Universidad de Oviedo
Universidá d'Uviéu
University of Oviedo

Departamento de Bioquímica y Biología Molecular

Programa Oficial de Doctorado en Biología Molecular y Celular

ADN ambiental y sus aplicaciones en la evaluación de conectividad en ecosistemas acuáticos

Environmental DNA and its applications to evaluate connectivity in aquatic ecosystems

Sara Fernández Fernández

Tesis Doctoral

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Obtenido de World Fish Migration Day (WFMD)
https://www.worldfishmigrationday.com y

Y sites Google; Socrasotware.

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y modificado por Sara Fernández Fernández

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: ADN ambiental y sus aplicaciones en la evaluación de conectividad en ecosistemas acuáticos	Inglés: Environmental DNA and its applications to evaluate connectivity in aquatic ecosystems

2.- Autor	
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RESUMEN (en español)

Los ríos son un recurso fundamental para la sociedad. A lo largo de la historia, el ser humano ha establecido sus poblaciones en el entorno de los ecosistemas fluviales para su aprovechamiento y la obtención de agua, energía y otros servicios ecosistémicos. Por ello, los ríos están entre los ecosistemas más explotados del planeta. Los impactos antropogénicos sufridos por los ecosistemas fluviales han producido en ellos una serie de cambios tanto a nivel biológico como fisicoquímico. Entre las alteraciones antropogénicas cabe destacar la fragmentación del hábitat debida a la construcción de barreras, como presas y embalses, que desde mediados del siglo XX son un componente fundamental de los programas de gestión del agua, pero suponen una gran amenaza a la conservación de las especies fluviales. La interrupción de la conectividad fluvial afecta al movimiento de materia, energía y organismos, teniendo gran impacto tanto en la calidad del agua como en la biodiversidad. El mayor impacto que sufren los ecosistemas fluviales a nivel mundial es la construcción de grandes presas y embalses, aunque las pequeñas barreras también pueden afectar de manera muy significativa a la conectividad fluvial y la calidad del agua.

Esta Tesis se centra en primer lugar en el desarrollo de herramientas basadas en ADN ambiental para su aplicación en el seguimiento de la calidad del agua fluvial, cumpliendo así con los requisitos de la Directiva Marco del Agua (DMA; 2000 CE). El uso del ADN ambiental, que implica un muestreo no invasivo, consiste en el seguimiento de las especies a través de los restos de ADN que dejan en el medio ambiente (agua, suelo o sedimentos) fruto de su actividad fisiológica y su interacción con el mismo. Se ha elegido el índice IBMWP (Iberian Bio-Monitoring Working Party), basado en la presencia de familias de macroinvertebrados y estandarizado dentro de los términos de la DMA, para su implementación en muestras ambientales. Se ha utilizado el método de secuenciación de última generación (Next Generation Sequencing, NGS) en ADN extraído de muestras de agua y se han comparado los resultados con los obtenidos mediante métodos convencionales de muestreo e identificación de visu de los macroinvertebrados. Los resultados obtenidos a partir de ambos métodos están significativamente correlacionados, y los datos de ADN ambiental permiten distinguir entre zonas fluviales contaminadas y no contaminadas, confirmando que esta técnica resulta apropiada para la evaluación biológica de la calidad de agua en los ríos.

El ADN ambiental y las técnicas de NGS se emplearon para la evaluación biológica de la calidad del agua en el Río Nalón (Asturias, Cornisa Cantábrica), que resultó ser menor en los tramos afectados por embalses que en los tramos libres. La composición

de familias de macroinvertebrados encontrada fue diferente aguas arriba y aguas abajo de los embalses. El incremento progresivo de biodiversidad aguas abajo esperable en un río íntegro sin alteraciones no se ha encontrado, ya que las zonas localizadas entre embalses mostraron un nivel severo de degradación. Los resultados de este estudio basados en ADN ambiental han demostrado el impacto que suponen los grandes embalses, tanto en la calidad del agua como en la conectividad fluvial.

Las zonas de interés para la conservación se definen legalmente como espacios naturales protegidos para reducir los impactos antropogénicos sobre ellas. Europa posee una de las redes de espacios protegidos más amplia, la Red Natura 2000. En esta Tesis se han aplicado técnicas basadas en ADN ambiental dentro del espacio Natura 2000 Reserva de la Biosfera y Parque Natural de Redes (Asturias, España), en el cual se incluyen dos grandes presas. Se usaron técnicas de PCR cuantitativa (qPCR) y de PCR-RFLPs para el seguimiento de las especies de salmónidos. La qPCR resultó tener una alta sensibilidad, revelando la presencia de ADN de la trucha exótica *Oncorhynchus mykiss* en agua corriente y siendo capaz de detectar la trucha nativa *Salmo trutta* en zonas donde la técnica de PCR-RFLP resultó ineficaz. El ADN ambiental también confirmó la existencia de escapes de piscifactorías de trucha arco iris (*Oncorhynchus mykiss*) dentro de la zona protegida en estudio.

También dentro de esta Reserva de la Biosfera de Redes se emplearon técnicas de muestreo convencionales como la electropesca, y se contó con la colaboración de pescadores locales que aportaron muestras de sus capturas deportivas. Combinando estos datos junto con análisis de ADN ambiental, se pudo demostrar la existencia de introducciones ilegales de especies exóticas de peces dentro del Parque Natural. Además, se ha visto que las especies de Ciprínidos introducidas se encuentran en expansión, probablemente debido a una combinación del aumento de temperaturas debido al cambio climático junto con repetidas sueltas ilegales de peces.

Los estudios realizados en esta Tesis permiten la recomendación de una serie de acciones para la gestión de los ecosistemas fluviales. La más sencilla es evaluar sistemáticamente los ecosistemas fluviales, vigilando tanto la calidad del agua como la introducción de especies. Una metodología basada en ADN ambiental similar a la empleada en este trabajo podría utilizarse en primera instancia como método exploratorio de elección, ya que es una técnica no invasiva y proporciona una visión global de la biota. Al aplicarla en muestras de agua del río se encuentra ADN no sólo de especies acuáticas obligadas, sino también de especies terrestres que viven en la cercanía del río, lo que sugiere que se podrían hacer evaluaciones a mayor escala espacial con un esfuerzo de muestreo relativamente pequeño. Otra recomendación que se desprende de los resultados de esta Tesis es la necesidad de un mayor control para evitar escapes de piscifactorías dentro de las áreas protegidas. Finalmente, la restauración de la conectividad mediante escalas para especies migratorias sería la principal medida de gestión recomendada para el mantenimiento de la biodiversidad, a la vista de los resultados obtenidos.

Este trabajo constituye un ejemplo de la utilidad potencial del ADN ambiental para la evaluación sistemática de los ecosistemas fluviales. Aunque aún hay que validar y estandarizar las técnicas de análisis en diversos aspectos, como la mejora de las bases de datos de referencia y de paneles de cebadores con amplio espectro taxonómico, se espera que los desarrollos futuros del ADN ambiental permitan su aplicación en numerosas áreas que van desde la evaluación de la conectividad hasta los inventarios de biota en espacios protegidos, e incluso en genética de poblaciones.

RESUMEN (en Inglés)

Rivers are an important resource for the society. Humans have historically established their populations around rivers to obtain water, energy and other ecosystem services. Consequently, they are among the most impacted ecosystems on earth. Anthropogenic impacts affect freshwater ecosystems changing both physio-chemical and biological components. Within these alterations, habitat fragmentation due to constructions of barriers such as dams and reservoirs, represents a threat to river species with multiple impacts, so it has taken importance over the years in conservation programs. The interruption of river connectivity affects movements of energy, matter and organisms with main ecological implications regarding water quality and biodiversity. The biggest impact caused on rivers' connectivity around the world is the construction of big dams and reservoirs; moreover, small barriers can also significantly affect river connectivity. This study focused first on the development of DNA-based methodologies aimed at routine monitoring to evaluate river water quality, complying with the requirements of the Water Framework Directive (WFD; 2000 EC). The use of environmental DNA (eDNA) that implies a non-invasive sampling technique was the choice method. It consists on monitoring species from the DNA traces they expel on the environment (water, soil or sediment samples). The WFD-standard Iberian Bio-Monitoring Working Party (IBMWP) index based on presence-absence of macroinvertebrate families was estimated from conventional and eDNA metabarcoding, and the results were highly correlated. The eDNA-based technique can distinguish between polluted and no polluted sites and was confirmed in this study as a sensitive tool for river water biomonitoring. The metabarcoding approach allowed the biological evaluation of water quality along River Nalón basin (Asturias, Bay of Biscay). Results showed a lower water quality in sites affected by dams. The composition of macroinvertebrate communities was different between sampling points located upstream and downstream dams. The progressive increase of biodiversity downstream expected in undisturbed rivers was not found, since the river areas located between dams were severely impacted. This study based on eDNA demonstrated the heavy impact of dams and reservoirs on both river connectivity and water quality.

To reduce human impacts, some spaces have been legally protected, especially in regions that are a focus of conservation efforts. Europe has one of the largest networks of protected spaces (Natura 2000 network). In the present PhD Thesis, eDNA technologies were applied within the Biosphere Reserve and Natural Park of Redes (Asturias, northern Spain), a protected area that has two big dams. Quantitative PCR (qPCR) and PCR-RFLPs techniques have been employed on eDNA to monitor dominant Salmonids. qPCR has proved to be highly sensitive, revealing the presence of exotic rainbow trout (*Oncorhynchus mykiss*) DNA in running waters and detecting the native brown trout (*Salmo trutta*) in areas where the PCR-RFLP technique was inefficient to do it. Using eDNA, escapes of *Oncorhynchus mykiss* from fish farms within the protected Reserve were confirmed.

Further work in the Biosphere Reserve involved conventional sampling techniques such as electrofishing and the collaboration of local anglers, who provided samples from their own catch. This combined methodological approach revealed the illegal

introduction of exotic species in Redes Natural Park. Introduced Cyprinids are in expansion there, likely due to climate warming and perhaps continuous illegal re-introductions.

These studies have served to recommend some management actions. Perhaps the easiest action is a regular monitoring of rivers, which is necessary to check both water quality and species introductions. The eDNA method here developed could be employed as a first approach because it is non-invasive and gives an overview of biota. When eDNA techniques are applied in river water, terrestrial species are also found, suggesting that larger biodiversity assessments are possible with little sampling effort. On the other hand, increasing efforts for controlling fish farm escapes inside protected areas is another recommendation. Indeed, restoring river connectivity through passages enabling migratory species to circulate up and down barriers is the main management action that can be recommended, from the results of this Thesis.

The results presented here are examples of the usefulness of eDNA for routinely river monitoring. Although further validation, standardization of methodologies and reduction of costs are still needed, upcoming research in the eDNA field would promote its use in a variety of study areas such as connectivity assessment, biodiversity inventories in protected spaces, and even in population genetics.

SR. PRESIDENTE DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO EN BIOLOGÍA MOLECULAR Y CELULAR

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FORMULARIO RESUMEN DE TESIS POR COMPENDIO

1.- Datos personales solicitante	
Apellidos: Fernández Fernández	Nombre: Sara

Curso de inicio de los estudios de doctorado	2015/2016

	SI	NO
Acompaña acreditación por el Director de la Tesis de la aportación significativa del doctorando	X	
Acompaña memoria que incluye		
Introducción justificativa de la unidad temática y objetivos	X	
Copia completa de los trabajos *	X	
Resultados/discusión y conclusiones	X	
Informe con el factor de impacto de la publicaciones	X	
Se acompaña aceptación de todos y cada uno de los coautores a presentar el trabajo como tesis por compendio	X	
Se acompaña renuncia de todos y cada uno de los coautores a presentar el trabajo como parte de otra tesis de compendio	X	

* Ha de constar el nombre y adscripción del autor y de todos los coautores asi como la referencia completa de la revista o editorial en la que los trabajos hayan sido publicados o aceptados en cuyo caso se aportará justificante de la aceptación por parte de la revista o editorial

Artículos, Capítulos, Trabajos	
Trabajo, Artículo 1	
Titulo (o título abreviado)	Environmental DNA for freshwater fish monitoring: insights for conservation within a protected area.
Fecha de publicación	6/03/2018
Fecha de aceptación	20/02/2018
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Sí
Factor de impacto	2.118

Coautor2 ☐ Doctor ☒ No doctor . Indique nombre y apellidos	Miguel Menéndez Sandín
Coautor3 ☐ Doctor ☒ No doctor . Indique nombre y apellidos	Paul G. Beaulieu
Coautor4 ☒ Doctor ☐ No doctor . Indique nombre y apellidos	Laura Clusa Cuesta
Coautor5 ☒ Doctor ☐ No doctor . Indique nombre y apellidos	Jose Luis Martínez Fernández
Coautor6 ☒ Doctor ☐ No doctor . Indique nombre y apellidos	Alba Ardura Gutiérrez
Coautor7 ☒ Doctor ☐ No doctor . Indique nombre y apellidos	Eva García Vázquez

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Trabajo, Artículo 2

Titulo (o título abreviado)	Evaluating freshwater macroinvertebrates from eDNA metabarcoding: A river Nalón case study.
Fecha de publicación	08/08/2018
Fecha de aceptación	20/07/2018
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Sí
Factor de impacto	2.766

Coautor2	☐ Doctor	☒ No doctor .	Saúl Rodríguez Martínez
Coautor3	☒ Doctor	☐ No doctor .	Jose Luis Martínez Fernández
Coautor4	☒ Doctor	☐ No doctor .	Yaisel Juan Borrell Pichs
Coautor5	☒ Doctor	☐ No doctor .	Alba Ardura Gutiérrez
Coautor6	☒ Doctor	☐ No doctor .	Eva García Vázquez
Coautor7	☐ Doctor	☐ No doctor .	Indique nombre y apellidos

Trabajo, Artículo 3

Titulo (o título abreviado)	How can eDNA contribute in riverine macroinvertebrate assessment? A metabarcoding approach in the Nalón River (Asturias, Northern Spain).
Fecha de publicación	En prensa
Fecha de aceptación	En prensa (20/04/2018)
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Sí
Factor de impacto	4.122

Coautor2	☐ Doctor	☒ No doctor .	Saúl Rodríguez Martínez
Coautor3	☒ Doctor	☐ No doctor .	Jose Luis Martínez Fernández
Coautor4	☒ Doctor	☐ No doctor .	Eva García Vázquez
Coautor5	☒ Doctor	☐ No doctor .	Alba Ardura Gutiérrez
Coautor6	☐ Doctor	☐ No doctor .	Indique nombre y apellidos
Coautor7	☐ Doctor	☐ No doctor .	Indique nombre y apellidos

Trabajo, Artículo 4

Titulo (o título abreviado)	Non-indigenous fish in protected spaces: trends in species distribution mediated by illegal stocking.
Fecha de publicación	En revisión
Fecha de aceptación	En revisión
Inclusión en Science Citation Index o bases relacionadas por la CNEAI (indíquese)	Sí
Factor de impacto	2.988

INFORME DE FACTORES DE IMPACTO

(según Journal Citation Reports® 2017)

- **Fernandez S**, Sandin MM, Beaulieu PG, Clusa L, Martinez JL, Ardura A, García-Vázquez E. (2018) Environmental DNA for freshwater fish monitoring: insights for conservation within a protected area. Publicado en *PeerJ.*

  Índice de impacto: 2,118

- **Fernández S**, Rodríguez S, Martínez JL, Borrell YJ, Ardura A, García-Vázquez E (2018) Evaluating freshwater macroinvertebrates from eDNA metabarcoding: A river Nalón case study. Publicado en PLoS ONE.

  Índice de impacto: 2,766

- **Fernández S**, Rodríguez-Martínez S, Martínez J.L, Garcia-Vazquez E, Ardura A. (2018) How can eDNA contribute in riverine macroinvertebrate assessment? A metabarcoding approach in the Nalón River (Asturias, Northern Spain). En prensa en Scientific Reports.

  Índice de impacto: 4,122

- **Fernández S**, Arboleya E, Dopico E, Ardura A, Garcia-Vazquez E. (2018). Non-indigenous fish in protected spaces: trends in species distribution mediated by illegal stocking. En revisión en Aquatic Conservation: Marine and Freshwater Ecosystems.

  Índice de impacto: 2,988

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Directora: Eva García Vázquez

Co-directora: Alba Ardura Gutiérrez

Oviedo, 2018

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A lo largo del camino que me ha llevado a realizar esta Tesis me he encontrado con el apoyo de muchas personas a las que me gustaría agradecer que hayan dedicado parte de sí mismos para que esto fuera posible:

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A Ellas,

A Adri por creer en mi,

Papá, mamá y Sergio

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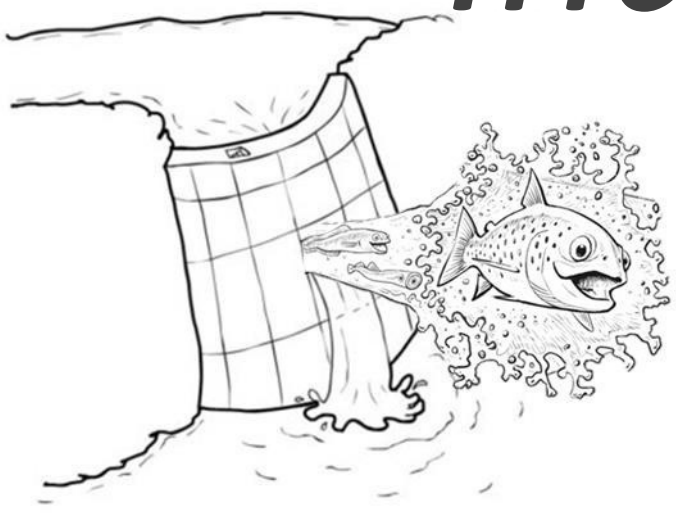

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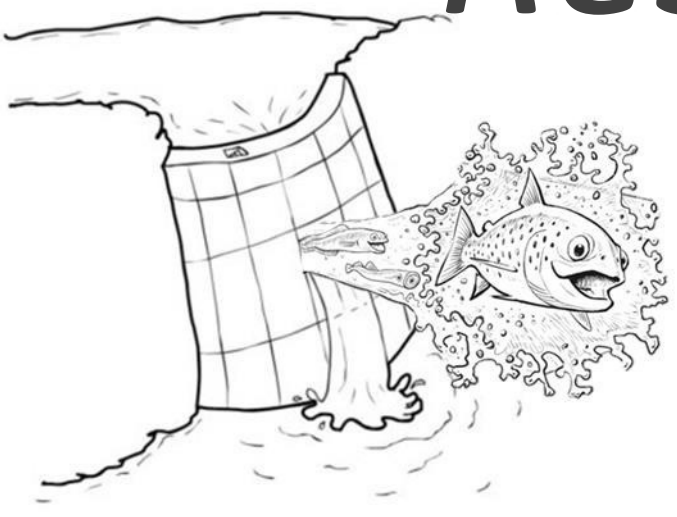
Resumen



Los ríos son un recurso fundamental para la sociedad. A lo largo de la historia, el ser humano ha establecido sus poblaciones en el entorno de los ecosistemas fluviales para su aprovechamiento y la obtención de agua, energía y otros servicios ecosistémicos. Por ello, los ríos están entre los ecosistemas más explotados del planeta. Los impactos antropogénicos sufridos por los ecosistemas fluviales han producido en ellos una serie de cambios tanto a nivel biológico como fisicoquímico. Entre las alteraciones antropogénicas cabe destacar la fragmentación del hábitat debida a la construcción de barreras, como presas y embalses, que desde mediados del siglo XX son un componente fundamental de los programas de gestión del agua, pero suponen una gran amenaza a la conservación de las especies fluviales. La interrupción de la conectividad fluvial afecta al movimiento de materia, energía y organismos, teniendo gran impacto tanto en la calidad del agua como en la biodiversidad. El mayor impacto que sufren los ecosistemas fluviales a nivel mundial es la construcción de grandes presas y embalses, aunque las pequeñas barreras también pueden afectar de manera muy significativa a la conectividad fluvial y la calidad del agua.

Esta Tesis se centra en primer lugar en el desarrollo de herramientas basadas en ADN ambiental para su aplicación en el seguimiento de la calidad del agua fluvial, cumpliendo así con los requisitos de la Directiva Marco del Agua (DMA; 2000 CE). El uso del ADN ambiental, que implica un muestreo no invasivo, consiste en el seguimiento de las especies a través de los restos de ADN que dejan en el medio ambiente (agua, suelo o sedimentos) fruto de su actividad fisiológica y su interacción con el mismo. Se ha elegido el índice IBMWP (Iberian Bio-Monitoring Working Party), basado en la presencia de familias de macroinvertebrados y estandarizado dentro de los términos de la DMA, para su implementación en muestras ambientales. Se ha utilizado el método de secuenciación de última generación (Next Generation Sequencing, NGS) en ADN extraído de muestras de agua y se han comparado los resultados con los obtenidos mediante métodos convencionales de muestreo e identificación de visu de los macroinvertebrados. Los resultados obtenidos a partir de ambos métodos están significativamente correlacionados, y los datos de ADN ambiental permiten distinguir entre zonas fluviales contaminadas y no contaminadas, confirmando que esta técnica resulta apropiada para la evaluación biológica de la calidad de agua en los ríos.

El ADN ambiental y las técnicas de NGS se emplearon para la evaluación biológica de la calidad del agua en el Río Nalón (Asturias, Cornisa Cantábrica), que resultó ser menor en los tramos afectados por embalses que en los tramos libres. La composición de familias de macroinvertebrados encontrada fue diferente aguas arriba y aguas abajo de los embalses. El incremento progresivo de biodiversidad aguas abajo esperable en un río íntegro sin alteraciones no se ha encontrado, ya que las zonas localizadas entre embalses mostraron un nivel severo de degradación. Los resultados de este estudio basados en ADN ambiental han demostrado el impacto que suponen los grandes embalses, tanto en la calidad del agua como en la conectividad fluvial.

Las zonas de interés para la conservación se definen legalmente como espacios naturales protegidos para reducir los impactos antropogénicos sobre ellas. Europa posee una de las redes de espacios protegidos más amplia, la Red Natura 2000. En esta Tesis se han aplicado

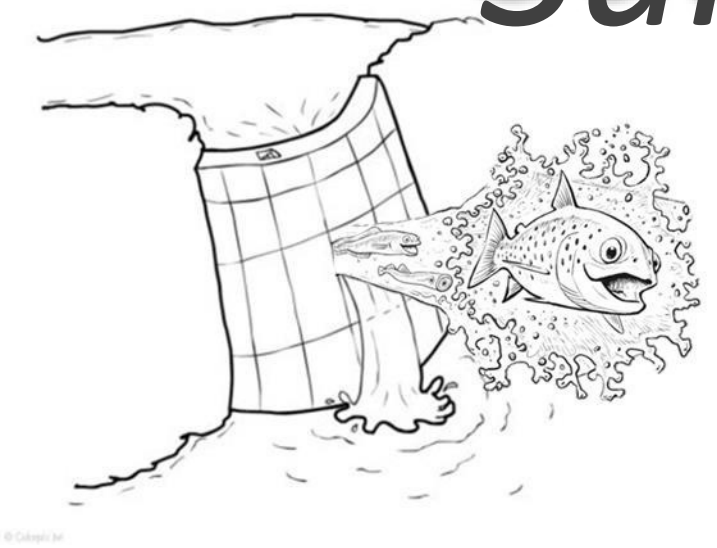
técnicas basadas en ADN ambiental dentro del espacio Natura 2000 Reserva de la Biosfera y Parque Natural de Redes (Asturias, España), en el cual se incluyen dos grandes presas. Se usaron técnicas de PCR cuantitativa (qPCR) y de PCR-RFLPs para el seguimiento de las especies de salmónidos. La qPCR resultó tener una alta sensibilidad, revelando la presencia de ADN de la trucha exótica *Oncorhynchus mykiss* en agua corriente y siendo capaz de detectar la trucha nativa *Salmo trutta* en zonas donde la técnica de PCR-RFLP resultó ineficaz. El ADN ambiental también confirmó la existencia de escapes de piscifactorías de trucha arco iris (*Oncorhynchus mykiss*) dentro de la zona protegida en estudio.

También dentro de esta Reserva de la Biosfera de Redes se emplearon técnicas de muestreo convencionales como la electropesca, y se contó con la colaboración de pescadores locales que aportaron muestras de sus capturas deportivas. Combinando estos datos junto con análisis de ADN ambiental, se pudo demostrar la existencia de introducciones ilegales de especies exóticas de peces dentro del Parque Natural. Además, se ha visto que las especies de Ciprínidos introducidas se encuentran en expansión, probablemente debido a una combinación del aumento de temperaturas debido al cambio climático junto con repetidas sueltas ilegales de peces.

Los estudios realizados en esta Tesis permiten la recomendación de una serie de acciones para la gestión de los ecosistemas fluviales. La más sencilla es evaluar sistemáticamente los ecosistemas fluviales, vigilando tanto la calidad del agua como la introducción de especies. Una metodología basada en ADN ambiental similar a la empleada en este trabajo podría utilizarse en primera instancia como método exploratorio de elección, ya que es una técnica no invasiva y proporciona una visión global de la biota. Al aplicarla en muestras de agua del río se encuentra ADN no sólo de especies acuáticas obligadas, sino también de especies terrestres que viven en la cercanía del río, lo que sugiere que se podrían hacer evaluaciones a mayor escala espacial con un esfuerzo de muestreo relativamente pequeño. Otra recomendación que se desprende de los resultados de esta Tesis es la necesidad de un mayor control para evitar escapes de piscifactorías dentro de las áreas protegidas. Finalmente, la restauración de la conectividad mediante escalas para especies migratorias sería la principal medida de gestión recomendada para el mantenimiento de la biodiversidad, a la vista de los resultados obtenidos.

Este trabajo constituye un ejemplo de la utilidad potencial del ADN ambiental para la evaluación sistemática de los ecosistemas fluviales. Aunque aún hay que validar y estandarizar las técnicas de análisis en diversos aspectos, como la mejora de las bases de datos de referencia y de paneles de cebadores con amplio espectro taxonómico, se espera que los desarrollos futuros del ADN ambiental permitan su aplicación en numerosas áreas que van desde la evaluación de la conectividad hasta los inventarios de biota en espacios protegidos, e incluso en genética de poblaciones.

Summary



Rivers are an important resource for the society. Humans have historically established their populations around rivers to obtain water, energy and other ecosystem services. Consequently, they are among the most impacted ecosystems on earth. Anthropogenic impacts affect freshwater ecosystems changing both physio-chemical and biological components. Within these alterations, habitat fragmentation due to constructions of barriers such as dams and reservoirs, represents a threat to river species with multiple impacts, so it has taken importance over the years in conservation programs. The interruption of river connectivity affects movements of energy, matter and organisms with main ecological implications regarding water quality and biodiversity. The biggest impact caused on rivers' connectivity around the world is the construction of big dams and reservoirs; moreover, small barriers can also significantly affect river connectivity. This study focused first on the development of DNA-based methodologies aimed at routine monitoring to evaluate river water quality, complying with the requirements of the Water Framework Directive (WFD; 2000 EC). The use of environmental DNA (eDNA) that implies a non-invasive sampling technique was the choice method. It consists on monitoring species from the DNA traces they expel on the environment (water, soil or sediment samples). The WFD-standard Iberian Bio-Monitoring Working Party (IBMWP) index based on presence-absence of macroinvertebrate families was estimated from conventional and eDNA metabarcoding, and the results were highly correlated. The eDNA-based technique can distinguish between polluted and no polluted sites and was confirmed in this study as a sensitive tool for river water biomonitoring. The metabarcoding approach allowed the biological evaluation of water quality along River Nalón basin (Asturias, Bay of Biscay). Results showed a lower water quality in sites affected by dams. The composition of macroinvertebrate communities was different between sampling points located upstream and downstream dams. The progressive increase of biodiversity downstream expected in undisturbed rivers was not found, since the river areas located between dams were severely impacted. This study based on eDNA demonstrated the heavy impact of dams and reservoirs on both river connectivity and water quality.

To reduce human impacts, some spaces have been legally protected, especially in regions that are a focus of conservation efforts. Europe has one of the largest networks of protected spaces (Natura 2000 network). In the present PhD Thesis, eDNA technologies were applied within the Biosphere Reserve and Natural Park of Redes (Asturias, northern Spain), a protected area that has two big dams. Quantitative PCR (qPCR) and PCR-RFLPs techniques have been employed on eDNA to monitor dominant Salmonids. qPCR has proved to be highly sensitive, revealing the presence of exotic rainbow trout (*Oncorhynchus mykiss*) DNA in running waters and detecting the native brown trout (*Salmo trutta*) in areas where the PCR-RFLP technique was inefficient to do it. Using eDNA, escapes of *Oncorhynchus mykiss* from fish farms within the protected Reserve were confirmed.

Further work in the Biosphere Reserve involved conventional sampling techniques such as electrofishing and the collaboration of local anglers, who provided samples from their own

catch. This combined methodological approach revealed the illegal introduction of exotic species in Redes Natural Park. Introduced Cyprinids are in expansion there, likely due to climate warming and perhaps continuous illegal re-introductions.

These studies have served to recommend some management actions. Perhaps the easiest action is a regular monitoring of rivers, which is necessary to check both water quality and species introductions. The eDNA method here developed could be employed as a first approach because it is non-invasive and gives an overview of biota. When eDNA techniques are applied in river water, terrestrial species are also found, suggesting that larger biodiversity assessments are possible with little sampling effort. On the other hand, increasing efforts for controlling fish farm escapes inside protected areas is another recommendation. Indeed, restoring river connectivity through passages enabling migratory species to circulate up and down barriers is the main management action that can be recommended, from the results of this Thesis.

The results presented here are examples of the usefulness of eDNA for routinely river monitoring. Although further validation, standardization of methodologies and reduction of costs are still needed, upcoming research in the eDNA field would promote its use in a variety of study areas such as connectivity assessment, biodiversity inventories in protected spaces, and even in population genetics.

Introducción

1. Los ecosistemas fluviales y su integridad

Los ríos representan una fracción muy pequeña del agua de la biosfera, en torno a un 0.006% del total de agua dulce; sin embargo, juegan un papel fundamental como recurso hídrico para la sociedad. Prueba de ello es que a lo largo de la historia, el ser humano ha localizado sus asentamientos en el entorno de masas fluviales que le han servido como fuente de recursos, proveyéndole de una serie de bienes y servicios entre los que destacan la obtención de agua potable y energía, los usos industriales y el desarrollo de actividades lúdicas o recreativas (Malmqvist & Rundle 2002). El intenso aprovechamiento de los ríos, no sólo como fuente directa de recursos (como agua y alimentos) sino también por la amplia gama de servicios ecosistémicos que proporcionan (p. ej. regulación de inundaciones, depuración del agua) (Costanza et al. 1997), los ha situado dentro de los ecosistemas más explotados del planeta.

Las alteraciones antropogénicas de los ecosistemas fluviales conllevan una serie de impactos sobre su estructura y funcionamiento, tanto a nivel fisicoquímico como biológico. Entre las principales alteraciones cabe destacar la fragmentación del cauce, que impide la conectividad aguas arriba y abajo del río. En las últimas décadas, restaurar la conectividad fluvial se ha convertido en una de las grandes prioridades de los programas de conservación (Hermoso et al. 2012; Correa Ayram et al. 2016).

La conectividad fluvial puede definirse como la transferencia de materia, energía y organismos dentro de las unidades espaciotemporales del sistema hidrológico (Wohl 2017). Las alteraciones producidas en esta conectividad tienen efectos muy complejos, y sus consecuencias dependerán del sistema afectado y su composición ecológica, de manera que la conectividad se verá afectada en diferente forma según las características de la red fluvial y los ciclos de vida de las especies que en ella habitan.

Las alteraciones en la conectividad afectan al ecosistema tanto a nivel de flujos energéticos y de materia, como a nivel biológico. Respecto a este último, el grado en el que las especies se ven afectadas será distinto según sus mecanismos y capacidad de dispersión. Por ejemplo, para las que disponen de sistemas de dispersión terrestre, la conectividad poblacional dependerá en menor medida de la estructura de la red fluvial que en el caso de especies cuya dispersión es dependiente de la corriente de agua (Crook et al. 2015). Si se construye una barrera en el río, los ciclos de vida de las especies con dispersión exclusiva a través de la red fluvial se verán más comprometidos.

A pesar de la gran variedad de ciclos vitales y mecanismos de dispersión, Crook et al. (2015) han identificado varios impactos antropogénicos que afectan de forma común a la mayoría de las especies al alterar significativamente la conectividad de los ecosistemas fluviales. No siempre se ve afectada de la misma manera, sino que la acción humana sobre la conectividad fluvial puede modificar la conectividad en diferente dirección, unas veces aumentándola y otras disminuyéndola. Los impactos mencionados por Crook et al. (2015) son los siguientes:

- El desarrollo urbano, agrícola, industrial y forestal: deriva en alteraciones físicas y químicas del hábitat, incluyendo el de los ríos, y en las zonas más afectadas impide el establecimiento de especies sensibles, dificultando por tanto la conectividad espacial de sus poblaciones a lo largo de la cuenca.
- El cambio climático: aunque no hay muchos ejemplos de cambios en los patrones de distribución de especies asociados directamente a los efectos del cambio climático en los ríos, la alteración de regímenes fluviales y aumento de las temperaturas están bien documentados y son conductores potenciales del cambio en la distribución de especies. Su efecto podría ser tanto aumentar como disminuir la conectividad poblacional, dependiendo de la latitud y las circunstancias concretas.
- Los trasvases entre cuencas son otro ejemplo de la acción humana, y actuarían aumentando la conectividad. Permiten la dispersión de especies entre cuencas, algo que no tendría lugar de manera natural.
- La regulación de caudales: provoca cambios en la composición del hábitat y de las especies, reconectando y aislando metapoblaciones alternativamente.
- La construcción de presas y embalses (pequeñas y grandes barreras): contrariamente a lo que ocurre en los ecosistemas terrestres y marinos, en los que hay múltiples rutas para la dispersión, las características de los ríos, que son esencialmente una masa de agua relativamente estrecha moviéndose continuamente en la misma dirección, hacen que los efectos de la interrupción de la conectividad tengan más impacto sobre las especies acuáticas que habitan en ellos. La construcción de barreras, por muy pequeñas que sean, puede dificultar muy significativamente el movimiento de estas especies tanto aguas arriba como aguas abajo. Esta Tesis se centra en estas barreras, siempre teniendo en cuenta que no pueden desligarse unos factores de otros y que probablemente el impacto de un embalse será diferente en una zona baja muy urbanizada que en un paisaje poco habitado de alta montaña.

2. Presas y embalses

Las presas y embalses se han usado a lo largo de la historia para el almacenamiento de agua, como recurso imprescindible para el desarrollo de la sociedad, siendo parte integral de las infraestructuras de la Humanidad (Lafuente et al. 2006). Se ha calculado que existen cerca de unos 50.000 embalses operativos en el mundo con una altitud de presa mayor de 15 m y una capacidad entre 7.000 y 8.300 km^3, y por su elevado número y densidad en la mayoría

de las cuencas representan una de las principales alteraciones de los ecosistemas fluviales tanto a nivel europeo como mundial (Lehner et al. 2011).

El nivel real de fragmentación de las cuencas fluviales aún no se conoce bien. Además de las grandes presas existen otras muchas barreras bloqueando los ríos, de las cuales la mayoría no está documentada. En Europa se estima que hay más de un millón de barreras en cauces fluviales (Figura 1).

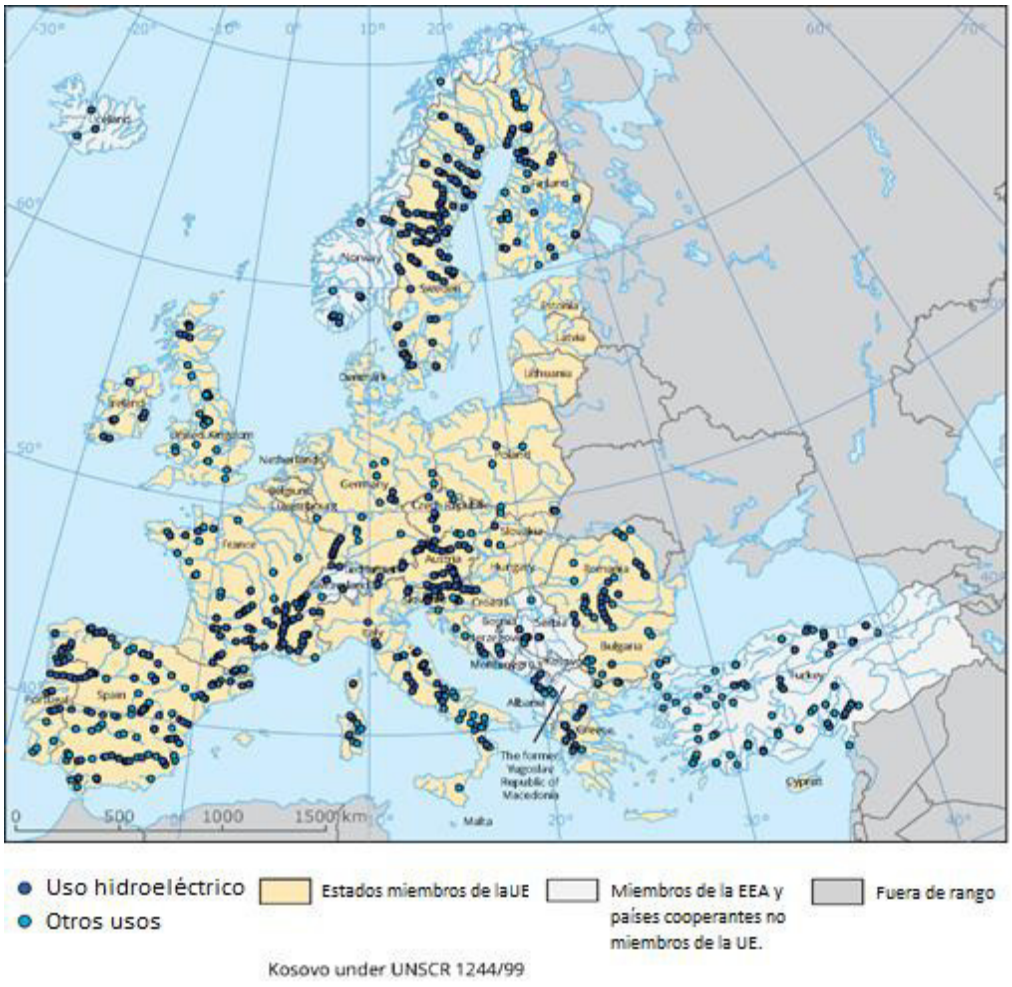


Figura 1. Mapa de embalses y barreras en Europa. Se representan aquellos embalses situados en ríos con una cuenca fluvial de más de 10.000 km^2. Imagen modificada desde: European Environment Agency (https://www.eea.europa.eu/data-and-maps/figures/dams-with-reservoirs-on-rivers).

La presencia de estas barreras en los ríos genera una serie de alteraciones en la biodiversidad y en la calidad del agua de las zonas afectadas (McCartney 2009). Los impactos que se producen como resultado de la obstaculización de la continuidad de los intercambios que tienen lugar a lo largo del sistema (Teoría del río continuo; Vannote et al. 1980), han sido clasificados en dos grandes categorías: impactos en la calidad del agua, que están provocados por las variaciones de caudal y flujos térmicos, cambios en la composición química y en la sedimentación; e impactos en la biodiversidad, que se producen a través de cambios en la producción primaria, de alteraciones en las condiciones del hábitat de las

especies, como por ejemplo los macroinvertebrados bentónicos como se verá más adelante, o la pérdida de hábitats terrestres tras la construcción de embalses. También, obviamente, se producen por el bloqueo de las rutas migratorias de las especies que viven exclusivamente en el agua.

Además de los efectos directos como los cambios fisicoquímicos y el bloqueo de movimientos de especies, hay otros efectos derivados de la construcción de embalses, entre los que se encuentran los vertidos ilegales y las repoblaciones incontroladas. Al tratarse de sistemas creados por el hombre, no se realizan esfuerzos para la conservación del medio y de las especies que se establecen en él; por ello, los embalses suelen ser receptores de introducciones de diversos organismos entre los que se incluyen especies exóticas (Johnson et al. 2008), que son en muchos casos empleadas en la pesca deportiva de forma directa o como cebo. Las condiciones particulares de los embalses, en los que la calidad y temperatura del agua, la profundidad y la modificación de la corriente no son favorables para muchas especies nativas, sí que pueden serlo sin embargo para las exóticas, creándose así un efecto sinérgico entre las sueltas incontroladas y el deterioro medioambiental que hace que se vea incrementada la capacidad invasora de estas especies resistentes por las condiciones degradadas de los embalses (Mcallister et al. 2001).

3. Espacios protegidos

Los espacios protegidos son zonas del ecosistema en las que las que se reduce la presión humana reduciendo su uso y las actividades que en ellos se desarrollan, lo que permite, teóricamente, que el sistema se recupere y vuelva su estado original o al menos se reduzca la huella ecológica humana (Suski & Cooke 2007). Cualquier zona del río se puede ver afectada como consecuencia de las alteraciones causadas por el hombre, por eso se han creado zonas de especial protección en las que la explotación humana se reduce o prohíbe totalmente, tratando de evitar así su degradación. En general, estas áreas protegidas tienen como función principal preservar la biodiversidad y conservar el ecosistema (Gaston et al. 2008). Las estrategias básicas para la creación de espacios protegidos suelen estar basadas en las amenazas predominantes que sucedan en los ecosistemas objeto de protección. En el caso de los ríos se han descrito como amenazas predominantes las perturbaciones por uso del suelo, la alteración hidrológica y las introducciones de especies no nativas (Saunders et al. 2002).

Muchas de las más de 100.000 zonas protegidas a nivel mundial incluyen ecosistemas fluviales con un alto valor de conservación. Entre ellas, algunas han sido designadas específicamente para la conservación del río (Kingsford et al. 2011). Normalmente, estas zonas se corresponden con una pequeña fracción del sistema, y es común que abunden los arroyos de alta montaña, al tratarse de territorios tradicionalmente poco poblados que se adecuan en gran medida a las figuras de protección (Aparicio et al. 2000).

Europa posee una de las redes de áreas protegidas más extensa del mundo, que se conoce como red Natura 2000 (Gallardo et al. 2017). Dentro esta red hay diferentes tipos de figuras de protección, que varían según el nivel de restricción. Por ejemplo, hay espacios protegidos en los que está permitida la pesca y/o los usos agrarios y ganaderos, de manera más o menos sostenible, y otros sin embargo en los que no se permite ningún tipo de uso o explotación (Eagles et al. 2002). Por ello, las implicaciones de gestión dependerán mucho del tipo de zonas a gestionar.

Para designar una zona protegida y planificar su posterior seguimiento, se debe determinar en primer lugar su estado de conservación. Para ello es fundamental, entre otras cosas, la caracterización biológica: saber qué especies están presentes y cuál es el estado de sus poblaciones. Las potenciales alteraciones dentro de las zonas protegidas, tanto en la biota como en el hábitat, cobran gran importancia por su mayor vulnerabilidad o interés de conservación (Nagendra et al. 2013). En el caso concreto del hábitat acuático, en Europa y en el resto del mundo se emplean indicadores de calidad que miden el estatus ecológico del río y que se comentan brevemente a continuación.

4. Indicadores biológicos de calidad de agua

En Europa se ha establecido la Directiva Marco del Agua (DMA en adelante; 2000 CE) con el fin de conservar los ecosistemas acuáticos mediante su uso sostenible. Dentro de esta Directiva hay un objetivo principal que consiste en alcanzar un estatus de buena calidad ecológica en todas las masas de agua europeas. Con el fin de implementar esta Directiva, los Estados miembro han desarrollado una serie de medidas para la evaluación rutinaria de la calidad del agua, que es el componente fundamental para la conservación de un buen estado ecológico de los ecosistemas acuáticos (Birk 2003). Esta serie de medidas están basadas normalmente en la información proporcionada por índices multimétricos. Dentro de estos índices una de las herramientas más comúnmente utilizadas son los indicadores biológicos (AQEM Consortium 2002), cuyo uso está ampliamente extendido en todos los programas europeos de evaluación de la calidad del agua.

Los indicadores biológicos son organismos que, o bien acumulan sustancias tóxicas, o bien responden de manera diferencial al estrés mediambiental (por ejemplo, son más o menos tolerantes a la contaminación), y por ello pueden ser utilizados como indicadores del estado de un ecosistema (Birk et al. 2012). Los peces, las diatomeas y los macroinvertebrados acuáticos son los grupos utilizados como indicadores biológicos del estatus de los ecosistemas fluviales. Los macroinvertebrados bentónicos son el grupo más utilizado para el cálculo de la calidad del agua (Buss et al. 2015). Es un grupo taxonómicamente heterogéneo que está compuesto fundamentalmente por moluscos, anélidos y artrópodos, siendo estos últimos los más abundantes en la mayoría de las zonas fluviales. Estos indicadores biológicos reflejan la degradación de los ecosistemas acuáticos gracias a que presentan distintos grados de tolerancia a la contaminación del agua. Basados en estas tolerancias diferenciales, se calculan los índices de calidad del agua que indican el grado de degradación o

conservación de un determinado sistema (Wallace et al. 1996). En la Península Ibérica, el índice utilizado con este propósito es el IBMWP (Iberian Bio-Monitoring Working Party), que puntúa la presencia de las distintas familias de invertebrados bentónicos según su tolerancia a la contaminación y tiene en cuenta la tipología de los ríos ibéricos (Alba et al. 2005; Pujante et al. 2011).

5. El ADN ambiental

Los organismos dejan rastros de ADN en el medio ambiente que son producto de su actividad fisiológica, de sus movimientos y de la interacción con el medio, y se encuentran en forma de restos celulares, tejidos, secreciones, etc. A partir de muestras ambientales como suelo, sedimentos o agua, y mediante el empleo de diferentes técnicas moleculares, este ADN se puede utilizar para la identificación de especies (Thomsen & Willerslev 2015) (Figura 2).

Figura 2. Proceso para la aplicación de técnicas de ADN ambiental.

Inicialmente empleadas para el inventario de comunidades procariotas (Zhou et al. 1996; Cowan et al. 2005; Daniel 2005), las técnicas basadas en la secuenciación masiva de amplicones obtenidos a partir de ADN ambiental se aplican para identificar organismos eucariotas en las últimas décadas. Los usos del ADN eucariota ambiental son muy variados. En los últimos años de desarrollo de esta herramienta, las mejoras biotecnológicas han permitido su aplicación en diversos campos que van desde la antropología física (Slon et al. 2017) hasta los inventarios de biodiversidad (Ji et al. 2013; Valentini et al. 2016), el seguimiento de especies individuales (Zhan et al. 2013; Goldberg et al. 2011), o la detección de organismos invasores (Ardura et al. 2015; Sigsgaard et al. 2015; Clusa et al. 2017).

El uso del ADN ambiental está siendo tan extenso porque llega donde a veces las técnicas convencionales no pueden (Rees et al. 2014). Se trata de una herramienta genética muy sensible que resulta útil para la detección de especies que se encuentran en baja densidad, por ejemplo aquellas que están amenazadas o en peligro de extinción cuyo número suele ser muy bajo en la naturaleza, o en la localización temprana de especies invasoras cuando se encuentran en las primeras etapas de la invasión, cuando tienen unas densidades de población todavía reducidas y son por tanto difíciles de detectar (Jerde et al. 2011). Además de su rápido análisis (cuando se analiza mediante PCR y cebadores especie-específicos) y su alta sensibilidad, el muestreo de ADN ambiental conlleva la ventaja añadida de ser no invasivo, al utilizar muestras del medio como agua o sedimentos; por lo tanto, la integridad de los organismos eucariotas objeto de estudio no se ve comprometida. Otra ventaja es que puede cubrir áreas mucho más extensas con menor esfuerzo de muestreo que los muestreos convencionales (Deiner et al. 2016).

Todas estas características hacen que el ADN ambiental sea una herramienta idónea para su aplicación en espacios protegidos, donde es esencial evitar el impacto sobre las especies objeto de estudio. Dos ejemplos muy claros de la utilidad del ADN ambiental para el seguimiento de especies dentro de estos ecosistemas fluviales protegidos son los peces y los macroinvertebrados bentónicos. En el caso de los peces, muchos tienen hábitos nocturnos o se camuflan con el ambiente, por lo que son difíciles de localizar de visu. Además, las técnicas que se utilizan rutinariamente para el muestreo de peces, como la electropesca, tienen gran impacto sobre los individuos y el medio en que se realizan los muestreos (Snyder 2004). Los macroinvertebrados, por su parte, son un grupo muy extenso que incluye diferentes taxones. Se describen comúnmente como invertebrados que se ven a simple vista por el ojo humano, sin necesidad de utilizar óptica de aumentos (Alba et al. 2005). Las características diagnósticas para su identificación son a veces difíciles de distinguir y su caracterización requiere extraer a los individuos del río (Birk 2003). Tanto los peces como los macroinvertebrados son muy importantes a la hora de caracterizar cualquier sistema fluvial, ya que los peces contienen algunas especies que están en la cima de la cadena trófica y son indicadores de la conectividad longitudinal del sistema, mientras que los macroinvertebrados representan un recurso trófico imprescindible de los primeros, y además son indicadores de la calidad del agua, como se ha visto anteriormente.

6. El río Nalón y el Parque Natural de Redes

Para desarrollar herramientas de ADN que permitan analizar el impacto de las barreras fluviales sobre la conectividad del ecosistema, es conveniente elegir con cuidado un estudio de caso en el cual se encuentren tanto tramos de río afectados por embalses como tramos libres, así como zonas degradadas medioambientalmente junto con otras bien conservadas, con escaso o nulo estrés medioambiental. En esta Tesis se ha elegido como estudio de caso el río Nalón en Asturias, que incluye tanto una zona montañosa de alto valor ecológico

aguas arriba como varios embalses, y áreas agrícolas e industriales muy degradadas aguas abajo.

El Nalón es uno de los ríos más grandes e importantes de la vertiente cantábrica de la Península Ibérica, con 140.8 km de longitud y 55.18 m^3/s de caudal medio anual. A lo largo de su cauce hay cinco embalses, dos de ellos con escalas para peces, cuyos usos van desde el abastecimiento y el control de riadas hasta la realización de actividades lúdicas (principalmente pesca deportiva). La cuenca del Nalón-Narcea ha sufrido una degradación histórica fruto de la presencia de minas de carbón, que se ha visto reducida desde su cierre progresivo tras la entrada de España en la Comunidad Europea. Esta degradación es evidente en las zonas más bajas del río a pesar de los esfuerzos realizados para su restauración. Se puede encontrar una descripción general del estado del ecosistema de este río en https://www.chcantabrico.es/rios/nalon (accesible en octubre de 2018).

En contraste con la degradación en los tramos medio y bajo, la zona alta del río Nalón se encuentra protegida con la figura de Parque Natural, El Parque Natural de Redes. La declaración de esta zona como tal tuvo lugar en el año 1996, fruto del valor atribuido a su gran diversidad faunística y extensiones de bosque, pero también por albergar dos grandes embalses, que abastecen de agua potable a la zona central de Asturias (Figura 3). Además de ser Parque Natural, este espacio protegido también fue declarado Reserva de la Biosfera por la UNESCO en 2001, Zona de Especial Protección para Aves (ZEPA) en 2003, y Lugar de Importancia Comunitaria (LIC) en 2004.

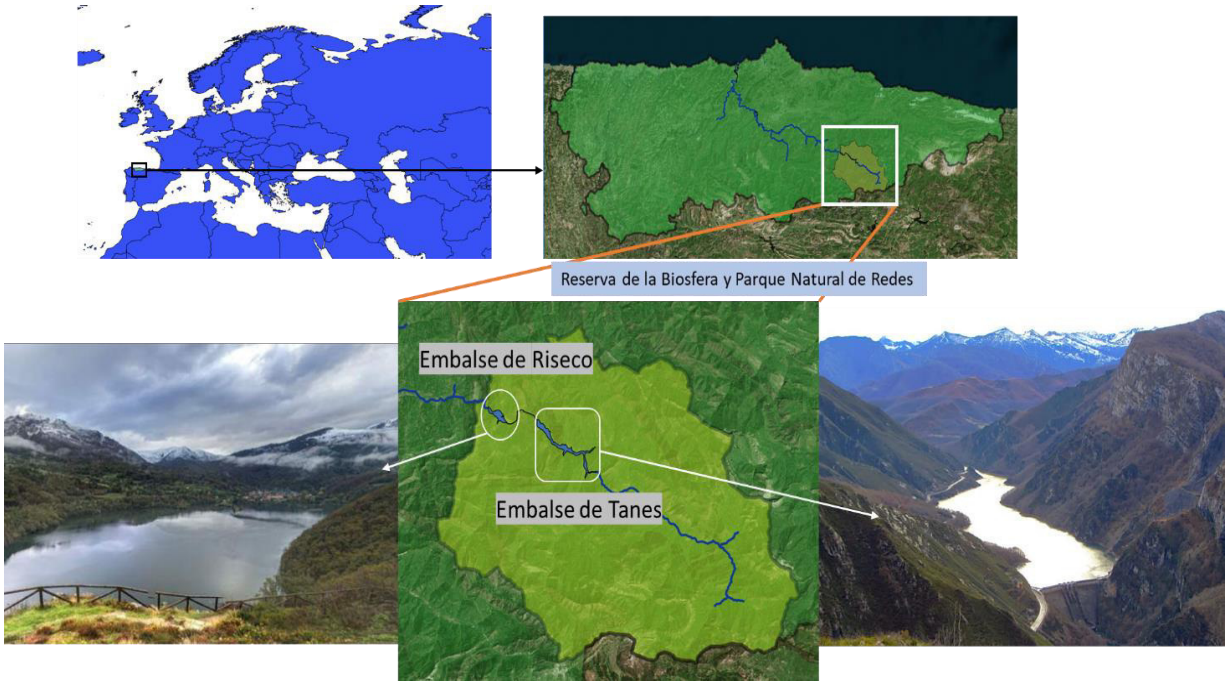


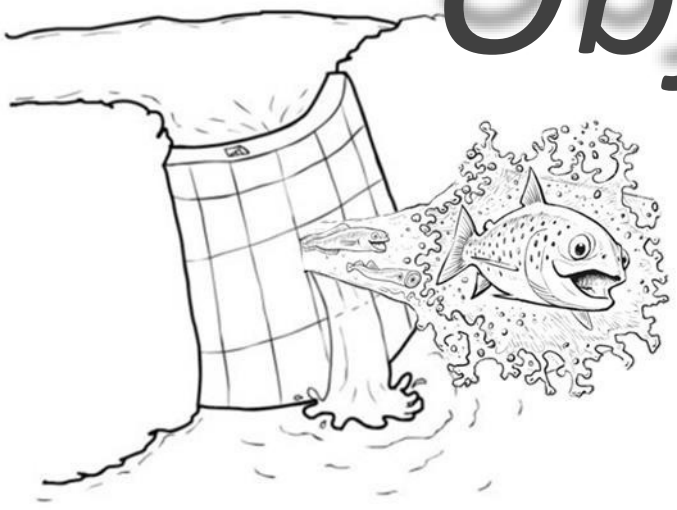
Figura 3. Embalses de Tanes y Rioseco dentro del Parque Natural de Redes (Fuente de las imágenes: Embalse de Rioseco: www.20minutos.es; Embalse de Tanes: www.rutasmontanaasturias.com).

