of sufficient quality for PCR amplification and PCR inhibitors did not occur (Fig 2A). *Corbicula* specific primers amplified from the three Ebro River samples (Fig 2B, Table 2); one individual was sampled in point 2 in the city centre (GenBank accession number MF401395). *M. tuberculata* specific primers amplified from Alhama de Aragón lake only (Fig 2C), where several individuals were collected (GenBank accession number MF401394). *M. leucophaeata* primers provided positive PCR amplification in two of the three samples from Guadalquivir River (near Sevilla) (Fig 2D, Table 2), where several individuals were collected (GenBank accession number MF401396). *S. woodiana* primers amplified DNA fragments from Santillana reservoir sample (Fig 2E). Negative control for extraction (1L of milliQ water filtrated and extracted at the same time as the rest of eDNA samples, NC1 in Fig 2; Table 2) was clean for every marker, indicating the absence of contamination during the filtration and eDNA extraction process. Also negative control for PCR (distilled water added to the PCR mix instead of DNA, NC2 in Fig 2) was clean, indicating the absence of

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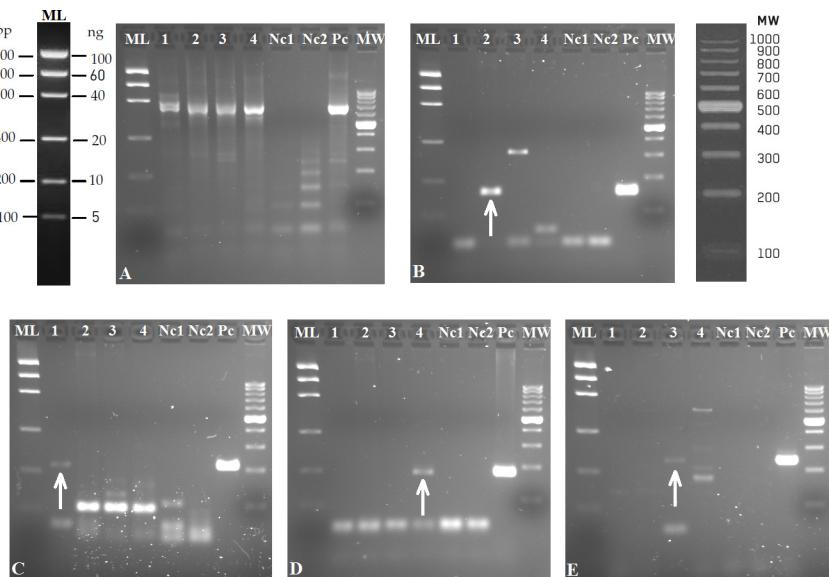
Fig 2. Agarose gels (2%) showing amplification products obtained from eDNA with the designed specific markers. PCR amplicons with: A) universal primers [48]; and specific primers for *Corbicula sp.* (B), *Melanoides tuberculata* (C), *Mytilopsis leucophaeata* (D); *Sinanodonta woodiana* (E). Lanes in all gels are: Mass ladder, 1-Alhama de Aragón thermal lake, 2-Ebro River (Zaragoza), 3-Santillana reservoir (Madrid), 4-Guadalquivir River (Sevilla), Nc1- Negative control for extraction, Nc2 negative control for PCR and Pc positive control with tissue DNA of each species.

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contamination while preparing the PCR. There are some unspecific bands in the eDNA analysis, but not of the same size of the target species (Fig 2). The bands marked with an arrow on Fig 2 were excised from the gel, the amplicons sequenced, and the species was confirmed by BLAST (S2 Table) (DDBJ accession numbers LC310741-LC310751). From BLAST tests, the species amplified from Ebro River samples was *Corbicula fluminea*. Regarding urban river areas, only the river zone around Madrid did not provide positive PCR amplification for any of the assayed markers; in the two other urban areas positive amplification was found for one of the assayed species (Table 2).

Discussion

In this study we have developed a useful PCR-based tool to detect four of the main freshwater molluscs invasive to Europe from water samples. Positive amplification of the expected species from water samples was found for the places where the species had been sampled, such as *Melanoides tuberculata* in Alhama de Aragón lake [17] and *Sinanodonta woodiana* in Santillana reservoir within Manzanares River (Madrid Community Official Bulletin Decreto 102/2014 of 8 September 2014). Although the primers were tested only in Spanish waters they are species- or genus-specific and did not show any cross amplification with other species either in the BLAST assays or in the *in vitro* tests. Thus they could be potentially useful to monitor these molluscs across Europe and other regions where they are invasive, as well as in native areas as proposed for markers developed for other species [39]. Unspecific bands were observed in the eDNA analysis, but not of the same size, similar to other studies [38, 39]. For that reason positive control in the PCR is highly recommended, first to correctly identify the band size of the target species and secondly to discard false negatives due to failure in the PCR [49]. Additionally the sequencing of the possible positive could help to elucidate the species amplified [49]. In the case described here, all the eDNA PCR results were purified and sequenced identifying

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Table 2. PCR amplification using the new taxon-specific primers on eDNA obtained from water samples of the considered Iberian rivers.

	Manzanares River basin				Guadalquivir River basin			Ebro River basin				
	Santillana reservoir MR	Madrid M1	Madrid M2	Madrid M3	Sevilla S1	Sevilla S2	Sevilla S3	Jalón River lake EL	Zaragoza Z1	Zaragoza Z2	Zaragoza Z3	Extraction negative control
Coordinates	40.719003N, 3.855379W	40.404968N, 3.722536W	40.400108N, 3.718048W	40.326673N, 3.654334W	37.404152N, 5.998669W	37.404307N, 5.998946W	37.403653N, 6.006897W	41.294383N, 1.898593W	41.736952N, 0.992233W	41.658574N, 0.878066W	41.632217N, 0.837865W	
Corbicula sp	-	-	-	-	-	-	-	-	X	X	X	-
Melanoides tuberculata	-	-	-	-	-	-	-	X	-	-	-	-
Mytilopsis leucophaeata	-	-	-	-	X	X	-	-	-	-	-	-
Sinanodonta woodiana	X	-	-	-	-	-	-	-	-	-	-	-
COI [48]	X	X	X	X	X	X	X	X	X	X	X	-

Positive PCR amplification is shown with X. Negative PCR is indicated as "–".

https://doi.org/10.1371/journal.pone.0188126.t002

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the species present in the samples as the target species confirming the correct detection of the invasive species.

Two of these species were found in urban river areas nearby two of the most populated Spanish cities. *Corbicula fluminea* DNA was found from all the sampling points tested from Ebro River. Oscoz *et al.* [17] indicated that the invasion of this species had not reached Zaragoza city in 2010, but in this study, carried out six years later, we found it in the city and 5.44 kilometres downstream (sampling points Z1-Z3) (Table 2), demonstrating the expansion of this invasive species. The rest of the rivers tested seem to be free of the *Corbicula* invasion but it is necessary to continue the surveillance and control the possible introduction of the species.

The other species associated with city areas was *Mytilopsis leucophaeata*, found in the section of Guadalquivir River crossing Sevilla (points S1 and S2). The species had been described in this river 14 years ago, restricted to an enclosed channel of water distribution for refrigeration [7]. Although this species is considered to have relatively reduced dispersal capacity [7, 53], its presence in open river waters would indicate the species started already spreading along the basin, at least in the urban zone sampled in this study.

The occurrence of *Melanoides tuberculata* in the Ebro River basin seems to be still restricted to the place where it had been already described; which is a lake with high water temperature as preferred by this species [54, 55]. In 2010 Jarillo and Salgado [56] reported its presence in L'Aldea in the Ebro's Delta, but the survival rate was too low. If climate change continues raising water temperatures, *M. tuberculata* would be a threat for the rest of the species in the region, as already happened in Alhama de Aragón lake where it is displacing the local native *Melanopsis penchinatti*, now classified as critically endangered [57].

On the other hand, *Sinanodonta woodiana* seems to be also restricted to an enclosed area (a reservoir) within Manzanares River and has not reached downstream running waters yet (or at least its DNA, if present, is at a very low concentration below the detection limit of 0.202 ng/ml found for this marker). Perhaps the reservoir dam represents a barrier to the expansion of this species, as it is for the migration of other species e.g. [58]. *S. woodiana* was not detected in Ebro River, although it is reported in Ter River and Daró River (north East Rivers) [59]. Numerous invasive species have been translocated from these Rivers to Ebro River and vice versa as an example *Misgurnus anguillicaudatus* [60]. In any case, since it may spread upstream the presence of the species in the basin is a potential threat for native molluscs as *Margaritifera auricularia* [61].

Finally, eDNA-based methodologies are not perfect for river faunal inventories and could be considered exploratory or early-detection systems instead. Floating DNA (not actual individuals) may be transported downstream creating false positives [62]. To deal with the possibility of false positives in the eDNA analysis an unambiguous detection approach was used as described in Lahoz-Monfort *et al.* [63]. Conventional sampling and/or fully referenced and reliable official reports confirmed the presence of the species in all the places where positive results were obtained for eDNA. *Corbicula fluminea* individuals were found in Ebro River Z2 sample, *Melanoides tuberculata* were found in Alhama de Aragón Lake, *Mytilopsis leucophaeata* were found in Sevilla samples and *Sinanodonta woodiana* has been reported in Santillana reservoir sample. Moreover replicates were considered in each place. Two samples from each sampling site were collected and extracted separately in time. In each eDNA sample two PCRs were done, thus a total of four replicates were used. Finally, all the positive results from eDNA were sequenced to confirm the species.

On the other hand, when a species is very scarce in a place and its DNA has very low concentration false negatives may occur, especially if the detection limit is not too low. In addition sampling design is also important when working with eDNA. The possible false negatives could derive from the life cycle of the target species, since many species vary their activity

depending on season [64, 65]. eDNA will be more effective when sampling is done according to seasonal activity of the species. Ardura *et al.* [36, 37] collected eDNA samples during spawning session for two invasive molluscs, *Rangia cuneata* and *Dreissena polymorpha* respectively, in order to use specific markers to detect them. The use of replicates, both temporal and spatial (from the same sampling point) is highly recommendable [66]. In any case, detecting eDNA of an- unreported potentially invasive species in a location should be followed by intensive conventional sampling to corroborate the invasion status. The new markers developed here could serve for the early detection step. Although the method described is non-quantitative, since it only determines the presence-absence of the invasive species, it is cheaper and faster than qPCR [67] and as reliable as qPCR or ddPCR to inference species presence in a sample [68]. It could be adapted to be used with qPCR but as it is it would be useful to help managers to control the spread of these invasive species, especially in places where only presence data is required or with limited resources.

Conclusion

Four eDNA-based markers were successfully designed, validated and applied in situ in Iberian rivers for detecting DNA from the highly invasive molluscs *Corbicula spp*, *M. tuberculata*, *M. leucophaeata* and *S. woodiana*. The new tools are ready to be used in other regions where these species are also invasive and could help to control their spreading.

Supporting information

S1 Table. Taxonomy of the species used in the cross amplification test. Different molluscs and fishes from a variety of taxa were chosen.
(DOCX)

S2 Table. Sequences obtained with the new specific primers from positive control (tissue DNA) and environmental samples (eDNA).
(DOCX)

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S1 Table. Taxonomy of the species used in the cross amplification test. Different molluscs and fishes from a variety of taxa were chosen.

Kingdom	Phylum	Class	Order	Family	Genus	Species
Animalia	Mollusca	Bivalvia	Myida	Dreissenidae	*Dreissena*	*Dreissena polymorpha*
Animalia	Mollusca	Bivalvia	Myida	Dreissenidae	*Mytilopsis*	*Mytilopsis leucophaeata*
Animalia	Mollusca	Bivalvia	Myida	Myidae	*Mya*	*Mya arenaria*
Animalia	Mollusca	Bivalvia	Unionoida	Unionidae	*Sinanodonta*	*Sinanodonta woodiana*
Animalia	Mollusca	Bivalvia	Veneroida	Corbiculidae	*Corbicula*	*Corbicula fluminea*
Animalia	Mollusca	Bivalvia	Veneroida	Mactridae	*Rangia*	*Rangia cuneata*
Animalia	Mollusca	Gastropoda	Sorbeoconcha	Thiaridae	*Melanoides*	*Melanoides tuberculata*
Animalia	Mollusca	Gastropoda	Littorinimorpha	Bithyniidae	*Bithynia*	*Bithynia tentaculata*
Animalia	Mollusca	Gastropoda	Littorinimorpha	Tateidae	*Potamopyrgus*	*Potamopyrgus antipodarum*
Animalia	Chordata	Actinopterygii	Cypriniformes	Cyprinidae	*Phoxinus*	*Phoxinus phoxinus*
Animalia	Chordata	Actinopterygii	Cypriniformes	Cyprinidae	*Carassius*	*Carassius auratus*
Animalia	Chordata	Actinopterygii	Perciformes	Centrarchidae	*Micropterus*	*Micropterus salmoides*
Animalia	Chordata	Actinopterygii	Salmoniformes	Salmonidae	*Salmo*	*Salmo trutta*

S2 Table. Sequences obtained with the new specific primers from positive control (tissue DNA) and environmental samples (eDNA).

Sample	Species	Source	forward primer name	forward primer sequence (5'-3')	reverse primer name	reverse primer sequence (5'-3')	SEQUENCE (5'-3')
CoFl-sp	*Corbicula fluminea*	Tissue – individual Z2 point	CoFl-16S-F	GAATAAC TTAAATG TAGGT	CoFl-16S-R	AGCAA ACTTCT TCTTAA ATAT	Cf-E2: ATTGGGGCAATAGAAAATGAAATGAATCATTTTTTTTAT TATAAGGATCCAGTTTTGACTGAAAAAAGCAAAGCTAC CGCGGGGATAACAGGGTAATTTTTTCTGAGAGTTCATAT TTAAGAAGAAGTTTGCT
Ebro1_CF	*Corbicula fluminea*	Ebro River eDNA Z1 point	CoFl-16S-F	GAATAAC TTAAATG TAGGT	CoFl-16S-R	AGCAA ACTTCT TCTTAA ATAT	Cf-eE1: GAAATGAATCATTTTTTTTATTATAAGGATCCAGTTTTGA CTGAAAAAAGCAAAGCTACCGCGGGGATAACAGGGTA ATTTTTTCTGAGAGTTCATATTTAAGAAG
Ebro2_CF	*Corbicula fluminea*	Ebro River eDNA Z2 point	CoFl-16S-F	GAATAAC TTAAATG TAGGT	CoFl-16S-R	AGCAA ACTTCT TCTTAA ATAT	Cf-eE2: AAATGAAATGAATCATTTTTTTTATTATAAGGATCCAGTT TTGACTGAAAAAAGCAAAGCTACCGCGGGGATAACAG GGTAATTTTTTCTCGTAGAG
Ebro3_CF	*Corbicula fluminea*	Ebro River eDNA Z3 point	CoFl-16S-F	GAATAAC TTAAATG TAGGT	CoFl-16S-R	AGCAA ACTTCT TCTTAA ATAT	Cf-eE3: AATGAATCATTTTTTTTATTATAAGGATCCAGTTTTGACT GAAAAAAGCAAAGCTACCGCGGGGATAACAGGGTAAT TTTTTCTGAGAGTTCATATT
MeTu-sp	*Melanoides tuberculata*	Tissue – individual EL point	MeTu-16S-F	GGTCTRA CGAAAG CAATACT	MeTu-16S-R	GCTTTG CTKGAT CTAAA YYT	Mt-EL: GTAGGTGAAGAGGCCTATATTATATTGAAGGACAAGAA GACCCTGTCGAGCTTTAAAATTAAATATAGGTGTAAATT ATTAACAAAATCAATGAACTGCATTATATTTTTAGTTGG GGCGACGAAGGAACAAACAAAGCTTCTTTTATTTTTATA AATTTATAGGTTTAGATCCAGCAAAGC
Alh-MT	*Melanoides tuberculata*	Lake eDNA EL point	MeTu-16S-F	GGTCTRA CGAAAG CAATACT	MeTu-16S-R	GCTTTG CTKGAT CTAAA YYT	Mt-eEL: TATTGAAGGACAAGAAGACCCTGTCGAGCTTTTAAATTA AAGTAGGTGTAAATTATTAACAAAATCAATGAACTGCAT TACATTTTTAGTTGGGGCGACGAAGGAACAAACAAAGCT TCTTTTATTTTTATAAATTTATAGGTTTAGATCCAGCAAA GC
MyLe-sp	*Mytilopsis leucophaeata*	Tissue – individual S1 point	MyLe-COI-F	GGTTGTA ACAACGC ACGGTTT AG	MyLe-COI-R	CACCTT CTCTGA AAGCC GAGC	Ml-G1: CCTATAATGATGGGTGGTTTTGGAAATTGATTAGTTCCA ATAATACTAGCAGTGCCTGATATAGGATTTCCTCGTTTA AATAATGTTAGGTTTTGGGTGTTACCTGTATCTATAGGTC TTTTATTTTGCTCGGCTTTCAGAGAAGGTG
Sevilla1_ML	*Mytilopsis leucophaeata*	Guadalquivir River eDNA S1 point	MyLe-COI-F	GGTTGTA ACAACGC ACGGTTT AG	MyLe-COI-R	CACCTT CTCTGA AAGCC GAGC	Ml-eG1: TGGGTGGTTTTGGAAATTGATTAGTTCCAATAATACTAG CAGTGCCTGATATAGGATTTCCTCGTTTAAATAATGTTAG GTTTTGGGTGTTACCTGTATCTATAGGTCTTTTATTTTGCT CGGCTTTCAGAGAAGGTG
Sevilla2_ML	*Mytilopsis leucophaeata*	Guadalquivir River eDNA S2 point	MyLe-COI-F	GGTTGTA ACAACGC ACGGTTT AG	MyLe-COI-R	CACCTT CTCTGA AAGCC GAGC	Ml-eG2: GATGGGTGGTTTTGGAAATTGATTAGTTCCAATAATACT AGCAGTGCCTGATATAGGATTTCCTCGTTTAAATAATGTT AGGTTTTGGGTGTTACCTGTATCTATAGGTCTTTTATTTT GCTCGGCTTTCAGAGAAGGTG
SiWo-sp	*Sinanodonta woodiana*	Tissue – museum specimen	SiWo-COI-F	GGGTCAG CCMGGR AGGCTTT TA	SiWo-COI-R	TGTTCA CCCTGT ACCAA CRCCC	Sw-voucher: TGTTACGGCTCATGCTTTTATAATAATTTTCTTCTTAGTT ATACCTATAATGATTGGAGGGTTTGGGAATTGATTAATT CCTTTAATAATTGGGGCTCCTGATATGGCTTTTCCTCGAT TGAATAATTTAAGGTTTTGGTTACTTGTGCCAGCGCTATT TTTATTATTAAGGTCTTCTTTGGTGGAAAGGGGCGTTGGT ACAGGGTGAACA
Sant_SW	*Sinanodonta woodiana*	Reservoir eDNA MR point	SiWo-COI-F	GGGTCAG CCMGGR AGGCTTT TA	SiWo-COI-R	TGTTCA CCCTGT ACCAA CRCCC	Sw-eMR: ATAATTTTCTTCTTAGTTATACCTATAATGATTGGAGGGT TTGGGAATTGATTAATTCCTTTAATAATTGGGGCTCCTGA TATGGCTTTTCCTCGATTGAATAATTTAAGGTTTTGGTTA CTTGTGCCAGCGCTATTTTTATTATTAAGGTCTTCTTTGG TGGAAAGGGGCGTTGGTACAGGGTGAACA

Capítulo 5:

Assessing performance of species-specific primers for detecting aquatic invasive species in Lake Constance region using eDNA

Clusa L, García-Vázquez E and Machado-Schiaffino G

Molecular Ecology

Assessing performance of species-specific primers for detecting aquatic invasive species in Lake Constance region using eDNA

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ABSTRACT:

Biological invasions are a global threat to biodiversity especially for aquatic resources. The distribution of alien species is associated with human activities; exotic species tend to accumulate near big urban areas, where most invasion vectors are: ballast water, hull fouling, aquarium and pet releases. The Rhine River region is one of the most important in Europe. 58 million people live in the river basin and the river is connected with nearly all large rivers Europe via the Rhine–Main–Danube shipping canal. On the other hand, in the Alpine Rhine region is the main reservoir for Rhine River, Constance Lake the second largest subalpine lake in Europe.

Two different approaches were used in order to identify the non-indigenous species hotspot and update invasive species records in the region. First to control the spread of ten common European fish and mollusc invasive species through PCR with specific primers, and the second one to apply NGS technologies amplifying a fragment of 313 bp from the cytochrome oxidase subunit I gene to detect any other invasive species and obtain global biodiversity in the region but also to compare the performance of the specific primers. From the application of the ten specific primers two invasive species were identified *Potamopyrgus antipodarum* and *Corbicula fluminea* and the use of specific primers allowed elucidating the invasion pattern of these two invasive species. In the uppermost sample none of the species tested was identified, the absence of NIS could be due to the impassable dam downstream, which may prevent the arrival of exotic species. From the NGS analysis nine exotic species were detected including two arthropods, two fishes, one cnidario and four molluscs. The use of specific primers was more sensitive than NGS. NGS failed to detect the exotic species identified with specific primers in some samples; but in this case it helped with the detection of multiple invasive species. The areas with the highest number of exotic species are port areas, where ships may facilitate the spread of exotic species and the higher temperature due to boat transport can aid to their survival and downstream areas near hydropower plants and big urban areas.

Better management measures should take into account the surveillance of these areas to avoid the spread of NIS, and to develop stricter regulations of ornamental species and aquaculture but also the surveillance of recreational boats would be advisable since they could spread these NIS to other water bodies nearby. Moreover establish a common regulation and management actions by all the countries implied in the region is highly recommendable.

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Keywords: eDNA, NGS, specific-primers, Rhine River, Constance Lake, NIS

Introduction

Biological invasions are a global threat to biodiversity especially for aquatic resources (Chown *et al.* 2015). Most of aquatic organisms' translocations derived from human activities (Leprieur *et al.* 2008). Alien species have been introduced into streams worldwide for recreational fishing, aquaculture or derived from aquarium trade (Havel *et al*. 2015). At the same time, global transport is helping the spread of many species out of their native distribution through ballast water (Alonso and Castro-Díez 2008; Thomaz *et al.* 2014). This global transport together with the

increase of temperature due to climate change may have benefit dispersion of invasive species; an example is the spread of zebra mussel (*Dreissena polymorpha*) in the Baltic Sea (Holopainen *et al.* 2016), or the expansion of *Corbicula fluminea* in northern areas (Gollasch and Nehring 2006; Crespo *et al.* 2015). In Europe there are three main routes of alien species introduction, one is through the UK, the second one is France as the main donor species to southwestern countries, and the third one is Germany as the main donor to northern countries. 13 fish species from North America were introduced to Germany and distributed to the Netherlands, Denmark, Hungary and Poland.

Moreover the 100% of the established exotic species in Germany are causing adverse ecological effects (García-Berthou *et al.* 2005). The distribution of alien species is associated with human activities (Wolter and Röhr 2010, Spear *et al.* 2013); exotic species tend to accumulate near big urban areas, where most invasion vectors are: ballast water, hull fouling, aquarium and pet releases (Strayer 2010, Duggan 2010).

The Rhine River is the most important river in Germany. From its 1,250 km length, 825 km of the river are navigable from the port of Rotterdam in the North Sea coast until Basel in Switzerland. 58 million people live in the river basin and the river supplies drinking water for more than 30 million people (Plum and Schulte-Wülwer-Leidig 2014). Moreover it is connected with nearly all large rivers in south- western, southern, central and eastern Europe. Together with the Danube, they are the rivers that are most invaded in Europe. They are connected via the Rhine–Main–Danube shipping canal since 1992 which works as an entrance of aquatic alien species. 26 alien species in German waters can be directly related with this canal, for example the entrance of the amphipod *Dikerogammarus villosus* in the Rhine basin (Gollasch and Nehring 2006). This pathway is the main vector for recent invaders in Germany and Austria, especially species from Ponto-Caspian region (Rabitsch *et al.* 2013). The number of non-indigenous macroinvertebrate species in the Rhine River increased over the period from 1800 to 2005, from 1 to more than 13 species per decade. The rapid dispersion of exotic species is facilitated by shipping activities and the interconnection of river basins (Leuven *et al.* 2009). In addition the river has plain hydrological power plants in its way long, for example in the high part of the river (High Rhine) from Lake Constance to Basel there are 12 in-stream barriers due to hydropower plants (N'Guyen *et al.* 2016), which altered the river flow and whose cooling waters can be the perfect habitat for invasive species, as it happened with the gobies in this region (Kalchhauser *et al.* 2013) or with the invasive *Mytilopsis leucophaeata* in southern Bothnian Sea, Sweden (Florin *et al.* 2013). On the other hand, the presence of reservoirs has been associated with higher number of exotic species introductions (Clavero *et al.* 2004; Johnson *et al.* 2005). In fact, Havel *et al.* (2015) suggested that once an exotic species is established in one lake, it could colonize easier nearby lakes. Precisely in this region, Constance Lake, the second largest subalpine lake in Europe, is situated at the northern fringe of the European Alps and is bordering Germany, Switzerland and Austria. It is the main reservoir for Rhine River. The lake itself is an important drinking water source for southwestern Germany and

economically important for recreational and commercial fisheries and for tourism (N'Guyen *et al.* 2016). Twenty-nine species occur in the lake of which only a few are of commercial interest and exploited intensively: two forms of lake whitefish (*Coregonus clupeiformis*); perch (*Perca fluviatilis);* European eel (Anguilla Anguilla); brown trout (*Salmo trutta*); pike (*Esox lucius*); Arctic charr (*Salvelinus alpinus*) and pike perch (*Sander lucioperca*) (Eckmann and Rosch 1998). The Lake Constance trout (*Salmo trutta*) was almost extinct in the 1950s due to dam construction in the alpine Rhine, but thanks to protective measures they have made a significant return (Ruhlé 1996). The lake was the home of the now extinct species of trout *Salvelinus profundus*, as well as of the Lake Constance whitefish (*Coregonus gutturosus*) (Freyhof and Kottelat 2008). One reason could be the introduction of exotic species. Usually these exotic invasive species are a leading cause of animal extinctions (Clavero and García-Berthou 2005).

Prevention and early detection of new invasions is the best way to control dispersion of invasive alien species (Thomaz *et al.* 2014). In the last few years the develop of environmental DNA (eDNA) techniques has become a promising tool to early detect and survey alien species in aquatic ecosystems (Goldberg, Strickler and Pilliod 2015), there are numerous examples of using eDNA to successfully detect invasive species (Ficetola *et al.* 2008; Ardura *et al.* 2015; Clusa *et al.* 2016; Borrell *et al.* 2017).

The control of alien invasive species both in the lake and the river is thus crucial, especially since they could serve as a reservoir and entrance for many invasive species that could distribute all over Europe. On the other hand the management of this region is not efficient since there are decentralized political structures from the countries surrounded (Austria, Germany and Switzerland) (Essl *et al.* 2011), which makes no sense especially when the river acts as border between Germany and Switzerland in the High Rhine region. Although there is a new European regulation concerning invasive species, the EU regulation No 1143/2014 of 22 October 2014 on Invasive Alien Species (http://ec.europa.eu/environment/nature/invasivealien/index_en.htm) the list of invasive alien species of Union concern are only 26 animal, from which the only freshwater fish included is *Pseudorasbora parva*. There is not a common list of invasive species for Switzerland, Austria and Germany (Wittenberg *et al.* 2005; Nehring *et al.* 2010; Gollasch and Nehring 2006). When searching the three regions in EASIN (European Alien Species Information Network) database (https://easin.jrc.ec.europa.eu/) the list of invasive

species in each is different with only a few species in common.

The main objective was to update the invasive species records in the region. Two different approaches were done, first to control the spread of ten common European fish and mollusc invasive species through PCR with specific primers, and the second one to apply NGS technologies in order to compare the performance of the specific primers and to detect any other invasive species in the region. Derived from the results the hotspot of non-indigenous species (NIS) in the region will be identified for developing better management actions.

Methodology

Study area

Constance Lake is 63 km long, and at its widest point, nearly 14 km. It covers approximately 571 km² and is 395 m above sea level. The greatest depth is 252 meters in the middle of the eastern part. It consists of two basins: Deep Upper Lake Constance (ULC) and Lower Lake Constance (LLC), which is smaller (Jeppesen *et al.* 2012).

Daily, car ferries link Romanshorn to Friedrichshafen and Constance to Meersburg (Gergs and Rothhaupt 2015) in the Upper Lake Constance. The Rhine River is divided in six sections: the Alpine Rhine and the Lake Constance; the high Rhine from lower Lake Constance to Basel, where many barriers are, including a 23 m waterfall situated 30km downstream the lake (Rheinfall) and many hydrological power plant dams; the Upper Rhine that extends from Basel to Bingen; the Middle Rhine; the Lower Rhine from Bonn to Lobith and the Delta Rhine in the Netherlands (Leuven *et al.* 2009). The alpine part of the Rhine River flows into the lake in the southeast (near Bregenz) and flows out near Stein am Rhein in the LLC. The Rhine River is the primary artery of one of the most important economic regions of Europe. It has a total length of about 1,250 km, a drainage area of circa 185,260 km² and an average discharge of about 2,300 m³ s⁻¹ (Rabitsch *et al.* 2013).

Between October and November 2017, 15 sampling points were selected in the region: from the Alpine Rhine (R0) to the Upper Rhine downstream Basel (R8), including the main ports areas of Constance Lake (Table 1, Figure 1).
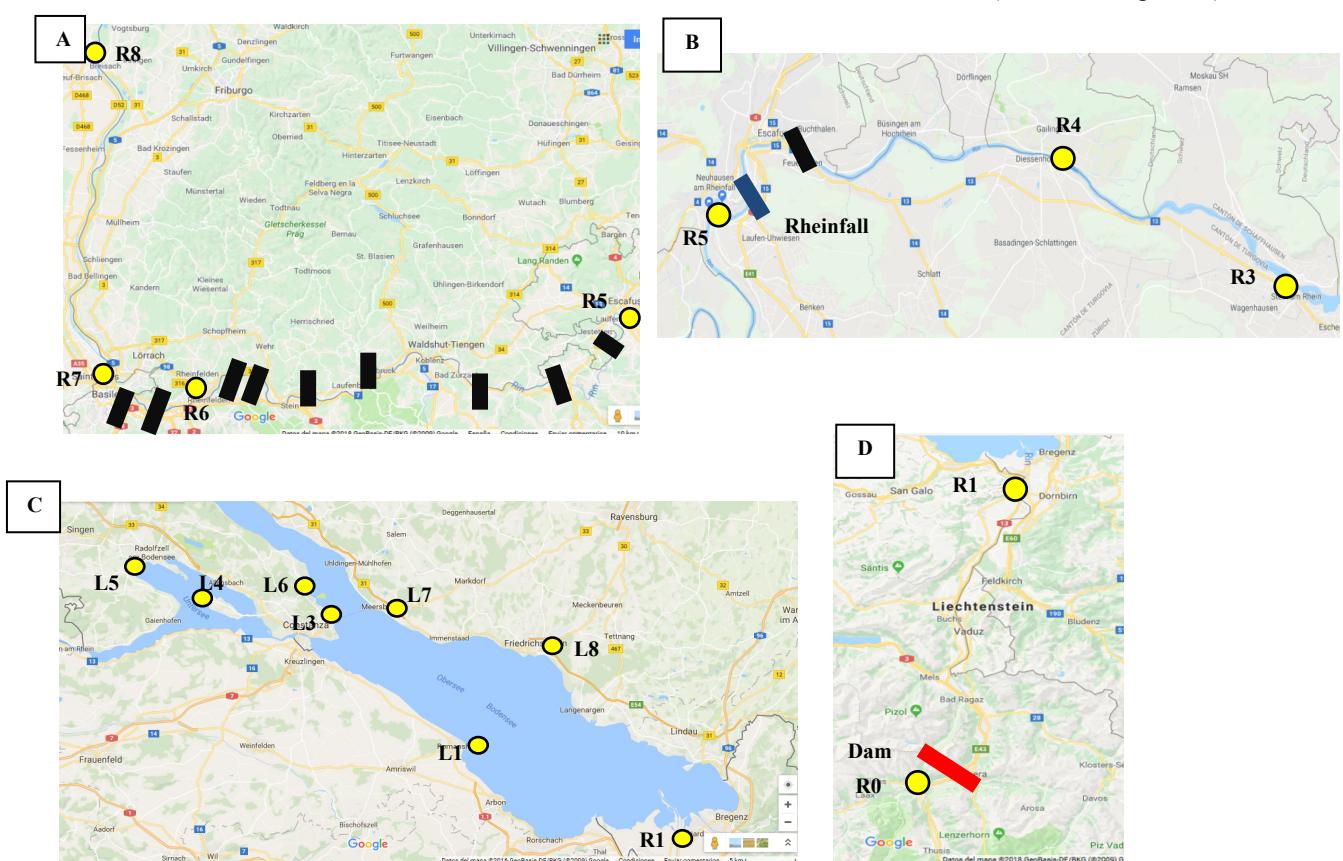
Figure 1. **Map showing the sampling points**. The region is divided in four sections from downstream to upstream: A) Upper part of the river, from Breisach to Rheinfall; B) upper part of the river from Rheinfall until Steim am Rhein; C) Constance Lake; D) Alpine part of the river. Sampling points are indicated with a yellow circle and dams are pointed with a solid bar, in black ink are the power stations, in blue ink the natural falls of Rheinfall and in red ink the impassable dam in the alpine part of the Rhine River. Maps obtained from Google maps.

