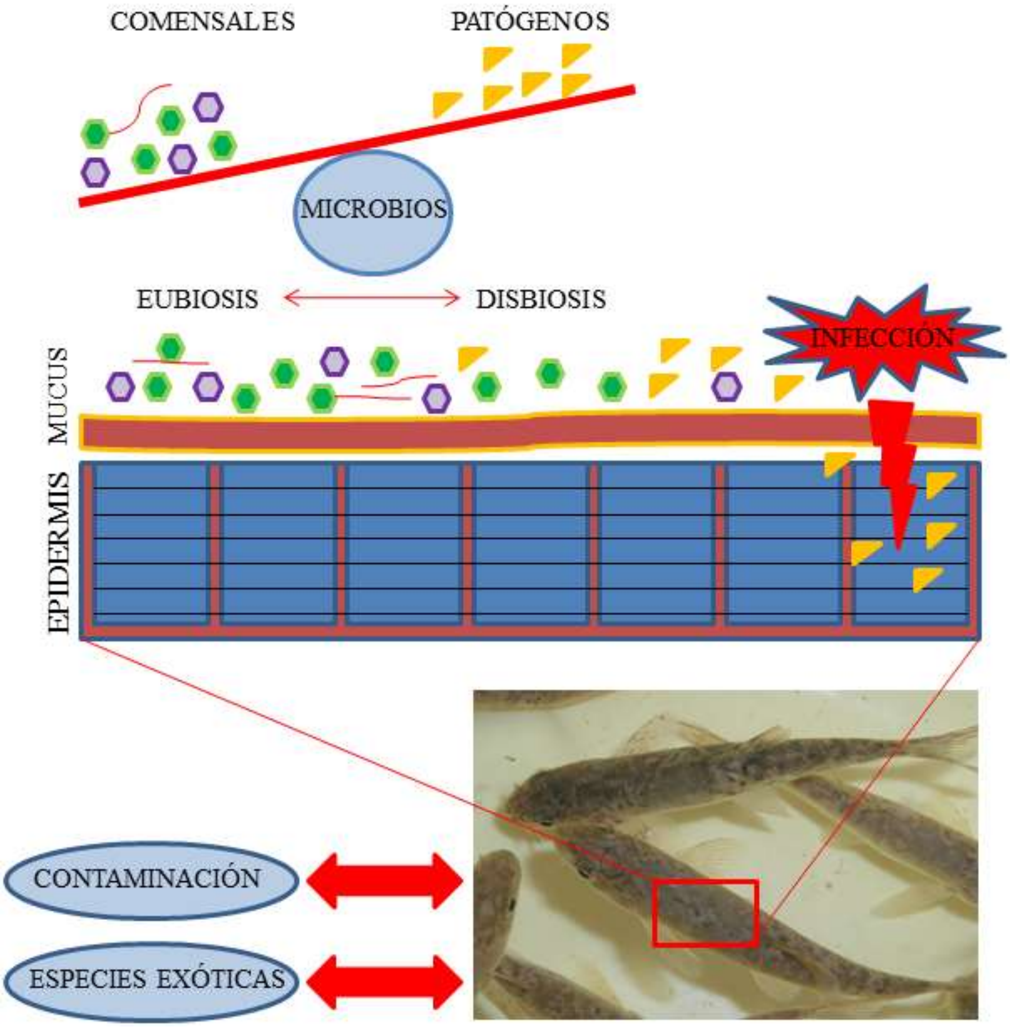
El microbioma epidérmico como nueva herramienta para evaluar los efectos de la contaminación y las invasiones biológicas sobre la salud de los peces de los ríos mediterráneos



INFORME FINAL

DICIEMBRE 2017

ALBERTO MACEDA-VEIGA

ÍNDICE

1. Breve reseña del marco del proyecto y sus objetivos

El objetivo general de este proyecto financiado por la Fundación Barcelona Zoo era determinar el impacto de las especies exóticas invasoras sobre la fauna nativa, principalmente peces, utilizando una aproximación fisiológica, parasitológica y microbiológica. Entendemos por microbiota al conjunto de microorganismos que viven sobre o dentro de un hospedador, en nuestro caso los peces. En sentido amplio, el presente proyecto abarca desde las bacterias comensales y patógenas hasta los protistas y los micrometazoos parásitos. No existe un estudio similar en la fauna nativa de las cuencas catalanas que haga un estudio de la microbiota tan exhaustivo a la par que determine variables fisiológicas. De hecho, son escasos este tipo de estudios en ecología de peces continentales a escala mundial.

Los resultados de este proyecto tienen una gran importancia en conservación si tenemos en cuenta que el número de especies exóticas establecidas no hace más que aumentar a escala mundial, que los peces continentales son de los vertebrados más amenazados y que los ríos mediterráneos son uno de los más afectados por las invasiones biológicas. En particular, destacan por su grado de amenaza las especies exóticas llamadas ingenieras de ecosistemas porque a la par de competir por los recursos, introducir patógenos y otros problemas derivados de su impacto directo sobre las especies nativas, tienen la capacidad de alterar el hábitat lo que causa múltiples impactos indirectos. Otro aspecto muy interesante del presente proyecto es su aplicabilidad a la Directiva Marco del Agua Europea. Esta directiva, aprobada por el parlamento europeo en el año 2000, requiere que todos los estados determinen el estado ecológico de sus aguas utilizando indicadores biológicos. Nuestro proyecto incrementa el número de técnicas no letales para evaluar el estado de salud de los peces que son uno de los cinco grupos faunísticos y florísticos al uso en los programas de biomonitoreo oficiales en ríos europeos. Los procedimientos tradicionales en peces -los llamados índices de calidad biótica- sólo permiten detectar impacto cuando hay variaciones drásticas en las abundancias de las especies. Con fines de conservación es vital que los gestores se anticipen a los efectos letales y actúen con medidas de gestión a la que se detectan los primeros efectos perniciosos sobre las poblaciones de peces. A fin de incrementar la relevancia de nuestros resultados, y dado el papel ecológico que juegan los peces en los ecosistemas, muchos de nuestros resultados contemplan efectos directos e indirectos sobre otras especies acuáticas, tales como mamíferos y anfibios.

2. Situación actual de los trabajos propuestos

El presente proyecto ha avanzado a buen ritmo y ha dado lugar a cuatro artículos científicos publicados o en fase de revisión durante la vigencia de la beca de la Fundación Zoo. Queda pendiente de realizar parte del trabajo de análisis estadístico de las muestras de bacterias epidérmicas debido a problemas logísticos que se detallan a continuación. Como se declaraba en la memoria original, la mejor época para muestrear los peces es durante la bajada estival de caudal de los ríos. Sin embargo, la concesión de este proyecto fue muy encima de la mejor época de muestreo en 2016 y, ante la imposibilidad de organizar la campaña de forma adecuada, decidimos moverla al otoño de este año aprovechando el inusual estiaje que afecta a los ríos. Las muestras ya han sido recogidas, secuenciadas y están pendientes de análisis exhaustivo por equipo de microbiología de la UB, aunque podemos presentar unos resultados preliminares (ver PUBLICACIÓN 5).

A continuación se detallan las publicaciones científicas y sus principales resultados en forma de texto y gráficos. El artículo completo se incluye como anexo al final de la memoria en caso de haberse enviado a una revista internacional indexada o estar ya publicado. Para el caso del artículo pendiente sobre la composición bacteriana sólo comentamos brevemente los resultados preliminares obtenidos. Por motivos obvios, rogamos no hacer difusión de los trabajos no publicados. Como puede leerse a continuación las cinco publicaciones resultantes del presente proyecto están completamente relacionadas y maximizan los *outputs* de este proyecto gracias a la financiación de la Fundación Barcelona Zoo.

3. Resultados y discusión presentados en forma de las cinco publicaciones científicas resultantes

PUBLICACIÓN 1

Maceda-Veiga, A., Baselga, A., Sousa, R., Vilà, M., Doadrio, I., & de Sostoa, A. (2017). Fine-scale determinants of conservation value of river reaches in a hotspot of native and non-native species diversity. *Science of the Total Environment*, 574, 455-466. Área científica: Ciencias Ambientales. **Factor de impacto: 4.9**

Este trabajo ha permitido conocer cuáles son los determinantes abióticos de la presencia o dominancia (en abundancia y riqueza) de las especies nativas e introducidas en todas las cuencas catalanas incluyendo a toda la cuenca del Ebro. Además, ha evaluado si los tramos de ríos donde las especies nativas gozan de mejor estado de salud a nivel de comunidad (es decir, en los tramos donde dominan en abundancia o riqueza) son las que también tienen una mayor presencia de especies introducidas lo cual plantea un serio problema de conservación. En este sentido, se han identificado los tributarios -pequeños afluentes- como zonas prioritarias de conservación para los peces nativos. Son tramos donde todavía hay poblaciones de peces nativos relativamente bien conservadas y baja presencia de especies exóticas. Otro problema de vital importancia en conservación de los ecosistemas acuáticos y que destacamos en este artículo es que no se tienen en cuenta las interacciones entre especies a la hora de establecer medidas de conservación como las áreas protegidas. Por ejemplo, las relaciones hospedador-parásito vitales para la conservación de las almejas nativas de agua dulce parecen no haberse contemplado en el diseño de dichas áreas. Teniendo en cuenta que los bivalvos nativos tienen un papel muy importante en la depuración del agua de los ríos, y son de las especies más amenazadas del planeta, que duda cabe que hace falta un rediseño de estas áreas protegidas. De hecho, resaltamos en nuestro artículo que las áreas protegidas actuales ofrecen una cobertura de protección limitada para los peces nativos y otros componentes de la biodiversidad fluvial. Sin embargo, nuestro estudio aboga por combinar la creación de nuevas áreas protegidas con una protección integral a escala de cuenca que tenga en cuenta la calidad del hábitat físico y del agua. Debe pensarse que hay especies nativas en nuestros ríos que son migradoras y, por tanto, capaces de transportar contaminantes de tramos contaminados a tramos limpios con lo cual de poco sirve hacer protecciones parciales a tramos sin tener en cuenta que los ríos son sistemas integrados cabecera-desembocadura. Asimismo, conviene no ignorar que otros organismos como las aves acuáticas realizan largos desplazamientos y también viajan entre cuencas transportando nutrientes e incluso a otros organismos. Por tanto, la salvaguarda de la biota nativa de nuestros ríos pasa ineludiblemente por actuar a escala global. A efectos del presente proyecto de la Fundación Barcelona Zoo, este estudio ha sido vital para conocer la respuesta a gran escala de buenos indicadores del estado de conservación de las comunidades de peces, tales como la riqueza de especies endémicas, amenazadas, invasoras, protegidas según la la legislación y una lista de hasta 20 indicadores a fin de conocer cuál es su relación con la degradación de la calidad del agua y del hábitat físico (**Figura 1**). No en vano, estudios más específicos como los de microbiota deben realizarse en condiciones ambientales en las cuales a priori se sospecha que pudiera haber un efecto sobre el estado de salud de los peces. Un primer paso es saber si existe dicha evidencia correlativa de efectos negativos entre parámetros que usualmente se utilizan para determinar la calidad del agua, tales como conductividad, las concentraciones de amonio, nitrito, nitrato, fosfato y los valores de temperatura y pH e indicadores a nivel de comunidad tal y como se realiza en los programas de biomonitorización oficiales.

Figura 1. Esquema del marco de trabajo de la Publicación 1. Se determina la relación entre las condiciones ambientales de los ríos y la comunidades de peces en base a su valor ecológico per se o para otros grupos acuáticos tales como aves y almejas de agua dulce. En base a estos resultados se establece el valor de conservación del tramo de río y se proponen medidas de conservación de acuerdo a cuáles son las variables ambientales que tuvieron un efecto consistente positivo o negativo sobre los indicadores del estado de conservación de las comunidades de peces.