La fauna ribereña de este espacio protegido está compuesta por una variedad de especies entre las que destacan la nutria (*Lutra lutra*) y el desmán (*Galemys pyrenaicus*), catalogados como casi amenazado y vulnerable respectivamente por la IUCN (Unión Internacional por la Conservación de la Naturaleza), además de los invertebrados bentónicos, que son parte fundamental en la cadena trófica, y la trucha común (*Salmo trutta*), especie piscícola predominante (García-Ramos et al. 2006).

Dentro de la cuenca, el salmón atlántico (*Salmo salar*) también es de gran importancia como especie nativa, tanto económica como ecológicamente, pero sus poblaciones no remontan el río Nalón hasta el espacio protegido aguas arriba, porque los tres embalses superiores no tienen escalas y son impasables.

Por todas las características aquí descritas el río Nalón y especialmente su zona alta constituye un estudio de caso muy adecuado para el objetivo principal de esta tesis, que es la aplicación de herramientas de ADN para investigar el impacto de los embalses en la conectividad de los ríos, ya que cuenta con zonas de conectividad interrumpida dentro de un área protegida de alto valor ecológico.

Objetivos



Objetivos

1. Evaluar las poblaciones de salmónidos nativos y exóticos mediante técnicas de PCR cuantitativa y ADN ambiental en espacios naturales protegidos, usando la Reserva de la Biosfera de Redes (cuenca alta del río Nalón, Asturias, España) como estudio de caso.

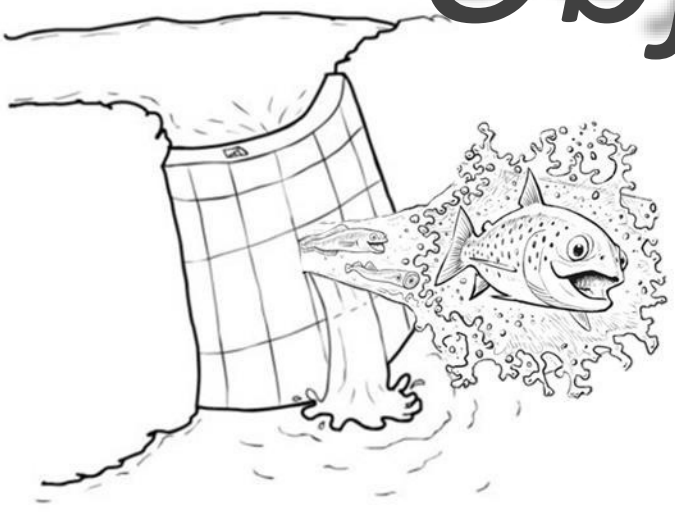
2. En la cuenca del Nalón como estudio de caso, validar la herramienta Metabarcoding a partir de ADN ambiental para evaluar la calidad del agua con el método estandarizado IBMWP (Iberian Bio-Monitoring Working Party), índice que implementa la Directiva Marco del Agua europea en la Península Ibérica.

3. Aplicando herramientas de ADN ambiental, evaluar en el río Nalón el efecto de los embalses sobre la calidad del agua y la conectividad de especies y comunidades, mediante el índice estandarizado IBMWP.

4. En la Reserva de la Biosfera de Redes como estudio de caso, estudiar el impacto de los embalses en la biodiversidad piscícola dentro de espacios protegidos, utilizando una estrategia multidisciplinar mediante herramientas de ADN ambiental en combinación con otros métodos.

5. Recomendar acciones de gestión y manejo de la conectividad fluvial a partir de datos obtenidos mediante ADN ambiental, e identificar posibles mejoras para superar las limitaciones técnicas y metodológicas del análisis de este tipo de ADN en ecosistemas fluviales con objeto de evaluaciones de conectividad.

Objectives



Objectives

1. To assess the populations of native and exotic Salmonids in natural protected areas employing environmental DNA (eDNA) and quantitative PCR techniques, using the Biosphere Reserve of Redes (Upper Nalón river, Asturias, Spain) as a case study.

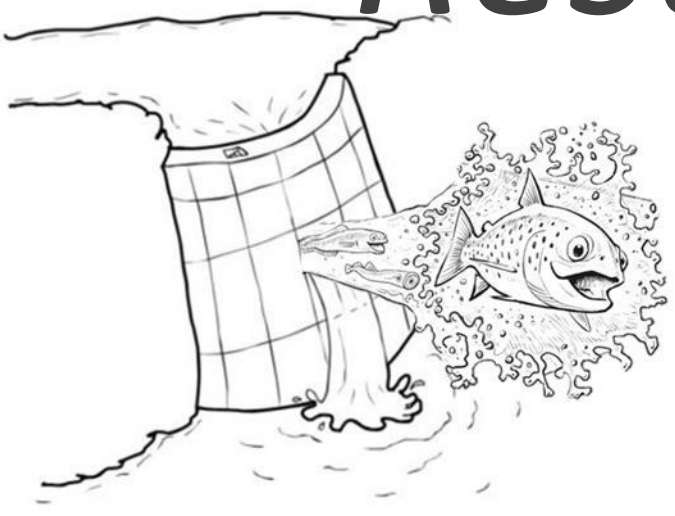
2. To validate eDNA metabarcoding as a tool for the bioassessment of river water quality using WFD-approved indices. The index chosen was the Biological Monitoring Working Party currently employed in Iberian waters, and the case study was River Nalón basin.

3. To apply eDNA tools for the assessment of the effect of dams in river water quality and connectivity at species and community level, employing the standardised method IBMWP (Iberian Bio-Monitoring Working Party).

4. To study the impact of dams in fish biodiversity within protected spaces, using a combination of eDNA and other methodologies. The case study was the Biosphere Reserve and Natural Park of Redes (Upper River Nalón, Asturias, Spain).

5. To recommend management actions where the use of eDNA can be applied for monitoring river connectivity, and to identify current knowledge gaps and technical and methodological improvements for a better application of eDNA-based technology in river waters.

Resultados



Resultados

Capítulo 1. Fernandez S, Sandin MM, Beaulieu PG, Clusa L, Martinez JL, Ardura A, García-Vázquez E. (2018) Environmental DNA for freshwater fish monitoring: insights for conservation within a protected area. Publicado en *PeerJ.*

Capítulo 2. Fernández S, Rodríguez S, Martínez JL, Borrell YJ, Ardura A, García-Vázquez E (2018) Evaluating freshwater macroinvertebrates from eDNA metabarcoding: A river Nalón case study. Publicado en PLoS ONE.

Capítulo 3. Fernández S, Rodríguez-Martínez S, Martínez J.L, Garcia-Vazquez E, Ardura A. (2018) How can eDNA contribute in riverine macroinvertebrate assessment? A metabarcoding approach in the Nalón River (Asturias, Northern Spain). En prensa en Scientific Reports.

Capítulo 4. Fernández S, Arboleya E, Dopico E, Ardura A, Garcia-Vazquez E. (2018). Non-indigenous fish in protected spaces: trends in species distribution mediated by illegal stocking. En revisión en Aquatic Conservation: Marine and Freshwater Ecosystems.

Capítulo 1

Environmental DNA for freshwater fish monitoring: insights for conservation within a protected area.

Fernández S, Sandin M.M, Beaulieu P.G, Clusa L, Martinez JL, Ardura A and Garcia-Vazquez E.

PeerJ

Environmental DNA for freshwater fish monitoring: insights for conservation within a protected area

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ABSTRACT

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

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

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

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

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Subjects Aquaculture, Fisheries and Fish Science, Conservation Biology, Genetics, Freshwater Biology
Keywords PCR-RFLP, River, *Oncorhynchus mykiss*, Genetics, qPCR, Protected areas, Environmental DNA, *Salmo trutta*

INTRODUCTION

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

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

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

For the reasons above, the evaluation of native and invasive fish populations is essential in monitoring the health of an ecosystem (*Arlinghaus, 2006*). This can be difficult, especially when their density is low, using conventional sampling methods such as electrofishing and netting (*Clusa et al., 2017*). Moreover, these types of sampling encompass some mortality (*Snyder, 2004*) and are not suitable for some ecosystems such as those located within protected areas. On the other hand, environmental DNA (eDNA), defined as the genetic material obtained directly from environmental samples such as soil, sediment, water, etc. (*Thomsen & Willerslev, 2015*), can enable the detection of species that can be elusive or

difficult to sample. This technique is commonly used today for species detection (*Dejean et al., 2012*; *Ardura et al., 2015*; *Ardura et al., 2016*; *Clusa et al., 2016*; *Devloo-Delva et al., 2016*) and biodiversity inventory (*Zaiko et al., 2015*; *Civade et al., 2016*). It is a non-invasive sampling technique that avoids distress to the fish allowing for compliance with the European Code of Conduct for Research Integrity (*Drenth, 2011*).

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

METHODS

Ethics statement

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

Study area-upper Nalón Basin

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

Field methods and macroinvertebrates sampling

Sampling took place in November 2015. Targeted sampling areas were typical juvenile habitat: shallow, well oxygenated and with moderate current velocity. At the sampling time of the year, in the particular river zones sampled trout juveniles around one-year-old are expected to be by far the most abundant life stage (*Alonso, Gortázar & García de Jalón, 2010*). Two sampling points were chosen along the upstream Nalón River (points n1, n2) and six on one of its head tributaries, Caleao river (c1 to c6; Fig. 1). The two streams are

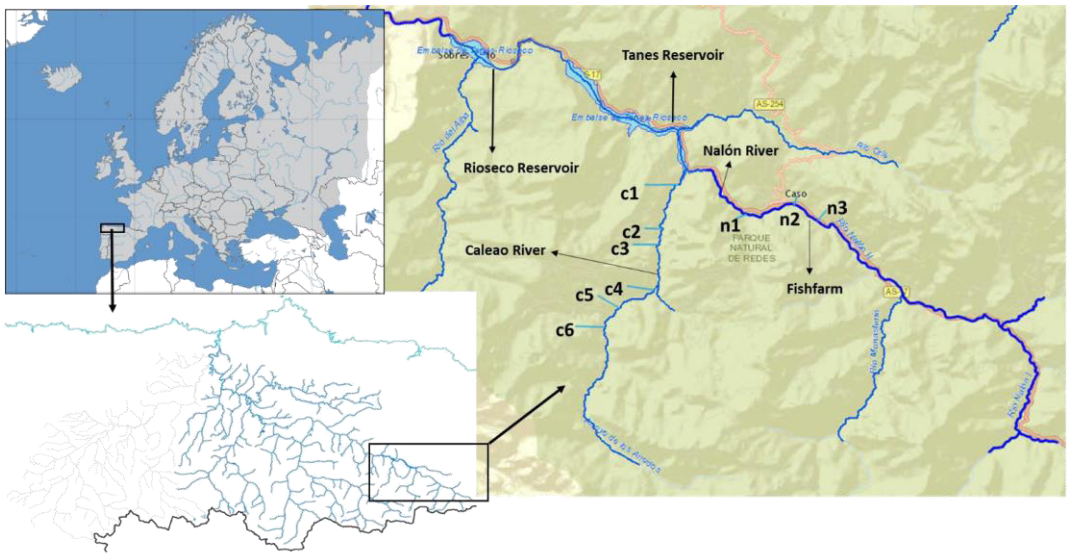

Figure 1 Upper Nalón River Basin. Upper Nalón Basin Map showing sampling points in Nalón River (n1 n3) and its tributary Caleao River (c1 c6) where the study was developed. The impounded areas are marked (Tanes and Rioseco reservoirs).
Full-size DOI: 10.7717/peerj.4486/fig-1

connected at the tail end of Tanes reservoir (Fig. 1). In each sampling point, the habitat was characterized based on the official protocol of the Spanish Ministry of Environment, Feeding and Agriculture (*Alba et al., 2005*), to check if it was appropriate for salmonids. A 20-meter transect was analysed per site. A profile of the substrate was drawn, characterized as percentage of blocks, boulders, gravel, sand and silt. The dominant vegetation type covering the river (river canopy) was identified as well as the shade percentage and continuity. Three measures of depth and width were taken within the 20-meter transect at each sampled site. From these data, the total amount of juvenile salmonid (`rearing') habitat units were estimated for each site based on the model described by *Juanes et al. (2011)*. All profile variables were considered to assign a percentage of juvenile's habitat to each sampling point. Then, it was standardized to 100 m2 (estimated rearing units or ERU) (Table S1).

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

In addition to abiotic measures, macroinvertebrates communities were studied to calculate water quality indices. Individuals were sampled following the kick-net sampling methodology described in the protocol employed (*Alba et al., 2005*). Collected specimens were identified down to Family level using an identification key (*Oscoz, Galicia & Miranda, 2011*), and family presence-absence was recorded. Ecological quality ratio (EQR) and

Table 1 Sampling Stations (November 2015). Physical-chemical features (TEMP: Temperature. TDS: Total Dissolved Solids). ERU: Estimated Rearing Units for Brown Trout. EQR: Ecological Quality Ratio, based on macroinvertebrate families. The ecological state based on macroinvertebrates was classified following the official proto-col of Environment, Feeding and Agriculture Ministry (NIPO: 770- 11-308-X), and is highlighted in yellow for Moderate and green for Good (*Alba et al., 2005*). Detection (Nested PCR-RFLP) and quantification (qPCR) of Trout eDNA.

Station	Local name	Water course	Coordinates	TEMP (C)	pH	TDS (g/L)	ERU	EQR	Ecological state	Number of trout DNA molecules (qPCR)		Nested PCR-RFLP	
										Rainbow trout DNA molecules	Brown trout DNA molecules	Rainbow trout eDNA presence/ no presence	Brown trout eDNA presence/ no presence
c1	Arrudos	Caleao river	43 08^{0}46.8N 5 24^{0}47.9W	9.07	7.90	0.11	20.09	0.62	Good	0	1,964.85	0	X
c2	Caliao	Caleao river	43 09^{0}11.2N 5 24^{0}24.0W	9.41	8.13	0.11	69.78	0.48	Moderate	0	3,119.54	0	X
c3	Encruceyada	Caleao river	43 09^{0}24.9N 5 23^{0}39.4W	9.60	8.27	0.12	17	0.48	Moderate	0	1,125.65	0	X
c4	Puentepiedra	Caleao river	43 10^{0}12.2N 5 23^{0}32.3W	10.48	8.36	0.13	20.11	0.48	Moderate	0	4,394.81	0	X
c5	NA	Caleao river	43 10^{0}26.8N 5 23^{0}29.2W	10.73	8.44	0.13	27.39	0.48	Moderate	0	2,231.15	0	X
c6	NA	Caleao river	43 11^{0}16.2N 5 23^{0}01.5W	10.92	8.39	0.13	53	0.48	Moderate	0	1,459.83	0	X
n3	Veneros	Nalón river	43 10^{0}35.2N 5 19^{0}55.6W							6,121.93	1,775.02	X	X
n2	El Campu	Nalón river	43 10^{0}51.3N 5 20^{0}27.7W	10.89	8.35	0.12	86.17	0.66	Good	5,456.07	1,679.35	X	0
n1	Cueva Deboyu	Nalón river	43 10^{0}41.0N 5 21^{0}36.3W	10.96	8.38	0.12	75	0.55	Good	94,342.76	2,307.53	X	0

the ecological state of each point were calculated based on macroinvertebrates family's composition, giving a punctuation to each family based on their tolerance to contamination (*Alba et al., 2005*) (Table 1).
One extra point was selected in Nalón River to collect only water samples (n3). Taken upstream n2 (where a rainbow trout fish farm is located, see Fig. 1), this water sample (n3) was collected 20 m upstream fish farm discharges as a control to discard the possibility that rainbow trout DNA comes only from fish farm runoff.

Water sample collection, filtration and eDNA extraction

Water samples of 1.5 L each were collected in sterile plastic bottles from the sampling points. Water samples were vacuum-filtered using a Supor R -200 Membrane Filter (Pall Corporation, Life Sciences, Ann Arbor, MI, USA) with 0.2 mm pore size. The filtration room was free of external sources of contamination as it was separated from the molecular laboratory. The filtration system was cleaned up with 10% commercial chlorine based-bleach between samples to avoid contamination between sampling points. 1.5 L of Milli-Q water that was previously transported with the rest of the water samples from the field, was filtered at last following the same steps, and was considered as an extra sample to monitor possible field or filtering contaminations. Finally, filters were placed into 15mL tubes using sterile forceps and stored at 20 C until DNA extraction.
DNA was extracted from filters with PowerWater R DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA) and preserved at 20 C until further processing. The DNA extractions were conducted under sterile conditions inside a laminar flow PCR-cabinet, following the manufacturer's instructions. A negative control was added at this step to monitor contaminations during the extraction process.

Quantitative PCR procedures

Quantitative PCR from eDNA using specific primers has been validated for brown (*Gustavson et al., 2015*) and rainbow trout (*Wilcox et al., 2015*) (Table 2). Details about qPCR protocols are included in this section, as recommended by *Bustin et al. (2009)*:
Two specific TaqMan assays were selected as molecular markers: for brown trout, a 61 base pairs (bp) fragment of the mitochondrial cytochrome oxidase I gene (COI: Cytochrome Oxidase Subunit I) (*Gustavson et al., 2015*); and a 102 bp fragment of the NADH gene for rainbow trout (*Wilcox et al., 2015*). In silico analysis were done using the Primer Blast application included in the NCBI webpage (*Ye et al., 2012*) to check the specificity of the markers. No coincidences were found with related nor cohabiting species. Pre-PCR analyses of eDNA samples were carried out in a room separated from the molecular laboratory where there is no DNA nor tissue samples, inside a flow PCR-cabinet. Negative controls from the field, filtration and extraction processes were included in PCR runs as well as a PCR negative control.
The qPCR (quantitative Polymerase Chain Reaction) runs were performed using 7,900 HT Fast Real-Time PCR System (Life Technologies, Inc., Applied Biosystems, Carlsbad, CA, USA). Amplification reaction mixtures for brown trout included: 10 ml of TaqMan Environmental Master Mix 2.0 (Life Technologies, Carlsbad, CA, USA), 0.4 ml of each

Table 2 qPCR molecular markers. TaqMan assays employed in the qPCR analysis for each targeted species. Primers' and hydrolysis probes' se-quences.

			qPCR		
Species	**Source**	**Gene**	**Primer**	**Sequence (5^0-3^0)**	**Amplicon(bp)**
Brown trout	*Gustavson et al. (2015)*	COI	Forward	TTTTG TTTGGGCCGTGTTAGT	61
			Reverse	TGCTAAAACAGGGAGGGAGAGT	
			Probe	ACCGCCGTCCTCT	
Rainbow trout	*Wilcox et al. (2015)*	NADH	Forward	AGTCTCTCCCTGTATATCGTC	102
			Reverse	GATTTAGTTCATGAAGTTGCGTGAGTA	
			Probe	6FAM-CCAACAACTCTTTAACCATC-MGBNFQ	

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

As positive controls, DNA from tissue samples of each species were extracted with E.Z.N.A. ^{R} Tissue DNA Kit (Omega, Bio-Tek, Norcross, GA, USA) following the manufacturer's instructions.

The two molecular markers were tested first on control DNAs. A mixture of control DNAs from rainbow and brown trout at known concentrations were PCR amplified with the two specific markers to check for possible co-amplification or interference between them (Fig. S1). On each qPCR run, a positive control from tissue extractions of the targeted species was also added to monitor PCR inhibition.

PCR amplicons were generated with the two primer sets from tissue DNA in a total volume of 20 ml, including Green GoTaq ^{R} Buffer 1X, $MgCl_2$, 0.25 mM dNTPS, 0.25 mM of each primer, 4 ml of template DNA and 0.65U of DNA Taq polymerase (Madison, WI, USA). PCR conditions were 95 C for 5 min, followed by 35 cycles at 94 C for 30 s, 57 C for 30 s and 72 C for 30 s, and a final step of elongation at 72 C for 10 min.

The PCR amplicons obtained were quantified by fluorimetry using Qubit ^{R} dsDNA BR Assay Kit (Thermofisher Scientific, Carlsbad, CA, USA). The amount of DNA was transformed into molecules per ml, calculated from the known base composition of the amplicon sequence. A standard curve was constructed including a serial dilution (from 2:34 10^9 to 2.34 10^3 molecules/mL for rainbow trout and from 6.3 10^9 to 6:3 10^3 molecules/mL for brown trout) and used as reference for DNA molecules quantification in water samples. A dilution series from tissue DNA of each species was done to determine the lowest copy number of target DNA per litre of water detectable from each assay.

All the analyses of tissue and amplicon samples were conducted separately from environmental samples, keeping them away from any source of contamination.

Table 3 PCR-RFLP Markers. Primers' sequences and amplicon's length employed in the nested PCR assay for rainbow and brown trout eDNA de-tection.

Nested PCR-RFLP				
Gene	**Primer**	**Source**	**Sequence (5^0 3^0)**	**Amplicon (bp)**
First-PCR 16S	Forward	*Clusa et al. (2017)*	GCCTGCCCTGTGACTATGG	567
	Reverse	*Palumbi et al. (2002)*	CCGGTCTGAACTCAGATCACGT	
Nested-PCR	Forward	*Zaiko et al. (2015)*	AAGACCTGTATGAATGGCATC	377
	Reverse	*Zaiko et al. (2015)*	TCGATAGGGACTCTGGGAGA	

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

Species-specific PCR-RFLP

The nested PCR-RFLP method was developed as described in *Clusa et al. (2017)* for detecting the two targeted species (brown and rainbow trout). Briefly: a first PCR was carried out to amplify a 567 bp fragment of the 16S rRNA gene, using as forward the 16S-new-F primer designed in the cited research (Table 3), and the reverse 16S-Br universal primer from *Palumbi et al. (2002)*. A nested PCR amplification was then performed with the pair of Salmonidae-specific primers described in *Zaiko et al. (2015)* (Table 3). The nested PCR product was digested with TaaI and Tru1I FastDigest enzymes (Thermo Fisher Scientific Inc., Waltham, MA, USA) that produce diagnostic band patterns in agarose gel allowing to identify the two targeted species. With TaaI enzyme, brown trout DNA gives two bands of 205 and 272 bp, and with Tru1I, rainbow trout DNA gives a band of 66 bp.

RESULTS

Habitat quality measures

The physical-chemical characteristics of the sampling points were within the optimum range for Salmonid juveniles (*Raleigh et al., 1984*; *Raleigh, Zuckerman & Nelson, 1986*). The numbers of juvenile salmonid habitat units (ERU) ranged between 17 in c4 and 86.17 in n2 (Table 1).
The ecological state measured from macroinvertebrate communities (EQR) was good at the points from the upper zone of Caleao River and in Nalon River (c6, n1 and n2), and moderate in the rest of the points. Detailed results of macroinvertebrate families along sampling stations are summarized in Table S2.

qPCR assays

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

Standard curves for brown and rainbow trout fitted the equations y= 3.819x C45:141, R2= 0.999 and y= 3.3128x C39:2113, R2 = 0,999 respectively. The lowest detectable

number of copies was 7 10^2 and 2.6 10^3 molecules per liter for brown and rainbow trout assays respectively. From eDNA, negative controls were clean as expected i.e., no evidence of amplification. As positive amplification was obtained from all eDNA extraction samples for brown trout assay (see below), PCR inhibition testing was not performed.
Values of quantification obtained from river water samples varied from 94:34102 to 11:26 102 trout DNA molecules (Table 1). Brown trout eDNA was found from all nine sampling sites. Rainbow trout eDNA occurred from the three points located in Nalón River (Table 1), one 20 m upstream (Veneros) and two downstream from the rainbow trout fish farm located in that river. The amount of rainbow trout eDNA molecules was considerably higher than that of brown trout in the Nalón River sampling points (n1 to n3), especially in n1 where the highest amount (94342.76 eDNA molecules) was found. This point is located in the lowest zone of the sampling area (Fig. 1).

PCR-RFLP

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

DISCUSSION

In this study, we have detected eDNA signals of rainbow trout on running waters that can be interpreted as fish farm escapes within a protected area. As in other studies, environmental DNA again proved its value as a tool for species detection in freshwater environments (*Jerde et al., 2011*). Moreover, the probe-based qPCR was confirmed to be a highly sensitive

technique that has the potential to offer quantification and biomass estimations (*Gustavson et al., 2015*). Although the PCR-RFLP technique employed here had demonstrated its sensitivity for salmonids detection in water samples (*Clusa et al., 2017*), it was not able to detect brown trout DNA in all the sampling points of the current study (n1 and n2), where it was detected from qPCR. The high amount of rainbow trout eDNA in these two sampling points, clearly more abundant than brown trout DNA, was a likely cause of the false brown trout negative found using PCR-RFLP technique. That technique is based on initial PCR amplification of a salmonids-specific DNA fragment which is the template for enzymatic digestion; the initial PCR amplification of the much more abundant rainbow trout DNA probably led to very weak undetectable brown trout bands (at least on agarose gels). PCR-RFLP technique has also another limitation compared with qPCR, which is the probability of cross-contamination, more probable when using a gel-based system rather than an enclosed system as qPCR is. Indeed, the results obtained using PCR-RFLP reinforced the conclusion of widespread rainbow trout occurrence in the upper Nalón River (n1, n2 and n3) found from qPCR. The two methods have detected rainbow trout DNA from running waters upstream of an impassable dam, where rainbow trout is not expected to occur naturally. Moreover, fish farm escapes have not been officially reported in this area, nor have established populations.

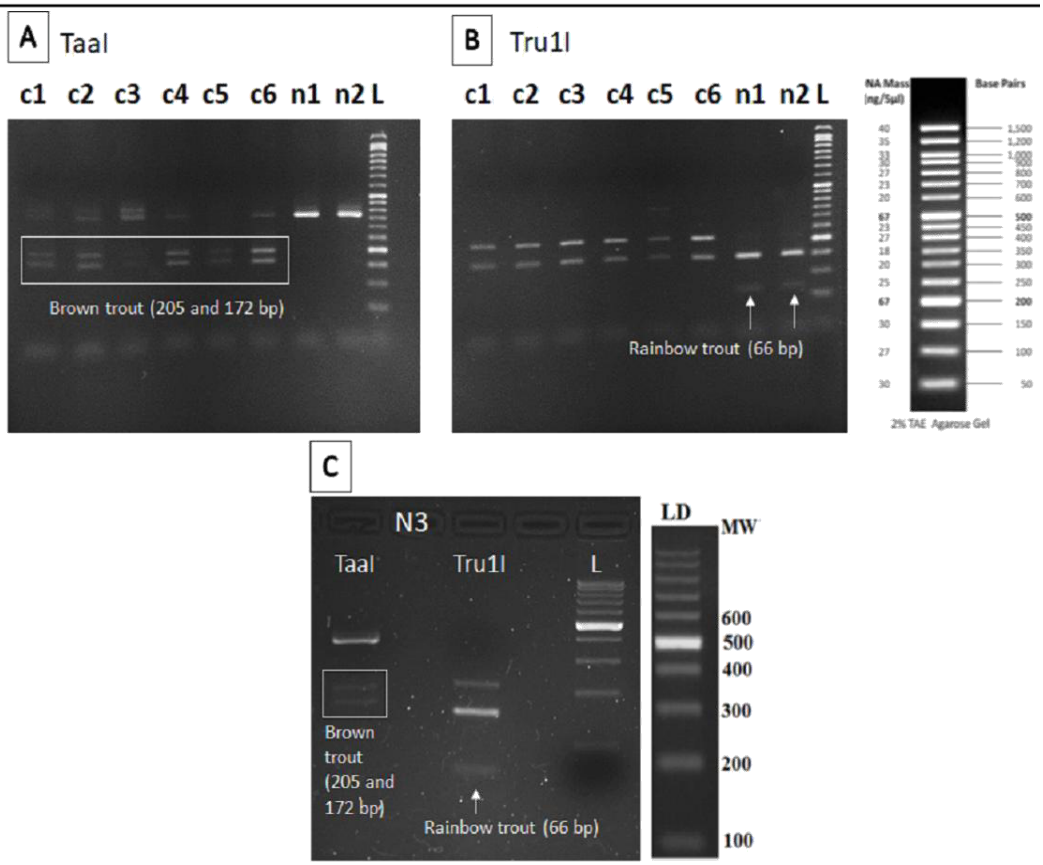

Figure 2 RFLP results of brown and rainbow trout. Diagnostic fragments of each species are indicated (c1 c6: sampling points in Caleao River; n1 n3: sampling points in Nalón River; L, Ladder to estimate fragment size; TaaI and Tru1I: restriction enzymes). (A) Restriction pattern obtained for brown trout de-tection (205 and 172 bp) with TaaI enzyme digestion from eDNA samples. (B) Restriction pattern ob-tained for rainbow trout (66 bp) with TruI enzyme digestion from eDNA samples. (C) Restriction pat-terns obtained with TaaI and Tru1I enzymes characteristic of brown (205 and 172 bp) and rainbow (66 bp) trout respectively, found in sampling Point n3, Veneros, from eDNA sample (upstream fish farm dis-charges).

Full-size DOI: 10.7717/peerj.4486/fig-2

been documented. There are rainbow trout fish farms downstream of Tanes reservoir, but it is an impassable barrier, thus arrival of escapes from downstream fish farms is impossible. Unreported fish farm escapes within the Biosphere Reserve, or DNA runoff from the fish farm (not individuals) would be alternative explanations. It is also possible that eDNA comes from predator transfers via depositing of carcasses or defecation (*Merkes et al., 2014*), but much more improbable. The occurrence of rainbow trout individuals (not just trout DNA from fish farm water discharges or runoffs) was strongly suggested here from rainbow trout DNA obtained in the sample taken as a control upstream of the fish farm drainage (n3), because runoff goes with the river current and floating DNA cannot move upstream. Thus, unreported escapes were a likely explanation of these results, which show how important is the location of sampling points in eDNA studies from running waters (*Deiner & Altermatt, 2014*; *Jane et al., 2015*). In theory, if escapes occur the escapees may interact with native brown trout inside the protected area, where the introduction of exotic

species is strictly prohibited from Spanish legislation (BOPA Decree 48/2006, of 18 of May, implementing the Spanish Law 5/1991, of 5 of April, for Protection of Natural Spaces).

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

The results of the present study could be taken as an alert signal of the presence of rainbow trout in the ecosystem. Although the presence of other exotic species is documented along Nalón River, the information about rainbow trout occurrence in the ecosystem is clearly insufficient. It is generally believed that there are no self-sustaining rainbow trout populations in Spain, but there are no specific studies about its naturalization (*Stankovi¢ et al., 2015*). The methodology employed in this study could serve for a wide and systematic monitoring of this species from all the Iberian rivers basins. One of the benefits of eDNA is allowing to monitor more sites in a faster and cheaper manner (*Deiner et al., 2016*; *Bista et al., 2017*), screening environments for potential species from which ground truthing (i.e., electrofishing or netting in the present case) can be conducted later to verify. The river part studied here exhibits a good habitat for salmonids (*Juanes et al., 2011*). The sampling points where rainbow trout eDNA was found had 161.17 ERUs and the best water quality. Since salmonids habitat is abundant and the water quality high, if escapes of rainbow trout occurred the species establishment would be favoured by those good conditions. This would have both direct and indirect impacts at multiple ecological levels (*Mack et al., 2009* Stankovi*¢ et al., 2015*), and would be an enormous threat for the protected ecosystem because the impacts of invasive fish propagate rapidly beyond the habitat initially occupied (*Baxter et al., 2004*). One of the possible consequences could be the fragmentation of native fish populations (*Fausch, 2007*). Brown trout populations would be especially affected in this case because River Nalón is already dammed, and upstream populations are forcedly isolated from downstream settings. On the other hand, the introduction of farmed salmonids in the wild (escapes or deliberate releases) is often followed by interspecific hybridization with wild individuals, due to altered behavior of farmed fish (e.g., *Horreo et al., 2014*; *Horreo et al., 2018*). Since the survival of brown trout x rainbow trout hybrids is extremely low (*Scheerer & Thorgaard, 1983*), if hybridization happened the reproductive potential of native brown trout would be diminished. Considering all the risks above together, and the fact that small and isolated brown trout populations are especially

vulnerable (*Mooney & Cleland, 2001*), their conservation within the protected area might be endangered if rainbow trout escapes are not carefully controlled.

Conclusion and implications for conservation

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

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

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Competing Interests

Paul G. Beaulieu is an employee of Tighe & Bond, Trout Unlimited.

Author Contributions

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

Field Study Permissions

The following information was supplied relating to field study approvals (i.e., approving body and any reference numbers):
This project and the sampling carried out in protected spaces were authorized by the entity legally entitled to do so in Spain: The Government of the Asturias Principality, with the permit reference 101/16.

Data Availability

The following information was supplied regarding data availability:

The raw data are provided in the Supplemental Files.

Supplemental Information

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

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1 **1. Supplemental information.**

2 **S1 Table.** Estimated Rearing Units (ERU). %Juvenile habitat calculation based on Juanes

3 *et al.*(2012).

Sampling point	Length (m)	Width (m)	Area (m2)	%Juvenile habitat	ERU	ERU/100m^2
C1_1	7	7.2	50.4	0.2	10.08	
C1_2	7	7	49	0	0	
C1_3	7	7.1	49.7	0.4	19.88	20.09
Total_C1			149.1		29.96	
C2_1	7	6	42	0.25	10.5	
C2_2	7	6.06	42.42	0.85	36.057	
C2_3	7	7	49	0.95	46.55	69.78
Total_C2			133.42		93.107	
C3_1	7	4.25	29.75	0.5	14.875	
C3_2	7	8.5	59.5	0.1	5.95	
C3_3	7	4.75	33.25	0	0	17.00
Total_C3			122.5		20.825	
C4_1	7	6	42	0.1	4.2	
C4_2	7	12	84	0	0	
C4_3	7	12.13	84.91	0.45	38.2095	20.11
Total_C4			210.91		42.4095	
C5_1	7	4.6	32.2	0.35	11.27	
C5_2	7	7.2	50.4	0.2	10.08	
C5_3	7	7	49	0.3	14.7	27.39
Total_C5			131.6		36.05	
C6_1	7	11.5	80.5	0	0	
C6_2	7	12.5	87.5	0.8	70	
C6_3	7	16	112	0.7	78.4	53.00
Total_C6			280		148.4	
N1_1	7	11	77	0.95	73.15	
N1_2	7	9	63	0.6	37.8	
N1_3	7	10	70	1	70	86.17
Total_N1			210		180.95	
N2_1	7	12	84	0.8	67.2	
N2_2	7	10	70	0.9	63	
N2_3	7	14	98	0.6	58.8	75.00
Total_N2			252		189	

4

S2. Table. Macroinvertebrates family's occurrence in sampling stations along River Nalón (1= Presence; 0= No Presence).

Sampling points	c1	c2	c3	c4	c5	c6	n1	n2
Preferred Preys								
***O.mykiss* preferences**								
Tipulidae	0	0	1	0	0	1	0	0
Stratiomyidae	0	0	0	0	0	0	0	0
Nemouridae	0	0	0	0	0	0	1	0
***S.trutta* preferences**								
Limnoniidae	0	1	0	0	0	0	1	0
Simuliidae	0	1	1	0	0	1	0	0
Perlidae	1	1	1	0	1	0	0	0
Hydropsychidae	0	0	1	0	0	1	1	1
Both salmonids preferences								
Chironomidae	1	0	1	1	1	1	1	0
Heptagenidae	1	1	1	1	1	0	1	0
Baetidae	1	1	1	1	1	1	1	0
Rhyacophilidae	0	0	0	0	1	1	0	1
No Preferred Preys								
Planaridae	1	0	0	0	1	0	0	0
Hydracarina	1	1	1	0	1	1	0	0
Hydrophilidae /Hydraenidae	1	1	0	0	1	0	0	0
Gyrinidae	1	0	0	0	0	1	1	0
Ephermerellidae	1	0	0	0	0	0	1	1
Leuctridae	1	0	0	0	0	0	1	0
Perlodidae	1	1	1	0	1	0	1	1
Lepidostomatidae	1	0	0	1	0	0	1	0
Leptoceridae	1	0	0	1	0	0	0	0
Odontoceridae	1	0	0	1	1	1	1	0
Perlidae	0	0	0	1	0	0	1	1
Leuctridae	0	0	1	1	0	1	0	0
Hydropsychidae	1	0	0	0	0	0	0	0
Phyganeidae	0	1	0	1	0	0	0	0
Chloroperlidae	0	1	1	0	1	1	1	0
Ancylidae	0	1	0	0	0	0	0	0
Glossiphonidae	0	1	1	0	0	0	0	0
Lumbricidae	0	1	0	0	0	0	1	0
Dytiscidae	0	1	0	0	0	1	0	1
Limephilidae	0	0	1	0	0	0	0	0
Erpobdellidae	0	0	1	0	0	0	0	0
Elmidae	0	0	0	1	1	0	0	0
Leptophlebidae	0	0	0	0	0	1	0	1
Polycentropoditae	0	0	0	1	1	0	0	0
Sphaeridae	0	0	0	0	0	0	1	0
Tubificidae	0	0	0	0	0	0	1	0
Hydrometridae	0	0	0	0	0	0	1	0
Sericostomatidae	0	0	0	0	0	0	0	1
Goeridae	0	0	0	0	1	0	0	0

Capítulo 2

Evaluating freshwater macroinvertebrates from eDNA metabarcoding: A river Nalón case study

Fernández S, Rodríguez S, Martínez J.L, Borrel Y.J, Ardura A and Garcia-Vazquez E.

Plos One

RESEARCH ARTICLE

Evaluating freshwater macroinvertebrates from eDNA metabarcoding: A river Nalon case study

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Abstract

Rivers are a vital resource for human wellbeing. To reduce human impact on water bodies, the European Union has established an essential regulatory framework for protection and sustainable management (WFD; 2000/60/EC). In this strategy, reliable and economic bioindicators are a fundamental component. Benthic macroinvertebrates are the group most commonly used as bioindicators through all European countries. However, their conven-tional assessment currently entails serious cost-efficiency limitations. In this study, we have tested the reliability of metabarcoding as a tool to record river macroinvertebrates using samples from a mock community (*in vitro* validation) and eDNA extracted for field validation from water from six sites within a north Iberian river (River Nalon, Asturias, Spain). Two markers (V4 region within the nuclear 18S rDNA and a fragment of the mitochondrial COI gene) were amplified and sequenced using an Illumina platform. The molecular technique has proven to be more sensitive than the visual one. A cost-benefit analysis shows that the metabarcoding approach is more expensive than conventional techniques for determining macroinvertebrate communities but requires fewer sampling and identification efforts. Our results suggest metabarcoding is a useful tool for alternative assessment of freshwater quality.

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Introduction

Rivers are one of the most important resources for human society, supplying the population with different goods and services: from drinking and industrial water to fisheries to recrea-tional activities [1]. Due to these anthropogenic uses, running water ecosystems are constantly changing and have generally experienced a reduction in the ecosystem services they provide [2]. As an attempt to reduce the impacts on European water bodies, the European Water Framework Directive (WFD; 2000/60/EC) has established a framework for their protection and sustainable management, with the aim of achieving at least a `good water status' [3]. Good water quality is one of the essential requirements to accomplish the status required within this directive.

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Multiple indicator groups (macrobenthic fauna, fish fauna, and aquatic flora) have been widely used to measure the ecological quality of rivers across Europe [4±8]. Benthic macroinvertebrates are biotic indicators of water quality because they reflect a diversity of anthropogenic perturbations, thus serving to detect both habitat and overall stream degradation [9]. They are organisms that usually inhabit the bottom substrates and are large enough to be seen without magnification. The dominant groups are arthropods, mollusks, and annelids [10]. Their use as bioindicators is widespread across Europe, and, together with algae, they are the most common biological water quality assessment indicators [9]. For these reasons, the monitoring of resident macroinvertebrate communities has become a primary component of water-resource evaluations with regard to the WFD [11].

Collection and identification of macroinvertebrates with traditional methodologies is generally costly. It requires a high sampling effort and the contribution of expert taxonomists for morphological identification that is sometimes difficult to obtain because of the lack of diagnostic characteristics for many macrozoobenthic larvae [12].

However, the use of environmental DNA (eDNA), where the genetic material is obtained directly from environmental samples (soil, sediment, water, etc.) [13], could overcome these cost-efficiency limitations. The samples needed for applying eDNA-based methodologies are easy to collect without the need for sampling individuals from the river, which can be difficult in river zones with no accessibility to the river bottom or in areas where netting is inefficient because of a low or nonexistent current. Due to the substantial number of taxa that compose `benthic macroinvertebrates', from arthropods to annelids, the use of a metabarcoding approach appears to be a good option. Metabarcoding has been defined as the combi-nation of high-throughput sequencing (HTS) platforms and DNA sequence association with taxonomic information to surveying [14]. Although it requires next-generation sequencing (NGS) technologies and the use of expensive platforms, the process can be externalized to specialized companies, reducing costs and becoming relatively affordable for monitoring aquatic communities [15]. NGS has been used to assess macroinvertebrates in a few studies [16±19], demonstrating its potential ability to monitor such a varied group of organisms. Within the mentioned studies, some authors have used a metabarcoding approach to assess benthic macroinvertebrates from tissue samples [19,20], showing its feasibility and higher sensitivity than morphological methods. Others validated the use of NGS for environmental samples to evaluate water quality in marine ecosystems [16] and in biodiversity studies in freshwater ecosystems [17], including macroinvertebrate species assessment. The application of these technologies to environmental samples is increasing [21]. Most of the recently developed studies have been based on advancing eDNA based approaches implementation (e.g., [13,21,22,23]), focusing on field validation, platform and barcode choice or database limitations [24±26]. However, there is a lack of information about the reliability of taxonomic assignment criteria. In this study, we tested the reliability of next-generation sequencing (NGS) for the detection and identification of macroinvertebrate families from running water samples using two different metabarcodes for checking the consistency of the taxonomic assignments and determining the proportion of positive and negative results by comparison of eDNA results with physical macroinvertebrate samples from the field, and a mock com-munity created *in vitro* from known DNA samples. Field samples obtained along a river will also serve to test the hypothesis of rivers being like conveyer belts of biodiversity [17]. From this hypothesis, DNA from terrestrial species will be found in water samples as well, so the assessment using eDNA could cover landscapes. And it is expected that the species diversity will increase downstream for macroinvertebrates and for the whole community identified from eDNA.

Methods

Ethics statement

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

Sample collection

Water samples were collected in November 2016 from six sites along the upper zone of the Nalon River (Fig 1), a river area belonging to the Nalon-Narcea basin in Asturias in the north of Spain. Study sites are located within the UNESCO (United Nations Educational, Scientific and Cultural Organization) Biosphere Reserve and the Redes Natural Park, a protected area with high faunal diversity [27]. In this area, river connectivity is interrupted by the presence of two big dams (Fig 1).

At each site, four liters of water were collected with a sterile bottle placed at the river bottom without disturbing the sediment. One liter of Milli-Q water was transported to the field and analyzed in the laboratory with the rest of the samples to monitor for contamination. After water sample collection, macroinvertebrate individuals were sampled after superficially kick-ing the riverbed substrates about for one minute (Kick-net method), as is performed in con-ventional macroinvertebrate sampling [28]. The released individuals were then collected with a 0.09 m^2 stainless steel sieve (1-μm mesh). The specimens collected were identified down to the family level using an identification key [29].

Processing and next-generation sequencing

Four one-liter samples were analyzed per sampling point. These water samples and the Milli-Q negative control were vacuum filtered using a Supor[1] 200 Membrane Filter (Pall Corporation,

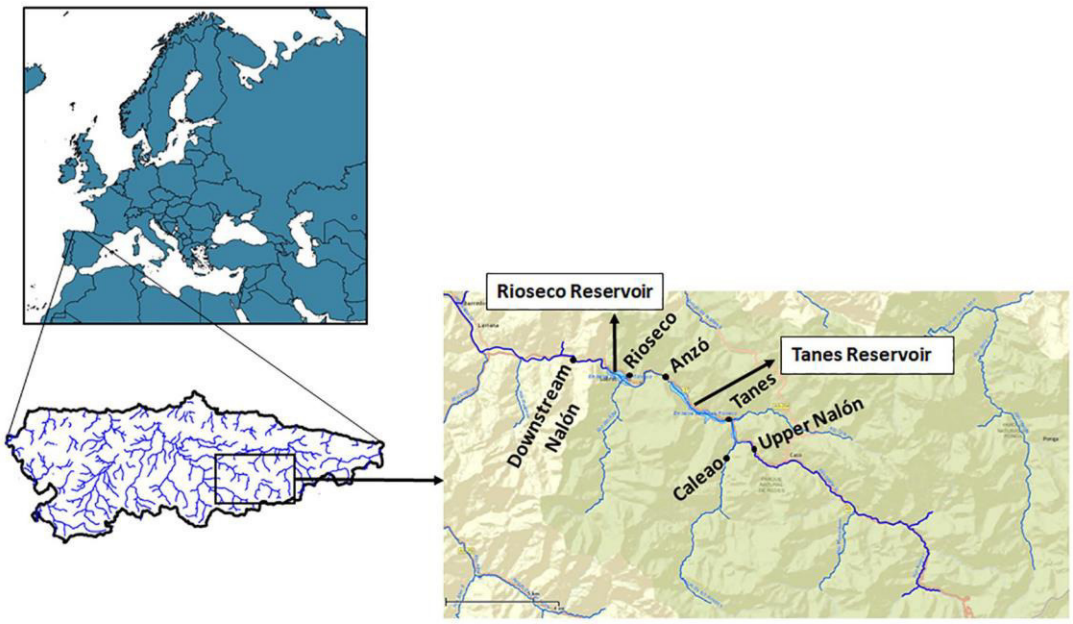

Fig 1. Upper Nalon basin. Distribution of sampling points along the Upper Nalon River. The two reservoirs in the area are indicated (Rioseco and Tanes reservoirs).

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Life Sciences, Ann Arbor, MI, USA) with 0.2-μm pore size. The filtration room was free of external sources of contamination, and it was separate from the molecular laboratory. The fil-tration system was cleaned with 10% commercial chlorine-based bleach between samples to avoid contamination between sampling points. Milli-Q water was filtered as the last sample, following the same steps to monitor for filtration cross-contamination. Lastly, the filters were placed into 15 mL tubes using sterile forceps and stored at −20 ÊC until DNA extraction.

DNA was extracted from filters with the PowerWater1 DNA Isolation Kit (QIAGEN laboratories) under sterile conditions inside a laminar flow PCR-cabinet following the manufactur-er's instructions. A negative control was added at this step to monitor contamination during the extraction process.

Metabarcoding molecular work was performed at the Cawthron Institute (www.cawthron.org.nz). PCR was performed for two target genes, the eukaryotic V4 region of the nuclear small subunit ribosomal DNA (18S rRNA gene, 18S from now) using the universal primers Uni18SF and Uni18SR [30] and a mitochondrial COI gene region using the universal primers COI NexF-mlCOIintF and NexR-jgHCO2198 [31]. The primers were modified to include Illu-mina™ overhang adaptors.

PCR for the 18S gene was performed on an Eppendorf Mastercycler (Eppendorf, Germany) in a total volume of 35 μl containing 18 μl of AmpliTaq Gold1 360 PCR Master Mix (Life Technologies, USA), 5 μl of AmpliTaq PCR Enhancer (Life Technologies, USA), 2 μl of BSA,1μM of each primer, and 3 μl of template DNA. The reaction cycling conditions were as fol-lows: 95 ÊC for 3 min; followed by 35 cycles of 94 ÊC for 30 s, 52 ÊC for 30 s, and 72 ÊC for 90 s;and a final extension at 72 ÊC for 8 min. PCR of the COI gene was performed in a total volume of 35 μl containing 1x MyTaq™Red Mix (Bioline, USA), 1 μM of each primer and 3 μl of tem-plate DNA. The reaction cycling conditions were as follows: 95 ÊC for 1 min; followed by 35 cycles of 95 ÊC for 15 s, 46 ÊC for 15 s, and 72 ÊC for 10 s; and a final extension at 72 ÊC for 3 min. Negative and positive controls were included for all PCR reactions. The amplification success was visually assessed on a 1.5% agarose gel.PCR amplicons were purified using the AMPureTM XP system (Agenecourt, USA), quanti-fied using the QuBit BR dsDNA kit (Invitrogen, USA), diluted to a concentration of 3 ng/μl and sent to New Zealand Genomics Limited (University of Auckland) for library preparation and sequencing. Sequencing adaptors and sample-specific indices were added to each ampli-con via a second round of PCR using the Nextera™ Index kit (Illumina™) following the manu-facturer's instructions. Amplicons were pooled into a single library and paired-end sequences (2 × 250) were generated on a MiSeq instrument using the TruSeq™ SBS kit v3 (Illumina™). The MiSeq Control Software Version 2.2 including MiSeq Reporter 2.2 was used for raw read primary analysis and demultiplexing and to assign the forward and reverse reads to the samples.

Bioinformatics analyses

Run quality was assessed using three processes, SolexaQA++, fastQC and fastQscreen. Using the VSEARCH tool [32], the pair-end reads from each sample were merged, filtered (discarding all reads with >1 error per assembled read and reads that were too long and too short compared to the expected amplicon length) and dereplicated into unique sequences. Chimeras were identified and removed in de novo mode using the UCHIME algorithm [33]. All the sequence reads were assessed for quality by applying a Phred quality score threshold of 30 (Table 1; Cleaned). Then, BLAST alignment was completed for the 18S rDNA dataset (maximum E-value = 10−50 and minimum percent identity = 80.0) against NCBI 18S sequences using QIIME [34].

Table 1. HTS and pipeline output. The number of sequences obtained along the process in the six samples analyzed and the Mock community for each gene. The sequences remaining after bioinformatics filtering (Merged and Cleaned) and the following different assignment criteria: #1 (maximum E-value = 10^{-10} and minimum per-cent identity = 97.0); #2 (maximum E-value = 10^{-50} and minimum percent identity = 97.0); #3 (maximum E-value = 10^{-10} and minimum percent identity = 90.0); #4 (max-imum E-value = 10^{-50} and minimum percent identity = 90.0); and 18S Assigned (maximum E-value = 10^{-50} and minimum percent identity = 80.0).

	18S				COI						
Sample	Raw	Merged	Cleaned	Assigned	Raw	Merged	Cleaned	Assigned			
								Criteria#1	Criteria#2	Criteria#3	Criteria#4
C	91464	58671	32413	29253	126701	116138	113204	32507	5218	94383	15889
N	127708	85499	49037	45067	137023	127237	124397	29890	7550	108993	22052
T	95941	64785	53671	43050	199512	158686	154766	31224	1956	117332	12327
EE	114814	77896	44074	38823	254680	228560	222835	108083	16100	187941	32368
R	56441	32407	24433	23191	56074	52845	51497	29517	25076	70618	34884
NB	112483	88051	70862	64364	149794	139942	136701	39918	8269	114634	16589
Mock community	30604	10490	8739	8728	34132	32468	31781	628	4613	31746	31593
% of Assigned OTU				89%				32%	8%	87%	20%

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For COI, BLAST alignment was also performed against NCBI COI sequences using QIIME, but with four different threshold criteria to further determine the most adequate for macroinvertebrate family assignation: Criteria #1 (maximum E-value = 10^{-10} and minimum percent identity = 97.0); Criteria #2 (maximum E-value = 10^{-50} and minimum percent iden-tity = 97.0); Criteria #3 (maximum E-value = 10^{-10} and minimum percent identity = 90.0); and Criteria #4 (maximum E-value = 10^{-50} and minimum percent identity = 90.0). The E-value or Expect value is the number of different alignments with scores equivalent to or better than S (the raw alignment score), which is expected to occur in a database search by chance. The lower the E-value, the more significant the score and the alignment. The percentage of identity measures the extent to which two sequences have the same nucleotides at the same positions in an alignment [35]. The two partial NCBI databases (for 18S and COI genes) were built using the algorithm described by Baker [36] in 2017. Genetic assignments for both markers were performed by employing the ``assign_taxonomy. pyº python script. Reference databases were constructed using the work flow developed by Baker [36]. Finally, OTU (Oper-ational Taxonomic Unit) tables, a list of OTUs obtained for each sample and the number of sequences assigned to them, were constructed with the `fromTaxassignments2OtuMap.py' algorithm.