Table 1. **Sampling points both from Rhine River and Constance Lake.** Coordinates, visual appearance and current speed based on relative impression during water sampling are shown.

Sample	Watershed	Location	Coordinates	Appearance	Current speed
R0	Rhine River	Reichenau (Tamins), alpine Rhine River (Switzerland)	46.82453N, 9.41161E	Sandy soil, few stones	+
R1	Rhine River	Alter Rhein (Austria)	47.45600N, 9.64358E	Small stones and channeled	+
R3	Rhine River	Stein am Rhein (Switzerland)	47.65852N, 8.86141E	Sandy soil, many small stones and shells from bivalves	+
R4	Rhine River	Diessenhofen (Switzerland)	47.69024N, 8.75222E		+
R5	Rhine River	After Rheinfall (Switzerland)	47.67615N, 8.61020E		++
R6	Rhine River	Stein (Germany)	47.55143N, 7.95023E	Large stones and algae, deep (~2m) near the shore	+++
R7	Rhine River	Basel (Switzerland)	47.56290N, 7.58853E	Sandy with large stones, deep (~2m) near the shore	+++
R8	Rhine River	Breisach am Rhein (Germany)	48.04345N, 7.57271E	Large stones and algae, deep (~2m) near the shore	+++
L1	Constance Lake	Romanshorn port (Switzerland)	47.56456N, 9.38158E	Large rocks in the depths, many algae and vegetation	--
L3	Constance Lake	Constance port (Germany)	47.68337N, 9.21094E	Small stones with a lot of moss, there were traces of *Corbicula sp* (shells)	--
L4	Constance Lake	Reichenau Insel (Constance) (Germany)	47.68671N, 9.06711E	Small stones	-
L5	Constance Lake	Radolfzell am Bodensee (Germany)	47.73523N, 8.96839E	Small stones	-
L6	Constance Lake	Constance Natural Area (Germany)	47.69559N, 9.19326E	Sandy soil, small stones	-
L7	Constance Lake	Meersburg port (Germany)	47.69669N, 9.26180E	Small stones and sandy	--
L8	Constance Lake	Friedrichshafen port (Germany)	47.650778N, 9.483804E	Large blocks of cement and stones, deep (~2m) near the shore	--

Samples collection

1L of water per replica (three replicas per point) was collected in sterile bottles in each sampling point. The samples collection was done in different days and always from upstream to downstream. All the personal equipment was cleaned with 50% bleach between points and new gloves and sterile bottles were used in each point. In every sampling point, water was collected 30cm below the surface, since recent DNA is located in the surface whereas in the sediments old eDNA can be accumulated and preserved long time at low temperatures even when the source of DNA has disappeared (Turner *et al*. 2015; Goldberg *et al*. 2016).

All the samples were transported to the laboratory, stored at 4°C and immediately or the day after they were filtrated using an Acetate cel. membrane (Fisher Scientific) with 0.22 μm pore size and a filter holder. Filtration took place inside a laminar flow cabinet previously treated with UV light to avoid any contamination. The filter holder was dismantled, clean with 50% bleach, rinse with distilled water and treated with UV for 20 minutes before use and between each sample. Moreover a negative control consisted in 1L of milliQ water was filtrated between two problem samples and included in all the posterior analysis. Filters were stored at -20°C until extraction. All the collection, filtration, extraction and analysis process were done following the recommendations from Goldberg *et al*. (2016) to avoid any cross contamination in the different steps.

eDNA extraction

DNA from water samples was extracted with the PowerWater® DNA Isolation Kit (Mobio laboratories) following the manufacture's protocol. Every replica from each sampling point was extracted separately in time, therefore all the analysis and extraction from the same water sample was done in different weeks, minimizing the possibility of contamination. In addition, the whole extraction process was done inside the laminar flow cabinet. And also two negatives control were included in each extraction and in all posterior PCRs analysis; the negative control for filtration (previously described) and a negative control for extraction which consisted in a clean membrane.

Specific primers

A selection of 10 fish and molusc species were used to control their invasion in the region (Table S1). All the pre-PCR steps were done inside the

laminar flow cabinet after 20 minutes of UV light decontamination, and the post-PCR steps were done in other laboratory unit. In order to test the quality of the DNA in each sample and discard false negatives due to excessive DNA degradation, inhibitors or other reasons; the cytochrome oxidase subunit I (COI) gene was amplified with general primers (Geller *et al.* 2013) in all water samples. Furthermore an internal positive control was included in the analysis, it consisted in the amplification of the three spine sticklebacks (*Gasterosteus aculeatus*) using the specific primers designed by Thomsen *et al.* (2012), since the species is native to the region and known to habit the Constance Lake and the Rhine River. Also a positive control with tissue sample was included in each PCR to discard errors, but it was added outside the cabinet, in the last minute when all the samples tubes were closed inside the PCR machine. As negative controls, three negative controls were done. The filtration and extraction negative controls (explained above) and a PCR negative control, using milliQ water as template instead of DNA.

The PCRs conditions used are the same described by Clusa *et al* (2016; 2017; 2018) and Thomsen *et al.* (2012) (Table S1). All the PCRs products were visualized in 2% agarose gel with DNA Stain clear G (SERVA). From the three replicates per sampling point, the whole panel of specific primers was used in two replicates. To consider a sample positive o negative, the two samples had to be positive or negative respectively. The last replicate was used for running an extra PCR for the positive results obtained with specific primer to confirm the presence of the species; for the dubitative cases, when only one sample was positive; and for the NGS analysis. Every positive PCR band was purified either with the enzymes (Fast AP and ExoI) or with the gel extraction kit Zymoclean™ Gel recovery kit and sequenced with ABI 3130 sequencer to confirm the species detected.

Inhibitors test

The presence of inhibitors in one sample can cause false negatives (Thomsen and Willerslev 2015). To control the presence of inhibitors in the samples, DNA from the species *Gambusia holbrooki* was added to one replicate from all the sampling sites in two different concentrations similar to the experiment done by Clusa *et al.* (2016). For the high concentration test 1 µl of Gambusia DNA from 10ng/mL was added to 5 µl of eDNA; and for the low concentration assay 1 µl of Gambusia DNA from 10pg/mL, closed to the detection limit of the specific primers, was added to 5 µl of eDNA. In both cases PCR was performed as described in 2.4 section.

NGS library preparation

Ten samples were selected for the NGS analysis to obtain a global view of the biodiversity in the region and evaluate the influence of ports, big urban areas and barriers. The third replicate of water samples was used for this purpose. The region amplified was a fragment of 313 bp from the COI gene, using the primers mlCOIlintF and jgHCO2198 described by Leray *et al.* (2013) and adapted to Illumina platform. The protocol used was the one described for Illumina platforms (Illumina), which consisted in double PCR. The first was done using general primers with a barcode and a tag and the second PCR using primers with the tag and adapters for Illumina (Table S2). Finally the pair-end sequences were generated on MiSeq platform. The negative control for filtration and extraction were used in the PCR but they showed no quantifiable DNA and they were not processed further.

NGS bioinformatics' analysis

The Fastq files were split by barcodes, allowing obtaining all the sequences from each sample. All the Fastq files were checked in the FastQC version 0.11.3 visor. A small subset of 5000 sequences was used to obtain the pipeline to analyze the rest of the samples, different merge and assignment conditions were tested and finally at least 5 sequences from all the species in the OTU table resulting were manually checked to confirm the good performance of the assignment (data not shown). The merged of the pair-end files was done using the script *join_paired_ends* (Aronesty 2011) included in QIIME (Quantitative Insights Into Microbial Ecology) (Caporaso *et al*. 2010), with a minimum overlap of 100 bp and a maximum error of 15%. After that the sequences were trimmed left and right using PrinSeq version 0.20.4 (Schmieder and Edwards 2011) to remove primer sequences and simultaneously the sequences were filtrated by length and quality, allowing a maximum length of 340 nucleotides and a minimum length of 230 and sequences with a mean quality score lower than 25 were removed.

To create a reference taxonomic database, an exhaustive search for "mitochondrial COI gene" sequences was done in the NCBI website in June 2017. All the cytochrome oxidase subunit I sequences available were downloaded with the script *entrez qiime.py* (Baker 2016). After that a blast was done using the script *assign taxonomy* from QIIME (Caporaso *et al*. 2010) using as database the file generated with all the COI sequences downloaded from GenBank. The assignment was done using a 97% of identity and an e-value of 10^{-50}. Finally the OTU table was obtained using the script for python *fromTaxassignment2Otutable*.The protocol is

similar to the one used by Galal-Khallaf *et al.* (2016).

Biodiversity analysis

With the OTU table, the Shannon index was calculated with the script *alpha diversity* from QIIME (Caporaso *et al.* 2010), using all the taxonomic groups but removing duplicated species and sequences assigned as invertebrate environmental sample.

After that, to localize the hotspot of invasive species, all the exotic species presented in each sample from the OTU table and the specific primers tests were recorded. To analyze the percentage of non-indigenous species in the samples only sequences from chordate and mollusc species were taken into account, excluding sequences from human and avian DNA. And the percentage of NIS was calculated as the number of exotic species identified by both methods divided by the total number of species detected.

Results

eDNA results with specific primers

From the application of the ten specific primers two species were identified (Table 2). All the positive results were sequenced and the species confirmed by Blast in the NCBI webpage. All the sequences are available in Table S3. The native fish *Gasterosteus aculeatus* was detected in eight out of the fifteen samples analyzed both in lake and river samples.

The invasive *Potamopyrgus antipodarum* is the most distributed species from the ones tested, being found in eleven water samples, with the exception of the alpine part (R0), the Lower Lake Constance (L4, L5) and Basel (R7) (Table 2). Furthermore one unique haplotype was found in all eDNA samples and it correspond to the European haplotype *t* described by Städler *et al.* 2005 and found by Clusa *et al.* (2016) in Nora River in Asturias (Table S3).

The invasive *Corbicula fluminea* was found only in Upper Lake Constance samples (L1, L3, L6 and L8) including three of the four ports analyzed and downstream Rhine River (R6, R7 and R8) near Basel. It was not found in High Rhine region (R3, R4 and R5), neither in Lower Lake Constance (L3, L4) or in the Alpine Rhine (R0), although the ecological niche seem similar to places where it was found (Table 1).

None of the invasive fish species analyzed were found. And in the sample R0 (the Alpine Rhine) none of the species tested was identified. But the amplification of the COI gene with universal primers confirmed the presence of good quality DNA in all eDNA samples (Table 2). In addition the spike test to discard the presence of

inhibitors in the samples was a success, positive amplification of the *Gambusia holbrooki* DNA was obtained in all eDNA samples in both PCRs, one with high concentration of Gambusia DNA and one with low concentration of Gambusia DNA, discarding the presence of inhibitors in the samples.

NGS results

In each sample a minimum of 500000 raw sequences were obtained. After the merged and filtration steps approximately the 76.80±11.1 % of sequences remained from raw data. Afterwards, only the 12.6±7.6% of the raw data was possible to be assigned with the 97% of identity. The sample with less sequences assigned was R8 with only the 2.6% of raw data, whereas the sample with the higher number of sequences assigned was L5 (29.1% of raw data) (Table S4).

The main taxa amplified from the NGS analysis was Porifera (more than 50% of the sequences) followed by Arthropoda (25.85%), and protista (11.32%); only a 1.87% correspond to mollusc and a 0.42% to chordate species (Figure 2A). Although the percentage vary in each sample, as an example in R0 the 81.3% of the sequences correspond to arthropods, in R1 the 81% correspond to protista species or in R5 the 12.2% are molluscs (Figure 2B). From the assigned sequences 9 exotic species were detected (Table 3, Table S5) including two arthropods, two fishes, one cnidario and four molluscs: The killer shrimp *Dikerogammarus villosus*, the Caspian slender shrimp *Limnomysis benedeni*, the whitefish *Coregonus lavaretus*, the rainbow trout *Oncorhynchus mykiss*, the freshwater jellyfish *Craspedacusta sowerbyi*, the zebra mussel *Dreissena polymorpha,* the Asiatic clam *Corbicula fluminea,* the New Zealand mudsnail *Potamopyrgus antipodarum* and the mollusc *Physella acuta.*

When comparing the performance of the NGS analysis with the specific primers, *C. fluminea* was identified in one sample (L3) and not in the rest of samples where it was detected with the specific primers (L8, R6 and R8) (Table 3). In L3 the proportion of mollusc sequences (2.3%) are higher than in the other samples (0.56% in L8, 0.27% in R6 and 0% in R8) (Figure 2B). And the same happened with *P. antipodarum*, which was identified in three samples (L3, L8 and R4), and not in the rest of samples previously detected (R1, R5, R6, R8) (Table 3), where the proportion of mollusc sequences are 0 in R1 and R8, 0.27% in R6 and 12% in R5 but all the sequences corresponding to the *Ancylus* sp in this last sample (Table S4) (Figure 2B). Furthermore the number of sequences assigned in these samples varies between each other (Table S4). A curious case was the presence of *Homo sapiens* DNA in sample L4.

The chance of a possible contamination source from the laboratory is really low, since all the negative controls were carefully taken and it is only present in one sample.

Table 2. **eDNA results from specific primers.** Results from specific primers are shown; positive results are highlighted in grey.

	Reference	Species	control mQ H$_2$O	R0	R1	R3	R4	R5	R6	R7	R8	L1	L3	L4	L5	L6	L7	L8
Invasive fish species	Clusa *et al.*, 2018	*Ameiurus sp*	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
		Gambusia sp	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
		Lepomis gibbosus	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
		Micropterus salmoides	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
		Pseudorasbora parva	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
Native fish species	Thomsen *et al.*, 2012	*Gasterosteus aculeatus*	0/3	0/3	3/3	0/3	0/3	3/3	0/3	0/3	3/3	0/3	3/3	3/3	3/3	0/3	3/3	3/3
Invasive mollusc species	Clusa *et al.*, 2017	*Corbicula fluminea*	0/3	0/3	0/3	0/3	0/3	0/3	3/3	3/3	3/3	3/3	3/3	0/3	0/3	3/3	0/3	3/3
		Melanoides tuberculata	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
		Sinanodonta woodiana	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
		Mytilopsis leucophaeata	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	Clusa *et al.,* 2016	*Potamopyrgus sp*	0/3	0/3	3/3	3/3	3/3	3/3	3/3	0/3	3/3	3/3	3/3	0/3	0/3	3/3	3/3	3/3
Universal primers	Geller *et al.*, 2013	COI gene	0/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3
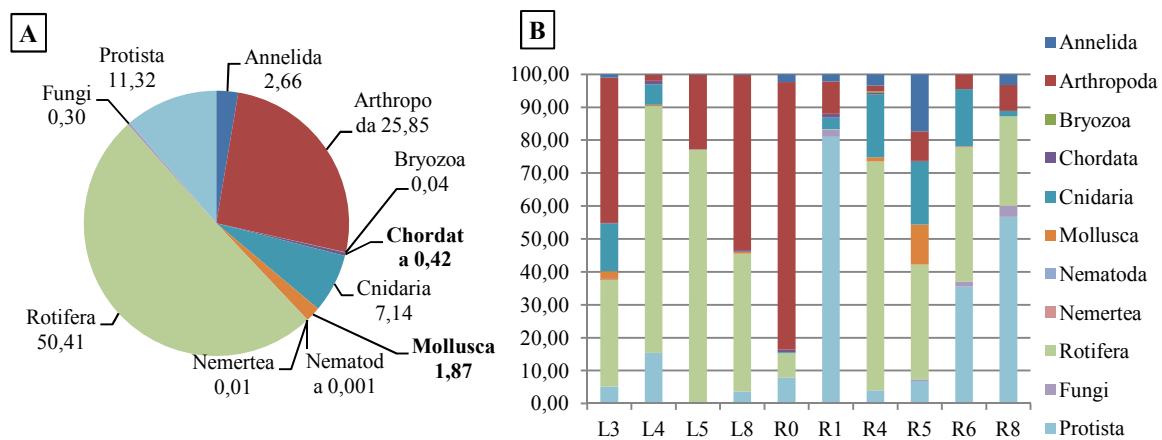
Figure 2. **Sequences from NGS analysis by taxon**. A) Percentage of all sequences obtained from the NGS analysis classified by taxon. B) Percentage of sequences from each taxon per sampling point.

Table 3. **Invasive species detected classified by sample**. The positive detection with NGS analysis is shown with an "X" and shadowed in grey are invasive species only detected with the specific primers. The total number of NIS in each sample is shown.

	Constance Lake samples				Rhine River samples					
Species	L3	L4	L5	L8	R0	R1	R4	R5	R6	R8
Dikerogammarus villosus	X							X	X	
Limnomysis benedeni		X		X						
Coregonus lavaretus		X								
Oncorhynchus mykiss						X				
Craspedacusta sowerbyi	X	X					X	X		
Corbicula fluminea	X			X					X	X
Dreissena polymorpha	X		X						X	
Potamopyrgus antipodarum	X			X		X	X	X	X	X
Physella acuta				X						
Total NIS	5	3	1	4	0	2	2	3	4	2

Biodiversity and % NIS

The biodiversity test based on the calculation of the Shannon index taking into account all the sequences from all the taxa identified by NGS showed that the highest value was found in samples R8 (3.880), R1 (3.771) and R6 (3.272) (Table 4). From the analysis of both methods together, the places with more invasive species were L3, where 5 out of the 9 NIS identified in the region were found, followed by L8 (4 species) and R6 (4 species). On the other hand the lowest number of NIS was found in R0 and L5 with none and one exotic species respectively (Table 3). Respecting the percentage of NIS in the samples, taking into account only mollusc and fish species detected by both methods, the samples with higher proportion are R6 (100%) and R8 (66.7%), while the samples with less proportion of NIS are R0 (0%), L4 (16.7%) and R5 (16.7%) (Table 4).

Discussion

The use of specific primers in this case allowed elucidating the invasion pattern of two invasive species (*C. fluminea* and *P. antipodarum*). The first record of *Corbicula* in the Rhine was in 1985 in the lower Rhine region in Netherlands, after that in 1995 was recorded in Basel, and in 2003 it was found 22km upstream Basel in Rheinfelden but not any further. It was not found between Rheinfelden and Lake Constance (Schmidlin and Baur 2007). The first detection of this species in Constance Lake was in 2003 in a sandy shallow-water near Bregenz (Werner and Mörtl 2004). Schmidlin and Baur (2007) suggest that both introductions are independently from each other and the results from this paper would confirm it. *Corbicula* larvae and small individuals can travel attached to avian feet or feathers and to fishes and might be transported over large physical barriers such as dams from hydroelectrical power plants (Schmidlin and Baur 2007). Moreover the absence of the species in Lower Lake Constance (L4 and L5) and High Rhine region (R3, R4, and R5) (Table 2), could be

due to the invasion did not reach these two regions or perhaps the presence of the species is limited to ports (samples L1, L3 and L8) and protected areas near big cities (R7, R8) or power plants (R6) where water temperature can be higher in winter conditions, since low winter temperatures and water level decrease produced a massive mortality in the *C. fluminea* population (Werner and Rothhaupt 2008).

The same happened with *Potamopyrgus antipodarum*, it is able to travel through animal vectors (Alonso and Castro-Díez 2008). In this paper, it was found almost in every sample (Table 2) except in the alpine Rhine (R0), in Basel (R7) and in the Lower Lake Constance (L4 and L5), although the ecological niche seemed similar to other sampling sites where it was found (Table 1). It could be that it did not reach this region, or the population if present is in low density and the primers are not able to detect it. In previous studies Gergs and Rothhaupt (2015) only found 2 *Potamopyrgus antipodarum* individuals in 2005, 0 in 2006 and 3 in 2007 in this region (near sampling point L4). In any case a posterior surveillance should be done in this region to confirm its absence and to control its spread. Different from *C. fluminea*, *P. antipodarum* is able to survive winter conditions, water temperatures from 0-28°C and even resist short times of desiccation (Moffitt and James 2012; Alonso and Castro-Díez 2012), which can explain its spread.

Furthermore climate change may benefit warmwater invaders (Rahel and Olden 2008; Chown *et al.* 2015), as an example the latter introductions of *Lepomis gibbosus* and *Pseudorasbora parva* are restricted to sampling sites with high temperature (Rabitsch *et al.* 2013) as well as the common carp (*Cyprinus carpio*) (Jeppesen *et al.* 2012). In this region the average water temperature increased 0.22°C per decade between 1965 and 2009 (Jeppesen *et al.* 2012), this change may be responsible of the expansion of *C. fluminea* in the lake from Bregenz in 2003 (Werner and Mörtl 2004) to Constance, the other side of the lake, in 2017 (Table 3).

Table 4. **Percentage of exotic fish and mollusc species detected.** In each point the percentage of exotic fish and mollusc detected based on both methods (NGS and specific primers) were calculated (% of NIS), as well as the global biodiversity expressed with the Shannon index.

	Constance Lake samples				Rhine River samples					
	L3	**L4**	**L5**	**L8**	**R0**	**R1**	**R4**	**R5**	**R6**	**R8**
Number of species detected	6	6	2	6	2	5	3	6	3	3
Number of NIS detected	3	1	1	3	0	2	1	1	3	2
% of NIS	50,00	16,67	50,00	50,00	0,00	40,00	33,33	16,67	100,00	66,67
Shannon index (whole sample)	2,909	1,598	1,048	2,107	2,059	3,771	2,140	3,062	3,272	3,880

In the case of fish invasive species tested, the absence of any results could be due hopefully to the absence of these species in the region or derived from the sampling strategy (Comtet *et al.* 2015). Sampling was done in the shore of the river and the lake with 1 meter depth, although *Gasterosteus aculeatus* was found in eight of the fifteen samples both in river and lake (Table 2).

The use of specific primers seemed to be more sensitive than NGS; all the species found with NGS and tested with specific primers were found with the specific markers but not the opposite. On the contrary, the use of NGS in this case helped with the detection of multiple invasive species (Table 3), as other projects (Rius *et al.* 2015; Borrell *et al.* 2017), but failed in some cases with the identification of exotic species previously detected by specific primers in the same sample. Kelly *et al.* (2014) already described this situation; they only were able to identify the turtle (*Chelonia mydas*) using specific markers because the NGS failed to do so. The false negative results in NGS could be due to the primer bias; the primers used in NGS can have different affinity with respect to the variety of species present in the samples (Deagle *et al.* 2014). The COI gene primers amplified higher proportion of arthropods and porifera species, similar to the studies done by Leray *et al.* (2013) and Deiner *et al.* (2016). Preference of the primers could explain the detection by NGS of *P. antipodarum* in L8 but not the detection of *C. fluminea* (Table 3). Also the absence of the two molluscs in sample R8 (Table 3) could be explain by a high number of protista sequences in the sample (56%) (Figure 2B). Another reason for NGS failure could be the data processing (Thomsen and Willerslev 2015), R8 is the sample with less assigned sequences, only the 2.6% of the raw data (Table S4), while the rest of the samples is about 12% of the raw data (Table S4), similar to other NGS studies (Deiner *et al.* 2016). Better bioinformatics tools could provide better assignment to the samples, but also better reference database is needed since the main limitation to assign the sequences is the presence of the species in the database (Comtet *et al.* 2015; Goldberg et al. 2016). False negatives can also result from failures in the sequencing process (Kelly *et al.* 2014; Thomsen and Willerslev 2015), failures in the PCR conditions (Ushio *et al.* 2017, Pochon *et al.* 2013) or even in the amount of DNA released by the different species of the environment (Minamoto *et al.* 2017). The use of several samples to do NGS and the use of several genes is highly recommended to avoid the errors mentioned (Kelly *et al.* 2014; Shaw *et al.* 2016).

In the case of R0, none exotic species was detected by any of the two methods used. It is the sample with the lowest number of exotic species and the lower proportion of non-indigenous fish and mollusc species (Table 3, Table 4). This absence of exotic species in R0 could be caused by the absence of NIS in this region or by NGS failure as explained before, since 81.3% of the sequences found in R0 are arthropods (Figure 2B). Perhaps the absence of NIS is influenced by the impassable dam downstream. The dam may prevent the arrival of exotic species as many authors described (Fausch *et al.* 2006; McLaughlin *et al.* 2007), as an example Dana *et al.* (2011) stopped the expansion of the invasive crayfish *Procambarus clarkii* in a Mediterranean stream by constructing small dams, but at the same time it could block the migration route of diadromous species and can cause the decrease of diversity and abundance upstream (Nislow *et al.* 2011; Limburg and Waldman 2009; Britton *et al.* 2011), although it can work as a refuge for imperilled native species (Beatty *et al.* 2017). In this case study was the only sampling point where the native Brown trout (*Salmo trutta*) was found (Table S5) but also with low biodiversity index (Shanon index 2.059) (Table 4). Furthermore this region is the alpine part of the river with less big urban areas and invasive species tend to accumulate in degraded areas near human population (Havel *et al.* 2015; Johnson *et al.* 2008; Spear *et al.* 2013). This last assumption may be happening in L5, which is the other sample with the lowest number of exotic species detected (Table 3) and in L4 which is the other sample with the lowest percentage of non-indigenous fish and mollusc species (Table 4). They are a natural protected areas and less degraded by human effects. At the same time natural resistance may impede the establishment of exotic species as shown in other aquatic systems (Stachowicz *et al.* 1999; Miralles *et al.* 2016).

The areas with the highest number of exotic species are L3, L8 and R6 (Table 3), and the samples with higher proportion of exotic fish and mollusc species are R6 and R8 (Table 4). L3 and L8 are both port areas, where ships may facilitate the spread of exotic species and the higher temperature due to boat transport can aid in the survival of these exotics (Strayer 2010; Gollasch and Nehring 2006). In fact the daily ferries crossing the lake from Constance to Meersburg (L3 to L7) and from Romanshorn to Friedrichshafen (L1 to L8) in the Upper Lake Constance (Gergs and Rothhaupt 2015) may contribute to this transport. In spite of the presence of barriers in the High Rhine region (12 hydropower dams and a 23meter waterfall) many species have colonized the Constance Lake by unknown routes (Eckmann *et al.* 2008). Including *Dreissena polymorpha*, *Craspedacusta sowerbyi*, *Corbicula fluminea, Potamopyrgus antipodarum*... (Table 3). Human recreational activities can aid dispersion of invasive species, for example all the in-stream barriers in the High Rhine region are

crossed by recreational boats that could work as a transport for exotic species, such as round gobies (N'Guyen *et al.* 2016) as well as for exotic invertebrates attached to the boat hull or in bilge water to other water bodies in the region (Ricciardi 2015; De Ventura *et al.* 2016). In the case of the presence of *Physella acuta* and *C. fluminea* in the lake the possible origin is through aquarium releases (Schmidlin and Baur 2007).

Regarding the downstream river areas, R6 and R8, both with the highest number of exotic species and the highest percentage of non-indigenous fish and mollusc species respectively (Table 3, Table 4); they are surrounded by hydropower plant dams which may confer a refuge for exotic species, and by big urban areas. Precisely, the presence of these dams altered water temperatures and flow regimes in High Rhine and generated the perfect environment for the invasive goby *Neogobius melanostomus* (Kalchhauser *et al.* 2013).

Something to consider is that all the analysis done are based on eDNA, which in some cases can cause false positives originated from exogenous eDNA, from aquarium releases, avian feces… (Kelly *et al.* 2014; Miya *et al.* 2015; Merkes *et al.* 2014), from contamination in the laboratory (Thomsen and Willerslev 2015) and importantly to know is that it is not necessarily detecting living animals (Comtet *et al.* 2015). To overcome this problem it is necessary to replicate the analysis in different seasons and obviously, all the eDNA analysis should be followed by an exhaustive search using traditional methods to confirm the presence of the species in the region (Comtet *et al.* 2015). In our case all the exotic species found have been previously reported in the region (Leuven *et al.* 2009; Bernauer and Jansen 2006; Gergs and Rothhaupt 2015; Aquatic neozoen im Bodensee webpage). On the other hand, eDNA techniques can lead to false negatives results. These can derived from the different life cycle of the target species (Dostine *et al.* 2013; de Souza *et al.* 2016), when a species is scarce and its DNA is in low concentration (Ficetola *et al.* 2008), because of the DNA degradation (Barnes et al. 2014) or due to the presence of inhibitors (Goldberg *et al.* 2015). In this case a good laboratory practices were followed including a test for inhibitors in the eDNA samples, and several replicates. The use of replicates, both temporal and spatial (from the same sampling point) is highly recommendable to avoid this kind of false results (Ficetola *et al.* 2015). In any case eDNA methodologies are cheaper, easier and in some cases more sensitive than traditional survey (Davy *et al.* 2015; Evans *et al.* 2017), and should be considered an useful extra tool to establish the biodiversity in the region without taking the place of traditional methods.

As a conclusion, first of all the specific primers were more sensible than NGS analysis regarding the species analyzed and emphasizing their utility in establishing the invasive pattern of the species. The ports and near big urban areas were identified as the hotspots of NIS in the region, therefore better management measures should take into account the surveillance of these areas to avoid the spread of the invasive species already established in the region, and also the surveillance of recreational boats would be advisable since they could spread these NIS to other water bodies nearby, especially in this region with high volume of tourists in summer who visit multiples lakes over short periods of time. In Germany most of the non native species introduction came with intentional releases, apart from the new channelling waterways. Prevention measures have to be focus on human behaviour; educational efforts should reduce intentional releases. On the other hand stricter regulations of ornamental species and aquaculture should be improved, in order to reduce contamination of stocks and pet releases. Any garden pond or aquarium is a potential threat especially when global warming is causing the increase of winter water temperatures which would promote the establishment of ornamental species. Moreover establish a common regulation and management actions by all the countries implied in the region is highly recommendable.

Acknowledgements

Data accessibility

Specific primers used are available in Table S1.
Primers used in the NGS analysis are available in Table S2.
eDNA sequences obtained with specific primers are available in Table S3.
Filtered sequences in NGS process are available in Table S4.
OTU table from NGS analysis is available in Table S5.

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Table S1. Specific primers used**.** Sequences and PCR conditions for each specific primer set used in this study are shown.

Species	Primer	Sequence (5'-3')	Annealing Temperature	[Mg²⁺]	Product size	Reference
Ameiurus sp	Am-16S-F	CGTCAAGAACYCAGTTRAACT	65°C	1mM	134bp	Clusa *et al.* 2018
	Am-16S-R	GWTTCTGYGACTTAGAGTTGTCA				
Gambusia sp	Ga-16S-F	GRAACCAACTGACCCCTGCTT	68°C	1mM	117bp	
	Ga-16S-R	GTTTTGTGAGCTGCGGCTCTWTA				
Lepomis gibbosus	LeGi-16S-F	GGACACGGGGCTAAACCAAAT	68°C	1mM	113bp	
	LeGi-16S-R	GGGCTCTTAGTTGTGGAATTGCA				
Micropterus salmoides	MiSa-16S-F	WCATCCCRAAACAAAGGGCY	68°C	2mM	142bp	
	MiSa-16S-R	AATTCTGTTCATTAGAGCGGAGG				
Pseudorasbora parva	PsPa-16S-F	GTTTAAYCATGTTAAACAACTTAT	58°C	2,5mM	192bp	
	PsPa-16S-R	TTCGTTGATCGACTATGTGT				
Gasterosteus aculeatus	GaacCBL	ACGCCACCTTAACACGTTTC	62°C	1mM	101bp	Thomsen *et al.* 2012
	GaacCBR	AGAGCCTGTCTGGTGAAGGA				
Corbicula fluminea	CoFl-16S-F	GAATAACTTAAATGTAGGT	55°C	2mM	165bp	Clusa *et al.* 2017
	CoFl-16S-R	AGCAAACTTCTTCTTAAATAT				
Melanoides tuberculata	MeTu-16S-F	GGTCTRACGAAAGCAATACT	58°C	2mM	230bp	
	MeTu-16S-R	GCTTTGCTKGATCTAAAYYT				
Mytilopsis leucophaeata	MyLe-COI-F	GGTTGTAACAACGCACGGTTTAG	66°C	1 mM	193 bp	
	MyLe-COI-R	CACCTTCTCTGAAAGCCGAGC				
Sinanodonta woodiana	SiWo-COI-F	GGGTCAGCCMGGRAGGCTTTTA	68°C	0.5 mM	258 bp	
	SiWo-COI-R	TGTTCACCCTGTACCAACRCCC				
Potamopyrgus sp	16SAr	CGCCTGTTTATCAAAAACAT	60°C	2,5mM	380bp	Clusa *et al.* 2016
	16SPA-R	TCAAAGATTTTGGATCATAGCT				

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Table S2. NGS primers.