Otro aspecto importante a destacar de este trabajo que es determina cuáles son los mecanismos responsables del cambio en la composición taxonómica de las comunidades de peces. Estos cambios se pueden generar por sustitución de especies, es decir, las especies más sensibles desaparecen de la comunidad y son reemplazadas por otras más tolerantes (del inglés *turn-over*). Por otro lado, puede darse un proceso de anidamiento (del inglés *nestedness*) según el cual las comunidades en ambientes alterados son simplificaciones estructurales de las comunidades más ricas pero no se incorporan especies nuevas a la comunidad. Teniendo en cuenta que estos dos procesos no son excluyentes, nuestros resultados abogan por que predominaría el *turn-over* sobre el anidamiento en la estructura de las comunidades de peces en los ríos mediterráneos expuestos a condiciones de estrés, siendo las variables geográficas las más influyentes por delante de las de calidad de agua y de hábitat físico, aunque ambas correlacionan ligeramente con el mecanismo de anidamiento (**Figura 2**). Cuando los datos de la composición bacteriana estén completamente analizados será interesante comprobar si los mecanismos de ensamble de la comunidad de hospedadores (peces, **PUBLICACIÓN 1**) y de sus microbios (microbiota, futura **PUBLICACIÓN 5**) son los mismos. Es decir, determinar si es predecible que las comunidades acuáticas, ya sean de microbios o vertebrados bajo estrés respondan siguiendo un patrón compatible con el anidamiento.

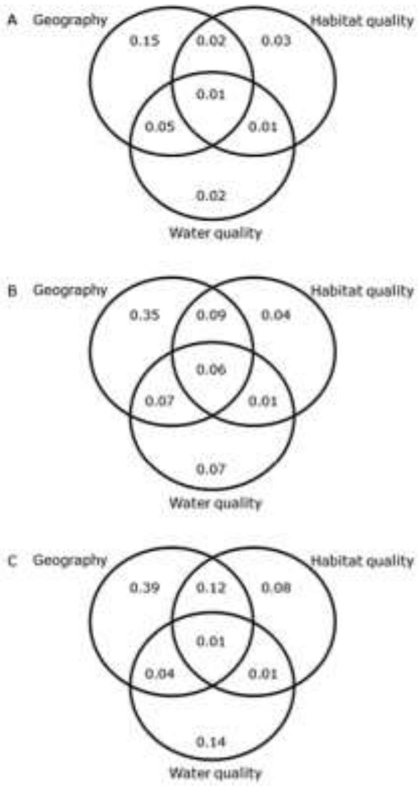


Figura 2. Diagramas de Venn que muestran el análisis partido de la varianza de la estructura de las comunidades de peces en función de variables geográficas, de calidad de agua y de hábitat físico. A) Indica la variación total en la estructura de la comunidad; B) Indica la variación en el componente *turn-over* y C) Indica la variación en el componente *nestedness*. Como puede verse, la importancia de las variables de calidad de hábitat físico y de agua incrementa para el componente *nestedness*, lo que implica que existe un cierto soporte en nuestros datos para que estas alteraciones de hábitat causen comunidades pobres en especies que sean una sub-comunidad de las comunidades ricas.

Si bien en los párrafos precedentes hemos hecho un resumen expandido de los resultados principales de este trabajo, a continuación presentamos el resumen en inglés de esta publicación en *Science of the Total Environment* para detalles complementarios:

“Global freshwater biodiversity is declining at unprecedented rates while non-native species are expanding. Examining diversity patterns across variable river conditions can help develop better management strategies. However, many indicators can be used to determine the conservartion value of aquatic communities, and little is known of how well they correlate to each other in making diagnostics, including when testing for the efficacy of protected areas. Using an extensive data set (99,700 km2, n = 530 sites) across protected and unprotected river reaches in 15 catchments of NE Spain, we examine correlations among 20 indicators of conservation value of fish communities, including the benefits they provide to birds and threatened mammals and mussels. Our results showed that total native fish abundance or richness correlated reasonably well with many native indicators. However, the lack of a strong congruence led modelling techniques to identify different river attributes for each indicator of conservation value. Overall, tributaries were identified as native fish refugees, and nutrient pollution, salinization, low water velocity and poor habitat structure as major threats to the native biota. We also found that protected areas offered limited coverage to major components of biodiversity, including rarity, threat and host-parasite relationships, even though values of non-native indicators were notably reduced. In conclusion, restoring natural hydrological regimes and water chemical status is a priority to stem freshwater biodiversity loss in this region. A complementary action can be the protection of tributaries, but more studies examining multiple components of diversity are necessary to fully test their potential as fluvial reserves in Mediterranean climate areas.”

Fuente: http://www.sciencedirect.com/science/article/pii/S0048969716320253

No menos importante que la calidad científica de esta **PUBLICACIÓN 1** es que éste ha permito establecer nuevas colaboraciones nacionales e internacionales al equipo investigador liderado por el Dr Maceda. Nunca antes habíamos colaborado con los siguientes investigadores:

- **Dr Andrés Baselga**. Departamento de Zoología, Facultad de Biología, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, España
- **Dr Ronaldo Sousa**. CBMA - Centre of Molecular and Environmental Biology, Department of Biology, University of Minho, 4710-057 Braga, Portugal.
- **Dr Ignacio Doadrio**. Department of Biodiversity and Evolutionary Biology, Museo Nacional de Ciencias Naturales-CSIC, E-28006 Madrid, Spain.

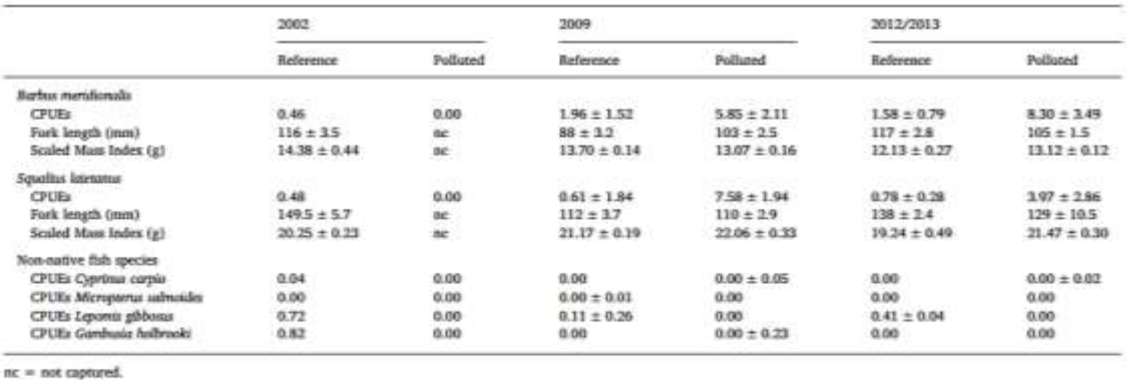
Estos investigadores, y en especial el Dr Baselga, serán cruciales para determinar cómo se estructura la comunidad de microbios en la **PUBLICACIÓN 5**.

PUBLICACIÓN 2

Colin, N., **Maceda-Veiga, A.**, Monroy, M., Ortega-Ribera, M., Llorente, M., & de Sostoa, A. (2017). Trends in biomarkers, biotic indices, and fish population size revealed contrasting long-term effects of recycled water on the ecological status of a Mediterranean river. *Ecotoxicology and Environmental Safety*, 145, 340-348. Área de trabajo: Ciencias Ambientales. **Factor de impacto: 3.7**

Este trabajo tiene en común con la **PUBLICACIÓN 1** que estudia cómo responden las comunidades de peces a la degradación del hábitat, tanto del hábitat físico como de la calidad del agua. Sin embargo, incluye una particularidad que escasea en los estudios de ecología de peces, y es el disponer de una serie temporal de datos que contemple una aproximación multidisciplinar. En concreto, en este trabajo analizamos durante el periodo 2009-2011 los cambios poblacionales en la comunidad de peces (cambios en la abundancia y estructura poblacional) y su relación con los parámetros fisiológicos de estos peces, tales como fórmula leucocitaria y el porcentaje de anomalías eritrocitarias. El primero es un buen indicador de estrés general y de la presencia de infecciones, mientras que el segundo está relacionado con la presencia de compuestos genotóxicos en el medio ambiente (es decir, que causan daño en el ADN). Nuestros resultados indican que, si bien estas variables sanguíneas detectan impactos sub-letales, todavía no conocemos bien cómo estos efectos se traducen a escala de población a largo plazo. Es decir, era predecible por las anomalías eritrocitarias observadas en el barbo de montaña (*Barbus meridionalis*) en 2009 que la abundancia de esta especie disminuyera en el tiempo, mientras que la bagra catalana (*Squalius laietanus*), con menos anomalías en el 2009, hiciera lo opuesto en 2011. Sin embargo, los resultados fueron opuestos para ambas especies lo que denota que todavía no conocemos bien los parámetros de normalidad de los análisis de sangre en peces continentales (**Tabla 1**).

Tabla 1. Datos biométricos (longitud, peso y condición física) y de abundancia (capturas por unidad de esfuerzo, CPUE) de las dos especies nativas del río Ripoll, el barbo de montaña (*Barbus meridionalis*) y la bagra catalana (*Squalius laietanus*), en los tramos de referencia y contaminados durante el periodo 2002 a 2013. Nótese que también se muestran las abundancias de las especies exóticas presentes (encabezados en inglés al ser una tabla extraída de la publicación original).



	2002		2009		2012/2013	
	Reference	Polluted	Reference	Polluted	Reference	Polluted
Barbus meridionalis						
CPUEs	0.46	0.00	1.96 ± 1.52	5.85 ± 2.11	1.58 ± 0.79	8.30 ± 3.49
Fork length (mm)	116 ± 3.5	nc	88 ± 3.2	103 ± 2.5	117 ± 2.8	105 ± 1.5
Scaled Mass Index (g)	14.38 ± 0.44	nc	13.70 ± 0.14	13.07 ± 0.16	12.13 ± 0.27	13.12 ± 0.12
Squalius laietanus						
CPUEs	0.48	0.00	0.61 ± 1.84	7.58 ± 1.94	0.78 ± 0.28	3.97 ± 2.86
Fork length (mm)	149.5 ± 5.7	nc	112 ± 3.7	110 ± 2.9	138 ± 2.4	129 ± 10.5
Scaled Mass Index (g)	20.25 ± 0.23	nc	21.17 ± 0.19	22.06 ± 0.33	19.24 ± 0.49	21.47 ± 0.30
Non-native fish species						
CPUEs *Cyprinus carpio*	0.04	0.00	0.00	0.00 ± 0.05	0.00	0.00 ± 0.02
CPUEs *Micropterus salmoides*	0.00	0.00	0.00 ± 0.01	0.00	0.00	0.00
CPUEs *Lepomis gibbosus*	0.72	0.00	0.11 ± 0.26	0.00	0.41 ± 0.04	0.00
CPUEs *Gambusia holbrooki*	0.82	0.00	0.00	0.00 ± 0.23	0.00	0.00

nc = not captured.