In vitro and field validation

***In vitro* validation.** A mock community was set up to verify that our laboratory methods and bioinformatics pipeline were able to correctly detect the taxa of interest (Table 2). It was composed of a known DNA mixture of nine species from different taxonomic groups (one crustacean, one insect, two acorn barnacles, two goose barnacles, and three fish) that occur in water samples at any life stage. This mock community was analyzed together with the set of eDNA samples obtained from the field. The taxonomic assignation of raw sequences for the mock community was manually checked with the BLAST tool included on the NCBI webpage [35] to confirm the assignations were correctly done using our pipeline or if there were errors or incongruences.

Field validation and statistics. The field validation was based on the coincidences between families found from the direct individual sampling of macroinvertebratesÐtaxo-nomically classified *de visu*Ðand the families found from metabarcoding at the six sampling points.

Table 2. Mock community results for the COI and 18S genes. Assignation results after different assignment methods (1±4 and 18S) and manual blast checking using the BLAST tool on the NCBI webpage [39]. Filtering criteria: #1 (maximum E-value = 10^{-10} and minimum percent identity = 97.0); #2 (maximum E-value = 10^{-50} and minimum percent identity = 97.0); #3 (maximum E-value = 10^{-10} and minimum percent identity = 90.0); #4 (maximum E-value = 10^{-50} and minimum percent identity = 90.0); and 18S (maximum E-value = 10^{-50} and minimum percent identity = 80.0).

MOCK COMMUNITY			NGS					Manual BLAST								
Family	Species	Quantity (ng)	COI				18S	COI					18S			
			Criteria #1	Criteria #2	Criteria #3	Criteria #4		Best match species	Accession number	E-value	Identity (%)	N Seqs	Best match species	Accession number	E-value	identity (%)
Caprellidae (crustacean)	*Caprella andreae*	0.05	-	-	-	-	-	-	-	-	-	120	-	-	-	-
Heptageniidae (insect)	*Rhithrogena sp.*	5	0	0	26941	26464	7829	*Rhithrogena sp.*	HM481023	e-133	94	450	*Rhithrogena sp.*	DQ008182	e-102	89
Salmonidae (fish)	*Salmo trutta*	5	4005	4002	4011	4011	-	*Salmo trutta*	HM480831	e-174	98	289	-	-	-	-
Lepadidae (goose barnacle)	*Lepas anatifera*	0.5	449	447	449	449	3	*Lepas anatifera*	GU993620	e-164	98	47	*Lepas anatifera*	FJ906773	e-146	96
Salmonidae (fish)	*Oncorhynchus mykiss*	0.5	20	20	22	22	-	*Oncorhynchus mykiss*	KU867889	e-175	98	304	-	-	-	-
Salmonidae (fish)	*Salmo salar*	0.05	18	18	18	18	772	*Salmo salar*	HM480828	e-178	98	305	*Oncorhynchus mykiss and other species.*	KY115616	0	99
Lepadidae (goose barnacle)	*Lepas pectinata*	0.05	69	69	68	68	-	*Lepas pectinata*	GU993658	e-164	98	17	-	-	-	-
Chthamalidae (acorn barnacle)	*Chthamalus stellatus*	5	28	28	29	29	2	*Chthamalus stellatus*	EU699251	e-166	98	34	*Chthamalus dalli and other species*	KM974371	0	99
Austrobalanidae (acorn barnacle)	*Austrominius modestus*	0.5	14	14	22	22	-	*Austrominius modestus*	KT209230	e-162	98	19	-	-	-	-

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rest of the species assignations. Even though the same amount of DNA (0.5 ng) was added for *Lepas anatifera*, *Oncorhynchus mykiss* and *Austrominius modestus*, the number of assigned sequences was much higher for *Lepas anatifera* than for the other two species (Table 2). For *Salmo salar* and *Lepas pectinata* species, the number of sequences assigned to the detected species were 18±19 and 69, respectively (Table 2). Finally, 0.05 ng of *Caprella andreae* DNA was not detected.

Regarding the assignment criteria tested here, only one was able to correctly detect eight of the species present in the mock community with no false positives, Criteria#4 (E-value of $e10^{-50}$ and 97% identity thresholds; Table 2, left). Using an E-value of $e10^{-10}$, false positives appeared for 97% (Criteria#1, 47 sequences were incorrectly assigned to the fish *Myctophum lychnobium*) and 90% (Criteria#3, 19 sequences wrongly assigned to the arachnid *Teutonia cometes*) identity thresholds. Regarding false negatives, the insect *Rhitrogena* sp. could not be detected from Criteria#1 or #2 (Table 2, left).

18S gene. For the 18S gene, five species from the DNA added to the mock community were not assigned (*Caprella andreae*, *Salmo trutta*, *Oncorhynchus mykiss*, *Lepas pectinata*, and *Austrominius modestus)*. There were 12 sequences for one nematode species that were wrongly assigned (*Eumonhystera cf. hungarica)*, and two of the assignments were under low quality criteria (*Salmo salar* and *Chthamalus stellatus*). Low quality criteria refers to sequences that were aligned using the BLAST tool on the NCBI webpage [39] (Manual BLAST). The real added species (Query) had the same punctuation of assignment (score, identity and coverage) with various species (best match species) (Table 2), so it was not possible to determine the best match. For the 18S gene, the number of sequences assigned correctly to the reference species from the mock community were roughly proportional to the DNA quantity of each species, but we can only refer to *Rhithrogena sp*. and *Lepas anatifera*, as incongruences were not found.

Field validation

The overall taxonomic composition found in the analyzed sampling points was different depending on the genetic barcode employed (Fig 2).

More taxonomic groups were found with COI barcodes, which detected red algae, diatoms, and fungi; these organisms remained undetected with the 18S barcode. In decreasing order of abundance, the more relevant macroinvertebrate groups detected with the COI gene are as follows: Arthropoda > Cnidaria > Annelida > Mollusca. The order was different for the 18S bar-code, as follows: Nematoda > Porifera > Arthropoda > Cnidaria (Fig 2). Many terrestrial species were found in the water from the two metabarcodes (S1 and S2 Tables), such as the birds *Cincla cincla* (European dipper) and *Passer domesticus* (sparrow) and many insects with-out an aquatic phase (Lepidoptera, etc.) that can be found on the river banks or nearby.

The community composition was different at the different sampling points. For example, the fungi Ascomycota were much more abundant at the Tanes sampling point for the COI marker than at the other points, while the abundance of Mollusca DNA was much higher at Anzo than at the other points (Fig 2 and S1 Table).

Considering only freshwater Metazoans for a more homogenous biota profile when com-paring the two barcodes and genus richness given the less accurate taxonomic identification of the 18S barcode, the taxa richness was different at the six sampling points using COI and 18S as barcodes (Fig 3).

The diversity decreased at one (18S barcode) or more (COI barcode) points within the area affected by reservoirs, with a minimum at Rioseco and Anzo in the respective datasets. For the COI marker, the decrease at Anzo was so sharp that this point was significantly different from the diversity at all the other points, except upstream at Caleao (Table 3).

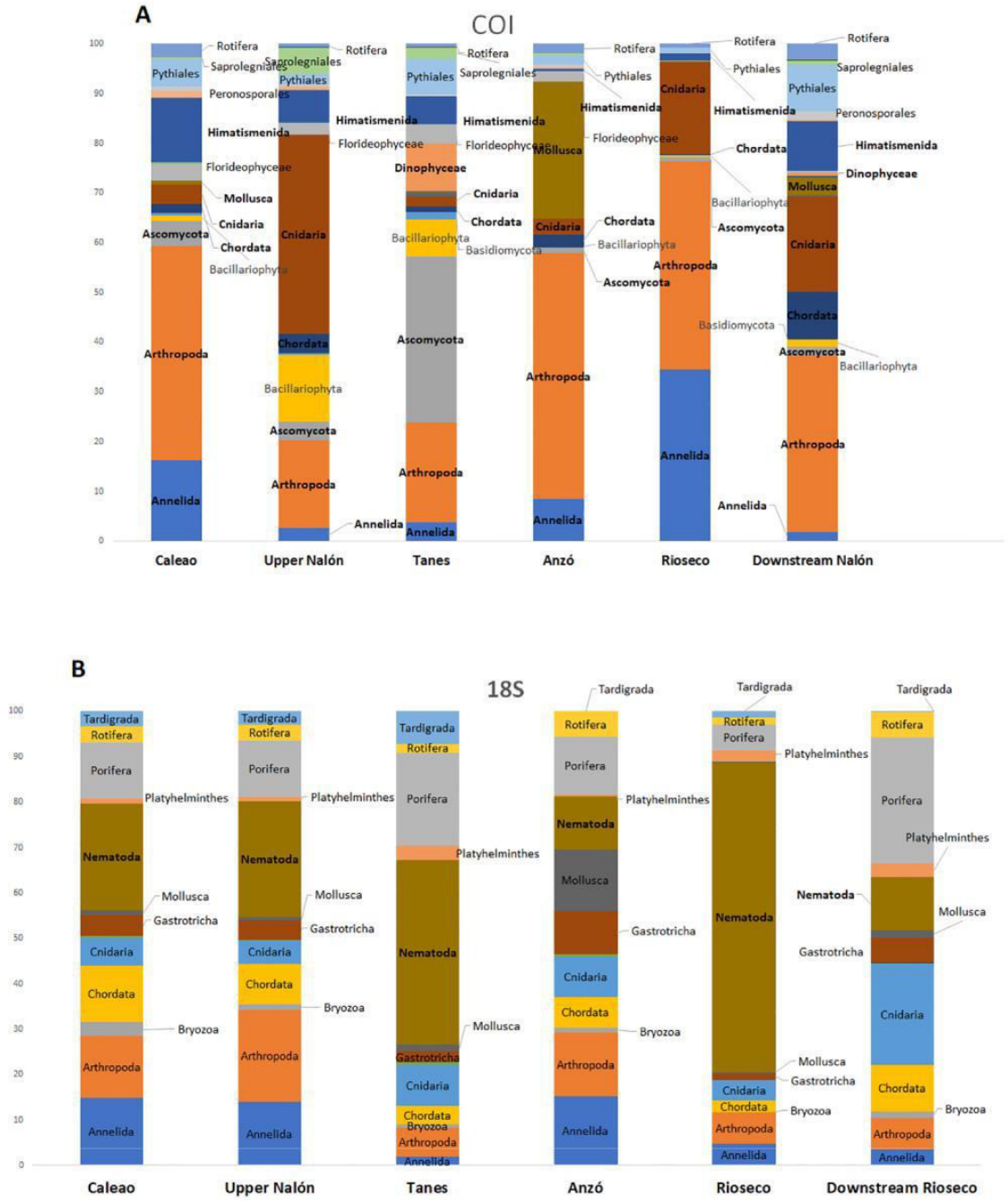

Fig 2. Taxonomic composition of the community identified from eDNA in the six sampling points considered from the Upper Nalon river. A: Percentage of sequences for each taxonomic group found per sampling point with the COI gene. **B**: Percentage of sequences for each taxonomic group found per sampling point with the 18S gene.

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For the 18S metabarcode (Table 3, above diagonal), no significant differences were found for any pairwise comparisons after applying Bonferroni correction (threshold of P = 0.0083 for significance). The point located downstream exhibited the highest diversity in the two datasets, but this was not significantly different from several points upstream for any metabarcode.

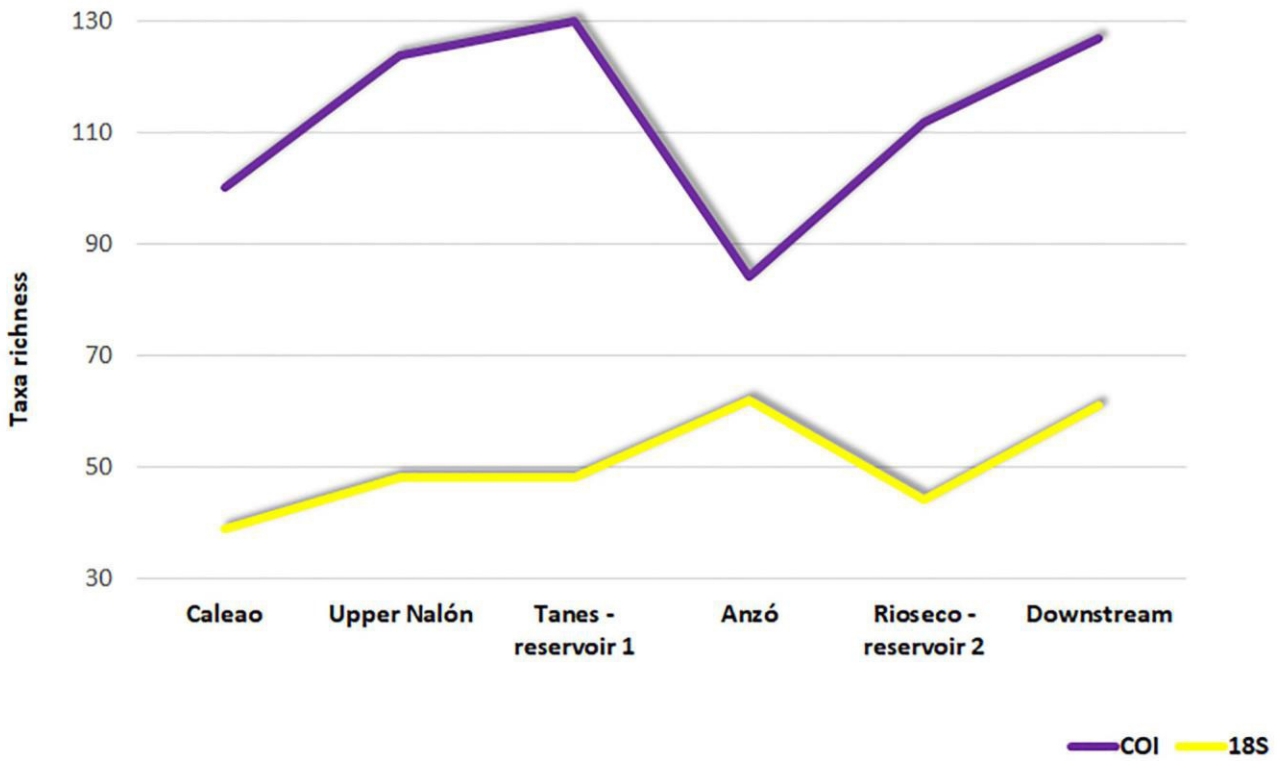

Fig 3. Genus richness at the six sampling points analyzed in this study within the Nalon river using COI and 18S metabarcodes. The points are ordered with downstream on the right.

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Regarding the macroinvertebrate indicators of water quality for the EU WFD, nineteen families were found by visual observation at the sampling points from the River Nalon basin (Table 4).

The same or a higher number of families than those detected by visual identification were found from each sampling point by employing the COI gene as the barcode (Table 4). Using the 18S gene, fewer families were found than with COI and from conventional sampling.

The consistency between eDNA-based family detection and visual observation was higher for COI than for the 18S gene (56.25% and 20.59%, respectively). Considering all the sites, the differences in the number of positives for each family detected from the three methods were statistically significant (Chi-square of contingency value of 44.515 for 19 rows and 3 columns, Fisher's exact test with P-value = 0.009). The 18S barcode was able to detect only 8 of the 19 families sampled from the river using the conventional methodology, while the COI barcode

Table 3. P-values obtained by permutations for pairwise differences in genus richness between the sampling points considered in the Nalon river. Significant values after Bonferroni correction are marked in bold.

COI/18S	Caleao	Upper Nalon	Tanes	Anzo	Rioseco	Downstream
Caleao	-	0.0173	0.2029	0.032	0.3623	0.0359
Upper Nalon	**0.0001**	-	1	0.0464	0.4309	0.185
Tanes	**0.0001**	0.0153	-	0.0506	0.3952	0.2086
Anzo	0.1003	**0.0013**	**0.0003**	-	0.0341	0.8509
Rioseco	0.1622	0.185	0.0855	**0.0001**	-	0.0569
Downstream	0.0127	0.5654	0.5715	**0.0001**	**0.0001**	-

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Table 4. Comparisons between methods. Macroinvertebrate families found by visual observation (visu) and through next-generation sequencing employing the 18S and COI genes, with Assignment criteria #4 for the latter, at each sampling point (marked with ªXº). Proportion of false negatives considering all the sampling sites. Number of positives: the number of times each family was detected through sampling points with each methodology (COI,18S and visual); employed to calculate Fishers exact test.

	Caleao			Upper Nalon			Tanes			Anzo			Rioseco			Downstream Nalon			Number of positives		
Family	**visu**	**COI**	**18S**	**visu**	**COI**	**18S**	**visu**	**COI**	**18S**	**visu**	**COI**	**18S**	**visu**	**COI**	**18S**	**visu**	**COI**	**18S**	**visu**	**COI**	**18S**
Baetidae	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X		5	6	5
Caenidae						X							X						1	0	1
Chironomidae		X	X	X	X	X		X	X	X	X	X	X	X	X		X	X	3	6	6
Chloroperlidae				X									X						2	0	0
Elmidae										X			X						2	0	0
Ephemerellidae	X	X	X		X	X			X			X			X	X		X	2	2	6
Heptageniidae	X	X	X	X	X	X					X	X		X			X		2	5	3
Hydropsychidae		X			X					X	X	X		X			X	X	1	5	2
Leptoceridae	X									X				X					2	1	0
Leuctridae	X	X		X	X									X					2	2	0
Lumbricidae	X																		1	0	0
Lymnaeidae						X				X	X	X							1	1	2
Phylopotamidae													X						1	0	0
Planorbidae		X			X			X					X	X	X			X	1	4	2
Polycentropodidae				X							X			X		X	X		2	3	0
Sericostomatidae	X	X			X								X						2	2	0
Simuliidae		X			X		X	X			X			X			X		1	6	0
Sphaeriidae																X			1	0	0
Tipulidae		X			X		X	X						X		X			2	4	0
N	**7**	**10**	**4**	**6**	**10**	**6**	**2**	**5**	**3**	**6**	**7**	**6**	**8**	**9**	**4**	**5**	**6**	**4**	**34**	**47**	**27**
False negatives	**-**	**2**	**3**	**-**	**2**	**3**	**-**	**0**	**2**	**-**	**2**	**2**	**-**	**5**	**5**	**-**	**3**	**4**			
False negative proportion	**COI = 29.8%**																				
	18S = 70.4%																				

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detected 13 of them. The Chloroperlidae, Elmidae, Lumbricidae, Phylopotamidae, and Sphaer-iidae families remained undetected by the eDNA methodology (Table 4).

For false negatives, as expected from the previous results, the number of families found by visual observation at each site that were not detected by the metabarcoding approach was indeed higher for 18S than for the COI gene. However, the significant difference was only mar-ginally ($p<0.1$) significant (Chi-square of 19.927 for 14 rows and 2 columns, Fisher's exact test with P = 0.097, Monte Carlo P = 0.072).

CBA results

The metabarcoding approach required less effort for sampling and identification (in time) than the morphological approach for sampling and sample processing (53 and 250 min, respectively) (Table 5).

The time estimated for bioinformatics assumes that only one criteria (Criteria#4 as deter-mined in this study) is used; thus, it includes the time necessary for writing commands and retrieving the OTU table in the pipeline employed here. The whole price for the metabarcod-ing analyses was 61.04 euros per sample, which is higher than that estimated for the morpho-logical approach in the current study. The CBA was calculated considering the number of minutes employed, the real metabarcoding costs, and the salaries of technicians in Spain.

Table 5. CBA. Cost estimates for effort and measurements for the metabarcoding and morphological approaches in Spain, where the study took place. Currency: euros (€).

Effort per sample	Metabarcoding	Morphological
Sampling in the river (time)	3 min	10 min
DNA extraction (time)	30 min	-
DNA extraction products (€)	12.7	-
Individual identification (time)	-	240 min
Sequencing cost (€)	40.58	-
Bioinformatics (time)	10 min	-
Total time	43 min	250 min
Total cost	61.04	45.12

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Discussion

Although uses of eDNA-based tools are continuously increasing [21,40±42], the molecular techniques employed, such as the metabarcoding approach, need to be validated depending on the research purposes. It is important to consider the choice of platform, barcode, and thresh-old criteria for bioinformatics analyses before the application of those procedures in real-life cases. In this study, we tested partial COI and 18S genes, two common barcodes for NGS anal-ysis [20,43,44], and a combination of different assignation criteria. Here, we have been able to demonstrate the higher accuracy of the COI gene by employing exigent criteria, such as an E-value (10^{-50}) and 90% identity. All the species were correctly assigned in the mock community, and assignment incongruences were not observed (Table 2). Although higher identity is gener-ally employed for species assignation in normal barcoding using this gene [45,46], it should be considered that the taxonomic level analyzed for water quality indices is family [9,47], not spe-cies, and 90% appears to be enough to assign invertebrate sequences to the family level [42]. Using a more restrictive identity threshold (97%), we would lose some information [48], such as in the case of *Rhithrogena sp*. from the mock community (Table 2). In the mock community, the number of sequences assigned to a species was not proportional to the amount of DNA for that species. This could be explained from primer biases: some primers anneal preferentially to DNA from some taxonomic groups, a bias that has been reported by different authors [24,25]. In other cases, the lack of assignment could be explained from the few reference sequences

in the current databases. This problem of reference scarcity has been repeatedly reported in many studies [21,49±51]. Expanding databases with barcodes from different regions, especially for underrepresented species, should be a priority for enabling the application of metabarcod-ing methodologies in real life environmental analysis.

The nuclear 18S gene did not provide reliable results in this study, and the reasons may be varied. After the quality filtering processes, a high proportion of COI sequences were left for assignation (87.2%; 835,181 sequences), while assignation of the 18S gene was only possible for 44% of the raw sequences (283,229 sequences). Despite the assignation criteria for the 18S gene being quite permissive (minimum percent identity = 80.0), 5 of the 9 species in the mock community could not be assigned (false negatives). Two of the nonassigned species, *Lepas pec-tinata* and *Austrominius modestus*, have 2 and 3 18S gene sequences, respectively, in the data-base; thus, they were probably not assigned because of the lack of reference sequences in the NCBI database. However, the same explanation does not fit for the lack of assignation for *Salmo trutta* and *Oncorhynchus mykiss*, as 18S gene sequences for these two species are more abundant in the database (211 and 495 sequences, respectively). Moreover, incongruent assig-nations were found for *Salmo salar* and *Chthamalus stellatus* in the mock community using the 18S gene (Table 2), with higher identity thresholds for various species. The results derived

from the mock community showed that the 18S gene is not an appropriate barcode for meta-barcoding analyses for our purpose. Additionally, the number of taxonomic groups assigned using the 18S marker was lower than the number assigned with the COI gene. A higher num-ber of Arthropods were assigned with the COI marker; thus, for our purpose of identifying benthic macroinvertebrates that are mostly arthropods, the COI gene marker has been shown to be more appropriate.

The field results supported the choice of the COI fragment as the metabarcode for macroin-vertebrate assessment, as it had a relatively low proportion of false negatives, at least in com-parison with 18S (29.8% for the COI gene and 70.4% for the 18S gene).

In contrast, in the field results, though significant differences were not found between the markers and techniques (molecular or visual), more families were obtained from COI meta-barcoding than from *de visu* analysis. Thus, the genetic techniques are generally more sensitive than conventional sampling [52,53]. It is possible that some invertebrates escaped manual sampling, especially if they were scarce or very small. Alternatively, it is possible that some floating DNA molecules were released from macroinvertebrates upstream. Another possibility that cannot be ruled out is that DNA is being released from carcasses or dead individuals deposited in the substrate. In any case, the presence of a species' DNA indicated the species were or had been present at or near the sampling point.

The taxonomic composition of the sampled river points also contained terrestrial species (i.e., arachnids belonging to the arthropod group) (S1 Table), confirming the hypothesis that river eDNA incorporates biodiversity for a larger scale or whole landscapes [17]. However, in our study, the reservoirs interrupted the expected progressive increase in downstream diversity. Strong diversity decreases were observed in the zones with reservoirs; these results were more acute for COI than for the 18S metabarcode dataset. The differences between the two datasets can be explained by two factors. First, the COI metabarcode detected more genera than the 18S metabarcode; thus, greater statistical significance was obtained in pairwise comparisons. Sec-ond, some taxa more represented in the COI dataset, such as Mollusca and Annelida, do not have terrestrial life stages. Thus, they move into the water and their connectivity is interrupted by dams, while other taxa, like insects (more represented in the 18S dataset), can fly over the dam or pass it from the river's edge in their adult phase. This suggests that the interruption of river connectivity, which is considered one of the worst ecological effects of dams and reser-voirs [54±57], will differentially affect aquatic organisms depending on their life history.

From a more practical perspective, CBA estimation suggested that the conventional tech-nique for macroinvertebrate assessment is costlier than the metabarcoding approach in effort, but not in monetary terms (metabarcoding approach is 15.92 euros more expensive than the conventional approach). Similar costs have been suggested by other authors [58,59], and the technical improvements and wider uses of metabarcoding will likely make the sequencing costs to go down. The use of an eDNA-based tool would therefore improve the effectivity and effi-ciency of water body assessment, allowing for the routine evaluation of freshwater ecosystems.

Finally, the results obtained in the present study regarding metabarcodes and taxonomic assignation criteria will lead the way for using metabarcoding in water samples as an alterna-tive or complementary method for freshwater quality evaluation. As macroinvertebrates are most commonly used as bioindicators, standardizing this approach [13] will allow for increased efficiency and time management [43].

Supporting information

S1 Table. COI OTU Table. Raw data obtained with COI marker clustered in family OTUs (Operational Taxonomic Units). N_genus: Number of genus per family within sampling points. NA: non-asignment at that level.
(XLSX).

S2 Table. 18S OTU Table. Raw data obtained with 18S marker clustered in family OTUs
(Operational Taxonomic Units). N_genus: Number of genus per family within sampling
points. NA: non-asignment at that level.
(XLSX)

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S2 Table. 18S OTU Table. Raw data obtained with 18S marker clustered in family OTUs (Operational Taxonomic Units). N_genus: Number of genus per family within sampling points. NA: non-asignment at that level.

Consensus Lineage				Sampling points (N_genus)					
Phylum	**Class/Subclass**	**Order**	**Family**	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Nalón
Annelida	Clitellata	Haplotaxida	Aeolosomatidae	2	2	1	2	1	1
Annelida	Clitellata	Haplotaxida	Enchytraeidae	1	1	0	1	1	0
Annelida	Clitellata	Haplotaxida	Tubificidae	7	8	4	7	5	8
Annelida	Clitellata	Lumbriculida	Lumbriculidae	0	0	0	1	0	0
Annelida	Clitellata	Rhynchobdellida	Glossiphoniidae	0	0	0	1	2	0
Annelida	Polychaeta	Eunicida	Amphinomidae	0	0	0	1	0	0
Annelida	Polychaeta	Golfingiida	Golfingiidae	0	0	0	0	1	0
Annelida	Polychaeta	Phyllodocida	Sphaerodoridae	1	1	1	1	1	1
Annelida	Polychaeta	Phyllodocida	Syllidae	2	2	1	1	1	1
Annelida	Polychaeta	Terebellida	Ampharetidae	1	1	0	0	0	1
Arthropoda	Arachnida	Araneae	Linyphiidae	1	1	0	0	0	0
Arthropoda	Arachnida	NA	Bdellidae	1	1	0	0	0	0
Arthropoda	Arachnida	NA	Hydryphantidae	0	1	0	2	0	0
Arthropoda	Arachnida	NA	Hygrobatidae	2	2	0	2	0	2
Arthropoda	Arachnida	NA	Labidostommatidae	0	1	0	0	0	0
Arthropoda	Arachnida	NA	Lebertiidae	1	1	0	0	1	1
Arthropoda	Arachnida	NA	Pionidae	0	0	0	1	0	0
Arthropoda	Arachnida	NA	Sperchontidae	0	1	0	1	0	1
Arthropoda	Arachnida	NA	Unionicolidae	0	0	0	1	0	0
Arthropoda	Arachnida	Opiliones	Leiobunidae	0	1	0	0	0	0
Arthropoda	Arachnida	Opiliones	Phalangiidae	0	0	1	0	0	0
Arthropoda	Arachnida	Oribatida	Brachychthoniidae	0	0	1	0	0	0
Arthropoda	Arachnida	Oribatida	Camisiidae	1	1	0	0	1	0
Arthropoda	Arachnida	Oribatida	Hydrozetidae	0	0	0	1	0	1
Arthropoda	Arachnida	Oribatida	Mycobatidae	0	0	0	1	0	1
Arthropoda	Arachnida	Oribatida	Nothridae	0	0	0	1	0	0
Arthropoda	Arachnida	Oribatida	Oppiidae	1	1	0	0	0	0
Arthropoda	Arachnida	Oribatida	Oribatulidae	0	0	0	0	0	1
Arthropoda	Arachnida	Oribatida	Phenopelopidae	0	0	0	1	0	0
Arthropoda	Arachnida	Oribatida	Phthiracaridae	0	0	0	0	0	1
Arthropoda	Collembola	NA	Hypogastruridae	0	0	0	0	0	1
Arthropoda	Diplopoda	Julida	Blaniulidae	1	1	0	0	0	0

Consensus Lineage				Sampling points (N_genus)					
Phylum	**Class/Subclass**	**Order**	**Family**	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Nalón
Arthropoda	Diplopoda	Julida	Julidae	0	0	0	1	0	0
Arthropoda	Diplopoda	Spirobolida	Pachybolidae	0	0	0	0	1	0
Arthropoda	Insecta	Coleoptera	Dytiscidae	1	1	1	1	1	2
Arthropoda	Insecta	Diptera	Chironomidae	1	1	1	2	3	2
Arthropoda	Insecta	Ephemeroptera	Baetidae	1	1	2	3	3	0
Arthropoda	Insecta	Ephemeroptera	Behningiidae	0	0	1	1	0	0
Arthropoda	Insecta	Ephemeroptera	Caenidae	0	1	0	0	0	0
Arthropoda	Insecta	Ephemeroptera	Ephemerellidae	1	1	1	1	1	1
Arthropoda	Insecta	Ephemeroptera	Heptageniidae	1	1	0	1	0	0
Arthropoda	Insecta	Ephemeroptera	Polymitarcyidae	0	0	1	1	0	0
Arthropoda	Insecta	Hemiptera	Aepophilidae	0	0	0	0	0	1
Arthropoda	Insecta	Hemiptera	Delphacidae	0	0	1	0	0	1
Arthropoda	Insecta	Hemiptera	Scutelleridae	0	0	1	1	1	1
Arthropoda	Insecta	Hemiptera	Velocipedidae	0	0	0	0	0	1
Arthropoda	Insecta	Lepidoptera	Bombycidae	1	1	1	1	1	1
Arthropoda	Insecta	Lepidoptera	Nepticulidae	1	1	1	1	1	1
Arthropoda	Insecta	Megaloptera	Corydalidae	1	1	1	1	1	1
Arthropoda	Insecta	Orthoptera	Anostostomatidae	0	0	1	0	0	1
Arthropoda	Insecta	Orthoptera	Cooloolidae	1	0	1	1	1	1
Arthropoda	Insecta	Phthiraptera	Pthiridae	0	0	0	0	0	1
Arthropoda	Insecta	Trichoptera	Hydropsychidae	0	1	0	1	0	1
Arthropoda	Malacostraca	Decapoda	Penaeidae	0	1	1	1	0	1
Arthropoda	Malacostraca	Mysida	Mysidae	0	0	0	0	0	1
Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae	0	1	1	3	1	2
Arthropoda	Maxillopoda	Cyclopoida	Oithonidae	0	1	0	0	0	1
Arthropoda	Maxillopoda	Harpacticoida	Ameiridae	1	0	1	0	0	0
Arthropoda	Maxillopoda	Harpacticoida	Canthocamptidae	2	2	1	2	0	0
Arthropoda	Ostracoda	Podocopida	Candonidae	1	1	1	2	1	1
Arthropoda	Ostracoda	Podocopida	Cyclocyprididae	0	0	2	1	0	1
Arthropoda	Ostracoda	Podocopida	Cyprididae	2	2	0	3	1	0
Chordata	Actinopteri	Anguilliformes	Anguillidae	0	0	0	0	0	1
Chordata	Actinopteri	Blenniiformes	Blenniidae	1	1	1	1	1	1
Chordata	Actinopteri	Characiformes	Characidae	1	1	1	1	1	1
Chordata	Actinopteri	Salmoniformes	Salmonidae	1	1	1	1	0	1

Consensus Lineage				Sampling points (N_genus)					
Phylum	**Class/Subclass**	**Order**	**Family**	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Nalón
Chordata	Amphibia	Anura	Ranidae	0	0	0	1	0	0
Chordata	Aves	Psittaciformes	Loriidae	1	1	1	1	0	0
Chordata	Mammalia	NA	Cervidae	2	2	2	2	2	2
Chordata	Mammalia	Primates	Hominidae	1	1	1	1	1	1
Cnidaria	Anthozoa	Actiniaria	Actinernidae	0	0	0	1	0	1
Cnidaria	Anthozoa	Actiniaria	Actiniidae	2	2	4	2	2	4
Cnidaria	Anthozoa	Actiniaria	Actinoscyphiidae	1	2	1	1	1	1
Cnidaria	Anthozoa	Actiniaria	Actinostolidae	1	1	2	1	1	4
Cnidaria	Anthozoa	Actiniaria	Aiptasiidae	0	0	0	0	0	1
Cnidaria	Anthozoa	Actiniaria	Aliciidae	1	1	1	0	0	1
Cnidaria	Anthozoa	Actiniaria	Andvakiidae	0	0	0	0	0	1
Cnidaria	Anthozoa	Actiniaria	Diadunenidae	1	1	1	1	0	1
Cnidaria	Anthozoa	Actiniaria	Edwardsiidae	1	1	1	1	0	1
Cnidaria	Anthozoa	Actiniaria	Halcampidae	0	0	0	1	1	1
Cnidaria	Anthozoa	Actiniaria	Halcampoididae	1	0	1	0	0	1
Cnidaria	Anthozoa	Actiniaria	Haloclavidae	0	0	1	0	0	0
Cnidaria	Anthozoa	Actiniaria	Hormathiidae	0	0	0	0	0	2
Cnidaria	Anthozoa	Actiniaria	Isanthidae	1	1	1	1	0	1
Cnidaria	Anthozoa	Actiniaria	Metridiidae	1	1	1	1	1	1
Cnidaria	Anthozoa	Actiniaria	Sagartiidae	0	1	0	1	0	1
Cnidaria	Anthozoa	Actiniaria	Stichodactylidae	0	1	0	0	0	2
Cnidaria	Anthozoa	Alcyonacea	Alcyoniidae	1	1	1	1	1	1
Cnidaria	Anthozoa	Alcyonacea	Anthothelidae	1	1	1	1	0	0
Cnidaria	Anthozoa	Alcyonacea	Briareidae	1	0	0	1	0	1
Cnidaria	Anthozoa	Alcyonacea	Chrysogorgiidae	1	1	2	2	1	2
Cnidaria	Anthozoa	Alcyonacea	Ellisellidae	1	1	2	2	1	2
Cnidaria	Anthozoa	Alcyonacea	Ifalukellidae	1	1	1	1	1	1
Cnidaria	Anthozoa	Alcyonacea	Isididae	3	2	3	1	2	3
Cnidaria	Anthozoa	Alcyonacea	Melithaeidae	0	0	0	0	0	1
Cnidaria	Anthozoa	Alcyonacea	Plexauridae	7	1	1	1	1	1
Cnidaria	Anthozoa	Alcyonacea	Primnoidae	1	5	6	7	5	7
Cnidaria	Anthozoa	Antipatharia	Antipathidae	0	0	0	1	0	0
Cnidaria	Anthozoa	Ceriantharia	Cerianthidae	0	0	2	1	0	1
Cnidaria	Anthozoa	Pennatulacea	Funiculinidae	1	1	1	1	1	1

Consensus Lineage				Sampling points (N_genus)					
Phylum	**Class/Subclass**	**Order**	**Family**	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Nalón
Cnidaria	Anthozoa	Pennatulacea	Protoptilidae	1	0	1	1	0	1
Cnidaria	Anthozoa	Pennatulacea	Renillidae	1	1	1	1	1	1
Cnidaria	Anthozoa	Pennatulacea	Virgulariidae	3	3	3	3	2	3
Cnidaria	Anthozoa	Ptychodactiaria	Ptychodactiidae	0	0	0	0	0	1
Cnidaria	Anthozoa	Scleractinia	Oculinidae	0	0	0	1	0	1
Cnidaria	Anthozoa	Scleractinia	Pocilloporidae	1	1	0	1	1	1
Cnidaria	Anthozoa	Scleractinia	Poritidae	1	1	1	1	1	1
Cnidaria	Anthozoa	Telestacea	Telestidae	1	1	1	1	0	1
Cnidaria	Cubozoa	Chirodropida	NA	1	1	0	0	0	0
Cnidaria	Hydrozoa	Actinulida	Halammohydridae	0	0	1	1	0	1
Cnidaria	Hydrozoa	Anthoathecata	Bougainvilliidae	1	1	1	1	1	2
Cnidaria	Hydrozoa	Anthoathecata	Hydractiniidae	1	1	1	1	0	1
Cnidaria	Hydrozoa	Anthoathecata	Pennariidae	1	1	1	1	0	1
Cnidaria	Hydrozoa	Anthoathecata	Stylasteridae	0	0	1	0	2	0
Cnidaria	Hydrozoa	Leptothecata	Aglaopheniidae	0	0	1	0	0	0
Cnidaria	Hydrozoa	Limnomedusae	Monobrachidae	0	0	0	0	0	1
Cnidaria	Hydrozoa	Limnomedusae	Olindiidae	1	4	3	4	3	4
Cnidaria	Hydrozoa	Narcomedusae	Aeginidae	0	0	1	0	1	2
Cnidaria	Hydrozoa	Siphonophorae	Prayidae	1	0	1	1	0	1
Cnidaria	Hydrozoa	Trachymedusae	Geryoniidae	1	1	1	1	1	1
Cnidaria	Hydrozoa	Trachymedusae	Rhopalonematidae	1	1	1	1	1	1
Cnidaria	Scyphozoa	Coronatae	Linuchidae	1	1	1	1	1	1
Cnidaria	Scyphozoa	Coronatae	Nausithoidae	0	1	1	1	0	1
Cnidaria	Scyphozoa	Rhizostomeae	Cassiopeidae	0	0	1	1	0	1
Cnidaria	Scyphozoa	Rhizostomeae	Mastigiidae	0	0	0	1	0	1
Cnidaria	Scyphozoa	Rhizostomeae	NA	0	0	0	0	0	1
Cnidaria	Scyphozoa	Semaeostomeae	Drymonematidae	1	1	1	1	0	0
Cnidaria	Scyphozoa	Semaeostomeae	Ulmaridae	0	0	1	1	0	1
Cnidaria	Staurozoa	Stauromedusae	Craterolophidae	0	0	0	1	0	0
Cnidaria	Staurozoa	Stauromedusae	Haliclystidae	0	0	0	1	0	1
Gastrotricha	NA	Chaetonotida	Chaetonotidae	3	3	1	5	1	1
Mollusca	Bivalvia	Mytiloida	Mytilidae	1	1	0	0	0	0
Mollusca	Bivalvia	Veneroida	Mactridae	1	1	1	1	1	1
Mollusca	Gastropoda	NA	Aclididae	1	1	1	1	1	1

Consensus Lineage				Sampling points (N_genus)					
Phylum	**Class/Subclass**	**Order**	**Family**	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Nalón
Mollusca	Gastropoda	NA	Anabathridae	0	0	0	1	0	0
Mollusca	Gastropoda	NA	Ancylidae	1	2	0	1	0	3
Mollusca	Gastropoda	NA	Caecidae	0	1	0	1	1	1
Mollusca	Gastropoda	NA	Cerithiidae	0	1	0	1	0	0
Mollusca	Gastropoda	NA	Conidae	0	0	0	1	0	2
Mollusca	Gastropoda	NA	Ebalidae	1	1	1	1	1	1
Mollusca	Gastropoda	NA	Epitoniidae	0	0	0	1	0	0
Mollusca	Gastropoda	NA	Hydrobiidae	0	0	0	1	0	0
Mollusca	Gastropoda	NA	Littorinidae	0	0	0	1	0	0
Mollusca	Gastropoda	NA	Planorbidae	0	0	0	0	1	0
Mollusca	Gastropoda	NA	Raphitomidae	0	0	0	1	0	0
Mollusca	Gastropoda	NA	Rissoinidae	0	0	0	1	0	0
Mollusca	Gastropoda	NA	Turridae	0	0	0	1	0	0
Mollusca	Gastropoda	NA	Volutidae	0	0	0	1	0	0
Nematoda	Chromadorea	Araeolaimida	Cylindrolaimidae	0	0	1	0	0	0
Nematoda	Chromadorea	Araeolaimida	Plectidae	2	2	1	0	2	2
Nematoda	Chromadorea	Araeolaimida	Rhabdolaimidae	0	0	1	1	1	1
Nematoda	Chromadorea	Chromadorida	Cyatholaimidae	1	1	1	0	2	0
Nematoda	Chromadorea	Desmodorida	Microlaimidae	0	0	0	0	1	0
Nematoda	Chromadorea	Diplogasterida	Neodiplogasteridae	0	0	1	1	0	0
Nematoda	Chromadorea	Monhysterida	Monhysteridae	1	2	1	3	2	3
Nematoda	Chromadorea	Rhabditida	Rhabditidae	0	0	1	0	0	1
Nematoda	Chromadorea	Rhabditida	Teratocephalidae	0	1	1	0	2	0
Nematoda	Chromadorea	Tylenchida	Anguinidae	0	0	1	0	0	0
Nematoda	Chromadorea	Tylenchida	Criconematidae	0	0	1	0	0	0
Nematoda	Chromadorea	Tylenchida	Tylenchidae	2	2	1	2	1	0
Nematoda	Chromadorea	Tylenchida	Tylenchulidae	0	0	1	0	0	0
Nematoda	Enoplea	Dorylaimida	Dorylaimidae	0	0	1	0	1	2
Nematoda	Enoplea	Dorylaimida	Longidoridae	0	0	1	0	0	0
Nematoda	Enoplea	Dorylaimida	NA	0	0	1	1	0	0
Nematoda	Enoplea	Dorylaimida	Tylencholaimidae	0	0	1	0	0	0
Nematoda	Enoplea	Enoplida	Alaimidae	0	0	0	0	0	1
Nematoda	Enoplea	Enoplida	Bastianiidae	0	0	1	0	0	0
Nematoda	Enoplea	Mermithida	Mermithidae	0	1	3	4	0	2

Consensus Lineage				Sampling points (N_genus)					
Phylum	**Class/Subclass**	**Order**	**Family**	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Nalón
Nematoda	Enoplea	Mononchida	Mononchidae	1	1	1	1	1	1
Nematoda	Enoplea	NA	NA	1	1	0	1	1	0
Nematoda	Enoplea	Triplonchida	Prismatolaimidae	1	1	1	0	1	1
Nemertea	Enopla	Monostilifera	Tetrastemmatidae	0	0	0	0	1	0
Onychophora	NA	NA	Peripatopsidae	1	1	1	1	1	1
Placozoa	NA	NA	NA	0	0	0	0	1	1
Platyhelminthes	Catenulida	NA	Stenostomidae	1	1	1	1	1	1
Platyhelminthes	NA	Lecithoepitheliata	Prorhynchidae	1	1	0	0	0	0
Platyhelminthes	NA	Macrostomida	Microstomidae	2	1	0	1	1	1
Platyhelminthes	NA	NA	Bothrioplanidae	0	0	0	1	1	0
Platyhelminthes	NA	Proseriata	Archimonocelididae	1	1	0	0	0	0
Platyhelminthes	NA	Proseriata	Calviriidae	1	1	0	0	0	0
Platyhelminthes	NA	Proseriata	Coelogynoporidae	1	1	0	0	0	0
Platyhelminthes	NA	Rhabdocoela	Dalyelliidae	0	1	0	1	1	1
Platyhelminthes	NA	Rhabdocoela	Koinocystididae	0	0	1	0	0	0
Platyhelminthes	NA	Rhabdocoela	Polycystididae	0	0	1	0	0	0
Platyhelminthes	NA	Rhabdocoela	Promesostomidae	1	1	1	0	0	0
Platyhelminthes	NA	Rhabdocoela	Typhloplanidae	0	0	1	0	1	0
Platyhelminthes	NA	Tricladida	Dugesiidae	0	0	0	0	1	1
Platyhelminthes	NA	Tricladida	Planariidae	0	1	0	1	0	0
Porifera	Demospongiae	Axinellida	Axinellidae	0	0	0	0	0	1
Porifera	Demospongiae	Biemnida	Biemnidae	1	1	1	1	1	1
Porifera	Demospongiae	Bubarida	Dictyonellidae	0	1	1	2	0	1
Porifera	Demospongiae	Chondrillida	Chondrillidae	1	1	1	1	1	1
Porifera	Demospongiae	Dendroceratida	Darwinellidae	2	2	3	2	1	3
Porifera	Demospongiae	Dendroceratida	Dictyodendrillidae	2	2	2	2	1	2
Porifera	Demospongiae	Dendroceratida	Halisarcidae	0	1	1	1	0	0
Porifera	Demospongiae	Desmacellida	Desmacellidae	1	1	0	1	0	1
Porifera	Demospongiae	Dictyoceratida	Dysideidae	1	1	2	3	2	2
Porifera	Demospongiae	Dictyoceratida	Irciniidae	1	1	1	1	0	2
Porifera	Demospongiae	Dictyoceratida	NA	1	0	0	1	0	1
Porifera	Demospongiae	Dictyoceratida	Spongiidae	2	2	2	3	2	3
Porifera	Demospongiae	Haplosclerida	Chalinidae	0	0	0	1	0	1
Porifera	Demospongiae	Haplosclerida	Niphatidae	0	0	0	0	0	1

Consensus Lineage				Sampling points (N_genus)					
Phylum	**Class/Subclass**	**Order**	**Family**	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Nalón
Porifera	Demospongiae	Merliida	Hamacanthidae	1	1	1	1	0	1
Porifera	Demospongiae	NA	NA	0	0	1	0	0	0
Porifera	Demospongiae	NA	NA	1	0	0	1	0	0
Porifera	Demospongiae	NA	NA	1	1	1	1	1	1
Porifera	Demospongiae	Poecilosclerida	Cladorhizidae	2	1	1	2	1	2
Porifera	Demospongiae	Poecilosclerida	Coelosphaeridae	1	1	2	1	1	2
Porifera	Demospongiae	Poecilosclerida	Crellidae	0	0	0	0	0	1
Porifera	Demospongiae	Poecilosclerida	Esperiopsidae	0	0	1	0	1	1
Porifera	Demospongiae	Poecilosclerida	Guitarridae	0	0	1	0	0	1
Porifera	Demospongiae	Poecilosclerida	Hymedesmiidae	1	2	1	2	1	1
Porifera	Demospongiae	Poecilosclerida	Isodictyidae	0	1	1	1	0	1
Porifera	Demospongiae	Poecilosclerida	Microcionidae	1	2	2	2	2	2
Porifera	Demospongiae	Poecilosclerida	Mycalidae	1	1	1	1	1	1
Porifera	Demospongiae	Poecilosclerida	NA	1	1	1	1	1	1
Porifera	Demospongiae	Sphaerocladina	Vetulinidae	1	1	1	1	1	1
Porifera	Demospongiae	Spongillida	Lubomirskiidae	0	0	1	0	0	0
Porifera	Demospongiae	Spongillida	Potamolepidae	1	1	1	1	1	1
Porifera	Demospongiae	Spongillida	Spongillidae	1	1	3	2	1	2
Porifera	Demospongiae	Suberitida	Halichondriidae	2	4	4	4	4	4
Porifera	Demospongiae	Suberitida	NA	1	1	1	1	1	1
Porifera	Demospongiae	Suberitida	Suberitidae	2	2	2	2	2	2
Porifera	Demospongiae	Tethyida	Tethyidae	1	1	0	0	0	1
Porifera	Demospongiae	Tetractinellida	Scleritodermidae	0	0	1	1	0	1
Porifera	Demospongiae	Verongiida	Aplysinidae	1	1	1	1	1	1
Porifera	Demospongiae	Verongiida	Ianthellidae	1	1	1	1	1	1
Porifera	Hexactinellida	Hexactinosida	Aphrocallistidae	0	1	2	2	1	1
Porifera	Hexactinellida	Hexactinosida	Farreidae	0	1	1	0	0	1
Porifera	Hexactinellida	Hexactinosida	Tretodictyidae	0	1	1	1	0	1
Porifera	Hexactinellida	Lyssacinosida	Euplectellidae	0	0	0	0	0	1
Porifera	Hexactinellida	Lyssacinosida	Leucopsacidae	1	1	1	1	1	1
Porifera	Hexactinellida	Lyssacinosida	Rossellidae	0	0	1	1	0	2
Porifera	Homoscleromorpha	Homosclerophorida	Plakinidae	2	2	2	3	2	3
Rotifera	Bdelloidea	Adinetida	Adinetidae	1	1	1	1	0	0
Rotifera	Bdelloidea	NA	NA	1	1	1	1	1	1

Consensus Lineage				Sampling points (N_genus)					
Phylum	**Class/Subclass**	**Order**	**Family**	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Nalón
Rotifera	Bdelloidea	Philodinida	Philodinidae	0	0	0	0	0	1
Rotifera	Monogononta	Collothecaceae	Atrochidae	0	0	0	0	1	0
Rotifera	Monogononta	Flosculariacea	Flosculariidae	1	0	0	1	1	4
Rotifera	Monogononta	Flosculariacea	Hexarthridae	0	0	0	0	0	1
Rotifera	Monogononta	Ploima	Brachionidae	3	1	3	3	2	5
Rotifera	Monogononta	Ploima	Dicranophoridae	2	1	2	1	1	1
Rotifera	Monogononta	Ploima	Epiphanidae	0	0	1	0	1	0
Rotifera	Monogononta	Ploima	Gastropidae	0	0	1	1	1	1
Rotifera	Monogononta	Ploima	Lecanidae	1	2	2	2	1	2
Rotifera	Monogononta	Ploima	Lepadellidae	1	1	1	1	0	1
Rotifera	Monogononta	Ploima	Lindiidae	0	0	1	1	1	1
Rotifera	Monogononta	Ploima	Notommatidae	2	2	2	2	2	2
Rotifera	Monogononta	Ploima	Proalidae	1	1	1	1	1	1
Rotifera	Monogononta	Ploima	Synchaetidae	2	1	2	2	2	2
Rotifera	Monogononta	Ploima	Trichocercidae	1	1	1	0	0	1
Rotifera	Monogononta	Ploima	Trichotriidae	1	2	1	0	0	2
Tardigrada	Eutardigrada	Parachela	Hypsibiidae	3	4	4	1	2	2
Tardigrada	Eutardigrada	Parachela	Macrobiotidae	1	1	0	0	0	0

S1 Table. COI OTU Table. Raw data obtained with COI marker clustered in family OTUs (Operational Taxonomic Units). N_genus: Number of genus per family within sampling points. NA: non-assignment at that level.