1st PCR

Fwd primer name:	Pad	5xN	region specific part	Full primer sequence
1st_PCR_COI_for	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	NNNNN	TANACYTCNGGRTGNCCRAARAAYCA	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNNNNNTANACYTCNGGRTGNCCRAARAAYCA
1st_PCR_COI_rev	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	NNNNN	GGWACWGGWTGAACWGGTWTAYCCYCC	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGNNNNNGGWACWGGWTGAACWGGTWTAYCCYCC

2nd PCR

Fwd primer name:	Adapter	index	Pad	Full Fwd primer sequence (Adapter+Index+Pad+Link+Primer)
NGS_i5_S502	AATGATACGGCGACCACCGAGATCTACAC	CTCTCTAT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGTC
NGS_i5_S503	AATGATACGGCGACCACCGAGATCTACAC	TATCCTCT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGTC
NGS_i5_S505	AATGATACGGCGACCACCGAGATCTACAC	GTAAGGAG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGTC
NGS_i5_S506	AATGATACGGCGACCACCGAGATCTACAC	ACTGCATA	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGTC
NGS_i5_S507	AATGATACGGCGACCACCGAGATCTACAC	AAGGAGTA	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGT
NGS_i5_S508	AATGATACGGCGACCACCGAGATCTACAC	CTAAGCCT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACCTAAGCCTTCGTCGGCAGCGTC
NGS_i5_S510	AATGATACGGCGACCACCGAGATCTACAC	CGTCTACT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACCGTCTAATTCGTCGGCAGCGTC
NGS_i5_S511	AATGATACGGCGACCACCGAGATCTACAC	TCTCTCCG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTCTCTCCGTCGTCGGCAGCGTC
NGS_i5_S513	AATGATACGGCGACCACCGAGATCTACAC	TCGACTAG	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTCGACTAGTCGTCGGCAGCGTC
NGS_i5_S515	AATGATACGGCGACCACCGAGATCTACAC	TTCTAGCT	TCGTCGGCAGCGTC	AATGATACGGCGACCACCGAGATCTACACTTCTAGCTTCGTCGGCAGCGTC

Reverse primer name:	Adapter	index	Pad	Full Fwd primer sequence (Adapter+Index+Pad+Link+Primer)
NGS_i7_N701	CAAGCAGAAGACGGCATACGAGAT	TCGCCTTA	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGG
NGS_i7_N702	CAAGCAGAAGACGGCATACGAGAT	CTAGTACG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGG
NGS_i7_N703	CAAGCAGAAGACGGCATACGAGAT	TTCTGCCT	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGG
NGS_i7_N704	CAAGCAGAAGACGGCATACGAGAT	GCTCAGGA	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGG

Reverse primer name:	Adapter	index	Pad	Full Fwd primer sequence (Adapter+Index+Pad+Link+Primer)
NGS_i7_N705	CAAGCAGAAGACGGCATACGAGAT	AGGAGTCC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGG
NGS_i7_N706	CAAGCAGAAGACGGCATACGAGAT	CATGCCTA	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGG
NGS_i7_N707	CAAGCAGAAGACGGCATACGAGAT	GTAGAGAG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGG
NGS_i7_N710	CAAGCAGAAGACGGCATACGAGAT	CAGCCTCG	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGG
NGS_i7_N711	CAAGCAGAAGACGGCATACGAGAT	TGCCTCTT	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGG
NGS_i7_N712	CAAGCAGAAGACGGCATACGAGAT	TCCTCTAC	GTCTCGTGGGCTCGG	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGG

Table S3. Sequences of the amplicons obtained using species-specific primers from water samples.

Sample	Species	Source	Forward primer name	Forward primer sequence (5'-3')	Reverse primer name	Reverse primer sequence (5'-3')	SEQUENCE (5'-3')	Query cover (%)	Ident (%)	BlastN (Best Match)
L1_CF	*Corbicula fluminea*	L1_eDNA	CoFl-16S-F	GAATAACTTAAATGTAGGT	CoFl-16S-R	AGCAAACTTCTTCTTAAATAT	TTATTGGGCAATAGAAATGAAATGAATCATTTTTTTATTATATAAGGATCCAGTTTTGACTGAAAAAAGCAAAAGCTACCGCGGGGATAACAGGGTAATTTTTTCTGAGAGTTCATATTTAAGAAGAAGTTTGCT	100	100	KY426904.1
L3_CF	*Corbicula fluminea*	L3_eDNA	CoFl-16S-F	GAATAACTTAAATGTAGGT	CoFl-16S-R	AGCAAACTTCTTCTTAAATAT	GAAAATGAAATGAATCATTTTTTATTATATAAGGATCCAGTTTGACTGAAAAAGCAAAAGCTACCGCGGGGATAACAGGGTAATTTTTTCTGAGAGTTCATATTTAAGAAGAAGTTTGCT	100	100	LC310741.1
L6_CF	*Corbicula fluminea*	L6_eDNA	CoFl-16S-F	GAATAACTTAAATGTAGGT	CoFl-16S-R	AGCAAACTTCTTCTTAAATAT	TTATTGGGCAATAGAAATGAAATGAATCATTTTTTTATTATATAAGGATCCAGTTTGACTGAAAAAAGCAAAAGCTACCGCGGGGATAACAGGGTAATTTTTTCTGAGAGTTCATATTTAAGAAGAAGTTTGCT	100	100	KY426904.1
L8_CF	*Corbicula fluminea*	L8_eDNA	CoFl-16S-F	GAATAACTTAAATGTAGGT	CoFl-16S-R	AGCAAACTTCTTCTTAAATAT	AAAATGAAATGAATCATTTTTTTATTATATAAGGATCCAGTTTGACTGAAAAAGCAAAAGCTACCGCGGGGATAACAGGGTAATTTTTTCTGAGAGTTCATATTTAAGAAGAAGTTTGCT	100	100	LC310741.1
R6_CF	*Corbicula fluminea*	R6_eDNA	CoFl-16S-F	GAATAACTTAAATGTAGGT	CoFl-16S-R	AGCAAACTTCTTCTTAAATAT	TGGGGCAATAGAAATGAAATGAATCATTTTTTTATTATAAGGATCCAGTTTGACTGAAAAAGCAAAAGCTACCGCGGGGATAACAGGGTAATTTTTTCTGAGAGTTCATATTTAAGAAGAAGTTTGCT	100	100	LC310741.1
R7_CF	*Corbicula fluminea*	R7_eDNA	CoFl-16S-F	GAATAACTTAAATGTAGGT	CoFl-16S-R	AGCAAACTTCTTCTTAAATAT	CAATAGAAAATGAAATGAATCATTTTTTTTATTATATAAGGATCCAGTTTGACTGAAAAAAGCAAAAGCTACCGCGGGGATAACAGGGTAATTTTTTCTGAGAGTTCATATTTAAGAAGAAGTTTGCT	100	100	LC310741.1
R8_CF	*Corbicula fluminea*	R8_eDNA	CoFl-16S-F	GAATAACTTAAATGTAGGT	CoFl-16S-R	AGCAAACTTCTTCTTAAATAT	AAAATGAAATGAATCATTTTTTTTATTATATAAGGATCCAGTTTGACTGAAAAAAGCAAAAGCTACCGCGGGGATAACAGGGTAATTTTTTCTGAGAGTTCATATTTAAGAAGAAGTTTGCT	100	100	LC310741.1
L1_PA	*Potamopyrgus antipodarum*	L1_eDNA	16SAr	CGCCTGTTTATCAAAAACAT	16SPA-R	TCAAAGATTTTGGATCATAGCT	CCTGCCCAGTGATATATATTTAACGGCCGCGGTACTCTGACCGTGCAAAGGTAGCATAATCATTTGCCTTATAATTGAAGGCTAGTATGAATGGTTTGACGAAAACAATACTGTCTCTCTAATTTTATAGAACTTGATTTTAGGTGAAGAAGCCTAAATAAAATTGAAAGACAAGAAGACCCTATCGAGCTTAAAAAAATTTGTTAAAATAAAATGACTATAAAAGAACATCTGTACCAAAAATTTTAGTTGGGGGACTAAGGAACATACAAAGCTTCCATCACAGTAATAAAAGGCTTTTAGCTATGATCCAAAATCTTTGA	100	100	JQ346709.1
L3_PA	*Potamopyrgus antipodarum*	L3_eDNA	16SAr	CGCCTGTTTATCAAAAACAT	16SPA-R	TCAAAGATTTTGGATCATAGCT	GCCTGCCCAGTGACATTGATATATATTTAACGGCCGCGGTACTCTGACCGTGCAAAGGTAGCATAATCATTTGCCTTATAATTGAAGGCTAGTATGAATGGTTTGACGAAAACAATACTGTCTCTCTAATTTTATAGAACTTGATTTTAGGTGAAGAAGCCTAAATAAAATTGAAAGACAAGAAGACCCTATCGAGCTTAAAAAAATTTGTTAAAATAAAATGACTATAAAAGAACATCGTACCAAAAATTTTAGTTGGGGGACTAAGGAACATACAAAGCTTCCATCACAGTAATAAAAGGCTTTTAGCTATGATCCAAAATCTTTGA	100	100	JQ346709.1
L6_PA	*Potamopyrgus antipodarum*	L6_eDNA	16SAr	CGCCTGTTTATCAAAAACAT	16SPA-R	TCAAAGATTTTGGATCATAGCT	GCCTGCCCAGTGATATATATTTAACGGCCGCGGTACTCTGACCGTGCAAAGGTAGCATAATCATTTGCCTTATAATTGAAGGCTAGTATGAATGGTTTGACGAAAACAATACTGTCTCTCTAATTTTATAGAACTTGATTTTAGGTGAAGAAGCCTAAATAAAATTGAAAGACAAGAAGACCCTATCGAGCTTAAAAAAATTTGTTAAAATAAAATGACTATAAAAGAACATCGTACCAAAAATTTTAGTTGGGGGACTAAGGAACATACAAAGCTTCCATCACAGTAATAAAAGGCTTTTAGCTATGATCCAAAATCTTTGA	100	100	JQ346709.1
L7_PA	*Potamopyrgus antipodarum*	L7_eDNA	16SAr	CGCCTGTTTATCAAAAACAT	16SPA-R	TCAAAGATTTTGGATCATAGCT	AGCCTGCCCAGTGATATATATTTAACGGCCGCGGTACTCTGACCGTGCAAAGGTAGCATAATCATTTGCCTTATAATTGAAGGCTAGTATGAATGGTTTGACGAAAACAATACATGTCTCTCTAATTTTATAGAACTTGATTTTTAGGTGAAGAAGCCTAAATAAAATTGAAAGACCAAGAAGACCCTATCGAGCTTAAAAAAATTTGTTAAAATAAAATGACTATAAAAGAACATCGTACCAAAAATTTTAGTTGGGGGACTAAGGAACATACAAAGCTTCCATCACAGTAATAAAAGGCTTTTAGCTATGATCCAAAATCTTTGA	100	100	JQ346709.1
L8_PA	*Potamopyrgus antipodarum*	L8_eDNA	16SAr	CGCCTGTTTATCAAAAACAT	16SPA-R	TCAAAGATTTTGGATCATAGCT	CCTGCCCAGTGATATATATTTAACGGCCGCGGTACTCTGACCGTGCAAAGGTAGCATAATCATTTGCCTTATAATTGAAGGCTAGTATGAATGGTTTGACGAAAACAATACTGTCTCTCTAATTTTATAGAACTTGATTTTAGGTGAAGAAGCCTAAATAAAATTGAAAGACAAGAAGACCCTATCGAGCTTAAAAAAATTTGTTAAAATAAAATGACTATAAAAGAACATCGTACCAAAAATTTTAGTTGGGGGACTAAGGAACATACAAAGCTTCCATCACAGTAATAAAAGGCTTTTAGCTATGATCCAAAATCTTTGA	100	100	JQ346709.1
R1_PA	*Potamopyrgus antipodarum*	R1_eDNA	16SAr	CGCCTGTTTATCAAAAACAT	16SPA-R	TCAAAGATTTTGGATCATAGCT	GCCTGCCCAGTGATATATATTTAACGGCCGCGGTACTCTGACCGTGCAAAGGTAGCATAATCATTTGCCTTATAATTGAAGGCTAGTATGAATGGTTTGACGAAAACAATACTGTCTCTCTAATTTTATAGAACTTGATTTTTAGGTGAAGAAGCCTAAATAAAATTGAAAGACAAGAAGACCCTATCGAGCTTAAAAAAATTTGTTAAAATAAAATGACTATAAAAGAACATCGTACCAAAAATTTTAGTTGGGGGACTAAGGAACATACAAAGCTTCCATCACAGTAATAAAAGGCTTTTAGCTATGATCCAAAATCTTTGA	100	100	JQ346709.1

Sent

Sample	Species	Source	Forward primer name	Forward primer sequence (5'-3')	Reverse primer name	Reverse primer sequence (5'-3')	SEQUENCE (5'-3')	Query cover (%)	Ident (%)	BlastN (Best Match)
R3_PA	*Potamopyrgus antipodarum*	R3_eDNA	16SAr	CGCCTGTTTAT CAAAAACAT	16SPA-R	TCAAAGATTTTG GATCATAGCT	AGTGATATATATTTAACGGCCGCGGTACTCTGACCGTGCAAAGGTAGCATAATCATTTGCCTTATATAATTG AAGGCTAGTAGTAGGCAGGAAGCCTAAATAATACTGTCTCTCTCAATTTTATAGAACTTGATTTTTAGGT GAAGAAGCCTAAATAAAATTGAAAGACAAGAAGACACCCTATCGAGCTTAAAAAAATTTTGTTAAAAATAAA ATGACTATAAAAGAACATCTGTACCAAAAAATTTTAGTTGGGGGACTAAGGAACATACAAAGCTTCCAT CACAGTAATAAAAGGCTTTTTAGCTATGATCCAAAATCTTTGA	100	100	KU93009.1
R4_PA	*Potamopyrgus antipodarum*	R4_eDNA	16SAr	CGCCTGTTTAT CAAAAACAT	16SPA-R	TCAAAGATTTTG GATCATAGCT	GCCTGCCCAGTGATATATATTTAACGGCCGCGGTACTCTGACCGTGCAAAGGTAGCATAATCATTTGCCTT ATAATTGAAGGCTAGTAGTATGAAATGGTTTGACGAAAACAATACTGTCTCTCTCAATTTTATAGAACTTGATT TTTAGGTGAAGAAGCCTAAATAAAATTGAAAGACAAGAAGACCCTATCGAGCTTAAAAAAAATTTTGTTA AAATAAAAATGACTATAAAAGAACATCTGTACCAAAAAATTTTAGTTGGGGGACTAAGGAACATACAAA GCTTCCATCACAGTAATAAAAGGCTTTTTAGCTATGATCCAAAATCTTTGA	100	100	JQ346709.1
R5_PA	*Potamopyrgus antipodarum*	R5_eDNA	16SAr	CGCCTGTTTAT CAAAAACAT	16SPA-R	TCAAAGATTTTG GATCATAGCT	AGCCTGCCCAGTGATATATATTTAACGGCCGCGGTACTCTGACCGTGCAAAGGTAGCATAATCATTTGCC TTATAATTGAAGGCTAGTAGTATGAAATGGTTTGACGAAAACAATACTGTCTCTCTCAATTTTATAGAACTTGA TTTTTAGGTGAAGAAGCCTAAATAAAATTGAAAGACAAGAAGACCCTATCGAGCTTAAAAAAATTTTGTT AAAATAAAAATGACTATAAAAGAACATCTGTACCAAAAAATTTTAGTTGGGGGACTAAGGAACATACAA AGCTTCCATCACAGTAATAAAAGGCTTTTTAGCTATGATCCAAAATCTTTGA	100	100	JQ346709.1
R6_PA	*Potamopyrgus antipodarum*	R6_eDNA	16SAr	CGCCTGTTTAT CAAAAACAT	16SPA-R	TCAAAGATTTTG GATCATAGCT	GCCCAGTGATATATATTTAACGGCCGCGGTACTCTGACCGTGCAAAGGTAGCATAATCATTTGCCTTATA ATTGAAGGCTAGTAGTATGAAATGGTTTGACGAAAACAATACTGTCTCTCTCAATTTTATAGAACTTGATTTTT AGGTGAAGAAGCCTAAATAAAATTGAAAGACAAGAAGACCCTATCGAGCTTAAAAAAATTTTGTTAAAA TAAAATGACTATAAAAGAACATCTGTACCAAAAAATTTTAGTTGGGGGACTAAGGAACATACAAAGCT TCCATCACAGTAATAAAAGGCTTTTTAGCTATGATCCAAAATCTTTGA	100	100	JQ346709.1
R8_PA	*Potamopyrgus antipodarum*	R8_eDNA	16SAr	CGCCTGTTTAT CAAAAACAT	16SPA-R	TCAAAGATTTTG GATCATAGCT	TCGAGCCTGCCCAGTGATATATATTTAACGGCCGCGGTACTCTGACCGTGCAAAGGTAGCATAATCATTT GCCTTATATTGAAGGCTAGTAGTATGAAATGGTTTGACGAAAACAATACTGTCTCTCTCAATTTTATAGAACT TGATTTTTAGGTGAAGAAGCCTAAATAAAATTGAAAGACAAGAAGACCCTATCGAGCTTAAAAAAATTTT GTTAAAATAAAAATGACTATAAAAGAACATCTGTACCAAAAAATTTTAGTTGGGGGACTAAGGAACACATA CAAAGCTTCCATCACAGTAATAAAAGGCTTTTTAGCTATGATC	100	100	JQ346709.1
L3_GA	*Gasterosteus aculeatus*	L3_eDNA	GaacCBL	ACGCCACCTTA ACACGTTTC	GaacCBR	AGAGCCTGTCT GGTGAAGGA	TTTGTCATTGCTGGTGCCACACTTGTTCACCTTCTTTTCCTTCACCAGACAGGCTCTA	100	100	KX910761.1
L4_GA	*Gasterosteus aculeatus*	L4_eDNA	GaacCBL	ACGCCACCTTA ACACGTTTC	GaacCBR	AGAGCCTGTCT GGTGAAGGA	CCATTTGTCATTGCTGGTGCCACACTTGTTCACCTTCTTTTCCTTCACCAGACAGGCTCTA	100	100	KX910761.1
L5_GA	*Gasterosteus aculeatus*	L5_eDNA	GaacCBL	ACGCCACCTTA ACACGTTTC	GaacCBR	AGAGCCTGTCT GGTGAAGGA	TTGCTGGTGCCACACTTGTTCACCTTCTTTTCCTTCACCAGACAGGCTCTA	100	100	KX910761.1
L7_GA	*Gasterosteus aculeatus*	L7_eDNA	GaacCBL	ACGCCACCTTA ACACGTTTC	GaacCBR	AGAGCCTGTCT GGTGAAGGA	ACTTGTTCACCTTCTTTTCCTTCACCAGACAGGCTCTA	100	100	KX910761.1
L8_GA	*Gasterosteus aculeatus*	L8_eDNA	GaacCBL	ACGCCACCTTA ACACGTTTC	GaacCBR	AGAGCCTGTCT GGTGAAGGA	CCATTTGTCATTGCTGGTGCCACACTTGTTCACCTTCTTTTCCTTCACCAGACAGGCTCTA	100	100	KX910761.1
R1_GA	*Gasterosteus aculeatus*	R1_eDNA	GaacCBL	ACGCCACCTTA ACACGTTTC	GaacCBR	AGAGCCTGTCT GGTGAAGGA	CCATTTGTCATTGCTGGTGCCACACTTGTTCACCTTCTTTTCCTTCACCAGACAGGCTCTA	100	100	KX910761.1
R5_GA	*Gasterosteus aculeatus*	R5_eDNA	GaacCBL	ACGCCACCTTA ACACGTTTC	GaacCBR	AGAGCCTGTCT GGTGAAGGA	CCATTTGTCATTGCTGGTGCCACACTTGTTCACCTTCTTTTCCTTCACCAGACAGGCTCTA	100	100	KX910761.1
R8_GA	*Gasterosteus aculeatus*	R8_eDNA	GaacCBL	ACGCCACCTTA ACACGTTTC	GaacCBR	AGAGCCTGTCT GGTGAAGGA	CCATTTGTCATTGCTGGTGCCACACTTGTTCACCTTCTTTTCCTTCACCAGACAGGCTCTA	100	100	KX910761.1

Table S4. Filtered sequences in NGS process. Number of sequences filtered in each step of the process and percentage of sequences remained from raw data are shown.

	Raw	Merge		PrinSeq			Assigned (97%, e 1-50)				After manual filtration (Remove of sequences assigned to invertebrate environmental sample)				
	Sequences	Sequences	% of remained	Sequences	% of remained vs raw	% of remained vs merge	Sequences	% of remained vs raw	% of remained vs merge	% of remained vs PrinSeq	Sequences	% of remained vs raw	% of remained vs merge	% of remained vs PrinSeq	% of remained vs assigned
L3	655481	519569	79,27	496625	75,76	95,58	92886	14,17	17,88	18,70	92457	14,11	17,79	18,62	99,54
L4	724988	482761	66,59	481309	66,39	99,70	110744	15,28	22,94	23,01	109514	15,11	22,68	22,75	98,89
L5	669523	528983	79,01	528559	78,95	99,92	194819	29,10	36,83	36,86	194584	29,06	36,78	36,81	99,88
L8	629312	436992	69,44	436748	69,40	99,94	120587	19,16	27,59	27,61	120144	19,09	27,49	27,51	99,63
R0	679386	604367	88,96	601918	88,60	99,59	54428	8,01	9,01	9,04	53409	7,86	8,84	8,87	98,13
R1	661891	534745	80,79	533911	80,66	99,84	42786	6,46	8,00	8,01	40746	6,16	7,62	7,63	95,23
R4	568708	498263	87,61	498059	87,58	99,96	62604	11,01	12,56	12,57	62076	10,92	12,46	12,46	99,16
R5	681981	625343	91,70	624010	91,50	99,79	93914	13,77	15,02	15,05	89439	13,11	14,30	14,33	95,24
R6	605877	445397	73,51	444903	73,43	99,89	43140	7,12	9,69	9,70	39390	6,50	8,84	8,85	91,31
R8	597330	333067	55,76	333066	55,76	100,00	15328	2,57	4,60	4,60	10397	1,74	3,12	3,12	67,83
Mean			77,26		76,80	99,42		12,66	16,41	16,52		12,37	15,99	16,10	94,48
Sd			11,17		11,10	1,35		7,59	10,08	10,10		7,77	10,36	10,39	9,76

Table S5. OTU table from NGS analysis. Species in red ink are invasive to the region based on EASIN webpage (https://easin.jrc.ec.europa.eu/), DAISIE webpage (http://www.europe-aliens.org/), Aquatic alien species in German inland and coastal waters webpage (http://aquatic-aliens.de/), Nehring *et al.* (2010), Wittenberg *et al.* (2005) and Aquatic neozoen im Bodensee webpage (http://www.neozoen-bodensee.de/).

#Full OTU Counts

#OTU ID	L3	L4	L5	L8	R0	R1	R4	R5	R6	R8	Philo	Class	Order	Family	Genus	Species
											| Consensus Lineage					
KU894119.1	0	0	0	0	65	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Enchytraeidae	Lumbricillus	*Lumbricillus rivalis*
KP420564.1	0	0	0	0	0	0	341	0	0	0	Annelida	Clitellata	Haplotaxida	Lumbricidae	Dendrodrilus	*Dendrodrilus rubidus*
KY284242.1	0	0	0	0	213	10	0	0	0	0	Annelida	Clitellata	Haplotaxida	Lumbricidae	Eiseniella	*Eiseniella tetraedra*
JQ519897.1	0	0	0	0	110	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Chaetogaster	*Chaetogaster diaphanus*
GQ355367.1	0	0	0	0	50	0	0	723	0	338	Annelida	Clitellata	Haplotaxida	Tubificidae	Chaetogaster	*Chaetogaster diastrophus*
KY369481.1	175	0	0	3	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Limnodrilus	*Limnodrilus hoffmeisteri*
LN999148.1	85	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	NA	*Tubificinae sp. 1 RV-2016*
GU902104.1	0	0	0	0	13	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais alpina*
JQ519864.1	0	0	103	0	93	476	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais barbata*
LN810267.1	0	163	0	0	92	0	0	14517	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais bretscheri*
JQ519837.1	0	0	0	0	197	0	0	3	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais christinae*
JQ519873.1	0	0	0	0	0	267	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais communis/variabilis complex sp. A1*
JQ519832.1	0	0	0	0	81	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais communis/variabilis complex sp. A2*
JQ519875.1	0	0	0	0	87	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais communis/variabilis complex sp. A3*
JQ519853.1	0	0	0	0	118	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais elinguis*
JQ519894.1	0	0	0	0	45	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais stolci*
LN810257.1	0	0	70	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Ophidonais	*Ophidonais serpentina*
KF366633.1	447	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Potamothrix	*Potamothrix moldaviensis*
AF534862.1	242	0	0	229	0	0	1497	237	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Stylaria	*Stylaria lacustris*
KF366638.1	0	0	0	0	90	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Tubifex	*Tubifex tubifex*
KT924117.1	0	0	0	0	0	0	1	0	0	0	Annelida	Clitellata	Lumbriculida	Sparganophilidae	Sparganophilus	*Sparganophilus tamesis*
KY331586.1	0	0	0	0	0	0	258	0	0	0	Annelida	Clitellata	NA	NA	NA	*Oligochaeta sp. DC-2016*
EU719117.1	8501	0	0	0	0	0	0	0	0	0	Arthropoda	Branchiopoda	Diplostraca	Chydoridae	Paralona	*Paralona pigra*
JF821190.1	0	0	0	217	0	0	0	0	0	0	Arthropoda	Branchiopoda	Diplostraca	Daphniidae	Daphnia	*Daphnia cucullata*
JF821193.1	0	0	0	0	71	0	0	0	0	0	Arthropoda	Branchiopoda	Diplostraca	Daphniidae	Daphnia	*Daphnia longispina*
EF189666.1	0	0	0	50650	0	0	0	0	0	0	Arthropoda	Branchiopoda	Diplostraca	Sididae	Diaphanosoma	*Diaphanosoma brachyurum*

#OTU ID	L3	L4	L5	L8	R0	R1	R4	R5	R6	R8	Philo	Class	Order	Family	Genus	Species
DQ310658.1	0	0	0	227	0	0	0	0	0	0	Arthropoda	Branchiopoda	Diplostraca	Sididae	Diaphanosoma	*Diaphanosoma sp. JRdW-2005*
KR921646.1	0	0	0	0	6	0	0	0	0	0	Arthropoda	Insecta	Diptera	Calliphoridae	Lucilia	*Lucilia cuprina*
HQ824523.1	0	0	0	0	0	0	0	0	927	348	Arthropoda	Insecta	Diptera	Ceratopogonidae	Forcipomyia	*Forcipomyia sp. CW-2011*
JN016822.1	0	1	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Chironomus	*Chironomus curabilis*
DQ910573.1	0	565	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Chironomus	*Chironomus nuditarsis*
KC250765.1	220	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cladotanytarsus	*Cladotanytarsus mancus*
KY837605.1	0	0	0	154	0	0	43	244	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus bicinctus*
KT609027.1	0	0	0	0	0	0	0	719	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus triannulatus*
LN897621.1	0	0	0	0	67	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa cinerella/tonsa group sp. 1 MM-2015*
LN897666.1	0	0	0	0	5	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa latitarsis*
LN897655.1	0	0	0	0	359	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa tonsa*
KR626558.1	0	0	0	0	59	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Micropsectra	*Micropsectra logani*
AM398706.1	0	0	0	0	0	0	0	0	0	357	Arthropoda	Insecta	Diptera	Chironomidae	Micropsectra	*Micropsectra notescens*
GU073175.1	0	0	0	0	62	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Micropsectra	*Micropsectra radialis*
KU374403.1	0	0	0	0	100	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius frigidus*
HQ105229.1	0	0	0	0	0	518	264	896	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius fuscimanus*
KR632335.1	0	0	0	0	0	169	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius oblidens*
KR759879.1	0	0	0	0	388	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius rivulorum*
LN897586.1	0	0	0	0	6917	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius sp. 1 MM-2015*
KC250823.1	0	187	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Paratanytarsus	*Paratanytarsus dissimilis*
HQ105361.1	0	0	0	0	0	0	0	0	323	0	Arthropoda	Insecta	Diptera	Chironomidae	Tanytarsus	*Tanytarsus heusdensis*
KT248932.1	0	0	0	0	0	0	0	269	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Tvetenia	*Tvetenia calvescens*
KP730850.1	0	0	0	0	0	126	0	0	0	0	Arthropoda	Insecta	Diptera	Drosophilidae	Drosophila	*Drosophila immigrans*
KJ163370.1	0	0	0	0	0	617	0	0	0	0	Arthropoda	Insecta	Diptera	Psychodidae	Psychoda	*Psychoda alternata*
HE577313.1	0	0	0	0	0	9	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium (Hellichiella) sp. IBE-GS5*
KP861022.1	0	0	0	0	0	226	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium intermedium*
KX673597.1	0	0	0	0	0	319	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium kiritshenkoi*
GU203469.1	0	0	0	0	0	0	5	274	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium lineatum*
KP861031.1	0	0	0	0	0	184	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium ornatum*
KP861040.1	0	0	0	0	0	302	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium trifasciatum*
KP861017.1	0	0	0	0	63	0	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium variegatum*