Pese a las limitaciones de los estudios de hematología en peces, el hecho de muestrear durante las cuatro estaciones del año en el presente artículo, en condiciones de referencia y contaminadas, ha contribuido significativamente a conocer los parámetros de normalidad de la sangre de estos peces que, como es bien sabido de medicina humana y veterinaria de animales domésticos, es clave para un buen diagnóstico (**Figura 3**). Otro resultado relevante de este artículo es que ninguna de las dos especies presentó protistas ecto-simbiontes ni macroparásitos de ningún tipo, algo que contrasta con los estudios previos realizados por el equipo de investigación en años anteriores. Es posible que la calidad del agua del río mejorara o que los peces se hayan adaptado a la contaminación volviéndose menos vulnerables a las infecciones. En cualquier caso, el río Ripoll, donde se ha realizado este estudio se confirma como uno de los mejores candidatos para estudiar potenciales efectos sub-letales a través de la composición de bacterias comensales. Es por ello, que fue uno de los seleccionados para la recogida de muestras este otoño. Junto con avances importantes en el campo de la hematología de peces, este estudio también ha permitido confirmar que los índices de calidad biótica al uso en los ríos catalanes tienen dificultades para identificar de forma certera cuadros de contaminación en ríos mediterráneos. Y, no sólo eso, si no que confunden los problemas de calidad de agua con problemas de invasiones biológicas por parte de especies exóticas, al reducir ambos los valores del índice y ser éste, por tanto, una medida integrada de impacto. Otro aspecto relevante de esta publicación científica es que ha contribuido a la formación de la doctoranda Nicole Colin, y de los estudiantes de grado Mireia Llorente y Martí Ortega-Ribera, quien ha iniciado sus estudios de doctorado en inmunología en el Hospital Clínico.

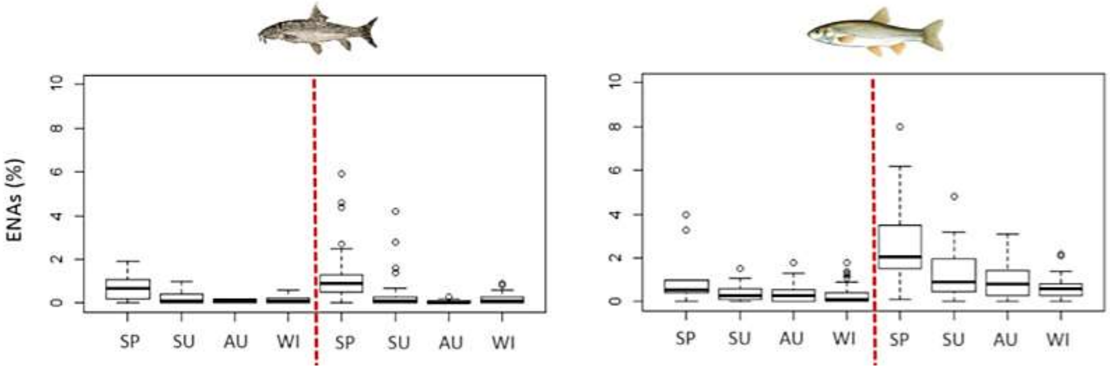


Figura 3. Cambios en el porcentaje de anomalías de glóbulos rojos en las dos especies de peces nativas del río Ripoll, el barbo de montaña (*Barbus meridionalis*, izquierda) y la bagra catalana (*Squalius laietanus*, derecha) a lo largo de todo un año (SP, primavera; SU, verano; AU, otoño; WI, invierno; acrónimos del inglés). Véase que el porcentaje de anomalías en los glóbulos rojos, relacionadas con la presencia de compuestos carcinógenos en el agua, incrementa en las localidades contaminadas (derecha de la línea roja) en ambas especies en relación con los tramos control pero con una estacionalidad muy marcada.

De igual modo que para la **PUBLICACIÓN 1**, el lector puede encontrar el resumen en inglés de la presente publicación a continuación para información complementaria.

"Recycled water is important for maintaining river flow in semi-arid regions. However, it has ecological risk, as suggested by comparison of habitat and white and red blood cell count in two wild fish species (*Barbus meridionalis* and *Squalius laietanus*) before and after an input of recycled water in Ripoll River (NE Spain) in 2009. Due to the lack of normal ranges for blood variables in wild fish, we surveyed seasonally the same river reaches in 2013 to test if blood alterations from 2009 compromised the viability of the fish populations. By examining other indicators of river health in baseline and polluted sites (fish abundance, mass-length relationships, and community indices in fish, diatoms and invertebrates), we tested for the superior utility of blood tests in biomonitoring. The comparison of water quality and scores of diatoms and invertebrate indices between polluted and reference sites showed that polluted sites improved from 2009 to 2013. The abundance of *B. meridionalis* also increased in polluted sites, but that of *S. laietanus* declined in 2013 compared to 2009. These results contrast with results of blood analyses in 2009, which suggested that *B. meridionalis* was more seriously affected by pollution than *S. laietanus*. The fish index did not reveal the risk of recycled water to fish health, whereas fish mass-length relationships suggested that *S. laietanus* individuals in 2013 had a better body condition in polluted than in reference sites. Given that the two fish species had opposite results in reference sites, and that the physical habitat was more suitable for *B. meridionalis* in polluted sites in 2013 than was for *S. laietanus*, trends in population size are not only explained by pollution. The role of phenology is suggested by peaks in blood disorders during the breeding season. However, more long-term studies combining indicators of river health at the individual and community scales are needed to fully assess the ecological risk of recycled water in this river. These studies will also help to develop blood tests as reliable health indicators in wild fish populations."

Fuente: http://www.sciencedirect.com/science/article/pii/S0147651317304608

Si bien esta publicación no ha dado lugar a nuevas colaboraciones nacionales o internacionales, ha sido fundamental para consolidar la línea de investigación del grupo de investigación. Una línea que desarrolló de forma pionera el Dr Maceda durante su tesis doctoral en 2011. Sin embargo, la **PUBLICACIÓN 2** no ha permitido testar eficientemente la influencia relativa de la calidad del agua y las especies exóticas invasoras sobre el estado de salud de las poblaciones de peces nativos porque sólo se detectaron en los tramos control. Es bien sabido que la naturaleza no siempre tiene los diseños experimentales que los investigadores necesitamos pese a que nuestros datos de años interiores indicaban que las especies exóticas estaban presentes en los tramos de referencia y contaminandos.

PUBLICACIÓN 3

Maceda-Veiga, A., Mac Nally, R., & de Sostoa, A. (2017). The presence of non-native species is not associated with native fish sensitivity to water pollution in greatly hydrologically altered rivers. *Science of The Total Environment*, 607, 549-557. Área científica: Ciencias Ambientales. **Factor de impacto: 4.9**

Este trabajo aborda el hito del marco general del presente proyecto que consiste en evaluar cómo afecta la presencia de las especies exóticas en la sensibilidad de las especies nativas a la contaminación del agua. Se utilizaron como indicadores del estado de salud de los peces nativos una serie de indicadores a nivel individual, tales como la presencia de cada una de las especies, la presencia de enfermedades en general de cualquier índole, así como la variación de cada uno de estos parámetros en las localidades de "presencia" (**Figura 4**). Esta aproximación estadística se hizo para abordar el problema de la heterogeneidad de varianzas dado que los datos tenían distribuciones muy extremas (mucha asimetría). De los resultados de este trabajo se deriva que la presencia de especies invasoras parece tener un efecto poco relevante sobre el conjunto de variables examinadas en los peces nativos. Esto no quiere decir que no haya efectos a largo plazo o que los efectos negativos ya se hayan producido en la población y no fueran detectados en los análisis. Es por ello que en el resto de trabajos seguimos ahondando en esta línea y varios de los puntos de muestreo de este trabajo han sido seleccionados para el estudio de posibles disbiosis.

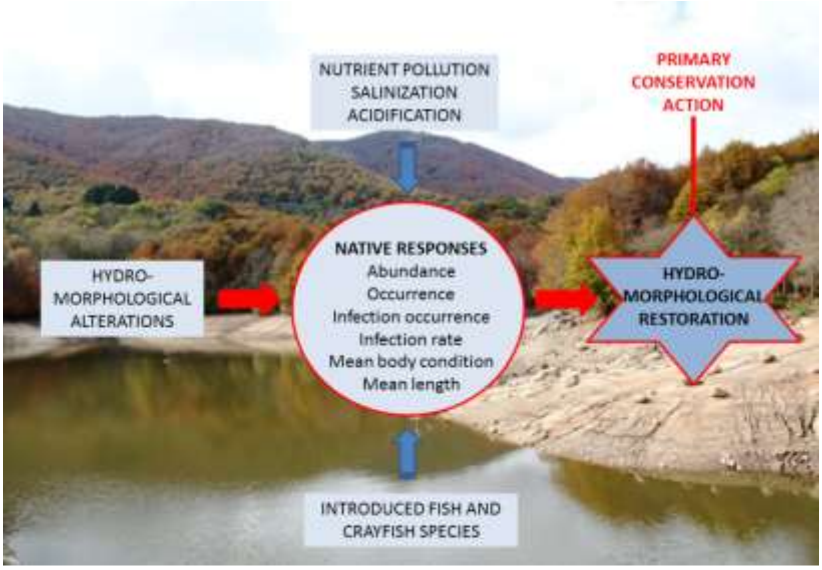


Figura 4. Esquema general del marco de trabajo de la **PUBLICACIÓN 3** en el cual se analizó la importancia relativa de los problemas de calidad de agua y de las invasiones por especies exóticas en relación a los problemas hidromorfológicos de los ríos mediterráneos. El resultado fue que estos últimos parecen ser el factor principal que afecta a los peces nativos en base a los seis indicadores examinados lo que a su vez implicó que no encontramos suficiente evidencia que las especies exóticas modulen la respuesta de las especies nativas a la contaminación.

A fin de cuentas, una infección bacteriana o de cualquier otra índole tiene lugar cuando las bacterias comensales que forman parte, junto con el mucus y las escamas, de la barrera física y química de los peces ante agresiones externas se rompe. Por tanto, a la que hay infección se dice que se ha producido una disbiosis de la microbiota comensal de la piel de los peces que si bien no la hemos analizado directamente en este trabajo si lo hemos hecho indirectamente. La ventaja ha sido poder hacer un uso más razonable del dinero en aquellos puntos que los análisis estadísticos han indicado que podían ser más interesantes. Sin embargo, uno de los puntos débiles de esta **PUBLICACIÓN 3** es que ha abordado el análisis a escala de población, es decir, en forma de frecuencias en relación al número total de peces capturados en cada punto corregido por las especies presentes. La crítica de un estudio de este tipo es que no contempla la variabilidad inter-individual, por ejemplo, de tamaño corporal, no así la poblacional definida como promedio de tamaño. Es por ello que, dados los resultados de este artículo, y para estar seguros de la relación entre condiciones ambientales y el riesgo de infección decidimos abordar más en profundidad este tema en la **PUBLICACIÓN 4**.

La **PUBLICACIÓN 3** también ha permitido determinar con datos cuantitativos los rangos de tolerancia estimados de muchas especies de peces nativas de los ríos catalanes. De forma tradicional, estas tolerancias se han determinado de acuerdo con el criterio de experto que, si bien puede aproximarse a la realidad, no da confianza a los gestores a actuar en consecuencia. Gracias a este artículo, los gestores de aguas disponen de la media ponderada de los valores de pH, conductividad, amonio, nitrito, nitrato y fosfatos en los cuales se han capturado la mayoría de las especies nativas. Estas condiciones fueron ~560 µS/cm, <2 mg NO_3^{-1}, <0.1 mg PO_4^{3-}P. Ahora falta comprobar si estos valores realmente no llevan asociados ninguna disbiosis u otra alteración fisiológica para darlos realmente como condiciones de referencia en pro de la conservación de las especies nativas de los ríos catalanes. Lo que sí parece confirmarse, de acuerdo con nuestras investigaciones previas, es que realmente existen especies nativas que parecen ser tan tolerantes a la contaminación como las exóticas. Asimismo, parece que los esfuerzos de conservación debieran centrarse en mejorar la hidromorfología de los ríos, incluyendo su caudal, por sus múltiples beneficios sobre el ecosistema que incluye una mejor calidad de agua y una reducción de las poblaciones de especies invasoras que típicamente gustan de aguas calmadas. En este sentido, nuestro trabajo también aboga por evitar sobrestimar el riesgo de complejas interacciones entre los impactos ambientales en los ecosistemas. No parece, a la luz de nuestros resultados, que tal complejidad sea tan aparente en los ríos continentales catalanes.