Consensus Lineage				Sampling points (N_genus)					
Phylum	**Class/Subclass**	**Order**	**Family**	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Nalón
Annelida	Hirudinida	Rhynchobdellida	Glossiphoniidae	0	0	0	1	1	2
Annelida	NA	Haplotaxida	Enchytraeidae	2	2	2	0	1	1
Annelida	NA	Haplotaxida	Lumbricidae	0	1	1	0	0	0
Annelida	NA	Haplotaxida	Naididae	4	5	7	2	5	6
Annelida	NA	Lumbriculida	Lumbriculidae	1	1	1	0	0	0
Annelida	Polychaeta	Capitellida	Capitellidae	1	1	1	0	1	1
Annelida	Polychaeta	Phyllodocida	Polynoidae	0	1	1	0	0	0
Arthropoda	Arachnida	Araneae	Anyphaenidae	1	1	1	0	0	1
Arthropoda	Arachnida	Araneae	Araneidae	0	0	0	0	0	1
Arthropoda	Arachnida	Araneae	Linyphiidae	0	1	1	0	0	0
Arthropoda	Arachnida	Araneae	Oxyopidae	1	1	1	1	1	1
Arthropoda	Arachnida	Araneae	Pisauridae	1	1	1	1	1	1
Arthropoda	Arachnida	Araneae	Salticidae	3	4	8	3	2	0
Arthropoda	Arachnida	Araneae	Tetragnathidae	1	1	1	0	0	0
Arthropoda	Arachnida	Araneae	Theridiidae	1	5	0	0	1	0
Arthropoda	Arachnida	Oribatida	Brachychthoniidae	1	1	1	1	1	1
Arthropoda	Branchiopoda	Diplostraca	Chydoridae	0	0	0	1	3	3
Arthropoda	Branchiopoda	Diplostraca	Daphniidae	0	0	1	0	0	0
Arthropoda	Branchiopoda	Diplostraca	Macrotrichidae	1	1	1	1	1	0
Arthropoda	Insecta	Coleoptera	Carabidae	0	0	0	1	1	1
Arthropoda	Insecta	Coleoptera	Cerambycidae	1	1	1	0	0	0
Arthropoda	Insecta	Coleoptera	Chrysomelidae	2	2	2	1	2	3
Arthropoda	Insecta	Coleoptera	Endomychidae	0	0	0	0	1	1
Arthropoda	Insecta	Coleoptera	Gyrinidae	0	1	1	1	0	0
Arthropoda	Insecta	Coleoptera	Staphylinidae	1	1	1	1	1	1
Arthropoda	Insecta	Diptera	Agromyzidae	3	5	5	2	3	4
Arthropoda	Insecta	Diptera	Anthomyiidae	1	1	1	0	0	0
Arthropoda	Insecta	Diptera	Calliphoridae	3	5	5	3	3	3
Arthropoda	Insecta	Diptera	Cecidomyiidae	4	4	4	1	2	2
Arthropoda	Insecta	Diptera	Ceratopogonidae	2	2	2	2	2	2

Consensus Lineage				Sampling points (N_genus)					
Phylum	**Class/Subclass**	**Order**	**Family**	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Nalón
Arthropoda	Insecta	Diptera	Chironomidae	18	20	21	18	20	21
Arthropoda	Insecta	Diptera	Culicidae	2	2	2	3	3	3
Arthropoda	Insecta	Diptera	Dolichopodidae	1	1	1	0	0	0
Arthropoda	Insecta	Diptera	Drosophilidae	2	3	3	3	4	4
Arthropoda	Insecta	Diptera	Hybotidae	1	2	2	1	1	1
Arthropoda	Insecta	Diptera	Muscidae	2	3	3	3	3	3
Arthropoda	Insecta	Diptera	NA	1	1	1	1	1	1
Arthropoda	Insecta	Diptera	Nycteribiidae	1	1	1	0	1	1
Arthropoda	Insecta	Diptera	Pediciidae	1	1	1	0	0	0
Arthropoda	Insecta	Diptera	Psychodidae	1	1	1	1	1	1
Arthropoda	Insecta	Diptera	Sciomyzidae	0	0	0	0	0	1
Arthropoda	Insecta	Diptera	Simuliidae	1	2	2	1	1	1
Arthropoda	Insecta	Diptera	Sphaeroceridae	0	2	2	0	0	0
Arthropoda	Insecta	Diptera	Syrphidae	3	4	4	5	5	5
Arthropoda	Insecta	Diptera	Tabanidae	0	1	1	0	1	1
Arthropoda	Insecta	Diptera	Tachinidae	6	7	7	3	5	5
Arthropoda	Insecta	Diptera	Tipulidae	1	1	1	1	1	1
Arthropoda	Insecta	Ephemeroptera	Baetidae	1	2	2	2	2	2
Arthropoda	Insecta	Ephemeroptera	Ephemerellidae	1	1	1	0	0	0
Arthropoda	Insecta	Ephemeroptera	Ephemeridae	1	1	1	1	1	1
Arthropoda	Insecta	Ephemeroptera	Heptageniidae	1	2	2	0	1	1
Arthropoda	Insecta	Hemiptera	Anoeciidae	0	1	1	0	0	1
Arthropoda	Insecta	Hemiptera	Aphididae	3	5	4	2	2	4
Arthropoda	Insecta	Hemiptera	Aphrophoridae	1	1	1	1	1	1
Arthropoda	Insecta	Hemiptera	Cicadidae	1	3	3	1	1	2
Arthropoda	Insecta	Hemiptera	Clastopteridae	0	1	1	0	0	1
Arthropoda	Insecta	Hemiptera	Drepanosiphidae	0	0	0	0	0	1
Arthropoda	Insecta	Hemiptera	Mesovellidae	1	1	1	1	1	1
Arthropoda	Insecta	Hemiptera	Gelastocoridae	1	1	1	1	1	1
Arthropoda	Insecta	Hemiptera	NA	0	0	0	0	0	1
Arthropoda	Insecta	Hemiptera	NA	1	1	1	1	1	1
Arthropoda	Insecta	Hemiptera	Pentatomidae	0	1	1	0	0	0
Arthropoda	Insecta	Hymenoptera	Apidae	3	1	1	1	1	0

Consensus Lineage				Sampling points (N_genus)					
Phylum	**Class/Subclass**	**Order**	**Family**	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Nalón
Arthropoda	Insecta	Hymenoptera	Braconidae	5	10	11	5	6	6
Arthropoda	Insecta	Hymenoptera	Cephidae	1	1	1	0	0	0
Arthropoda	Insecta	Hymenoptera	Chrysididae	1	1	3	2	2	2
Arthropoda	Insecta	Hymenoptera	Colletidae	27	2	2	2	3	2
Arthropoda	Insecta	Hymenoptera	Eucharitidae	0	1	1	1	1	1
Arthropoda	Insecta	Hymenoptera	Formicidae	2	3	2	0	0	1
Arthropoda	Insecta	Hymenoptera	Ichneumonidae	1	1	1	0	0	0
Arthropoda	Insecta	Hymenoptera	Pamphiliidae	0	0	1	0	0	0
Arthropoda	Insecta	Hymenoptera	Pteromalidae	1	1	1	0	0	1
Arthropoda	Insecta	Hymenoptera	Tenthredinidae	1	1	1	0	2	2
Arthropoda	Insecta	Lepidoptera	Elachistidae	1	1	1	1	1	1
Arthropoda	Insecta	Lepidoptera	Erebidae	1	1	2	1	1	2
Arthropoda	Insecta	Lepidoptera	Geometridae	7	10	10	4	5	5
Arthropoda	Insecta	Lepidoptera	Gracillariidae	2	2	2	1	1	1
Arthropoda	Insecta	Lepidoptera	Hesperiidae	2	1	2	1	1	1
Arthropoda	Insecta	Lepidoptera	Lycaenidae	1	6	9	4	6	7
Arthropoda	Insecta	Lepidoptera	Nepticulidae	1	1	1	1	0	0
Arthropoda	Insecta	Lepidoptera	Noctuidae	4	6	8	1	1	4
Arthropoda	Insecta	Lepidoptera	Notodontidae	1	1	1	1	1	1
Arthropoda	Insecta	Lepidoptera	Nymphalidae	2	2	2	3	4	3
Arthropoda	Insecta	Lepidoptera	Oecophoridae	0	0	0	1	1	1
Arthropoda	Insecta	Lepidoptera	Papilionidae	2	2	3	2	2	2
Arthropoda	Insecta	Lepidoptera	Plutellidae	0	1	1	0	0	0
Arthropoda	Insecta	Lepidoptera	Pyralidae	0	1	1	0	0	1
Arthropoda	Insecta	Lepidoptera	Tortricidae	1	1	1	1	1	1
Arthropoda	Insecta	Lepidoptera	Uraniidae	1	1	1	1	1	1
Arthropoda	Insecta	Mecoptera	Panorpidae	1	1	1	0	1	1
Arthropoda	Insecta	Neuroptera	Berothidae	1	1	1	0	0	0
Arthropoda	Insecta	Neuroptera	Chrysopidae	2	2	2	0	0	0
Arthropoda	Insecta	Neuroptera	Hemerobiidae	1	2	2	1	1	1
Arthropoda	Insecta	Odonata	Libellulidae	0	0	0	0	1	1
Arthropoda	Insecta	Plecoptera	Leuctridae	1	1	1	0	1	1
Arthropoda	Insecta	Plecoptera	Nemouridae	1	1	1	0	0	0

Consensus Lineage				Sampling points (N_genus)					
Phylum	**Class/Subclass**	**Order**	**Family**	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Nalón
Arthropoda	Insecta	Psocoptera	NA	1	1	1	0	1	1
Arthropoda	Insecta	Trichoptera	Brachycentridae	1	1	1	1	1	1
Arthropoda	Insecta	Trichoptera	Hydropsychidae	1	2	2	1	2	2
Arthropoda	Insecta	Trichoptera	Hydroptilidae	0	0	0	1	1	1
Arthropoda	Insecta	Trichoptera	Lepidostomatidae	1	1	1	0	0	0
Arthropoda	Insecta	Trichoptera	Leptoceridae	0	0	0	0	1	1
Arthropoda	Insecta	Trichoptera	Philopotamidae	1	1	1	1	1	1
Arthropoda	Insecta	Trichoptera	Polycentropodidae	0	0	0	1	1	1
Arthropoda	Insecta	Trichoptera	Psychomyiidae	1	1	1	2	2	1
Arthropoda	Insecta	Trichoptera	Rhyacophilidae	1	1	1	1	1	1
Arthropoda	Insecta	Trichoptera	Sericostomatidae	1	1	1	0	0	0
Arthropoda	Malacostraca	Isopoda	Cirolanidae	0	1	1	0	0	0
Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae	0	1	1	1	2	1
Arthropoda	Ostracoda	Podocopida	Cyprididae	0	0	0	0	1	1
Arthropoda	Ostracoda	Podocopida	NA	0	1	1	1	1	1
Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	1	1	1	1	1	1
Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	1	2	2	2	2	2
Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	0	1	1	1	1	1
Ascomycota	Leotiomycetes	NA	Pseudeurotiaceae	1	1	1	1	1	1
Ascomycota	Sordariomycetes	Hypocreales	Bionectriaceae	2	2	2	1	2	2
Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	1	1	1	1	0	1
Ascomycota	Sordariomycetes	Hypocreales	NA	1	1	1	0	0	1
Ascomycota	Sordariomycetes	Hypocreales	NA	1	1	1	0	1	1
Ascomycota	Sordariomycetes	Hypocreales	NA	1	1	1	1	1	1
Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	4	6	6	1	5	5
Ascomycota	Sordariomycetes	Sordariales	Sordariaceae	1	1	1	1	1	1
Bacillariophyta	Bacillariophyceae	Naviculales	Naviculaceae	1	3	3	0	1	1
Bacillariophyta	Bacillariophyceae	Naviculales	Sellaphoraceae	1	2	2	1	1	1
Bacillariophyta	Coscinodiscophyceae	Chaetocerotales	Chaetocerotaceae	1	1	1	0	0	0
Bacillariophyta	Coscinodiscophyceae	Melosirales	Hyalodiscaceae	1	0	0	1	1	1
Bacillariophyta	Coscinodiscophyceae	NA	Stephanodiscaceae	1	1	1	0	0	1
Bacillariophyta	Fragilariophyceae	Fragilariales	Fragilariaceae	1	1	1	0	0	0
Bacillariophyta	Fragilariophyceae	Striatellales	Striatellaceae	1	1	1	0	0	0

Consensus Lineage				Sampling points (N_genus)					
Phylum	**Class/Subclass**	**Order**	**Family**	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Nalón
Bacillariophyta	Mediophyceae	Cymatosirales	Cymatosiraceae	1	1	1	0	0	0
Bacillariophyta	Mediophyceae	Lithodesmiales	Lithodesmiaceae	1	1	1	0	0	0
Basidiomycota	Agaricomycetes	Agaricales	Agaricaceae	1	0	1	1	1	1
Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	1	1	1	0	0	1
Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	1	1	1	0	0	0
Basidiomycota	Agaricomycetes	Agaricales	Physalacriaceae	1	1	1	0	0	0
Basidiomycota	Agaricomycetes	Agaricales	Pluteaceae	1	1	1	0	0	0
Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	1	1	1	1	1	1
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	2	2	2	1	3	3
Basidiomycota	Agaricomycetes	Boletales	Boletaceae	1	2	2	0	0	0
Basidiomycota	Agaricomycetes	Boletales	Paxillaceae	1	1	1	0	0	0
Basidiomycota	NA	Pucciniales	Melampsoraceae	1	1	1	1	1	1
Bryozoa	Gymnolaemata	Cheilostomatida	Candidae	1	1	1	0	0	0
Chordata	Actinopterygii	Cypriniformes	Cyprinidae	1	1	2	1	1	1
Chordata	Actinopterygii	Salmoniformes	Salmonidae	1	2	1	1	1	2
Chordata	Amphibia	Anura	Bufonidae	1	1	1	0	0	1
Chordata	Aves	Passeriformes	Cinclidae	1	1	1	0	0	0
Chordata	Aves	Passeriformes	Passeridae	1	1	1	0	0	0
Chordata	Mammalia	Carnivora	Canidae	1	1	1	0	0	0
Chordata	Mammalia	NA	Suidae	1	0	0	1	1	1
Chordata	Mammalia	Primates	Hominidae	1	1	1	1	1	1
Chordata	Mammalia	Rodentia	Dipodidae	1	0	1	0	0	1
Cnidaria	Hydrozoa	Hydroida	Aequoreidae	1	1	1	0	0	0
Cnidaria	Hydrozoa	Hydroida	Campanulariidae	2	4	4	3	4	3
Cnidaria	Hydrozoa	Hydroida	Corymorphidae	0	0	5	0	0	0
Cnidaria	Hydrozoa	Hydroida	Hydridae	1	1	1	1	1	1
Cnidaria	Hydrozoa	Hydroida	Olindiidae	1	1	1	1	1	1
Cnidaria	Hydrozoa	Trachylina	Rhopalonematidae	1	1	1	1	1	1
Gastrotricha	NA	Chaetonotida	Chaetonotidae	1	1	1	0	1	1
Mollusca	Gastropoda	NA	Achatinellidae	1	1	1	0	0	1
Mollusca	Gastropoda	NA	Agriolimacidae	0	0	0	0	0	1
Mollusca	Gastropoda	NA	Ancylidae	1	1	1	1	1	1
Mollusca	Gastropoda	NA	Bithyniidae	0	0	0	0	0	1

Consensus Lineage				Sampling points (N_genus)					
Phylum	**Class/Subclass**	**Order**	**Family**	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Nalón
Mollusca	Gastropoda	NA	Bulimulidae	0	0	1	0	0	0
Mollusca	Gastropoda	NA	Helicarionidae	0	0	1	0	0	0
Mollusca	Gastropoda	NA	Helicidae	0	0	0	1	1	1
Mollusca	Gastropoda	NA	Hydrobiidae	0	1	1	2	1	2
Mollusca	Gastropoda	NA	Limacidae	1	1	1	0	0	0
Mollusca	Gastropoda	NA	Lymnaeidae	0	0	0	2	2	2
Mollusca	Gastropoda	NA	Physidae	0	0	0	0	1	1
Mollusca	Gastropoda	NA	Planorbidae	2	2	3	0	2	2
NA	Dinophyceae	Gonyaulacales	Amphidomataceae	1	2	2	1	1	2
NA	Dinophyceae	Lophodiniales	Lophodiniaceae	0	0	1	1	1	1
NA	Dinophyceae	NA	NA	0	1	1	1	1	1
NA	Dinophyceae	NA	NA	0	0	1	0	0	2
NA	Dinophyceae	Peridiniales	Oxytoxaceae	0	0	1	0	0	1
NA	Dinophyceae	Peridiniales	Peridiniaceae	0	0	0	0	1	2
NA	Dinophyceae	Peridiniales	Protoperidiniaceae	0	0	0	0	0	1
NA	Dinophyceae	Suessiales	Suessiaceae	0	0	1	0	0	1
NA	Dinophyceae	Suessiales	Symbiodiniaceae	0	1	1	0	0	1
NA	Dinophyceae	Thoracosphaerales	NA	0	0	1	0	0	1
NA	Florideophyceae	Acrochaetiales	Acrochaetiaceae	1	1	1	1	1	1
NA	Heterotrichea	Heterotrichida	Stentoridae	0	0	1	0	0	0
NA	NA	Arcellinida	Lesquereusiidae	1	1	1	0	0	0
NA	NA	Euglyphida	Cyphoderiidae	1	1	1	0	1	1
NA	NA	Himatismenida	Cochliopodiidae	1	1	1	1	1	1
NA	NA	Himatismenida	NA	0	1	1	0	0	0
NA	NA	Lagenidiales	Lagenidiaceae	1	1	1	1	1	1
NA	NA	Leptomitales	NA	1	1	1	0	1	1
NA	NA	Mucorales	Mucoraceae	1	1	1	0	0	1
NA	NA	NA	Vannellidae	1	1	1	1	1	1
NA	NA	Peronosporales	Salisapiliaceae	1	1	1	1	1	1
NA	NA	Physariida	Didymiaceae	1	1	1	1	1	1
NA	NA	Pythiales	Pythiaceae	4	4	4	3	3	4
NA	NA	Saprolegniales	Saprolegniaceae	3	6	7	3	5	6
Nemertea	NA	NA	NA	0	0	0	1	1	1

Consensus Lineage				Sampling points (N_genus)					
Phylum	**Class/Subclass**	**Order**	**Family**	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Nalón
Porifera	Demospongiae	Haplosclerida	Spongillidae	0	0	0	1	1	1
Porifera	Demospongiae	Homosclerophorida	Plakinidae	0	1	1	1	0	0
Rotifera	Bdelloidea	Adinetida	Adinetidae	1	1	1	1	1	1
Rotifera	Bdelloidea	Philodinida	Philodinidae	1	3	3	3	3	4
Rotifera	Monogononta	Ploimida	Gastropidae	0	0	0	0	0	1
Rotifera	Monogononta	Ploimida	Lecanidae	1	1	1	1	1	1
Rotifera	Monogononta	Ploimida	Synchaetidae	1	2	2	1	2	2

Capítulo 3

How can eDNA contribute in riverine macroinvertebrate assessment? A metabarcoding approach in the Nalón River (Asturias, Northern Spain).

Fernández S, Rodríguez S, Martínez J.L, Ardura A and Garcia-Vazquez E.

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How can eDNA contribute in riverine macroinvertebrate assessment? A metabarcoding approach in the Nalón River (Asturias, Northern Spain).

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Rivers' bioassessment is a fundamental component of surface waters surveying. One of the groups most commonly employed as bioindicators of impacted aquatic ecosystems are benthic macroinvertebrates. Their conventional assessment based on morphological identification entails several limitations, such as being time-consuming and expertise-demanding. The use of genetic tools in environmental samples (eDNA) offers an alternative way to evaluate rivers status. The use of eDNA metabarcoding has increased in recent years for different purposes, but its use in water quality evaluation needs to be tested. Here, morphological and eDNA based inventories were compared from the same seven sampling sites in the Upper Nalón River Basin (Asturias, Spain). High Throughput Sequencing (HTS) analyses were carried out with DNA from water samples using an Ion Torrent platform and a region of cytochrome oxidase subunit 1 (COI) gene as barcode marker to assess macroinvertebrate families. Biotic water quality IBMWP indices were calculated from morphological and molecular data and compared with independent physical-chemical habitat assessment to validate the eDNA based approach as an alternative way to monitor the water quality of rivers. The similarity of results from the two approaches, with significant correlation between them, and highly significant correlation between molecular and habitat quality indices, suggest that eDNA performs as well as conventional methods for calculating biotic indices, opening a transformation on river monitoring programs.

Running waters have historically provided a wide range of ecosystem services for human societies[1]. Since they have been the focus of human settlements, rivers are heavily exploited for diverse uses, such as water supply, irrigation, and electricity generation; thus, being amongst the most impacted ecosystems on earth[2]. Nowadays, many restoration and conservation initiatives aim at rivers to reach a good ecological state, for over-time sustainable use of these essential ecosystems[3].

The preservation of aquatic ecosystems is legally binding for public Administrations and private owners in most countries. In Europe, the main instrument for this purpose is the Water Framework Directive (WFD) (Directive 2000/60/EC)[4] that was established to achieve a good ecological status in all surface waters[5]. The accomplishment of WFD requirements implies a regular river monitoring, with water quality assessment as one of its main elements.

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Similarly, in North America the National Water-Quality Assessment (NAWQA) Program was implemented to support national, regional, and local information needs and decisions related to water-quality management and policies[6]. Several Multi-Metric Indices (MMIs) are employed across countries to measure water quality [5–11], and biological indicators are central in the panel of MMIs. Benthic macroinvertebrates are the most widely used species for bioassessment metrics, since they are key indicators of aquatic ecosystem health[12], and as such they are commonly used to identify impacted sites[13]. Water monitoring programs usually involve macroinvertebrate sampling in Europe, North America, and many other regions worldwide[14]. Macroinvertebrates are collected from the river benthos and morphologically identified. Water quality indices are then calculated based on the presence, abundance or proportion of indicator taxa [15]. Many water quality indices use families – instead of species - as biotic indicators: for example, RIVPACS in the UK, and BMWP. The latter (BMWP, Biological Monitoring Working Party) is one of the most widely employed indices in Europe (e.g. the British BMWP/ASPT; IBMWP, adapted to the Iberian Peninsula, and others). It applies different scores to macroinvertebrate families depending on their tolerance to environmental disturbances: the lesser tolerance the higher score [16]. The index value is the sum of the scores of the families present in a site.

These biomonitoring protocols based on macroinvertebrate species have to deal with logistic and financial limitations derived from sampling and taxonomic identification. They are time-consuming and expertise-demanding[17]. Moreover, conventional sampling methods are often invasive because the individuals are removed from the river for identification in the laboratory under a magnifying glass or the microscope[18]. The use of environmental DNA (eDNA), i.e. DNA that macroinvertebrates expel or release in the environment (soil, water, sediments) could deal with these limitations of conventional assessments. Environmental DNA can be amplified through molecular techniques to detect a species' presence, among other applications[19]. Metabarcoding is the combination of high-throughput sequencing (HTS) with the taxonomic assignment of the obtained DNA sequences to reference sequence databases[20]. This approach allows the non-invasive detection of many species from the same environmental sample[21]. The use of eDNA metabarcoding in ecological projects has increased over the last years, and many studies have successfully tested its use for different purposes, such as the detection of invasive[21] and nuisance species[22], biodiversity monitoring[23,24], and others. The use of eDNA for calculating water quality indices based on diatoms has been described[25], but to the best of our knowledge, no one study has been focused on the use of eDNA for indices based on benthic macroinvertebrates, in spite of their application for river water quality monitoring worldwide.

Some authors have employed DNA barcoding and metabarcoding to identify benthic macroinvertebrates and compared molecular techniques with the morphological identification approach (i.e. identification based on diagnostic morphological traits) [26–29]. Emilson *et al*.[27] concluded from their results that DNA barcoding and morphological identification give the same key gradients of water quality in stream conditions. Stein *et al*. [28] found that DNA barcoding gives a deeper ecological signal than morphology, providing higher taxonomic richness as a result of the improvement of assignations in some groups (midges, mayflies, non-insects, caddis flies, and black flies), since from DNA individuals from those groups were assigned to a species level. Elbrecht *et al.*[29] used DNA extracted from tissue samples to demonstrate that metabarcoding represents a feasible method to identify these organisms, and if applied in streams it would give results comparable to conventional protocols based on morphological identification for water quality assessment. On the other hand, the combination of molecular approaches (HTS techniques) on eDNA samples has shown to be useful for evaluating macroinvertebrate diversity in marine and freshwater ecosystems[30–33]. Notwithstanding it, the metabarcoding technique itself has still some limitations that should be addressed[34]. One is the lack of universal primer sets[35]. Although there are some tools available to find the most appropriate primer set for a range of organisms[36], sometimes it is difficult to adjust the

recommended primers to the real target taxonomic groups. The difficult choice of bioinformatics pipelines, or the still incomplete status of reference databases, are two of the most debated issues for application of metabarcoding in real studies of aquatic biodiversity[37].

Water quality assessments based on macroinvertebrates, such as BMWP indices, need taxonomic identification at only a family level. The results of conventional bioassessments would be, in theory, easily improved from eDNA metabarcoding because the problem of incomplete databases for species identification would be less important (it's easier to have all families than all species covered). However, in practice the technique is still immature. Although it has been recently demonstrated that it is more sensitive than conventional morphological approaches for identifying macroinvertebrate families[33,38], the results may vary considerably depending on the specific genes and assignment criteria applied, strict E-value thresholds being necessary in bioinformatics pipelines for preventing false positives[33]. In real river samples, Fernandez et al[33] found cytochrome oxidase I gene (COI) was better suited than ribosomal 18S DNA for this purpose, at least partially due to the fact that there are more COI sequences of freshwater macroinvertebrates in reference databases. From the current state of the field [20,33,38,39], it is clear that further validation is needed for the application of eDNA metabarcoding in real water quality surveys (BMWP or others). Given the scarcity of real river data, the validation should be focused on field studies, comparing eDNA-based biological indices with the same indices obtained from conventional (morphology-based) methodology. Comparisons with independent indicators of river water quality are needed, in order to confirm the validity of the technique for real life river monitoring.

In this study, morphological and molecular approaches were used to calculate water quality indices based on benthic macroinvertebrates as bioindicators, in particular BMWP. The results were compared between methods, and with independent indices estimated from physical and chemical indicators of habitat quality. The upstream area of River Nalón basin (south central Bay of Biscay, northwest of Iberian Peninsula) was considered for field validation, as in previous studies[33,40], because it contains locations of very different river water quality located at a short distance from each other. Some samples were taken from pristine well conserved streams inside the Biosphere Reserve of Redes (Natura 2000[41]), and others from degraded river zones affected by dams, thus different water quality scores were expected. The departure hypothesis of this study was that BMWP indices obtained from eDNA metabarcoding and de visu conventional methods would be positively and significantly correlated to each other, and that the more sensitive eDNA approach would provide stronger correlation with non-biological indicators of water quality than the conventional biological method.

Results

HTS output

Raw sequencing data comprised a total of 2,650,693 sequences distributed across 21 water samples – three replicates per site, seven sites - and one positive control (Supplementary table S1). All the negative controls were clean (undetectable DNA). The raw sequences are available on NCBI's Sequence Read Archive (SRA accession: SRP128681) with the BioSample number SAMN08295300. After application of quality and size filters, 58.42% of the sequences were recovered ("*Filtered*"). Out of these sequences, 27.21% were assigned down to a family level with the settings chosen for taxonomic assignation (Supplementary Table S2). A considerably high number of sequences (51.50% average) were from macroinvertebrate groups (Table 1).

Table 1. High-throughput sequencing output. Number of raw, filtered (after quality and size filtering) and assigned sequences. Macroinvertebrates assigned: total of sequences assigned to target macroinvertebrate groups (maximum E-value = 10^{-50} and minimum percent identity = 90.0 in BLAST alignment tool within Qiime pipeline[42]).

Sampling point	Sample	Raw	Filtered	Assigned	Macroinvertebrates assigned
Caleao	C1	127,379	74,816	11,259	7,441
	C2	137,869	71,586	12,099	3,661
	C3	113,859	53,644	10,092	6,918
Upper Nalón	N1	133,220	85,908	30,348	24,568
	N2	124,524	70,749	6,934	2,562
	N3	70,068	35,737	7,002	708
Tanes	T1	125,211	74,247	2,916	419
	T2	55,575	28,054	1,331	59
	T3	112,924	68,646	1,946	58
Anzó	A1	119,638	69,257	24,045	14,764
	A2	81,893	46,929	20,334	16,916
	A3	55,250	29,982	6,433	5,478
Rioseco	R1	156,094	105,070	92,117	91,528
	R2	143,941	80,729	16,123	11,234
	R3	39,810	23,620	7,192	5,339
Downstream Rioseco	DR1	173,045	106,887	13,962	6,468
	DR2	165,446	99,494	6,409	2,222
	DR3	56,160	32,446	2,675	740
El Condao	EC1	153,362	81,701	4,465	1,018
	EC2	264,839	148,637	9,654	1,605
	EC3	47,683	28,271	2,243	425
Positive control	PC	192,903	132,026	131,825	126,857
Total		**2,650,693**	**1,548,436**	**421,404**	**330,988**

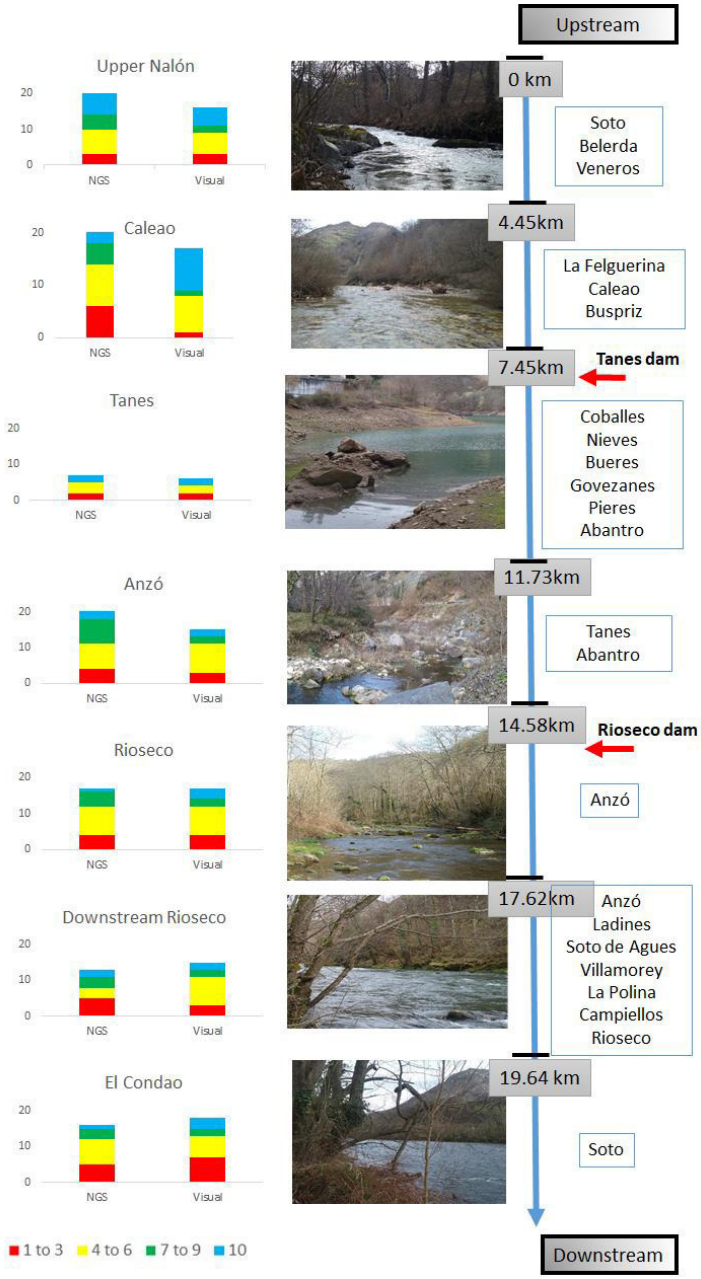
Positive control

All the species included in the positive control were detected (Supplementary Table S1), confirming the robustness of the sequencing and analytical pipeline. The number of sequences assigned to a species was not proportional to the DNA quantity of such species added in the positive control. For example, although the same amount of DNA (5 ng) from the insect *Rhithrogena sp.* and the acorn barnacle *Chthamalus stellatus* was added in the positive control, the number of sequences assigned to the insect species were >120,000, while only 5 sequences were assigned to the acorn barnacle (Supplementary Table S1). A rough association between the DNA quantity and the number of sequences occurred only for Salmonids in this positive control. For *Salmo trutta*, with 5 ng DNA, 3,341 sequences were assigned to any of its subspecies or varieties[43], while 17 sequences were assigned to *Oncorhynchus mykiss* (0.5 ng) and only two to *S. salar* (0.05 ng) (Supplementary Table S1).

Detection of macroinvertebrate families in field samples

A total of 57 macroinvertebrate families listed in the Iberian version of the BMWP index[16], one of the most employed in the Iberian Peninsula for water quality assessment (Figure 1), were detected from either molecular and/or morphological methods in the field samples (Supplementary Table S3). Of these 57 families, 26 were identified from both molecular and visual methods, while 13 and 18 were detected only from visual and eDNA techniques respectively (Supplementary Table S3).

Figure 1. Macroinvertebrate families. Number of macroinvertebrate families in each sampling point, with their correspondent punctuations from the IBMWP index (1 to 10, for most to least tolerant families so worst to best water quality) in different colors. Results from eDNA (NGS) and morphological traits (Visual) are presented. The villages discharging along the surveyed river sectors are shown at right. The situation of the dams is marked with red arrows. The distance (km) between the Upper Nalón point and the rest of the sampled points is shown.



With the morphological method, between 6 and 17 families were found from each sampling site, and between 7 and 25 with the molecular one (Supplementary Table S3). The number of families found from each technique along sampling points was not significantly different (Wilcoxon test W=16, normal approximate t = 1.156, P = 0.247).

Biotic Indices and environmental stressors.

The correlation between index values inferred from conventional and eDNA techniques was positive and statistically significant (r = 0.798, 5 degrees of freedom d.f., P = 0.031) (Figure 2). The water quality obtained from molecular data was equal or higher than those obtained from conventional sampling (Supplementary Fig. 1).

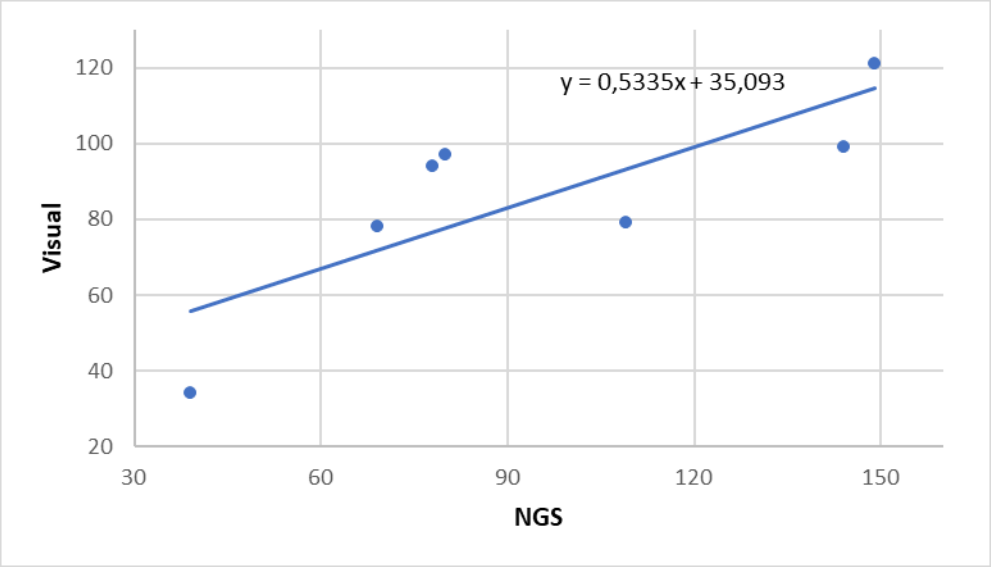


Figure 2. Water quality indices correlation. Correlation between values of water quality indices calculated based on IBMWP index value (NGS: values obtained with the eDNA technique; Visual: values obtained from conventional sampling). The equation is given.

The correlations between the index obtained from physical environmental stressors (Table 2) and the biological water quality indices were negative and significant for both eDNA and visual assessments, rho-value being higher and P-value much lower for the former (Stress score & eDNA, ρ = -0.927 and P = 0.006; Stress score & Visual, ρ = -0.873 and P = 0.017) (Figure 3).

Table 2. Environmental stressors in the river sites considered. Physical-chemical variables: total physical- chemical stress was calculated as ΣNs/6 being Ns the number of parameters that do not fit within the reference values classified as "Good ecological state" for this type of river [44] (highlighted in grey shadow); **Human population pressure**: number of inhabitants of the villages discharging in the river up to 5 km upstream a sampling site (In bold, the sites with >300 inhabitants; scoring one point); **Substrate modification** and **water regime alterations** (In Bold the sites scoring one point). **Total stress score:** sum of stressor scores.

Sampling point	Caleao	Upper Nalón	Tanes	Anzó	Rioseco	Downstream Rioseco	El Condao
pH	7.38	**8.04**	**8.12**	**8.02**	**9**	**8.12**	**8.5**
Conductivity (µS)	125.7	127.1	168,6	123.6	134.2	120.5	121.9
Dissolved 0_2 (mg/L)	8.9	9.2	8	8.76	10	9.3	8.9
Temperature (°C)	8.3	8.5	10.4	8.3	10.3	8.5	8.8
O_2 Saturation (%)	80	86	76	77	94	83	80
NH_4 (mg/L)	**1.73**	0.39	0.39	0.27	**3.4**	**0.56**	0.26
Total physical-chemical stress	*0.16*	*0.16*	*0.16*	*0.16*	*0.33*	*0.33*	*0.16*
Human population	293	203	**330**	187	8	**870**	51
Substrate modification	No	No	**Yes**	No	No	No	No
Water regime alteration	No	No	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
Total stress score	*0.16*	*0.16*	*3.16*	*1.16*	*1.33*	*2.33*	*1.16*

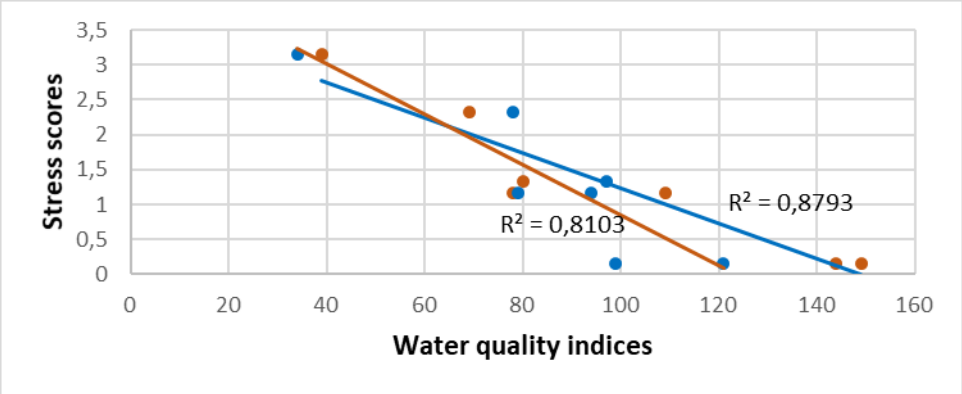
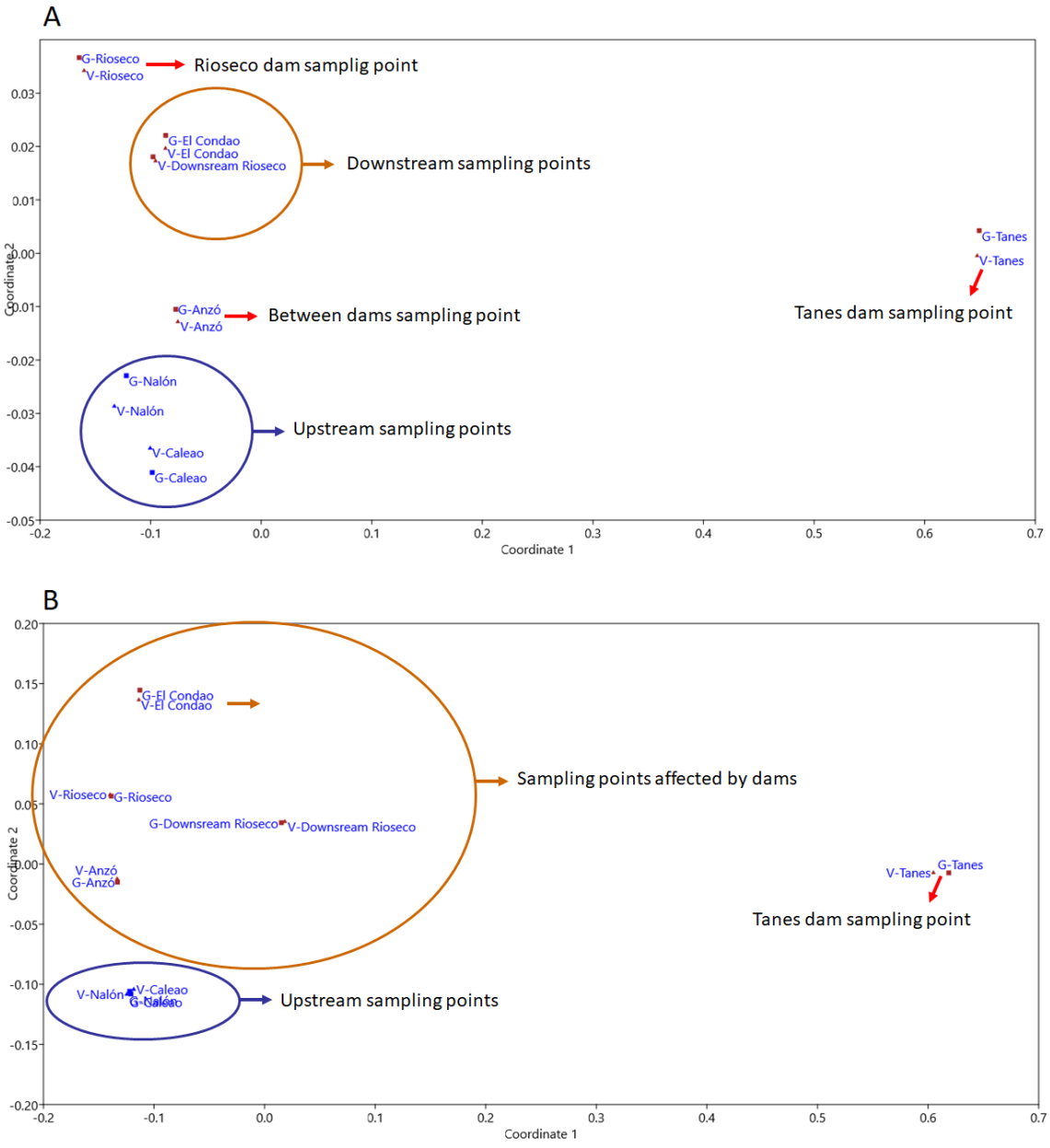


Figure 3. Stress scores and water quality indices. Plots are as follows: Orange, environmental stress scores based on eDNA-IBMWP. Blue: environmental stress scores based on visual-IBMWP.

The differences in ecological status among sampling points are evidenced in the MDS graph (Figure 4). The R^2 values for axis 1 and 2 were 0.762 and 0.029 respectively. The Shepard plot (Supplementary Figure S2) had a stress value of 0.089 (Supplementary Figure 2). The ecological values obtained from eDNA and visual methods for each sampling point were closely grouped together (Figure 4). The samples were roughly grouped according to their situation above or below

the dams, being together the two upstream samples (Upper Nalón and Caleao) and relatively close to each other those located between and below dams, while the two samples directly affected by impounded waters (Tanes and Rioseco) were apart (Figure 4A). The analysis made without the environmental stressors gave a similar picture (Figure 4B; this analysis has a stress value of 0.092, axis 1 with R^2 = 0.499 and axis 2 with R^2 = 0.149), although only Tanes was clearly apart and Rioseco was grouped with the rest of points affected by dams.

Figure 4. Scatter plot of the communities inhabiting the different samples obtained from multidimensional scaling analysis, with (A, above) and without (B, below) environmental stressors (V-sampling point: visual; G-sampling point: eDNA).



For the investigation of the specific effect of dams on the water quality measured from macroinvertebrates, the IBMWP index was compared between the group of samples located upstream the dams (Caleao, Upper Nalón) and the rest of points affected by them. For the eDNA dataset the difference of means was highly significant ($t = 3.796$ with 5 d.f. and $P = 0.012$). For the visual dataset the difference of medians was marginally significant with $p < 0.10$ (Mann-Whitney U with $z = -1.74$, $P = 0.08$).

Discussion

The results of the current study, in particular the highly significant correlation obtained between eDNA BMWP values and independent indices based on environmental stressors in a dammed river basin, demonstrate the usefulness of eDNA metabarcoding to assess river water quality through indices based on macroinvertebrate communities. Supporting Andujar et al[38], we could consider that the eDNA approach has been convincingly tested for the evaluation of the benthic macroinvertebrates employed in current biomonitoring of freshwater ecosystems. The metabarcoding technique here employed, based on COI amplicons obtained from water samples, gave a good taxonomic coverage with an overall 70.5% of the assigned sequences belonging to the targeted groups of macroinvertebrates. Similar BMWP indices were obtained from eDNA and visual techniques in the considered river, with no significant differences in the number of families found by each method and a highly significant correlation between the water quality values obtained from the two methods. However, the IBMWP values obtained using eDNA were higher than those calculated by the visual technique in some points, especially in clean waters (Caleao, Upper Nalón and Anzó), and correlated better with the independent assessment based on environmental stressors. In this sense, the eDNA tool would comply the requirements established by WFD to distinguish clean and highly degraded areas, for their respective conservation and restoration as management priorities.

Our study revealed a big effect of damming in the studied area, from both datasets (eDNA and visual), with the site values grouped together with or without consideration of the environmental stressors (Figure 4). Using the eDNA technique, significant differences were detected between the water quality indices found in the upstream and downstream groups of samples, while the visual indices provided a lower t-value between them – as expected from less marked differences in visual IBMWP indices between clean and disturbed sites. Again, this supports the higher sensitivity of eDNA revealed in other studies[32,33,45,46].

Indeed, some differences between eDNA and visual IBMWP assessment can be explained by the fact that the information obtained from each technique is different. While the visual assessment is based on evaluating macroinvertebrates inhabiting several river meters, the eDNA could, in theory, come from a broader spatial scale because it may be transported downstream suspended in the running water[32]. The eDNA method could thus be employed to bioassess longer river sections with lower sampling effort. The eDNA methodology has also other potential advantages. For some authors it is considered more cost-efficient than the visual assessment, that may be more expensive [21], although the analytical costs of HTS are higher than those of the examination of specimens under the microscope[33]. Besides, conventional sampling has limitations in some sites, for example where the access to the river bottom is difficult, or where trapping macroinvertebrates with a net is impractical due to low or inexistent current. In contrast, sampling eDNA only requires taking water samples, which is much easier and less invasive than kick-sampling macroinvertebrates. Finally, the metabarcoding approach does not rely upon taxonomist expertise [47] and is life stage - and body size - independent.

The differences commented above could explain some dissimilarities in the macroinvertebrates detected from each method. Some families amplified with the eDNA technique were not detected by the visual one, likely because of the higher sensitiveness of eDNA that can detect scarce and low-density populations[45,46,48]. On the other hand, some families physically found

from the sampling sites were not detected by eDNA (Glossiphoniidae, Chrysomelidae, Haliplidae, Gyrinidae, Gammaridae, Athericidae, Ephemeridae, Calamoceratidae, Lepidostomatidae, Leptoceridae, Ferrisia, Planorbidae, Sphaeridae). This could be explained by several reasons. For example, some families may shed less DNA into the water because they have hard exoskeletons/shells (e.g. Planorbidae and Sphaeridae), or perhaps they have a low metabolic activity (i.e. less secretions) and leave less DNA traces. On the other hand, some technical problems still persist in HTS. It is important to remark some limitations of the current technique's state of the art. Possible primer mismatches or template competition for primers affinity may limit amplification success. PCR inhibition, although prevented in our study, could still cause false negatives of scarce sequences. Perhaps the most frequently reported problem is the scarcity of reference sequences in databases for some taxa [22,33,49,50]. The use of COI as a barcode marker has also been contested in some studies [35]. However, for the taxonomic level (family) and groups (river macroinvertebrates including Annelida, Mollusca, Insecta) required for water quality indices, it seems that the molecular approach using a region of COI gene as barcoding marker is robust[12,30–33,38,50]. This robustness is not substantially established for eDNA metabarcoding in general, but from the outcome of our study, it gives results comparable to the morphological approach. Thus, at least in this case, all the reference database employed, barcode marker and bioinformatics pipeline do not seem to limit water quality bioassessment using BWMP index in water samples.

Both eDNA and visual techniques may give false positives. False positives attributed to conventional methodologies such as BMWP are mainly due to inaccurate taxonomic classification of juveniles and larvae, and the proportion ranges between 22.1% and 33.8%[32]. For eDNA, they could come from individuals inhabiting far upstream in cases of strong current, or from remains of dead individuals, although the average life of eDNA in freshwater has been estimated to be quite short (a few days to two weeks)[51]. False positives may also occur in other steps of the eDNA analysis, for example in the laboratory or in the bioinformatics pipeline[52]. In this study we have prevented them in the laboratory working in strict sterile conditions and using positive and negative controls. In the bioinformatics pipeline we did set up a positive control and filtered sequences by quality, as recommended for this type of studies[52]. However, too strict filters could produce false negatives. The results from the positive control analysis showed that, although singletons should indeed be removed from OTU tables for downstream analysis, removing species represented by less than 10 sequences could produce false negatives, since we have obtained less than 10 sequences from four of the nine species of the positive control.

Considering together our study and previous works, a recalibration of molecular indices would be recommended for adapting biotic water quality indices to molecular data, as proposed for marine water quality indices [30]. Some international projects are already developing tools to apply metabarcoding in bioassessment, as DNAAquanet (http://dnaqua.net/). However, this is a huge task that may be impractical in rivers at European level immediately, due to the high costs and the amount of time required. So, while the technique is growing, the existing techniques based on visual assessment could be complemented with eDNA, after their validation and implementation in different areas. Indeed, the specific characteristics of the eDNA in running waters should be considered, such as higher sensitivity and a broader spatial scale application, before applying the results in management actions.

As a technical remark, in the present study quality indices were calculated following the Spanish official protocol for water quality assessment[16], based on presence/absence data, where family is the taxonomic level aimed. For this reason, 90% identity was the cutoff selected for sequence assignation against reference databases, since Hebert el al. [53] deemed it enough for family assignments through COI gene barcoding for most taxonomic groups. However, not all water quality indices are based on families, and the taxonomic level demanded for monitoring programs varies significantly among countries[54]. If the index requires species level assignation, 97% identity will be

more suitable[53]. On the other hand, quantitative elements are also currently required within the WFD. Although some studies have tried to relate the sequence reads with individual abundance metrics[55,56], it is still not possible to do it with the metabarcoding analysis carried out in this study. Nevertheless, the presence/absence of data seems to be sufficient for a precise assessment in many current indices[30,31,39,57], thus the approach here employed has potential for application in the calculation of other indices employed in different regions of the world for river bioassessment [8,10,58]. Despite abundance, metrics as number of individuals or biomass could not be done (see the positive control, where there is no correspondence between number of sequences and DNA quantity), the method would be as informative as conventional methodology for many indices because it is possible to obtain other abundance data (i.e. Total number of taxa, Number of EPT (Ephemeroptera, Plecoptera, Trichoptera) taxa, species richness, etc.). In the current study estimating abundance was not an aim, because the macroinvertebrates index IBMWP is calculated from presence/absence data in Spain [59].

In conclusion, having seen the reliable results obtained in the current study for macroinvertebrate assessment in water samples, eDNA will expectedly transform river biomonitoring. Even though this new approach has some limitations and its implementation still requires an effort, such as the comparison with the metabarcoding of bulk samples, it also has huge advantages, like minimum sampling effort, high sensitivity, species level resolution that barcoding often provides, and non-invasive sampling. Implementing a quick standardized protocol that could be done routinely is a challenge, but we are on the way of improving river monitoring with the use of eDNA based tools.

Methods

Sample collection and processing. Samples were collected on March 2017 from seven sites along the Upper Nalón River Basin (Figure 5). Located in the central part of the region of Asturias (Bay of Biscay, Spain), Nalón-Narcea is the largest basin in the area. The upper zone of the Nalón River belongs to the UNESCO (United Nations Educational, Scientific and Cultural Organization) Biosphere Reserve and Natural Park of Redes. There are two big dams and associated reservoirs (Tanes and Rioseco) interrupting river connectivity.

At each sampling site, physical and chemical variables were recorded using a multiparametric probe (YSI Professional Plus Multiparameter Water Quality Instrument). Three replicates of 1L of water were collected from the bottom of the water column in separate sterile bottles. Immediately after taking the water samples, two macrobenthic samples were collected using a Surber net following the official protocol of the Spanish Ministry of Agriculture for river water quality monitoring[59]. Briefly: One surber sample was taken per each habitat type present in the sampling area, finally obtaining two surber samples per sampled point (as there were two dominant habitats in all of them). Water samples were stored refrigerated until filtration while macroinvertebrate tissue was conserved in 100% ethanol.

Samples were brought to the laboratory and macroinvertebrate specimens were identified by a taxonomist expert in their classification, who categorized them down to a family level using an identification key [60]. For both morphological and molecular data, IBMWP index was calculated as described in the protocol[59]. IBMWP (Iberian Biological Monitoring Working Party) index was chosen for water quality bioassessment because it is the index employed in Spain, where the study took place. Briefly: Each macroinvertebrate family has a score depending on its tolerance to habitat disturbances. The scores are from 1 to 10 points, 1 being the most and 10 the least tolerant. The final value of the index is the sum of the scores of all the families present in a sample.

To control possible contamination during the sampling, all the gear, waders and researchers' clothes that were in contact with the river water and banks were carefully cleaned with 10% bleach

before and after sampling each site. A closed bottle containing sterile water was transported together with the sampling gear and processed with the rest of eDNA-water samples as a sampling negative control to monitor contaminations.

Molecular analyses. *eDNA extraction.* Water samples were vacuum filtered the same day of collection, immediately after arriving to the lab. A Supor® 200 Membrane Filter (Pall Corporation, Life Sciences, Ann Arbor, MI, USA) with 0.2 µm pore size was used. The filtration process followed the protocol described in Clusa *et al.* [18] to prevent contamination. Briefly: water samples were filtered in a room separated from the molecular laboratory in which only water samples are analyzed. The filtration apparatus was cleaned with 10% bleach and then exposed to 20 minutes of UV light in a PCR cabinet (normally utilized for pre-PCR experiments) between samples to prevent contaminations. The sterile water carried to the field (negative control of sampling) was filtered last, after the rest of river water samples. Filters were manipulated with sterile forceps to place them in storage tubes. The tubes were stored at -20ºC until DNA extraction. Environmental DNA was extracted from filters (one extraction per filter) with the PowerWater® DNA Isolation Kit (MoBio Laboratories, CA, USA) under sterile conditions inside a laminar flow PCR-cabinet, following the manufacturer´s instructions. Three extraction replicates per sampling point (one litre each), without any filter clogged, and extraction negative controls (1L of sterile water) plus sampling negative controls were obtained at the end of the process.

Positive control. A positive control was set up to verify that our laboratory methods and bioinformatics pipeline were able to correctly detect the taxa of interest. It was a known DNA mixture of nine species from different taxonomic groups and origin (one crustacean, one insect, two acorn barnacles, two goose barnacles, three fish) that may occur in aquatic environments at any life stage. This positive control was amplified together with the set of eDNA samples obtained from the field in order to have an assignment baseline.

Next generation sequencing. Metabarcoding analyses were done in the Scientific-Technique Services of the University of Oviedo (Spain). Polymerase Chain Reaction (PCR) was carried out under sterile conditions inside a laminar flow PCR-cabinet. Negative controls from filtration, extraction and PCRs were analysed at the same conditions as the rest of the samples and no evidences of contamination were found.