Sent

#OTU ID	L3	L4	L5	L8	R0	R1	R4	R5	R6	R8	Philo	Class	Order	Family	Genus	Species
KR668323.1	0	0	0	0	0	128	0	0	0	0	Arthropoda	Insecta	Diptera	Syrphidae	Syrphus	*Syrphus ribesii*
KR430871.1	0	0	0	0	0	398	0	0	0	0	Arthropoda	Insecta	Diptera	Syrphidae	Syrphus	*Syrphus vitripennis*
LT626102.1	0	0	0	0	0	125	0	0	0	0	Arthropoda	Insecta	Ephemeroptera	Baetidae	Baetis	*Baetis buceratus*
GU812339.1	0	0	0	0	35233	0	0	0	0	0	Arthropoda	Insecta	Ephemeroptera	Baetidae	Baetis	*Baetis rhodani*
LN734709.1	0	0	0	0	0	0	242	273	0	0	Arthropoda	Insecta	Ephemeroptera	Caenidae	Caenis	*Caenis pusilla*
KY306872.1	0	0	0	0	0	222	0	0	0	0	Arthropoda	Insecta	Hemiptera	Aphididae	Euceraphis	*Euceraphis betulae*
KR581272.1	0	0	0	0	0	223	0	0	0	0	Arthropoda	Insecta	Hemiptera	Aphididae	Periphyllus	*Periphyllus testudinaceus*
KF639623.1	0	0	0	0	0	139	0	0	0	0	Arthropoda	Insecta	Hemiptera	Aphididae	Rhopalosiphum	*Rhopalosiphum insertum*
KR567973.1	0	0	0	0	0	1	0	0	0	0	Arthropoda	Insecta	Hemiptera	Aphididae	Rhopalosiphum	*Rhopalosiphum sp. BOLD:AB26546*
JQ736346.1	0	0	0	0	58	0	0	0	0	0	Arthropoda	Insecta	Plecoptera	Nemouridae	Protonemura	*Protonemura nimborella*
KR143613.1	0	0	0	0	0	1	0	0	0	0	Arthropoda	Insecta	Psocoptera	Caeciliusidae	NA	*Caeciliusidae sp. BOLD:AAN8447*
KR380641.1	0	0	0	0	0	289	0	0	0	0	Arthropoda	Insecta	Psocoptera	Caeciliusidae	Valenzuela	*Valenzuela flavidus*
KX295519.1	0	0	0	0	0	0	554	472	191	111	Arthropoda	Insecta	Trichoptera	Hydropsychidae	Hydropsyche	*Hydropsyche incognita*
KX141395.1	0	0	0	0	0	0	0	0	1	0	Arthropoda	Insecta	Trichoptera	Hydropsychidae	Hydropsyche	*Hydropsyche sp. BIOUG16210-D02*
KX140791.1	0	0	0	0	0	0	1	2	2	1	Arthropoda	Insecta	Trichoptera	Hydropsychidae	Hydropsyche	*Hydropsyche sp. BIOUG17118-A04*
KX144015.1	0	0	0	0	0	0	0	1	0	0	Arthropoda	Insecta	Trichoptera	Hydropsychidae	Hydropsyche	*Hydropsyche sp. BIOUG17466-C10*
KX104695.1	0	0	0	0	0	0	0	3619	0	0	Arthropoda	Insecta	Trichoptera	Psychomyiidae	Psychomyia	*Psychomyia pusilla*
KJ019852.1	12	0	0	0	0	0	0	405	328	0	Arthropoda	Malacostraca	Amphipoda	Gammaridae	Dikerogammarus	*Dikerogammarus villosus*
KJ676735.1	0	0	106	0	0	0	0	0	0	0	Arthropoda	Malacostraca	Isopoda	Asellidae	Asellus	*Asellus aquaticus*
FJ197651.1	0	1274	0	231	0	0	0	0	0	0	Arthropoda	Malacostraca	Mysida	Mysidae	Limnomysis	*Limnomysis benedeni*
KC627287.1	0	0	0	4071	0	0	0	0	0	0	Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae	Cyclops	*Cyclops abyssorum*
KC627307.1	5347	0	0	0	0	0	0	0	0	0	Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae	Eucyclops	*Eucyclops cf. serrulatus ZISP 11SNM-532*
KC627308.1	485	0	0	0	0	0	0	0	0	0	Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae	Eucyclops	*Eucyclops cf. serrulatus ZISP 11SNM-533*
KX160820.1	26209	0	232	8321	18	0	0	103	0	0	Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae	Mesocyclops	*Mesocyclops leuckarti*
KF357726.1	0	0	43799	0	0	0	0	716	0	0	Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae	Thermocyclops	*Thermocyclops crassus*
KC275673.1	0	0	124	409	201	0	0	565	1449	2542	Arthropoda	NA	NA	NA	NA	*Arthropoda environmental sample*
FJ590523.1	0	0	0	0	0	0	0	252	0	341	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	*Cladosporium bruhnei*
FJ590522.1	0	0	0	0	0	276	0	0	335	0	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	*Cladosporium tenuissimum*
EF180155.1	0	0	0	0	0	0	0	0	250	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium bialowiezense*
FJ501229.1	0	0	0	0	0	263	0	0	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium boothii*
FJ501230.1	0	0	0	0	76	0	0	223	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium cf. avenaceum HY046-07*

#OTU ID	L3	L4	L5	L8	R0	R1	R4	R5	R6	R8	Philo	Class	Order	Family	Genus	Species
FJ501244.1	0	0	0	0	0	107	0	0	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium solani*
EF164941.2	121	0	0	0	0	0	0	0	0	0	Bacillariophyta	Bacillariophyceae	Naviculales	Sellaphoraceae	Sellaphora	*Sellaphora bacillum*
EF164929.1	0	0	0	0	1	0	0	0	0	0	Bacillariophyta	Bacillariophyceae	Naviculales	Sellaphoraceae	Sellaphora	*Sellaphora cf. minima*
HQ317100.1	0	0	0	0	46	0	0	0	0	0	Bacillariophyta	Bacillariophyceae	Naviculales	Sellaphoraceae	Sellaphora	*Sellaphora pupula*
GQ844251.1	0	0	0	0	0	264	0	177	0	0	Bacillariophyta	Coscinodiscophyceae	Thalassiosirales	Stephanodiscaceae	Cyclotella	*Cyclotella cryptica*
JN029451.1	0	0	0	0	0	274	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrophorus	*Hygrophorus agathosmus*
FJ196106.1	0	0	0	0	0	0	312	0	0	0	Bryozoa	Phylactolaemata	NA	Cristatellidae	Cristatella	*Cristatella mucedo*
FJ196107.1	0	0	0	0	0	0	0	2	0	0	Bryozoa	Phylactolaemata	NA	Lophopodidae	Lophopus	*Lophopus crystallinus*
KT716359.1	0	0	0	385	0	0	0	0	0	0	Chordata	Actinopteri	Cypriniformes	Cyprinidae	Abramis	*Abramis brama*
KM286491.1	0	0	0	0	0	0	279	50	0	0	Chordata	Actinopteri	Cypriniformes	Cyprinidae	Barbus	*Barbus barbus*
KU302619.1	0	0	0	0	0	0	0	242	0	0	Chordata	Actinopteri	Cypriniformes	Cyprinidae	Squalius	*Squalius cephalus*
HM592232.1	0	390	0	0	0	0	0	0	0	0	Chordata	Actinopteri	Esociformes	Esocidae	Esox	*Esox lucius*
KJ553084.1	0	0	0	0	0	286	0	0	0	0	Chordata	Actinopteri	Perciformes	Cottidae	Cottus	*Cottus gobio*
KM286617.1	0	0	0	0	0	1	0	0	0	0	Chordata	Actinopteri	Perciformes	Cottidae	Cottus	*Cottus microstomus*
KR862762.1	157	90	0	0	0	0	0	0	0	0	Chordata	Actinopteri	Perciformes	Gasterosteidae	Gasterosteus	*Gasterosteus aculeatus*
KR862803.1	0	2	0	0	0	0	0	0	0	0	Chordata	Actinopteri	Perciformes	Gasterosteidae	Gasterosteus	*Gasterosteus gymnurus*
KJ553493.1	1	5	0	0	0	0	0	0	0	0	Chordata	Actinopteri	Perciformes	Gasterosteidae	Gasterosteus	*Gasterosteus sp. BOLD:AAI8488*
AB034824.1	0	162	0	0	0	0	0	0	0	0	Chordata	Actinopteri	Salmoniformes	Salmonidae	Coregonus	***Coregonus lavaretus***
FJ999067.1	0	0	0	0	0	195	0	0	0	0	Chordata	Actinopteri	Salmoniformes	Salmonidae	Oncorhynchus	***Oncorhynchus mykiss***
KJ554701.1	0	0	0	0	0	0	0	0	0	0	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	*Salmo letnica*
KU933676.1	0	0	0	0	443	0	0	0	0	0	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	*Salmo trutta*
KT803613.1	0	0	0	246	0	0	0	0	0	0	Chordata	Aves	Gruiformes	Rallidae	Fulica	*Fulica atra*
AP008737.1	0	447	0	0	0	0	0	0	0	173	Chordata	Mammalia	Primates	Hominidae	Homo	*Homo sapiens*
GU722851.1	832	57	141	0	0	0	0	0	0	0	Cnidaria	Hydrozoa	Anthoathecata	Hydridae	Hydra	*Hydra circumcincta*
KP895118.1	3035	6018	112	0	136	1082	11182	16171	6760	0	Cnidaria	Hydrozoa	Anthoathecata	Hydridae	Hydra	*Hydra oligactis*
AB565092.1	0	24	0	0	0	1	0	0	112	0	Cnidaria	Hydrozoa	Anthoathecata	Hydridae	Hydra	*Hydra robusta*
GU722909.1	0	0	0	0	0	341	0	0	0	0	Cnidaria	Hydrozoa	Anthoathecata	Hydridae	Hydra	*Hydra vulgaris*
FJ423620.1	9560	727	0	0	0	0	652	879	0	0	Cnidaria	Hydrozoa	Limnomedusae	Olindiidae	Craspedacusta	***Craspedacusta sowerbyi***
KU906067.1	232	0	0	0	0	0	0	0	0	0	Mollusca	Bivalvia	Veneroida	Corbiculidae	Corbicula	***Corbicula fluminea***
AM749000.1	396	0	106	0	0	0	0	0	105	0	Mollusca	Bivalvia	Veneroida	Dreissenidae	Dreissena	***Dreissena polymorpha***

#OTU ID	L3	L4	L5	L8	R0	R1	R4	R5	R6	R8	Philo	Class	Order	Family	Genus	Species
KY012101.1	87	0	0	0	0	0	0	10834	0	0	Mollusca	Gastropoda	NA	Ancylidae	Ancylus	*Ancylus sp. C1*
AY350511.1	0	0	0	1	0	0	0	49	0	0	Mollusca	Gastropoda	NA	Ancylidae	Ancylus	*Ancylus sp. H6*
FJ160291.1	0	0	0	0	0	0	138	0	0	0	Mollusca	Gastropoda	NA	Bithyniidae	Bithynia	*Bithynia tentaculata*
KT373703.1	1414	0	0	224	0	0	676	0	0	0	Mollusca	Gastropoda	NA	Hydrobiidae	Potamopyrgus	***Potamopyrgus antipodarum***
HG932231.1	0	448	0	0	0	0	0	0	0	0	Mollusca	Gastropoda	NA	Lymnaeidae	Radix	*Radix ampla*
KF737952.1	0	0	0	442	0	0	0	0	0	0	Mollusca	Gastropoda	NA	Physidae	Physella	***Physella acuta***
KJ579388.1	0	0	0	0	64	0	0	0	0	0	NA	Chrysophyceae	Chromulinales	Dinobryaceae	Dinobryon	*Dinobryon bavaricum*
FN663974.1	0	0	0	0	133	0	0	0	0	0	NA	Chrysophyceae	Chromulinales	Dinobryaceae	Dinobryon	*Dinobryon divergens*
JQ247703.1	75	0	0	0	0	0	0	0	0	0	NA	Dinophyceae	Gonyaulacales	Amphidomataceae	Azadinium	*Azadinium caudatum*
GQ47923.1	3	0	0	376	0	0	0	0	0	0	NA	Dinophyceae	Gonyaulacales	Ceratiaceae	Ceratium	*Ceratium hirundinella*
GQ501214.1	0	1	0	0	0	0	0	0	0	0	NA	Dinophyceae	Gonyaulacales	Goniodomataceae	Gambierdiscus	*Gambierdiscus toxicus*
GQ501119.1	3480	504	0	1463	0	0	0	0	0	0	NA	Dinophyceae	Gonyaulacales	Gonyaulacaceae	Alexandrium	*Alexandrium affine*
GQ501128.1	7	3	0	0	0	0	0	0	0	0	NA	Dinophyceae	Gonyaulacales	Gonyaulacaceae	Alexandrium	*Alexandrium catenella*
GQ501157.1	1	0	0	0	0	0	0	0	0	0	NA	Dinophyceae	Gonyaulacales	Gonyaulacaceae	Alexandrium	*Alexandrium minutum*
GQ501159.1	1	0	0	1	1	0	0	0	0	0	NA	Dinophyceae	Gonyaulacales	Gonyaulacaceae	Alexandrium	*Alexandrium ostenfeldii*
GQ501173.1	0	0	0	1	0	0	0	0	0	0	NA	Dinophyceae	Gonyaulacales	Gonyaulacaceae	Alexandrium	*Alexandrium sp. CC4P1119/14*
GQ501142.1	1	0	0	0	0	0	0	0	0	0	NA	Dinophyceae	Gonyaulacales	Gonyaulacaceae	Alexandrium	*Alexandrium sp. RFS-2009a*
GQ501178.1	6	2	0	8	0	0	0	0	0	0	NA	Dinophyceae	Gonyaulacales	Gonyaulacaceae	Alexandrium	*Alexandrium tamarense*
GQ501301.1	0	1	0	0	0	0	0	0	0	0	NA	Dinophyceae	Gonyaulacales	Gonyaulacaceae	Protoceratium	*Protoceratium reticulatum*
GQ501256.1	0	15118	134	1513	0	0	0	0	0	0	NA	Dinophyceae	Gymnodiniales	Kareniaceae	Karlodinium	*Karlodinium veneficum*
GQ501539.1	0	161	1	10	0	0	0	0	0	0	NA	Dinophyceae	NA	NA	NA	*uncultured Peridinium*
GQ501269.1	0	0	0	0	0	0	305	0	1	0	NA	Dinophyceae	Peridiniales	Peridiniaceae	Peridinium	*Peridinium inconspicuum*
GQ502055.1	0	2	0	0	0	0	0	0	0	0	NA	Dinophyceae	Peridiniales	Peridiniaceae	Peridinium	*Peridinium sp. ES18-106*
GQ501195.1	1	1	0	0	0	0	0	0	0	0	NA	Dinophyceae	Peridiniales	Pfiesteriaceae	Cryptoperidiniopsis	*Cryptoperidiniopsis sp. CCMP2784*
GQ501194.1	0	0	0	0	0	0	0	0	0	2	NA	Dinophyceae	Peridiniales	Pfiesteriaceae	Cryptoperidiniopsis	*Cryptoperidiniopsis sp. CCMP2786*
GQ501198.1	385	0	0	0	36	0	0	0	452	670	NA	Dinophyceae	Peridiniales	Pfiesteriaceae	NA	*Pfiesteriaceae sp. CCMP1874*
GQ501203.1	0	0	0	0	0	0	0	0	1	0	NA	Dinophyceae	Peridiniales	Pfiesteriaceae	NA	*Pfiesteriaceae sp. CCMP1876*
GQ501199.1	0	0	0	0	0	0	0	0	3	4	NA	Dinophyceae	Peridiniales	Pfiesteriaceae	NA	*Pfiesteriaceae sp. CCMP1881*
GQ501202.1	0	0	0	0	0	0	1	0	0	0	NA	Dinophyceae	Peridiniales	Pfiesteriaceae	NA	*Pfiesteriaceae sp. CCMP1958*
GQ501400.1	0	0	0	0	0	0	220	0	651	0	NA	Dinophyceae	Peridiniales	Thoracosphaeraceae	Thoracosphaera	*Thoracosphaera heimii*
KC130152.1	0	0	0	0	230	0	0	651	0	0	NA	Florideophyceae	Acrochaetiales	Acrochaetiaceae	Audouinella	*Audouinella hermannii*

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#OTU ID	L3	L4	L5	L8	R0	R1	R4	R5	R6	R8	Philo	Class	Order	Family	Genus	Species
KC596294.1	0	0	0	0	166	4882	0	695	3900	1212	NA	Florideophyceae	Batrachospermales	Batrachospermaceae	Sheathia	*Sheathia arcuata*
JX669710.1	0	0	0	0	0	809	0	0	0	0	NA	Florideophyceae	Batrachospermales	Batrachospermaceae	Sheathia	*Sheathia boryana*
JX669692.1	0	0	0	0	0	226	0	0	0	0	NA	Florideophyceae	Batrachospermales	Batrachospermaceae	Sheathia	*Sheathia involuta*
JX669618.1	0	0	0	0	163	0	0	0	0	0	NA	Florideophyceae	Batrachospermales	Lemaneaceae	Lemanea	*Lemanea fucina*
JN604926.1	0	4	0	0	2272	11595	230	0	1495	420	NA	Florideophyceae	Batrachospermales	Lemaneaceae	Paralemanea	*Paralemanea annulata*
KX506070.1	0	0	0	0	0	186	0	0	0	0	NA	Heterolobosea	Schizopyrenida	Vahlkampfiidae	Naegleria	*Naegleria fultoni*
KX506069.1	0	0	0	0	0	0	0	0	0	442	NA	Heterolobosea	Schizopyrenida	Vahlkampfiidae	Naegleria	*Naegleria sp. 13 CF-2016*
HM187654.1	0	0	0	0	0	0	0	0	236	0	NA	NA	Euglyphida	Cyphoderiidae	Cyphoderia	*Cyphoderia ampulla*
KJ781464.1	56	480	51	556	0	72	0	275	0	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium actinophorum*
KJ781461.1	0	0	0	0	0	0	0	0	215	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium arabianum*
GQ354207.1	0	0	0	1	0	0	0	0	0	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium cf. actinophorum*
KJ569728.1	0	0	0	0	0	178	0	0	0	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium kieliense*
KJ781466.1	75	0	0	0	0	0	0	0	0	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium larifeili*
KJ569707.1	0	0	0	0	229	0	0	217	0	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium minus*
KJ569724.1	0	0	0	0	0	0	0	2901	0	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium sp. SG-2014*
LC137888.1	429	1230	111	34	818	2040	528	3910	2301	2389	NA	NA	NA	NA	NA	*invertebrate environmental sample*
KU659850.1	0	0	0	0	0	0	144	0	150	0	NA	NA	NA	Paramoebidae	Korotnevella	*Korotnevella heteracantha*
KU659820.1	0	0	0	0	243	268	0	0	0	0	NA	NA	NA	Paramoebidae	Korotnevella	*Korotnevella stella*
GQ354155.1	0	0	0	0	159	2701	0	134	5403	111	NA	NA	NA	Vannellidae	Vannella	*Vannella simplex*
HQ708209.1	0	0	0	0	0	7	0	0	1	3	NA	Oomycetes	Lagenidiales	Lagenidiaceae	Lagenidium	*Lagenidium caudatum*
HQ708199.1	0	0	0	0	0	0	0	0	0	1	NA	Oomycetes	Leptomitales	NA	Apodachlya	*Apodachlya minima*
HQ708296.1	0	0	0	0	0	486	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora gonapodyides*
KP749417.1	0	0	0	0	0	15	0	0	2	9	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora lacustris*
HM535007.1	0	0	0	0	0	6	1	0	1	1	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora parsiana*
HQ261403.1	0	0	0	0	0	1	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora psychrophila*
HQ261454.1	0	0	0	0	19	8330	0	0	325	1361	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora sp. AL-2010b*
HQ708400.1	0	0	0	0	0	372	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora sp. BOLD:AAO6744*
KP749428.1	0	0	0	0	0	236	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora sp. NJB-2015*
HQ708426.1	0	0	0	0	0	1	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytopythium	*Phytopythium citrinum*
HQ708432.1	0	0	0	0	0	0	0	0	1	0	NA	Oomycetes	Peronosporales	NA	Phytopythium	*Phytopythium litorale*
HQ171167.1	0	0	0	0	0	1	0	0	0	0	NA	Oomycetes	Peronosporales	Salisapiliaceae	Salisapilia	*Salisapilia tartarea*

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#OTU ID	L3	L4	L5	L8	R0	R1	R4	R5	R6	R8	Philo	Class	Order	Family	Genus	Species
JN660054.1	333	0	48	8	117	0	1060	789	0	0	NA	Oomycetes	Pythiales	NA	NA	*uncultured Pythium*
HQ708219.1	0	0	0	0	0	0	0	0	1	0	NA	Oomycetes	Pythiales	Pythiaceae	Halophytophthora	*Halophytophthora avicenniae*
HQ708462.1	0	145	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium adhaerens*
KT692793.1	0	0	0	0	0	2	0	0	1	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium aff. acanthophoron JEB-2016*
KT692281.1	0	0	0	0	0	0	0	0	1	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium aff. intermedium JEB-2016*
HQ708492.1	0	0	0	0	0	3	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium aquatile*
HQ708560.1	0	4	0	0	0	205	0	2	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium contiguanum*
HQ708573.1	0	0	0	0	0	2	0	0	0	1	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium dissimile*
HQ708580.1	1	0	0	189	28	361	0	6	15	201	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium flevoense*
HQ708712.1	0	0	0	0	0	1	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium irregulare*
KJ995598.1	0	0	0	0	0	14	0	0	0	3	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium junctum*
HQ708741.1	0	0	0	0	0	0	0	0	25	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium monospermum*
KT692768.1	0	0	0	0	0	1	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium nodosum*
KF761143.1	0	0	0	0	0	1	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium oligandrum*
KX387369.1	0	0	0	0	0	238	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium oopapillum*
HQ708766.1	0	0	0	0	196	226	0	0	167	991	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium pachycaule*
HQ708784.1	0	0	0	0	0	3	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium periplocum*
HQ708814.1	0	0	0	0	0	0	0	0	1	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium senticosum*
HQ261487.1	0	205	0	0	10	93	0	2	6	32	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium sp. AL-2010*
HQ708857.1	0	0	0	0	0	1	0	0	0	110	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium sp. CAL-2011c*
KF761145.1	0	0	0	0	0	1	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium ultimum*
KJ995588.1	0	0	0	0	0	241	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium utonaiense*
HQ708159.1	0	0	0	0	0	5	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Achlya	*Achlya bisexualis*
HQ708173.1	0	0	0	184	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Achlya	*Achlya heterosexualis*
HQ708194.1	0	0	0	0	0	0	0	0	1	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Aphanomyces	*Aphanomyces iridis*
HQ708212.1	0	0	0	0	0	459	556	240	654	323	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Leptolegnia	*Leptolegnia sp. BOLD:AAX5717*
HQ709017.1	0	0	0	0	33	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia delica*
KM361513.1	0	2	0	0	0	212	0	0	296	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia ferax*
HQ709030.1	259	226	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia hypogyna*
HQ709045.1	0	1	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia parasitica*
FN397754.1	0	0	0	0	0	8	0	0	0	0	Nematoda	Chromadorea	Rhabditida	NA	NA	*Rhabditida sp. 304ed*

#OTU ID	L3	L4	L5	L8	R0	R1	R4	R5	R6	R8	Philo	Class	Order	Family	Genus	Species
JF287388.1	82	0	0	0	0	0	0	0	0	0	Nemertea	NA	NA	NA	NA	Nemertea sp. BOLD:AAG3607
DQ167167.1	0	0	0	450	0	0	0	0	0	1	Porifera	Demospongiae	Spongillida	Lubomirskiidae	Baikalospongia	Baikalospongia bacillifera
GQ411060.1	0	0	0	1	0	0	0	0	1	0	Porifera	Demospongiae	Spongillida	Spongillidae	Ephydatia	Ephydatia fluviatilis
GQ411061.1	0	0	0	1639	0	0	0	0	969	908	Porifera	Demospongiae	Spongillida	Spongillidae	Ephydatia	Ephydatia muelleri
DQ176779.1	0	0	0	0	0	0	0	0	0	3	Porifera	Demospongiae	Spongillida	Spongillidae	Eunapius	Eunapius fragilis
HQ379431.1	0	0	0	495	0	0	0	0	0	1453	Porifera	Demospongiae	Spongillida	Spongillidae	Spongilla	Spongilla lacustris
KC618848.1	1460	0	424	0	0	0	0	0	3335	0	Rotifera	Monogononta	Ploima	Brachionidae	Keratella	Keratella cochlearis
KC618788.1	0	0	0	0	0	0	1945	0	0	0	Rotifera	Monogononta	Ploima	Gastropidae	Ascomorpha	Ascomorpha ecaudis
JN936506.1	21877	53478	59615	23710	0	0	24485	11001	10949	0	Rotifera	Monogononta	Ploima	Synchaetidae	Polyarthra	Polyarthra dolichoptera
KC618999.1	6491	24646	84528	23455	3783	1	12871	19024	795	0	Rotifera	Monogononta	Ploima	Synchaetidae	Polyarthra	Polyarthra dolichoptera complex sp. UO-2013
KC619289.1	3	3970	2028	4	0	1	3813	905	2	466	Rotifera	Monogononta	Ploima	Synchaetidae	Polyarthra	Polyarthra sp. UO-2013
JN936549.1	0	0	0	221	0	0	0	0	0	0	Rotifera	Monogononta	Ploima	Synchaetidae	Synchaeta	Synchaeta cf. tremula/oblonga UO-2012
JN936520.1	0	0	0	0	67	0	0	0	0	0	Rotifera	Monogononta	Ploima	Synchaetidae	Synchaeta	Synchaeta grandis
JN936573.1	302	0	0	0	91	0	0	245	0	0	Rotifera	Monogononta	Ploima	Synchaetidae	Synchaeta	Synchaeta kitina
KP875614.1	0	0	3086	445	0	0	0	0	0	0	Rotifera	Monogononta	Ploima	Synchaetidae	Synchaeta	Synchaeta pectinata
Total sequences	92886	110744	194819	120587	54428	42786	62604	93914	43140	15328						

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Capítulo 6:

The role of barriers in aquatic fauna diversity: a case study in Nalón River, north of the Iberian Peninsula

Clusa L, Fernández S, Dopico E and García-Vázquez E

Scientific Reports

The role of barriers in aquatic fauna diversity: a case study in Nalón River, north of the Iberian Peninsula

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ABSTRACT:

Aquatic ecosystems are at risk due to the introduction of exotic species. Studies suggest that reservoirs are more susceptible to biological invasions than natural systems. We explored the effect of damming on aquatic fish fauna, taking Nalón River (south Bay of Biscay) as a case study. We combined environmental DNA analysis, data from sport fishing catch and conventional electrofishing surveys to determine the aquatic fish diversity in river areas differently affected by five dams. Four native species were identified from electrofishing and the native *S. salar* was detected from eDNA and confirmed from anglers' catch. All of them are diadromous species and were found only in the accessible river area, except the brown trout. Six exotic species were also identified and the exotic *Squalius carolitertii* was monitored upstream the impassable dams from anglers' catch. Species scoring higher in ecosystem services were found principally in the accessible zone of the river. On the other hand > 50% individuals from low-score species were found in the inaccessible zone between dams. The proportion of exotic species was positively associated to the accessibility of river areas. The uppermost dam could be acting as a barrier preventing the access of exotic species to head tributaries.

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Introduction

In the last decades, the introduction of invasive species to aquatic ecosystems is increasing and threatens local diversity (*1*). The number of threatened freshwater fish species in Spain is amongst the highest in Europe, with a total of 81 species from which 52 are native and 29 introduced (*2*). On the other hand, the most frequently introduced species worldwide are freshwater fishes (*3*). Some of them are leading to a homogenization of the ecosystems: *Carassius auratus, Cyprinus carpio, Micropterus salmoides, Gambusia holbrooki, Oncorhynchuss mykiss* (*4*). The five cited species were introduced to Spanish waters in the last decades (*5*), mainly from aquaculture, sport fishing (*6*) and aquarium trade (*7*). The impacts caused by those introduced species are numerous: they reduce water quality, displace native species through competition and predation, transmit diseases, hybridize with natives and other effects (e.g. *6, 8*).

As many as 50% of the freshwater ecoregions are obstructed by dams (*9*). Dams have indeed a negative effect on migratory fishes, especially on diadromous fish (such as *Salmonidae, Gobiidae*); because they are barriers that impede fish migration (*10*). The presence of barriers has been associated with upstream decreases of fishes' diversity and abundance (*11*). Diadromous species are declining in the North Atlantic hemisphere due, at least partially, to habitat loss caused by damming (*12*). From 12 eastern north Atlantic diadromous fish species, one is already extinct (*Coregonus oxyrinchus*), two critically endangered (*Anguilla anguilla, Acipenser sturio*) and one is considered vulnerable (*Coregonus lavaretus*) by the IUCN (International Union for Nature Conservation Red List of Threatened Species www.iucnredlist.org downloaded on 15 September 2017). For their role in biological invasions, however, the impact of dams is not so clear. Some studies suggest that reservoirs are more susceptible to biological invasions than natural aquatic systems. The shift from free-flowing rivers (lotic habitat) to standing waters (lentic habitat) affects the water flow, temperature, sediment transport and biological communities (*13*). These altered conditions may facilitate the spread of invasive species which can easily adapt to degraded ecosystems (*1*). Spreading of non-native fishes such as *Cyprinidae* and *Centrarchidae* as a consequence of dams has been reported (*10*). Clavero et al. (*14*) found dams were positively correlated with the number of introduced species in Iberian rivers, while being the main factor affecting negatively native fishes; introduced species were more successful invading artificial water bodies, mainly reservoirs. In the Caribbean area, impassable barriers eliminated native fish and shrimp fauna from upstream waters (*15*). In Australia the artificial reservoirs facilitate

biological invasions by supplying refuges for the invasive cane toad (*Rhinella marina*) and allowing its spread to waterless areas (*16*). Santos et al. (*17*) observed in Portugal a change in aquatic fauna after the construction of two reservoirs, with a final decline of native fish species, invasion of exotic species and different adverse environmental changes.

However, some authors have a different perspective about the role of river barriers regarding biological invasions, envisaging them as a protection measure against them, instead of a potential reservoir and spreading facilitator of invaders. Barrier removal enhances the risk of invasive species expansion, thus barriers could be considered a containment of such species blocking them downstream (*18, 19*). Implementing artificial barriers in selected tributaries has been recommended in New Zealand to limit exotic trout predation on native Galaxiidae (*20*). More recently, Dana et al. (*21*) demonstrated the efficiency of constructing small dams for preventing the expansion of the invasive crayfish *Procambarus clarkii* in a Mediterranean stream that contains the southernmost natural population of the endangered *Austropotamobius pallipes*. When fish are (or were) stocked, dams can impede the arrival of domestic or exotic lineages in upstream river areas, thus preventing undesired genetic introgression in indigenous lineages. This has been reported for trout species in different regions (*22, 23, 24*). Indeed, physical barriers (dams, electric barriers or other types) will also block the passage of other species and so may be inappropriate in rivers that provide significant migratory routes for native fishes (*25*).

Ecosystems provide a range of services that contributes to human well-being. Constanza et al. (*26*) estimated the global value of 17 ecosystem services for 16 biomes as $33 trillion per year. One decade later, the value was more than three times higher (*27*). Holmlund and Hammer (*28*) showed that human societies benefit from ecosystem services provided from fish populations, since fish are part of the food chain, help nutrient cycling, provide employment and leisure and can be used for disease control among others. With a growing need for natural resources it is crucial to establish the value of the ecosystem services. For example, in Scotland the contribution of salmon and sea trout rod fishery was £56.7 million (*29*). However, in many cases people are not aware about the ecosystem services that are contributing to their well-being (*30*). In southern Chile, the increase of salmon aquaculture has improved the economic situation in the region but also consumed many natural ecosystem services, whose regulation is key to preserve them (*31*). Therefore, the identification of vulnerable areas which exploitation could lead to losses of ecosystem

services will help to focus management efforts (*32*).

For all the reasons above, evaluating the positive and negative effect of river barriers on native and exotic freshwater, fish diversity is more important than ever, in order to design appropriate approaches to river barrier removal, as those suggested by Kemp and O'Hanley (*33*) that consider adequately the important issue of biological invasions. Here we have studied a case within the Iberian Peninsula; where less than 50% of the total watercourse length is flowing free (*9*). The basin studied was Nalón River (north of the Iberian Peninsula), with five dams and reservoirs along the river. The occurrence of native and non-indigenous species (NIS) in different river zones (upstream, downstream, within and between dams) has been evaluated using a suite of methodologies that have proven to be useful for fish inventory from the field. We have relied upon different methodologies to ensure all the species were identified: conventional electrofishing surveys used in Spain to implement the EU Water Framework Directive (2000/60/CE); environmental DNA, employed in other regions to detect rare species, exotic species in low density, with nocturnal habits or in waters where conventional sampling is not possible (*34, 35*); citizen science, where volunteers provide samples and help scientist to obtain relevant data in real time with less cost and effort (*36, 37*). In this particular case, anglers collaborated to obtain non-lethal fish tissue samples as in Williams et al. (*38*). Thus data sources were a combination of standardized electrofishing surveys, eDNA-based methods, and tissue samples from fish catch obtained from recreational fishing.

Results

Fish biodiversity in Nalón River

Regarding the physicochemical indicators (Table 1), the six sampling points exhibited noticeable differences in temperature and conductivity. The two parameters were lower upstream than downstream, as expected for clean mountain streams. For the rest of environmental traits considered the results were quite similar in all the points. The locations between dams exhibited less vegetation coverage than the other sampling points that were fully covered.

The aquatic fish inventory (Table 2) revealed that all except two of the species reported from the river any year since 1922 were found in 2016. From the electrofishing survey four of the six native species were identified: the brown trout *Salmo trutta*, the European eel *Anguilla anguilla*, the thicklip grey mullet *Chelon labrosus* and the sea lamprey *Petromyzon marinus*. Atlantic salmon

Salmo salar, well known to inhabit the lower river reach, was found from eDNA in point #1. Environmental DNA of the widespread *S. trutta* was found from all the sampling points. On the other hand, five of the six Nalon River NIS were found from electrofishing: the northern straight-mouth nase *Chondrostoma duriense*, the cyprinid *Cobitis paludica*, the Iberian gudgeon *Gobio lozanoi*, the minnow *Phoxinus bigerri* and the northern Iberian chub *Squalius carolitertii*. Finally, angler volunteers provided samples of *Salmo trutta* and *Squalius carolitertii* from the river area between D4 and D5, and of *Salmo salar* from point #1.