Para acabar, y como hemos hecho con las anteriores publicaciones, a continuación pueden encontrar el resumen en inglés de la **PUBLICACIÓN 3** para más detalles.

“There is a risk of ‘ecological surprises’ if multiple potentially interacting stressors are managed individually, which is a question attracting significant current interest. Habitat degradation and species introductions are major threats to global biodiversity, and riverine fish are among the most threatened taxa in the world. Our interest was whether the presence of non-native species can affect native fish sensitivity to water quality deterioration in a large region in northeastern Spain (99,700 km^2, 15 catchments, 530 sites). We used a ‘base model’ with geographical and hydro-morphological variables, which are the major shaping factors in rivers. We tested whether water pollution, non-native species, or their interaction provided an improved understanding of patterns of distributions and health measurements of the twelve most common native species. There was little evidence that variation in native species abundance, where they occurred, the presence of diseases and changes in mean fish length or body condition was affected by water deterioration, the presence of non-native species, or their interaction. The disease rate and occurrence of native species might be affected, to a minor degree, by water quality changes and the presence of non-native species. Environmental conditions between sites with and without non-native fish differed in the condition of riparian areas and in water quality. Based on presence-absence data and changes in abundances through weighted average equations we also derived potential safe levels of salinization, nutrient pollution, and pH for the native fish. Overall, additive effects of stressors prevail over interactions, and the restoration of natural hydro-morphology in rivers is likely to be the most effective management approach to improving the prospects for the native fish fauna."

Extraído de: https://www.ncbi.nlm.nih.gov/pubmed/28709090

PUBLICACIÓN 4

Artículo no publicado (en revisión) por lo tanto se hace copia del encabezado del manuscrito y de su resumen en el formato enviado a la revista. Se ruega nuevamente no hacer difusión de esto resultados por ser datos inéditos y no publicados. La revista donde se encuentra en proceso de revisión es *Global Change Biology*. Área Científica: Ciencias Ambientales. **Factor de impacto: 8.5**

Maceda-Veiga, A., Mac Nally, R., Green, A.J., Poulin, R., & de Sostoa, A. Environmental stressors and the transmission of a widespread alien parasite among riverine fish: additive vs interactive effects (2ª revisión).

En este trabajo se examinan los factores que determinan la expansión de un parásito, el copépodo *Lernaea cyprinacea*, en las poblaciones de peces de los ríos mediterráneos catalanes y toda la cuenca del Ebro. Este parásito se cree que fue introducido en medio mundo con *stocks* de carpa (*Cyprinus carpio*) y dado su capacidad para infectar hospedadores diversos, incluyendo anfibios, se ha expandido por todas las cuencas. El objetivo de este trabajo fue determinar hasta qué punto mejorar las condiciones ambientales de los ríos podría ayudar a disminuir la expansión de este copépodo parásito tan generalista y qué especies de peces, en función de sus características biológicas y ecológicas, serían las más afectadas. Nuestros resultados, sin embargo, indican que los factores del hospedador (es decir, su biología y ecología) juegan un papel menor en la expansión de este patógeno a excepción de su tamaño corporal. Observamos que los peces de menor tamaño están entre los más afectados, una vez controlamos por el resto de factores bióticos tales como especie, cuenca o punto de muestreo. Nuestro estudio no permite dilucidar las causas de este patrón, pero suponemos que se deba a la mayor susceptibilidad de los peces pequeños a los impactos ambientales, tales como una calidad de agua pobre, es decir, una elevada salinidad por causa de vertidos de aguas residuales urbanas e industriales y una excesiva concentración de nutrientes, principalmente nitrógeno y fósforo. De hecho, los modelos indican que una reducción en la concentración de estos últimos podría reducir la expansión del parásito como también una reducción en la salinidad. Otro aspecto relevante del presente trabajo, y que refuerza los resultados de la **PUBLICACIÓN 3**, es que no encontramos evidencia alguna de la existencia complejas interacciones entre los factores ambientales analizados. Por tanto, es esperable que su manejo de forma aislada reduzca la expansión de la enfermedad sin dar lugar a sorpresas inesperadas derivadas que la respuesta no linear entre los factores ambientales.

Aunque la introducción del copépodo parásito es atribuible a especies de peces exóticas, y en particular de la carpa, no encontramos evidencia alguna que a día de hoy estuviera relacionado con el status nativo o introducido de los peces. Por tanto, todo apunta a que la gestión cuidadosa de la calidad de agua de los ríos parece ser la manera más eficaz de controlar la expansión de este patógeno el cual, por otro lado, parece estar poco limitado por factores naturales como la altitud. No en vano especies de aguas frías como la trucha (*Salmo trutta*) se vieron igual de afectadas que especies más termófilas como el barbo del Ebro (*Luciobarbus graellsii*), lo cual es alarmante teniendo en cuenta que la temperatura del agua se incrementará aún más si cabe con el cambio climático. Un aspecto final a destacar del presente trabajo es que, pese a que en la actualidad hay un creciente interés en los llamados contaminantes emergentes, todavía no está solucionado en muchos ríos europeos el problema de la contaminación por nutrientes. Un problema que ya era bien conocido por los ecólogos de principios de siglo y que sigue estando presente en nuestros ríos. No obstante, es cierto que con la entrada en vigor de la Directiva Marco del Agua los problemas de contaminación aguda por amoníaco y nitrito parecen ir a la baja en relación con los vertidos de nitratos y fosfatos, dos contaminantes en auge derivados de la intensificación de la agricultura y la ganadería. Ahora falta, y nuestro trabajo quiere contribuir a ello, que se incorporen el tratamiento terciario a las depuradoras del territorio a fin de hacer la gestión de los residuos producidos por las zonas urbanas y agrícolas más sostenibles desde un punto de vista medio ambiental.

Como ya hicimos con las anteriores publicaciones, a continuación se incluye el resumen en inglés de esta publicación para que los lectores conozcan más detalles de nuestro trabajo. No hemos incluido soporte gráfico de esta publicación en el informe porque parte de los co-autores del trabajo han preferido mantener su confidencialidad.

“The increased rate of outbreaks of infectious diseases in ecosystems is among the most dramatic consequences of global change, particularly when it affects highly imperilled taxa such as freshwater fish. However, the links between disease-inducing epizootics and widespread environmental stressors, including nutrient pollution and salinization, in freshwater organisms are largely unexplored. Ours is the first study to examine additive and interactive effects of globally pervasive environmental stressors on a riverine host-parasite system. We explored the host and environmental factors that singly, or in combination, are likely to influence the spread of a worldwide invasive parasite, the anchor worm *Lernaea cyprinacea*, throughout an extensive region of north-eastern Spain (99,700 km^2, 15 river catchments, N = 530 sites). Smaller fish experienced higher rates of infection, and lower altitudes, river salinization and nutrient pollution (nitrate and phosphates) promoted infection rates of *L. cyprinacea* in 19 endemic and widely distributed fish species. We found no evidence that interactive effects among major riverine stressors were related to the spread of *L. cyprinacea* among fish, despite the current emphasis on the importance of accounting for interactions among stressors in biodiversity management; our results suggest that management can be effective on a stressor-by-stressor basis. Given that nutrient pollution and salinization were two of the major factors contributing to the increasing infection rates of *L. cyprinacea*, the improvement of wastewater treatment, optimization of agricultural practices, and the restoration of riparian areas would probably reduce the spread of this widespread parasite among native fish."

PUBLICACIÓN 5

El objetivo de este trabajo fue estudiar en detalle los cambios que se producen en la composición bacteriana de la piel de los peces expuestos a condiciones de estrés, tales como la presencia de especies exóticas invasoras y un empeoramiento en la calidad del agua. Como indicábamos al inicio de esta memoria, fue necesario realizar toda una serie de trabajos previos a fin de refinar el diseño experimental de este trabajo con garantías. Además dicho análisis exhaustivo nos ha permitido maximizar los resultados de este proyecto a la par que analizar alguna de nuestras hipótesis como si la presencia de las especies exóticas invasoras afecta a la susceptibilidad de los peces nativos a la enfermedad de forma mucho más robusta, esto es, con un mayor número de puntos de muestreo.

Como ocurre con la **PUBLICACIÓN 4** los resultados de este trabajo no pueden plasmarse de forma exhaustiva en la presente memoria por motivos de confidencialidad hasta que no estén publicados en revistas indexadas tal y como acordaron los co-autores. Un equipo investigador que está constituido por el Prof Ralph Mac Nally de la Universidad de Camberra (Australia), el Prof Robert Poulin de la Universidad de Otago (Nueva Zelanda), el Prof Julian Marchesi del Imperial College de Londres (Reino Unido), el Dr Gordon Webster de la Universidad de Cardiff (Reino Unido) y un equipo de microbiólogos de la Universitat de Barcelona encabezados por el Dr Anicet Blanch. Así pues, además de a la calidad científica del trabajo resultante, la Fundación Barcelona Zoo ha contribuido a consolidar y crear nuevas colaboraciones entre el investigador responsable del presente proyecto y unas de las más prestigiosas instituciones académicas internacionales.

A falta de este análisis más exhaustivo que comentamos en la memoria que falta para dar por finalizada esta publicación, presentamos a continuación la estructura taxonómica que presenta la comunidad bacteriana de acuerdo a tres factores: temperatura y presencia de nutrientes en el agua siguiendo un diseño experimental cruzado de estos dos factores. El factor temperatura lo hemos analizado siguiendo un gradiente altitudinal comparando tramos limpios o contaminados por nutrientes en zonas de montaña y zonas de baja altitud, intentando que los tramos fluviales tuvieran aproximadamente la misma estructura hidromorfológica (**Figura 5**). Asimismo, es imposible encontrar tramos fluviales que estén contaminados exclusivamente por un tipo de contaminante, en nuestro caso los nutrientes. Sin embargo, creemos que los tramos analizados permiten desligar de forma suficientemente adecuada el impacto de la contaminación por nutrientes de otros impactos que pudieran estar presentes.