PCRs were carried out using the following primers for the mitochondrial region of COI gene: mlCOIintF and jgHCO2198 [61] modified with a PGM sequencing adaptor, the barcodes (one per sample) needed to differentiate the reads belonging to each water sample, and a ‘‘GAT” spacer (Supplementary Table S4). Amplification was carried out in a total volume of 20 µl including Green GoTaq® Buffer 1X, 2.5mM MgCl2, 0.25mM dNTPS, 20pmol of each primer, 4 µl of template DNA, 200ng/µl of BSA[62] (bovine serum albumin), and 0.65 U of DNA Taq polymerase (Promega, Madison, Wisconsin, USA). PCR conditions in the Veriti Thermal Cycler (Applied Biosystems, Foster City, California) were 95°C for 1min, followed by 35 cycles of 95 °C for 15s, 46 °C for 15s, 72 °C for 10s, and a final extension of 72 °C for 3min. Negative and positive controls were included at any instance. The amplification success was visually assessed on 2% agarose gel. PCR amplicons were purified from agarose gel using the Montage DNA Gel Extraction Kit (Millipore, Massachusetts, USA); quantified using the QuBit BR dsDNA kit (Thermofisher Scientific, Carlsbad, CA, USA); and double-checked in a Bioanalyser 2100 (Agilent Technologies, USA) to confirm the fragment size, the absence of by-products, and to do a more precise quantification. All the samples were diluted down to 26pmol for preparing an equimolar pool with them. The pool was processed by liquid emulsion PCR in the One Touch System using the Ion PGM™ OT2 Supplies Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer’s instructions. Then the sample was loaded in the Ion ‘‘314” Chip (Life Technologies, Carlsbad, CA, USA), and sequenced employing the Ion Torrent Personal Genome Machine (Life Technologies, Carlsbad, CA, USA), following the specifications in the protocol Ion

PGM™ Sequencing kit. Low quality and polyclonal sequences were filtered automatically and the PGM adaptor was trimmed within the software.

Environmental Stressors. Some stressors affecting the environmental status of the river in the sampled points were considered.

Six physical-chemical variables (pH, Conductivity, Dissolved 0_2, Temperature, O_2 Saturation and NH_4) were measured for each sampling point at the moment of sample collection. These values were categorised as "good/not good" based on the reference values defined by Pardo et al. for Cantabrian Confederation Rivers[44]. For each sampling point, the physical-chemical stress was calculated from the number of variables not accomplishing a 'good status' divided by the total number of variables (ΣNs/6, being Ns the number of parameters that did not fit under the classification of "good ecological status") (Table 2) (e.g.: In Caleao sampling point, all variables fit with a "good status" value except NH_4, so the score for physical-chemical stress is 0.16 (1/6)).

Three other stressors were considered: the number of inhabitants in the nearest villages (up to 5 km upstream) discharging waste waters in the river: score 0-1, 1 for >300 inhabitants; substrate modification: score 0-1, 1 for modifications such as excessive sediments caused from impounded waters or works, artificial river bed (e.g. concrete), etc.; water regime disturbances caused by damming (i.e. water releases to control reservoir water levels), also scoring 0-1.

The total environmental stress score was calculated as the sum of the four environmental stressors considered.

Bioinformatics analysis. Low quality and polyclonal sequences were automatically filtered and the PGM adaptor was trimmed within the PGM software. Qiime software[42] 1.9.1 version was used to split the 'fastq' files into constituent. fna and .qual files using ''convert_fastqual_fastq. py" python script, and to filter sequences by quality and size (minimum and maximum size of 250-400 and quality score of 25) using "split_libraries.py" python script. Then, primer trimming was done with PRINSEQ v0.20.4 software[63].

COI gene reference database was constructed from NCBI COI sequences using the work flow developed by Baker [64]. Then, BLAST alignment was done against this database with the settings described by Fernandez *et al.* [33] as optimal for this gene and taxonomic groups (maximum E-value = 10^{-50}; minimum percent identity = 90.0), employing ''assign_taxonomy. py" python script without clustering or dereplication, taking into account all the sequences and haplotypes obtained. Finally, OTU (Operational Taxonomic Unit) tables, a list of OTUs obtained in each sample and the number of sequences assigned to them (Supplementary Table S2), were constructed with the algorithm 'fromTaxassignments2OtuMap.py'. In downstream analysis, families represented by less than 1 sequence (singletons) were removed from the OTU table.

OTUs corresponding to the taxonomic groups Annelida, Arthropoda, Mollusca, Cnidaria, and Platyhelminthes were filtered from the OTU table in Microsoft excel (2013), then they were given the corresponding family scores and IBMWP index was calculated as the sum of all the family scores, following IBMWP methodology.

All the 21 field samples were processed separately, and the results combined per sampled point after OTU table construction (Supplementary table S2).

Statistical analysis. Data normality was checked from Shapiro-Wilk tests. According to the results, parametric (ANOVA, t-tests of independent or paired groups) or non-parametric (Kruskal-Wallis, Mann-Whitney, Wilcoxon) tests were employed to compare groups of samples. Similarly, Pearson's r or Spearman's rho tests were employed for determining correlations between normal and non-normal datasets respectively.

For multivariate analysis we used non-metric multidimensional scaling (MDS). A general vision and graphic representation of the communities present in the different samples from genetics and visual methods was obtained through a 2D scatter plot, employing Bray-Curtis similarity index for the distances and 9999 bootstrap. A Shepard plot was also constructed and the stress and r2 of the two axes were calculated (Supplementary Figure S2). The dataset included the macroinvertebrate families (1 presence, 0 absence) and five physical-chemical parameters: pH, conductivity dissolved 02 (mg/L), temperature and NH4 (mg/L).

The statistical analyses were implemented in PAST software[65].

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Author Contributions

S.F. conceived and designed the experiments, performed the experiments, analyzed the data and wrote the main manuscript. S.R-M. performed the experiments and analyzed the data. JL.M. conceived and designed the experiments, performed the experiments and analyzed the data. A.A. designed the experiments and revised the manuscript. E.G-V. conceived and designed the experiments, analyzed the data and wrote the main manuscript. All authors reviewed the manuscript.

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Additional information

The author(s) declare no competing interests.

Supplementary information

Supplementary table S1. Positive control assignation results. Number of sequences assigned from the alignment of the positive control.

Organism	Family	Species	DNA quantity (ng)	Number of assigned sequences
Insect	Heptageniidae	*Rhithrogena sp.*	5	126,854
Crustacean	Caprellidae	*Caprella andreae*	0.05	8
Goose barnacle	Lepadidae	*Lepas anatifera*	0.5	1,334
Goose barnacle	Lepadidae	*Lepas pectinata*	0.5	248
Acorn barnacle	Austrobalanidae	*Austrominius modestus*	0.5	8
Acorn barnacle	Chthamalidae	*Chthamalus stellatus*	5	5
Fish	Salmonidae	*Oncorhynchus mykiss*	0.5	17
Fish	Salmonidae	*Salmo salar*	0.05	2
Fish	Salmonidae	*Salmo trutta*	5	3,341

Supplementary Table S2. Raw OTU sequences per sampling replicate (C:Caleao; N:Upper Nalón; T:Tanes; A:Anzó;R:Rioseco;DR:Downstream Rioseco; EC:El Condao; PC:positive control). **Anexo 1.**

Supplementary Table S3. Benthic macroinvertebrates. Macroinvertebrate families found per sampling point with molecular (G: number of sequences amplified) and morphological (V: number of individuals visually assessed) approaches, and their punctuation following the official protocol for IBMWP index calculation. **Anexo 1.**

Supplemetary Table S4. Metabarcoding primers sequences (5'-3').

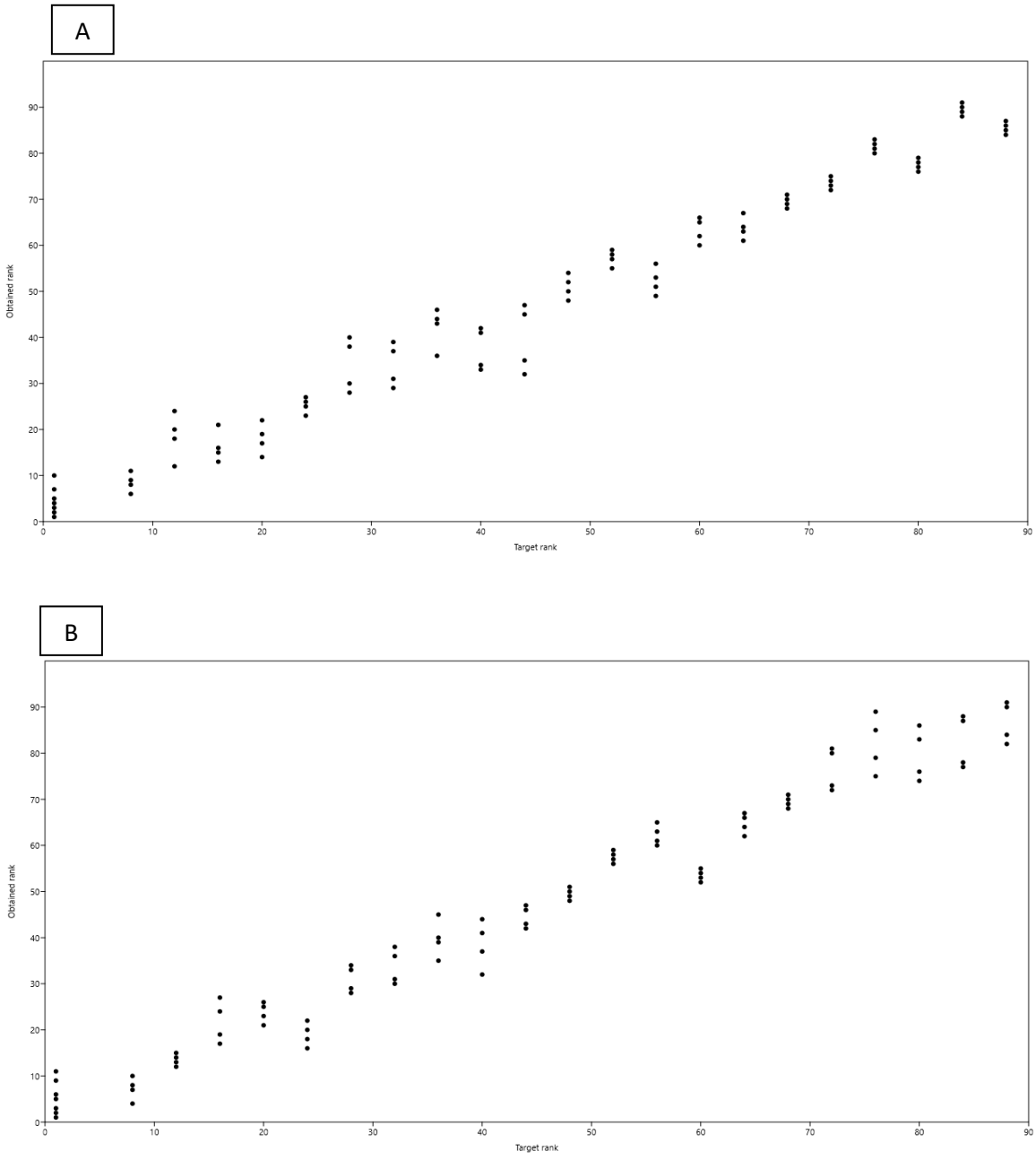
Primer name	Sequence
mlCOIintF_22	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGAGACGCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_23	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGCCACGAACGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_24	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCTCATTCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_25	CCATCTCATCCCTGCGTGTCTCCGACTCAGCCTGAGATACGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_26	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTACAACCTCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_27	CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCATCCGCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_28	CCATCTCATCCCTGCGTGTCTCCGACTCAGATCCGGAATCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_29	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGACCACTCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_30	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGAGGTTATCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_31	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCAAGCTGCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_32	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTTACACACGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_33	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCTCATTGAACGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_34	CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCATCGTTCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_35	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGCCATTGTCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_36	CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGGAATCGTCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_38	CCATCTCATCCCTGCGTGTCTCCGACTCAGTGGAGGACGGACGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_39	CCATCTCATCCCTGCGTGTCTCCGACTCAGTAACAATCGGCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_40	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACATAATCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_41	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCACTTCGCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_42	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACGAATCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_43	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTTGACACCGCGATGGWACWGGWTGAACWGTWTAYCCYCC
mlCOIintF_44	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTGGAGGCCAGCGATGGWACWGGWTGAACWGTWTAYCCYCC

Supplementary Figure S1. Water quality indices. Values of water quality indices calculated based on IBMWP index value. A: Range of quality classes from Alba et al. (2005) [40]; B: Quality IBMWP indices obtained for the sampling points of this study (NGS: values obtained using eDNA; Visual: values obtained from conventional tecnique).

A Class	IBMWP value	Meaning	Color
I	>101	Clean water	Blue
II	61-100	Some pollution effects are visible	Green
III	36-60	Polluted water	Yellow
IV	16-35	High polluted water	Orange
V	<15	So high polluted water	Red

B Sampling Points	NGS-IBMWP	Visual-IBMWP
Caleao	149	121
Upper Nalón	144	99
Tanes	39	34
Anzó	109	79
Rioseco	80	97
Downstream Rioseco	69	78
El Condao	78	94

Supplementary Figure S2. Shepard plots of the MDS analyses (A: including physical-chemical parameters; B: Without physical-chemical parameters).



Capítulo 4

Non-indigenous fish in protected spaces: trends in species distribution mediated by illegal stocking.

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Non-indigenous fish in protected spaces: trends in species distribution mediated by illegal stocking.

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ABSTRACT

Many freshwater NIS (non-indigenous species) are stocked for recreational fishing, in some cases illegally as it happens in protected areas. Here we monitored fish communities in a mountainous Biosphere Reserve in Asturias (Northern Spain) where stocking is forbidden, using environmental DNA, electrofishing and anglers' catch as sources of samples. Three exotic species were illegally introduced in the protected space and show increasing trends in the last two decades. Two of them used as fishing bait, the chub (*Squalius carolitertii)* and the minnow (*Phoxinus phoxinus)*, are expanding in running waters. So it does the rainbow trout (*Oncorhynchus mykiss)*, likely introduced for angling and/or from fish farm escapes. The results suggest that sustained illegal stocking is contributing to the increase of the three NIS. Opposite to them, brown trout (*Salmo trutta*) of northern European lineages –identified from *90 alleles at LDH-C1 locus and formerly legally stocked for angling- is decreasing, probably due to climate warming. The same climate warming would contribute to the expansion to formerly much colder upstream areas of the two non-native Cyprinids. Through the application of a social survey we found that unlike other population groups, anglers in the region significantly preferred stocking over environmental improvement for management of fish populations. The results obtained suggest raising angler's awareness about the importance of safeguarding local fish could help to prevent the spread of non-native species in protected spaces.

KEY WORDS: Protected spaces; monitoring; eDNA; exotic species; brown trout lineages; citizen science.

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INTRODUCTION

Species distribution in rivers follows the river´s continuum theory, in which structural and functional characteristics of communities are adapted to conform to the most probable position or mean state of the physical system (Vannote *et al.*, 1980). However, changes in freshwater species occupancy are sometimes human mediated. The society uses rivers as source of energy, water and other ecosystem services, consequently, there are many impacts derived from human action that affects rivers. Stocking, aquarium releases, and international shipping have been identified as main activities contributing to the spreading of exotic freshwater species (Havel *et al.*, 2015). The construction of dams, canals and reservoirs also creates new paths for species dispersal (Rahel and Olden, 2008). In some cases, reservoirs can act as shelters for non-indigenous species.

Fish contain the most abundant group of non-indigenous species (NIS) transported by humans in freshwater ecosystems (Gozlan, 2010). Fish development, spawning and growth are heavily influenced by water temperature (Sharma *et al.*, 2007), thus not all the exotic species can successfully establish self-sustained populations in the recipient ecosystem (Moyle and Maechetti, 2006), because of the differences on water temperature compared with native ecosystems. Their adaptation and spreading are highly dependent on suitable thermal conditions, and global warming is likely enhancing only some introduced species, like Cyprinidae and Percidae (Lehtonen, 1996). Apart from warming temperatures, other consequences of climate change may favor the spreading of exotic species across freshwater ecosystems. Examples are the alteration of flow regimes that increases the probability of aquaculture escapes, and the salinity decrease in estuaries due to increased river flow that facilitates NIS introduction therein (Holopainen *et al.*, 2016).

Many economic and ecological consequences of NIS introductions have been reported worldwide (i.e. Simon and Townsend 2003; Gozlan 2010; Strayer 2010; Ardura *et al.* 2016; Wissinger *et al.* 2006). However, the real impact of NIS is still unknown since it should be considered at different levels, over the long term, and this type of studies about NIS impact are scarce. In the Iberian Peninsula (South Europe), a 36% of NIS are fish species (Gozlan, 2010). One of the most important impacts of fish NIS on Iberian freshwater ecosystems is the alteration of native populations (Rincon *et al.*, 1990; Garcia-Marin *et al.*, 1999; Aparicio *et al.*, 2000; Ayllon *et al.*, 2006; Horreo and Garcia-Vazquez, 2011). Since Iberian native fish are amongst the most threatened species in Europe (Miranda and Pino-del-Carpio, 2016), impacts caused from NIS aggravate the condition of these vulnerable populations.

The creation of protected areas may help to reduce the spreading of non-indigenous species because the anthropogenic impacts should be minimized there, but in some cases the protection does not

involve the prohibition of some human uses as angling. The success of protected areas to prevent NIS introduction has been reported for marine protected areas (Lubchenco *et al.* 2003; Ardura *et al.* 2016). However, few spatial protection figures have been created specifically for freshwater; many protected river sections have such status because they flow within terrestrial reserves (Skelton *et al.* 1995). A river included in a protected area is not a synonym of specific protection of freshwater. Dams or water diversion for agriculture can occur outside the boundaries of protected areas and still have negative consequences for freshwater habitats. An example of mismanagement of freshwater ecosystems within protected spaces is the practice of stocking non-native species for sport fishing (Saunders *et al.*, 2002). Although stocking has caused enormous negative impacts in native wild fish populations (e.g. Levin *et al.* 2001), it was and still is demanded by anglers in many regions worldwide, and the Iberian Peninsula is not an exception: stocking is currently driving introgression of exotic brown trout in Spain (e.g. Horreo *et al.* 2015).

The anglers themselves are an important part of the problem. Illegal stocking often happens for purposes of increasing recreational fishing, through releases of fishing targets (Hickley and Chare, 2004; Canonico *et al.*, 2005; Johnson *et al.*, 2009) and/or of their preys (Elvira *et al.*, 2001; McPhee *et al.*, 2002). Illegal stocking is suspected even in areas declared genetic refuges of native fish (Araguas *et al.*, 2009). Angler's awareness of the importance of preserving native biodiversity is thus essential for excluding stocking (Araguas *et al.*, 2009, 2017), together with strict control and surveillance of the protected spaces.

Given the importance of preserving the highly vulnerable native fish communities, it is important to avoid illegal stocking in protected spaces. The several factors that mediate the expansion of NIS should be considered. In this study, fish communities were assessed in the upper zone of a river basin, located within a Biosphere reserve area in Asturias (northwestern Spain) where the introduction of non-indigenous fish and any type of stocking is strictly forbidden (Spanish Directive 162/2014, 29 December). In this area introduction of exotic rainbow trout *Oncorhynchus mykiss*, likely from aquaculture escapes, has been recently detected from environmental DNA (Fernandez *et al.*, 2018).

The objective of this study was to monitor the trends in NIS and native fish distribution and to infer the NIS origin from a combination of electrofishing, environmental DNA and volunteer donation of samples by anglers. The trends (expansion or contraction) of non-indigenous fish in the last decades were determined by comparison with previous inventories. The opinion of anglers and non-anglers in the region was tackled, as proposed by Araguas *et al.* (2009), to infer the local pressure/s for (illegal) stocking that is generally assumed to be conducted

by anglers (e.g. McPhee *et al.*, 2002; Johnson *et al.*, 2009). Expectations were: i) Following Lehtonen et al. (1996), Cyprinids would increase, and cold-water non-indigenous salmonids would decrease; ii) Illegal stocking, detected in other protected areas in the region (Horreo & Garcia-Vazquez, 2011), would contribute to increase the two types of exotic fish; iii) Farm escapes (Fernandez *et al.*, 2018) and reservoirs (Rahel, 2007) would contribute to enhance exotic Salmonids.

METHODS

Study area

Nalón River, located in the central part of the province of Asturias (northern Spain), is the largest freshwater system and one of the most important in the Iberian Bay of Biscay (140.8 km length and an average discharge of 55.18 m3/s) (Figure 1).

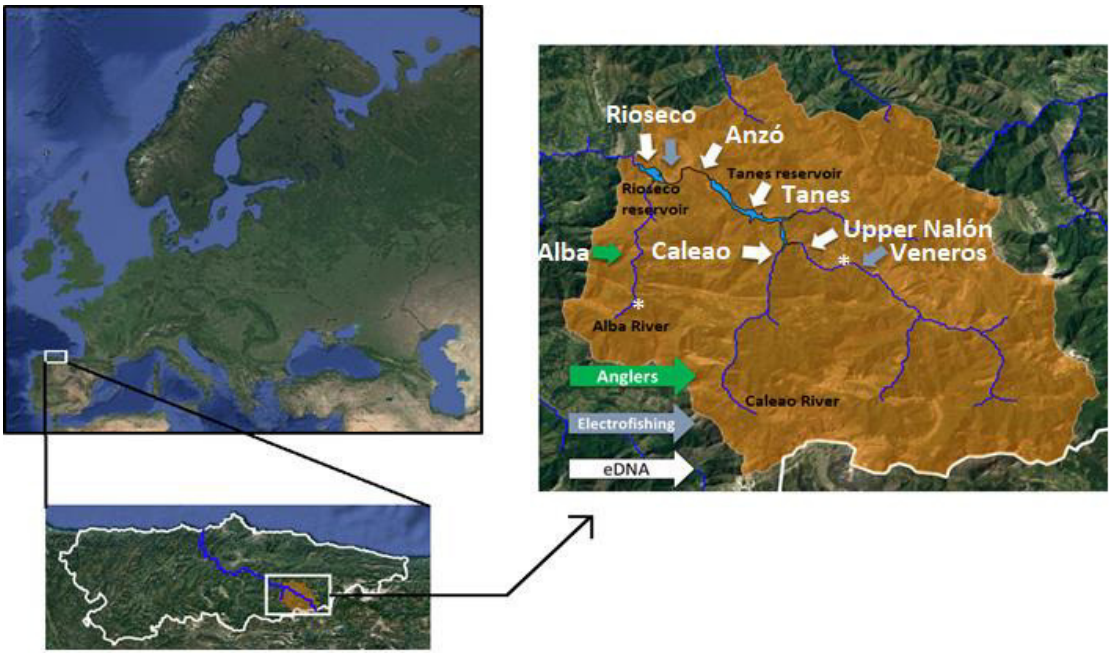

Figure 1. Natural Park of Redes. Map of the protected area in Asturias (Spain) and sampling points (names included). The three techniques employed for fish detection are marked as: Green arrow: anglers' catch; White arrows: environmental DNA from water samples; Blue arrows: electrofishing. Rainbow trout fish farms are marked as an asterisk.

This study took place in the upper zone of the river, a protected space that belongs to the UNESCO (United Nations Educational, Scientific and Cultural Organization) Biosphere Reserve and Natural Park of Redes. The protection started officially in 1996 as a Natural Park, and in 2001 it was declared a Biosphere Reserve (Spanish law 8/1996, 27 December). Streams inside this

mountainous protected area are steep, fast, with cold and well oxygenated waters. They hold a highly diverse fauna (García-Ramos *et al.*, 2006) where the dominant fish species is brown trout (*Salmo trutta),* an appreciated species in the region for having important economic and ecological value as the main target of recreational fishing.

As in the rest of temperate zones in the planet, climate is changing in the region, with a notable increase of 1.2 ºC average in temperatures within the interval between 1996 and 2017 (data taken from the meteorological station 80150 in Oviedo: 43°21'00.0"N 5°51'36.0"W).

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

Non-indigenous fish

Freshwater fish inventories from the rivers of Asturias have been published in 1996, just when the area was protected first (Reyes-Gavilan *et al.*, 1996). The species of this Biosphere Reserve, including fish, were published later in 2006 (Lopez Fernandez *et al.* 2006). In addition to these publications, the Regional Government conducts electrofishing surveys for fish species inventories that are sporadically updated. The last one was carried out in 2014 (updated form De la Hoz, 2006). In the current study we have searched for the accessible information from official reports, scientific articles and media about non-indigenous fish within the study area, in order to compare them with our inventory. The internet, newspapers archives and official depositories of the Regional Government of Asturias were searched. Using Boolean operators for Internet repositories we used the following search terms: the names of the rivers and reservoirs inside the protected area (Caleao, Alba and Nalón Rivers; Tanes and Rioseco reservoirs); fish; and/or exotic, non-native, introduced, native, inventory. The searches were conducted in Spanish and English.

Four exotic fish had been reported from the studied protected area before the current survey (Table 1). They were: The Cyprinids Iberian chub *Squalius carolitertii* and minnow *Phoxinus phoxinus*, and the Salmonids north American rainbow trout *Oncorhynchus mykiss* and a center European brown trout *Salmo trutta* lineage imported from Germany (Morán *et al.*, 1991). The two cyprinids are native to other Iberian regions but naturally absent from the studied one and are employed as bait.

***Table 1. Non-indigenous fish reports**. Records of exotic fish in the Upper Nalón River inside the Biosphere Reserve.*

Exotic fish species					
SPECIES	**Geographic origin**	**Year of first report**	**Introduction purpose**	**Reported habitat**	**Source**
Non-indigenous lineages of brown trout ***(Salmo trutta)***	Germany (domestic trout lineages)	1990	Recreational fishing	Reservoir & river	(Morán *et al.*, 1991)
Iberian chub (***Squalius carolitertii***)	Iberian Peninsula (Galicia and Portugal)	2008 (absent in 2006)	Bait	Rioseco reservoir	News in media (El Comercio Local Newspaper, March/2008); Lopez Fernandez *et al.* (2006); De la Hoz (2014)
Minnow (***Phoxinus phoxinus***)	France	2006 (absent above Rioseco in 1996)	Bait	Reservoir & river	Reyes-Gavilán *et al.* (1996); Lopez Fernandez *et al.* (2006)
Rainbow trout (***Oncorhynchus mykiss***)	America	2006 (absent in 1996)	Recreational fishing, aquaculture escapes	Reservoir & river	Reyes-Gavilán *et al.* (1996), Lopez Fernandez *et al.* (2006), Fernandez *et al.* (2018)

Other sources of information (i.e. fishermen associations, media releases) may anticipate the report of some exotic species.

Another source of exotic fish are exotic lineages of native species. Before the area was protected there were introductions of brown trout hatchery lineages from central Europe to enhance wild brown trout populations, and introgression of exotic lineages, detected from genetic analysis, has been already published (Morán *et al.*, 1991; Izquierdo *et al.*, 2006).

Sampling

Fish individuals and/or their DNA were sampled from six locations within the protected area (Figure 1) in 2016-2017 using three different techniques: electrofishing, which is a conventional widely employed sampling methodology; water samples for extracting environmental DNA (eDNA); and citizen collaboration, from local anglers specifically.

Electrofishing surveys

Fish species were surveyed from accessible river areas with running water, one in the most upstream zone (Veneros) and the other just upstream the impounded area of Rioseco reservoir, in the reservoir tail (Figure 1, grey arrows), in November 2016. The official protocol for electrofishing of the Spanish Ministry of Agriculture, Fisheries and Environment -implementing the EU Water Framework

Directive 2000/60/CE- was employed (Protocol ML-R-FI-2015, NIPO: 280-15-122-6). Fish individuals were taxonomically identified to species level. A small piece of adipose fin was excised from brown trout individuals and stored in ethanol for DNA analysis.

Water sampling for eDNA

Three liters of water were sampled using sterile bottles from five locations inside the protected area on March 2017: Rioseco reservoir, Anzó (between the reservoirs), Tanes reservoir, and Caleao and Upper Nalón streams above Tanes reservoir (Figure 1, white arrows). The protocol of water sampling followed Fernandez *et al*. (2018). A bottle containing only water was transported during all the samplings and later processed together with the river water samples, to confirm contamination did not occur during the travel. To prevent contamination across sites, new sterile gloves were used to get the water samples in each site. Waders were carefully rinsed with 10% bleach before and after each sampling point.

Anglers collaboration

We have also obtained samples from the collaboration of local anglers who contributed to this research providing scales from their own catch and trophies, all of them from the small fast River Alba above Rioseco reservoir (Figure 1, green arrow). Local anglers were contacted in an open talk given in Rioseco's social center during the 2016 World Fish Migration Day (https://www.worldfishmigrationday.com/about; accessed April 2017). They were trained to sample scales from the anterior dorsal part of fish using tweezers, to be stored in paper envelopes provided by the researchers. They were asked to record the catch date and location (river zone) in writing on the envelopes. The council of Rioseco, a village in the targeted area, allowed anglers to deposit envelopes with scales in its premises for collection by researchers after 2016 angling season.

Genetic analysis Genomic DNA was extracted from scale and adipose fin samples following a Chelex resin protocol (Estoup *et al.*,1996). For species identification from scale samples, PCR amplification of a COI gene fragment was performed employing the primers designed by Kochzius *et al.* (2010). The PCR final volume was 20µL, including Green GoTaq® Buffer 1X, 2.5mM $MgCl_2$, 0.25mM dNTPS, 10pmol of each primer, 2µl of template DNA and 0.65 U of DNA Taq polymerase (Promega, Madison, WI, USA). PCR conditions were 94°C for 4min, followed by 40 cycles at 94°C for 50 s, 59°C for 50 s and 72°C for 90 s, and a last step of elongation at 72 °C for 7 minutes. Generated amplicons were sequenced in the Sequencing Unit of University of Oviedo´s Scientific-Technical services. The resulting sequences were edited with Sequence Scanner Software v1.0 (Applied Biosystems, Sussex, UK). Then, each sequence was assigned to a species by comparison with public DNA databases using the BLAST tool included in the NCBI webpage (Altschul *et al.*, 1990) with the

following settings: best match with minimum 99% identity and 99% coverage.

The PCR-RFLP technique described by McMeel et al. (2001) based on the LDH-C1* locus analysis was applied to distinguish between non-indigenous and native individuals identified as brown trout, following Izquierdo *et al.* (2006) methodology. Nucleotide sequences of the *LDH-C1*90* and **100* alleles of brown trout differ at position 308 with A in the **100* allele and G in the **90* allele. Digestion with *Bsl*I restriction enzyme of the DNA of *LDH-C1*90/90* individuals generates two fragments, one of 360 and other of 80 nucleotides. Digestion of DNA from **100/100* homozygotes generates a single uncut fragment of 440 bp, while the *90/100* heterozygotes generate all three bands, 440 bp, 360 bp and 80 bp. Allele *100 is typical of autochthonous populations, while *90 allele is from introduced lineages (Izquierdo *et al.*, 2006). For obtaining the *LDH-C1** gene fragment, the primers LDHxon3F and LDHxon4R were employed for PCR amplification. PCR was carried out in a final volume of 20 µl, including Green GoTaq® Buffer 1X, 1.5mM $MgCl_2$, 0.25mM dNTPS, 10pmol of each primer, 2µl of template DNA and 0.65 U of DNA Taq polymerase (Promega, Madison, WI) on DNA extracted from scales and adipose fin clips. PCR conditions were: 95°C for 5min, followed by 30 cycles at 95°C for 1 minute, 70°C for 1 minute and 72°C for 1 minute, and a last step of elongation at 72 °C for 10 minutes. For obtaining the RFLPs, 10 µl aliquot of the PCR amplicon was digested with *Bsl*I (New England BioLabs, MA, USA) in a total volume of 20 µl according to the enzyme supplier´s instructions. The resultant fragments were separated by electrophoresis on a 2.5% agarose gel. LDH-C1* locus was genotyped in brown trout individuals from the band pattern obtained in agarose gels (Fig S1).

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

eDNA extraction and analysis

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

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

Metabarcoding analyses were done in the Scientific-Technical Services of the University of Oviedo (Spain), using universal primers for the mitochondrial region of COI gene: mlCOIintF and jgHCO2198 (Leray *et al.*, 2013) as barcode markers to detect fish species.

Amplification was carried out in a total volume of 20 µl including Green GoTaq® Buffer 1X, 2.5mM MgCl2, 0.25mM dNTPS, 20pmol of each primer, 4 µl of template DNA, 200ng/µl of BSA (bovine serum albumin) and 0.65 U of DNA Taq polymerase (Promega, Madison, Wisconsin, USA). PCR conditions in the Veriti Thermal Cycler (Applied Biosystems, Foster City, California) were 95°C for 1min, followed by 35 cycles of 95 °C for 15s, 46 °C for 15s, 72 °C for 10s, and a final extension of 72 °C for 3min. Negative controls to monitor contamination and positive controls to check for inhibition were included in the amplification process. The amplification success was visually assessed on 2% agarose gel. PCR amplicons were purified from agarose gel using the Montage DNA Gel Extraction Kit (Millipore, Massachusetts, USA), quantified using the QuBit BR dsDNA kit (Thermofisher Scientific, Carlsbad, USA) and double-checked in a Bioanalyser 2100 (Agilient Technologies, USA) to confirm the fragment size, the absence of by-products and to do a more precise quantification. All the samples were diluted down to 26pmol for preparing an equimolar pool with all samples. The pool was processed by liquid emulsion PCR in the One Touch System using the Ion PGM™ OT2 Supplies Kit (Life Technologies, Carlsbad, CA, USA) following the instructions of manufacturer. Then the sample was loaded in the Ion ‘‘314” Chip (Life Technologies, Carlsbad, CA, USA), and sequenced employing the Ion Torrent Personal Genome Machine (Life Technologies, Carlsbad, CA, USA), following the specifications in the protocol Ion PGM™ Sequencing kit. Low quality and polyclonal sequences were filtered automatically and the PGM adaptor was trimmed within the PGM software.

Qiime software (Caporaso *et al.*, 2011) 1.9.1 version was used for bioinformatic analysis. To split the ‘fastq’ files into constituent .fna and .qual files ‘‘convert_fastqual_fastq. py” python script was used. To filter sequences by quality and size (minimum and maximum size of 250-400 and quality score of 25) “split_libraries.py” python script was used. Then, primer trimming was done with PRINSEQ v0.20.4 software (Schmieder and Edwards, 2011). COI gene database was constructed using the work flow developed by Baker (2017), and then BLAST alignment was done against this database of NCBI COI sequences (maximum e-value = 10^{-50}; minimum percent identity = 97.0) employing ‘‘assign_taxonomy. py” python script. Finally, OTU (Operational Taxonomic Unit) tables, a list of OTUs obtained for each sample and the number of sequences assigned to them, were constructed with the algorithm ‘fromTaxassignments2OtuMap.py’. This table was filtered to obtain fish read counts.

Social survey

A face-to-face survey (N= 218, approx. 0.1% of the adult population of central Asturias) was conducted in different years, asking for the preferred management actions to improve river fish

populations. Anglers (N = 117) on the river (Horreo et al. 2015) and non-anglers (N=101) in neighboring areas were directly contacted. After acceptance (verbal consent) to participate in the survey, the subjects were informed it was for research purposes solely and anonymity was guaranteed. The answers were recorded in writing and each participant checked the notes reflected accurately her/his opinion.

Statistical analysis

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

RESULTS

Trends of non-indigenous fish inside the protected area

Before 1996 only minnow (Reyes-Gavilan *et al.*, 1996) and exotic brown trout lineages (Morán *et al.*, 1991) were reported from the area now occupied by the Biosphere Reserve (Table 1). At that time minnow was restricted in the region to altitudes lower than 500m, and its limit here was below Rioseco reservoir (Reyes-Gavilan *et al.*, 1996). Non-native brown trout was legally stocked in the area for enhancing native populations before the area was protected, with ultimate purpose of sport fishing (Morán *et al.*, 1991). Rainbow trout was reported from rivers and reservoirs of the Biosphere Reserve in 2006 (Lopez Fernandez *et al*. 2006). It was likely introduced for sport fishing, with additional inputs from aquaculture escapes (Fernandez *et al.*, 2018). The Iberian chub was the most recent introduction, being reported from Rioseco reservoir in regional newspapers in 2008 (de la Hoz, 2014) (Table 1).

Fish inventory in 2016-2017

The four fish species previously reported inside the Biosphere Reserve were found in 2016-2017 inventory from different sampling methods (Table 2). Summarizing the data obtained from each method, brown trout was detected from all the sampling sites and with all the sampling methods employed (volunteer anglers, electrofishing, eDNA; Table 2). Minnow was detected from both electrofishing and eDNA, and rainbow trout was inferred from its eDNA (Table 2). Finally, Iberian chub was only detected from scale samples provided by anglers.

By sampling site, the scale samples provided by anglers, obtained from fish caught in River Alba running waters upstream Rioseco reservoir, were genetically identified as brown trout *Salmo trutta* and Iberian chub *Squalius*

carolitertii. Their Barcodes are available under GenBank Accession numbers KY492314, KY49231425, KY492328-KY492330, KY492326 and KY492327. Two species were detected from electrofishing: *Salmo trutta* from the two sites surveyed (Veneros and Rioseco reservoir tail), and *Phoxinus phoxinus* from Rioseco reservoir tail (Table 2).

Table 2. Results of 2016-2017 surveys. *Fish (DNA or individuals) found within the protected area from electrofishing, scales provided by anglers, and next-generation DNA sequencing (NGS). The original distribution (native, or Northern/Southern in relation with the studied area), and the trend by comparison with previous reports are showed. *: cannot differentiate between native and non-indigenous brown trout; **: could be escapes from a hatchery.*

Sampling sites per technique		Electrofishing		eDNA						Fishermen	Trend of exotic
Species	Distribution	Veneros	Rioseco	Veneros	Upper Nalón	Caleao	Tanes	Anzó	Rioseco	Alba	
Indigenous brown trout (*Salmo trutta*)	Native	X		X*	X*	X*	X*	X*	X*	X	-
Non-indigenous brown trout (*Salmo trutta*)	Northern		X							X	Contraction
Chub (*Squalius carolitertii*)	Southern									X	Expansion
Minnow (*Phoxinus phoxinus*)	Northern		X				X	X			Expansion
Rainbow trout (*Oncorhynchus mykiss*)	Northern			X		X	X**				Expansion

For the eDNA inventory, raw sequences from NGS data are available on NCBI's sequence read archive (SRA accession: SRP128681) with the BioSample number SAMN08295300. A total of 2,650,693 reads were originated, and after quality filters 1,548,436 sequences were retained. Three fish species were identified in the dataset (Supplementary Table 1): brown trout, minnow and rainbow trout. Brown trout DNA was found from all the sites. Minnow DNA was found from the two reservoirs (Rioseco and Tanes) and the running water between them (Anzó). Rainbow trout DNA was found in the two rivers upstream and in Tanes reservoir (Table 3).

Table 3. Results of eDNA samples. *Number of sequences assigned to different fish species from metabarcoding analysis after NGS of eDNA samples, in the five sampling locations considered.*

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

For the exotic brown trout lineage, four of the 55 brown trout samples analyzed in our study (7.3%) possessed the LDH-C1*90 allele, indicator of non-native origin (Table 4), all of them caught by electrofishing from Rioseco reservoir tail (Figure 1). The scales provided by anglers from River Alba, and the individuals analyzed from other sampling points, were all homozygotes *100/100.

Table 4. LDHC1* genotypes. *Number of brown trout individuals of each genotype found in the current (2016) and previous studies in the area: 1990: Data taken from* Morán et al. (1991)*; 1997-2003: Data taken from* Izquierdo et al. (2006)*. q (90*): Frequency of the non-native allele LDH-C1*90. N: sample size.*

Year	1990			1997-2003			2016		
LDH-C1* genotypes	*90/90	*90/100	*100/100	*90/90	*90/100	*100/100	*90/90	*90/100	*100/100
Upstream rivers	0	22	55	0	15	264	0	0	28
Rioseco reservoir	100	0	0	50	0	0	0	4	23
N	177			329			55		
q (90*)	0.63			0.18			0.04		

The 90* allele frequency (q) decreased over time and was clearly lower in 2016 than in previous years (Table 4). The difference in the distribution of genotypes among the three moments considered (1990, 1997-2003 and 2016) was highly significant (Chi-Square of 113.46, 4 d.f., $p < 0.0001$). A posteriori tests did reveal a highly significant difference between river samples caught in 1990 and 1997-2003 (Chi-Square of 44.9, 2 d.f., $p < 0.0001$), and between that period and 2016 (Chi-Square of 20.9, 2 d.f., $p < 0.0001$). For the trout sampled from the reservoir, significant change was found only between 2003 and 2016 (Chi-Square of 87, 2 d.f., $p < 0.0001$).

Regarding the trend (expansion or contraction), and the inference of illegal stocking of the non-indigenous fish here detected, the non-indigenous brown trout is in regression in this Biosphere Reserve (Table 2), and there are no signals of illegal stocking. In the past they were found in upstream rivers and in the reservoirs, but now they are only associated to Rioseco reservoir, and the frequency of individuals carrying non-indigenous *90 alleles has decreased significantly.

For rainbow trout, DNA was found from Tanes reservoir water and in the two streams upstream (Table 2). In 1996 the species was absent from the area, and the presence of its DNA in Caleao stream, where there are no rainbow trout farms, suggests expansion either natural (from the reservoir) or due to illegal stocking. The Iberian Chub absent in 1996 and 2006, was found here in running waters (Alba River) for the first time, thus it could be considered in expansion (Table 2), and indeed due to illegal stocking. Finally, the minnow was absent from waters >500m in 1996. In the present survey its DNA was found in Tanes reservoir, showing an expansive trend from its initial distribution in the area. Since Tanes dam is impassable, its presence upstream the dam can be due solely to illegal stocking.

Social survey

Survey results (Figure 2) revealed significant differences among the groups of participants (anglers and non-anglers), with a contingency Chi-square of 17.333, 1 d.f., $P = 0.0001$. More than 50% of anglers chose ‘stocking’ as the preferred measure to improve fish populations, whereas ‘Improving river conditions’ was the type of action preferred by the other group of participants interviewed in this study.

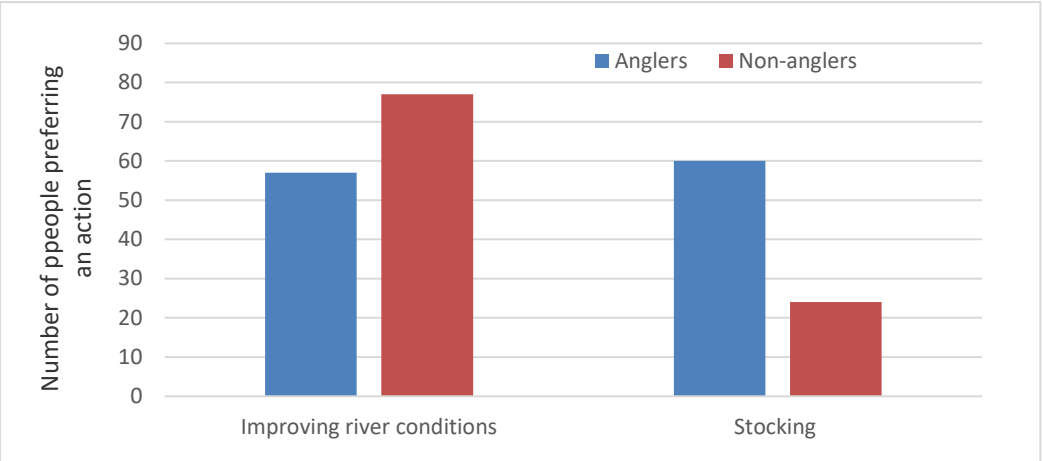

Figure 2. Results of the social survey. Number of individuals preferring stocking or improving river conditions for managing fish populations.

DISCUSSION

The results of this study revealed a change of fish communities inside a temperate Biosphere Reserve in the last two decades. First, the number of species doubled from two (one indigenous, one non-indigenous) to four, and second, the two new species are non-indigenous. The population trends of the three non-indigenous species are expansive in the sense they were found in more areas and outside the reservoirs, while an exotic lineage of the native brown trout stocked in the area is strongly declining.

In this fish survey we have used three techniques that allowed us to obtain different information contributing differently to fish inventory. The use of eDNA for species inventories has increased a lot in the last few years, being often employed as a sensitive complementary tool for conventional monitoring (Zaiko *et al.*, 2015; Clusa *et al.*, 2017; Evans *et al.*, 2017; Kelly *et al.*, 2017). Here, it has detected all species except the chub, that was only detected in anglers' samples. eDNA was able to detect one more species than electrofishing, obtaining comparable results and being even more sensitive. Electrofishing, although invasive, is in some cases necessary to confirm individuals' presence and to obtain tissue samples for genetic analysis. The stress generated by this technique would be avoided by the involvement of anglers, obtaining tissue samples from their own catch. In addition to avoid animal´s distress caused by electrofishing (Buckland-Nicks *et al.*, 2012), anglers contribution would decrease the sampling effort and cover lotic areas, where electrofishing is not effective. The introduction of a citizen science approach could also deal with logistic and financial limitations in monitoring purposes. Also, anglers' samples can be used for detecting rare or low-density species like the chub found in this study that otherwise we had not

detected. Although anglers prefer bigger preys, so they are not representative of the population (Gledhill *et al.*, 2015), in our study they caught chub, a species of little or null value as a trophy, for helping in our research.

The expansion of non-indigenous species in a zone always start with (legal or illegal, deliberate or inadvertent) introductions (García-Berthou *et al.*, 2005; Ribeiro and Leunda, 2012; Maceda-Veiga *et al.*, 2013; Chown *et al.*, 2015). In most studies where illegal stocking is reported it is suspected (e.g. Johnson *et al.*, 2009; Araguas *et al.*, 2017), but in the present case the isolated condition of the water bodies, restricted to a mountainous area by impassable dams, ensures that the introduction of new species or its expansion above dams cannot occur naturally as in other cases (e.g. Horreo and Garcia-Vazquez, 2011; Araguas *et al.*, 2017). Although physical and environmental conditions in reservoirs may favor the establishment of exotic species (Clavero *et al.*, 2004; Han *et al.*, 2008; Havel *et al.*, 2015), dams have blocked the NIS spreading to upper zones in Nalón River (Clusa *et al.*, 2018). Indeed, natural expansion upstream the dams of the species that are already in the reservoirs may happen. From the results of our study, where new species have appeared upstream impassable dams, illegal stocking seems to be the main driver of the increase of non-native fish in the studied Reserve, providing further support to the suspects of this factor in other studies (Araguas *et al.*, 2009; Johnson *et al.*, 2009). The main factor involved in fish NIS introgression in this case study is therefore illegal stocking, with farm escapes as a secondary factor (Fernández *et al.* 2018). Anglers pressure for stocking in the region was deduced from the social survey and is reflected in the reality that they have released, illegally, both bait and target catch species in the protected space (Cyprinid and Salmonids respectively). The presence of Iberian chub, and minnow in Tanes reservoir where it never was, are a proof of this.

On the other hand, climate change is altering aquatic ecosystems due to water warming and modifications in stream flow patterns (Rahel and Olden, 2008). As a consequence, shifts occur in the distribution and abundance of aquatic species (Hughes, 2000). This type of changes has been documented, mostly by depth and latitude, for marine ecosystems (Perry *et al.*, 2010). In continental waters shifts in community composition are also expected. For fish communities, Lehtonen (1996) pointed out that global warming would result in an increase on cyprinid and percid dominance, and a decline of salmonids and other cold-water fish. This global warming paradigm where the spread of southern species is being facilitated (Walther *et al.*, 2009) would be also supported in our case, but only partially, in the case of the southern Iberian chub it would be true since it is spreading into natural running waters in River Alba; but rainbow trout seems to be expanding in the system as well, opposite to Lehtonen *et al.* (1996) predictions. Rainbow trout is a cold-water species and

we would expect it was in recession; however, since Caleao stream is colder than the reservoirs because it is higher up in the mountains, it is possible that rainbow trout naturally enters its waters looking for a colder environment. In mountain streams cold-water fish may move to colder waters, expanding its distribution in the upper river reaches. Alternatively, or concomitantly to natural movements, illegal stocking could also explain the presence of rainbow trout in Caleao. Climate would also explain a reduction of the imported stocks of brown trout. The frequency of carriers of the non-native allele LDHC1*90 is steadily decreasing in the Reserve since 1990. Since the foreign stock of brown trout introduced in the region was of north European origin (Germany; Moran et al. 1991), its reduction under current climate warming would be logically expected.

The presence of exotic species in rivers would affect native populations. Native brown trout is catalogued as vulnerable in Spain because natural populations were reduced by 20% due to habitat losses, exotic lineages introductions and overfishing (Doadrio, 2001). The presence of exotic fish species cohabiting rivers with brown trout could result in a threat for their native populations, affecting their behavior, genetic background and distribution, among other effects (Cucherousset and Olden, 2011), so in this basin there is a threat to brown trout native populations that needs to be highlighted in order to avoid populations´ decline.

Anglers have historically contributed to rivers management, in this (Horreo et al. 2015) and other regions. Nowadays they seem to play a dual role: on the one hand their activity is the main reason for exotic species introduction, but on the other hand they contribute on rivers conservation being involved in research programs (Couvet *et al.*, 2008; Granek *et al.*, 2008; Gledhill *et al.*, 2015; Williams *et al.*, 2015). In this study, surveyed anglers prefer to invest money in supporting breeding than in improving river environment, contrary to the other surveyed group. The intentional introductions on this study were done mainly for fishing activities as it has occurred in other regions worldwide (i.e. Arlinghaus 2006; Wissinger et al. 2006; Fausch 2007; Crawford and Muir 2008), so it is important to promote anglers' role in conservation issues, encouraging them to stop illegal introductions. Considering that 42% of all aquatic introductions are deliberate (Gozlan, 2010), anglers' involvement is crucial to avoid the spread of non-native species.

CONCLUSION

The illegal introduction of exotic species is a frequent practice and has been demonstrated here in a Biosphere Reserve. To monitor these introductions and take appropriate measurements, a regular evaluation of the rivers including anglers´ involvement is needed. To do so, an integrated assessment including routine sampling and non-invasive techniques for early NIS detection should be implemented, especially in protected

areas. The use of integrated assessment would be the way to have comprehensive ecosystem information. A first approach based on eDNA tools is an appropriate choice for being non-invasive and giving an overview of the fish fauna. For a deeper evaluation such as population genetics or demographic studies individuals are needed. Electro-fishing and/or the collaboration of anglers that obtain captures every season would be methods of choice for this purpose, and as demonstrated in this study, they are good for detecting introduced fish species. Finally, the involvement of anglers in conservation programs is recommended to deal with illegal stocking.

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SUPPORTING INFORMATION

Table S1. Raw data of NGS analysis from Chordata phylum.

Figure S1. Digestion pattern from S.trutta individuals. R=Rioseco; V: Veneros. C+: positive control of *90/90 allele digestion pattern; L: Ladder.

Table S1. Raw data of NGS analysis from Chordata phylum. Number of reads per sampling point and extraction replicate (1, 2 and 3).