Table 1. **Physicochemical data**. Physical water quality indicators from the electrofishing survey by Taxus S.L.

	Dissolved oxygen (mg/l)	Water temperature (°C)	Conductivity (µS/cm)	% of vegetation in the riverbank	% shadow	Current velocity	Accessibility	Depth (m)
Point 1_Aranguín_Pravia	10.27	10.12	459.5	100	10	3	2	0.5
Point 2_San Román-Grado	9.47	13.7	521	100	95	1	2	0.8
Point 3_Casa Aurina	10	14.85	544	100	95	1	2	0.8
Point 4_Bueño	11.67	14	518	60	90	1	1	0.7
Point 5_Rioseco Casa del agua	10.33	6.48	159.5	90	40	2	0	0.5
Point 6_Veneros	10.33	6.48	159.5	100	40	2	0	0.6

Table 2. **Fish inventory**. Number of individuals of each fish species identified from electrofishing survey (shaded in grey), salmonid eDNA (presence-absence marked with a cross or 0, shaded in blue) and samples from anglers (shaded in orange) in each sampling point along Nalón River, from the list of official species records in any part of the river any year since 1922 (*68*). The species in bold are exotic to the region. The sampling points affected by damming are on the right of the black column.

Species	Method	#1	#2	#3		#4	#5	#6
Anguilla anguilla	Electrofishing	0	5	5		0	0	0
Chelon labrosus	Electrofishing	0	0	2		0	0	0
Chondrostoma duriense	Electrofishing	0	0	0		6	0	0
Cobitis paludica	Electrofishing	0	0	0		5	0	0
Gobio lozanoi	Electrofishing	0	59	7		9	0	0
Petromyzon marinus	Electrofishing	0	0	1		0	0	0
Phoxinus bigerri	Electrofishing	59	64	1		160	37	0
Salmo salar	Electrofishing	0	0	0		0	0	0
	eDNA	X	0	0		0	0	0
	Anglers	14						
Salmo trutta	Electrofishing	8	1	0		1	62	21
	eDNA	X	X	X		X	X	X
	Anglers	-	-	-		-	15	-
Squalius carolitertii	Electrofishing	0	20	6		28	0	0
	Anglers	-	-	-		-	2	-
Fish diversity - Shannon index	Electrofishing	0.366	1.147	1.554		0.826	0.661	0
% of NIS	Any method	33.33%	60%	42.86%		83.33%	66.67%	0%
% of NIS individuals	Electrofishing & anglers	72.84%	95.97%	63.63%		99.52%	33.62%	0%

Table 3. **Pairwise Kendall's tau correlations between ecological and physicochemical features of the sampling points**. The rs and p values are below and above the diagonal, respectively. Significant correlations are marked in bold and their p-values in grey.

	Fish diversity - Shannon index	% of NIS	% of NIS individuals	Dissolved oxygen (mg/l)	Water temperature (°C)	Conductivity (µS/cm)	% shaded area	Current velocity	Accessibility	% of vegetation in the riverbank	Depth (m)
Fish diversity - Shannon index		0.348	0.348	0.437	0.052	0.020	0.026	0.048	0.125	0.808	0.069
% of NIS	0.333		0.188	0.697	0.437	0.697	0.545	0.273	0.826	0.029	0.840
% of NIS individuals	0.333	0.467		0.697	0.120	0.243	0.545	0.273	0.273	0.467	0.545
Dissolved oxygen (mg/l)	-0.276	0.138	-0.138		0.421	0.227	0.531	0.820	0.112	0.045	0.403
Water temperature (°C)	0.690	0.276	0.552	-0.286		0.016	0.144	0.112	0.112	1.000	0.095
Conductivity (µS/cm)	**0.828**	0.138	0.414	-0.429	**0.857**		0.060	0.112	0.041	0.616	0.037
% shaded area	**-0.788**	-0.215	-0.215	0.222	-0.519	-0.667		0.010	0.239	1.000	0.009
Water speed	**-0.701**	-0.389	-0.389	0.081	-0.564	-0.564	**0.920**		0.442	0.571	0.018
Accessibility	0.545	-0.078	0.389	-0.564	0.564	**0.725**	-0.418	-0.273		0.257	0.157
% of vegetation in the riverbank	-0.086	**-0.775**	-0.258	**-0.713**	0.000	0.178	0.000	0.201	0.402		0.602
Depth (m)	0.645	0.072	0.215	-0.297	0.593	**0.741**	**-0.923**	**-0.836**	0.502	0.185	

Table 3 presents Kendall's tau correlations. The fish diversity was positively correlated with the conductivity and negatively with the percentage of shaded area and with water speed. The percentage of NIS was significantly and negative correlated with vegetation coverage.

The multiple linear model (Table 4) showed a significant positive effect of accessibility on the percentage of NIS individuals, and a negative effect of water temperature, vegetation coverage and current velocity.

Regarding river zonation, the highest number of native species and individuals occurred in the accessible region, where 62.5% of the total sample was individuals from native species. The NIS were concentrated mainly between dams. In the upstream area all the individuals sampled were native *Salmo trutta* (Table 5). For the provision of services by species, the current fishing regulation in the region allows sport fishing of seven of the species detected (Table 6). Only one species (*Anguilla anguilla*) is subject of commercial fishing (glass eels arriving in the river mouth). Two species are used as bait (*Cobitis paludica* and *Phoxinus bigerri*) (*39*). Two species (*Salmo salar* and *Salmo trutta*) are of tourism value, since anglers travel to the region to fish these two species (only recreational fisheries). For ecosystem regulation, the species with the highest trophic level are *Salmo salar* and *Petromyzon marinus*, and the lowest are *Chondrostoma duriense*, *Phoxinus bigerri* and *Cobitis paludica*. The latter is employed for pest control because it feeds on insect larvae (*39*). None of the species detected here have significant impact on water turbidity.

Table 4. **Multiple linear model analysis.** Relation between the percentage of NIS individuals and the independent environmental variables: Water temperature, current velocity, accessibility and percentage of vegetation in the riverbank. Significant p values are highlighted in grey.

%NIS individuals	Coeff.	Std.err.	t	p	R^2
Constant	639.200	11.362	56.256	0.011	
Water temperature (°C)	-28.510	0.664	-42.924	0.015	0.697
Current velocity	-62.678	1.449	-43.255	0.015	0.170
Accessibility	119.490	2.025	59.005	0.011	0.537
% of vegetation in the riverbank	-3.288	0.048	-68.853	0.009	0.172

Dependent variable:	% of NIS individuals
N:	6.00000
Multiple R	0.99997
Multiple R^2	0.99995
Multiple R^2 adjusted	0.99973
ANOVA	
F	4680.3
df_1, df_2	4, 1
p:	0.010962

Table 5. **Summary of native and NIS species by river zone**. Native and NIS species richness (SR), number of native and NIS individuals, percentage of native species and individuals are shown for each zone.

Zone	Native SR	NIS SR	Native individuals	NIS individuals	% native SR	% native individuals
Accessible from the sea	5	3	36	216	62.50	14.29
Inaccessible-between dams	1	5	78	247	16.67	24.00
Inaccessible - upstream dams	1	0	21	0	100.00	100.00

Regarding the cultural services, for public appreciation 39 participants in the survey identified *Salmo salar* as native, 114 *Salmo trutta*, 25 *Anguilla anguilla* and only one *Petromyzon marinus*. Five persons misidentified *Phoxinus bigerri* as native, and so did one with *Squalius caroliertii*. The rest of species were not named by the volunteers. For educational and cultural activities carried out in the region around freshwater fish, the World Fish Migration Day is celebrated around migratory brown trout (*Salmo trutta*) in the river basin (https://worldfishmigrationblog.wordpress.com/portfolio/spain-2/; accessed December 2017). There is a museum of the Atlantic salmon (*Salmo salar*) in Pravia, a town located in the downstream zone of the basin (https://www.ayto-pravia.es/casa-del-salmon; accessed December 2017). The elements here explained justified the scores of the different species in the cultural services.

In summary, the species with the higher scores for the ecosystem considered were the native species *Salmo salar*, *Anguilla anguilla*, *Salmo trutta* and *Petromyzon marinus*. They can be found only in the accessible zone of the river downstream, except *Salmo trutta* that was found in the three zones. For this species, only 8.3% was sampled from the lower accessible zone, while 72.2% occurred between dams and 19.5% upstream. The species with lower scores in the ecosystem services considered were the exotic *Chondrostoma duriense*, *Phoxinus bigerri* and *Squalius caroliertii*. More than 50% individuals of these species were found between dams. Moreover, all the individuals of two exotic species (*Chondrostoma duriense* and *Cobitis paludica*) were sampled from this zone (Table 6).

Table 6. **Ecosystem services provided by the fish species detected, and their spatial distribution**. For each species the global value of the services provided and the distribution in the three river zones considered are shown. Species found from only one zone are highlighted in grey.

Species	Provision of services					Regulation				Cultural		Total value per species	Distribution (%)		
	Sport fishing	Commercial fishing	Bait	Tourism	Commercial aquaculture	Top-down (Trophic level as indicator)	Pest control	Turbidity increase	Native species	Public appreciation	Museums and educational material		Accessible	Inaccessible-between dams	Inaccessible - upstream dams
Salmo salar	1	0	0	1	0	1	0	0	1	1	1	6	100	0	0
Salmo trutta	1	0	0	1	0	0.75	0	0	1	1	1	5.75	8.33	72.22	19.44
Anguilla anguilla	0	1	0	0	1	0.75	0	0	1	1	0	4.75	100	0	0
Chelon labrosus	1	0	0	0	0	0.5	0	0	1	0	0	2.5	100	0	0
Petromyzon marinus	0	0	0	0	0	1	0	0	1	1	0	3	100	0	0
Chondrostoma duriense	1	0	0	0	0	0.5	0	0	0	0	0	1.5	0	100	0
Cobitis paludica	0	0	1	0	0	0.5	1	0	0	0	0	2.5	0	100	0
Gobio lozanoi	1	0	0	0	0	0.75	0	0	0	0	0	1.75	88	12	0
Phoxinus bigerri	1	0	1	0	0	0.5	0	0	0	-1	0	1.5	38.63	61.37	0
Squalius caroliertii	1	0	0	0	0	0.75	0	0	0	-1	0	0.75	46.43	53.57	0

Discussion

The results obtained in this study are contradictory in some ways. On one hand, the disruption of migratory pathways of fish species due to damming is well known (*10, 12*). In our case study, all the native species are diadromous, and their occurrence was limited to the area downstream the first dam, Valduno (D1 in Figure 1). Three of the four species with ≥3 score in ecosystem services (*Salmo salar*, *Anguilla anguilla* and *Petromyzon marinus)* were only found in the accessible region of the river (Table 6). Although the dams D1 and D3 have fish passages (ladders), apparently they are not sufficient for restoring upstream populations of all the native species of this river. Dam removal or construction of side channels could help the restoration of migratory native fauna (*15, 40*), and would be especially important for the European eel, nowadays a critically endangered species, which lost 80% of its original range in the Iberian Peninsula (*41*).

On the other hand, in our case study it seems that the dams represent a barrier to the expansion of exotic fish, and this effect could be beneficial to native fauna by blocking pollutants, parasites or diseases that come together with exotics (*9*). Upstream dam areas could act as refuges for imperilled freshwater fishes where exotic species cannot accede (*42*). In the present study, most individuals from exotic species were found between dams (Table 6), and exotic cyprinids were not detected upstream Tanes (D5 in Figure 1); that dam could be seen as a barrier protecting the upper part of the river from exotic species. Rahel (*43*) suggested that the dams could be helpful to keep under control non-native species and exotic diseases and prevent their spread, and this was actually the case of the sea lamprey in the Laurentian Great Lakes (*19*). However, all this should be taken with caution. Although reservoirs can stop the advance of exotic species in some cases, in other cases they maintain NIS close to uninvaded water bodies and facilitate their spread (*1*). It seems to be the case of the reservoirs located between D1 and D5, where most exotics were found.

Coinciding with a higher abundance of NIS and the absence of high-score native species of ecological value, the zone located between dams that contains most reservoirs in the basin seemed to provide less ecosystem services than downstream and upstream areas. Speaking only from the point of view of the fish community, that zone could be considered ecologically devaluated. Beier et al. (*32*) highlighted the importance of identifying vulnerable areas which exploitation could cause losses of ecosystem services. From our results, the zone between dams is clearly vulnerable. Controlling the introduction of exotic species in reservoirs should be recommended as a priority in River Nalón basin.

Other lessons can be learned from this case study. NIS abundance was negatively correlated with vegetation coverage in the riverbank –a signal of undisturbed rivers, and positively with water temperature. Exotic species adapt easily to degraded habitats and artificial water bodies (*1, 13, 14*). On the other hand, more abundant NIS in zones with higher water temperature is consistent with the hypothesis of current climate warming favouring the spread of exotic species (*44, 45*). A general recommendation to prevent exotics from becoming invaders could be to favour native biodiversity, since it confers a natural resistance against the spread of exotics in terrestrial (*46*) and aquatic ecosystems (*47, 48*).

From the technical point of view, in this study we have employed different methods for the inventory of fish fauna. Environmental DNA is gaining relevance as a tool for biodiversity monitoring (*49, 50*), since it is more efficient than conventional electrofishing, trapping and netting for detecting nocturnal or rare species (*34*). Here eDNA confirmed the presence of *S. salar* where electrofishing failed to do so. Evans et al. (*51*) showed that eDNA required less effort than electrofishing and was less expensive than multiple-pass electrofishing. According to this, in our study eDNA sampling effort was 10 minutes per sampling point plus 8 hours of laboratory analysis (one person), that is 9 person hours in total, while the electrofishing survey was 4 hours per sampling point and four people, 24 person hours in total. eDNA analysis costs were 13.4 euros per sample plus one day technician salary (*52*). In total the electrofishing survey costs were 1028 euros per sampling point, and waiting for proper weather conditions, clearly more expensive than eDNA costs. Nowadays the cost of eDNA analysis is decreasing making NGS studies for biodiversity inventory affordable and in some cases cheaper than conventional surveys (*53, 54*). A weakness of eDNA is the possibility of false positives due to contamination with DNA sources such as avian faeces, carcasses, discharges from fish farms etc. (*55, 56*). In this case a good knowledge of the region will help to solve this problem and if there is a contamination source nearby, an additional method of species detection should be employed to confirm the eDNA source was real individuals.

On the contrary, the contribution of citizens in conservation projects is very valuable and can be an additional information source (*57*). They help to detect exotic species (*58*), for example in the eBird program in North America (*59*), the mapping of invasive crabs in the U. S. (*37*), the detection of invasive bumblebees in Japan (*60*), the detection and control of biological

invasions in Alaska (*61*), in the Mediterranean Sea (*62*), in Australian islands (*63*) and others. Anglers can help in conservation researches (*38, 64*), and in the present case study they detected the NIS *Squalius carolitertii* near Rioseco reservoir (red circle in Figure 1), upstream the first impassable dam (D4). It makes sense to involve anglers in conservation studies, since releases for angling and aquaculture are the two main causes of introduction of non-native fish in Spain, where the 42% of all aquatic introductions are intentional (*65*). Collaborating with anglers and making them aware of the problems caused by exotic species will help to control and decrease intentional introductions (*61*).

Management recommendation

As a final remark, the dams occurring in this case study interrupt the passage of migratory fish upstream, but at the same time seem to prevent exotic fish from entering in the protected riverheads. On the other hand, the fish populations inhabiting the zones located between the first and the last dam seem to provide less ecosystem services than the fish inhabiting downstream accessible zones. Restoring connectivity in the mid reach of the river through appropriate infrastructures and controlling strictly the introduction of exotics in the reservoirs would be recommended. More studies of aquatic biodiversity in the Bay of Biscay region should be done to design management strategies to recover native fauna, avoid the spreading of exotic species and evaluate the effects of dams and reservoirs in the river basins.

Materials and methods

Study area, river barriers and sampling points

The study area was Nalón River (140.8 km long and average discharge of 55.18m^3/s) from the Nalón-Narcea basin (Principality of Asturias, Spain), which is the largest freshwater system and one of the most important in the Iberian Bay of Biscay. The main tributaries are Caudal River, Trubia River, Nora River, Narcea River and Aranguín River, Narcea River containing one of the largest Atlantic salmon (*Salmo salar*) natural populations. Nalón River has five dams (D1-D5 in Figure 1): Valduno, Priañes, Furacón, Rioseco and Tanes. Priañes is placed right where the tributary Nora River joins Nalón River. Valduno and Furacón have fish passages (ladders) but the rest are >10m height impassable dams (Table 7) (*66*). The upper part of the river basin is protected under Natura 2000 regulation and since 2001 is included in the global network of the Biosphere Reserves by UNESCO. The entire zone upstream D4 is included in the Asturian Natural Reserve of Redes. The sampling points were labelled #1 to #6 from down to upstream (in black in Figure 1). From their location in relation with barriers, three points were downstream in the accessible part of the river: #1 "Pravia", near the joining point of the tributary Aranguín River and the mainstream 43.491283N, 6.103837W; #2 "San Román-Grado" 43.447861N, 6.080278W; #3 "Casa Aurina" 43.401778N, 6.037111W. Two locations were located between dams: # 4 "Bueño" 43.311058N, 5.889788W between D3 and D4, and #5 "Rioseco" 43.218639N, 5.452556W between D4 and D5. Finally, point #6 "Veneros" (43.178948N, 5.337093W) was located upstream.

Table 7. **Dams in Nalón River with their coordinates and main characteristics.** Information retrieved from Ministerio de Agricultura y Pesca, Alimentación y Medio ambiente webpage (http://www.mapama.gob.es/es/agua/temas/seguridad-de-presas-y-embalses/inventario-presas-y-embalses/ accessed on September 2017).

Code	Dam name	River	Coordinates	Height (m)	Fish passages	Construction year	Use
D1	Valduno	Nalón	43.389091N, 6.002791W	13	Yes	1993-2000	Hydroelectric power
D2	Priañes	Nora (tributary of Nalón River)	43.383321N, 5.975038W	27	No	1953	Hydroelectric power
D3	Furacón	Nalón	43.367265N, 5.964046W	14	Yes	1952	Hydroelectric power
D4	Rioseco	Nalón	43.223583N, 5.459807W	28.5	No	1978	Flood prevention and hydroelectric power
D5	Tanes	Nalón	43.192474N, 5.382222W	95	No	1979	Water supply and hydroelectric power

Figure 1. **Nalón River basin**. Dams, sampling points (black ink), and anglers captures sites are shown. Image downloaded from Confederación Hidrográfica del Cantábrico (*66*) and modify with GIMP software version 2.6.

Official inventory of freshwater fish species in the region

The regional fauna has been inventoried and is sporadically monitored from the rivers by the competent authority in charge of freshwater ecosystem management in Spain, which is the Regional Government. The most recent official inventory of regional river native fauna and exotic species was published in 2014 by De la Hoz updated from De la Hoz (*67*) and is available in the Asturias Regional Government webpage (*68*) (http://movil.asturias.es/portal/site/webasturias/menuitem.4b280f8214549ead3e2d6f77f2300030/?vgnextoid=996842fe2c47e010VgnVCM100000b0030a0aRCRD&vgnextchannel=9362d22a18b6e210VgnVCM1000002f030003RCRD&i18n.http.lang=.%20). Six native species occur in Nalon River. Five of them are diadromous (*Alosa alosa*, *Anguilla anguilla*, *Chelon labrosus*, *Petromyzon marinus* and *Salmo salar*) and are restricted to the accessible part of the river downstream D3. Only the native brown trout *Salmo trutta* occupies the entire river. Six NIS have been reported sporadically any year since 1922: *Carassius auratus, Chondrostoma duriense, Cobitis paludica, Gobio lozanoi*, *Phoxinus bigerri* and *Squalius carolitertii*. They occurred in the lower part of the river (below D1 dam and in Trubia and Cubia tributaries, see Figure 1) with the exceptions of *Phoxinus bigerri* that is considered widespread, and *Squalius carolitertii* which was found from the middle reach of the river –between dams D3 and D4- and in D4 and D5 since 2006.

In addition to regular freshwater fauna inventories (presence/absence), more information is available about salmonids. Nalón River supports Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) sport fishing (rod and line). Atlantic salmon catches are recorded and the annual catch statistics for Nalón River (number of individuals caught from each river area) are publicly available http://fon-fishing.com/info/128/estadistica-oficial-capturas-salmon-asturias-2016/.

Electrofishing surveys

The local aquatic fauna occurring in the river was surveyed in November-December 2016 using the standard electrofishing protocol approved by the Spanish Ministry of Agriculture, Fisheries and Environment for implementing the EU Water Framework Directive 2000/60/CE: Protocol ML-R-FI-2015 (NIPO: 280-15-122-6). Physical water quality indicators were measured in each sampling point: water temperature, dissolved oxygen, conductivity, medium depth, percentage of vegetation in the riverbank, percentage of shadow (dark) areas, and water speed. The survey was carried out by Taxus S.L., a company authorized for aquatic biodiversity surveys in the Principality of Asturias. Electrofishing was carried out from six sampling sites along Nalón River described above (Figure 1).

Fish species detection from eDNA

Water (2L) was sampled in February 2016 from the six sampling points. The presence of salmonids was assessed from eDNA as in Clusa et al. (*52*). The eDNA was sampled and extracted as described therein, following Goldberg et al. (*69*) indications to avoid contamination of the water samples. Briefly, 16S rRNA gene was amplified from eDNA with general primers by PCR. Nested PCR with *Salmonidae* specific primers was done using as template the previous amplification. The PCR product from this nested-PCR was digested with restriction enzymes that provided species-specific RFLP patterns, allowing the identification of the different salmonids present in a sample.

Samples and information provided by anglers

Scales from fish catches from a small tributary located between D4 and D5 were kindly provided by anglers (see upstream red circle in Figure 1). Volunteers were asked to note the species of the first catch of the day in 21 May 2016. All the 2016 Atlantic salmon catch from Nalón River was recorded (downstream red circle in Figure 1).

Data analysis

In each point the percentage of NIS was calculated taking into account all the NIS identified by any of the methods described. The percentage of NIS individuals was calculated using the quantitative data from the electrofishing survey and information provided by anglers. Information based solely on eDNA cannot be employed for this

estimate because it detects DNA but not the number of individuals present of a species.

From the electrofishing survey results the fish diversity was calculated with Shannon diversity index as follows:

$$H = -\sum_{i=1}^{S} p_i * \ln(p_i)$$

p_i is the relative abundance for each species calculated as number of individuals of each species divided by the total number of individuals in a sample.

For the river current, or water velocity, scores were: 3 for fast, 2 for moderate and 1 for reduced flow. For accessibility the sampling points not affected by dams (#1, #2, #3) were scored with 2, sampling point number #4 (above dams with fish passages) was scored with 1, and points #5 and #6 were scored with 0 since there are impassable dams downstream.

The dataset was statistically analyzed using Past 3.15 free software (*70*). Pairwise correlations were calculated using the non-parametric Kendall´s tau test of ranks because the data ranges are very different among variables. Multiple linear model was applied to establish the relation between % of NIS, % of NIS individuals and Shannon diversity with independent environmental variables. Bonferroni correction was applied when necessary.

Ecosystem services provided by fish population in each zone

In order to evaluate the effect of the river dams on ecosystem services, the basin was divided in three zones: accessible from the sea, i.e. downstream area free of dams (sampling points 1-3); inaccessible zone between dams (points 4 and 5); inaccessible upstream dams (point 6). The species richness was calculated separately for native (native species richness) and the non-indigenous species (NIS species richness) as the number of species of each type. The percentage of native species and native individuals were also calculated. For each species, the percentage of individuals in each zone was calculated. The ecosystem services provided by each fish species was calculated as the sum of provision services, regulation services and cultural services, following Holmlund and Hammer (*28*) and Butler et al. (*29*). The categorization of the ecosystem services was based on Constanza (*30*) as it follows:

- Provision of services: the species can be used for sport fishing, commercial fishing, as bait, for tourism and commercial aquaculture. The official fishing regulation in the region can be found in: https://sede.asturias.es/portal/site/Asturias

/menuitem.1003733838db7342ebc4e1911 00000f7/?vgnextoid=d7d79d16b61ee010 VgnVCM1000000100007fRCRD&fecha =24/10/2016&refArticulo=2016- 11027&i18n.http.lang=es (accessed December 2017). Each service was scored with 1.

- Regulation: The trophic level was used as an indicator of top-down ecosystem regulation. The value was obtained from FishBase (*71*) and was scored as it follows: <1 trophic level, 0; 1-2, 0.25; 2-3, 0.5; 3-4, 0.75; >4, 1). The ability of each species for species control, scored with 1, and the increase of water turbidity, scored with -1, were taken from the literature. The native status was considered as positive for the ecosystem biodiversity and scored with 1.

- Cultural services: Public appreciation of each species was measured from 140 surveys conducted in the region, representing 0.05% of the population inhabiting the study areas. The interviewees were approached near the rivers in recreational promenades and were asked to identify the native and exotic species they know to occur in the region. Native species correctly identified as such scored with 1, and the species identified (or misidentified) as undesired exotics were scored with -1. A final category of museums and educational material was included considering the species that contribute with any educational material or are the main topic of museums or exhibitions, scored with 1.

Ethics statement

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Author contributions

Conceived and designed the experiments: LC, EGV.
Performed the experiments: LC SF.
Analyzed the data: LC, EGV.
Contributed reagents/materials/analysis tools: LC, SF, ED, EGV.
Wrote the paper: LC, EGV.
Revise the manuscript: LC, SF, ED, EGV.

Additional information

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Capítulo 7:

Public knowledge of alien species: a case study on aquatic biodiversity in North Iberian rivers.

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Public knowledge of alien species: a case study on aquatic biodiversity in North Iberian rivers

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ABSTRACT

Biological invasions have increased in recent decades due to globalization and human activities. These invasions are currently one of the main threats to biodiversity, and their early detection is essential for a rapid and effective response. Here, we explored the use of citizen science strategies to create an early alert to detect invasive species. Our main objective was to evaluate the general knowledge of volunteer participants of invasive freshwater species in Asturias (north of the Iberian Peninsula) and compare it with both real data from electrofishing surveys and official data from the regional government. A total of 140 volunteer surveys were conducted in four different rivers in Asturias. The largest group of participants consisted of males older than 50 years. Four species were identified as native to the four rivers: *Anguilla anguilla; Mugil cephalus; Salmo salar*; and, *Salmo trutta*. More than 50% of the native species surveyed by electrofishing were recognized by the locals in each river region. A total of 22.86% of the volunteers were able to correctly name an exotic species, and a total of 7 were correctly identified: *Procambarus clarkii; Trachemys scripta; Cyprinus carpio; Esox lucius; Salvelinus fontinalis; Carassius auratus;* and, *Oncorhynchus mykiss*. However, compared to the list of actual exotic species surveyed, less than 40% were recognized in the four rivers. Despite the poor correlation between local knowledge and real exotic aquatic fauna, citizens were able to detect one exotic species not yet found in the wild in this region (*T. scripta*). Finally, more than 70% of the volunteers were in favor of fighting against invasive species, although only 22.86% were able to identify any specific exotic species found in the region. The positive attitude to exotic species control was correlated with both the level of native species knowledge and the concern about the ability of exotic species to impact native fauna in the region. Better training will improve public awareness, reduce the non-intentional release of non-native species, and increase the detection of non-indigenous species. The attitudes of the citizens make the region a promising candidate for education efforts to reduce alien species introductions and help preserve fauna biodiversity.

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Introduction

In the last several decades, the number of non-indigenous species (NIS) has increased due to globalization and human activities (Hulme, 2009). NIS (exotic, alien, non-native species) are species outside their native range that are often introduced by human activities (introduced species). In some cases, these NIS can proliferate, undergo exponential population increases and spread quickly to become invasive species (Occhipinti-Ambrogi & Galil, 2004). Invasive species cause numerous impacts to the community structure and environment (Chown et al., 2015). Eradication of these exotic species may allow for the recovery of the native fauna, but if they become invasive,

eradication is nearly impossible; therefore, the main efforts should be performed before establishment. Early detection is essential for rapid response and prevention from further spread (Havel, Kovalenko, Thomaz, Amalfitano, & Kats, 2015).

During the last twenty years, the number of research articles based on citizen science has increased exponentially. There are many projects involving citizen science, including ones about climate change, conservation biology, ecological restoration, water quality, invasive species and many more topics (Silvertown, 2009). Technology has facilitated citizen science programs through new smartphone applications or on the Internet. Such applications can improve communication

between scientists and citizens, as daily citizen observations can be easily uploaded online and made accessible to researchers, thereby generating thousands of data records (Newman et al., 2012). In citizen science programs, many volunteers can cover large regions with their observations and help identify migration patterns, the spread of infectious diseases and other ecological phenomena at a large scale (Devictor, Whittaker, & Beltrame, 2010; Dickinson, Zuckerberg, & Bonter, 2010). For example, many citizen science programs are the basis of large bird inventories (Tulloch, Possingham, Joseph, Szabo, & Martin, 2013), such as the eBird program in North America that collects five million bird observations per month (Sullivan et al., 2014). Another example is the study of hosts' natural resistance to virulent forest pests, which was carried out in the USA with the help of citizen science (Ingwell & Preisser, 2011). Citizen science programs have been increasingly used to collect data for monitoring invasive species in real time (Crall et al., 2010; Dickinson et al., 2012), especially for early alerts of new NIS. Citizens can reach locations that may not be accessible to scientists; for example, some areas in South Florida where Burmese pythons (*Python molurus bivittatus*) have been found (Falk, Snow, & Reed, 2016). Mapping crab invasions (*Carcinus maenas* and *Hemigrapsus sanguineus*) along US coasts would not be possible without citizen science (Delaney, Sperling, Adams, & Leung, 2008) due to the large size and extent of the invaded spaces.

Many of the established citizen science programs are aimed at invasive plants. After training, citizen scientists are able to distinguish invasive plants and collect robust data, as has been shown in Texas (Gallo & Waitt, 2011). Other examples include the monitoring of invasive plants in a natural reserve in Georgia, which was carried out by citizens with the help of smartphones and a geo-referencing application (Hawthorne et al., 2015); the Invasive Plant Atlas of New England (IPANE) that was created in 2001 (Bois, Silander, & Mehrhoff, 2011; Crall et al., 2011); the Invasive Plant Atlas of the Mid-South (IPAMS); and the Cactus Moth Detection and Monitoring Network (CMDMN) (Simpson et al., 2009). Plants are not the only species studied; successful programs are also running for the detection of invasive animals, as reported above for serpents and crabs. In Japan, 300,000 bumblebees were removed from the wild within the monitoring program of the invasive *Bombus terrestris* (Kobori et al., 2016). In North Italy and Switzerland, it was possible to map the stink bug *Halymorpha halys* and develop identification guides to help track this invasive species in other regions (Maistrello, Dioli, Bariselli, Mazzoli, & Giacalone-Forini, 2016). The use of citizen science to detect invasive aquatic

species has also increased in recent years. In Alaska, citizen science was employed to control marine invasions, which are a threat to native marine resources (https://seagrant.uaf.edu/research/projects/summary.php?id=939). In Greece, 86 observations of 28 alien species reported in 2012 demonstrated the spread of more than 20 invasive species (Zenetos, Koutsogiannopoulos, Ovalis, & Poursanidis, 2013). Citizen scientists contributed to the detection of the invasive lionfish in the Caribbean Sea (Carballo-Cárdenas & Tobi, 2016). The first report of the sergeant major (*Abudefduf saxatilis*) in the Mediterranean Sea was collected through citizen science on the "Seawatchers" webpage (http://www.observadoresdelmar.es), where volunteers collect data and inform scientists about new invasive species (Azzurro, Broglio, Maynou, & Bariche, 2013).

Evaluating public knowledge of the specific taxa to be monitored is recommended before creating a citizen science program about NIS. García-Llorente, Martín-López, González, Alcorlo and Montes (2008) studied how different groups (tourists, conservation professionals, local users and others) perceived the impact caused by IAS (invasive alien species) in the Natural Reserve of Doñana (Southwest Spain) and their attitudes towards IAS eradication. As many as 97% of the people in all groups agreed that IAS eradication was necessary, but they were principally concerned with the recent invasions and the species that had been objects of particular campaigns and appeared in the news. The authors concluded that the general knowledge of citizens is crucial to generating public demand for actions against invasive species, and they emphasized the low concordance found between official data, real data and citizen perceptions.