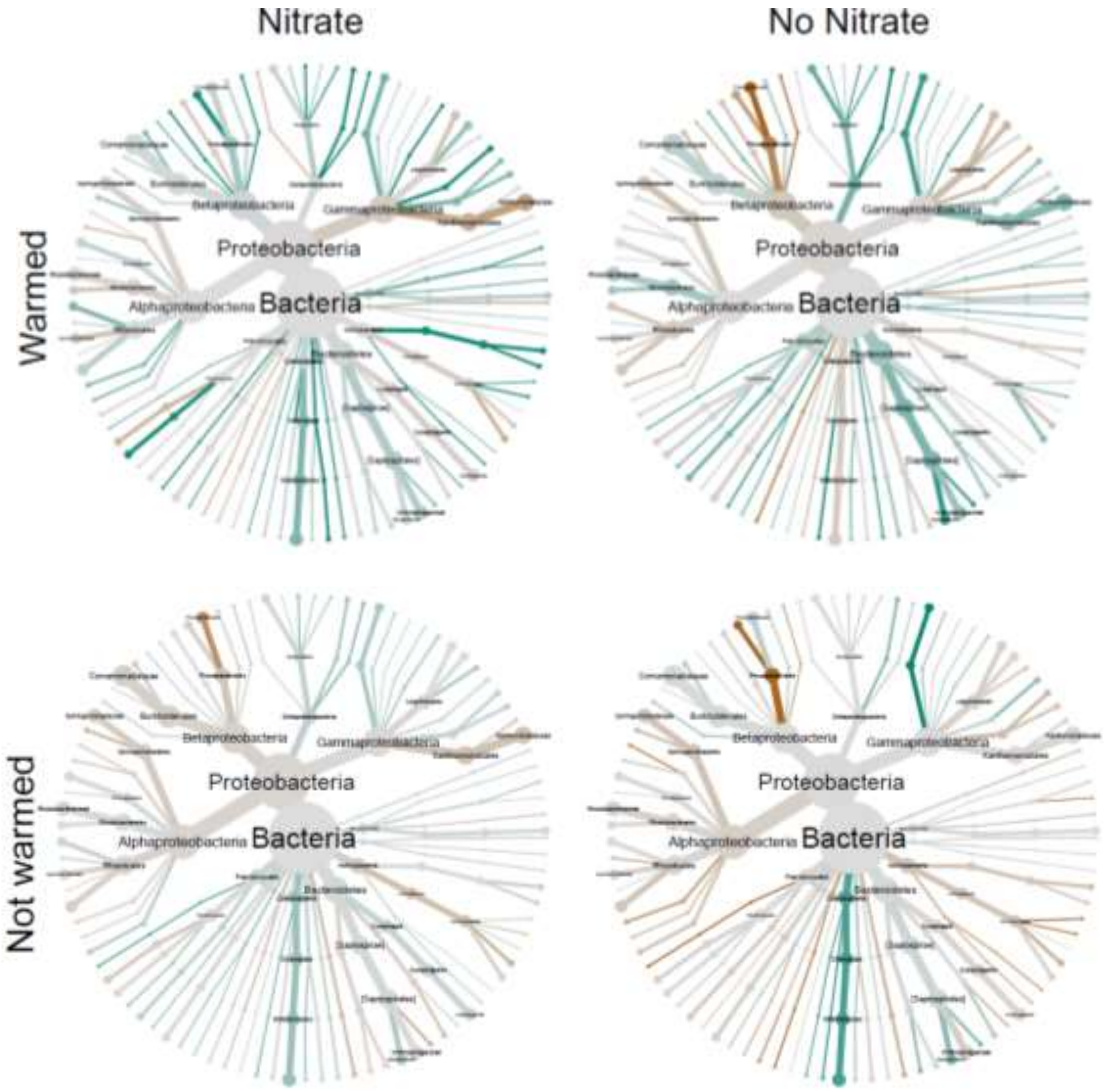


Figura 5. Los diagramas de agrupación muestran la composición bacteriana de los tramos de baja (*not warmed*) y alta altitud (*warmed*) con o sin contaminación por nutrientes (*nitrate*) siguiendo la jerarquía taxonómica. En el centro está el menor nivel de resolución taxonómica y en el círculo más exterior encontramos el mayor nivel, normalmente género o familia.

Tal y como muestra la **Figura 5**, la composición bacteriana parece ser bastante similar entre tramos con un predominio de taxa pertenecientes a las Alphaproteobacterias, Betaproteobacterias y Gammaproteobacterias. Ahora nos falta profundizar en el análisis y determinar cuáles son los determinantes intrínsecos y extrínsecos de esta composición bacteriana. Bien pudiera ser que la colonización del mucus de los peces no estuviera condicionada por las bacterias presentes en el medio si no por las presentes en otros peces, es decir, transferencia horizontal más que transferencia del ambiente. Nos falta incorporar los datos de estructura de la comunidad de peces y también los de patología de algunos de los peces muestreados. Estos presentaban ulceraciones y los cultivos microbiológicos han detectado colonias compatibles con bacterias filamentosas del tipo

Flavobacterium columnare. Además, durante este tiempo hemos establecido una colaboración con el Dr Jordi Bosch del Museo de Ciencias Naturales de Madrid-CSIC y vamos a intentar detectar la presencia del quitridio *Batrachochytrium dendrobatidis* en los peces gracias a la financiación propia del Dr Bosch.

Esperamos que esta publicación sirva para conocer cuáles son las bacterias comensales comunes de dos especies de peces nativas típicas de ríos mediterráneos, el barbo de montaña (*B. meridionalis*) y la bagra catalana (*S. laietanus*), y cuáles son las condiciones ambientales que las favorecen a fin de poder desarrollar estrategias de conservación antes que la disbiosis tenga efectos dramáticos sobre la conservación de las especies de peces. Asimismo, pensamos hacer un experimento de laboratorio complementario, con gastos a nuestra cuenta fuera de la Fundación Barcelona Zoo, pero que evidentemente beneficiará los resultados del presente proyecto. El objetivo es confirmar que la transferencia horizontal de bacterias, ya sea comensales o patógenas, se ve alterada por las condiciones ambientales. Es decir, que la introducción de especies de peces exóticos invasores puede tener un impacto negativo sobre las especies de peces nativas poco visible hasta la fecha en forma de este trasvase de bacterias polizones con consecuencias difícil de predecir a día de hoy para las poblaciones de nativas de peces.

4. Publicaciones en formato revista anexadas

A continuación se anexan las publicaciones resultantes de este proyecto en formato PDF tal y como aparecen en las revistas científicas donde se encuentra el trabajo original. Para el caso de los manuscritos en proceso de revisión adjuntamos el manuscrito en su última versión pero sin las figuras por ser esta la manera acordada con el resto de co-autores por razones de confidencialidad que escapan al criterio del investigador principal del presente proyecto (Dr Maceda). Para el último artículo, todavía no disponemos de manuscrito como tal pues estamos en fase de un análisis más exhaustivo de los datos.

Este informe que agrupa los resultados de las cinco publicaciones resultantes de este proyecto financiado por la Fundación Barcelona Zoo no tiene lista bibliográfica propia, al estar ésta la misma que se ha utilizado para cada uno de los trabajos científicos resultantes, evitándose así duplicar la información excesivamente. Junto con los artículos aquí presentados, los investigadores han hecho divulgación de los artículos aquí presentados a través de su canal de twitter @AMacedaVeiga o de la página web de sus instituciones (Institut de Recerca en Biodiversitat, IRBio) y Universitat de Barcelona vía su gabinete de prensa. En todos los artículos aquí mencionados aparece la Fundación Barcelona Zoo en los agradecimientos.

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Fine-scale determinants of conservation value of river reaches in a hotspot of native and non-native species diversity

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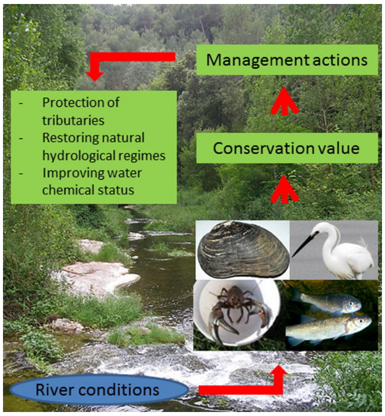
HIGHLIGHTS

- A moderate relationship was observed among indicators of conservation value.
- Protected areas offered limited coverage to imperilled freshwater fauna.
- River tributaries were identified as native fish refugees.
- Restoring water quality and the natural hydrological regime are priority tasks.
- Multiple components of diversity should be examined in resource management.

GRAPHICAL ABSTRACT



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ABSTRACT

Global freshwater biodiversity is declining at unprecedented rates while non-native species are expanding. Examining diversity patterns across variable river conditions can help develop better management strategies. However, many indicators can be used to determine the conservartion value of aquatic communities, and little is known of how well they correlate to each other in making diagnostics, including when testing for the efficacy of protected areas. Using an extensive data set (99,700 km^2, $n = 530$ sites) across protected and unprotected river reaches in 15 catchments of NE Spain, we examine correlations among 20 indicators of conservation value of fish communities, including the benefits they provide to birds and threatened mammals and mussels. Our results showed that total native fish abundance or richness correlated reasonably well with many native indicators. However, the lack of a strong congruence led modelling techniques to identify different river attributes for each indicator of conservation value. Overall, tributaries were identified as native fish refugees, and nutrient pollution, salinization, low water velocity and poor habitat structure as major threats to the native biota. We also

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Threatened taxa
Natura 2000
Environmental degradation

found that protected areas offered limited coverage to major components of biodiversity, including rarity, threat and host-parasite relationships, even though values of non-native indicators were notably reduced. In conclusion, restoring natural hydrological regimes and water chemical status is a priority to stem freshwater biodiversity loss in this region. A complementary action can be the protection of tributaries, but more studies examining multiple components of diversity are necessary to fully test their potential as fluvial reserves in Mediterranean climate areas.

1. Introduction

Biodiversity loss is occurring at unprecedented rates driven by global change (Foley et al., 2005; Halpern et al., 2008; Tittensor et al., 2014). Although global change effects are visible across a wide range of habitats, freshwater ecosystems are particularly affected (Strayer and Dudgeon, 2010). A good example are Mediterranean rivers, where many endemic species live and some of them are at the brink of extinction (Smith and Darwall, 2006; Marr et al., 2010). Human pressure in the Mediterranean area date back to ancient times when humans settled along main rivers and began to exploit water and biological resources, including on the riverbanks (Hooke, 2006). This pressure intensified with modern civilisations that also diversified the type of impacts, including emergent pollutants (Petrovic et al., 2011; Kuzmanović et al., 2015) and the release of non-native species (Leprieur et al., 2008a; Cobo et al., 2010). The situation is expected to worsen due to climate change and human population growth (Vörösmarty et al., 2010); therefore, conservation of freshwater diversity and the goods and services they provide to society requires urgent management actions.

Protected areas are considered as a mainstay of biodiversity conservation as well as contributing to human well-being (Gaston et al., 2008). In rivers, the most effective conservation strategy is proposed to be framed at the basin scale (Allan et al., 1997; Saunders et al., 2002; Linke et al., 2012). This framework considers that basins are biogeographic units (Doadrio, 1988; Reyjol et al., 2007), and that rivers are linear systems through which major threats to freshwater diversity such as pollution can easily propagate (Allan et al., 1997; Nel et al., 2007). Environmental quality standards have been proposed at the basin scale driven by international legislation, such as EU's River Basin Management Plans (Directive 2000/60/EC). At this scale, however, a strict protection is unrealistic. It generates many socio-economic conflicts and is logistically unfeasible for large basins (Saunders et al., 2002); therefore, river reaches need to be prioritised according to their conservation value (Margules and Usher, 1981; Filipe et al., 2004; Hermoso et al., 2015). Nevertheless, this raises the question of which are the best indicators to assess the conservation value of a community.

Traditionally, conservation priorities have been based on indicators such as species richness, rarity, and threatened status (Margules and Usher, 1981). The threatened status is often based on the International Union for the Conservation of Nature (IUCN) Red List (http://www.iucnredlist.org/). However, the conservation status of a species can be unknown or vary across regions due to discrepancies in classifications; for example, the river blenny *Salaria fluviatilis* is listed as least concern in the IUCN Red list and as endangered in the Spanish Red Data Book (Doadrio et al., 2011). Therefore, the focus on international criteria can bias setting conservation priorities at the national level; the target of most conservation actions since they are more politically than biogeographically driven (O'Riordan and Stoll-Kleeman, 2002; Battisti and Fanelli, 2015). Likewise, prioritising rarity to reduce extinction risk may leave unprotected species with a less restricted distribution, including species of major importance for other threatened taxa as food source (e.g. Ruiz-Olmo et al., 2001; Lopes-Lima et al., in press) or for the functioning of the fluvial ecosystem (Winfield and Townsend, 1991; Flecker et al., 2010). Thus, the ideal conservation action would be one that secures threatened species while maximising the protection of species diversity at the basin scale.