Taxonomic information						Sampling points (Samples: extraction replicates)																	
						Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao		
Phylum	Species	Family	Genus	Class	Order	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Chordata	*Phoxinus bigerri*	Cyprinidae	Phoxinus	Actinopteri	Cypriniformes	0	0	1	1	0	0	0	0	90	21	46	13	10	9	0	161	73	8	4	13	9
Chordata	*Phoxinus phoxinus*	Cyprinidae	Phoxinus	Actinopteri	Cypriniformes	0	0	0	0	0	0	0	0	1	0	1	1	1	0	0	2	1	0	3	1	0
Chordata	*Squalius carolitertii*	Cyprinidae	Squalius	Actinopteri	Cypriniformes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0
Chordata	*Oncorhynchus mykiss*	Salmonidae	Oncorhynchus	Actinopteri	Salmoniformes	5,905	12,664	0	0	0	11,791	0	12	0	0	0	0	0	0	0	44	26	0	15	21	8
Chordata	*Salmo trutta*	Salmonidae	Salmo	Actinopteri	Salmoniformes	78	57	37	26	29	50	0	118	0	11	19	6	14	7	1	0	5	2	6	0	0
Chordata	*Salmo salar*	Salmonidae	Salmo	Actinopteri	Salmoniformes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chordata	*Gallus gallus*	Phasianidae	Gallus	Aves	Galliformes	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chordata	*Cinclus cinclus*	Cinclidae	Cinclus	Aves	Passeriformes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0
Chordata	*Fringilla coelebs*	Fringillidae	Fringilla	Aves	Passeriformes	0	0	0	0	27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chordata	*Passer domesticus*	Passeridae	Passer	Aves	Passeriformes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0	0
Chordata	*Ardea cinerea*	Ardeidae	Ardea	Aves	Pelecaniformes	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chordata	*Phalacrocorax lucidus*	Phalacrocoracidae	Phalacrocorax	Aves	Pelecaniformes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Chordata	*Bos taurus*	Bovidae	Bos	Mammalia	NA	15	34	0	0	0	17	0	0	0	0	0	0	0	0	0	1	1	0	5	0	0
Chordata	*Capra aegagrus*	Bovidae	Capra	Mammalia	NA	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0
Chordata	*Cervus elaphus*	Cervidae	Cervus	Mammalia	NA	0	0	0	0	0	8	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0
Chordata	*Sus scrofa*	Suidae	Sus	Mammalia	NA	0	1	8	0	55	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
Chordata	*Homo sapiens*	Hominidae	Homo	Mammalia	Primates	9	26	46	12	36	5	363	42	12	47	6	16	1	1	1	9	10	5	10	45	7
Chordata	*Apodemus flavicollis*	Muridae	Apodemus	Mammalia	Rodentia	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Chordata	*Mus musculus*	Muridae	Mus	Mammalia	Rodentia	0	0	0	0	0	0	0	21	0	0	0	0	0	0	0	0	0	0	0	0	0
Chordata	*Rattus norvegicus*	Muridae	Rattus	Mammalia	Rodentia	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0

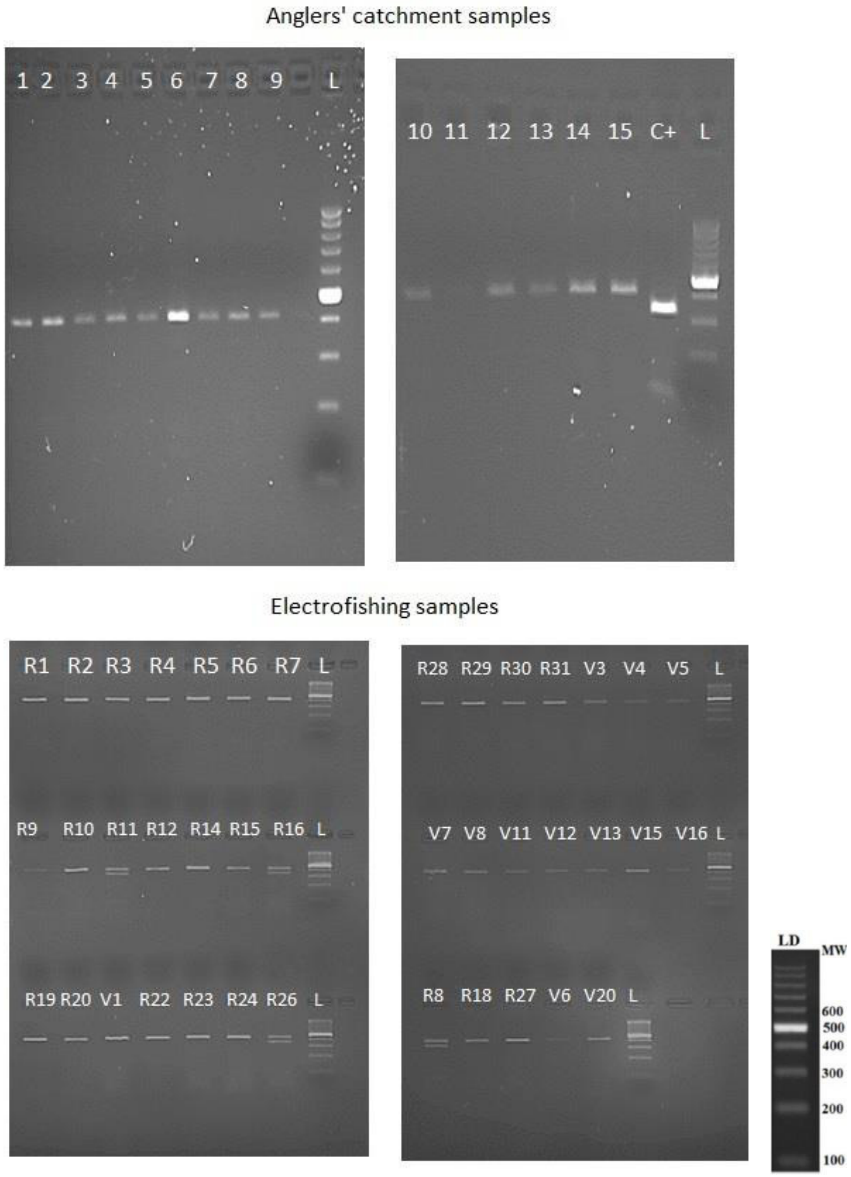
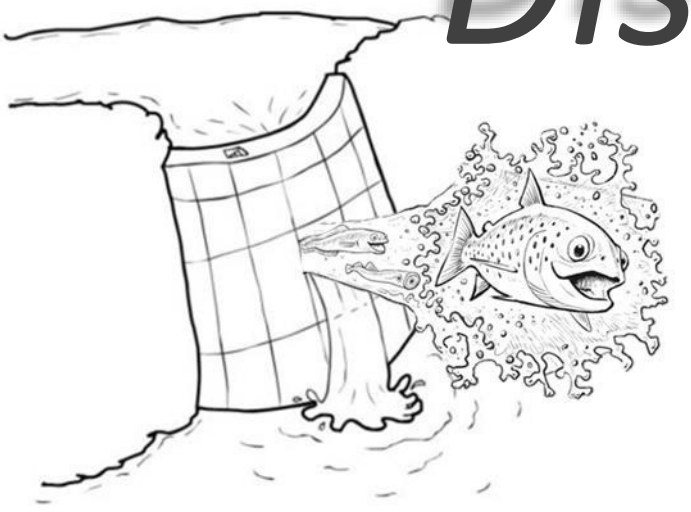


Figure S1. Digestion pattern from *S.trutta* individuals. R=Rioseco; V: Veneros. C+: positive control of *90/90 allele digestion pattern; L: Ladder

Discusión



1. Usos del ADN ambiental en ríos fragmentados

Los resultados obtenidos en la presente Tesis son un ejemplo de la utilidad del ADN ambiental en el estudio de las alteraciones de las comunidades bióticas causadas por la construcción de barreras en los ecosistemas fluviales. La biodiversidad y la calidad del agua son dos de los componentes del ecosistema más afectados cuando la conectividad del río se ve interrumpida (Vörösmarty et al. 2010), y en los trabajos desarrollados aquí se ha demostrado que el ADN ambiental permite reconocer alteraciones en ambos componentes en situaciones reales. Se comenta a continuación cada componente.

1.1 Calidad del agua

Se ha podido comprobar en este trabajo que los métodos de secuenciación masiva, con los marcadores moleculares apropiados y aplicados a muestras de ADN ambiental, proporcionan medidas de índices biológicos de calidad del agua comparables a las que se obtienen con los métodos convencionales de muestreo e identificación de macroinvertebrados. Es la primera vez que se demuestra la eficacia del ADN ambiental para medir el índice estandarizado IBMWP cumpliendo con la Directiva Marco del Agua (DMA; 2000/60/CE), y es una aportación muy significativa de esta Tesis.

Los resultados han demostrado que, en el momento actual y con las bases de datos de referencia disponibles, el gen COI (subunidad 1 de la citocromo oxidasa) es un buen marcador para su uso en la caracterización de comunidades de macroinvertebrados bentónicos, al contrario que el gen del ARN ribosomal 18S (18S rDNA región V4), que ha demostrado ser ineficaz para la caracterización de la mayoría de los grupos incluidos en el índice IBMWP. Sin embargo, en estudios realizados en otras zonas geográficas ha demostrado ser útil para caracterizar algunos grupos como por ejemplo los nemátodos (Pawlowski et al. 2018). Su uso podría complementar el del gen COI en los casos en los que la caracterización mediante el uso único de este marcador no sea suficiente para abarcar todos los organismos objeto de estudio, por ejemplo, en el caso de que haya pocas secuencias de referencia de determinados organismos en las bases de datos. Este resultado contradice en principio otros trabajos que postulan el 18S rDNA como marcador de elección en Metabarcoding (Keeley et al. 2018; Laroche et al. 2018). Es probable que la región V4 del 18S rDNA, que es muy variable dentro de especie y por supuesto de familia, no tenga el suficiente grado de conservación para diferenciar entre familias de macroinvertebrados de forma consistente. En cambio, los resultados son coherentes con el gran desarrollo de las bases de datos para el gen COI, que fue el primer marcador de elección como código de barras genético para grupos taxonómicos animales (Hebert et al. 2003).

Los índices biológicos más frecuentemente empleados en la actualidad para implementar la DMA se basan en presencia/ausencia de familias de macroinvertebrados. Como se comentó en la Introducción, los macroinvertebrados bentónicos son los grupos más utilizados como bioindicadores de la calidad del agua porque las distintas familias tienen diferentes grados de tolerancia a la contaminación (Armitage et al. 1983). Su uso, al igual que el de otros bioindicadores, está ampliamente extendido dentro de las medidas de evaluación de la calidad del agua que han sido adoptadas por los diferentes países europeos para cumplir con los requisitos de la DMA (Birk et al. 2012). La caracterización convencional de estos organismos requiere un muestreo exhaustivo y la identificación taxonómica de los individuos a partir de caracteres diagnóstico, una tarea que en muchas ocasiones resulta difícil y de forma particular en juveniles (Haase et al. 2010). A este respecto, el uso del ADN ambiental proporciona una serie de ventajas importantes. Al tratarse de una técnica basada en la identificación molecular, no es necesaria la muy laboriosa y a veces inexacta identificación morfológica (Bohmann et al. 2014). Por otra parte, el nivel taxonómico al que se pueden identificar muchos individuos, sobre todo jóvenes, mediante el método visual convencional suele ser más alto (familia o género) que el que permite la asignación molecular, que llega al nivel de especie en casi todos los casos (Elbrecht & Leese 2017). Este ha sido el caso en los resultados de la presente Tesis.

Según los resultados de esta Tesis, el ADN ambiental cumple con los requisitos de la DMA por haber sido capaz de distinguir entre zonas degradadas y zonas con buena calidad del agua. En principio parecería ser una herramienta ideal para medir la calidad del agua. Sin embargo, presenta una limitación en cuanto a las métricas cuantitativas se refiere. Dentro de la DMA se indica la conveniencia de contar con métodos de evaluación cuantitativos (abundancia de individuos), algo que con este método basado en Metabarcoding no se ha logrado alcanzar por el momento. En los últimos años se han desarrollado algunos estudios para establecer una relación entre la cantidad de ADN ambiental presente en una muestra y el número de lecturas o secuencias obtenidas mediante NGS (Elbrecht and Leese 2017; Ushio et al. 2018). Sin embargo, aunque esta correspondencia fuera significativa y la cantidad de ADN fuera extrapolable a partir del número de lecturas de una secuencia, dicha cantidad de ADN no es necesariamente sinónimo de número de individuos porque el ADN liberado al medio dependerá del tamaño, edad y estado fisiológico del individuo (Tréguier et al. 2014; Deiner et al. 2015; Jane et al. 2015). Este es un problema difícil de resolver con las herramientas moleculares y bioinformáticas actuales. Sin embargo, refiriéndose en particular a la implementación de la DMA, algunos programas europeos de seguimiento de la calidad del agua, como es precisamente el caso del utilizado en España, no utilizan elementos cuantitativos. Sus índices de calidad están basados únicamente en la presencia de los organismos indicadores de calidad (Prat et al. 2013). Por lo tanto, según los resultados de esta Tesis en los que utilizando ADN ambiental se han obtenido resultados comparables a aquellos obtenidos con el uso de técnicas convencionales para el cálculo de índices de calidad de agua en la Península Ibérica (IBMWP), el empleo del ADN ambiental sí

que podría ser actualmente aplicado en la evaluación de la calidad del agua en ecosistemas fluviales en España, así como en otros países en los que los elementos cuantitativos no son necesarios.

Recientemente, Elbrecht et al. (2017) han propuesto realizar la caracterización de macroinvertebrados bentónicos usando NGS para la identificación de individuos obtenidos a partir de muestreos convencionales. No cabe duda de que esta alternativa puede mejorar mucho el nivel de resolución taxonómica y evitar problemas relacionados con especies crípticas. Sin embargo, para la aplicación en medidas biológicas de calidad de agua y para inventarios en espacios naturales protegidos, donde los muestreos deben ser mínimamente invasivos, no sería una solución adecuada porque supone recurrir al muestreo clásico.

En la Tabla 1 se presenta una comparación de diferentes metodologías aplicadas a la evaluación de macroinvertebrados bentónicos para la evaluación de calidad de agua. Como se puede observar, ningún método parece perfecto con la biotecnología actual, pero el ADN ambiental parece especialmente prometedor. La introducción del ADN ambiental en el seguimiento biológico de la calidad del agua está siendo estudiada y validada a nivel mundial (Mächler et al. 2014; Apothéloz-Perret-Gentil et al. 2017; Laroche et al. 2017; Hering et al. 2018; Pawlowski et al. 2018). Al igual que su aplicación en otros muchos campos de la biología, aún necesita madurez y mejoras técnicas para en un futuro poder ser incorporado a los programas de evaluación y seguimiento de la calidad del agua.

Tabla 1. Comparación de técnicas para la evaluación de macroinvertebrados bentónicos. Ventajas e inconvenientes de la aplicación de las diferentes técnicas moleculares y convencionales para la evaluación de macroinvertebrados bentónicos como indicadores de calidad del agua.

ADN ambiental		**Técnicas y muestras convencionales**		**Técnicas moleculares con muestras convencionales**	
Ventajas	Inconvenientes	Ventajas	Inconvenientes	Ventajas	Inconvenientes
Muestreo no invasivo			Muestreo invasivo		Muestreo invasivo
Esfuerzo de muestreo bajo			Esfuerzo de muestreo alto		Esfuerzo de muestreo alto
Evaluación global		Evaluación localizada		Evaluación localizada	
Identificación sencilla			Dificultades en la identificación	Identificación sencilla	
Identificación independiente de experiencia en taxonomía			Identificación dependiente de experiencia en taxonomía	Identificación independiente de experiencia en taxonomía	
	Riesgo de contaminaciones				
	Posibles sesgos en la identificación		Posibles sesgos en la identificación		Posibles sesgos en la identificación

ADN ambiental		Técnicas y muestras convencionales		Técnicas moleculares con muestras convencionales	
Ventajas	Inconvenientes	Ventajas	Inconvenientes	Ventajas	Inconvenientes
Caracterización a nivel de especie en la mayoría de los casos			No siempre alcanza el nivel de especie	Caracterización a nivel de especie en la mayoría de los casos	
	No incluye elementos cuantitativos en su estado actual	Incluye elementos cuantitativos			No incluye elementos cuantitativos en su estado actual

Lo que sin duda parece desprenderse de los resultados de este trabajo es que la calidad del agua dentro del Parque Natural de Redes es menor en aquellas zonas que se encuentran afectadas por embalses que en las zonas no afectadas aguas arriba. Esto pone de manifiesto el impacto directo de las barreras fluviales en la calidad del hábitat, un hecho sobradamente conocido (Magilligan et al. 2016; Santos et al. 2017; Quevedo et al. 2018) y que aquí ha sido evidenciado mediante el uso del índice IBMWP calculado a partir del ADN ambiental. Hay muchas razones por las cuales se altera la calidad del agua en los embalses, que representan una interrupción forzada del flujo normal de la corriente del río en un espacio que tiene una pendiente y una dinámica hidrológica natural normalmente muy alejada de la correspondiente a las aguas embalsadas. Entre estas razones se encuentran en primer lugar las descargas continuadas de agua con el fin fundamental de satisfacer necesidades de riego o de generación de energía, pero también para liberar su acumulación aguas arriba o para favorecer actividades recreativas aguas abajo que requieran mayores caudales. Estas descargas hacen que los cambios de flujo en las zonas situadas aguas abajo del embalse sucedan a tasas mucho mayores que como lo harían de forma natural, lo que tiene consecuencias en estas zonas que van desde la destrucción de hábitats hasta el cambio del régimen térmico (McCartney 2009). Por otro lado, la presencia de embalses también provoca una reducción en la velocidad del flujo de la corriente y por lo tanto una intensificación de la sedimentación; como consecuencia, se acumulan grandes concentraciones de sedimentos aguas abajo de los embalses (Malmqvist & Rundle 2002). Otro de los cambios consecuencia del agua embalsada es la eutrofización, que suele darse como consecuencia del aporte alto de nutrientes fruto de la actividad humana y que nuevamente altera la composición aguas abajo del embalse (Rossel & De La Fuente 2015). Todos estos cambios en la composición física y química del agua hacen que varíe también la composición de comunidades y que la calidad del agua se vea afectada, como se ha evidenciado en este trabajo.

1.2 Biodiversidad

Las alteraciones en la conectividad fluvial hacen que se alteren los patrones de diversidad. Según la teoría del río continuo (Vannote et al. 1980), la diversidad será menor en la cabecera de los ríos por la escasa heterogeneidad fluvial, luminosidad y nutrientes que estas zonas presentan. Sin embargo, aumentará en los tramos intermedios porque los recursos de agua y luz son los adecuados para ello, y el sustrato conforma un mosaico de hábitats que favorece este aumento (Ward 1998). La diversidad de las comunidades biológicas es uno de los elementos que junto con los procesos biogeoquímicos naturales de los ecosistemas fluviales regulan la cantidad y la calidad del agua (Arthington et al. 2010); es un recurso

natural muy valioso tanto a nivel científico como cultural y económico (Dudgeon et al. 2006) que debe ser preservado.

La zona de estudio de esta Tesis está localizada en la cabecera del río a unos 1.400 metros sobre el nivel del mar. Se espera un cambio en la diversidad biológica aunque sea pequeño, aumentando ésta en las zonas más bajas (Ward 1998). Sin embargo, las zonas del río intermedias y más bajas que se evaluaron en este estudio no presentaron mayor diversidad que las zonas altas. Además, las áreas fluviales situadas entre embalses mostraron grandes diferencias en cuanto a diversidad se refiere, algo esperable porque están muy afectadas por las barreras y como se ha visto antes están bastante degradadas. La composición de la comunidad, a nivel taxonómico de género que es más conservador en el set de datos de NGS obtenido, también varía entre las zonas afectadas por los embalses y aquellas que no lo están. Por ejemplo, la zona intermedia tiene un contenido de hongos ascomicetos mucho mayor que el resto de las zonas estudiadas en las que el grupo predominante es el de artrópodos (Figura 2 del Capítulo 2). Todos estos resultados evidencian el gran impacto que tienen los embalses presentes en la zona de estudio. La interrupción en la conectividad longitudinal de los ríos supone una de las amenazas más importantes a la persistencia de la biodiversidad (Hermoso et al. 2018), algo que ha quedado reflejado en los resultados de esta Tesis.

Dentro del estudio de caso presentado, se ha podido comprobar que mediante el uso de técnicas de *metabarcoding* en muestras de agua no sólo se han detectado especies acuáticas, sino que también se ha encontrado ADN de especies terrestres. Se confirma en estos datos la teoría de que mediante el ADN ambiental se pueden detectar organismos muy variados en los ríos, sin que necesariamente su localización esté restringida a la corriente de agua (Deiner et al. 2016). Empleando un esfuerzo de muestreo mucho menor que con los métodos tradicionales, tal como se ve en la Tabla 1, sería posible una evaluación menos localizada y a gran escala de la biodiversidad a nivel de cuenca, o al menos en las zonas colindantes a los ecosistemas acuáticos (Deiner et al. 2017).

2. Seguimiento de especies piscícolas en espacios protegidos.

Los espacios protegidos son zonas en las que se reduce al máximo el impacto humano y que a su vez requieren de una evaluación sistemática para su conservación (Stolton et al. 1999). Por eso, el ADN ambiental parece una herramienta idónea para el muestreo rutinario de la biota en este tipo de ecosistemas. Como se ha visto, es útil para la caracterización de las comunidades de macroinvertebrados, y también de otras especies acuáticas y no acuáticas que aprovechan recursos de los ríos (Deiner et al. 2016). Sin embargo, las técnicas de *metabarcoding* tienen la limitación de que tienden a subestimar las especies que se encuentran en bajas densidades (Ardura et al. 2016). Esta desventaja no es aplicable a otras

técnicas basadas en cebadores especie-específicos para detectar especies concretas a partir de ADN ambiental. En esta Tesis, la PCR cuantitativa (qPCR) ha demostrado ser un método muy sensible en la detección de especies cuando su densidad es baja. Se ha detectado ADN de la especie de trucha arco iris (*Oncorhynchus mykiss*), que es exótica en Europa, y de la nativa trucha común (*Salmo trutta*) dentro del Parque Natural de Redes. Mediante el uso de qPCR fue posible detectar el ADN de ambas especies, mientras que mediante PCR-RFLP no se detectó la trucha nativa en todas las muestras, pese a que se comprueba *de visu* su presencia en todos los cursos fluviales del Parque. Este resultado puede tener implicaciones para la gestión si se pretende aplicar el ADN ambiental para muestreos de especies piscícolas.

Además de la mayor sensibilidad de la qPCR, gracias a estos resultados se ha puesto de manifiesto la presencia de *Oncorhynchus mykiss* en agua corriente, confirmándose la sospecha de que se producen escapes de esta especie desde las piscifactorías localizadas en el área protegida objeto de estudio. La introducción de especies exóticas es una de las causas que ha llevado a la trucha común a un estado de vulnerabilidad (IUCN; Doadrio 2001), por lo que evitar los escapes de trucha arco iris y las introducciones de especies exóticas resulta crucial. Para vigilar estos escapes e introducciones y poder evitarlos a tiempo, se recomienda el uso del ADN ambiental en las evaluaciones rutinarias, ya que permite la detección temprana de estas especies (Clusa et al. 2017).

Los resultados presentados en este trabajo han mostrado la utilidad de la aplicación del ADN ambiental como herramienta para el seguimiento de especies piscícolas dentro del Parque Natural de Redes, y también para inferir su evolución en una zona aislada de la cuenca, cuya conectividad con los tramos bajos del río se ha interrumpido debido a la presencia de grandes embalses impasables (Introducción, Figura 3). El uso combinado del ADN ambiental, la electropesca y la colaboración de los pescadores locales ha hecho posible este seguimiento. Los resultados apuntan a la intervención de varios factores para explicar la distribución actual de las especies que se encuentran en el Parque. Se ha podido documentar que las dos especies exóticas de ciprínidos (*Squalius carolitertii* y *Phoxinus phoxinus)* se encuentran en expansión, ya que su presencia había sido detectada únicamente en los embalses y ahora se encuentran ya en agua corriente. Es posible que los embalses actúen como reservorio para estas especies exóticas al inicio de su introducción, y que las condiciones de temperatura y flujo de corriente favorezcan su establecimiento (Clavero et al. 2004; Liermann et al. 2012), aunque, como se desprende de los resultados de este trabajo, es evidente que las introducciones de especies exóticas en el espacio protegido sólo han podido ser deliberadas, probablemente llevadas a cabo por pescadores locales.

Las poblaciones de la especie dominante, la trucha común (*Salmo trutta*), han sufrido un descenso notable en toda la región fruto de los impactos antropogénicos (Doadrio 2001). Años atrás se llevaron a cabo repoblaciones con stocks importados de truchas alemanas, linajes exóticos al nativo de la zona, para aumentar el tamaño de las poblaciones de trucha y

continuar con las prácticas de pesca deportiva (Morán et al. 1991; Izquierdo et al. 2006). Se ha comprobado en esta Tesis que la frecuencia de alelos de los linajes de trucha común noreuropeos ha disminuido significativamente en el Parque Natural de Redes, posiblemente por el cese de estas introducciones y el aumento de temperaturas debido al cambio climático global, que seguramente no favorecen a los linajes norteños. Este estudio específico se llevó a cabo sobre muestras individuales de truchas, no sobre ADN ambiental. Como se ve, el uso del ADN ambiental como primera aproximación en inventarios de especies no implica que se dejen de utilizar los muestreos convencionales para algunos propósitos. En este caso concreto, se podría haber empleado ADN ambiental si, por ejemplo, se hubieran desarrollado cebadores específicos de linaje, que permitieran la hibridación en la PCR solamente sobre uno u otro tipo de trucha, quizás con algún marcaje diferencial como se hace en la identificación de especies a partir de SNP (p.e. Machado-Schiaffino et al. 2008). Sin embargo, el ADN ambiental sólo permitiría detectar presencia/ausencia del linaje exótico, no calcular la frecuencia real de cada alelo.

Tras la exposición de estos resultados, cabe preguntarse hasta qué punto es sostenible la gestión actual del Parque Natural de Redes. Es cierto que para llevar a cabo una gestión sostenible en una zona poblada, es necesario involucrar a la población y que esta obtenga beneficios (Hirschnitz-Garbers & Stoll-Kleemann 2010), pero también hace falta concienciarse de que en este espacio protegido hay actividades como la pesca o la explotación hidroeléctrica del río que están afectando en gran medida al ecosistema.

3. Limitaciones y futuros retos para el uso del ADN ambiental

A la vista de los resultados obtenidos en este trabajo, se puede deducir que la incorporación del ADN ambiental en el seguimiento del estatus de ecosistemas fluviales se convertirá en un hecho en un futuro no muy lejano. Por un lado, se ha demostrado que su aplicación dentro de los espacios protegidos resulta de gran utilidad como un primer acercamiento que proporciona una visión global de composición de la biota y también del estado de calidad del ecosistema. Y, además, se ha visto cómo sus aplicaciones para evaluar la conectividad fluvial y los impactos derivados de su interrupción ofrecen resultados prometedores que abren la puerta a diferentes usos y aplicaciones de esta herramienta, no sólo para la gestión de espacios protegidos sino también para su incorporación a programas de conservación de todo tipo en los ríos.

Sin embargo, aún quedan algunas limitaciones asociadas a la técnica que han de ser resueltas en posteriores estudios para el desarrollo de este campo. En primer lugar, los costes. Como es lógico pensar, varían mucho dependiendo del objetivo del estudio y lo que éste requiera experimentalmente. Sin embargo, en los trabajos desarrollados durante los últimos años se ha recalcado que los costes asociados a las herramientas genéticas son

inferiores que los que conlleva el uso de técnicas convencionales de muestreo y análisis (Senapati et al. 2018). Esto se podría afirmar siempre y cuando se tenga en cuenta el número de muestras a analizar, ya que para estudios con tamaños muestrales pequeños los costes del método molecular siguen siendo superiores a aquellos calculados con el método convencional (Aylagas et al. 2018).

El uso del ADN ambiental, como el de cualquier otra técnica, también conlleva la aparición de falsos positivos y negativos (Ficetola et al. 2015); por lo tanto, es necesario tomar medidas para reducirlos al máximo, como se ha hecho en esta Tesis en todos los puntos críticos tanto de muestreo como de laboratorio, para evitar contaminaciones y conseguir un muestreo representativo de los sitios muestreados, también en el posterior análisis de datos aplicando los filtros de calidad apropiados. Pese a todos estos cuidados, hay que señalar igualmente que el ADN ambiental no supone una solución perfecta para todos los análisis de diversidad. Una de las limitaciones que presenta el ADN ambiental en cuanto a estudios de diversidad es la cuantificación individual. Mientras que sí es posible hacer estimas de índices de diversidad de especies a partir de esta herramienta (Bohmann et al. 2014), el cálculo de índices de diversidad intraespecífica a fecha de hoy no puede ser extrapolado a partir de muestras ambientales, como tampoco pueden ser calculados índices de biodiversidad basados en la abundancia relativa de las distintas especies.

Estrechamente relacionada con este problema está la aplicación del ADN ambiental en genética de poblaciones. La técnica no ha alcanzado un estado de madurez suficiente como para ser aplicada y obtener resultados semejantes a los obtenidos utilizando metodologías convencionales de muestreo y análisis independiente de cada individuo, precisamente por la naturaleza de las muestras ambientales, en las que hay una mezcla de material genético de distintos organismos con distintas características. Sin embargo, esto podría resolverse en un futuro próximo. Por ejemplo, se ha desarrollado un método que permite inferir la variabilidad genética intraespecífica a partir de datos de *metabarcoding* en muestras de macroinvertebrados (Elbrecht et al. 2018). Cuando se desarrolle un método analítico semejante para el análisis de datos de muestras ambientales, se podrán obtener resultados de variabilidad genética intraespecífica. Lo que parece todavía muy lejano, y quizás no sea posible empleando solamente ADN ambiental, es distinguir entre individuos maduros e inmaduros, entre cohortes o entre diferentes clases de edad. Parece que los muestreos individuales, prioritariamente no destructivos, seguirán siendo por tanto necesarios para el conocimiento de poblaciones y especies.

El uso estandarizado del ADN ambiental en estudios de diversidad requiere a su vez de la estandarización de todos los métodos, incluyendo el procesamiento de las muestras, ya que se ha visto que los resultados en la recuperación de ADN varían según el método empleado (Majaneva et al. 2018). También hay que estandarizar los análisis molecular y bioinformático, que pueden suponer grandes sesgos y dificultar o impedir la comparación entre estudios diferentes si no son cuidadosamente replicados (Baird & Hajibabaei 2012).

Como ejemplo de esto, se han comparado los resultados obtenidos en esta Tesis en la caracterización de familias de macroinvertebrados. Se han utilizado dos plataformas de secuenciación de última generación diferentes dentro del desarrollo de la Tesis, Illumina en el Capítulo 2 e Ion Torrent en el Capítulo 3. En la Figura 4 se presenta el porcentaje de familias de macroinvertebrados de diferentes órdenes encontrado con las distintas plataformas de secuenciación mencionadas y las obtenidas mediante la identificación visual convencional. Como los puntos de muestreo y la época del año fueron los mismos, se esperaría que no hubiera grandes diferencias en los resultados de las dos plataformas. Sin embargo, se ve que, aunque los perfiles encontrados mediante los tres métodos son parecidos, no son iguales. El mayor número de familias se obtuvo con el método molecular, concretamente en el set de datos de Ion Torrent. En el set de datos de Illumina el mayor número de familias correspondió a los tricópteros, seguidos de los dípteros, al igual que lo obtenido con el método visual; sin embargo, en el set de IonTorrent las familias de dípteros fueron más numerosas que las de tricópteros (Figura 4). Esta diferencia podría suponer un sesgo en el caso del set de Ion Torrent hacia familias con menor puntuación de calidad, ya que el grupo de dípteros contine más familias de este tipo, con altas tolerancias a la contaminación de las aguas. A pesar de que estas diferencias no son muy notables y se han obtenido valores de calidad de aguas semejantes a los obtenidos con el método visual, es necesaria la estandarización de metodologías antes de su incorporación a los programas de evaluación, ya que el estatus de una determinada masa de agua podría cambiar en función a los sesgos derivados de la elección de plataforma y de los procesos bioinfórmaticos.

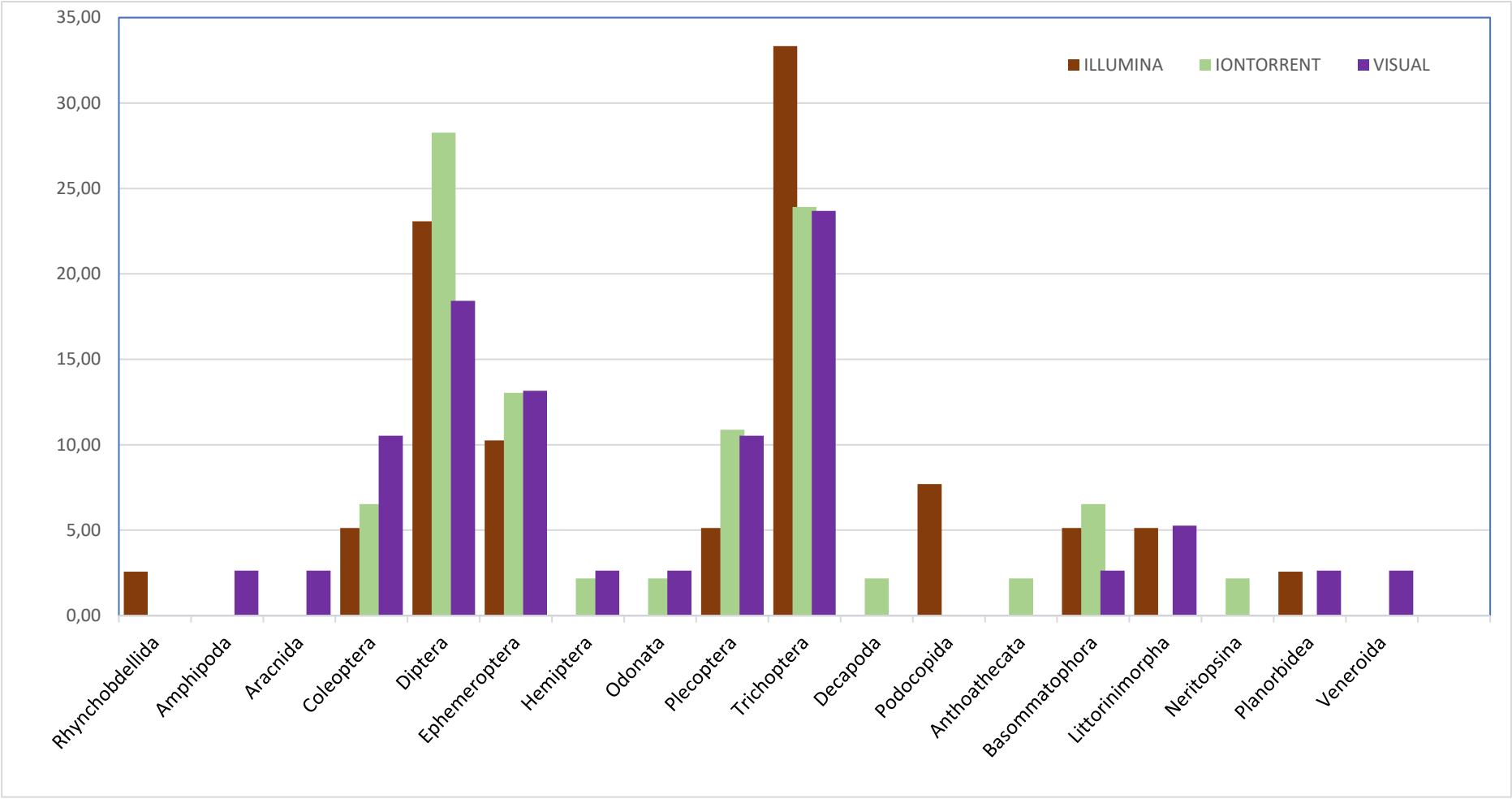


Figura 4. Comparación de Plataformas de NGS. Porcentaje de familias de macroinvertebrados bentónicos en cada uno de los órdenes encontrados en este trabajo, para las plataformas NGS Illumina e Ion Torrent y con el método visual tradicional.

Otra de las limitaciones del *metabarcoding* es la abundancia relativa de secuencias de referencia en las bases de datos, que pueden ser insuficientes, o incluso puede darse el caso de que no haya secuencias de referencia de alguna de las especies presentes en la muestra (Shokralla et al. 2012). Las bases de datos se actualizan constantemente, y si se desean comparar estudios realizados en momentos diferentes hay que tener en cuenta esta circunstancia. En concreto en esta Tesis, pese a la escasa diferencia temporal entre los estudios realizados en los Capítulos 2 y 3, se pudo comprobar que las bases de datos de referencia no eran iguales. Fue realizado antes el estudio basado en la plataforma Illumina, y cuando se hizo analizó el set de datos obtenido con Ion Torrent varios meses después el número de referencias había aumentado notablemente. Algo parecido sucede con las versiones de la herramienta bioinformática (*pipeline*) empleada. Cuando los programas se actualizan, los datos pueden dejar de ser comparables dependiendo de lo significativos que sean los cambios en cada actualización. Dentro de una misma *pipeline*, en los resultados influye mucho también la elección de los diferentes algoritmos, pudiendo producirse sesgos ligados bien a fallos en versiones previas de la *pipeline* o a simples diferencias entre los algoritmos empleados.

4. Recomendaciones para la gestión de la conectividad fluvial derivadas de los resultados de esta Tesis

Estos resultados dejan patente el impacto de los embalses, que han favorecido el establecimiento y proliferación de las especies exóticas encontradas en la zona alta de la cuenca del río Nalón, mientras que impiden el acceso de especies bandera como el salmón atlántico o la muy vulnerable anguila europea *Anguilla anguilla*, extirpadas ambas aguas arriba y ausentes en el Parque Natural de Redes. La situación de esta zona protegida en una cuenca donde hay especies migradoras tan importantes como la anguila europea, la trucha común o el salmón atlántico, debería motivar la adopción de medidas para la recuperación de estas especies, lo que a su vez beneficiaría enormemente al estado ecológico de la zona. Por una lado la trucha común tiene poblaciones sedentarias en la zona; sin embargo, estas se encuentran aisladas y en un estado vulnerable por los impactos antropogénicos. La anguila estaba presente en el Parque antes de que se construyeran los embalses (Fernández et al. 2006), por lo que no hay razón para dudar que volvería a establecerse si se dieran las condiciones adecuadas. Al igual que en el caso del salmón atlántico, que al ser especie bandera su conservación significaría igualmente la mejora del resto del ecosistema. Además de la importancia ecológica de estas especies por encontrarse en la zona alta de la cadena trófica, también tienen una gran importancia económica y cultural en la región (Doadrio

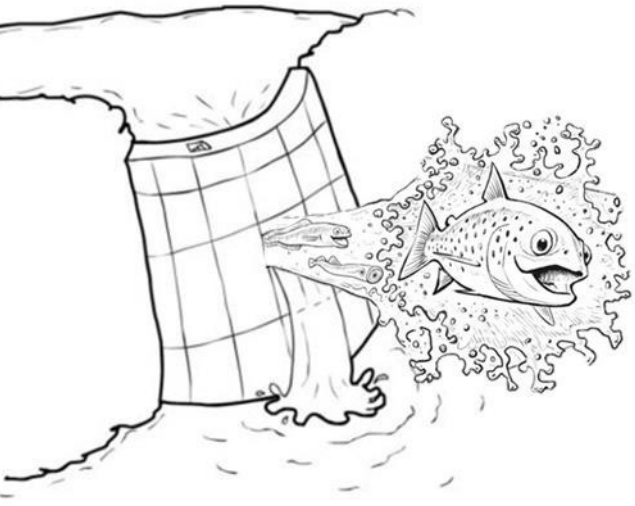
2001). Una posible medida de mitigación de los impactos de estas barreras sobre las especies nativas sería la reconexión de las áreas fluviales afectadas por los embalses mediante la construcción de escalas, que permitan el contacto de sus poblaciones y el restablecimiento de sus rutas migratorias. El posible riesgo que supondría esta reconexión es que las especies exóticas localizadas en la zona baja del río también podrían acceder a la cabecera (Carbajo 2017). Sin embargo, aunque pueda parecer en un principio que los embalses están actuando como barreras en el espacio protegido, el hecho es que según se desprende de esta Tesis y de otros estudios son los propios pescadores los que introducen estas especies aguas arriba de los grandes embalses, por lo que la reconexión de zonas aisladas junto con una mejora de la calidad del agua podría hacer que las poblaciones nativas mejorasen su estatus y las exóticas se vieran desplazadas, al requerir condiciones de temperatura y corriente que no se darían en estos tramos altos (Havel et al. 2015).

Otra recomendación que podría derivarse de los resultados de esta Tesis es el replanteamiento de los usos del espacio protegido de Redes. La gestión del Parque Natural de Redes en particular y de la cuenca en general no aboga por la conservación de las especies de peces nativas antes mencionadas. La anguila se reproduce en el mar y el salmón no tiene poblaciones sedentarias en esta región, por lo que simplemente no podrán recuperar sus poblaciones en el río Nalón y en el Parque Natural de Redes si no se facilita su acceso a través de las presas. En el Plan de Gestión no se consideran medidas de reconexión de sus rutas migratorias, y además en las zonas de influencia de los embalses la calidad del agua no es la adecuada para su supervivencia. Aunque para la trucha común, una especie relativamente resistente, no parecería ser tan perjudicial, de hecho, no se ha encontrado en uno de los puntos de muestreo, el próximo al embalse de Tanes cuya calidad ecológica (medida a partir de los macroinvertebrados) es muy pobre. Mejorar la calidad del agua controlando los vertidos y la sedimentación en los embalses sería otra acción deseable. Por su parte, si se comprueba que la pesca deportiva está facilitando la suelta ilegal de especies exóticas en los embalses, quizás habría que replantearse el uso de los mismos para este fin, aumentar la vigilancia en la zona por parte de la autoridad medioambiental competente, y/o promover programas de educación medioambiental que sensibilicen a los pescadores y el público en general.

Finalmente, desde el punto de vista técnico se ha visto que las herramientas biotecnológicas basadas en ADN ambiental no están aún totalmente maduras para poder aplicarse como único método de inventario biológico en exclusiva. Mientras esta técnica se encuentre en desarrollo, una buena opción sería su combinación con otras metodologías de muestreo, al igual que se ha hecho en este trabajo con la electropesca y las muestras de los pescadores. Una solución intermedia durante este periodo de crecimiento del ADN ambiental es la convivencia de técnicas de muestreo, mientras tiene lugar la validación biotecnológica del *metabarcoding* para su uso en diferentes campos, y su desarrollo alcanza el nivel suficiente como para sustituir, en algunos casos, y complementar en otros, a las técnicas

convencionales de medida de la biodiversidad. Según los resultados de este Tesis, el uso de la qPCR para detectar especies concretas es recomendable en el caso de que las especies objeto de estudio se encuentren en bajas densidades, o cuando se sospeche que el ADN de esas especies pueda competir en la PCR por los marcadores moleculares empleados.

Conclusiones



Conclusiones

1. Mediante el uso de PCR cuantitativa (qPCR) ha sido posible detectar la presencia de las dos especies de salmónidos que se encuentran en el Parque Natural de Redes, la trucha arco iris (*Onconrhynchus mykiss*) que es exótica en Europa, y la especie nativa trucha común (S*almo trutta*). La qPCR demostró ser más sensible que otras técnicas como la PCR-RFLP que no fue eficaz en la detección de la trucha común. El uso de la qPCR es recomendable para la detección de especies que se encuentran en baja abundancia.

2. Se ha demostrado la eficacia del gen COI (subunidad 1 de la citocromo oxidasa) como marcador molecular para la caracterización de macroinvertebrados bentónicos mediante técnicas de *metabarcoding* aplicadas a muestras de agua. Utilizando el gen COI como código de barras genético en el estudio de caso del alto Nalón, la cantidad de secuencias obtenidas pertenecientes a los grupos taxonómicos de interés ha sido suficiente para el cálculo de índices de calidad del agua basados en la presencia de familias indicadoras.

3. La aplicación de técnicas de *metabarcoding* sobre ADN ambiental ha servido para estimar la calidad del agua en el Río Nalón mediante el índice IBMWP empleado en la Península Ibérica para el cumplimiento de la Directiva Marco del Agua europea. Los resultados se correlacionan positiva y significativamente con los obtenidos con el método *de visu* convencional. Su aplicación en otros países se vería dificultada al no poder ser utilizada para métricas cuantitativas con la metodología aquí desarrollada.

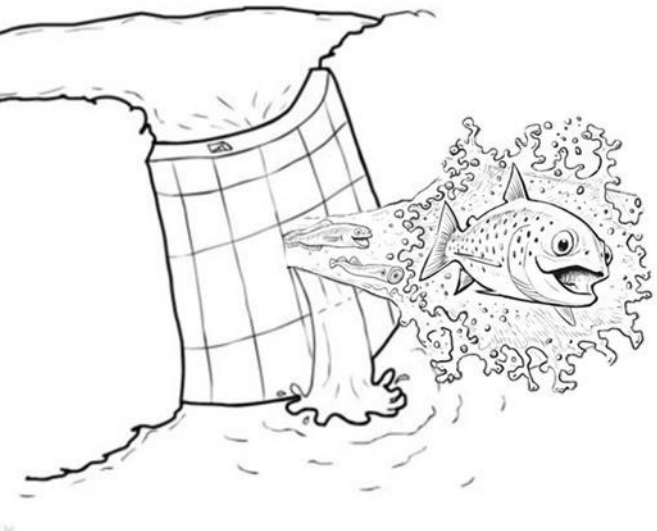
4. Se ha evidenciado el impacto de los embalses de Tanes y Rioseco presentes en el Parque Natural de Redes sobre la calidad del agua, medida tanto a partir de ADN ambiental y *metabarcoding* como con el método de muestreo convencional. Se han visto reducciones en la diversidad tanto de las comunidades de macroinvertebrados como de otros grupos taxonómicos en los tramos del río afectados por los embalses, alterándose el gradiente normal de la diversidad que debería crecer progresivamente aguas abajo.

5. Se ha demostrado mediante ADN ambiental y otros muestreos convencionales que existen introducciones ilegales de especies exóticas dentro de la Reserva de la Biosfera y Parque Natural de Redes. La trucha arcoiris (*Oncorhynchus mykiss*) procede de escapes de piscifactoría, y el bordallo (*Squalius carolitertii*) y el piscardo (*Phoxinus phoxinus*), detectados aguas arriba de barreras impasables, se han tenido soltado de forma deliberada y se están expandiendo aguas arriba en el espacio protegido, quizás favorecidas por el cambio climático.

6. Los resultados de esta Tesis permiten recomendar la aplicación del ADN ambiental para el seguimiento de la calidad del agua en espacios protegidos, al conllevar un muestreo no invasivo y sensible. En la actualidad debería combinarse con otras técnicas convencionales mientras se mejora el estado de las bases de datos de referencia y se estandarizan los protocolos moleculares y el tratamiento bioinformático de las secuencias obtenidas.

7. Se recomienda replantear la gestión del Parque Natural y Reserva de la Biosfera de Redes, considerando la reconexión de la conectividad del río Nalón y el control de la calidad del agua y la introducción de especies exóticas piscícolas.

Conclusions



Conclusions

1. The study carried out in Upper River Nalón for detecting Salmonid DNA from running waters revealed the sensitiveness of the qPCR technique applied on eDNA makes it more effective for the detection of fish species than other PCR-based techniques. In conditions of low abundance of target species, the use of qPCR is recommended.

2. COI (Cytochrome oxidase subunit 1) gene has showed a high accuracy as DNA barcode for the evaluation of benthic macroinvertebrates using a metabarcoding approach in water samples from River Nalón. It has given a sufficient taxonomic coverage to be applied in estimates of water quality indices based on presence-absence of invertebrate families.

3. The usefulness of eDNA metabarcoding for bioassessments of river water quality based on macroinvertebrates evaluation has been proved in River Nalón waters. Except for quantitative elements such as the abundance of a species, this approach complies with WFD requirements.

4. The impact of dams has been confirmed in River Nalón using eDNA-based methodology. Dams and reservoirs affected taxa richness and the taxonomic composition of invertebrate communities above and between dams. The protected area upstream the river basin has been therefore significantly affected by the barriers downstream.

5. The illegal introduction of exotic fish species within the Biosphere Reserve and Natural Park of Redes has been demonstrated as well as expansive trends in their distribution to upstream running waters. Rainbow trout (*Oncorhynchus mykiss*), Iberian chub (*Squalius carolitertii*) and minnow (*Phoxinus phoxinus*) have been detected above impassable dams, proving their introduction was deliberate. Warming temperatures may be favouring the establishment of these exotic species, but the main driver of introductions is likely from farm escapes (*O. mykiss*) or as bait for angling (the other two).

6. The use of eDNA is recommended to monitor river water quality within protected spaces, for being non-invasive, sensitive, and usually requiring less effort than conventional monitoring techniques. If improved in sensitivity, capacity of

quantitative analysis, and taxonomic coverage of reference databases, its use would much improve the efficacy and efficiency of bioassessment in water bodies.

7. The management of the Biosphere Reserve and Natural Park of Redes should be reconsidered. It is recommendable to reconnect Nalón River´s connectivity and to control water quality and exotic fish species introductions.

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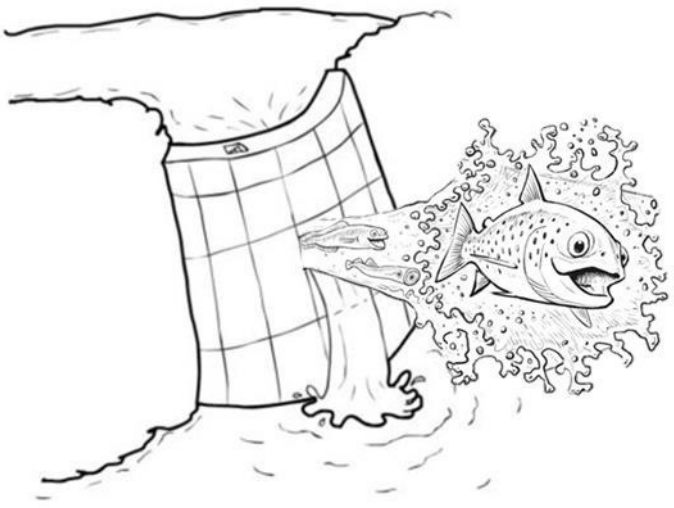

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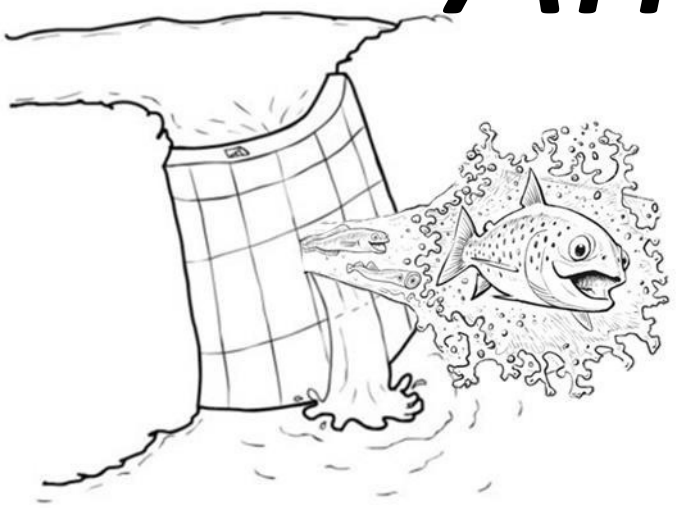
Anexo



Anexo 1

Supplementary Table S2. Raw OTU sequences per sampling replicate (C: Caleao; N: Upper Nalón; T: Tanes; A: Anzó; R: Rioseco; DR: Downstream Rioseco; EC: El Condao; PC: positive control).