The main objective of this study was to evaluate the public's knowledge about freshwater NIS in Asturias (north of the Iberian Peninsula) through a survey on species reports, and the survey results were compared with actual local fauna and official data from the regional and national environmental authorities. The results served to identify knowledge gaps that could be used to focus training efforts in future citizen science programs on aquatic biodiversity inventories.

Materials and Methods

Sampling sites and river biota

During 2016, four different rivers in Asturias (south-central Bay of Biscay) were selected for social and biodiversity surveys: Raíces; Piles; Negro; and, Nalón (Figure 1). Three of the rivers are short coastal streams (the Negro, Piles and Raíces rivers are 20, 16 and 15 km in length,

respectively), and the Nalón River (140 km long) originates from the Nalón-Narcea basin, which is the largest freshwater system in the region. Sampling sites were set within river towns at the following coordinates: Luarca (43.544240N, 6.535308W) on the Negro River; Salinas (43.566852N, 5.962669W) on the Raíces River; Gijón (43.537846N, 5.639280W) on the Piles River; Las Caldas (43.330988N, 5.930960W) and, Rioseco (43.218977N, 5.454763W) on the Nalón River.

The most recent official inventory of the native fauna and NIS of the regional rivers was published by De la Hoz (2006).
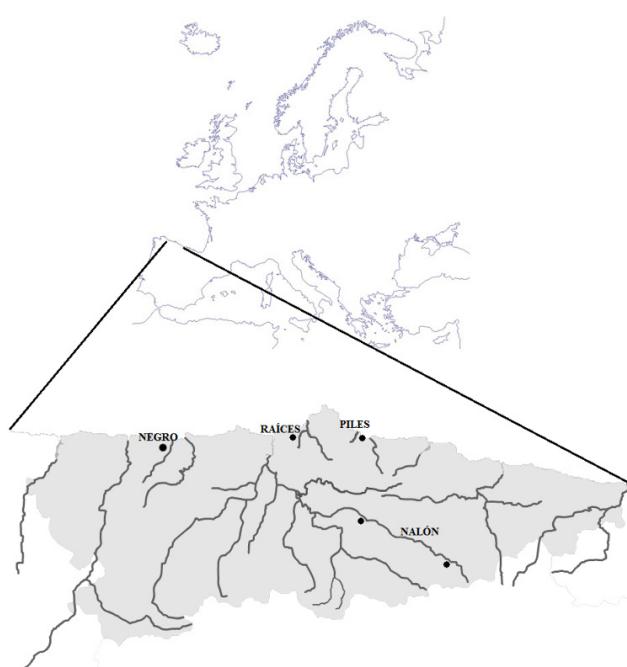
Figure 1. **Map showing the sampling sites of the four rivers**: Nalón, Negro, Piles and Raíces

Social survey

A total of 140 local participants were interviewed across the study region, including males and females older than 20 years. The samples represented 0.05% of the population inhabiting the study areas. Potential interviewees were approached along recreational promenades near the rivers, and eligible and willing participants were interviewed in Spanish, their native language. Interview sessions were no longer than 5 minutes per person to facilitate easy and spontaneous responses.

The questionnaire was inspired by García-Llorente et al. (2008). The interview was formulated as a conversation to help the volunteers feel more comfortable and answer without any

pressure. The survey was divided into two sections (Supplementary file 1), as follows:

1) General knowledge of aquatic species in the region, where the volunteers listed the species they remembered from the local river by their common names and classified them as native or exotic using their knowledge. The translation from common name to scientific name for each species was performed by the researchers. There was no possibility of error since the common names are unique for each species in this region, and there are no local variants in different valleys;

2) Awareness and concerns about exotic species, which contained four questions (Supplementary file 1). A final open question about the perceived changes in the river ecosystem, if any, was posed.

Pictures of animals inhabiting Iberian rivers were available if needed for recognition of a species but were not offered beforehand. The survey was previously tested in a pilot sample (N=10) to refine the questions and ensure the content was clear and easy to understood.

The word "invasive" was avoided in the interview because it has a negative connotation, so the answers from the participants were not influenced. If needed to clarify a participant's understanding, exotic species were defined as "species that are not native to this place".

The participant's answers were recorded in writing. After finishing the interview, the participants were asked to check their answers and confirm they were correctly recorded.

Ethics statement

All volunteers agreed to participate in the study and signed the informed consent for the use of their answers in research. The study was approved by the Ethics Committee from the Principality of Asturias with the permit of reference number 99/16.

Electrofishing surveys

The actual local aquatic fauna occurring in the four rivers considered was surveyed in March 2017. The standard protocol approved by the Spanish Ministry of Agriculture, Fisheries and Environment for implementing the EU Water Framework Directive 2000/60/CE was employed. This protocol, ML-R-FI-2015 (NIPO: 280-15-122-6), is based on electrofishing. The survey was carried out by Taxus S.L., a company authorized for aquatic biodiversity surveys in the Principality of Asturias. Due to the different river sizes, electrofishing was carried out from one sampling

site in each of the three small rivers and six sampling sites along the Nalón River.

Data analysis

Participants were grouped by river (Nalón, Negro, Piles and Raíces), age (older or younger than 50 years) and gender. Some participants provided additional information about terrestrial species, but answers about only aquatic species were considered.

Knowledge was measured as the concordance between a participant's answer and the official list of native and exotic species in Asturias, which is available in De la Hoz (2006). Four measurements were obtained: the number of native species correctly identified (correct natives, C_N); the number of exotic species correctly identified (correct exotics, C_E); the number of exotic or absent species mistaken as native (incorrect natives, I_N); and, the number of native or absent species mistaken as exotics (incorrect exotics, I_E).

A knowledge index (Ki) was calculated as the mathematically averaged knowledge (scored as correct – incorrect species) of native and exotic species, using the following formula:

$$K_i = \frac{(C_N - I_N) + (C_E - I_E)}{2}$$

In the second section of the survey, the scores were 0 (I don't know), 1 (No), and 3 (Yes) for the questions with three answer choices; and 0 (I don't know), 1 (No), 2 (Sometimes, depending on the species), and 3 (Yes) for the questions with four answer choices. In question C (changes in the ecosystem), the answers were classified into four large groups: "Water quality", including changes in water quality (cleaner or more polluted water, more or less algae, increase of floods, more sediments, lower water flow…); "Fauna", including changes in aquatic fauna (for example, reduced trout spawning, changes in species abundance such as an increase of *Mugil cephalus* and a decrease of *Salmo trutta*); "Infrastructure", including new ponds, dams and promenades; "Environment", including cleaner or dirtier surrounding environment and changes in riverbank vegetation and excluding changes in the water considered in the above category.

Statistical analysis

The data were analyzed with the program Past 3.15 (Hammer, Harper, & Ryan, 2001). Normality was checked using Shapiro-Wilk W and Anderson-Darling A tests. Comparisons among groups (rivers, ages or gender) were conducted using ANOVA or Kruskal-Wallis to test for differences among the means or medians of the groups, respectively (the latter in case of significant deviation from normality). Pairwise correlations (between questions, or between knowledge and perception/opinion) were calculated using Spearman's rs. Statistical significance was set at p<0.05. Bonferroni correction of the significance level was applied for multiple comparisons.

Results

In total, 58 women (41.43%) and 82 men (58.57%) participated in the survey (Table 1). The largest age group (34.29% of the total sample) was older than 60 years.

Table 1. **Sample for social survey**. Number of citizens classified by gender and age in each river is shown.

	Nalón		Negro		Piles		Raíces		Total by age
	Men	Women	Men	Women	Men	Women	Men	Women	
20-30	0	2	0	0	3	2	0	2	9
30-40	5	5	2	2	1	2	3	3	23
40-50	6	3	4	2	2	4	3	5	29
50-60	5	1	4	4	5	3	6	3	31
>60	8	0	11	6	7	6	7	3	48
Total by river	35		35		35		35		140

The native species identified by participants were the European freshwater crayfish ("cangrejo de río" in Spanish) *Austropotamobius pallipes,* the European eel ("anguila") *Anguilla anguilla,* the sea lamprey ("lamprea") *Petromyzon marinus*, the European sea bass *Dicentrarchus labrax* ("lubina"), the flathead gray mullet *Mugil cephalus* ("muil", an Asturias linguistic variant, or "mújol" in standard Spanish)*,* the Atlantic salmon *Salmo salar* ("salmón"), the brown trout *Salmo trutta* ("trucha") and the gilthead sea bream *Sparus aurata* ("dorada") (Supplementary file 2). More than 23 participants in each river recognized *S. trutta* as a native species. Several exotic species introduced from other Spanish regions were considered to be native by some participants: the cyprinid ("madrilla") *Parachondrostoma miegii*; the minnow ("piscardo") *Phoxinus* spp.; the Iberian barbel ("barbo") *Luciobarbus bocagei*; and, the Iberian chub ("cacho") *Squalius carolitertii*. Only one person interviewed in the Raíces River region reported a native species to be an exotic species (*Anguilla anguilla*) (Table S1).

A total of 32 participants (22.86% of the total) correctly identified at least one exotic

species. Seven exotic species were identified: the American crayfish ("cangrejo americano") *Procambarus clarkii;* the pond slider ("tortuga de Florida") *Trachemys scripta;* the common carp ("carpa") *Cyprinus carpio;* the northern pike ("lucio") *Esox lucius*; the goldfish ("carpín") *Carassius auratus*; the brook trout ("salvelino") *Salvelinus fontinalis;* and, the rainbow trout ("trucha arco iris") *Oncorhynchus mykiss* (Table S1). *P. clarkii* was reported as an exotic species by 10, 2, 2 and 3 participants from the Piles, Nalón, Negro and Raíces river regions, respectively. In contrast, C. *carpio* was more frequently reported as an exotic species in the Nalón River (8 participants) than in the Negro River (1 participant) and the other two rivers (no participants).

A few participants (1 in Nalón, 1 in Negro and 3 in Piles) considered the Asian carp *C. carpio* as a native species, and two participants (1 in Nalón and 1 in Piles) regarded the American rainbow trout *O. mykiss* as being native (Table S1). The most frequently reported NIS was *P. clarkii,* which was cited in the four rivers by a total of 17 citizens, followed by *C. carpio* (14 participants in three rivers) and *T. scripta* (6 participants in two rivers). These three species were also the most cited in recent media releases (Table S2).

Significant differences in the knowledge about river species were not found between genders and age groups (data not shown) with the exception of the number of incorrect exotics (I_E). The mistakes about exotic species (I_E) were significantly different between age groups (Kruskal-Wallis Hc=3.97; 3 degrees of freedom (df); P=0.046), and there were clearly fewer mistakes by younger participants (I_E=0±0) than by older participants (I_E=0.06±0.24). The rest of the data were pooled and organized by river. Knowledge on native species (C_N) was significantly different among rivers (Kruskal-Wallis Hc=41.87; 3 df; P=4.27x10^{-9}) (Table 2), and the knowledge levels were clearly lower in the Raíces River region (C_N=0.86±0.60) than in the

rest of the regions (Figure 2). The knowledge of exotic species (C_E) was significantly lower (Kruskal-Wallis Hc=14.13; 3 df; P=0.003) in Negro and Raíces than in the Nalón and Piles river regions (Figure 2). Mistakes about exotic and native species (I_E and I_N, respectively) were generally lower than those about correct species assigned to these categories, and significant differences among rivers were not found (Kruskal-Wallis of Hc=0.62; 3 df; P=0.892 and Hc=4.07; 3 df; P=0.254, respectively). The knowledge index, Ki, was significantly different among river regions (Kruskal-Wallis of Hc=32.97; 3 df; P=3.27x10^{-7}), and the Ki was lower in the Raíces River region than in the other river regions accordingly (Figure 2), as fewer native species were reported from the Raíces River. Significant differences between genders were not found for any question regarding perception/opinion about NIS (data not shown). The two-way ANOVA that considered age and river as factors revealed significant differences between ages and among rivers for Question A, which was about the potential of NIS to adapt in the rivers of the region (F=4.33 with P=0.039 for age; F= 2.86 with P=0.039 for river; F= 0.317 with P=0.813 for interaction).
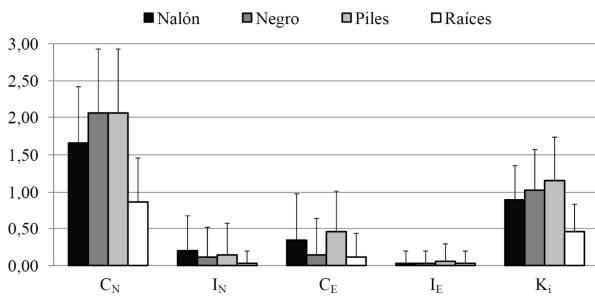

Figure 2. **Mean and standard deviation of public knowledge per basin**. Correct natives (C_N), incorrect natives (I_N), correct exotics (C_E), incorrect exotics (I_E) and knowledge index (K_i) are shown.

Table 2. **Kruskal-Wallis tests of the results analyzed by basin, age or gender.** Results are based on averages across the different sample groups. Significant *P* values for differences among or between sample medians are in bold. C_N and C_E are correct native and correct exotic species identified. I_N and I_E are incorrect native and incorrect exotic species identified.

	C_N	I_N	C_E	I_E	Knowledge index (Ki)	A- Exotic adaptation	B- Harm to natives	C- Ecosystem changes	D-Exotics's removal
Basin	**<0.001**	0.254	**0.003**	0.892	**<0.001**	**0.041**	0.238	0.061	**<0.001**
Age (<50 and >50)	0.625	0.942	0.262	**0.046**	0.660	**0.041**	0.557	0.308	0.784
Gender	0.490	0.283	0.297	0.947	0.385	0.310	0.269	0.634	0.780

Participants in the Nalón area and younger participants perceived, on average, a higher capacity for the adaptation of NIS than other participant groups (Figure 3). For Question D (demanding NIS eradication from Asturias rivers), highly significant differences among rivers were found (F=0.034 with P=0.853 for age; F=8.922 with P=2x10^{-5} for rivers; F=0.287 with P=0.835 for interaction). Participants interviewed in the Raíces River region were less supportive of the eradication of exotic species than those interviewed in other river regions (Figure 3). For the other two questions on how much exotics affect native species (Question B) and how intense the changes perceived in the river ecosystem are (Question C), significant differences were not found among rivers nor between ages (data not shown).
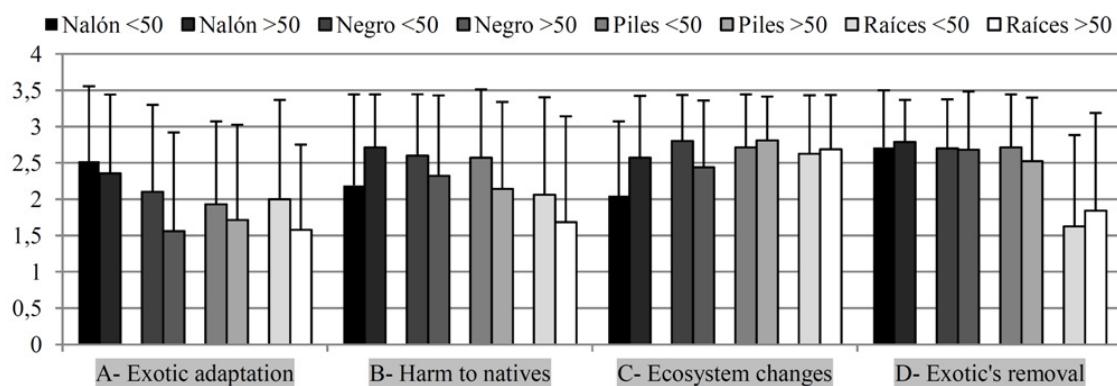

Figure 3. **Mean and standard deviation of perception issues about exotic species per basin and age.** A-"Exotic adaptation" is the answer to the ability of exotic species to adapt in Asturian rivers; B- "harm to natives" is the answer to the ability of exotic species to affect native fauna; C- "Ecosystem changes" is the answer to detection of changes in the ecosystem by the citizens and D- "Exotic's removal" is the answer to the necessity of taking action against exotic species.

Table 3. **Spearman's rs correlation results of the 140 surveys.** The rs and p values are below and above the diagonal, respectively. Significant correlations (after Bonferroni correction) are indicated in bold and significant p-values are highlighted in grey. C_N and C_E are correct native and correct exotic species identified. I_N and I_E are incorrect native and incorrect exotic species identified. Ki = knowledge index. A-Exotic adaptation is the answer to the ability of exotic species to adapt in Asturian rivers; B- harm to natives is the answer to the ability of exotic species to affect native fauna; C- Ecosystem changes is the answer to detection of changes in the ecosystem by the citizens and D-Exotic's removal is the answer to the necessity of taking action against exotic species.

	C_N	I_N	C_E	I_E	A- Exotic adaptation	B- Harm to natives	C- Ecosystem changes	D- Exotics's removal	Knowledge index (Ki)
C_N		0.439	0.150	0.143	0.466	0.083	0.032	3.08x10^{-5}	1.42x10^{-37}
I_N	0.066		0.012	0.452	0.468	0.116	0.442	0.084	0.023
C_E	0.122	0.213		0.391	2.55x10^{-3}	0.149	0.343	0.212	2.14x10^{-8}
I_E	0.125	-0.064	0.073		0.223	0.675	0.228	0.724	0.857
A- Exotic adaptation	-0.062	-0.062	**0.253**	0.104		0.022	0.715	0.021	0.347
B- Harm to natives	0.147	0.134	0.123	0.036	0.193		0.959	5.71x10^{-8}	0.087
C- Ecosystem changes	0.182	0.065	0.081	0.103	0.031	0.004		0.243	0.135
D- Exotics's removal	**0.344**	0.146	0.106	0.030	0.194	**0.439**	0.099		3.21x10^{-4}
Knowledge index (Ki)	**0.835**	-0.192	**0.452**	-0.015	0.080	0.145	0.127	**0.300**	

Table 3 presents pairwise Spearman's rs correlations in the dataset. The knowledge index, Ki, was positively correlated with the knowledge about native and exotic species (Table 3), as expected. Interestingly, after Bonferroni correction, the knowledge index was positively correlated with the demand for the control actions against exotic species, Question D (P=3.21x10^{-4}). The number of correctly identified native species (C_N) was also positively correlated with Question D (control of exotic species) (P=3.08x10^{-5}). The number of correctly identified exotic species (C_E) was consistently positively correlated with the perception of the adaptation ability of the exotic species, which was Question A (P= 2.55x10^{-3}). As also expected, Question B (opinion on how harmful NIS are to native species) was highly positively correlated with Question D, which was the demand for NIS control (P= 5.71x10^{-8}). The main changes detected in the ecosystem by participants (Figure 4) were changes in the river environment (59 participants). More participants from the Nalón River region (12) than from the other zones detected changes in water quality; 7 of them reported improved water quality. For river fauna, in the Negro River region, 11 participants noticed a decrease in the *S. trutta* population in the region. In the river environment category, more citizens in the Piles River region detected changes in the ecosystem, while in the river infrastructure category, many Raíces River participants (11) reported a new promenade near the riverbank.

Regarding the value of public knowledge used for early alerts of exotic species in river systems, in this case study, *Acipenser sturio, Esox lucius* and *Luciobarbus bocagei* were listed by different participants as occurring in the region although they have not yet been found in biodiversity surveys in Asturias rivers (Ministerio de Medio Ambiente 2007). On the other hand, the electrofishing survey detected the pond slider *Trachemys scripta* (Table S1) in the river where the participants reported it. The species is cataloged in the official list of exotic species, but until now it has been reported from only isolated artificial ponds in Gijón and La Granda (Pleguezuelos 2002). Thus, this is the first time the exotic pond slider was found in the wild in this region.

From the electrofishing survey, a total of 8 NIS were found in the region: *Chondrostoma duriense; Cobitis paludica; Gobio lozanoi, Phoxinus* spp.*; Squalius carolitertii; Carassius auratus; Procambarus clarkii;* and, *Trachemys scripta*. Seven native species were sampled: *Anguilla anguilla; Chelon labrosus; Dicentrarchus labrax; Mugil cephalus; Petromyzon marinus; Platichtys flesus;* and, *Salmo trutta* (Table S1). The brown trout *Salmo trutta* was the only species found in all four rivers. The Nalón River contained more NIS (five species), and the Raíces River exhibited the highest proportion of NIS (three NIS out of a total of four species, 75%) (Table 4).

Comparing the aquatic fauna found from the electrofishing survey with the knowledge of the local citizens (Table 4) revealed that the percentage of native species recognized by locals in the four river regions was higher than the percentage of NIS. In the Negro River region, where no NIS and only two native species were found from electrofishing (Table 1S), participants recognized all species surveyed. In the Piles River region, citizens were able to recognize 80% of the surveyed native species. In the Raíces River region, participants recognized the native species but only one of three exotic species. In the Nalón River region, citizens recognized 50% of the native species sampled from the river (the same percentage of the official records).
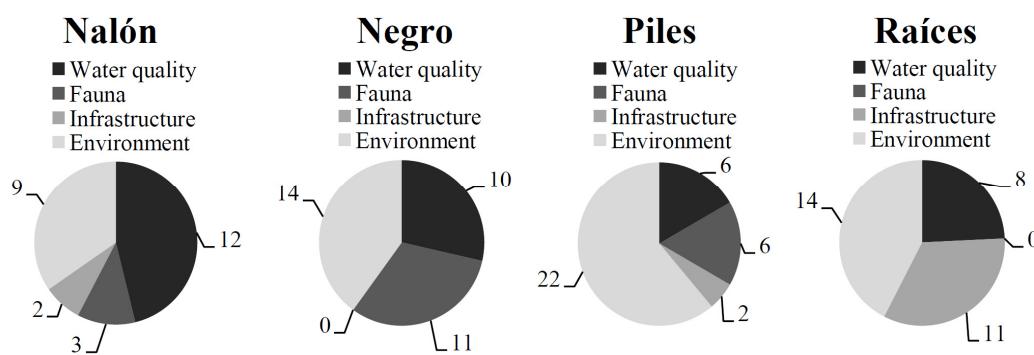

Figure 4. **Changes in the environment reported by the citizens in the four rivers surveyed**. Four groups of changes were considered: changes water quality (including water flow, algae, or sediments), changes regarding river fauna (less *S. trutta*, more *Mugil cephalus*); infrastructure (new ponds, dams, promenade); and environment (cleaner environment, more pollution, more vegetation). Number of citizens in each place expressing each kind of change is shown.

Table 4. **Comparison between real aquatic fauna, official records and local citizens' data**. Two results are considered per river, the number of correct species (native, exotic and total species) listed by volunteers over the total number of species in the region based on official records (over region), and the number of correct species listed by volunteers over the number of species found in the electrofishing survey (over survey). In parenthesis percentage of species recognized by locals over region and over survey is shown.

	Region (N)	Nalón			Negro			Piles			Raíces		
		Electrofishing Survey	Recognized by locals		Electrofishing Survey	Recognized by locals		Electrofishing Survey	Recognized by locals		Electrofishing Survey	Recognized by locals	
			Over region	Over survey		Over region	Over survey		Over region	Over survey		Over region	Over survey
Native	12	4	6 (50%)	2 (50%)	2	5 (41.7%)	2 (100%)	5	6 (50%)	4 (80%)	1	4 (33.3%)	1 (100%)
Exotic	16	5	6 (37.5%)	2 (40%)	0	6 (37.5%)	0 (0%)	2	4 (25%)	0 (0%)	3	2 (12.5%)	1 (33.3%)
Total	28	9	12 (42.9%)	4 (44.4%)	2	11 (39.3%)	2 (100%)	7	10 (35.7%)	4 (57.1%)	4	6 (21.4%)	2 (50%)

Discussion

This case study illustrates the importance of considering the knowledge of citizens and their opinions on treating biodiversity issues. Despite the relatively limited knowledge about NIS, citizens were generally aware of their potential risks. Although only 22.9% of the participants correctly recognized any NIS, as many as 73.6% were of the opinion it is necessary to act against exotic species, and 67.9% believed that NIS could affect native species. Accordingly, there was a positive attitude towards the eradication of the NIS that affect native aquatic fauna. The results were similar to those found in Scotland where 87% of the respondents supported the control and eradication of invasive species (Bremner & Park, 2007).

In our particular case, the correspondence between real data, government reports and citizen data was not accurate in relation to NIS; but, citizens recognized more than 50% of the native species surveyed by electrofishing in each river (Table 4), and they were able to detect one exotic species in running waters in the wild, *Trachemys scripta,* which was previously believed to only occur in artificial ponds (Pleguezuelos 2002) (Table S1). The occurrence of species in the Raíces River was confirmed by electrofishing in our study. This is a case where citizens reported a NIS that was overlooked in official reports, and, as emphasized by many other authors working with invasive species (Gallo & Waitt, 2011; Zenetos et al., 2013; Hawthorne et al., 2015; Kobori et al., 2016; Maistrello et al., 2016), this result reinforces the importance of counting on citizen scientists.

The local knowledge about native species was much greater than that about NIS. More than 80% of the participants listed brown trout as a native species. Brown trout is actually the dominant freshwater species in the region (e.g., Lobón-Cerviá, 2009) and was the only species found from all four rivers considered in this study (Table S1). Interestingly, such knowledge about the native fauna was highly and positively correlated with the demand for NIS eradication (Table 3). Positive correlations between local knowledge and awareness about biodiversity have been found by other authors in Scotland, Chile and the Pyrenees (Bremner & Park, 2007; Loyau & Schmeller, 2017; Zorondo-Rodríguez, Reyes-García, & Simonetti, 2014), and the results of this study are along the same lines.

In the Raíces River region, the knowledge of the native and exotic species was significantly lower than in the rest of river regions, as was the support of actions against NIS. This could be explained by the lower quality environmental conditions in this river. The Raíces River is a small narrow coastal stream (<2 meters wide), with very reduced water flow. The local people may believe that there is not aquatic fauna in the river and conservation efforts are not worthy there. The electrofishing survey revealed a population of the native species *S. trutta*. It also revealed that the Raíces River is invaded by NIS, since 75% of the species surveyed were exotics, including *Phoxinus* spp., *P. clarkii* and *T. scripta*.

Better environmental education will improve the public awareness of NIS and reduce the intentional release of some aquatic species (Zenetos et al., 2013). For instance, *Carassius auratus*, which was found in Gijón, can be purchased in any pet shop and is likely one of the cases of releases from pet owners (Elvira & Almodóvar, 2001; Maceda-Veiga, Domínguez-Domínguez, Escribano-Alacid, & Lyons, 2016), as reported in the Pacific Northwest (Strecker,

Campbell, & Olden, 2011), Iberian Peninsula (Maceda-Veiga, Escribano-Alacid, de Sostoa, & García-Berthou, 2013), and Czech Republic (Lusková, Lusk, Halačka, & Vetešník, 2010). The importance of good environmental education is undeniable. Jordan, Gray, Howe, Brooks, and Ehrenfeld (2011) showed a substantial change in behavior regarding invasive plants after citizens acquired new knowledge about them. Environmental education would reduce the misclassification of species and likely increase the reports of non-native species. In our study, the species officially cataloged as exotics in Spain were considered NIS by the participants, except for two fishes that were misidentified as native species by some respondents: *Cyprinus carpio;* and, *Oncorhynchus mykiss*. These species are old introductions since *C. carpio* was introduced to Spain in the 17th century (Elvira & Almodóvar, 2001) and *O. mykiss* has been farmed in the region for more than 50 years (Stanković, Crivelli, Snoj, Stankovi, & Snoj, 2015). People tend to be more aware of recent introductions (García-Llorente et al., 2008). The most cited exotic in our study was the American crayfish *P. clarkii,* which was identified in all four river regions. In Gijón (Piles River), 10 participants out of the 35 identified *P. clarkii* as an invasive species, compared with two participants in the Nalón and Negro river regions and three in the Raíces River region. This is consistent with the higher awareness about recent introductions (García-Llorente et al., 2008) because this species was found in an artificial pond in downtown Gijón in June 2016, and the discovery was highly publicized in the local newspapers (Table S2).

Although few people recognized any alien species in this study, 77.9% of the participants were able to notice changes in the river ecosystem. Increasing the local knowledge could help control non-native species. In general, citizen science programs are cheaper and more affordable than research programs where scientists obtain the data (Delaney et al., 2008). In our case, the cost would make the monitoring of the rivers and the aquatic fauna at every moment throughout the region impossible without the help of citizen science. As an example, in the Netherlands, Nunes and Van den Bergh (2004) calculated that the benefits of a marine protection program far exceeded the costs with the help of citizen science. Therefore, citizen science programs will help make research cheaper and profitable, especially in this era when mobile phones and applications are continuously renewed throughout the world (Newman et al., 2012). A strategy to develop better responses to invasive species is publicly sharing the information collected (Simpson et al., 2009), as is the case for the open-source atlas of invasive plants of New England created in 2001, which offers

presence/absence data and contributes to many studies (Bois et al., 2011). However, it is necessary to create good cyber infrastructure to manage the vast amounts of data from the citizen science programs (Dickinson et al., 2012; Kobori et al., 2016).

On the other hand, molecular methods, such as environmental DNA (eDNA), have been recently developed for the early detection of exotic species (Clusa et al., 2016; Ficetola, Miaud, Pompanon, & Taberlet, 2008; Thomsen & Willerslev, 2015). Together, eDNA and citizen science could be a promising tool to monitor and avoid the spread of non-native species. For example, researchers trained 20 volunteers to differentiate between the invasive pygmy mussel (*Xenostrobus securis*) and native mussels in Asturias. In one day, volunteers were able to clean the affected area. The eDNA tool made it possible to monitor the population in the region after the cleaning, and the results demonstrated the success of the eradication process (Miralles, Dopico, Devlo-Delva, & Garcia-Vazquez, 2016). Also, in the United Kingdom, the use of volunteers to collect eDNA samples across the country helped to monitor the status of the crested newt (*Triturus cristatus*) (Biggs et al., 2015).

Finally, efforts should focus on explaining the problems caused by NIS to the local population and collaborating with the media to quickly divulge this knowledge to the citizens, both of which could help detect new alien species that may come to the region. Sharing research results with managers will help provide a better understanding of the real fauna and the potential invaders, allowing for the design of better management programs. In addition, the involvement of the general public through citizen science, perhaps coupled with eDNA surveys, would be very helpful to prevent the future spread of present and upcoming NIS in the region.

Conclusion

In this work, we detected how local citizens could be the first to detect significant changes in the ecological environment of rivers and the introduction of any exotic species. Early alert networks will contribute to transferring knowledge of any changes detected to researchers and authorities for a rapid response. There is evidence that action by citizens at an ecosystem level could keep the presence of non-native species under control. For this reason, developing citizen science programs will increase public interest in NIS intervention and may keep citizens in contact with scientific knowledge. With better education about NIS, intentional releases may decrease, and people will be more vigilant about their environment. Moreover, taking advantage of their enthusiasm

and motivation to participate in scientific research is also a strong incentive to share scientific knowledge. In the case of Asturias, the local knowledge about non-indigenous species is not accurate; but, the attitude towards these species makes the region a promising candidate for focused education efforts to help preserve the fauna biodiversity and protect against exotic species.

Acknowledgements

Data Accessibility

- Questionnaire is available in "Supplementary material_1".
- Data obtained from the 140 surveys are available in "Supplementary material_2".
- List of aquatic species and their status in Asturias together with the results from the official inventory, electrofishing results and social survey in the 4 rivers are available in "Supplementary material_3_Table S1"
- Compilation of data from official inventory, electrofishing results, social survey and releases in public media regarding non indigenous species is available in "Supplementary material_4_Table S2".
- All the supplementary material is available in the online repository figshare: https://figshare.com/s/cb7524b36edc574de412 doi: 10.6084/m9.figshare.5357671

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Supplemental material 1.Survey

…………………………………Questionnaire #………………………

River………..

Place:………….Date……….

Personal information

♂	♀		From 20 to 30		From 40 to 50		Older than 60	
		Age	From 30 to 40		From 50 to 60			

Scientific information

1. **Knowledge of aquatic species in the region**

    1.1. *What species do you know that are native from Asturian rivers?*

    1.2. *What species have you ever seen you think are not native from this river?*

2. **Perception about river status and exotic species**

    A. *Do you think exotic species could adapt to Asturian Rivers?*

       Scoring: 3= Yes; 1= No; 0= I don't know

    B. *In your opinion, if an exotic species is able to adapt in these rivers do you think it will affect to native species?*

       Scoring: 3= Yes; 2= It depends on the species; 1= No; 0= I don't know

    C. *Have you noticed any change in biodiversity in this aquatic ecosystem in the last years?*

       Scoring: 3= Yes; 1= No; 0= I don't know

    D. *Do you think it should be necessary to take action against exotic species?*

       Scoring: 3= Yes; 2= "Only if they affect negatively the ecosystem"; 1= No; 0= I don't know.