Since a major ecological rule is that biodiversity increases with surface area (Lomolino, 2000; but see Allouche et al., 2012), and river size increases downstream (Strahler, 1964), protecting downstream areas could maximise the number of species protected at the basin scale. However, these reaches are usually neighboured by large urban areas and hence the most disturbed, including the presence of non-native species (Marchetti et al., 2004; Closs et al., 2015). As biological invasions pose a significant threat to biodiversity and ecosystem services (Vilà et al., 2009; Simberloff et al., 2013), the presence of non-native species may jeopardise conservation goals in rivers. Studies examining diversity patterns help identify hotspots of high conservation value, but also the mechanisms behind these patterns (Baselga, 2010; Gutiérrez-Cánovas et al., 2013). For instance, if turnover dominates diversity patterns, it suggests that stress generates new communities in which tolerant species may replace those sensitive (Baselga, 2010). In contrast, if species poor sites are a subset of species of those enriched (high degree of nestedness), it suggests that stress causes a progressive loss of sensitive species and that conservation efforts may focus on species rich sites (Baselga, 2010). However, hotspots of native species richness may not be congruent with rarity or threat (Orme et al., 2005; Collen et al., 2014), further increasing the complexity of setting conservation targets.

In this study, we examine indicators that can be used to determine the conservation value of fauna across 15 catchments (99,700 km^2) in the Western Mediterranean area, a world hotspot of biodiversity (Myers et al., 2000) but also highly prone to biological invasions (Leprieur et al., 2008a). The selected basins typify common threats to other Mediterranean-type rivers, including pollution, overharvesting, hydrological alterations, and riparian removal (Moyle et al., 2011). We mainly focus on fish because the distribution of many native species has markedly declined worldwide (Closs et al., 2015), including in the study area (Maceda-Veiga et al., 2010). Firstly, we used pair-wise correlations to test whether one indicator of conservation value could act as surrogate of the others to plan management actions, including measures of fish species diversity, rarity, and nativeness plus indicators of conservation value of fish for other fauna, such as host for freshwater mussels or prey for mammals and birds. Secondly, we tested whether current protected areas meet conservation indicators of the aquatic fauna because they were designed primarily to protect terrestrial taxa (Filipe et al., 2004; Lawrence et al., 2011; Hermoso et al., 2015). Finally, we examined relationships between these indicators of conservation value, and geographical, water and habitat variables to identify the river attributes in which management policies can act to enhance the conservation value of fish communities. These river attributes were further confirmed via a fish community analysis, which also identified the mechanisms behind community variation across rivers and their conditions.

2. Materials and methods

2.1. Study area

We assembled environmental and fish data from our own surveys performed in NE Spain from 2002 to 2009 (Maceda-Veiga et al., 2010; Maceda-Veiga and De Sostoa, 2011; Figuerola et al., 2012, and unpublished data). Briefly, this data set comprised 530 sampling sites that involved all Catalonian catchments from the Muga to Riudecanyes basins, plus the complete River Ebro and part of the Garonne basin (Fig. 1). Our

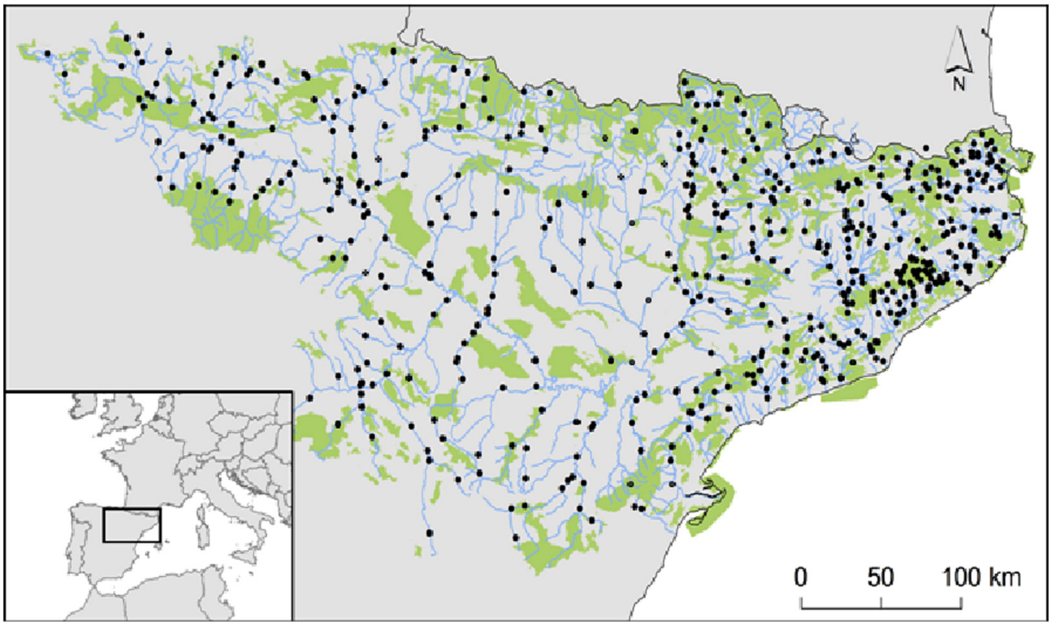

Fig. 1. Location of the 530 sampling sites surveyed for the current study in NE Spain with protected areas highlighted in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

data set accounted for all river typologies present in this region in terms of hydrological alterations, riparian characteristics, geology, water quality and flow. Most of these rivers are small and follow a typical Mediterranean hydrological regime, with severe droughts in summer and torrential floods in autumn. Large rivers, however, peak in flow in spring because of snowmelt. We surveyed in low flow conditions because this is when fish populations are more stable and can be properly sampled using electrofishing (see below). Low flow conditions also represent more intense stressful conditions in Mediterranean rivers (Gasith and Resh, 1999) and consequently, we could better identify the environmental drivers of fish fauna.

Sampled fish included species exclusively found in Iberian rivers (endemisms), such as the Ebro barbel (*Luciobarbus graellsii*) and the Iberian red-fin barbel (*Barbus haasi*), in addition to some of the world's worst invasive species, such as the largemouth bass (*Micropterus salmoides*) and the common carp (*Cyprinus carpio*). Only strictly freshwater fish were included in our analyses with the exception of the critically endangered European eel (*Anguilla anguilla*). We did not include brackish species (e.g. mugilids) that mostly occur in coastal lagoons or river mouths, which are more influenced by marine than freshwater conditions.

Other fauna included threatened freshwater mussels that depend on fish as host to complete their life cycle (Lopes-Lima et al., in press) and piscivorous animals, such as the European otter (*Lutra lutra*) and many waterbirds, including the common kingfisher (*Alcedo atthis*), the Grey heron (*Ardea cinerea*) and cormorants (*Phalacrocorax carbo*). We did not survey them but used their potential distribution in our study area (e.g. Palomo et al., 2007; Lopes-Lima et al., in press; SEO Birdlife, 2012a,b) to estimate the conservation value of fish for freshwater mussels, mammals and birds.

2.2. Fish survey

We followed an international standardised fish sampling method (CEN standards EN 14962 and EN 14011), as driven by the European Water Framework Directive. Fish were sampled by a single-pass electrofishing using a portable unit which generated up to 200 V and 3 A pulsed D.C in an upstream direction, covering the whole wetted width of the 100-m long reaches surveyed at each location (see also Maceda-Veiga et al., 2010; Benejam et al., 2012). We selected the location of each sampling site based on accessibility and representativeness, including a variety of habitat types (pools, rifles and runs). The same equipment was used across sites to avoid bias in fish captures (Benejam et al., 2012), and the crew had a standardised time devoted to the electrofishing passes according to their own experience and the reach features. Fish captures were standardised to captures per unit of effort (CPUE - fish abundance divided by fishing time in minutes and the area surveyed in square meters). Although sites were only surveyed once due to the vast geographical area covered, the methodological consistency across sites should accurately reveal relative changes in fish abundance or richness depending on river conditions. Our estimates of species richness and abundance from 4-pass electrofishing were reasonably high with 80–100% of the species detected and 50–90% of the individuals captured (A. Sostoa, unpublished data).

Fish were identified to species level, counted, and released in each site. Species nomenclature was updated from previous studies (Maceda-Veiga et al., 2010; Maceda-Veiga and de Sostoa, 2011) after an exhaustive examination of recent literature (Doadrio et al., 2011; Aparicio et al., 2013) and fish collections at the Natural History Museum of Madrid, Spain. Fish species were defined as non-native if they did not historically occur in a basin and in Spain, and as translocated if their presence is the result of an introduction from another basin within Spain where they are native (Table 1). Non-native and translocated species were grouped as introduced.

2.3. Indicators of conservation value

We calculated 20 indicators of conservation value to describe the fish community at each sampling site along the 15 basins surveyed in NE Spain. We defined an indicator of conservation value as any trait of the fish community composition that can be used to determine its conservation interest and guide management strategies (e.g. presence of threatened and non-native species, overall native richness). We first calculated the total abundance (captures per unit of effort) and richness for native, non-native, translocated and introduced fish species separately. We then calculated the proportion of native, non-native and translocated species in relation to the total fish abundance and richness in each site as a measure of the degree of nativeness and invasiveness of the fish community. We also calculated the number of species listed as the world's worst invaders (http://www.issg.org/). To better determine

Table 1
Occurrence (%) of freshwater fish species in NE Spain ($n = 530$ sites) with indication of their distribution (endemic, native, and non-native) and threatened status (catalogued as endangered in the IUCN red list, Habitats Directive, Spanish legislation or the Red data book of fish), the presence of spawning migratory behaviour in native fish, and the value of all fish species for waterbirds, threatened mussels and mammals (see methods for further details).

Scientific name	Occurrence	Threatened status	Distribution	Migratory	Mussel host	Piscivorous
Achondrostoma arcasii	**4.72**	Endangered	Endemism	No	No	No
Anguilla anguilla	11.51	Endangered	Native	Yes	Yes	No
Barbatula quignardi	10.5	Not endangered	Native	No	No	Yes
Barbus haasi	26.06	Endangered	Endemism	No	Yes	Yes
Barbus meridionalis	15.66	Endangered	Native	No	Yes	Yes
Cobitis calderoni	**2.45**	Endangered	Endemism	No	Yes	No
Cobitis palludica	**0.19**	Endangered	Endemism	No	Yes	No
Cottus hispaniolensis	**0.57**	Endangered	Native	No	No	No
Gobio lozanoi	19.06	Not endangered	Native	No	Yes	Yes
Luciobarbus graellsii	28.11	Not endangered	Endemism[t]	Yes	Yes	Yes
Parachondrostoma miegii	21.89	Endangered	Endemism[t]	Yes	Yes	Yes
Phoxinus bigerri	24.34	Not endangered	Native	No	Yes	Yes
Salaria fluviatilis	**4.53**	Endangered	Native	No	Yes	No
Salmo trutta	34.34	Not endangered	Native[t]	Yes	Yes	Yes
Squalius laietanus	16.23	Not endangered	Native	Yes	Yes	No
Gasterosteus aculeatus	**0.94**	Endangered	Native	No	Yes	No
Alburnus alburnus	9.06	–	Non-native	No	Yes	Yes
Ameirus melas	0.75	–	Non-native	No	Yes	Yes
Barbatula barbatula	1.5	–	Non-native	No	No	Yes
Carassius auratus	1.51	–	Non-native	No	No	No
Cyprinus carpio	14.91	–	Non-native[w]	No	Yes	Yes
Esox lucius	0.38	–	Non-native	No	Yes	No
Gambusia holbrooki	2.45	–	Non-native[w]	No	Yes	No
Gobio occitaniae	2.45	–	Non-native	No	Yes	No
Gobio spp.	5.40	–	Non-native[*]	No	Yes	No
Lepomis gibbosus	4.15	–	Non-native	No	Yes	No
Micropterus salmoides	1.32	–	Non-native[w]	No	Yes	No
Oncorhynchus mykiss	1.89	–	Non-native	No	No	No
Phoxinus spp.	6.60	–	Non-native[*]	No	Yes	No
Pseudorasbora parva	0.19	–	Non-native	No	No	No
Rutilus rutilus	0.38	–	Non-native	No	Yes	No
Sander lucioperca	0.38	–	Non-native	No	Yes	No
Scardinius erythrophthalmus	3.4	–	Non-native	No	Yes	No
Silurus glanis	1.89	–	Non-native	No	No	No

[t] translocated native species in some catchments of NE Spain.
[w] Listed as world's worst invasive species (http://www.issg.org/).
[*] Introduced taxa pending of genetic studies to confirm species identify in some catchments of Catalonia.

the contribution of native and non-native species richness in fish diversity hotspots, we also calculated the ratio between the current native species present in each site and the total number of native species historically occurring in each basin following Doadrio et al. (2011).