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
GU453367.1	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	Annelida	Clitellata	Haplotaxida	Enchytraeidae	Buchholzia	*Buchholzia appendiculata*
GU902040.1	0	0	1	104	477	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Enchytraeidae	Cernosvitoviella	*Cernosvitoviella aggtelekiensis*
KF672373.1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Enchytraeidae	Chamaedrilus	*Chamaedrilus aff. glandulosus B SM-2014*
KF672404.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Enchytraeidae	Chamaedrilus	*Chamaedrilus chalupskyi*
KX618734.1	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Enchytraeidae	Enchytraeus	*Enchytraeus coronatus*
GU902069.1	0	0	0	3	0	0	0	161	403	100	684	0	8	7	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Enchytraeidae	Fridericia	*Fridericia perrieri*
GU902091.1	0	0	2	7	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Enchytraeidae	Lumbricillus	*Lumbricillus tuba*
GU902092.1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Enchytraeidae	Marionina	*Marionina argentea*
KF672423.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Enchytraeidae	Mesenchytraeus	*Mesenchytraeus armatus*
KT706560.1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Enchytraeidae	NA	*Enchytraeidae gen. sp. BOLD:AAT8916*
LN999074.1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Enchytraeidae	NA	*Enchytraeidae sp. 1 RV-2016*
KM527620.1	0	0	5	954	268	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Glossoscolecidae	Martiodrilus	*Martiodrilus sp. 1 DP-2015*
KY633747.1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Lumbricidae	Aporrectodea	*Aporrectodea caliginosa*
AM774292.1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Lumbricidae	Aporrectodea	*Aporrectodea longa*
KP420564.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Lumbricidae	Dendrodrilus	*Dendrodrilus rubidus*
KT705452.1	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Lumbricidae	Dendrodrilus	*Dendrodrilus sp. BOLD:AAA7664*
JX908679.1	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Lumbricidae	Eisenia	*Eisenia andrei*
JX908671.1	3	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Lumbricidae	Eisenia	*Eisenia fetida*
KY284242.1	2	7	0	0	3	2	0	0	0	0	0	0	0	3	0	1	1	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Lumbricidae	Eiseniella	*Eiseniella tetraedra*
GU013996.1	8	0	0	0	0	0	0	1	0	12	20	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Lumbricidae	NA	*Lumbricidae sp. DPEW44204*
FJ374779.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Annelida	Clitellata	Haplotaxida	Lumbricidae	Octolasion	*Octolasion sp. Osp*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
JN869968.1	0	3	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Lumbricidae	Satchellius	*Satchellius mammalis*
GU902037.1	0	0	102	751	503	0	1	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Megascolecidae	Achaeta	*Achaeta unibulba*
AB425786.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Megascolecidae	Metaphire	*Metaphire sieboldi*
HM460268.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Aulodrilus	*Aulodrilus pluriseta*
JQ519897.1	6	23	0	0	0	4	0	0	0	0	0	0	0	0	0	2	6	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Chaetogaster	*Chaetogaster diaphanus*
GQ355367.1	6	3	0	1	1	9	0	0	0	2	0	0	2	20	61	7	13	8	1	18	1	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Chaetogaster	*Chaetogaster diastrophus*
KF952316.1	1	2	0	0	13	0	0	0	0	0	0	0	0	0	0	1	1	7	0	1	2	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Chaetogaster	*Chaetogaster limnaei*
KY369481.1	0	0	0	0	0	0	0	0	3	0	0	0	0	5	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Limnodrilus	*Limnodrilus hoffmeisteri*
GU902104.1	7	141	0	4	0	12	0	0	0	61	36	4	51	162	212	2	7	1	4	4	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais alpina*
JQ519864.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	2	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais barbata*
LN810267.1	0	5	0	0	4	0	0	0	0	6	4	0	8	54	10	7	8	0	0	7	1	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais bretscheri*
JQ519837.1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	10	2	0	5	0	1	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais christinae*
JQ519856.1	0	3	0	0	0	2	0	0	0	1	0	0	0	0	0	1	1	20	7	8	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais communis/variabilis complex sp. A4*
JQ519853.1	1	11	1	1	0	5	0	0	0	35	15	3	3	40	6	5	6	0	2	5	2	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais elinguis*
KF000146.1	1	0	0	0	0	0	0	0	0	0	0	0	1	3	1	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais sp. 5D-20118-3*
JQ519894.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	4	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Nais	*Nais stolci*
LN810257.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Ophidonais	*Ophidonais serpentina*
LN810362.1	0	0	0	0	0	0	0	213	8	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Potamothrix	*Potamothrix bavaricus*
GU902108.1	0	0	0	0	0	0	0	29	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Pristina	*Pristina longiseta*
AF534862.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Stylaria	*Stylaria lacustris*
KF366638.1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Haplotaxida	Tubificidae	Tubifex	*Tubifex tubifex*
FJ639303.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	Annelida	Clitellata	Lumbriculida	Lumbriculidae	Lumbriculus	*Lumbriculus variegatus*
JX993871.1	6	8	1	0	0	0	76	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Lumbriculida	Lumbriculidae	Stylodrilus	*Stylodrilus heringianus*
JX993901.1	3	2	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Annelida	Clitellata	Lumbriculida	Lumbriculidae	Stylodrilus	*Stylodrilus sp. AA-2012*
EU835658.1	0	5	14	12	3	1	62	52	11	1	0	0	1	7	0	4	7	2	1	7	4	0	Annelida	Polychaeta	Capitellida	Capitellidae	Dasybranchus	*Dasybranchus sp. DH1*
KP645942.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Arachnida	Araneae	Araneidae	Larinioides	*Larinioides sclopetarius*
KY017719.1	0	0	0	0	0	0	0	0	0	1	1	0	0	4	0	0	0	0	0	0	0	0	Arthropoda	Arachnida	Araneae	Eutichuridae	Eutichurus	*Eutichurus ravidus*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
KJ744482.1	3	1	10	18	15	3	0	0	1	3	1	2	1	4	2	6	1	3	3	5	0	0	Arthropoda	Arachnida	Araneae	Nemesiidae	Aname	*Aname sp. MYG271*
AB374044.1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Arachnida	Araneae	Pisauridae	Dolomedes	*Dolomedes raptor*
KC615687.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Arachnida	Araneae	Salticidae	Corythalia	*Corythalia sp. Jatun Sacha*
AY297369.1	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Arachnida	Araneae	Salticidae	Hypaeus	*Hypaeus mystacalis*
KY268889.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	Arthropoda	Arachnida	Araneae	Theridiidae	Paidiscura	*Paidiscura pallens*
KR099129.1	24	53	69	152	153	50	46	2	34	206	46	29	13	67	11	21	56	34	8	41	8	0	Arthropoda	Arachnida	NA	NA	NA	*Arachnida sp. BOLD:ACM9770*
KR102125.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Arachnida	NA	Tydeidae	NA	*Tydeidae sp. BOLD:ACJ0030*
KM829536.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	4	0	0	0	0	0	Arthropoda	Arachnida	Oribatida	Camisiidae	Platynothrus	*Platynothrus peltifer*
LC215480.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	Arthropoda	Branchiopoda	Diplostraca	Bosminidae	Bosmina	*Bosmina cf. longirostris WM-2017a*
KC020624.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Arthropoda	Branchiopoda	Diplostraca	Chydoridae	Chydorus	*Chydorus sp. PS-2013*
EU719132.1	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Branchiopoda	Diplostraca	Chydoridae	Chydorus	*Chydorus sphaericus*
EU702267.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	Arthropoda	Branchiopoda	Diplostraca	Chydoridae	Pleuroxus	*Pleuroxus varidentatus*
KU720109.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	17	17	3	3	6	4	0	Arthropoda	Branchiopoda	Diplostraca	Daphniidae	Daphnia	*Daphnia galeata*
KT705917.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	Arthropoda	Branchiopoda	Diplostraca	Daphniidae	Daphnia	*Daphnia sp. BOLD:ACW5340*
GU689249.1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	Arthropoda	Branchiopoda	Diplostraca	Eurycercidae	Eurycercus	*Eurycercus longirostris*
KC617066.1	3	10	4	9	4	4	0	20	68	42	41	21	3	75	21	16	14	6	23	38	10	0	Arthropoda	Branchiopoda	Diplostraca	Macrotrichidae	Macrothrix	*Macrothrix sp. HE-364*
KC479041.1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Branchiopoda	Diplostraca	Moinidae	Moina	*Moina sp. ARRV-2013*
KP697487.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	Arthropoda	Collembola	NA	Hypogastruridae	Ceratophysella	*Ceratophysella sp. BOLD:ACE6295*
HQ592701.1	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Collembola	NA	Isotomidae	Isotomurus	*Isotomurus maculatus*
KT808340.1	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	6	0	1	0	0	0	0	Arthropoda	Collembola	NA	Isotomidae	Isotomurus	*Isotomurus palustris*
KT808324.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	Arthropoda	Collembola	NA	Neanuridae	Anurida	*Anurida granaria*
KM443523.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Coleoptera	Brachyceridae	Dorytomus	*Dorytomus rufatus*
HG514707.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Coleoptera	Carabidae	Anophthalmus	*Anophthalmus tolminensis*
AF332947.1	0	0	9	4	0	0	0	0	0	0	0	0	0	0	1	7	3	2	6	3	0	0	Arthropoda	Insecta	Coleoptera	Cerambycidae	Prionus	*Prionus insularis*
AY242397.1	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Arthropoda	Insecta	Coleoptera	Chrysomelidae	Colasposoma	*Colasposoma sp. JJG318*
KJ381196.1	0	0	0	0	0	0	0	0	0	34	11	13	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Coleoptera	Elmidae	Elmis	*Elmis maugetti*
HF947938.1	2	0	0	0	0	0	0	0	0	8	24	4	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Coleoptera	Elmidae	Elmis	*Elmis perezi*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
HE970905.1	0	0	102	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Coleoptera	Hydraenidae	Hydraena	*Hydraena truncata*
KM445870.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	Arthropoda	Insecta	Coleoptera	Staphylinidae	Lesteva	*Lesteva pubescens*
EF104718.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Arthropoda	Insecta	Diptera	Agromyzidae	Phytobia	*Phytobia sp. Ptb-2*
EU367598.1	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Agromyzidae	Phytomyza	*Phytomyza gymnostoma*
KM855848.1	0	0	26	8	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Anthomyiidae	NA	*Anthomyiidae sp. BOLD:ACI3501*
JX416984.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	Arthropoda	Insecta	Diptera	Cecidomyiidae	Asteromyia	*Asteromyia carbonifera*
KR957130.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Cecidomyiidae	NA	*Cecidomyiidae sp. BOLD-2016*
KT091715.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Ceratopogonidae	Alluaudomyia	*Alluaudomyia sp. BOLD-2016*
HQ824523.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	3	7	0	0	Arthropoda	Insecta	Diptera	Ceratopogonidae	Forcipomyia	*Forcipomyia sp. CW-2011*
KR505584.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Ablabesmyia	*Ablabesmyia monilis*
KR494560.1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Ablabesmyia	*Ablabesmyia sp. BOLD-2016*
KM956233.1	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Acricotopus	*Acricotopus sp. BOLD:AAB9516*
JF286665.1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Brillia	*Brillia sp. BOLD:AAI3832*
KR615102.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Bryophaenocladius	*Bryophaenocladius sp. 7ES*
HQ105023.1	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Chaetocladius	*Chaetocladius dissipatus*
KR773709.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Chaetocladius	*Chaetocladius perennis*
KR157356.1	0	1	0	0	0	6	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Chaetocladius	*Chaetocladius sp. BOLD:ACF6903*
DQ910591.1	0	2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Chironomus	*Chironomus luridus*
DQ910573.1	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Chironomus	*Chironomus nuditarsis*
DQ393882.1	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cladotanytarsus	*Cladotanytarsus australomancus*
KT612855.1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cladotanytarsus	*Cladotanytarsus sp. BOLD:ACM0192*
KF489835.1	0	0	0	0	0	1	2	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Corynoneura	*Corynoneura fortispicula*
HQ105045.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Corynoneura	*Corynoneura lacustris*
HQ105049.1	3	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Corynoneura	*Corynoneura lobata*
KR654450.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Corynoneura	*Corynoneura scutellata*
KF489860.1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Corynoneura	*Corynoneura septadentata group sp. BOLD:AAW4943*
KF489866.1	0	1	42	4	0	1	3	0	0	0	0	0	0	2	0	1	1	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Corynoneura	*Corynoneura unicapsulata*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
KR760508.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus annulator complex sp. BOLD-2016*
KY837605.1	0	0	0	0	0	0	0	0	0	20	10	5	2	6	1	13	0	0	2	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus bicinctus*
JN887061.1	0	0	0	1	0	0	0	0	0	0	0	0	47	0	0	0	0	0	1	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus bimaculatus*
KP954810.1	0	1	0	0	0	0	0	0	0	0	9	1	0	1	0	1	1	0	4	0	1	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus conicornis*
KM934731.1	0	0	0	0	0	0	0	0	0	16	0	0	0	5	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus coronatus*
KP954806.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	1	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus draysoni*
KC130758.1	0	0	0	19	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus glacialis*
KR643612.1	1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus intersectus*
KP955060.1	0	0	0	0	0	0	0	0	0	3	4	1	0	0	0	1	1	0	1	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus parbicinctus*
KR612961.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus perniger*
KR625407.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus sp. 4ES*
KR613074.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus sp. 7ES*
KT612002.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus sp. BOLD:ACF9756*
KR648148.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus sp. BOLD-2016*
KM991965.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus sp. SK-2011*
KJ439932.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus sylvestris*
KP955110.1	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus tasmania*
KR762679.1	1	1	1	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus tremulus*
KT609027.1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	1	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus triannulatus*
KR633644.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus tricinctus*
KT611073.1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	1	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus trifascia*
JF764761.1	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Cricotopus	*Cricotopus trifascia group sp. AJ-2011*
KR426850.1	0	0	0	4	0	0	1	0	0	0	0	0	6	1	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa arctica*
KR957669.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa bertrami*
JF764769.1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa bohemani*
LN897667.1	0	1	0	587	0	0	10	0	0	0	0	0	12208	50	1	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa cinerella*
LN897621.1	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa cinerella/tonsa group sp. 1 MM-2015*

Anexo 1

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	**C1**	**C2**	**C3**	**N1**	**N2**	**N3**	**T1**	**T2**	**T3**	**A1**	**A2**	**A3**	**R1**	**R2**	**R3**	**DR1**	**DR2**	**DR3**	**EC1**	**EC2**	**EC3**	**PC**	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**
LN897650.1	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa dampfi*
KU373737.1	0	0	0	0	0	0	21	0	0	0	0	0	14	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa geminata*
JF764759.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa hyperborea*
LN897666.1	0	0	0	6	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa latitarsis*
KM941542.1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	1	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa nr. hyperborea BOLD:AAL5996*
KR586873.1	0	3	591	1138	0	2	1	0	0	4	1	1	1	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa sp. BOLD-2016*
LN897655.1	0	0	0	0	0	0	0	0	0	2	0	0	8	0	0	0	0	0	4	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa tonsa*
LN897653.1	0	0	0	0	0	0	0	0	0	0	1	0	4581	23	1	0	0	1	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Diamesa	*Diamesa zernyi*
JF869917.1	0	0	0	0	0	0	0	0	0	2	0	0	1	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Eukiefferiella	*Eukiefferiella sp. BOLD:AAN4988*
KR774202.1	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	1	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Eukiefferiella	*Eukiefferiella sp. BOLD-2016*
KR277841.1	0	0	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Gymnometriocnemus	*Gymnometriocnemus brumalis*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
KP050318.1	0	0	0	0	0	0	0	0	0	0	0	0	115	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Gymnometriocnemus	*Gymnometriocnemus kamimegavirgus*
KR177169.1	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Gymnometriocnemus	*Gymnometriocnemus sp. 2 TE-2010*
KR585243.1	0	0	0	1	0	0	0	0	0	0	0	0	53	0	0	0	0	0	0	0	0	1	Arthropoda	Insecta	Diptera	Chironomidae	Gymnometriocnemus	*Gymnometriocnemus sp. BOLD-2016*
KR721943.1	0	0	0	0	0	0	0	0	0	0	1	0	14	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Gymnometriocnemus	*Gymnometriocnemus volitans*
KT285296.1	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Hydrobaenus	*Hydrobaenus sikhotealinensis*
KY399219.1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Hydrobaenus	*Hydrobaenus sp. 1 EAM-2017*
JF870079.1	0	0	0	17	0	1	0	0	0	0	0	0	12	2	1	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Krenosmittia	*Krenosmittia sp. BOLD:AAN4358*
HQ105141.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Limnophyes	*Limnophyes pentaplastus*
KM902003.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	47	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Limnophyes	*Limnophyes sp. BOLD:AAQ0626*
KR715807.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Limnophyes	*Limnophyes sp. BOLD-2016*
HQ105192.1	0	2	34	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Metriocnemus	*Metriocnemus tristellus*
KC788682.1	0	2	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Micropsectra	*Micropsectra atrofasciata*
AM398692.1	0	6	0	0	0	15	0	0	0	19	15	1	0	0	0	1	5	0	0	15	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Micropsectra	*Micropsectra contracta*
AM398706.1	4	5	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Micropsectra	*Micropsectra notescens*
HQ105213.1	1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Micropsectra	*Micropsectra pallidula*
KC788649.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Micropsectra	*Micropsectra sp. 3 ES*
KC788719.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Micropsectra	*Micropsectra sp. ATNA53*
KT619453.1	0	0	0	0	0	0	0	0	0	1	6	1	0	0	0	0	1	0	0	0	1	0	Arthropoda	Insecta	Diptera	Chironomidae	Microtendipes	*Microtendipes pedellus*
HQ938459.1	0	0	0	0	0	0	0	0	0	1	3	0	0	0	0	0	0	1	0	2	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Microtendipes	*Microtendipes pedellus group sp. BOLD:AAN3037*
KY225367.1	0	0	0	0	0	0	0	0	0	3	3	0	0	0	0	0	0	0	0	3	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Microtendipes	*Microtendipes sp. PA6*
KR178221.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Monopelopia	*Monopelopia tenuicalcar*
KF000265.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	NA	*Chironomidae 20280-C7*
KR742419.1	9	393	1575	1060	4	15	1	0	10	1758	57	33	165	330	0	18	6	0	14	10	3	0	Arthropoda	Insecta	Diptera	Chironomidae	NA	*Chironominae sp. BOLD-2016*
KR728953.1	2	104	12	17	0	4	0	0	0	189	3	2	139	109	0	3	5	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	NA	*Orthocladiinae sp. BOLD-2016*
KR525261.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	Arthropoda	Insecta	Diptera	Chironomidae	NA	*Tanypodinae sp. BOLD-2016*
KR962217.1	0	0	0	0	0	0	0	0	0	53	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Nilotanypus	*Nilotanypus fimbriatus*
KR766010.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius dorenus*
KU374403.1	2	2	0	1	0	0	0	0	0	0	0	0	0	0	0	1	3	0	1	1	3	0	Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius frigidus*
HQ105229.1	0	0	0	0	0	0	0	0	0	1	0	0	0	2	5	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius fuscimanus*
KR632335.1	0	0	0	0	0	0	0	0	0	3	3	0	3	1	1	3	1	0	1	0	1	0	Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius oblidens*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
KT702472.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius oliveri*
KR760127.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	409	1	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius rivicola*
KR759879.1	0	0	0	11085	0	0	0	0	0	0	0	0	0	0	0	3	1	0	0	0	3	0	Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius rivulorum*
LN897586.1	1	1	0	0	0	0	0	0	0	0	0	392	0	1	0	0	0	0	0	2	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius sp. 1 MM-2015*
KR760341.1	2	0	2	207	0	3	0	0	0	8	14	3	1	9	0	4763	7	2	2	1	1	0	Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius sp. BOLD-2016*
JF764756.1	0	0	0	0	0	0	0	0	0	0	0	9	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Orthocladius	*Orthocladius telochaetus*
KT248926.1	0	0	0	0	0	0	0	0	0	0	2	0	1	2	0	0	0	0	2	6	3	0	Arthropoda	Insecta	Diptera	Chironomidae	Paracricotopus	*Paracricotopus sp. BOLD:ACP9613*
KR592072.1	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Parakiefferiella	*Parakiefferiella scandica*
KR960009.1	0	0	0	0	0	0	0	0	0	0	2	1	0	0	0	1	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Parametriocnemus	*Parametriocnemus boreoalpinus*
KR641614.1	1	5	0	0	0	9	61	0	0	2	0	0	71414	262	1	2	0	1	3	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Parametriocnemus	*Parametriocnemus sp. BOLD-2016*
KR685398.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Paraphaenocladius	*Paraphaenocladius irritus*
KR617974.1	0	0	0	9	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Paraphaenocladius	*Paraphaenocladius sp. BOLD-2016*
AM398738.1	0	0	0	0	0	0	0	0	0	10	5	1	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Parapsectra	*Parapsectra mendli*
KC250823.1	0	0	0	0	0	0	0	0	0	0	1	0	0	2	0	0	1	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Paratanytarsus	*Paratanytarsus dissimilis*
DQ393889.1	0	0	0	0	0	0	104	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Paratanytarsus	*Paratanytarsus kathleenae*
KP954934.1	0	0	0	0	0	0	0	0	0	156	4	2	0	17	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Paratrichocladius	*Paratrichocladius bifenestrus*
JN887090.1	0	0	0	0	0	0	0	0	0	0	0	0	0	4	2647	0	0	1	0	0	1	0	Arthropoda	Insecta	Diptera	Chironomidae	Paratrichocladius	*Paratrichocladius rufiventris*
KC750485.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	Arthropoda	Insecta	Diptera	Chironomidae	Paratrichocladius	*Paratrichocladius sp. 2 MEC-2013*
KU497112.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Polypedilum	*Polypedilum sp. 13 SC*
KP954646.1	0	0	0	0	0	0	0	0	0	3	1	0	0	0	0	3	4	0	2	7	3	0	Arthropoda	Insecta	Diptera	Chironomidae	Procladius	*Procladius bellus*
KR636269.1	0	59	0	0	0	0	0	0	0	17	8	2	12	0	0	4	3	0	0	0	1	0	Arthropoda	Insecta	Diptera	Chironomidae	Procladius	*Procladius sp. ES02*
KT248930.1	1	10	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Prodiamesa	*Prodiamesa olivacea*
KM989051.1	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Psectrocladius	*Psectrocladius barbimanus*
KR642048.1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Psectrocladius	*Psectrocladius cf. limbatellus BOLD-2016*
KR723134.1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Psectrocladius	*Psectrocladius obvius*
KC250849.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Psectrocladius	*Psectrocladius simulans*
KU374615.1	0	0	0	0	0	0	0	0	0	0	0	0	616	3	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Psectrocladius	*Psectrocladius sokolovae*
KR642004.1	0	0	0	0	0	0	0	0	0	0	0	0	456	1	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Psectrocladius	*Psectrocladius sp. BOLD-2016*
AB838636.1	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Rheocricotopus	*Rheocricotopus chalybeatus*
KR727104.1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Rheocricotopus	*Rheocricotopus sp. BOLD-2016*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	**C1**	**C2**	**C3**	**N1**	**N2**	**N3**	**T1**	**T2**	**T3**	**A1**	**A2**	**A3**	**R1**	**R2**	**R3**	**DR1**	**DR2**	**DR3**	**EC1**	**EC2**	**EC3**	**PC**	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**
JF287899.1	0	0	0	39	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Rheotanytarsus	*Rheotanytarsus sp. BOLD:AAH3855*
KP977110.1	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Smittia	*Smittia aterrima*
KR426785.1	0	28	2	0	0	0	0	0	0	0	0	0	0	1	0	0	2	0	1	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Smittia	*Smittia sp. ES6*
EF585415.1	0	0	0	0	0	0	44	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Stempellinella	*Stempellinella ciliaris*
KT618835.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Stempellinella	*Stempellinella fimbriata*
KR281539.1	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Synorthocladius	*Synorthocladius semivirens*
KM626934.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	8	342	5	10	2	0	Arthropoda	Insecta	Diptera	Chironomidae	Tanytarsus	*Tanytarsus bathophilus*
KT613648.1	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Tanytarsus	*Tanytarsus bispinosus*
HQ105359.1	0	0	0	0	0	0	0	0	0	0	1	2	1	3	23	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Tanytarsus	*Tanytarsus brundini*
KR636643.1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Tanytarsus	*Tanytarsus guerlus*
AM084265.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Tanytarsus	*Tanytarsus hjulorum*
KR962541.1	0	0	0	22	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Tanytarsus	*Tanytarsus sp. BOLD-2016*
KR272707.1	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Thienemanniella	*Thienemanniella lobapodema*
HQ105378.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Thienemanniella	*Thienemanniella minuscula*
JF288127.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Thienemanniella	*Thienemanniella sp. BOLD:AAG1615*
KR519415.1	0	0	1	0	0	0	0	0	0	43	1	0	0	5	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Thienemanniella	*Thienemanniella xena*
KT248932.1	8	3	0	2408	0	2	0	0	0	12	9	483	1	1	0	2	1	0	7	8	4	0	Arthropoda	Insecta	Diptera	Chironomidae	Tvetenia	*Tvetenia calvescens*
KR761439.1	4	5	180	0	3	7	0	0	0	787	16	7	0	126	0	2	1	0	1	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Tvetenia	*Tvetenia paucunca*
JF764757.1	0	0	0	367	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Tvetenia	*Tvetenia sp. AJ-2011*
KR514078.1	0	0	0	4	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chironomidae	Zavrelimyia	*Zavrelimyia sp. BOLD-2016*
KM911116.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chloropidae	Thaumatomyia	*Thaumatomyia pulla*
KR399960.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Chloropidae	Thaumatomyia	*Thaumatomyia trifasciata*
JQ615288.1	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles albitarsis*
KC330249.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles arthuri s. l. C MAS-2013*
JX205104.1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles benarrochi B*
KM068084.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles dthali*
KF202357.1	0	0	3	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles farauti*
JX020723.1	0	1	0	0	0	0	0	0	0	1	0	0	0	2	0	0	0	0	0	1	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles fluviatilis*
JQ291237.1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles homunculus*
JQ728235.1	0	14	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles jeyporiensis*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
JN413695.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	2	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles lutzii s. l. 2 RS19*
JQ728030.1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles minimus*
AB781760.1	0	0	0	0	1	0	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles nigerrimus*
AB715046.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles peditaeniatus*
KU671370.1	0	0	0	0	0	0	0	0	0	2	0	0	1	0	0	2	2	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles sawyeri*
AB738221.1	0	0	2	1	5	0	0	0	0	0	2	0	1	0	0	0	0	0	1	0	2	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles sinensis*
KJ522835.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	3	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles sp. 7 BSL-2014*
KU948655.1	0	0	0	0	0	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles sp. AGB-2016*
JQ728051.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles tessellatus*
JF923736.1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Anopheles	*Anopheles triannulatus*
JQ728188.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Culex	*Culex bicornutus*
AB738163.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Culex	*Culex ryukyensis*
KF000313.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Culiseta	*Culiseta impatiens*
GU908120.1	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Orthopodomyia	*Orthopodomyia alba*
JX260715.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Psorophora	*Psorophora howardii*
KU495089.1	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Tripteroides	*Tripteroides tasmaniensis*
JQ728222.1	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	2	0	0	0	0	0	Arthropoda	Insecta	Diptera	Culicidae	Uranotaenia	*Uranotaenia nivipleura*
KM928824.1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Diastatidae	NA	*Diastatidae sp. BOLD:AAN5689*
KU873322.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Dolichopodidae	NA	*NA*
FJ808412.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	Arthropoda	Insecta	Diptera	Dolichopodidae	Tachytrechus	*Tachytrechus tessellatus*
KJ082997.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Drosophilidae	Apsiphortica	*Apsiphortica melanogaster*
EU493625.1	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Drosophilidae	Drosophila	*Drosophila baimaii*
JF736070.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Drosophilidae	Drosophila	*Drosophila richardsoni*
AY154416.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Drosophilidae	Drosophila	*Drosophila subquinaria*
HQ631577.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Drosophilidae	Drosophila	*Drosophila tani*
KU600602.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	Arthropoda	Insecta	Diptera	Drosophilidae	Impatiophila	*Impatiophila chiasmosternata*
KX069324.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Drosophilidae	Leucophenga	*Leucophenga rhombura*
KT884772.1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Drosophilidae	Leucophenga	*Leucophenga sp. 3 YLW-2015*
KJ085134.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Drosophilidae	NA	*Drosophilidae sp. BOLD:ABA0755*
KJ130750.1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Drosophilidae	Phortica	*Phortica acongruens*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
KJ130862.1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Drosophilidae	Phortica	*Phortica subradiata*
FJ948765.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Arthropoda	Insecta	Diptera	Drosophilidae	Zaprionus	*Zaprionus beninensis*
KR652535.1	0	0	2388	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Empididae	Hemerodromia	*Hemerodromia sp. BOLD:AAF9865*
KP697425.1	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Empididae	NA	*Empididae sp. BOLD:ACG8766*
KF297865.1	0	0	1475	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Empididae	Wiedemannia	*Wiedemannia bohemani*
KR748859.1	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Ephydridae	Discocerina	*Discocerina obscurella*
KY837590.1	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Ephydridae	NA	*Ephydridae sp. BIOUG02205-F08*
KP041551.1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Fanniidae	NA	*Fanniidae sp. BOLD:AAM6400*
KM939745.1	0	0	1036	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Heleomyzidae	Suillia	*Suillia barberi*
KJ082967.1	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Hybotidae	Tachydromia	*Tachydromia annulimana*
KR397076.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	Arthropoda	Insecta	Diptera	Lauxaniidae	Sapromyza	*Sapromyza sp. BOLD:AAG6931*
KJ090456.1	0	0	108	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Limoniidae	Dicranomyia	*Dicranomyia frontalis*
KR517883.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Limoniidae	NA	*Limoniidae sp. BOLD-2016*
KC499755.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Muscidae	Hydrotaea	*Hydrotaea aenescens*
JX861422.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Muscidae	Hydrotaea	*Hydrotaea dentipes*
FJ025628.2	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Muscidae	Limnophora	*Limnophora olympiae*
KR465823.1	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Muscidae	Spilogona	*Spilogona sororcula*
KM679400.1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Mycetophilidae	Mycetophila	*Mycetophila lunata*
KP715944.1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Mycetophilidae	Neuratelia	*Neuratelia jabalmoussae*
JN289150.1	2	272	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	NA	NA	*Diptera sp. BOLD:AAC3216*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
JX196862.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Nymphomyiidae	Nymphomyia	*Nymphomyia rohdendorfi*
GU909457.1	0	0	0	14	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Psychodidae	Lutzomyia	*Lutzomyia evansi*
GU909505.1	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Psychodidae	Lutzomyia	*Lutzomyia longipalpis*
JQ349588.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Psychodidae	Pneumia	*Pneumia mutua*
KR989851.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Psychodidae	Psychoda	*Psychoda phalaenoides*
KT100389.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Psychodidae	Psychoda	*Psychoda sp. BOLD-2016*
KJ481113.1	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Psychodidae	Sergentomyia	*Sergentomyia antennata*
KR759524.1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Sarcophagidae	Oxysarcodexia	*Oxysarcodexia sp. BOLD-2016*
KR665426.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Arthropoda	Insecta	Diptera	Sciaridae	Scatopsciara	*Scatopsciara atomaria*
JF872917.1	3	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Prosimulium	*Prosimulium hirtipes*
KP861143.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Prosimulium	*Prosimulium latimucro*
KP861000.1	223	329	18	10	1	274	0	0	0	0	0	0	0	0	0	6	5	1	1	5	2	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium argyreatum*
GQ465960.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium aureum*
KJ649643.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium bullatum*
AY251528.1	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium chamlongi*
KP861022.1	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium intermedium*
KX673597.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium kiritshenkoi*
GU203469.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium lineatum*
KF640037.1	0	551	0	4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium margaritae*
FJ524751.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium noelleri*
KP861031.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium ornatum*
AY251497.1	0	0	0	1	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium tahanense*
KP252599.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium travisi*
KP861040.1	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	2	0	1	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium trifasciatum*
KP861017.1	8	32	1	2	0	23	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium variegatum*
GQ465954.1	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	2	0	1	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium velutinum*
GU073098.1	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Simuliidae	Simulium	*Simulium vernum*
KT609130.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Sphaeroceridae	Coproica	*Coproica ferruginata*
KM954809.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Sphaeroceridae	NA	*Limosininae sp. BOLD:AAG7312*
KJ092369.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Arthropoda	Insecta	Diptera	Sphaeroceridae	NA	*Sphaeroceridae sp. BOLD:ABV1178*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
KM643715.1	0	2	74	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Sphaeroceridae	Rachispoda	*Rachispoda sp. BOLD:ACG6399*
KR427383.1	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Sphaeroceridae	Spelobia	*Spelobia sp. BOLD-2016*
KM946555.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	4	0	0	0	0	0	Arthropoda	Insecta	Diptera	Sphaeroceridae	Spelobia	*Spelobia tufta*
KR758537.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	Arthropoda	Insecta	Diptera	Sphaeroceridae	Sphaerocera	*Sphaerocera curvipes*
DQ983520.1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Tabanidae	Hybomitra	*Hybomitra rhombica*
KX844419.1	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Tachinidae	Allophorocera	*Allophorocera lapponica*
GU142237.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Tachinidae	Chrysoexorista	*Chrysoexorista sp. Wood06*
EF181907.1	0	1385	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Tachinidae	Lespesia	*Lespesia sp. posticaDHJ05*
KM633286.1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Tachinidae	NA	*Exoristinae sp. BOLD:ABW0151*
KR436670.1	0	0	0	888	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Tachinidae	Peleteria	*Peleteria iterans*
HQ548265.1	0	1	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Tachinidae	Pseudochaeta	*Pseudochaeta sp. Janzen13*
JQ576364.1	1	0	0	0	0	0	0	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Tachinidae	Tachinidae gen. tachJanzen01	*Tachinidae gen. tachJanzen01 sp. Janzen01*
KT215933.1	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Thaumaleidae	Thaumalea	*Thaumalea truncata*
KM647581.1	0	0	1	0	0	0	83	0	0	0	1	0	0	0	0	0	0	0	0	13	0	0	Arthropoda	Insecta	Diptera	Tipulidae	NA	*Tipulinae sp. BOLD:AAI1200*
KR441804.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Tipulidae	Tipula	*Tipula limbata*
KU844263.1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Tipulidae	Tipula	*Tipula maoershanensis*
KP047556.1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Tipulidae	Tipula	*Tipula senega*
KR769911.1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Diptera	Tipulidae	Tipula	*Tipula sp. BOLD-2016*
JN164286.1	1	0	0	0	1	4	0	0	0	4	13	4	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Ephemeroptera	Baetidae	Baetis	*Baetis lutheri*
GU812339.1	0	2	2	4754	1658	5	0	1	0	9	9	2	5	4	0	0	0	0	0	0	0	1	Arthropoda	Insecta	Ephemeroptera	Baetidae	Baetis	*Baetis rhodani*
JN299135.1	0	0	0	0	0	0	0	0	0	3	0	0	2	14	20	0	0	0	0	0	0	0	Arthropoda	Insecta	Ephemeroptera	Baetidae	Centroptilum	*Centroptilum luteolum*
LN734678.1	2	3	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Ephemeroptera	Baetidae	Takobia	*Takobia muticus*
LN734709.1	0	0	0	0	0	0	0	0	0	0	0	0	2	16	7	1	0	0	0	13	1	0	Arthropoda	Insecta	Ephemeroptera	Caenidae	Caenis	*Caenis pusilla*
HG935102.1	11	12	0	0	0	0	0	0	0	1348	849	112	5	270	3	0	0	0	0	0	0	1	Arthropoda	Insecta	Ephemeroptera	Ephemerellidae	Serratella	*Serratella cf. ignita SE-36-FR(MV)*
LT626197.1	0	0	0	0	0	0	0	0	0	18	11	0	1	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Ephemeroptera	Ephemerellidae	Torleya	*Torleya major*
JX571926.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Arthropoda	Insecta	Ephemeroptera	Ephemeridae	Ephemera	*Ephemera danica*
HG935046.1	0	4	1	0	10	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Ephemeroptera	Heptageniidae	Ecdyonurus	*Ecdyonurus sp. EC-37-FR(MV)*
LN734726.1	15	15	7	6	34	5	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Arthropoda	Insecta	Ephemeroptera	Heptageniidae	Ecdyonurus	*Ecdyonurus venosus*
KX447081.1	0	0	0	0	0	0	0	0	0	1	7	2	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Ephemeroptera	Heptageniidae	Electrogena	*Electrogena lateralis*
LT626119.1	33	151	64	12	479	102	0	0	0	0	0	0	0	0	0	13	0	2	0	0	0	0	Arthropoda	Insecta	Ephemeroptera	Heptageniidae	Epeorus	*Epeorus assimilis*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
LT626159.1	6840	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Ephemeroptera	Heptageniidae	Rhithrogena	*Rhithrogena adrianae*
LT745900.1	25	34	4	9	11	30	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Ephemeroptera	Heptageniidae	Rhithrogena	*Rhithrogena cf. fiorii SC-2017*
LT745906.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Ephemeroptera	Heptageniidae	Rhithrogena	*Rhithrogena hercynia group sp. SC-2017*
HM481157.1	125	304	19	17	25	91	0	0	19	0	0	0	0	0	0	0	0	0	0	0	0	126854	Arthropoda	Insecta	Ephemeroptera	Heptageniidae	Rhithrogena	*Rhithrogena sp. 8 LV-2010*
LN734747.1	0	0	12	12	0	1	5	0	6	0	1	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Ephemeroptera	Leptophlebiidae	Habroleptoides	*Habroleptoides confusa*
LT626133.1	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Ephemeroptera	Leptophlebiidae	Habrophlebia	*Habrophlebia eldae*
GU447026.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Arthropoda	Insecta	Hemiptera	Aphrophoridae	Aphrophora	*Aphrophora permutata*
HE577314.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	Arthropoda	Insecta	Hemiptera	Cicadellidae	NA	*Erythroneurini sp. IBE-GS4*
JQ618320.1	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Hemiptera	Cicadidae	Platypleura	*Platypleura sp. BWP-2010e*
GQ922207.1	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Hemiptera	Halimococcidae	Colobopyga	*Colobopyga pritchardiae*
HQ846916.1	2	12	2	6	9	0	82	3	3	0	0	1	3	3	0	21	6	1	5	20	8	0	Arthropoda	Insecta	Hemiptera	Reduviidae	Rhynocoris	*Rhynocoris kumarii*
KP943643.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Hymenoptera	Braconidae	Crassomicrodus	*Crassomicrodus sp. H14964*
JQ736323.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	5	0	Arthropoda	Insecta	Hymenoptera	Braconidae	Utetes	*Utetes sp. XYL-2012*
KF642890.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Hymenoptera	Cephidae	Calameuta	*Calameuta pygmaea*
AJ514334.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Hymenoptera	Chrysididae	Hedychridium	*Hedychridium roseum*
JN578953.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Hymenoptera	Colletidae	Hylaeus	*Hylaeus sp. 11 PK-2011*
KU504831.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Hymenoptera	Formicidae	Anochetus	*Anochetus altisquamis*
KM995931.1	1	1	85	0	0	0	0	0	0	0	0	0	0	15	4	0	0	1	3	0	0	0	Arthropoda	Insecta	Hymenoptera	NA	NA	*Hymenoptera sp. ASAHY926-14*
JQ523385.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Lepidoptera	Elachistidae	Stenoma	*Stenoma sp. Janzen16*
KX041875.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Arthropoda	Insecta	Lepidoptera	Erebidae	Coscinia	*Coscinia cribraria*
KX049493.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Lepidoptera	Geometridae	Alsophila	*Alsophila aescularia*
GQ433528.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	Arthropoda	Insecta	Lepidoptera	Geometridae	Eois	*Eois nr. olivacea PS-2010*
NC_021427.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Arthropoda	Insecta	Lepidoptera	Hesperiidae	Erynnis	*Erynnis montanus*
AF279220.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Lepidoptera	Lycaenidae	Crudaria	*Crudaria leroma*
AB608851.1	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Lepidoptera	Micropterigidae	NA	*Micropterigidae sp. SP01*
KX281336.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Lepidoptera	Nepticulidae	Stigmella	*Stigmella longisacca*
JN305074.1	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Lepidoptera	Noctuidae	Drobeta	*Drobeta thacia*
KJ386078.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Lepidoptera	Noctuidae	Noctua	*Noctua pronuba*
GU333567.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Arthropoda	Insecta	Lepidoptera	Notodontidae	Rifargia	*Rifargia sp. felderiDHJ02*
KT073487.1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Lepidoptera	Nymphalidae	Polyura	*Polyura sp. POLYU141-15*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
KJ398262.1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Lepidoptera	Tortricidae	Cydia	*Cydia fagiglandana*
AB354079.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Neuroptera	Chrysopidae	Mallada	*Mallada desjardinsi*
JQ240181.1	0	0	0	5	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Neuroptera	Hemerobiidae	Micromus	*Micromus tasmaniae*
JN419454.1	0	0	48	0	0	0	0	0	0	22	3	0	0	3	7	0	0	0	12	0	0	0	Arthropoda	Insecta	Odonata	Calopterygidae	Calopteryx	*Calopteryx maculata*
KC107653.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Orthoptera	Acrididae	Chorthippus	*Chorthippus parallelus*
EU366097.1	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Orthoptera	Acrididae	Omocestus	*Omocestus haemorrhoidalis*
AY738363.1	0	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Orthoptera	Acrididae	Omocestus	*Omocestus viridulus*
HQ705646.1	0	0	0	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Plecoptera	Chloroperlidae	Siphonoperla	*Siphonoperla torrentium*
KT874610.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	Arthropoda	Insecta	Plecoptera	Leuctridae	Leuctra	*Leuctra digitata*
KT807852.1	0	0	0	1727	2	0	0	0	0	0	0	0	0	4	36	0	0	0	0	0	0	0	Arthropoda	Insecta	Plecoptera	Leuctridae	Leuctra	*Leuctra fusca*
KT874632.1	9	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Plecoptera	Leuctridae	Leuctra	*Leuctra hippopus*
JQ736341.1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Plecoptera	Leuctridae	Leuctra	*Leuctra major*
KT874605.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0	0	0	0	0	Arthropoda	Insecta	Plecoptera	Leuctridae	Leuctra	*Leuctra sp. BOLD:ACY3863*
KU955864.1	2	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Plecoptera	Nemouridae	Amphinemura	*Amphinemura sulcicollis*
KF881073.1	2	1	7	4	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Plecoptera	Nemouridae	Protonemura	*Protonemura meyeri*
JQ736346.1	1	1	4	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Plecoptera	Nemouridae	Protonemura	*Protonemura nimborella*
KF492802.1	14	1	0	2	5	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Plecoptera	Perlidae	Dinocras	*Dinocras cephalotes*
KU955914.1	0	0	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Plecoptera	Perlodidae	Isoperla	*Isoperla grammatica*
KR942123.1	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Psocoptera	Ectopsocidae	Ectopsocus	*Ectopsocus californicus*
KR147593.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Psocoptera	Ectopsocidae	NA	*Ectopsocidae sp. BOLD:AAN8452*
HQ978915.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Psocoptera	NA	NA	*Psocoptera sp. BOLD:AAN8452*
KP871429.1	0	0	1	0	4	0	0	2	0	0	0	1	4	10	2	3	3	3	2	2	0	0	Arthropoda	Insecta	Thysanoptera	Phlaeothripidae	Haplothrips	*Haplothrips tenuipennis*
KX143271.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Brachycentridae	Micrasema	*Micrasema moestum*
HQ150976.1	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Brachycentridae	Micrasema	*Micrasema sp. AMI 1*
KR144841.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Brachycentridae	Micrasema	*Micrasema sp. BOLD:AAB2409*
HM101977.1	3	3	0	0	0	13	0	0	0	0	13	0	0	0	0	0	12	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Brachycentridae	Micrasema	*Micrasema wataga*
KX143036.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Glossosomatidae	Glossosoma	*Glossosoma privatum*
KX291898.1	0	13	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	0	13	0	0	0	Arthropoda	Insecta	Trichoptera	Goeridae	Silo	*Silo nigricornis*
FM998448.1	0	0	0	0	239	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Hydropsychidae	Cheumatopsyche	*Cheumatopsyche persica*
KF255617.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Hydropsychidae	Hydropsyche	*Hydropsyche dinarica*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
KX294379.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	Arthropoda	Insecta	Trichoptera	Hydropsychidae	Hydropsyche	*Hydropsyche siltalai*
KX144581.1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Hydropsychidae	Hydropsyche	*Hydropsyche sp. BIOUG17466-C09*
KX293932.1	0	0	0	0	0	0	0	0	0	13	1	1	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Hydropsychidae	Hydropsyche	*Hydropsyche sp. WIA1*
KX104980.1	0	0	0	0	0	0	0	0	0	0	0	0	0	31	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Hydroptilidae	Hydroptila	*Hydroptila forcipata*
KX103526.1	0	0	0	0	0	0	0	0	0	0	15	0	0	1	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Hydroptilidae	Hydroptila	*Hydroptila vectis*
KX294360.1	0	2	0	0	0	1	0	0	0	2	0	0	1	12	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Limnephilidae	Halesus	*Halesus radiatus*
KX294127.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	12	0	1	12	0	0	Arthropoda	Insecta	Trichoptera	Limnephilidae	Limnephilus	*Limnephilus lunatus*
KX144783.1	59	34	6	8	3	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Limnephilidae	Potamophylax	*Potamophylax cingulatus*
KX292008.1	0	3	0	2	0	0	0	0	0	138	315	58	1	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Limnephilidae	Potamophylax	*Potamophylax latipennis*
KX142075.1	0	0	0	0	0	10	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Philopotamidae	Philopotamus	*Philopotamus montanus*
KX104260.1	0	0	0	0	0	0	0	0	0	0	11	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Philopotamidae	Philopotamus	*Philopotamus variegatus*
KX295948.1	0	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Polycentropodidae	Polycentropus	*Polycentropus flavomaculatus*
KX142952.1	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Psychomyiidae	Lype	*Lype reducta*
KX104695.1	0	0	0	0	0	0	0	0	0	2	0	1	11	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Psychomyiidae	Psychomyia	*Psychomyia pusilla*
GU667767.1	0	0	0	0	0	0	0	0	0	1	12	1	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Psychomyiidae	Psychomyia	*Psychomyia sp. CJGsp IQ1*
KX294471.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Psychomyiidae	Tinodes	*Tinodes waeneri*
KX296294.1	0	0	0	0	0	0	0	0	0	14	0	0	0	0	0	1	0	0	10	0	0	0	Arthropoda	Insecta	Trichoptera	Rhyacophilidae	Rhyacophila	*Rhyacophila fasciata*
KX144438.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Rhyacophilidae	Rhyacophila	*Rhyacophila meridionalis*
KX293765.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Rhyacophilidae	Rhyacophila	*Rhyacophila occidentalis*
KX140774.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2	0	0	Arthropoda	Insecta	Trichoptera	Rhyacophilidae	Rhyacophila	*Rhyacophila relicta*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
KY225396.1	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Sericostomatidae	Sericostoma	*Sericostoma personatum*
AF436535.1	6	11	2	13	0	6	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	Arthropoda	Insecta	Trichoptera	Sericostomatidae	Sericostoma	*Sericostoma sp. UMSP-Spain*
KC146138.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	Arthropoda	Malacostraca	Amphipoda	Caprellidae	Caprella	*Caprella andreae*
KU603483.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	Arthropoda	Malacostraca	Decapoda	Astacidae	Pacifastacus	*Pacifastacus leniusculus*
KC617188.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	36	0	1	0	0	0	0	Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae	Acanthocyclops	*Acanthocyclops americanus*
KC016184.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	67	0	1	0	0	1	0	Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae	Acanthocyclops	*Acanthocyclops robustus*
KR048975.1	0	0	0	0	0	0	0	3	0	2	0	0	0	0	0	190	0	11	2	1	0	0	Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae	Acanthocyclops	*Acanthocyclops vernalis*
KC627303.1	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Arthropoda	Maxillopoda	Cyclopoida	Cyclopidae	Eucyclops	*Eucyclops cf. serrulatus ZISP 11SNM-506*
GU993588.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1334	Arthropoda	Maxillopoda	Pedunculata	Lepadidae	Lepas	*Lepas anatifera*
GU993650.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	248	Arthropoda	Maxillopoda	Pedunculata	Lepadidae	Lepas	*Lepas pectinata*
KU695270.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	Arthropoda	Maxillopoda	Sessilia	Austrobalanidae	Austrominius	*Austrominius modestus*
EU699232.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	Arthropoda	Maxillopoda	Sessilia	Chthamalidae	Chthamalus	*Chthamalus stellatus*
FJ590523.1	0	10	5	18	6	0	98	12	3	0	0	3	4	5	0	17	11	5	7	8	0	0	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	*Cladosporium bruhnei*
FJ590525.1	7	0	0	0	0	0	0	0	0	15	0	1	9	36	1	52	8	56	4	0	0	0	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	*Cladosporium herbarum*
FJ590524.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	*Cladosporium sp. BM-2009-3*
FJ590526.1	0	0	2	0	1	0	0	2	0	0	0	0	0	1	2	1	0	1	0	0	0	0	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	*Cladosporium sp. BM-2009-5*
FJ590522.1	2	78	17	30	46	0	0	8	58	24	1	8	11	125	9	44	16	29	9	30	2	0	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	Cladosporium	*Cladosporium tenuissimum*
NC_018100.1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Aspergillus	*Aspergillus oryzae*
FJ590520.1	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Aspergillus	*Aspergillus terreus*
EF180133.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium antarcticum*
EF180150.1	0	1	0	1	0	0	0	0	0	0	2	0	0	1	0	2	0	0	0	1	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium aurantiogriseum*
EF180155.1	0	0	8	0	4	0	0	0	0	0	0	0	0	1	1	0	0	0	0	3	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium bialowiezense*
EF180182.1	0	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium chrysogenum*
EF180208.1	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium coprobium*
EF180222.1	0	0	3	0	14	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium crustosum*
HQ850896.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium digitatum*
EF180265.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium formosanum*
EF180275.1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium glabrum*
HQ850895.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium italicum*
EF180101.1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium kewense*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
EF180109.1	0	0	0	0	0	0	2	0	0	0	0	0	0	0	1	3	1	0	3	0	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium osmophilum*
EF180350.1	0	2	0	0	0	0	0	2	3	0	0	1	0	1	0	1	2	0	3	3	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium oxalicum*
EF180368.1	1	2	1	0	1	0	0	0	0	0	0	0	0	1	0	2	1	0	8	1	2	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium polonicum*
EF180380.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium sclerotigenum*
EF180393.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	Penicillium	*Penicillium soppii*
EF180447.1	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	3	0	1	2	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Talaromyces	*Talaromyces flavus*
EF180449.1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	Talaromyces	*Talaromyces trachyspermus*
FJ590558.1	20	126	79	516	289	20	27	9	27	15	11	7	10	133	23	809	351	73	36	73	7	0	Ascomycota	Leotiomycetes	NA	Pseudeurotiaceae	Pseudogymnoascus	*Pseudogymnoascus bhattii*
FJ590560.1	126	309	572	630	602	139	263	123	330	174	83	24	65	171	45	194	249	142	113	226	55	0	Ascomycota	Leotiomycetes	NA	Pseudeurotiaceae	Pseudogymnoascus	*Pseudogymnoascus roseus*
FJ590561.1	14	20	54	312	225	5	97	85	142	4	12	1	3	26	3	38	41	11	14	30	8	0	Ascomycota	Leotiomycetes	NA	Pseudeurotiaceae	Pseudogymnoascus	*Pseudogymnoascus sp. BM-2009-4*
FJ663052.1	2	4	1	5	3	0	0	1	3	0	0	0	0	0	0	4	0	2	0	3	1	0	Ascomycota	NA	NA	NA	Arthrosporium	*Arthrosporium sp. 'hyalospora'*
FJ501248.1	4	13	4	27	19	1	7	5	0	10	8	4	5	24	2	14	7	9	2	8	2	0	Ascomycota	NA	NA	NA	Monocillium	*Monocillium mucidum*
FJ663053.1	1	2	6	2	3	0	30	0	0	0	0	0	0	3	0	0	0	0	2	2	0	0	Ascomycota	Sordariomycetes	Hypocreales	Bionectriaceae	Clonostachys	*Clonostachys compactiuscula*
FJ663054.1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	2	1	0	0	0	0	Ascomycota	Sordariomycetes	Hypocreales	Bionectriaceae	Clonostachys	*Clonostachys rosea*
FJ663051.1	0	0	4	15	0	1	1	1	0	0	0	0	0	2	0	0	0	2	0	4	0	0	Ascomycota	Sordariomycetes	Hypocreales	Bionectriaceae	Gliomastix	*Gliomastix murorum*
FJ501247.1	3	26	18	13	38	0	63	27	25	0	2	0	3	17	1	4	17	9	10	2	0	0	Ascomycota	Sordariomycetes	Hypocreales	Clavicipitaceae	Metarhizium	*Metarhizium anisopliae*
FJ501224.1	3	170	16	305	11	1	0	14	4	1	0	1	1	2	0	2	11	7	7	6	12	0	Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	Beauveria	*Beauveria bassiana*
DQ311640.1	1	0	1	2	0	1	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	Cordyceps	*Cordyceps confragosa*
NC_004514.1	0	0	0	224	0	0	0	0	0	0	0	0	0	1	0	0	0	2	0	0	0	0	Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	Lecanicillium	*Lecanicillium muscarium*
FJ501246.1	0	2	0	1	0	0	0	5	5	0	0	0	0	0	0	0	0	3	0	0	0	0	Ascomycota	Sordariomycetes	Hypocreales	Hypocreaceae	Trichoderma	*Trichoderma reesei*
FJ501222.1	4	26	16	7	2	4	0	60	9	2	0	0	2	4	1	6	3	0	3	3	1	0	Ascomycota	Sordariomycetes	Hypocreales	NA	Acremonium	*Acremonium cf. chrysogenum DAOM 226667*
FJ501225.1	2	19	4	10	9	0	4	0	0	1	1	2	0	1	0	7	11	5	5	3	0	0	Ascomycota	Sordariomycetes	Hypocreales	NA	Emericellopsis	*Emericellopsis minima*
FJ663055.1	5	7	24	26	23	0	0	13	6	6	6	0	0	5	1	21	28	12	13	37	4	0	Ascomycota	Sordariomycetes	Hypocreales	NA	Gliocladium	*Gliocladium viride*
FJ663057.1	0	1	0	0	1	0	0	0	0	0	0	2	0	1	1	0	0	0	1	0	0	0	Ascomycota	Sordariomycetes	Hypocreales	NA	Stilbella	*Stilbella aciculosa*
JN574872.1	26	95	105	334	222	23	71	52	107	44	10	17	21	101	11	50	50	25	20	36	6	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Calonectria	*Calonectria colhounii*
JN574865.1	7	17	7	11	16	1	3	4	2	6	0	5	2	12	1	6	2	3	0	1	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Cosmospora	*Cosmospora gigas*
JN574859.1	7	69	19	23	14	7	0	8	0	1	0	2	2	22	1	5	1	1	3	32	1	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Cosmospora	*Cosmospora meliopsicola*
JN574861.1	3	28	24	43	118	8	0	3	37	2	0	0	2	16	1	6	3	13	1	1	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Cylindrodendrum	*Cylindrodendrum hubeiense*
JN574863.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Dialonectria	*Dialonectria episphaeria*
FJ501229.1	0	0	1	1	0	0	78	2	6	0	0	0	0	0	0	0	0	0	0	2	1	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium boothii*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
FJ501230.1	0	0	1	0	3	0	0	0	2	0	0	0	0	0	0	0	1	0	0	3	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium cf. avenaceum HY046-07*
FJ501231.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium circinatum*
JN574867.1	0	2	0	2	0	3	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium decemcellulare*
FJ501236.1	18	6	5	5	4	13	39	32	15	2	0	0	1	10	1	0	1	14	0	18	5	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium delphinoides*
FJ501239.1	1	0	2	0	0	0	12	1	2	0	0	0	0	1	0	0	0	0	0	1	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium graminearum*
FJ590533.1	2	0	2	4	2	0	67	0	5	0	0	0	0	0	0	0	1	0	0	2	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium oxysporum*
FJ590532.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium proliferatum*
FJ501244.1	0	19	0	0	10	0	1	0	5	0	0	0	0	0	0	0	0	1	0	3	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium solani*
FJ590530.1	0	0	0	0	2	0	0	0	1	0	0	0	0	0	0	0	2	0	0	0	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium	*Fusarium tricinctum*
FJ501249.1	1	5	4	4	9	0	66	1	0	0	0	3	1	0	0	3	1	4	1	6	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Neonectria	*Neonectria ditissima*
JN574871.1	0	0	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Pseudocosmospora	*Pseudocosmospora vilior*
JN574874.1	11	59	26	25	27	10	2	12	13	8	0	0	6	17	1	0	1	3	0	12	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Thelonectria	*Thelonectria discophora*
JN574862.1	27	94	53	133	57	39	0	3	47	3	0	0	2	21	0	18	5	22	8	0	3	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Thelonectria	*Thelonectria veuillotiana*
JN574873.1	0	6	2	2	9	0	0	1	0	1	0	0	5	4	2	0	1	2	0	0	0	0	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Volutella	*Volutella consors*
X14669.1	0	0	3	2	2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	3	0	0	Ascomycota	Sordariomycetes	Sordariales	Sordariaceae	Neurospora	*Neurospora crassa*
HQ317080.1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	Bacillariophyta	Bacillariophyceae	Bacillariales	Bacillariaceae	Fragilariopsis	*Fragilariopsis curta*
HQ317085.1	0	0	1	0	0	0	0	0	0	0	0	0	2	12	2	28	15	8	18	58	12	0	Bacillariophyta	Bacillariophyceae	Bacillariales	Bacillariaceae	Nitzschia	*Nitzschia sp. BOLD:AAO7110*
HQ317088.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	1	0	0	0	Bacillariophyta	Bacillariophyceae	Bacillariales	Bacillariaceae	Pseudo-nitzschia	*Pseudo-nitzschia subcurvata*
GQ844260.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	3	0	0	Bacillariophyta	Bacillariophyceae	NA	Entomoneidaceae	Entomoneis	*Entomoneis cf. alata*
HF562256.1	0	0	0	0	0	0	0	0	3	15	4	2	0	9	0	0	6	0	3	0	0	0	Bacillariophyta	Bacillariophyceae	Naviculales	Amphipleuraceae	Frustulia	*Frustulia vulgaris*
HQ317074.1	32	69	28	13	12	48	0	0	0	96	16	41	26	96	33	17	11	4	23	82	11	0	Bacillariophyta	Bacillariophyceae	Naviculales	Naviculaceae	Fistulifera	*Fistulifera pelliculosa*
HQ317071.1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Bacillariophyta	Bacillariophyceae	Naviculales	Naviculaceae	Navicula	*Navicula cryptocephala*
EF164938.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	3	0	34	0	0	Bacillariophyta	Bacillariophyceae	Naviculales	Pinnulariaceae	Pinnularia	*Pinnularia cf. gibba*
EF164941.2	0	2	0	0	1	0	6	0	4	2	1	1	0	3	1	3	0	1	1	2	0	0	Bacillariophyta	Bacillariophyceae	Naviculales	Sellaphoraceae	Sellaphora	*Sellaphora bacillum*
EF164929.1	33	68	67	192	106	33	0	0	0	201	65	81	20	365	70	104	33	125	26	89	20	0	Bacillariophyta	Bacillariophyceae	Naviculales	Sellaphoraceae	Sellaphora	*Sellaphora cf. minima*
EF164956.1	0	0	0	1	0	0	0	0	1	0	0	0	0	0	1	2	2	2	1	3	0	0	Bacillariophyta	Bacillariophyceae	Naviculales	Sellaphoraceae	Sellaphora	*Sellaphora cf. seminulum*
EF164953.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2	0	0	Bacillariophyta	Bacillariophyceae	Naviculales	Sellaphoraceae	Sellaphora	*Sellaphora lanceolata*
HQ317090.1	0	0	0	0	0	0	5	0	0	0	0	1	0	2	0	0	2	0	0	0	0	0	Bacillariophyta	Bacillariophyceae	Naviculales	Sellaphoraceae	Sellaphora	*Sellaphora obesa*
HQ317100.1	0	5	1	2	0	0	69	0	7	0	0	0	0	3	0	2	0	6	2	2	1	0	Bacillariophyta	Bacillariophyceae	Naviculales	Sellaphoraceae	Sellaphora	*Sellaphora pupula*
KM202108.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Bacillariophyta	Coscinodiscophyceae	Chaetocerotales	Chaetocerotaceae	Chaetoceros	*Chaetoceros sp. MBTD-CMFRI-S005*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
GQ844265.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	2	0	0	Bacillariophyta	Coscinodiscophyceae	Melosirales	Hyalodiscaceae	Podosira	*Podosira stelligera*
KJ671746.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	Bacillariophyta	Coscinodiscophyceae	Melosirales	Stephanopyxidaceae	Stephanopyxis	*Stephanopyxis turris*
KM202114.1	0	0	0	0	0	0	0	0	0	10	5	1	0	2	0	73	82	16	100	290	33	0	Bacillariophyta	Coscinodiscophyceae	Thalassiosirales	Skeletonemataceae	Skeletonema	*Skeletonema ardens*
KJ671745.1	0	0	0	0	0	0	0	0	0	33	52	10	4	11	3	670	923	126	1258	2699	351	0	Bacillariophyta	Coscinodiscophyceae	Thalassiosirales	Skeletonemataceae	Skeletonema	*Skeletonema tropicum*
GQ844251.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	11	17	4	2	5	2	0	Bacillariophyta	Coscinodiscophyceae	Thalassiosirales	Stephanodiscaceae	Cyclotella	*Cyclotella cryptica*
GQ844277.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	Bacillariophyta	Coscinodiscophyceae	Thalassiosirales	Thalassiosiraceae	Thalassiosira	*Thalassiosira weissflogii*
KJ671735.1	0	0	0	0	0	0	2	0	1	4	6	2	0	2	2	5	10	0	3	6	1	0	Bacillariophyta	Mediophyceae	Lithodesmiales	Lithodesmiaceae	Lithodesmium	*Lithodesmium variabile*
JN029464.1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Agaricaceae	Lacrymaria	*Lacrymaria lacrymabunda*
JN029470.1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Agaricaceae	Lepiota	*Lepiota sp. MUSH114-07*
JN029421.1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Bolbitiaceae	Conocybe	*Conocybe sp. TRTC156993*
JN029425.1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	Cortinarius	*Cortinarius cf. violaceus TRTC155606*
JN029422.1	0	0	0	6	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	2	0	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	Cortinarius	*Cortinarius sp. TRTC156946*
JN029439.1	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Cortinariaceae	Hebeloma	*Hebeloma crustuliniforme*
JN029428.1	0	0	0	5	0	0	0	21	0	0	0	3	0	0	0	6	0	0	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Entolomataceae	Entoloma	*Entoloma sp. TRTC150975*
JN029442.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Hygrophoraceae	Hygrocybe	*Hygrocybe conica*
EU593189.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Pleurotaceae	Pleurotus	*Pleurotus purpureo-olivaceus*
JN029486.1	0	0	0	15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Pluteaceae	Pluteus	*Pluteus cervinus*
JN029483.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	Basidiomycota	Agaricomycetes	Agaricales	Pluteaceae	Pluteus	*Pluteus sp. YYY002-10*
JN029498.1	0	0	0	1	0	0	2	0	0	0	0	0	0	0	0	3	5	1	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Psathyrella	*Psathyrella aff. gracilis TRTC155531*
JN029500.1	0	3	0	0	0	0	0	0	0	1	3	4	0	0	0	1	2	0	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Psathyrella	*Psathyrella candolleana*
JN029501.1	2	5	0	0	2	0	77	0	2	0	0	0	1	5	0	0	1	2	0	3	0	0	Basidiomycota	Agaricomycetes	Agaricales	Psathyrellaceae	Psathyrella	*Psathyrella cf. hydrophila TRTC155552*
JN029414.1	1	0	2	28	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Clitocybe	*Clitocybe robusta*
JN029415.1	0	0	0	0	0	0	0	0	17	0	0	0	0	0	0	0	0	0	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Clitocybe	*Clitocybe subditopoda*
JN029420.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Collybia	*Collybia tuberosa*
JN029462.1	1	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	8	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Laccaria	*Laccaria laccata*
JN029472.1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	Lepista	*Lepista flaccida*
JN029372.1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Basidiomycota	Agaricomycetes	Boletales	Boletaceae	Xerocomus	*Xerocomus badius*
JN029478.1	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Basidiomycota	Agaricomycetes	Boletales	Paxillaceae	Paxillus	*Paxillus involutus*
JN029437.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Basidiomycota	Agaricomycetes	Polyporales	Ganodermataceae	Ganoderma	*Ganoderma resinaceum*
GQ501094.1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	5	0	0	Basidiomycota	Pucciniomycetes	Pucciniales	Melampsoraceae	Melampsora	*Melampsora magnusiana*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
KJ554128.1	0	0	1	0	0	0	0	0	74	25	26	10	13	7	1	143	48	6	10	12	3	0	Chordata	Actinopteri	Cypriniformes	Cyprinidae	Phoxinus	*Phoxinus bigerri*
KM286916.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	Chordata	Actinopteri	Cypriniformes	Cyprinidae	Phoxinus	*Phoxinus phoxinus*
KJ554392.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	Chordata	Actinopteri	Cypriniformes	Cyprinidae	Phoxinus	*Phoxinus sp. BOLD:AAY8725*
KP218514.1	11	18	0	0	0	17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	Chordata	Actinopteri	Salmoniformes	Salmonidae	NA	*Oncorhynchus mykiss x Salmo salar*
FJ999067.1	2587	4879	0	0	0	5038	0	6	0	0	0	0	0	0	0	21	11	0	6	6	2	17	Chordata	Actinopteri	Salmoniformes	Salmonidae	Oncorhynchus	*Oncorhynchus mykiss*
KJ554871.1	1	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	183	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	*Salmo carpio*
KX241518.1	19	11	7	6	7	8	0	16	0	2	4	4	5	1	0	0	2	1	3	0	0	2360	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	*Salmo cettii*
KR477109.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	*Salmo labrax*
KJ554701.1	1	3	3	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	182	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	*Salmo letnica*
KJ554725.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	17	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	*Salmo marmoratus*
KJ554617.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	*Salmo obtusirostris*
KJ554684.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	15	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	*Salmo opimus*
KJ554776.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	*Salmo pellegrini*
FJ999484.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	*Salmo salar*
KJ554931.1	1	0	0	0	0	2	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	19	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	*Salmo sp. BOLD:AAB3872*
KU933676.1	3	3	0	1	3	1	0	4	0	0	0	0	0	1	0	0	0	0	0	0	0	562	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	*Salmo trutta*
KJ554629.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	Chordata	Actinopteri	Salmoniformes	Salmonidae	Salmo	*Salmo visovacensis*
AP003323.1	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Chordata	Aves	Galliformes	Phasianidae	Gallus	*Gallus gallus*
GU571402.1	0	0	0	0	23	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Chordata	Aves	Passeriformes	Fringillidae	Fringilla	*Fringilla coelebs*
JF498807.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	Chordata	Aves	Passeriformes	Passeridae	Passer	*Passer domesticus*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
JF499095.1	0	0	0	0	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Chordata	Aves	Pelecaniformes	Ardeidae	Ardea	*Ardea cinerea*
KF385966.1	28	36	0	0	0	24	0	0	0	0	0	0	0	0	0	1	1	0	4	0	0	0	Chordata	Mammalia	NA	Bovidae	Bos	*Bos taurus*
KF385963.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	Chordata	Mammalia	NA	Bovidae	Capra	*Capra aegagrus*
KJ205545.1	0	0	0	0	0	6	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	0	Chordata	Mammalia	NA	Cervidae	Cervus	*Cervus elaphus*
GU130593.1	0	1	0	0	14	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Chordata	Mammalia	NA	Suidae	Sus	*Sus scrofa*
AP008737.1	5	16	35	10	18	3	283	33	9	30	2	15	0	1	1	9	11	3	5	37	3	0	Chordata	Mammalia	Primates	Hominidae	Homo	*Homo sapiens*
JQ935785.1	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Chordata	Mammalia	Rodentia	Muridae	Apodemus	*Apodemus flavicollis*
KC709676.1	0	0	0	0	0	0	0	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Chordata	Mammalia	Rodentia	Muridae	Mus	*Mus musculus*
EF186576.1	0	0	0	0	0	0	0	0	0	0	12	0	0	0	0	0	0	0	0	0	0	0	Chordata	Mammalia	Rodentia	Muridae	Rattus	*Rattus norvegicus*
GU722851.1	1	1	0	0	0	12	0	0	0	0	11	0	0	0	0	1	0	0	0	0	0	0	Cnidaria	Hydrozoa	Anthoathecata	Hydridae	Hydra	*Hydra circumcincta*
KP895118.1	0	0	0	0	0	0	0	0	0	0	1	0	14	0	0	17	12	0	18	13	8	0	Cnidaria	Hydrozoa	Anthoathecata	Hydridae	Hydra	*Hydra oligactis*
AB565092.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	Cnidaria	Hydrozoa	Anthoathecata	Hydridae	Hydra	*Hydra robusta*
KT981921.1	0	0	12	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Cnidaria	Hydrozoa	Anthoathecata	Hydridae	Hydra	*Hydra viridissima*
EF540795.1	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Cnidaria	Hydrozoa	Anthoathecata	Oceaniidae	Turritopsis	*Turritopsis rubra*
AY530415.1	0	0	0	0	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	3	0	0	Cnidaria	Hydrozoa	Leptothecata	Campanulariidae	Obelia	*Obelia geniculata*
KF962167.1	0	0	0	0	1	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	Cnidaria	Hydrozoa	Leptothecata	Campanulariidae	Obelia	*Obelia sp. JRH-2014*
KF982160.1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Cnidaria	Hydrozoa	Leptothecata	Plumulariidae	Plumularia	*Plumularia setacea*
FJ423620.1	44	20	305	345	381	26	395	53	77	6920	1442	145	38	1353	935	122	87	27	58	62	37	0	Cnidaria	Hydrozoa	Limnomedusae	Olindiidae	Craspedacusta	*Craspedacusta sowerbyi*
GQ119954.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Cnidaria	Hydrozoa	Siphonophorae	Apolemiidae	Apolemia	*Apolemia sp. BO-2009*
AY937375.1	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Cnidaria	Hydrozoa	Siphonophorae	Prayidae	Praya	*Praya dubia*
FJ602530.1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	Cnidaria	Hydrozoa	Trachymedusae	Halicreatidae	Botrynema	*Botrynema brucei*
GQ120077.1	1	0	0	0	0	3	0	0	0	1	0	0	0	5	0	0	0	0	0	1	0	0	Cnidaria	Hydrozoa	Trachymedusae	Rhopalonematidae	Colobonema	*Colobonema sericeum*
CP001878.2	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	3	0	0	0	0	0	2	Firmicutes	Bacilli	Bacillales	Bacillaceae	Bacillus	*Bacillus pseudofirmus*
KU705230.1	0	0	0	16	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Gastrotricha	NA	Chaetonotida	Chaetonotidae	Chaetonotus	*Chaetonotus (Chaetonotus) sp. MD-2016*
JQ798693.1	0	0	2	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Gastrotricha	NA	Chaetonotida	Chaetonotidae	Chaetonotus	*Chaetonotus aemilianus*
JQ798709.1	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	1	0	Gastrotricha	NA	Chaetonotida	Chaetonotidae	Chaetonotus	*Chaetonotus cf. maximus TK191*
JQ798685.1	0	0	0	0	0	0	0	0	4	0	0	0	0	9	0	0	0	0	0	0	5	0	Gastrotricha	NA	Chaetonotida	Chaetonotidae	Chaetonotus	*Chaetonotus heideri*
JQ798710.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	63	15	0	0	0	0	0	Gastrotricha	NA	Chaetonotida	Chaetonotidae	Chaetonotus	*Chaetonotus similis*
JQ798730.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	Gastrotricha	NA	Chaetonotida	Chaetonotidae	Chaetonotus	*Chaetonotus sp. 1 TK-2012*
JQ798736.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Gastrotricha	NA	Chaetonotida	Chaetonotidae	Ichthydium	*Ichthydium squamigerum*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
KC353395.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Mollusca	Cephalopoda	Teuthida	Ommastrephidae	Dosidicus	*Dosidicus gigas*
AY044372.1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Mollusca	Gastropoda	NA	Achatinellidae	Achatinella	*Achatinella mustelina*
KF894287.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Mollusca	Gastropoda	NA	Agriolimacidae	Deroceras	*Deroceras reticulatum*
KP976443.1	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Mollusca	Gastropoda	NA	Agriolimacidae	Deroceras	*Deroceras sp. BOLD:AAI9663*
AY350517.1	5	6	28	12	9	5	0	0	0	50	47	5	30	171	28	0	1	0	5	11	6	0	Mollusca	Gastropoda	NA	Ancylidae	Ancylus	*Ancylus sp. H34*
AY987891.1	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Mollusca	Gastropoda	NA	Arionidae	Arion	*Arion intermedius*
KJ842875.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Mollusca	Gastropoda	NA	Arionidae	Arion	*Arion sp. 1509_4*
FJ160291.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	14	0	0	0	0	0	Mollusca	Gastropoda	NA	Bithyniidae	Bithynia	*Bithynia tentaculata*
KC955004.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Mollusca	Gastropoda	NA	Helicidae	Cepaea	*Cepaea nemoralis*
JX081824.1	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	0	0	0	0	Mollusca	Gastropoda	NA	Hydrobiidae	Corrosella	*Corrosella falkneri*
AF213345.1	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	Mollusca	Gastropoda	NA	Hydrobiidae	Horatia	*Horatia sturmi*
KT373703.1	13	0	0	0	0	0	0	0	0	1264	508	136	34	146	25	3	3	4	12	0	12	0	Mollusca	Gastropoda	NA	Hydrobiidae	Potamopyrgus	*Potamopyrgus antipodarum*
KP242623.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	Mollusca	Gastropoda	NA	Lymnaeidae	Galba	*Galba truncatula*
KP242321.1	0	0	0	0	0	0	0	0	0	49	28	0	1	0	0	0	0	0	0	0	0	0	Mollusca	Gastropoda	NA	Lymnaeidae	Radix	*Radix auricularia*
KP242451.1	1	0	0	1	0	1	0	0	0	26	23	7	1	3	0	3	0	0	0	0	0	0	Mollusca	Gastropoda	NA	Lymnaeidae	Radix	*Radix balthica*
AY771285.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	Mollusca	Gastropoda	NA	Neritidae	Theodoxus	*Theodoxus fluviatilis*
KF737952.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12	0	0	0	Mollusca	Gastropoda	NA	Physidae	Physella	*Physella acuta*
KT707824.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	Mollusca	Gastropoda	NA	Succineidae	NA	*Succineidae sp. BOLD:ACI9370*
FJ590550.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	Mucoromycota	NA	Mortierellales	Mortierellaceae	Mortierella	*Mortierella hyalina*
FJ590555.1	22	22	0	0	0	10	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	Mucoromycota	NA	Mucorales	Mucoraceae	Mucor	*Mucor circinelloides*
FJ590552.1	0	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	Mucoromycota	NA	Mucorales	Mucoraceae	Mucor	*Mucor hiemalis*
FJ590553.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	Mucoromycota	NA	Mucorales	Mucoraceae	Mucor	*Mucor sp. BM-2009-2*
FJ590551.1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	0	1	2	1	1	1	1	0	Mucoromycota	NA	Umbelopsidales	Umbelopsidaceae	Umbelopsis	*Umbelopsis ramanniana*
FN663974.1	0	0	0	0	0	0	0	0	0	1	0	0	0	2	0	0	0	1	3	0	0	0	NA	Chrysophyceae	Chromulinales	Dinobryaceae	Dinobryon	*Dinobryon divergens*
FN663989.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	3	0	0	NA	Chrysophyceae	Chromulinales	Dinobryaceae	Poteriospumella	*Poteriospumella lacustris*
FJ905159.1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Colpodea	Colpodida	Colpodidae	Colpoda	*Colpoda sp. CBOLN070-08*
JQ247715.1	0	2	2	1	1	1	0	0	2	0	0	1	2	3	0	0	1	0	0	1	0	0	NA	Dinophyceae	Gonyaulacales	Amphidomataceae	Amphidoma	*Amphidoma languida*
GQ501214.1	2	3	6	0	4	0	0	0	8	2	4	2	0	6	1	0	0	0	0	0	1	0	NA	Dinophyceae	Gonyaulacales	Goniodomataceae	Gambierdiscus	*Gambierdiscus toxicus*
GQ501616.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	NA	Dinophyceae	Gonyaulacales	Gonyaulacaceae	Adenoides	*Adenoides eludens*
GQ501119.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3	1	0	1	0	0	NA	Dinophyceae	Gonyaulacales	Gonyaulacaceae	Alexandrium	*Alexandrium affine*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
GQ501856.1	0	0	0	0	0	0	0	0	3	0	2	0	1	0	0	1	0	0	0	0	0	0	NA	Dinophyceae	Gonyaulacales	Gonyaulacaceae	Gonyaulax	*Gonyaulax sp. PL9-25*
GQ501301.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	NA	Dinophyceae	Gonyaulacales	Gonyaulacaceae	Protoceratium	*Protoceratium reticulatum*
GQ502111.1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	NA	Dinophyceae	Lophodiniales	Lophodiniaceae	Woloszynskia	*Woloszynskia sp. ES1-2*
GQ501756.1	0	1	5	1	1	0	0	0	4	0	3	1	0	2	0	2	0	1	0	1	0	0	NA	Dinophyceae	NA	NA	NA	*uncultured dinoflagellate*
GQ501186.1	1	1	4	4	4	0	0	0	6	3	0	0	0	2	2	0	2	0	0	0	0	0	NA	Dinophyceae	Peridiniales	Ostreopsidaceae	Coolia	*Coolia monotis*
GQ501436.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	0	0	1	0	0	NA	Dinophyceae	Peridiniales	Oxytoxaceae	Thecadinium	*uncultured Thecadinium*
GQ501269.1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	2	1	0	0	2	0	0	NA	Dinophyceae	Peridiniales	Peridiniaceae	Peridinium	*Peridinium inconspicuum*
GQ501194.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	NA	Dinophyceae	Peridiniales	Pfiesteriaceae	Cryptoperidiniopsis	*Cryptoperidiniopsis sp. CCMP2786*
GQ501198.1	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	8	5	0	2	1	0	0	NA	Dinophyceae	Peridiniales	Pfiesteriaceae	NA	*Pfiesteriaceae sp. CCMP1874*
GQ501400.1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	4	5	2	0	10	0	0	NA	Dinophyceae	Peridiniales	Thoracosphaeraceae	Thoracosphaera	*Thoracosphaera heimii*
GQ501297.1	0	1	0	3	0	1	0	0	2	2	1	0	4	1	2	0	0	0	0	0	0	0	NA	Dinophyceae	Prorocentrales	Prorocentraceae	Prorocentrum	*Prorocentrum minimum*
GQ501306.1	0	0	0	1	0	0	0	0	0	0	1	1	0	0	0	0	1	0	0	0	0	0	NA	Dinophyceae	Pyrocystales	Pyrocystaceae	Pyrocystis	*Pyrocystis lunula*
KC130152.1	7	1	0	14	9	2	0	0	0	2	10	2	0	0	0	7	4	0	1	9	19	0	NA	Florideophyceae	Acrochaetiales	Acrochaetiaceae	Audouinella	*Audouinella hermannii*
EU073843.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	NA	Florideophyceae	Batrachospermales	Batrachospermaceae	Batrachospermum	*Batrachospermum helminthosum*
KC596294.1	27	103	16	17	55	52	0	0	11	88	120	46	0	3	1	9	7	1	8	10	4	0	NA	Florideophyceae	Batrachospermales	Batrachospermaceae	Sheathia	*Sheathia arcuata*
JX669710.1	1	0	1	0	0	1	0	0	0	5	6	0	0	0	0	1	0	0	0	0	0	0	NA	Florideophyceae	Batrachospermales	Batrachospermaceae	Sheathia	*Sheathia boryana*
JN604926.1	18	26	16	7	33	25	13	10	18	10	36	1	6	7	0	18	36	5	76	133	23	0	NA	Florideophyceae	Batrachospermales	Lemaneaceae	Paralemanea	*Paralemanea annulata*
KR090577.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	NA	Florideophyceae	Ceramiales	Rhodomelaceae	Neosiphonia	*Neosiphonia paniculata*
KX506059.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	NA	Heterolobosea	Schizopyrenida	Vahlkampfiidae	Naegleria	*Naegleria sp. 7 CF-2016*
HM187654.1	1	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	1	0	0	0	NA	NA	Euglyphida	Cyphoderiidae	Cyphoderia	*Cyphoderia ampulla*
KJ781464.1	3	1	0	4	0	0	0	0	0	2	3	1	6	34	5	4	2	0	1	0	0	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium actinophorum*
KJ781461.1	0	0	0	1	0	0	0	0	1	7	2	4	1	34	3	3	0	0	0	2	0	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium arabianum*
GQ354207.1	0	0	0	0	0	0	0	0	0	0	1	0	0	12	1	0	0	0	0	0	0	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium cf. actinophorum*
KJ569728.1	5	21	0	2	1	0	28	0	0	0	8	2	2	34	3	0	0	2	0	0	0	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium kieliense*
KJ781466.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium larifeili*
KJ569707.1	16	84	2	4	8	13	9	0	0	11	5	8	22	202	23	8	10	4	10	36	4	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium minus*
KJ781453.1	25	98	0	4	0	17	52	1	2	23	15	15	36	369	59	22	15	7	22	57	20	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium minutoidum*
KJ569731.1	6	11	0	3	1	2	2	0	2	4	0	2	13	63	2	5	5	1	10	17	4	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium plurinucleolum*
KJ569724.1	20	22	0	2	6	0	0	0	1	9	3	5	8	64	6	2	1	4	4	20	2	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium sp. SG-2014*
KJ781457.1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	0	1	0	0	0	NA	NA	Himatismenida	Cochliopodiidae	Cochliopodium	*Cochliopodium spiniferum*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
DAAX01000003.1	1	0	1	6	1	1	0	0	0	4	0	1	1	5	0	2	2	0	1	4	0	0	NA	NA	NA	NA	NA	*NA*
KU659850.1	0	3	15	0	0	1	0	0	0	155	241	115	9	19	5	8	0	0	21	0	1	0	NA	NA	NA	Paramoebidae	Korotnevella	*Korotnevella heteracantha*
KU659820.1	79	124	12	16	18	28	29	0	55	9	11	4	11	123	47	63	46	44	90	96	15	0	NA	NA	NA	Paramoebidae	Korotnevella	*Korotnevella stella*
GQ354144.1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	NA	NA	NA	Vannellidae	Vannella	*Vannella persistens*
GQ354155.1	1	2	0	0	0	3	0	0	0	2	0	0	0	33	2	0	0	2	0	3	0	0	NA	NA	NA	Vannellidae	Vannella	*Vannella simplex*
KJ854448.1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	5	9	2	0	0	0	0	NA	NA	Physariida	Didymiaceae	Didymium	*Didymium minus*
AF239224.1	0	0	0	0	0	0	0	1	5	0	0	0	0	0	0	0	2	1	0	0	1	0	NA	NA	Physariida	Didymiaceae	Didymium	*Didymium nigripes*
AF239222.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	NA	NA	Stemonitida	Stemonitidae	Stemonitis	*Stemonitis flavogenita*
KC741457.1	1	1	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	NA	Oomycetes	Lagenidiales	Lagenidiaceae	Lagenidium	*Lagenidium aff. deciduum strain LEV5864*
HQ708209.1	0	2	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	NA	Oomycetes	Lagenidiales	Lagenidiaceae	Lagenidium	*Lagenidium caudatum*
KC741454.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	NA	Oomycetes	Lagenidiales	Lagenidiaceae	Lagenidium	*Lagenidium deciduum*
KF913690.1	0	1	0	0	1	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	NA	Oomycetes	Lagenidiales	Lagenidiaceae	Lagenidium	*Lagenidium giganteum*
KC741445.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	NA	Oomycetes	Lagenidiales	Lagenidiaceae	Lagenidium	*Lagenidium humanum*
KC741456.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	NA	Oomycetes	Lagenidiales	Lagenidiaceae	Lagenidium	*Lagenidium sp. strain LEV5863*
KC741450.1	0	0	14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Lagenidiales	NA	Paralagenidium	*Paralagenidium sp. C06-TW60*
HQ708199.1	0	0	2	0	4	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Leptomitales	NA	Apodachlya	*Apodachlya minima*
HQ261239.1	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora aff. primulae*
HQ261242.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora alni*
HQ708221.1	0	0	0	0	0	0	1	6	0	1	1	1	0	1	0	1	0	0	0	1	1	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora boehmeriae*
HQ261255.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora botryosa*
HQ708234.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora cactorum*
HQ708245.1	1	11	0	0	0	1	22	0	0	0	0	0	2	0	0	39	179	23	21	35	3	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora cambivora*
HQ708259.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora cinnamomi*
JN605978.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora citrophthora*
HQ261285.1	0	0	0	2	2	0	3	0	0	0	1	0	0	1	0	0	1	1	1	1	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora clandestina*
GQ847767.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora elongata*
HQ261303.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora europaea*
HQ261313.1	0	3	0	1	1	2	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora frigida*
HQ708296.1	2	10	2	4	39	4	0	0	0	0	0	0	0	0	0	0	2	0	3	5	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora gonapodyides*
HQ261321.1	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	2	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora heveae*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
HQ261325.1	6	9	0	1	0	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora humicola*
HQ708309.1	4	6	8	9	7	1	3	0	0	1	3	2	0	2	0	0	1	0	0	3	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora infestans*
HQ708311.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora inundata*
HQ261342.1	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora ipomoeae*
HQ261346.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora katsurae*
KP749417.1	2	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	7	0	1	5	2	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora lacustris*
HM534976.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora macrochlamydospora*
HQ261359.1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora megasperma*
HQ261366.1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora mengei*
HQ708337.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora mirabilis*
HQ261371.1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora multivesiculata*
HQ708357.1	0	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora palmivora*
HM535007.1	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora parsiana*
HM534980.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora polonica*
HQ708387.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora ramorum*
HQ261412.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora rubi*
KT886048.1	4	5	0	0	0	2	11	2	1	2	15	0	0	1	0	6	12	5	9	18	5	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora sp. GHJ-2016a*
HQ261458.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	4	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora sulawesiensis*
HQ708413.1	0	0	0	0	0	0	17	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora tentaculata*
HQ708417.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora tropicalis*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
HQ261469.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora uliginosa*
HQ708988.1	0	3	3	1	0	1	0	0	0	0	7	0	0	0	0	0	1	0	0	4	0	0	NA	Oomycetes	Peronosporales	NA	Phytophthora	*Phytophthora undulata*
KF853240.1	0	0	0	0	0	4	0	0	0	5	26	1	0	0	0	1	1	0	1	0	2	0	NA	Oomycetes	Peronosporales	NA	Phytopythium	*Phytopythium delawarense*
HQ708438.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	0	4	0	0	NA	Oomycetes	Peronosporales	NA	Phytopythium	*Phytopythium montanum*
HQ708443.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	NA	Phytopythium	*Phytopythium sindhum*
HM033192.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Peronosporales	Peronosporaceae	Peronospora	*Peronospora valerianellae*
HQ171167.1	0	4	0	4	0	0	0	5	0	0	0	2	0	0	0	3	2	0	3	1	0	0	NA	Oomycetes	Peronosporales	Salisapiliaceae	Salisapilia	*Salisapilia tartarea*
JN660054.1	6	19	2	2	0	16	0	0	4	10	11	2	3	33	16	15	10	5	4	13	0	0	NA	Oomycetes	Pythiales	NA	NA	*uncultured Pythium*
HQ708219.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Halophytophthora	*Halophytophthora avicenniae*
HQ708285.1	1	0	0	1	2	1	0	0	0	0	0	0	0	0	0	1	1	1	0	2	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Halophytophthora	*Halophytophthora epistomium*
HQ708205.1	0	0	0	0	0	0	3	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Halophytophthora	*Halophytophthora exoprolifera*
HQ708455.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium abappressorium*
HQ708458.1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium acanthicum*
HQ708460.1	0	0	1	0	0	0	0	3	1	0	0	0	0	0	0	0	3	0	0	2	1	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium acanthophoron*
HQ708462.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium adhaerens*
KT692793.1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	1	0	0	1	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium aff. acanthophoron JEB-2016*
KT692881.1	0	0	0	0	0	0	14	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium aff. intermedium JEB-2016*
HQ708483.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium anandrum*
HQ708489.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium aphanidermatum*
HQ708491.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium apleroticum*
HQ708492.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium aquatile*
HQ708520.1	0	1	0	0	0	1	1	0	0	0	0	0	0	0	0	3	8	0	3	5	2	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium attrantheridium*
FR774197.1	1	10	0	6	7	1	1	0	0	0	1	0	0	0	0	3	1	1	1	4	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium baisense*
KJ995591.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium biforme*
KJ995594.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium brachiatum*
FR774196.1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium breve*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
HQ708546.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium coloratum*
HQ708560.1	0	0	0	0	0	1	0	0	0	0	1	0	1	5	0	95	57	10	23	84	22	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium contiguanum*
GU071824.1	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium cylindrosporum*
HQ708573.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium dissimile*
EU350526.1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium echinulatum*
HQ708580.1	104	183	4	4	1	126	0	10	0	1	0	0	3	4	1	1	4	2	2	6	1	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium flevoense*
HQ708594.1	0	0	0	0	0	2	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium helicandrum*
KT692896.1	2	9	0	1	0	4	0	15	0	2	0	0	0	4	0	10	10	1	4	8	1	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium heterothallicum*
HQ708610.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium inflatum*
HQ708611.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium insidiosum*
HQ708712.1	2	5	0	0	0	2	0	0	0	0	0	0	0	0	0	0	2	0	1	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium irregulare*
HQ708713.1	1	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium iwayamai*
KJ995598.1	1	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium junctum*
KX387367.1	0	0	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium kashmirense*
HQ708730.1	1	1	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium macrosporum*
EU350523.1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium mastophorum*
HQ708738.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium middletonii*
HQ708741.1	16	31	1	2	0	22	7	0	0	0	0	0	2	7	3	5	10	3	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium monospermum*
KF761203.1	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium myriotylum*
KT692768.1	0	2	0	1	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium nodosum*
KF761143.1	0	1	0	0	0	0	2	2	0	1	1	0	0	2	0	1	0	0	0	2	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium oligandrum*
HQ708766.1	1	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	2	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium pachycaule*
HQ708773.1	75	105	0	0	0	118	0	0	0	0	0	0	0	2	0	0	3	0	0	2	1	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium paroecandrum*
HQ708787.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium phragmitis*
KT692814.1	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium pleroticum*
HQ708791.1	1	0	1	0	0	7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium plurisporium*
KX527563.1	0	0	0	0	0	0	0	0	0	0	2	0	1	1	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium porphyrae*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
HQ708797.1	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium radiosum*
HQ708805.1	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium rostratifingens*
HQ708812.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium scleroteichum*
HQ708813.1	0	1	0	0	0	1	0	4	0	0	0	0	0	0	0	1	0	0	0	0	1	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium segnitium*
HQ708814.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium senticosum*
KX266871.1	3	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium sp.*
HQ261487.1	15	29	1	0	0	32	6	2	2	0	5	0	0	0	0	8	8	0	3	24	3	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium sp. AL-2010*
HQ708835.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	3	1	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium spinosum*
HQ708877.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium sukuiense*
HQ708880.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium sulcatum*
KF761145.1	11	17	0	0	0	19	3	3	0	3	3	0	0	1	0	5	4	0	1	17	1	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium ultimum*
KJ995588.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium utonaiense*
HQ708991.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium vanterpoolii*
HE797904.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	NA	Oomycetes	Pythiales	Pythiaceae	Pythium	*Pythium viniferum*
HQ708452.1	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Pythiales	Pythiogetonaceae	Pythiogeton	*Pythiogeton zeae*
HQ708159.1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Achlya	*Achlya bisexualis*
KR264880.1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Achlya	*Achlya catenulata*
HQ708165.1	0	8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Achlya	*Achlya conspicua*
HQ708169.1	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Achlya	*Achlya flagellata*
HQ708173.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Achlya	*Achlya heterosexualis*
HQ708174.1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	6	0	4	5	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Achlya	*Achlya oligacantha*
HQ708178.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Achlya	*Achlya racemosa*
HQ708179.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Achlya	*Achlya radiosa*
KM888089.1	0	0	1	0	0	0	0	0	0	0	0	0	1	6	0	0	0	0	0	1	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Achlya	*Achlya recurva*
HQ708181.1	43	68	0	0	0	39	0	0	0	0	1	0	0	0	0	2	1	2	9	19	2	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Achlya	*Achlya sparrowii*
HQ708185.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Aphanomyces	*Aphanomyces cladogamus*
HQ708194.1	1	2	0	0	0	2	0	0	0	0	0	0	0	4	0	2	3	0	2	1	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Aphanomyces	*Aphanomyces iridis*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
HQ708195.1	2	0	0	0	0	1	0	0	0	2	2	0	0	3	0	0	1	0	1	2	1	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Aphanomyces	*Aphanomyces laevis*
HQ708201.1	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Brevilegnia	*Brevilegnia macrospora*
HQ708211.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Leptolegnia	*Leptolegnia caudata*
HQ708212.1	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Leptolegnia	*Leptolegnia sp. BOLD:AAX5717*
HQ708182.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Newbya	*Newbya spinosa*
HQ708453.1	1	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Pythiopsis	*Pythiopsis terrestris*
HQ709015.1	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia anisospora*
HQ709016.1	1	2	2	1	0	4	43	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia asterophora*
HQ709021.1	0	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia diclina*
HQ709022.1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia eccentrica*
KM361513.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia ferax*
HQ709030.1	0	1	0	2	3	0	0	0	0	0	4	0	1	0	0	1	0	0	0	2	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia hypogyna*
HQ709031.1	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia lapponica*
HQ709038.1	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia monilifera*
HQ709039.1	0	0	0	0	0	0	0	0	0	1	3	3	1	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia monoica*
HQ709045.1	3	16	1	0	0	3	12	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia parasitica*
HQ709050.1	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia subterranea*
HQ709052.1	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia terrestris*
HQ709053.1	0	1	0	0	0	0	3	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Saprolegnia	*Saprolegnia turfosa*
HQ709058.1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	NA	Oomycetes	Saprolegniales	Saprolegniaceae	Thraustotheca	*Thraustotheca clavata*
AB477081.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Nematoda	Chromadorea	Rhabditida	Diplogastridae	Acrostichus	*Acrostichus sp. RGD807*
KC130711.1	0	0	0	0	0	0	0	0	2	0	0	0	0	3	0	0	1	0	0	0	0	0	Nematoda	Chromadorea	Rhabditida	Molineidae	Oswaldocruzia	*Oswaldocruzia sp. sp. BOLD:AAY6341*
FN397754.1	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	5	1	0	0	0	0	Nematoda	Chromadorea	Rhabditida	NA	NA	*Rhabditida sp. 3004ed*
JN252515.1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	Nematoda	Chromadorea	Rhabditida	Strongylidae	Murshidia	*Murshidia africana*
AY508063.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Nematoda	Chromadorea	Tylenchida	Aphelenchoididae	Bursaphelenchus	*Bursaphelenchus sexdentati*
EF208981.1	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	Nemertea	Enopla	Monostilifera	Tetrastemmatidae	Prostoma	*Prostoma graecense*
LM995251.1	0	0	0	0	0	0	0	0	0	0	0	0	1	4	1	1	0	0	0	0	0	0	Phaeophyceae	NA	Ectocarpales	Chordariaceae	NA	*Chordariaceae sp. 7 AP-2014*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	C1	C2	C3	N1	N2	N3	T1	T2	T3	A1	A2	A3	R1	R2	R3	DR1	DR2	DR3	EC1	EC2	EC3	PC	Phylum	Class	Order	Family	Genus	Species
FJ873087.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	4	2	0	Phaeophyceae	NA	Fucales	Durvillaeaceae	Durvillaea	*Durvillaea potatorum*
HQ386111.1	0	0	0	0	0	0	0	0	0	8	0	3	7	35	9	35	22	9	40	50	23	0	Phaeophyceae	NA	Fucales	Durvillaeaceae	Durvillaea	*Durvillaea willana*
FJ646953.1	0	0	0	0	0	0	0	0	0	0	0	0	0	13	0	0	0	0	1	14	0	0	Platyhelminthes	NA	Tricladida	Dugesiidae	Dugesia	*Dugesia sp. 05 MR-2009*
GQ411060.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	Porifera	Demospongiae	Spongillida	Spongillidae	Ephydatia	*Ephydatia fluviatilis*
GQ411061.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	1	9	6	2	0	Porifera	Demospongiae	Spongillida	Spongillidae	Ephydatia	*Ephydatia muelleri*
CP006942.1	0	0	0	0	0	0	0	0	0	2	0	0	2	0	0	6	0	0	4	0	0	3	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Mannheimia	*Mannheimia sp. USDA-ARS-USMARC-1261*
CP006953.1	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	7	0	0	1	0	0	0	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Mannheimia	*Mannheimia varigena*
GQ465636.1	0	0	0	0	2	0	0	0	0	0	0	0	0	3	0	0	0	1	0	0	0	0	Rotifera	Bdelloidea	Adinetida	Adinetidae	Adineta	*Adineta gracilis*
EF173184.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	2	0	0	0	0	Rotifera	Bdelloidea	Adinetida	Adinetidae	Adineta	*Adineta grandis*
EF173203.1	11	0	0	7	1	0	0	0	0	0	0	0	0	7	0	11	0	0	0	0	0	0	Rotifera	Bdelloidea	Adinetida	Adinetidae	Adineta	*Adineta sp. MX.1.1*
GQ465605.1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Rotifera	Bdelloidea	Adinetida	Adinetidae	Adineta	*Adineta steineri*
GQ465608.1	0	5	14	24	26	0	0	0	1	0	0	0	0	6	0	1	1	11	0	1	2	0	Rotifera	Bdelloidea	Adinetida	Adinetidae	Adineta	*Adineta vaga*
KJ543667.1	0	0	0	0	1	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0	Rotifera	Bdelloidea	NA	NA	NA	*Bdelloidea sp. Bd41_01*
DQ656850.1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Rotifera	Bdelloidea	NA	NA	Rotaria	*Rotaria sordida*
JN660052.1	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	Rotifera	Bdelloidea	Philodinida	Philodinidae	Embata	*Embata environmental sample*
FJ426410.1	0	0	0	5	0	0	0	0	0	0	0	0	0	1	0	0	0	1	0	0	0	0	Rotifera	Bdelloidea	Philodinida	Philodinidae	Macrotrachela	*Macrotrachela quadricornifera*
KR133433.1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Rotifera	Bdelloidea	Philodinida	Philodinidae	NA	*Philodinidae sp. BBa*
KR133426.1	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	Rotifera	Bdelloidea	Philodinida	Philodinidae	NA	*Philodinidae sp. NCa*
DQ890128.1	1	148	0	22	24	0	0	0	1	0	0	0	0	1	0	0	1	1	0	0	2	0	Rotifera	Bdelloidea	Philodinida	Philodinidae	Philodina	*Philodina flaviceps*
FJ426463.1	0	1	2	6	4	0	7	0	0	0	0	0	2	9	0	1	1	2	0	0	0	0	Rotifera	Bdelloidea	Philodinida	Philodinidae	Pleuretra	*Pleuretra lineata*
AF416994.1	0	0	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	Rotifera	Monogononta	Ploima	Asplanchnidae	Asplanchna	*Asplanchna sieboldi*
KC618848.1	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	282	0	0	0	0	33	0	Rotifera	Monogononta	Ploima	Brachionidae	Keratella	*Keratella cochlearis*
JX216669.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	Rotifera	Monogononta	Ploima	Lecanidae	Lecane	*Lecane bulla AEG9*
KC618999.1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	41	8	8	5	21	13	0	Rotifera	Monogononta	Ploima	Synchaetidae	Polyarthra	*Polyarthra dolichoptera complex sp. UO-2013*
KC619289.1	0	0	0	0	0	0	0	0	0	104	0	2	9	0	0	3386	672	596	496	1506	734	0	Rotifera	Monogononta	Ploima	Synchaetidae	Polyarthra	*Polyarthra sp. UO-2013*
JN936549.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	Rotifera	Monogononta	Ploima	Synchaetidae	Synchaeta	*Synchaeta cf. tremula/oblonga UO-2012*
JN936573.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0	0	4	0	0	Rotifera	Monogononta	Ploima	Synchaetidae	Synchaeta	*Synchaeta kitina*