The information given is voluntary and anonymous. This survey has the objective to identify invasive species in aquatic ecosystems and the results will be used for that purpose.

Supplementary material Table S1. List of aquatic species surveyed and their status in Asturias. The results from the official species list, from the electrofishing inventory and from the local citizen interviews in the 4 rivers (Nalón, Negro, Piles and Raíces) are shown. "X" represents that the species was found. "N" means species considered native by the volunteers. "E" represents exotic species detected by the volunteers. In parenthesis, number of participants citing a species. Misidentifications are shown in bold and highlighted in grey.

Species	Common name	Status in Asturias	Native range	Official records[1]	Nalón River (140 km) Electro fishing (6 sites)	Nalón River (140 km) Survey (N=35)	Negro River (20 km) Electro fishing (1 site)	Negro River (20 km) Survey (N=35)	Piles River (16 km) Electro fishing (1 site)	Piles River (16 km) Survey (N=35)	Raíces River (15km) Electro fishing (1 site)	Raíces River (15km) Survey (N=35)
Acipenser sturio	Sturgeon	Not in the region[1]	Duero, Guadiana and Guadalquivir									**N (1)**
Chondrostoma duriense	Northern straight-mouth nase	Introduced[1]	Duero basin and Galicia	X	X							
Cobitis paludica		Introduced[1]	Central and South Spain	X	X							
Gobio lozanoi	Iberian gudgeon	Introduced[1]	Eastern Cantabrian corridor	X	X							
Luciobarbus bocagei	Iberian barbel	Not in the region[1]	Central Spain			**N (1)**				**N (1)**		
Parachondrostoma miegii		Introduced[1]	Eastern Cantabrian corridor	X		**N (1)**						
Phoxinus sp	minnow	Introduced[1]	France and eastern Cantabrian corridor	X	X	**N (2)**		**N (3)**	X		X	
Squalius carolitertii	Northern Iberian chub	Introduced[1]	Central Spain and Portugal	X	X	**N (1)**						
Tinca tinca	Tench or doctor fish	Introduced[1]	-	X								
Carassius auratus	Goldfish	Exotic[1]	Asia	X								E (1)
Cyprinus carpio	Common carp	Exotic[1]	Asia	X		**N (1)** E (8)		**N (1)**	X	**N (3)** E (1)		
Esox lucius	Northern pike	Not in the region[1]	North America	X		E (1)		E (1)		E (2)		
Gambusia holbrooki	Eastern mosquitofish	Invasive[1,2]	North America	X								
Micropterus salmoides	Largemouth bass	Invasive[1,2]	South of USA and Mexico	X								
Pascifastacus leniusculus	Signal crayfish	Invasive[1,2]	North America	X								
Procambarus clarkii	American crayfish	Invasive[1,2]	North America	X		E (2)		E (2)		E (10)	X	E (3)
Salvelinus fontinalis	Brook trout	Invasive[1,2]	North America	X				E (1)				
Trachemys scripta	Pond slider	Exotic[2,3]	North America	X				E (1)		E (5)	X	
Alosa alosa	Allis shad	Native[1]	-	X								
Anguilla anguilla	European eel	Native[1]	-	X	X	N (2)	X	N (12)	X	N (7)		N (4) **E (1)**

Species	Common name	Status in Asturias	Native range	Official records[1]	Nalón River (140 km)		Negro River (20 km)		Piles River (16 km)		Raíces River (15km)	
					Electro fishing (6 sites)	Survey (N=35)	Electro fishing (1 site)	Survey (N=35)	Electro fishing (1 site)	Survey (N=35)	Electro fishing (1 site)	Survey (N=35)
Chelon labrosus	Thicklip grey mullet	Native[1]	-	X	X							
Dicentrarchus labrax	European sea bass	Native[1]	-	X					X	N (1)		
Lampreta planeri	Brook lamprey	Native[1]	-	X								
Mugil cephalus	Flathead grey mullet	Native[1]	-	X		N (1)			X	N (25)		N (1)
Petromyzon marinus	Sea lamprey	Native[1]	-	X	X			N (1)				
Platichtys flesus	European flounder	Native[1]	-	X					X			
Salmo salar	Atlantic salmon	Native[1]	-	X		N (17)		N (13)		N (8)		N (1)
Salmo trutta	Brown trout	Native[1]	-	X	X	N (33)	X	N (33)	X	N (24)	X	N (24)
Sparus aurata	Gilt-head bream	Native[1]	-	X		N (1)						

1- De la Hoz, J. *Bol. Cien. Nat.* RIDEA, Oviedo, 2006. La gestión de la pesca continental en Asturias (España). Capturas, repoblaciones y actuaciones de mejora del medio. Demanda de cotos y licencias.

2- Spanish invasive species list: BOE 3 de agosto de 2013.

3- Pleguezuelos, J. M. 2002. Las especies introducidas de anfibios y reptiles. Pp: 503-532. En: Pleguezuelos, J.M., Márquez, R., Lizana, M. (Eds.). *Atlas y Libro Rojo de los Anfibios y Reptiles de España.* Dirección General de Conservación de la Naturaleza-Asociación Herpetológica Española (2ª impresión). Madrid

Supplementary material Table S2. Compilation of data from official inventory, electrofishing results, social survey and releases in public media regarding non indigenous species.

Non indigenous species	Official inventory[1,2]	Electrofishing (number of rivers)	Social survey (number of citizens)	Media releases
Chondrostoma duriense	Yes	1 (Nalón)	0	- **October 2014**: http://www.elcomercio.es/gijon/20081020/oviedo/miles-bogas-cangrejos-invaden-20081020.html - **April 2015**: http://www.elcomercio.es/oviedo/201504/13/nuevos-invasores-oviedo-20150413003040-v.html
Cobitis paludica	Yes	1 (Nalón)	0	- **April 2015**: http://www.elcomercio.es/oviedo/201504/13/nuevos-invasores-oviedo-20150413003040-v.html
Gobio lozanoi	Yes	1 (Nalón)	0	- **April 2015**: http://www.elcomercio.es/oviedo/201504/13/nuevos-invasores-oviedo-20150413003040-v.html
Parachondrostoma miegii	Yes	0	1	-
Phoxinus sp	Yes	3 (Nalón, Piles and Raíces)	5	- **December 2016**: http://www.elcomercio.es/asturias/oriente/201612/17/aguas-enol-pierden-claridad-20161217004150-v.html
Squalius carolitertii	Yes	1 (Nalón)	1	- **May 2016**: http://www.lavozdeasturias.es/noticia/asturias/2016/05/25/impuesto-linaje-trucha-exotica-autoctona-asturiana/00031464171435895420698.htm - **May 2016**: http://www.uniovi.es/-/la-universidad-lidera-en-espana-un-proyecto-europeo-para-analizar-los-ecosistemas-de-los-embalses
Tinca tinca	Yes	0	0	- **December 2016**: http://www.elcomercio.es/asturias/oriente/201612/17/aguas-enol-pierden-claridad-20161217004150-v.html
Carassius auratus	Yes	1 (Piles)	1	-
Cyprinus carpio	Yes	0	14	- **May 2016**: http://www.lavozdeasturias.es/noticia/asturias/2016/05/25/impuesto-linaje-trucha-exotica-autoctona-asturiana/00031464171435895420698.htm - **May 2016**: http://www.uniovi.es/-/la-universidad-lidera-en-espana-un-proyecto-europeo-para-analizar-los-ecosistemas-de-los-embalses - **August 2016**: http://www.lne.es/mar-campo/2016/08/18/aumenta-asturias-poblacion-mapaches-origen/1971560.html - **August 2016**: http://www.lne.es/sociedad/2016/08/10/principado-cambia-ley-obligar-pescadores/1968145.html - **February 2017**: http://www.lne.es/economia/2017/02/16/congreso-aprueba-descatalogar-especies-exoticas/2058766.html
Gambusia holbrooki	Yes	0	0	-
Micropterus salmoides	Yes	0	0	- **August 2016**: http://www.lne.es/mar-campo/2016/08/18/aumenta-asturias-poblacion-mapaches-origen/1971560.html - **May 2016**: http://www.lavozdeasturias.es/noticia/asturias/2016/05/25/impuesto-linaje-trucha-exotica-autoctona-asturiana/00031464171435895420698.htm
Oncorhynchus mykiss	Yes	0	5	- **May 2016**: http://www.uniovi.es/-/la-universidad-lidera-en-espana-un-proyecto-europeo-para-analizar-los-ecosistemas-de-los-embalses - **August 2016**: http://www.lne.es/mar-campo/2016/08/18/aumenta-asturias-poblacion-mapaches-origen/1971560.html - **August 2016**: http://www.lne.es/sociedad/2016/08/10/principado-cambia-ley-obligar-pescadores/1968145.html - **February 2017**: http://www.lne.es/economia/2017/02/16/congreso-aprueba-descatalogar-especies-exoticas/2058766.html
Pascifastacus leniusculus	Yes	0	0	- **April 2015**: http://www.elcomercio.es/oviedo/201504/13/nuevos-invasores-oviedo-20150413003040-v.html - **August 2016**: http://www.lne.es/sociedad/2016/08/10/principado-cambia-ley-obligar-pescadores/1968145.html

Non indigenous species	Official inventory[1,2]	Electrofishing (number of rivers)	Social survey (number of citizens)	Media releases
Procambarus clarkii	Yes	1 (Raíces)	17	- **October 2014**: http://www.elcomercio.es/gijon/20081020/oviedo/miles-bogas-cangrejos-invaden-20081020.html - **April 2015**: http://www.elcomercio.es/oviedo/201504/13/nuevos-invasores-oviedo-20150413003040-v.html - **June 2016**: http://www.rtpa.es/asturias:El-estanque-de-la-Plaza-de-Europa,-infestado-de-cangrejos-rojos-americanos_111465903685.html - **June 2016**: http://www.elcomercio.es/gijon/201606/14/aparecen-cangrejos-rojos-estanque-20160614115039.html - **June 2016**: http://www.lne.es/gijon/2016/06/14/detectan-ejemplares-cangrejo-rojo-americano/1942308.html - **June 2016**: http://cadenaser.com/emisora/2016/06/14/ser_gijon/1465927343_384701.html - **June 2016**: http://www.lne.es/gijon/2016/06/15/colonia-cangrejo-rojo-americano-invade/1942652.html - **June 2016**: http://www.elcomercio.es/gijon/201606/15/cangrejo-rojo-invade-plaza-20160615003759-v.html - **June 2016**: http://www.lne.es/gijon/2016/06/16/cangrejo-rojo-toma-isabel-catolica/1943316.html - **June 2016**: http://www.elcomercio.es/gijon/201606/16/ayuntamiento-descarta-presencia-cangrejos-20160616003435-v.html - **August 2016**: http://www.lne.es/sociedad/2016/08/10/principado-cambia-ley-obligar-pescadores/1968145.html - **February 2017**: http://www.elcomercio.es/gijon/201702/13/especies-invasoras-punto-mira-20170213013723-v.html
Salvelinus fontinalis	Yes	0	1	- **August 2016**: http://www.lne.es/mar-campo/2016/08/18/aumenta-asturias-poblacion-mapaches-origen/1971560.html - **August 2016**: http://www.lne.es/sociedad/2016/08/10/principado-cambia-ley-obligar-pescadores/1968145.html
Trachemys scripta	Yes	1 (Raíces)	6	- **August 2016**: http://www.lne.es/mar-campo/2016/08/18/aumenta-asturias-poblacion-mapaches-origen/1971560.html - **June 2016**: http://www.elcomercio.es/gijon/201606/16/ayuntamiento-descarta-presencia-cangrejos-20160616003435-v.html - **June 2016**: http://www.lne.es/oriente/2016/06/22/tortuga-invasora-rio-sella/1946138.html - **November 2016**: http://www.lavozdeasturias.es/noticia/asturias/2016/10/31/cerdoli/0003147793916984266526.htm - **February 2017**: http://www.elcomercio.es/gijon/201702/13/especies-invasoras-punto-mira-20170213013723-v.html

1- De la Hoz, J. *Bol. Cien. Nat.* RIDEA, Oviedo, 2006. La gestión de la pesca continental en Asturias (España). Capturas, repoblaciones y actuaciones de mejora del medio. Demanda de cotos y licencias.
2- Pleguezuelos, J. M. 2002. Las especies introducidas de anfibios y reptiles. Pp: 503-532. En: Pleguezuelos, J.M., Márquez, R., Lizana, M. (Eds.). *Atlas y Libro Rojo de los Anfibios y Reptiles de España*. Dirección General de Conservación de la Naturaleza-Asociación Herpetológica Española (2ª impresión). Madrid

Discusión
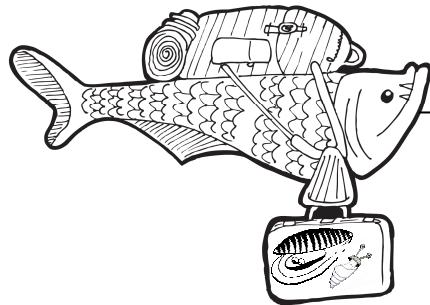
1. El uso del ADN ambiental

La presente Tesis Doctoral es un claro ejemplo de la versatilidad del ADN ambiental y su aplicación en genética de la conservación, pues en ella se ha conseguido diseñar y validar *in situ* marcadores específicos aplicables en ADN ambiental para diez especies invasoras en España y otro marcador para la familia Salmonidae.

Otros estudios resaltan la utilidad del ADN ambiental en diversos campos. Para citar algunos ejemplos en ecología y gestión de especies fluviales, Doi *et al.* (2017a) consiguieron detectar ADN de ayu o pez dulce *Plecoglossus altivelis*, muy apreciado en pesquerías asiáticas, en los lugares donde se había encontrado visualmente en el río Saba (Japón). Yamanaka y Minamoto (2016) comprobaron el avance de especies migradoras aguas arriba de algunas presas usando técnicas de ADN ambiental. En áreas protegidas se recomienda el uso de ADN ambiental porque evita la alteración de las poblaciones nativas que inevitablemente conllevan los métodos de muestreo convencionales (Civade *et al.* 2016). Esto es especialmente importante para el control de especies en peligro en sus lugares de origen, como *Margaritifera margaritifera* (Stoeckle *et al.* 2016), *Lepisosteus oculatus* (Boothroyd *et al.* 2016) y *Zearaja maugeana* (Weltz *et al.* 2017). Respecto a la detección de especies exóticas, Adrian-Kalchhauser y Burkhardt-Holm (2016) identificaron gobios invasores en el río Rin en Suiza. En la eficacia de programas de erradicación (Davison *et al.* 2017) son también provechosas las técnicas de ADN ambiental.

Por otro lado, la aplicación de estas metodologías en sistemas lóticos permite cubrir mayores distancias que los muestreos tradicionales con electro pesca (Evans *et al.* 2017). Sin embargo, hay que tener en cuenta que el uso del ADN ambiental en ríos tiene algunos inconvenientes, precisamente derivados del flujo de agua y el desplazamiento de ADN en ella. Goldberg *et al.* (2014) sugirieron que era imposible inferir la referencia espacial en un sistema lótico a partir de ADN ambiental, ya que este se puede transportar aguas abajo de la fuente de origen. Por ejemplo, Deiner y Altermatt (2014) encontraron ADN de dos invertebrados entre 9 y 12 km aguas abajo de sus poblaciones reales. En otros ejemplos esta distancia no es tan grande; Civade *et al.* (2016) sólo observaron un desplazamiento de unos 2-3 km en el río Tier (Francia). Esta diferencia podría deberse a la diferencia de corriente entre los ríos, pues Jane *et al.* (2015) confirmaron que el transporte de ADN se reducía con velocidades de corriente más pequeñas. En cualquier caso, el uso del ADN ambiental puede dar una visión general de la biodiversidad de un río y todo su ecosistema (Rius *et al.* 2015; Deiner *et al.* 2016).

En los estudios de caso concretos de esta Tesis, el principal objetivo fue detectar la presencia de especies exóticas. Localizar su situación concreta en un punto del río es interesante pero no es el objetivo del ADN ambiental en este caso, que es detectar rápidamente la presencia de una especie en un sistema fluvial. Una vez detectada se debe proceder a realizar muestreos convencionales, localizar las poblaciones o individuos concretos, estimar su abundancia y planificar su gestión (eliminación o contención), pero todos estos procesos no requieren ya el análisis del ADN ambiental, cuya aplicación se limitaría al sistema de alerta temprana.

1.1 Limitaciones del ADN ambiental: Falsos positivos y falsos negativos

Uno de los principales problemas cuando se trabaja con ADN ambiental es la posibilidad de que se produzcan falsos negativos y falsos positivos. Una fuente frecuente de falsos positivos es la posible contaminación en el laboratorio (Thomsen y Willerslev 2015); un exhaustivo protocolo de descontaminación, sumado al uso de controles negativos en cada paso del proceso, son esenciales para evitarlos (Goldberg *et al.* 2016). Estas precauciones se tomaron en este trabajo y

se puede descartar razonablemente que los resultados se deban a problemas de este tipo. También se puede dar el caso de amplificaciones inespecíficas de ADN si los cebadores hibridan en otras especies. Esto se puede descartar mediante la simple secuenciación de los amplicones, que confirmará la especie amplificada (Goldberg *et al.* 2016). Otro método para evitar falsos positivos es usar modelos estadísticos aplicados al número de muestras y al número de positivos o la confirmación con muestreos físicos (Lahoz-Monfort *et al.* 2016). En el presente trabajo, todas las amplificaciones positivas fueron confirmadas por secuenciación, y en muchos casos mediante muestreos físicos. Hay que tener en cuenta que los cebadores desarrollados en este trabajo fueron validados para descartar la amplificación cruzada con las especies que habitan los ecosistemas de aguas del continente europeo. Es posible que no puedan aplicarse directamente en aguas que posean perfiles diferentes de comunidades dulceacuícolas, pues no se puede descartar la amplificación cruzada con especies no ensayadas.

Excluyendo problemas técnicos del método (cebadores que hibriden en varias especies, amplificación inespecífica, etc.), los falsos positivos en una situación de monitoreo de especies corresponderían a ADN ambiental que no procede de organismos vivos. Puede provenir de individuos muertos, heces de depredadores de la especie objetivo, desechos de piscifactorías, restos de cebo y otras fuentes (Merkes *et al.* 2014; Hänfling *et al.* 2016; Clusa *et al.* 2017). El ADN es capaz de resistir por un tiempo en agua fría, incluso cuando los individuos ya no están presentes (Ficetola *et al.* 2008). Por ejemplo, Strickler *et al.* (2015) midieron la degradación del ADN ambiental en un ecosistema de charca, y obtuvieron detecciones positivas de ADN de la rana toro *Lithobates catesbeianus* hasta 54 días después de eliminar los individuos de la charca. En los resultados de esta Tesis, sería posible que el ADN de *Oncorhynchus mykiss* detectado en el Nalón proviniese de desagües de las piscifactorías de la zona y no de individuos reales sueltos en el río, ya que en el muestreo de electropesca posterior no se detectó ningún individuo. Sin embargo, la presencia de especie en libertad en el río Nalón está citada en las estadísticas medioambientales oficiales de Asturias como procedente de escapes de piscifactorías, por lo que es probable que la ausencia de muestras en la electropesca se haya debido a escasa eficacia de ese método físico.

Por otro lado, pueden producirse falsos negativos cuando la especie es poco abundante en un lugar y su ADN se encuentra en baja concentración (Ficetola *et al.* 2008), especialmente si el límite de detección del método no es muy bajo (es decir, es un marcador poco sensible). El diseño de toma de muestras también influye en los falsos negativos, pues a veces la cantidad de ADN liberado al medio cambia según el momento del ciclo de vida de la especie objetivo, y si la muestra medioambiental se toma en una época de poca actividad de la especie es posible que apenas haya moléculas de ADN en el agua (Dostine *et al.* 2013; De Souza *et al.* 2016). También pueden deberse simplemente a fallos en la PCR debido a la presencia de inhibidores en la muestra ambiental (Goldberg *et al.* 2014). Si bien los primeros tipos de falsos negativos son difíciles de evitar, esta última fuente de error fue controlada en esta Tesis empleando controles positivos (PCR a partir de ADN de calidad) en todos los casos. Por otra parte, la sensibilidad de los marcadores desarrollados en este trabajo fue muy elevada en todos los casos, con límites de detección muy bajos, lo que disminuye la probabilidad de que si la especie está presente y libera ADN al agua no se detecte con estos marcadores.

En definitiva, el estudio de la zona de muestreo, el uso de réplicas temporales y espaciales, y un buen protocolo de laboratorio para evitar las posibles contaminaciones pueden ayudar a controlar los falsos positivos y falsos negativos (Ficetola *et al.* 2015; Goldberg *et al.* 2016). Como se indicó anteriormente, es importante recordar que el ADN ambiental no implica la detección de organismos vivos (Comtet *et al.* 2015), por lo que es una herramienta adecuada

para la detección temprana pero en ningún caso reemplaza los métodos convencionales basados en la detección física de los individuos.

1.2 Comparación entre métodos de muestreo

Comparado con los muestreos convencionales físicos (Tabla 2), el uso del ADN ambiental es muy útil especialmente en zonas de difícil acceso o donde no se pueden aplicar estos métodos, como son los embalses (Yamanaka y Minamoto 2016). Los métodos tradicionales como electropesca, pesca con trampas o redes, son más laboriosos, requieren más tiempo y a veces son menos sensibles que el uso del ADN ambiental (Evans *et al*. 2017), en especial al detectar especies raras, poco abundantes o con hábitos nocturnos (Jerde *et al.* 2011). En nuestro estudio, el análisis de ADN ambiental en el Nalón costó 13,4 euros por muestra, sin contar el salario de un técnico por 8 horas de trabajo (Clusa *et al*. 2017), mientras que los muestreos con electropesca costaron 1028 euros por cada punto, además del inconveniente de tener que esperar a condiciones climatológicas favorables.

Refiriéndose a las diferentes opciones de muestreo a partir del ADN ambiental, los marcadores específicos desarrollados en la presente tesis se basan en PCR convencional, que aunque es un método no cuantitativo y sólo determina presencia/ausencia de las especies, es más barato y más rápido que la PCR cuantitativa (qPCR) (Darling y Blum 2007) y tan fiable como qPCR y ddPCR (digital droplet PCR) (Nathan *et al*. 2014). Podrían ser útiles para ayudar al control de las especies invasoras, en especial en lugares donde sólo se requieren datos de presencia/ausencia o con recursos limitados. Por otro lado, también son más rápidos y baratos que las técnicas basadas en secuenciación masiva (Tabla 2) (Comtet *et al*. 2015, Taberlet *et al.* 2012), ya que no necesitan análisis bioinformáticos para interpretar los resultados (Coissac *et al*. 2012). Más aún, en algunos casos el marcador universal amplificado en secuenciación masiva no permite la detección de algunas especies que sí son posibles de detectar con los marcadores específicos (Kelly *et al*. 2014). Este punto se ha comprobado en el presente estudio con varias muestras de la región del Rin, donde *Corbicula fluminea* y *Potamopyrgus antipodarum* fueron detectadas solamente mediante los marcadores específicos y no mediante NGS.

Las técnicas basadas en NGS son sin duda de utilidad cuando se trata de la exploración de comunidades completas. Hoy en día el coste de la secuenciación masiva está disminuyendo aceleradamente, de tal forma que permite hacer estudios de biodiversidad asequibles y en algunos casos más baratos que los muestreos tradicionales (Borrell *et al*. 2017; Sigsgaard *et al*. 2015). Los métodos basados en secuenciación masiva son buenos para la detección temprana de especies invasoras, siempre y cuando se tengan en cuenta las limitaciones del trabajo con ADN ambiental y las limitaciones propias del método, ya que como se ha comentado previamente, en algunos casos se producen falsos negativos derivados de la diferente afinidad de los cebadores por las especies presentes en la muestra (Deagle *et al*. 2014; Kelly *et al*. 2014). Los fallos (falsos negativos y positivos) en NGS se pueden originar también debido a problemas en el procesado bioinformático de los datos (Thomsen y Willerslev 2015), a carencias en las bases de datos de referencia (Comtet *et al*. 2015; Goldberg *et al*. 2016), a fallos en el proceso de secuenciación (Thomsen y Willerslev 2015), a las condiciones de la PCR (Pochon *et al*. 2013; Ushio *et al*. 2017) o incluso a la cantidad de ADN liberada por las distintas especies del ecosistema o por el estadío de vida en el que se encuentran (Thomsen y Willerslev 2015; Minamoto *et al*. 2017). Por todo ello, es recomendable el uso de varias réplicas y varios genes diferentes como Barcodes para estimar la biodiversidad de una muestra mediante NGS (Miya *et al*. 2015; Shaw *et al*. 2016). Al igual que con el uso de marcadores especie-específicos, en caso de obtener secuencias de ADN ambiental de alguna especie invasora que no esté reportada en

una zona, siempre es aconsejable la confirmación de su presencia mediante un método de muestreo convencional.

Tabla 2. Clasificación y principales ventajas y desventajas de cada uno de los métodos de detección utilizados.

				Ventajas	Desventajas
Métodos de detección temprana de especies exóticas	Convencionales	Muestreos físicos	Pesca eléctrica Uso de redes Uso de nasas Buceadores	- Detección visual - Permite recogida de muestras - Identificación de organismos vivos - Identificación de diferentes estadíos - Cuantificación de individuos exóticos	- Posible daño a fauna nativa - Fallo al detectar especies elusivas o de hábitos nocturnos - Alto coste - Dependiente de condiciones climatológicas - Necesidad de taxónomos expertos
	Emergentes	ADN ambiental	Marcadores moleculares específicos	- Rápido - Menos esfuerzo en el muestreo - Barato - Más sensible que muestreos físicos - Más sensible que NGS	- No identificación visual - Carencia en bases de datos - Detección de ADN no de organismos vivos - No permite distinguir estadíos
			Técnicas de secuenciación masiva (NGS)	- Menos esfuerzo en el muestreo - Obtención de biodiversidad global - Detección de mayor número de invasores en una sola PCR - Más sensible que muestreos físicos	- Más caro que el uso de marcadores moleculares - Menos sensible que el uso de marcadores moleculares - Carencia en bases de datos - Detección de ADN no de organismos vivos - No permite distinguir estadíos - Necesidad de usar varios cebadores de distintos genes - Preferencia de cebadores por algunas especies - Necesidad de conocimientos bioinformáticos
		Ciencia ciudadana	Uso de ciudadanos voluntarios	- Barato - Acceso a áreas mayores - Ayuda a reducir introducciones al incrementar conocimiento popular	- Más subjetivo, depende de ciudadanos - Necesidad de entrenamiento y educación - Depende del grado de motivación de los voluntarios - Depende de tecnologías para establecer comunicación entre científicos y voluntarios

1.3 Futuro del ADN ambiental

Las metodologías basadas en el uso del ADN ambiental para la detección de especies exóticas se han desarrollado muy rápidamente en tan sólo unos años; de hecho, los costes del uso de secuenciación masiva se han reducido enormemente (Sigsgaard *et al*. 2015). A pesar de las limitaciones mencionadas de estas técnicas, ya hay estudios que están explorando el uso del ARN ambiental como herramienta para detectar individuos vivos en una muestra (Pochon *et al*. 2017), y la amplificación de genomas completos a partir de ADN ambiental (Deiner *et al*. 2017), ya que los fragmentos más grandes de ADN se degradan antes y podrían ser indicadores de la presencia de organismos vivos (Jo *et al*. 2017).

También se ha conseguido la detección de especies terrestres a partir de ADN ambiental de muestras de agua, como es el caso de mamíferos en peligro en bosques tropicales en Borneo (Ishige *et al*. 2017). El continuo desarrollo de la tecnología está mejorando no solamente las técnicas de secuenciación masiva, sino también las de obtención de muestras, por ejemplo el uso

de drones para recoger muestras de agua en sitios no accesibles (Doi *et al.* 2017b). Un futuro no muy lejano permitirá el uso de estas técnicas de forma rutinaria para controlar la introducción y dispersión de especies exóticas. De hecho, ya han sido de utilidad en programas de erradicación para controlar la total eliminación de especies exóticas (Miralles *et al.* 2016; Davison *et al.* 2017), y algunas técnicas basadas en el uso de la genética ya están implementándose en algunos protocolos de bioseguridad (Amaral *et al.* 2013; Levy *et al.* 2014).

2. El papel de las barreras fluviales en la dispersión de especies invasoras

Es evidente el efecto negativo de las barreras en especies migradoras, ya que obstruyen su paso en el río (Han *et al.* 2008; Limburg *et al.* 2009). En el estudio de caso del río Nalón, con numerosas presas no remontables en su cauce, todas las especies nativas identificadas son diadromas y su presencia está limitada aguas abajo de la primera presa. La única especie nativa detectada por arriba de las barreras es la trucha (*S. trutta*), y parte de estas poblaciones probablemente provienen de introducciones durante el siglo XX creando una población sedentaria (Morán *et al.* 1991). Lo mismo pasa en la parte alpina del Rin, donde la construcción de una presa limita el desplazamiento de la trucha común (*S. trutta*) aguas abajo.

Por otro lado, la presencia de barreras podría tener un efecto positivo evitando la dispersión de especies exóticas (Liermann *et al.* 2012), bloqueando a su vez los contaminantes, parásitos y enfermedades asociados a ellas. Por ejemplo, Rahel (2003) sugirió que la construcción de barreras podría favorecer el ecosistema y mantener bajo control la dispersión de especies exóticas, como fue el caso de la lamprea en la región de los Grandes Lagos en Norteamérica (McLaughlin *et al.* 2007). En un río de Sierra Nevada se consiguió frenar la expansión del cangrejo rojo americano (*Procambarus clarkii*) mediante la construcción de pequeñas presas como método de contención (Dana *et al.* 2011). Las presas podrían proporcionar refugio para especies nativas en peligro aguas arriba, previniendo la dispersión de especies exóticas (Beatty *et al.* 2017). En el estudio de caso del río Nalón, la mayoría de las especies exóticas encontradas se localizan en la zona entre embalses. Aguas arriba de la presa de Tanes, que es la situada más arriba del río, no se encontró ningún pez exótico. De la misma manera, en la región del Rin, aguas arriba de la presa localizada cerca del nacimiento del río no se encontró ninguna especie exótica. La eliminación de las barreras podría ayudar a restablecer la fauna migradora local (Holmquist *et al.* 1998; Magilligan *et al.* 2016), especialmente en casos en los que la especie está amenazada, como puede ser la anguila europea (Clavero y Hermoso 2015), pero hay que tener en cuenta que esta eliminación también podría favorecer la dispersión de especies exóticas. Estos resultados no deberían tomarse como un argumento definitivo para apoyar el mantenimiento de presas impasables en los ríos, sino como un elemento más para evaluarlos caso por caso. Los embalses generados por las presas también pueden servir como reserva de especies exóticas y facilitar así su expansión a zonas cercanas (Havel *et al.* 2015), ya que muchas especies exóticas consiguen establecerse con más facilidad en agua estancada que en agua corriente (Johnson *et al.* 2008). Como ejemplo derivado de los resultados de esta Tesis Doctoral, se observa que la región del alto Rin (entre Basilea y el Lago Constanza), rodeada de numerosas presas de centrales hidroeléctricas, alberga un mayor número de especies exóticas entre las cuales se encuentran el anfípodo *Dikerogammarus villosus*, la almeja asiática *Corbicula fluminea*, el mejillón cebra *Dreissena polymorpha*, el caracolillo del cieno *Potamopyrgus antipodarum* y el gobio *Neogobius melanostomus* (presente Tesis Doctoral; Kalchhauser *et al.* 2013). Todas estas especies son menos abundantes aguas arriba. En este caso, la concentración de embalses actuaría como un albergue o reservorio de especies invasoras, como sucede en el Nalón en las zonas entre embalses. Es difícil predecir qué sucedería si se

restableciera la conectividad a lo largo de toda la cuenca del Rin y si el acceso de las especies nativas serviría para controlar a las exóticas. En cualquier caso, los planes de gestión fluvial deben considerar a las especies exóticas y, de forma muy especial, cuando se trate de cuencas con presas y embalses.