To assess the conservation value of fish communities based on their endangerment degree, we calculated the number of species in each site using three classifications: the IUCN red list of globally endangered species (http://www.iucnredlist.org/), the red data book of the fish of Spain (Doadrio et al., 2011), and the list of fish species protected by Spanish legislation (Real Decreto 139/2011). A species was considered as threatened if it was catalogued as 'critically endangered', 'endangered' or 'vulnerable'. We also considered the fish species listed in Annexes of the EU's Habitats Directive, which denotes threatened species at the European level. As a regional fish conservation index, we used the scores of species provided by Maceda-Veiga et al. (2010) that were summed to describe the conservation value of each location. Finally, we calculated the number of species whose range occupied <5% of our sites as a measure of rarity, and the number of native species exclusively found in Spain as a measure of endemicity (Table 1).

To further determine the importance of the fish fauna in each site, we calculated total richness of migratory species (Doadrio et al., 2011, Table 1). This indicator informs about river connectivity, as migratory species play a major role in energy transfer along rivers (Flecker et al., 2010). We also calculated the proportional abundance of fish suitable as hosts for freshwater mussels (see Lopes-Lima et al., in press in relation to the total fish abundance in each site (Table 1). Freshwater mussels that use fish as hosts for their larvae are worldwide-threatened taxa (Strayer et al., 2004; Lopes-Lima et al., in press). For this analysis, we only considered basins where we had historical evidence of occurrence of freshwater mussel species (see Lopes-Lima et al., in press). As fish are a key food item for the European otter and many waterbird species, we also calculated the total abundance of potential preferred prey based on diet studies of these consumers in our study area (e.g. Lekuona and Campos, 1997; Ruiz-Olmo et al., 2001; Vilches et al., 2012).

As a by-product of electrofishing, we also captured the red-swamp crayfish (*Procambarus clarkii*) and the signal crayfish (*Pacifastacus leniusculus*). Although both non-native species are a potential valuable food resource for mammals and waterbirds (Tablado et al., 2010), both cause several ecological impacts due to their trophic and non-trophic activities (Gherardi, 2006; Carvalho et al., 2016). Thus, the abundance (expressed as CPUE) of the two crayfish species was also included as a neutral indicator of conservation value in our analyses.

2.4. Geographical and environmental descriptors

We characterised each sampling site with 27 variables related to geography, habitat quality and water properties.

As geographical features, we recorded the basin name and elevation (m.a.s.l.) in each sampling site using Google Earth®. Elevation was used as a surrogate for the position of the sampling site in the river, and summarise the role of natural spatial gradients in fish indicators, as previously validated in this region (Maceda-Veiga et al., 2013; Murphy et al., 2013). We also calculated the Strahler stream order number on a map (1:50,000) as a measure of river size. It ranks rivers from a small, first order tributary all the way to the largest main river based on a hierarchy of tributaries. Strahler stream order number is directly proportional to relative watershed dimensions, channel size and stream discharge at that place in the system (Strahler, 1964). Because stream order number is dimensionless, two drainage basins differing greatly in linear scale can be easily compared with respect to corresponding points into their geometry.

Prior to each fish survey we analysed 7 water quality variables in situ. A digital multiparametric YSI® sonde was used for temperature (°C), conductivity (μS/cm) and pH, and the colourimetric test kit VISOCOLOR® for ammonium (NH_4^+, mg/l; detection limit (dl) = 0.2 mg/l), nitrite (NO_2^-, mg/l; dl = 0.02 mg/l), nitrate (NO_3^-, mg/l; dl = 1 mg/l) and phosphate (PO_4^{3-}-P, mg/l; dl = 0.2 mg/l) concentrations. To characterise habitat quality, we incorporated 17 variables from two widely used habitat quality indices in this region: the riparian vegetation quality index QBR (Munné et al., 2003), and a version of the U.S. Rapid Bioassessment (RBA) protocol (Barbour et al., 1999) for Mediterranean rivers. Briefly, RBA ranked 10 features of the local habitat (habitat structure, habitat diversity, river channelization, channel morphology, water flow, degree of silting, erosion of river margins, macrophyte coverage, and the coverage and width of riparian canopy) on an ordinal scale of 1–10 for RBA and 0–25 for QBR (score increases with quality). RBA includes more variables related to physical habitat for aquatic fauna than the QBR (total vegetation cover and structure, vegetation cover quality, and river channel alterations) but both consider the status of riparian vegetation.

To assess whether the sampling site was located in a protected area, we combined the layers of regional protected areas and the Natura 2000 network from the Autonomous Government of Catalonia, the Ebro Water Authority and the Spanish Government with the layer containing all our sampling points using the Geographical Information System (GIS) software ArcGis®. Subsequently, we obtained a matrix with our sampling points and the value of the landscape attribute, resulting in 27% of the sampling sites within protected areas ($n = 139$).

2.5. Statistical analyses

All statistical analyses were performed in R v.2.15.3 (R Core Team, 2013) using the libraries 'MASS' (Venables and Ripley, 2002), 'vegan' (Oksanen et al., 2015), 'lme4' (Bates and Maechler, 2009), 'car' (Fox and Weisberg, 2011), and 'betapart' (Baselga and Orme, 2012) and the functions outlined below. Spearman rank correlation (ρ) was used to examine congruence among indicators of conservation value in fish communities. Correlation coefficients around 0.1 were considered to be weak, 0.3 as moderate, 0.5 as moderately strong, and 0.7 and above as strong (modified from Lamoreux et al., 2006 and Tisseuil et al., 2013).

To assess if protected areas fulfil conservation values, we compared values of the 15 least correlated indicators of conservation value ($\rho < 0.7$) between protected and unprotected areas using a series of generalised linear mixed models (GLMMs) with the function 'lmer'. Basin was included as random factor in GLMMs to account for spatial autocorrelation of sites within each basin. Sampling year was also included as random factor to control possible inter-annual variation in fish captures, but it was removed from the final models because it did not influence the significance of predictors, as reported in a sub-set of the current data-set (Murphy et al., 2013).

To test whether indicators of conservation value identify the same river attributes, we determined relationships between the least correlated indicators of conservation value and the predictors related to geography, water and habitat quality (Appendix S1) using a series of GLMMs with the function and random term stated above. Elevation was included as fixed factor in models to account for the longitudinal position of the reach in the stream, and the role of spatial gradients in the fish community structure. Main and interactive effects of Strahler stream order number with elevation were also included to account for the differences in the conservation value of tributaries and main rivers at different elevations. For each indicator of conservation value we built a saturated model (containing all predictors) and followed a manual step-wise deletion of non-significant terms (Crawley, 2007; Zuur et al., 2009). Significance of predictors in GLMMs was tested using a likelihood-ratio test within the function 'Anova'. The comparison of nested models (models with and without a predictor) via a likelihood-ratio test led to the same minimum adequate model.

Final models were validated via diagnostic plots of model residuals against fitted values and against each predictor, Q-Q plots of model residuals and the Cox statistic to verify the assumptions of normality, homoscedasticity and detect unduly observations following Zuur et al. (2009) and Thomas et al. (2015). Relationships between each indicator and the selected or excluded predictors were also visually inspected to further determine their relevance. Log-transformation was applied to continuous predictors and arcsine squared root transformation to proportions to increase model fitting and meet statistical assumptions. Although we are aware of a vibrant debate on "the best" model selection procedure, all have pros and cons (e.g. Aho et al., 2014; Cade, 2015; Leek and Peng, 2015) and we considered a backward stepwise procedure is appropriate in our case given the clear effects of the selected predictors on the response variables and their ecological relevance.

To further determine the role of geography, habitat quality and water properties in the fish community, we examined relationships among the composition of the fish community (presence/absence) and the 15 least correlated predictors used in the GLMMs. For this analyses, predictors were grouped in three sets: (i) geographical features (basin, elevation and the Strahler river order number), (ii) water properties (conductivity, pH, concentrations of ammonia, nitrites, nitrate, and phosphate), and (iii) habitat quality (water velocity, aerial coverage, riparian coverage, habitat diversity, and macrophyte coverage, percentage of dead wood, and channel morphology. The variation in community composition attributed to each of these three sets of predictors was computed using variation-partitioning analyses (VP). Whilst causality cannot be determined in observational studies, VP decomposes the variation of dependent variables in independent and joined effects of a set of predictors (Borcard et al., 1992).

To determine the mechanisms behind community variation across sites, we used Baselga's method (2010) that decomposes total dissimilarity (i.e. beta diversity) in the community into its turnover (species replacement) and nestedness-resultant components (species loss). The relationship between community variation (either turnover or nestedness-resultant dissimilarity) and the predictors was assessed using distance-based Redundancy Analyses (db-RDA, function 'capscale' in R). We ranked the predictors within each group according to their unique explained variance (from greater to the least), introduced them in db-RDA models in this order, and tested for significance using the function 'anova.cca'. Only significant predictors were retained to avoid overfitting due to the inclusion of non-significant terms. Finally, we ran series of db-RDA models containing all combinations of the selected predictors per set to estimate the unique and shared fractions of explained variation. Significance was reached at $P \leq 0.05$ in all statistical procedures.

3. Results

We found 16 native and 18 non-native fish species in rivers of NE Spain, including two introduced uncertain taxa (*Phoxinus* spp. and *Gobio* spp.), and translocated native species from the Ebro basin (Table 1). Threatened species at national and international levels represented 56% of the fish fauna, including all endemic fish species (Table 1). However, five out of six are not listed as threatened at the European level (EU's Habitats Directive), even though they occurred in ≤5% of the sampling sites (Table 1).