	Caleao			Upper Nalón			Tanes			Anzó			Rioseco			Downstream Rioseco			El Condao				Consensus Lineage					
#OTU ID	**C1**	**C2**	**C3**	**N1**	**N2**	**N3**	**T1**	**T2**	**T3**	**A1**	**A2**	**A3**	**R1**	**R2**	**R3**	**DR1**	**DR2**	**DR3**	**EC1**	**EC2**	**EC3**	**PC**	**Phylum**	**Class**	**Order**	**Family**	**Genus**	**Species**
KU513417.1	0	0	0	0	0	0	0	0	0	0	0	0	0	6	0	0	0	0	0	0	0	0	Tardigrada	Eutardigrada	Parachela	Hypsibiidae	Diphascon	*Diphascon higginsi*
KU513418.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	Tardigrada	Eutardigrada	Parachela	Hypsibiidae	Hypsibius	*Hypsibius cf. dujardini DS-2016*
AY598771.1	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	24	0	0	0	0	Tardigrada	Eutardigrada	Parachela	Macrobiotidae	Dactylobiotus	*Dactylobiotus parthenogeneticus*
KJ856928.1	0	0	0	0	0	0	0	0	0	0	0	0	0	5	3	0	0	0	0	0	0	0	Tardigrada	Eutardigrada	Parachela	NA	NA	*Parachela sp. Ta9-01*
EU046189.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	Tardigrada	Heterotardigrada	Echiniscoidea	Echiniscidae	Echiniscus	*Echiniscus blumi*

Supplementary Table S3. Benthic macroinvertebrates. Macroinvertebrate families found per sampling point with molecular (G: number of sequences amplified) and morphological (V: number of individuals visually assessed) approaches, and their punctuation following the official protocol for IBMWP index calculation.

Consensus Lineage				Caleao		Upper Nalon		Tanes		Anzó		Rioseco		Downsream Rioseco		El Condao		Family Score
Phylum	**Class**	**Order**	**Family**	**G**	**V**	**G**	**V**	**G**	**V**	**G**	**V**	**G**	**V**	**G**	**V**	**G**	**V**	
Anellida	Clitellata	Hirudinea	Glossiphoniidae	0	0	0	0	0	0	0	0	0	0	0	0	0	2	3
Arthropoda	Insecta	Aracnida	Acariformes	0	0	0	0	0	0	0	0	0	1	0	0	0	0	4
Arthropoda	Insecta	Coleoptera	Chrysomelidae	0	5	0	1	0	1	0	1	0	14	0	1	0	1	4
Arthropoda	Insecta	Coleoptera	Elmidae	0	0	0	1	0	0	94	18	0	46	0	9	0	2	5
Arthropoda	Insecta	Coleoptera	Haliplidae	0	0	0	0	0	0	0	1	0	0	0	0	0	0	4
Arthropoda	Insecta	Coleoptera	Gyrinidae	0	0	0	3	0	0	0	0	0	0	0	0	0	1	3
Arthropoda	Insecta	Coleoptera	Hydraenidae	102	0	0	0	0	0	0	0	0	0	0	0	0	0	5
Arthropoda	Crustacea	Amphipoda	Gammaridae	0	0	0	0	0	0	0	0	0	2	0	2	0	0	2
Arthropoda	Insecta	Diptera	Anthomyiidae	26	0	10	0	0	0	0	0	0	0	0	1	0	0	4
Arthropoda	Insecta	Diptera	Athericidae	0	0	0	0	0	0	0	0	0	1	0	0	0	0	10
Arthropoda	Insecta	Diptera	Ceratopogonidae	0	5	0	0	0	0	0	0	10	0	10	0	10	0	4
Arthropoda	Insecta	Diptera	Chironomidae	3143	10	17103	33	269	4	4268	62	93585	125	5710	9	154	232	2
Arthropoda	Insecta	Diptera	Culicidae	40	0	13	0	0	0	14	0	13	0	10	0	10	0	2
Arthropoda	Insecta	Diptera	Empididae	3867	0	0	0	10	0	0	0	0	0	0	0	0	0	4
Arthropoda	Insecta	Diptera	Ephydridae	10	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Arthropoda	Insecta	Diptera	Limoniidae	109	3	0	1	0	0	0	1	0	5	0	1	0	1	4

Consensus Lineage				Caleao		Upper Nalon		Tanes		Anzó		Rioseco		Downsream Rioseco		El Condao		Family Score
Phylum	**Class**	**Order**	**Family**	**G**	**V**	**G**	**V**	**G**	**V**	**G**	**V**	**G**	**V**	**G**	**V**	**G**	**V**	
Arthropoda	Insecta	Diptera	Psychodidae	0	0	14	0	0	0	10	0	10	0	0	0	0	0	4
Arthropoda	Insecta	Diptera	Simuliidae	1169	0	323	15	10	1	10	21	10	0	25	0	11	0	5
Arthropoda	Insecta	Diptera	Thaumaleidae	10	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Arthropoda	Insecta	Diptera	Tipulidae	0	0	0	0	84	0	0	0	0	0	0	3	13	0	5
Arthropoda	Insecta	Ephemeroptera	Baetidae	10	101	6424	68	0	0	44	185	45	375	0	7	0	7	4
Arthropoda	Insecta	Ephemeroptera	Caenidae	0	1	0	0	0	0	0	0	25	1	0	0	14	15	4
Arthropoda	Insecta	Ephemeroptera	Ephemerellidae	23	0	0	0	0	0	2338	0	279	8	0	2	0	0	7
Arthropoda	Insecta	Ephemeroptera	Ephemeridae	0	1	0	0	0	0	0	0	0	1	0	0	0	26	10
Arthropoda	Insecta	Ephemeroptera	Heptageniidae	7642	17	832	116	19	1	10	4	0	0	16	0	0	0	10
Arthropoda	Insecta	Ephemeroptera	Leptophlebiidae	24	0	13	0	11	0	0	0	0	0	0	0	0	0	10
Arthropoda	Insecta	Odonata	Calopterygidae	48	0	0	0	0	0	25	0	10	0	0	0	12	2	8
Arthropoda	Insecta	Plecoptera	Chloroperlidae	0	3	14	25	0	1	0	0	0	0	0	0	0	0	10
Arthropoda	Insecta	Plecoptera	Leuctridae	17	4	1729	8	0	0	0	0	56	0	0	0	0	0	10
Arthropoda	Insecta	Plecoptera	Nemouridae	30	0	10	6	0	0	0	0	0	0	0	0	0	0	7
Arthropoda	Insecta	Plecoptera	Perlidae	15	4	10	3	0	0	0	0	0	0	0	0	0	0	10
Arthropoda	Insecta	Plecoptera	Perlodidae	0	0	16	0	0	0	0	0	0	0	0	0	0	0	10
Arthropoda	Insecta	Trichoptera	Brachycentridae	10	0	14	0	0	0	14	183	0	8	12	2	0	5	10
Arthropoda	Insecta	Trichoptera	Calamocerati	0	0	0	0	0	0	0	0	0	0	0	1	0	5	10

Consensus Lineage				Caleao		Upper Nalon		Tanes		Anzó		Rioseco		Downsream Rioseco		El Condao		Family Score
Phylum	Class	Order	Family	G	V	G	V	G	V	G	V	G	V	G	V	G	V	
			dae															
Arthropoda	Insecta	Trichoptera	Goeridae	13	0	11	0	0	0	0	0	0	0	0	0	13	0	10
Arthropoda	Insecta	Trichoptera	Hydropsychidae	0	1	240	15	0	0	15	44	0	9	0	3	0	0	5
Arthropoda	Insecta	Trichoptera	Hydroptilidae	0	0	0	0	0	0	15	0	32	0	0	0	0	0	6
Arthropoda	Insecta	Trichoptera	Lepidostomatidae	0	1	0	0	0	0	0	0	0	0	0	0	0	0	10
Arthropoda	Insecta	Trichoptera	Leptoceridae	0	1	0	0	0	0	0	0	0	0	0	0	0	0	10
Arthropoda	Insecta	Trichoptera	Limnephilidae	104	0	24	0	0	0	514	0	14	0	12	0	13	0	7
Arthropoda	Insecta	Trichoptera	Philopotamidae	0	0	10	0	0	0	22	0	0	0	0	0	0	0	8
Arthropoda	Insecta	Trichoptera	Polycentropodidae	0	0	0	0	0	0	13	1	0	8	0	0	0	21	7
Arthropoda	Insecta	Trichoptera	Psychomyiidae	0	1	12	0	0	0	16	0	11	0	12	0	0	0	8
Arthropoda	Insecta	Trichoptera	Rhyacophilidae	0	0	0	1	0	0	14	1	0	0	0	1	12	0	7
Arthropoda	Insecta	Trichoptera	Sericostomatidae	22	2	19	2	0	0	0	0	0	0	0	0	0	0	10
Arthropoda	Malacostraca	Decapoda	Astacidae	0	0	0	0	0	0	0	0	0	0	12	0	0	0	8
Cnidaria	Hydrozoa	Anthoathecata	Hydridae	14	0	12	0	0	0	12	0	14	0	306	0	40	0	X
Mollusca			Ferrisia	0	4	0	0	0	0	0	1	0	11	0	10	0	0	6
Mollusca	Gastropoda	NA	Ancylidae	39	0	26	0	0	0	102	0	229	0	0	0	22	0	6
Mollusca	Gastropoda	NA	Bithyniidae	0	0	0	0	0	4	0	4	0	6	14	0	0	16	3

Consensus Lineage				Caleao		Upper Nalon		Tanes		Anzó		Rioseco		Downsream Rioseco		El Condao		Family Score
Phylum	**Class**	**Order**	**Family**	**G**	**V**	**G**	**V**	**G**	**V**	**G**	**V**	**G**	**V**	**G**	**V**	**G**	**V**	
Mollusca	Gastropoda	NA	Hydrobiidae	13	0	0	0	0	0	1913	1	205	0	10	0	24	0	3
Mollusca	Gastropoda	NA	Neritidae	0	0	0	0	0	0	0	0	0	0	0	0	12	0	6
Mollusca	Gastropoda	NA	Physidae	0	0	0	0	0	0	0	0	0	1	0	4	12	28	3
Platyhelminthes	NA	Tricladida	Dugesiidae	0	0	0	0	0	0	0	0	13	0	0	0	15	0	5
Mollusca	Gastropoda	NA	Planorbidae	0	0	0	1	0	0	0	0	0	0	0	0	0	3	3
Mollusca	Bivalvia	Veneroida	Sphaeridae	0	0	0	0	0	0	0	0	0	0	0	0	0	6	3
Oligochaeta			All families	379	0	3165	0	914	0	992	0	660	0	138	0	76	0	1
Number of families				25	17	23	16	7	6	21	15	18	17	13	15	17	17	
Index Value				149	121	144	99	39	34	109	79	80	97	69	78	78	94	