3. Ciencia ciudadana para detección de especies exóticas

La colaboración de ciudadanos locales en estudios de biodiversidad ha resultado muy provechosa a nivel científico, tal y como demuestran diversos estudios (Granek *et al.* 2008; Williams *et al.* 2015). En el estudio de caso del río Nalón, las informaciones proporcionadas por pescadores deportivos sirvieron para detectar el exótico *Squalius carolitertii* cerca del embalse de Rioseco, y también para confirmar la presencia de salmón (*Salmo salar*) en la zona baja del río Nalón, que se había detectado con ADN ambiental pero no mediante pesca eléctrica (Clusa *et al.* 2017). Tiene sentido involucrar a pescadores en estudios de conservación, ya que, junto con los escapes de acuicultura, la introducción de especies para la pesca es razón principal de importación de peces exóticos en ecosistemas fluviales en España (Gozlan 2010). Colaborar con la comunidad de pescadores y hacerles conscientes del problema derivado de la liberación de especies exóticas en los ríos podría ayudar a su control y a reducir las introducciones intencionadas (Zenetos *et al*. 2013).

Los resultados del estudio social realizado dejaron patente la ausencia de conocimiento sobre especies fluviales invasoras en la región, acompañada no obstante de un conocimiento general de los riesgos de las invasiones biológicas y de una actitud positiva hacia la erradicación de estas especies. Solo el 22,9% de los participantes reconocieron alguna especie exótica, pero el 73,6% piensan que es necesario actuar para erradicarlas, y el 67,9% creen que pueden afectar a la ictiofauna nativa. Los resultados son muy coherentes con otro estudio realizado en la Península Ibérica (García-Llorente *et al.* 2008), como en él, no se encontró mucha correspondencia entre los datos reales, los datos oficiales y el conocimiento de los ciudadanos locales en relación a las especies exóticas. Sin embargo, su aportación fue muy valiosa, pues fueron capaces de identificar una especie exótica, la tortuga de Florida (*Trachemys scripta*), en la naturaleza, cuya presencia en los datos oficiales estaba restringida a estanques artificiales (Pleguezuelos 2002). La posterior localización de esta especie mediante electropesca en el río Raíces confirmó la importancia de la colaboración ciudadana para la detección de especies invasoras, algo que ha sido repetidamente confirmado por estudios realizados en otros países (Gallo y Waitt 2001; Hawthorne *et al.* 2015; Kobori *et al.* 2016; Maistrello *et al.* 2016).

La importancia de una buena educación ambiental para la conservación de la biodiversidad es innegable. Contribuiría a reducir la liberación no intencionada de alguna especie acuática, como puede ser el carpín (*Carassius auratus*), que se puede comprar en cualquier tienda de animales y a menudo se suelta en la naturaleza sin conciencia de estar causando un daño a la biota nativa (Elvira y Almodóvar 2001; Maceda-Veiga *et al*. 2013). Por otro lado, la repercusión de los medios en la opinión pública podría dirigirse a recabar colaboración para la detección de especies invasoras. En el presente estudio la especie más citada por los entrevistados fue el cangrejo rojo americano (*Procambarus clarkii*), que casualmente se encontró en un estanque en el centro de Gijón en Junio de 2016 (http://www.lne.es/gijon/2016/06/14/detectan-ejemplares-cangrejo-rojo-americano/1942308.html) y fue ampliamente publicitado en los medios.

La ciencia ciudadana es un método barato y asequible para obtención de datos sobre biodiversidad (Delaney *et al.* 2008), además de ser fácilmente accesible en esta nueva era de tecnología al estar constantemente conectados a través de *smartphones* y de internet (Newman

et al. 2012). La combinación de métodos basados en ADN ambiental junto con programas de ciencia ciudadana podría ser muy útil para la detección temprana y la erradicación y control de especies invasoras. Por ejemplo, Miralles *et al.* (2016) formaron a 20 voluntarios en Asturias para reconocer el mejillón pigmeo invasor *Xenostrobus securis* y distinguirlo de los mejillones nativos del género *Mytilus*. Con su colaboración fueron capaces de limpiar el área afectada, y el ADN ambiental sirvió para confirmar la eficacia del trabajo de los voluntarios para erradicar la especie. En el Reino Unido, Biggs *et al.* (2015) recogieron muestras de ADN ambiental para localizar el tritón (*Triturus cristatus*) gracias a la ayuda de voluntarios. La combinación de ambas estrategias de detección temprana podría ayudar a la localización de posibles invasores y así permitir actuar sobre ellos de una manera más rápida y eficaz. Para que los programas de ciencia ciudadana funcionen habría que centrar los esfuerzos en explicar a la población local los graves problemas causados por especies exóticas, y recabar la colaboración de los medios para distribuir de una manera fácil y rápida este conocimiento, ayudando a prevenir la entrada y dispersión de especies exóticas. En el caso particular de Asturias, el conocimiento público de especies exóticas no está muy acorde con la realidad, pero la actitud respecto a su eliminación y sobre todo hacia la protección de la fauna nativa recalca el potencial de los ciudadanos como medioambientalistas, y hace que esta región sea un buen candidato para centrar esfuerzos en la educación ambiental y, de este modo, ayudar a preservar la biodiversidad local.

4. Riesgos sanitarios y económicos derivados de las especies exóticas encontradas en Asturias:

La introducción de especies exóticas puede entrañar graves peligros sanitarios y ecológicos, además de un gran coste derivado de los procesos de erradicación o de paliación de los impactos producidos. Por ejemplo, en 1991 el desagüe de agua de lastre contaminada con el microbio *Vibrio cholerae* provocó un brote de cólera en Perú con un millón de personas infectadas y diez mil muertes (Kolar y Lodge 2001). En España la llegada del mosquito tigre (*Aedes albopictus*) podría llevar asociada la introducción de enfermedades tropicales como el Dengue (Santos-Sanz *et al.* 2014). En Asturias, las especies exóticas encontradas (Tabla 3), en principio parece que no conllevarían riesgos de salud directos para el ser humano, o al menos no se han descrito hasta ahora, aunque se necesitarían estudios experimentales para descartarlos completamente, teniendo en cuenta que aún se desconocen muchos mecanismos de transmisión de zoonosis entre vertebrados. Según la literatura consultada, sí que suponen riesgos para el resto de especies del ecosistema, y por tanto de forma indirecta para el bienestar del ser humano.

Tabla 3. Especies exóticas acuáticas encontradas en ríos de Asturias.

	Río Nalón	Río Nora	Río Piles	Río Raíces
Carassius auratus			X	
Chondrostoma duriense	X			
Cobitis paludica	X			
Gobio lozanoi	X			
Oncorhynchus mykiss	X			
Phoxinus bigerri	X		X	X
Squalius carolitertii	X			
Potamopyrgus antipodarum		X		
Procambarus clarkii				X
Trachemys scripta				X

Por ejemplo, el cangrejo rojo americano (*P. clarkii*), encontrado en el río Raíces (Tabla 3), es capaz de transmitir el parásito *Aphanomyces astaci* a los cangrejos autóctonos (Aquiloni *et al*. 2010), viéndose la población de estos gravemente disminuida en algunos países europeos en los últimos años debido a este parásito (Chucholl y Schrimpf 2016) (Tabla 4). El carpín (*C. auratus*), encontrado en el río Piles (Tabla 3), puede a su vez introducir numerosos parásitos como *Lernaea cyprinacea* que son capaces de afectar gravemente a otros peces (García-Berthou *et al*. 2007, Leunda 2010). El caracol del cieno (*P. antipodarum*) puede llegar a alcanzar grandes densidades de población, afectando a moluscos nativos por competencia, e incluso a los salmónidos, que lo comen pero resulta ser no apto como alimento para ellos (Zaranko *et al*. 1997; Vinson y Baker 2008).

Por otra parte, el escape de individuos de trucha arco iris (*O. mykiss*) podría acarrear la transmisión de parásitos y enfermedades a las poblaciones nativas de salmónidos a consecuencia de los procesos de vacunación que tienen lugar en piscifactorías, que al inmunizar a las truchas cultivadas contra las infecciones las convierten en portadores asintomáticos de los agentes patógenos (Tabla 4). Un ejemplo es la transmisión del parásito *Lepeophtheirus salmonis* que ha puesto en peligro a especies nativas. Esto ya ha sucedido en Canadá, donde la especie nativa afectada fue *Oncorhynchus gorbuscha* a partir de piscifactorías de salmónidos (Krkosek *et al*. 2007); en Irlanda la especie nativa en peligro fue la misma que hay en Asturias, *Salmo trutta* (Tully *et al*. 1999); en Noruega fue tristemente conocida la transmisión de *Aeromonas salmonicida* al salmón atlántico salvaje, *Salmo salar* (Johnsen y Jensen 1994). Cualquiera de estos casos podría reproducirse en las zonas estudiadas en este Tesis, donde al menos la trucha arco iris parece provenir de escapes de piscifactoría

Tabla 4. Riesgos asociados a las especies exóticas acuáticas encontradas en ríos de Asturias. Se detallan ejemplos de problemas sanitarios producidos en poblaciones piscícolas de otras partes del mundo a partir de la introducción de las especies exóticas halladas en Asturias.

Riesgo asociado	Especie	Efecto	Región	Referencia
Introducción de parásitos a partir de la introducción de especies exóticas	*Carassius auratus*	Transmisión del parásito *Lernaea cyprinacea* a otras especies de ciprínidos	Península Ibérica	García-Berthou *et al*. 2007 Leunda 2010
	Procambarus clarkii	Portador del parásito *Aphanomyces astaci*	Italia	Aquiloni *et al*. 2010
		Transmisión de parásitos al cangrejo autóctono *Austropotamobius torrentium*	Alemania	Chucholl y Schrimpf 2016
Introducción de parásitos como consecuencia de sueltas de piscifactorías	Salmónidos: *Oncorhynchus mykiss, Salmo salar*	Transmisión del parásito *Lepeophtheirus salmonis* a poblaciones nativas de *Oncorhynchus gorbuscha*	Canadá	Krkosek *et al*. 2007
		Transmisión del parásito *Lepeophtheirus salmonis* a poblaciones nativas de *Salmo trutta*	Irlanda	Tully *et al*. 1999
		Dispersión del patógeno *Aeromonas salmonicida*	Noruega	Johnsen y Jensen 1994
		Transmisión del Piscine reovirus (PRV) a salmones salvajes	Noruega	Garseth *et al*. 2013
Alteración de la cadena nutricional de otras especies	*Potamopyrgus antipodarum*	No apto como alimento para salmónidos	Norte América	Zaranko *et al*. 1997 Vinson y Baker 2008

En cuanto al coste económico derivado de la introducción de especies exóticas, en países como Estados Unidos las cifras para paliar sus impactos son astronómicas, rondando los 120.000 millones al año (Pimentel *et al.* 2005). En España la reciente eliminación de las carpas en la Casa de Campo de Madrid ha costado 4 millones de euros (http://www.abc.es/espana/madrid/abci-carmena-vaciara-lago-casa-campo-para-repararlo-porque-tiene-graves-danos-201703150108_noticia.html). Los costes derivados de la introducción del mejillón cebra entre 2001 y 2005 se estiman en más de 2,5 millones de euros en la cuenca del Ebro, y las previsiones llegan a alcanzar los 40 millones de euros en 2025; por ejemplo, en el pueblo de Fayón se destinaron 490.000 euros para la eliminación del mejillón cebra en las canalizaciones de agua potable (Durán *et al.* 2010; Pérez-y-Pérez y Chica-Moreu 2007). En Extremadura se han destinado más de 24 millones de euros en 10 años para la eliminación del calamote o jacinto de agua (*Eichhornia crassipes*), una planta acuática tremendamente invasora (http://www.eldiario.es/eldiarioex/sociedad/peligrosa-Extremadura-coloniza-kilometros-Guadiana_0_443056597.html). En el caso concreto de Asturias el cangrejo rojo americano ha causado ya costes directos a la comunidad al proceder a su erradicación en un estanque de Gijón (http://www.lne.es/gijon/2016/06/15/colonia-cangrejo-rojo-americano-invade/1942652.html). Es difícil estimar las consecuencias económicas de la pérdida del cangrejo nativo *Austropotamobius pallipes*, que se ha visto enormemente reducido tras la introducción del americano. También resulta prácticamente imposible en este momento hacer una estimación en euros del impacto de la invasión de *P. antipodarum* sobre las poblaciones de trucha. En la evaluación de servicios al ecosistema proporcionados por las especies presentes en los ríos no se han empleado valores monetarios sino aproximaciones a la calidad global basadas en los factores ambientales y en las características de las especies piscícolas. Se ha visto que la presencia de especies invasoras supone una degradación de los ecosistemas y suele ser sistémica con otros tipos de alteraciones medioambientales, por ejemplo entre embalses del mismo cauce, lo que empeora aún más la calidad medioambiental fluvial. Probablemente una mayor inversión en desarrollar técnicas de detección temprana y una mejor comunicación entre científicos, ciudadanía y responsables de la Administración ayudaría a reducir los costes ocasionados por la colonización de estas especies invasoras.

5. Recomendaciones para la gestión derivadas de los resultados de la Tesis:

Resulta obvio que la manera más efectiva de actuar contra las especies exóticas es evitar su introducción en un ecosistema. Por ello, una de las recomendaciones urgentes sería la vigilancia de los principales focos de entrada de especies invasoras. En esta Tesis Doctoral se ha observado que el mayor número de especies exóticas se localiza en hábitats degradados como son las zonas portuarias, zonas de embalses y áreas cercanas a grandes urbes. Estos puntos serían prioritarios para la vigilancia y control de EEI.

Otro de los puntos clave es la concienciación ciudadana y la educación medioambiental. Un mayor conocimiento por parte del público ayudaría a reducir las introducciones de especies exóticas e incluso podrían colaborar en su detección temprana.

Por otro lado, el listado de especies encontradas en este trabajo indica que sería necesaria una legislación más estricta respecto a especies ornamentales y de acuario, ya que muchas de las introducciones provienen seguramente de sueltas de mascotas. Por ejemplo, el carpín se puede adquirir en cualquier tienda de animales, además de soltarse como especie decorativa en numerosos estanques artificiales, a partir de los cuales puede acabar fácilmente invadiendo ríos (Maceda-Veiga *et al*. 2013). El continuo cambio climático está favoreciendo

que estas especies de aguas más cálidas se adapten a nuevos ambientes en latitudes anteriormente típicas de climas templados y amplíen su rango de distribución (Rahel y Olden 2008; Chown *et al.* 2015), como es por ejemplo el caso de la almeja asiática en la región del Rin (Crespo *et al.* 2015).

A nivel europeo se ha creado un consorcio de 26 países para desarrollar nuevas herramientas de detección temprana para la protección de los ecosistemas acuáticos en Europa: DNA AquaNet COST Action (http://dnaqua.net/) (Leese *et al.* 2016). En España, cabe destacar la reciente renovación en junio de 2016 del catálogo de especies exóticas invasoras (https://www.boe.es/buscar/act.php?id=BOE-A-2013-8565) debido al éxito del recurso contencioso-administrativo número 1/396/2013, promovido por CODA-Ecologistas en Acción, Sociedad Española de Ornitología (SEO) y Asociación para el Estudio y Mejora de los Salmónidos (AEMS-Ríos con Vida) que ha llevado a la inclusión en el catálogo de especies exóticas invasoras entre otras a las especies *Cyprinus carpio* y *O. mykiss*, antes eliminadas debido a la presión ejercida por pescadores y empresas que se benefician de su actividad (García-Berthou *et al*. 2015). Un pequeño triunfo para la conservación del ecosistema, pero aún hay muchas especies que no están incluidas y cuyo comercio y tráfico no está regulado, como el ya mencionado carpín (*C. auratus*) (Maceda-Veiga *et al.* 2013).

Por último y más importante, es necesario establecer una correcta comunicación entre científicos, políticos y gestores, ya que el estudio o el desarrollo de nuevas medidas de detección no es efectivo si estas no se consiguen implantar en los protocolos de bioseguridad.

Conclusiones/ Conclusions
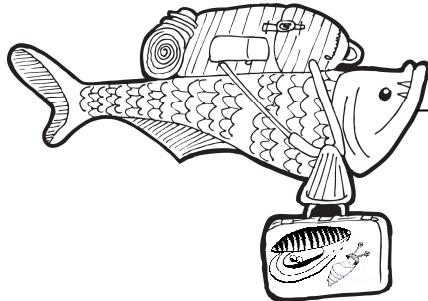
Conclusiones

1) Se ha desarrollado y validado *in vitro* e *in situ* un marcador específico para salmónidos altamente sensible, basado en cebadores específicos de familia, PCR anidada y polimorfismos de restricción, que permite diferenciar distintas especies de salmónidos a partir de una muestra de agua mediante un patrón de restricción característico.

2) Se han diseñado marcadores moleculares basados en cebadores específicos y PCR para la detección de trece especies de peces y moluscos invasores comunes a toda Europa: el pez gato *Ameirus* sp (tanto para *A. melas* como *A. nebulosus*), el pez mosquito *Gambusia* sp (*G. holbrooki* y *G. affinis*), la perca americana *Micropterus salmoides*, el pez sol *Lepomis gibbosus*, la rasbora *Pseudorasbora parva,* el caracolillo del cieno neozelandés *Potamopyrgus antipodarum*, la almeja asiática *Corbicula* sp (*C. fluminea* y *C. fluminalis*), el caracol trompeta *Melanoides tuberculata*, la almeja china del cieno *Sinanodonta woodiana* y el falso mejillón de Conrad *Mytilopsis leucophaeata*. Se han validado en ADN ambiental, y se ha comprobado su eficacia *in situ* en ríos y/o lagos con poblaciones de esas especies exóticas. También se han aplicado con éxito en la región del Rin (Alemania) para establecer el patrón de dispersión de las especies exóticas encontradas, resultando ser más sensibles que la secuenciación masiva.

3) Se ha detectado por primera vez la presencia del caracolillo del cieno neozelandés (*Potamopyrgus antipodarum*) en Asturias, en el río Nora, aplicando el marcador desarrollado sobre ADN ambiental. Se han encontrado dos linajes diferentes en el mismo río que pueden interpretarse como introducciones repetidas de esta especie.

4) Con las metodologías multidisciplinares, incluyendo el ADN ambiental, se han podido identificar los puertos, las zonas entre embalses y las áreas de los ríos cercanas a grandes urbes como los principales focos de introducción de especies exóticas; por ello se puede recomendar la vigilancia de estas zonas para controlar la llegada de nuevos invasores fluviales.

5) Empleando una combinación de metodologías se ha comprobado que las presas y embalses del río Nalón afectan a la diversidad piscícola, que disminuye aguas arriba. Los peces que ofrecen servicios al ecosistema de menor calidad se encuentran localizados entre embalses.

6) Mediante entrevistas a usuarios de los ríos se ha podido detectar en ríos asturianos la tortuga de Florida (*Trachemys scripta*) y el bordallo (*Squalius carolitertii*). Pese a un conocimiento inexacto sobre las especies exóticas existentes en la región, se ha encontrado una actitud favorable a la erradicación de estas especies y una alta concienciación para preservar la naturaleza autóctona.

7) Los riesgos sanitarios derivados de la presencia de las especies exóticas fluviales encontradas en Asturias, en este trabajo, son principalmente de salud medioambiental, destacando el riesgo de parásitos y enfermedades transmisibles a especies autóctonas de cangrejos y peces.

8) Los resultados de esta Tesis respecto a la detección de especies fluviales invasoras en diferentes cuencas y latitudes se han conseguido gracias a una combinación de metodologías basadas en ADN ambiental, muestreos convencionales y ciencia ciudadana. Se recomendaría esta integración metodológica para mejorar las estrategias de monitoreo y control de especies exóticas.

Conclusions

1) An extremely sensitive specific marker for salmonids was designed and validated *in vitro* and *in situ*. It is based on family specific primers, nested PCR and restriction polymorphism sites. It is possible to differentiate the salmonid populations from a water sample by a characteristic restriction pattern.

2) Molecular markers, based on specific primers and PCR, for the detection of thirteen fish and molluscs invasive species common to Europe were designed including: the catfish *Ameirus* sp (both for *A. melas* and *A. nebulosus*), the mosquito fish *Gambusia* sp (*G. holbrooki* and *G. affinis*), the black bass *Micropterus salmoides*, the pumpkinseed *Lepomis gibbosus*, the stone moroko *Pseudorasbora parva*, the New Zealand mudsnail *Potamopyrgus antipodarum*, the Asian clam *Corbicula* sp (*C. fluminea* and *C. fluminalis*), the red-rimmed melania *Melanoides tuberculata*, the Chinese pond mussel *Sinanodonta woodiana* and the Conrad's false mussel *Mytilopsis leucophaeata*. They were validated in environmental DNA and were successfully applied in rivers or lakes with population of these exotic species. They have been also applied in the Rhine region to establish the dispersal pattern of the exotic species found, resulting to be more sensitive than next generation sequencing.

3) For the first time in Asturias, the New Zealand mudsnail (*Potamopyrgus antipodarum*) was detected in Nora river samples through eDNA. In addition, two different lineages have been found in the same river, which may come from different introductions of the species.

4) Ports, areas between reservoirs and river areas near large cities have been identified as the main focus for exotic species introduction based on multidisciplinary methodologies including environmental DNA. It is advisable to monitor these regions to avoid the arrival of other exotic species in the region.

5) Using a combination of methodologies, it has been proved that dams and reservoirs of the Nalón River affect fish diversity, which decreases upstream. The fishes that offer ecosystem services of lower quality are located between reservoirs.

6) Citizen science helped with the detection of the pond slider (*Trachemys scripta*) and the Northern Iberian chub (*Squalius carolitertii*) in Asturian Rivers. Despite a lack in the local knowledge about exotic species, a positive attitude towards the eradication of these species and a high awareness to preserve the autochthonous species were detected.

7) The health risks derived from the presence of exotic species found in Asturias in this work are mainly environmental, highlighting the risk of parasites and transmissible diseases to native species of crabs and fish.

8) The results of this Thesis regarding the detection of invasive species in different basins and latitudes have been achieved thanks to a combination of methodologies based on environmental DNA, conventional sampling and citizen science. This methodological integration would be recommended to improve the monitoring and control strategies of exotic species.

Informe
Factores de Impacto
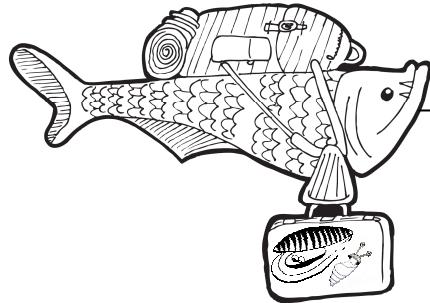
Informe de factores de impacto
(Según Journal Citation Reports ® 2016)

- **Clusa L**, Ardura A, Fernández S, Roca A and Garcia-Vazquez E. 2017. An extremely sensitive nested PCR-RFLP mitochondrial marker for detection and identification of Salmonids in eDNA from water samples. *Peer J* 5: e3045. DOI 10.7717/peerj.3045

Índice de impacto: 2,117

- **Clusa L** and Garcia-Vazquez E. 2018. A simple, rapid method for detecting seven common invasive fish species in Europe from environmental DNA. *Aquatic Conservation: Marine and Freshwater Ecosystems*. DOI: 10.1002/aqc.2890

Índice de impacto: 3,130

- **Clusa L**, Ardura A, Gower F, Miralles L, Tsartsianidou V, Zaiko A and 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

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- **Clusa L**, Miralles L, Basanta A, Escot C and Garcia-Vazquez E. 2017. eDNA for detection of five highly invasive molluscs. A case study in urban rivers from the Iberian Peninsula. *PLoS ONE* 12 (11): e0188126. DOI: 10.1371/journal.pone.0188126

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- **Clusa L**, García-Vázquez E and Machado-Schiaffino G. 2018. Assessing performance of species-specific primers for detecting aquatic invasive species in Lake Constance region using eDNA. Enviado: *Molecular Ecology*

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- **Clusa L**, Fernández S, Dopico E and García-Vázquez E. 2018. The role of barriers in aquatic fauna diversity: a case study in Nalón River, north of the Iberian Peninsula. En revisión: *Scientific reports*.

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- **Clusa L**, Miralles L, Fernández S, García-Vázquez E and Dopico E. 2018. Public knowledge of alien species: a case study on aquatic biodiversity in North Iberian rivers. *Journal for Nature Conservation*. DOI: 10.1016/j.jnc.2018.01.001

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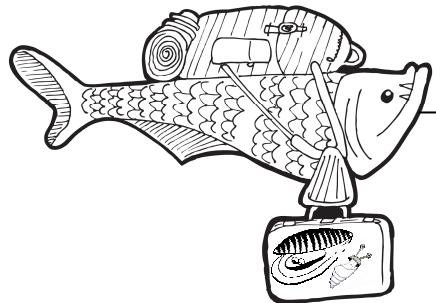
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Anexos
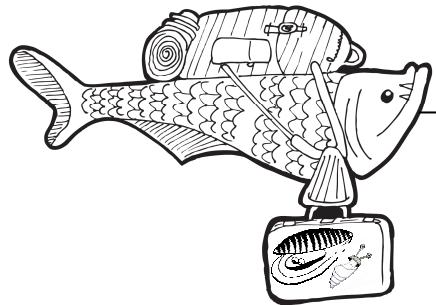
Anexo I. Especies exóticas de agua dulce o estuario presentes en España. Listado de especies exóticas dulceacuícolas presentes en España obtenido de Ministerio de Medio Ambiente (2007), Doadrio *et al.* (2011) y BOE (2013), se detalla cada especie, su origen, año de introducción y motivo así como la distribución de cada una de ellas en las principales cuencas hidrográficas españolas. También se señalan en rojo aquellas especies que particularmente inciden de una forma muy negativa en los ecosistemas ibéricos. (Elvira y Almodóvar 2001, Escot *et al.* 2003, Soler *et al.* 2006, Jarillo y Salgado 2010, Oscoz *et al.* 2010, Pou-Rovira *et al.* 2010, y Miranda y Pino del Carpio 2016).

Peces

Especie	Nombre común	Origen	Año de introducción	Motivo	Catálogo de especies exóticas invasoras: RD 630/2013	Norte (Costa Cantábrica: desde el río Navia hasta el río Bidasoa)	Galicia (Desde río Eo hasta río Limia)	Duero	Tajo	Guadiana	Guadalquivir	Sur (Desde río Guadalete hasta el río Segura)	Levante (Desde río Vinalopó hasta río Cenia, incluyendo cuenca del Júcar)	Ebro	Cataluña (Cuencas del Ter y Llobregat)
Abramis bjoerkna	Brema blanca	Europa	1955	Pesca deportiva									X		
Acipenser baeri	Esturión siberiano	Europa	1995	Acuicultura							X				
Alburnus alburnus	Alburno	Europa	1992	Pesca deportiva	Si			X	X	X	X	X	X	X	X
Ameiurus melas	Pez gato	Norte América	1910-1913	Mejorar poblaciones lago Bañolas	Si				X	X			X	X	X
Aphanius fasciatus	Fartet oriental	Europa	1997	Acuaristas										X	
Australoheros facetus (Cichlasoma facetum)	Chanchito	América del sur	1980-1986	Acuaristas	Si					X				X	
Barbatula barbatula	Lobo de río	Europa	1997	Pesca deportiva: Alimento para truchas				X							
Carassius auratus	Carpín	Asia	Siglo XVII	Ornamental		X	X	X	X	X	X	X	X	X	X
Cobitis bilineata	-	Europa	2000	Pesca deportiva									X		X
Cyprinus carpio	Carpa	Asia	Siglo XVII	Ornamental		X	X	X	X	X	X	X	X	X	X
Esox lucius	Lucio	Europa	1949	Pesca deportiva	Si		X	X	X	X	X	X	X	X	X
Fundulus heteroclitus	Fúndulo	Norte América	1970-1973	Acuaristas	Si						X	X		X	
Gambusia holbrooki	Pez mosquito	Norte América	1921	Control mosquitos (Malaria)	Si		X	X	X	X	X	X	X	X	X
Hucho hucho	Salmón del Danubio	Europa	1968	Pesca deportiva				X							
Ictalurus punctatus	Pez gato punteado	Norte América	1995	Acuicultura / Pesca deportiva	Si					X				X	
Lepomis gibbosus	Pez sol	Norte América	1910-1913	Mejorar poblaciones del lago Bañolas / Acuicultura / Pesca deportiva	Si	X	X	X	X	X	X	X	X	X	X
Micropterus salmoides	Perca americana, Black bass	Norte América	1955	Pesca deportiva	Si	X	X	X	X	X	X	X	X	X	X
Misgurnus anguillicaudatus	Misgurno	Asia	2001	Acuaristas / Cebo en pesca deportiva	Si									X	X
Oncorhynchus kisutch	Salmón del Pacífico	Norte América	1983-1984	Acuicultura				X							
Oncorhynchus mykiss	Trucha arco iris	Norte América	Siglo XIX	Pesca deportiva		X	X	X		X	X	X	X	X	X
Perca fluviatilis	Perca de río, perca Europea	Europa	1970-1979	Pesca deportiva	Si				X					X	X
Poecilia reticulata	Guppy	Norte América	2000	Acuaristas									X		

Grupo	Nombre científico	Nombre común	Origen	Fecha	Vía de entrada / Uso	Si										
	Pseudorasbora parva	Pseudorasbora	Asia	2002	Accidental con acuicultura	Si									X	X
	Rutilus rutilus	Rutilo	Europa	1910-1913	Mejorar poblaciones del lago Bañolas	Si				X					X	X
	Salvelinus fontinalis	Salvelino	Europa	XIX	Pesca deportiva	Si	X		X	X				X	X	
	Sander lucioperca	Lucioperca	Europa	1970-1979	Pesca deportiva	Si			X	X				X	X	
	Scardinius erythrophthalmus	Gardí	Europa	1910-1913	Mejorar poblaciones lago Bañolas	Si					X				X	X
	Silurus glanis	Siluro	Europa	1974	Pesca deportiva	Si			X	X					X	X
Mamíferos	Castor fiber	Castor Europeo	Europa	2003	Conservación										X	
	Mustela vison	Visón americano	Norte América	Década de 1960	Escapes de granjas peleteras	Si	X	X	X					X	X	X
	Myocastor coypus	Coipú	América del sur	Década de 1970	Escapes de granjas peleteras	Si	X									X
Reptiles	Pelodiscus sinensis	Tortuga china de caparazón blando	Asia	1990	Ornamental							X				
	Trachemys scripta	Tortuga de Florida	Norte América	1983	Ornamental	Si	X	X	X	X	X	X	X	X	X	X
	Corbicula fluminea	Almeja de río asiática	Asia	Década de 1980	Accidental asociada a la pesca	Si	X	X	X	X	X	X		X	X	X
	Cordilophora caspia	Hidroide esturialino	Europa	2001	Acuaristas Agua de lastre	Si	X					X		X	X	
Invertebrados no Artrópodos	*Dreissena polymorpha*	Mejillón cebra	Europa-Asia	2001	Accidental asociada a la pesca	Si			X				X		X	X
	Melanoides tuberculata	Caracol trompeta o caracol Malayo	Zonas tropicales África, Asia y Australia	Década de 1980	Acuaristas Accidental asociada a la pesca	Si									X	X
	Mytilopsis leucophaeat[2]	Mejillón de agua salobre/ Falso mejillón de Conrad	Norte América	1993	Agua de lastre	Si						X			X	
	Potamopyrgus antipodarum	Caracolillo del cieno	Nueva Zelanda	Siglo XIX	Accidental asociada la pesca Agua de lastre	Si	X		X	X			X		X	X
	Sinanodonta woodiana	Almeja china del cieno	Asia	2006	Accidental asociada a la pesca											X
	Artemia franciscana	Artemia	Norte América	Década de 1980	Accidental asociado a cultivos de peces								X		X	
Artrópodos crustáceos	Cherax destructor	Cangrejo australiano/ Yabbie	Australia	2003	Cultivo	Si									X	
	Eriocheir sinensis	Cangrejo chino	Asia	Década de 1980	Agua de lastre	Si			X	X		X		X		
	Lernaea cyprinacea	Gusano ancla	Asia	Década de 1960	Acuicultura		X	X	X	X	X	X	X	X	X	X
	Pascifastacus leniusculus	Cangrejo señal	Norte América	1974	Pesca comercial	Si	X	X	X	X					X	
	Procambarus clarkii	Cangrejo rojo americano	Norte América	1974	Pesca comercial	Si	X	X	X	X	X	X	X	X	X	X
					Total /cuenca		11	14	19	20	19	18	15	19	34	26