3.1. Relationships among the indicators of conservation value and their coverage by protected areas

Most pair-wise correlations among native-fish indicators of conservation value were moderately strong ($\rho \sim 0.5$) to moderate ($\rho \sim 0.3$). The strongest positive relationships ($\rho \geq 0.7$) occurred between richness

Table 2
Spearman rank correlation coefficients (ρ) among 20 indicators of conservation value used to describe the fish community of rivers in NE Spain (n = 530). Note that most indicators of conservation value were not strongly correlated to each other ($r \geq 0.70$ in bold). See methods for further details.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. Total native fish abundance																			
2. Total native fish richness	0.67																		
3. Endemic fish richness	0.49	0.61																	
4. IUCN threatened fish richness	0.36	0.32	0.47																
5. Local conservation index	0.63	**0.93**	**0.71**	0.35															
6. Legally threatened fish in Spain	0.28	0.35	0.29	0.28	0.31														
7. Spanish Red Book fish	0.40	**0.72**	0.10	0.08	0.64	0.20													
8. EU's threatened fish species	0.20	0.24	−0.21	0.13	0.16	0.29	0.45												
9. Rare fish richness	0.23	0.35	0.22	0.16	0.36	0.62	0.35	0.06											
10. Migratory fish richness	0.41	**0.74**	0.34	0.15	0.68	0.12	**0.80**	0.15	0.18										
11. Historical versus actual richness	0.40	0.19	0.02	0.32	0.15	−0.09	0.22	0.35	−0.03	0.12									
12. Non-native fish abundance	0.01	0.19	0.12	0.04	0.25	0.12	0.27	0.05	0.21	0.14	−0.29								
13. Non-native fish richness	0.01	0.22	0.15	0.06	0.28	0.14	0.30	0.04	0.23	0.18	−0.29	**0.96**							
14. Traslocated fish abundance	−0.13	−0.07	−0.06	0.00	0.09	−0.06	0.21	0.28	−0.06	0.14	0.08	0.14	0.13						
15. Traslocated fish richness	−0.08	−0.09	0.01	0.06	0.14	−0.04	0.15	0.21	−0.03	0.10	0.00	0.17	0.17	**0.90**					
16. Introduced fish abundance	−0.05	0.12	0.05	0.00	0.25	0.07	0.34	0.18	0.15	0.18	−0.19	**0.81**	**0.80**	0.60	0.54				
17. Introduced fish richness	−0.01	0.15	0.13	0.07	0.30	0.10	0.33	0.11	0.19	0.19	−0.26	**0.86**	**0.88**	0.45	0.53	**0.91**			
18. Worst invasive fish richness	0.18	0.42	0.37	0.04	0.48	0.20	0.42	0.00	0.20	0.25	−0.25	0.65	0.68	0.02	0.08	0.49	0.59		
19. Preferred fish prey	**0.94**	0.61	0.48	0.34	0.62	0.24	0.41	0.22	0.19	0.41	0.32	0.15	0.14	0.07	0.09	0.15	0.15	0.25	
20. Mussel hosts	0.48	0.43	0.32	0.36	0.47	0.04	0.36	0.45	0.03	0.21	0.61	0.07	0.07	0.22	0.17	0.16	0.13	0.08	0.47

and abundance-based indicators, and between total native richness and that of migratory and threatened species in the Spanish Red Book (Table 2). A highly positive relationship was also found between total native richness and the local conservation index score, which also correlated well with endemic species richness (Table 2). However, total native richness was moderately related to that of threatened species according to the IUCN, European and Spanish legislation (Table 2). The latter only correlated well with the richness of rare species. A moderately strong relationship also occurred between total native richness and the number of potentially preferred prey for birds and mammals and world worst invasive species (Table 2). Hotspots of introduced and non-native fish species correlated well to each other. However, both were weakly related to mussel hosts, as opposed to native fish indicators, including the current hotspots of richness in relation to the historical fish occurring in each basin (Table 2).

The lack of strong congruence ($\rho \geq 0.7$) among the majority of indicators suggests that factors affecting them differ markedly, including the effect of protected area (Table 3). The abundance and total richness of native fish species, together with the proportion of native fish species, increased in protected compared to unprotected areas (Table 3). Protected areas also had a higher abundance of potential preferred fish prey for birds and mammals, which was strongly correlated with total native fish richness (Table 2). Protected status was negatively associated with all indicators related to introduced fish species (Table 3) but neutral for the two introduced crayfish species. This was also the case of the indicator related to threatened mussels and most indicators of the native fish fauna, including number of threatened and rare species (Table 3).

3.2. Influence of geography, habitat quality and water properties on the indicators of conservation value

The relative influence of geography, habitat and water quality variables varied with the indicators of conservation value (Table 4). Overall, both native and introduced fish indicators were negatively related to elevation. A negative relationship was also found between native fish indicators and nutrient pollution (ammonium, nitrites, nitrate and phosphate), whereas introduced-fish indicators increased in reaches

Table 3
Estimates and their associated standard errors and tests for the effect of protected area on indicators of conservation value of river reaches in NE Spain (n = 530). The direction of the effect on each indicator is shown based on the sign of estimate and significance (at $P \leq 0.05$) in generalised linear mixed models.

	Estimate	SE	χ^2	P value	Effect
Native fish					
Total native fish abundance (Nat abun)	0.20	0.05	15.46	<**0.001**	**Positive**
Total native fish richness (Nat rich)	0.12	0.05	4.90	**0.02**	**Positive**
Endemic fish richness (End rich)	0.04	0.09	0.18	0.66	Neutral
IUCN threatened fish richness (IUCN rich)	0.09	0.12	0.65	0.41	Neutral
Legally threatened fish in Spain (Leg Spain)	−0.15	0.25	0.33	0.56	Neutral
EU's threatened fish species (Leg Habitats)	0.04	0.18	0.04	0.85	Neutral
Rare fish richness (Rare rich)	0.29	0.27	1.19	0.28	Neutral
Historical versus actual richness (Hist rich)	0.33	0.04	62.86	<**0.001**	**Positive**
Ratio native:total fish richness (Ratio nat rich)	0.05	0.03	4.26	**0.03**	**Positive**
Introduced fish					
Non-native fish richness (Non-native rich)	−0.86	0.24	12.57	<**0.001**	**Negative**
Traslocated fish richness (Tras rich)	−1.19	0.23	27.06	<**0.001**	**Negative**
Worst invasive fish richness (Worst inv. rich)	−0.42	0.18	5.43	**0.02**	**Negative**
Ratio non-native:total fish richness (Ratio nnative rich)	−1.03	0.27	14.58	<**0.001**	**Negative**
Ratio introduced:total fish richness (Ratio intr rich)	−1.08	0.21	25.17	<**0.001**	**Negative**
Other categories					
Procambarus clarkii abundance (Proc abun)	−0.10	0.19	0.27	0.60	Neutral
Pacifastacus leniusculus abundance (Pacif abun)	0.45	0.29	2.33	0.13	Neutral
Mussel hosts	0.19	0.27	4.01	0.06	Neutral

Table 4
Predictors related to geography, habitat quality and water properties retained as having a significant effect (at $P \leq 0.05$) on the indicators of conservation value of fish communities in NE Spain according to generalised linear mixed models with basin as random factor (see methods for further details). The direction of effect is based on regression coefficients of predictors for each indicator of conservation value (+, positive; −, negative; +/−, inconsistent). See Table 3 for Acronyms and Appendix S2 for descriptive statistics of the predictors.

Indicators of conservation value	Elevation	Stream order	Aerial coverage	Channel morphology	Dead wood	Habitat diversity	Macrophytes	Riparian coverage	Water velocity	Ammonia-Nitrites	Conductivity	Nitrate	pH	Phosphate
Native fish fauna														
Nat abun	ns	ns	ns	ns	ns	−	+	ns	+	−	ns	ns	ns	−
Nat rich	−	ns	ns	ns	ns	+	+	ns	ns	−	ns	ns	+	−
End rich	−	+/−	ns	ns	ns	ns	+	ns	ns	−	+	ns	+	ns
IUCN rich	−	ns	−	ns	ns	ns	ns	ns	ns	ns	−	ns	ns	−
Leg Spain	ns	+/−	−	ns	ns	+/−	ns	ns	+	ns	+	ns	ns	−
Leg Habitats	ns	ns	−	ns	ns	ns	ns	ns	ns	−	+	ns	ns	ns
Rare rich	−	ns	ns	ns	ns	ns	ns	ns	+	−	+	ns	ns	−
Migr rich	ns	+	ns	ns	ns	+/−	ns	ns	ns	−	−	ns	+	ns
Hist rich	ns	−	ns	ns	ns	+/−	+	ns	ns	ns	ns	−	ns	ns
Ratio nat rich	ns	−	ns	+/−	ns	ns	+/−	ns	ns	ns	−	ns	−	ns
Introduced fish fauna														
Exo rich	−	+	ns	+/−	ns	ns	ns	ns	−	ns	+	ns	+	ns
Tras rich	ns	+	ns	ns	ns	+	ns	ns	ns	ns	ns	ns	ns	ns
Worst rich	−	+	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	+	ns
Ratio exo rich	−	+	ns	−	ns	ns	ns	ns	ns	ns	+	ns	+	ns
Ratio intr rich	ns	+	ns	−	ns	ns	−	ns	ns	ns	+	ns	+	ns
Other categories														
Proc abun	−	+	ns	ns	ns	−	ns	ns	ns	ns	ns	+	ns	ns
Pacif abun	ns	ns	+	+/−	+	+	ns	ns	ns	ns	−	+	+	ns
Mussels' hosts	ns	ns	ns	ns	ns	+	+	ns	ns	ns	+	ns	ns	ns

with low water velocity, low macrophyte coverage, and high pH and conductivity, as proxy of salinity (Fig. 2). Interestingly, the nativeness of fish communities declined with conductivity and river size, as defined by Strahler stream order (Table 4). Small streams typically had the most well-preserved native fish fauna (Appendix S3), including the largest proportion of native species historically present in each basin (Fig. 3). As for introduced fish, the abundance of the crayfish *P. clarkii* increased with river size and nitrate pollution (Table 4). The latter was also related to the presence of the crayfish *Pacifastacus leniusculus*, which was also associated with reaches with a higher habitat complexity and lower salinity (Table 4).

The total variation in the composition of fish communities ($R^2 = 24\%$) was mostly driven by geographical features (15%) followed by habitat quality (3%) and water properties (2%, Fig. 4). The total explained variance by the dissimilarity analysis (beta diversity) was higher, either in its turnover (69%) or nestedness-resultant component (79%). Geographical features made the largest contribution to variation in the two components of beta diversity, representing 35% for turnover and 39% for the nestedness-resultant dissimilarity (Fig. 4). However, the latter was more related to water (14%) and habitat quality (8%) than was turnover (7 and 4%, respectively), suggesting that disturbed river reaches had a subset of species of those enriched.

4. Discussion

Our intensive survey used 20 indicators of conservation value to assess fish communities and the associated benefits for other riverine taxa. We found that hotspots of native and introduced fish richness were weakly correlated, but that the former encapsulated at least reasonably well variation in one indicator of rarity and threatened status and the benefits of fish for mussels, birds and mammals. However, protected areas had a neutral effect on most native indicators, as opposed to the introduced ones, highlighting the need to increase their value for the former. We identified tributaries as native fish refugees, and nutrient pollution, salinization, low water velocity and poor habitat structure as major threats to the native biota at the basin scale.

4.1. Low congruence among most indicators of conservation value and the limited coverage of imperilled fauna by protected areas

Our results are consistent with previous data showing that regional and Natura 2000 protected areas did not markedly favour European and IUCN threatened aquatic taxa (Abellán and Sánchez-Fernández, 2015; Guareschi et al., 2015). Our study expands fish research in the Natura 2000 network by Hermoso et al. (2015) including regional protected areas and introduced species, and showing the congruence among 20 indicators of conservation value. Since many native indicators did not strongly correlate to each other, we suggest that the design of new protected areas should use indicators that balance richness and rarity, such as the conservation index by Filipe et al. (2004). In our study though, this index generated a similar pattern of total native fish richness, highlighting the difficulties of setting conservation priorities and nourishing the debate of what to conserve (Wilson et al., 2006; Polasky et al., 2008). This debate also applies to the use of richness or abundance data in ecological research (Brotons et al., 2004; Howard et al., 2014). Despite their strong correlation in our study, it is advisable that the former provides unique information, such as population viability (Morris et al., 2002).

In our study, total native fish richness and abundance also correlated reasonably well with the number of potential preferred fish prey for birds and mammals compared to mussel hosts. This can be attributed to the host specificity of mussel's larvae, which also varies across species (Lopes-Lima et al., in press). However, it may be related to the fact that this aspect of mussels' biology is still poorly studied (Lopes-Lima et al., in press). Differences in congruence among these indicators also resulted in a different coverage by protected areas, having a neutral effect on the mussel hosts and a positive effect on the prey for birds and mammals. These results support the notion that current protected areas were designed primarily to protect terrestrial taxa (Lawrence et al., 2011; Hermoso et al., 2015). Likewise, they illustrate that the protection of species interactions is largely neglected in conservation (Valiente-Banuet et al., 2015), even though freshwater mussels are one of the most imperilled faunal groups (Lopes-Lima et al., in press). For instance,

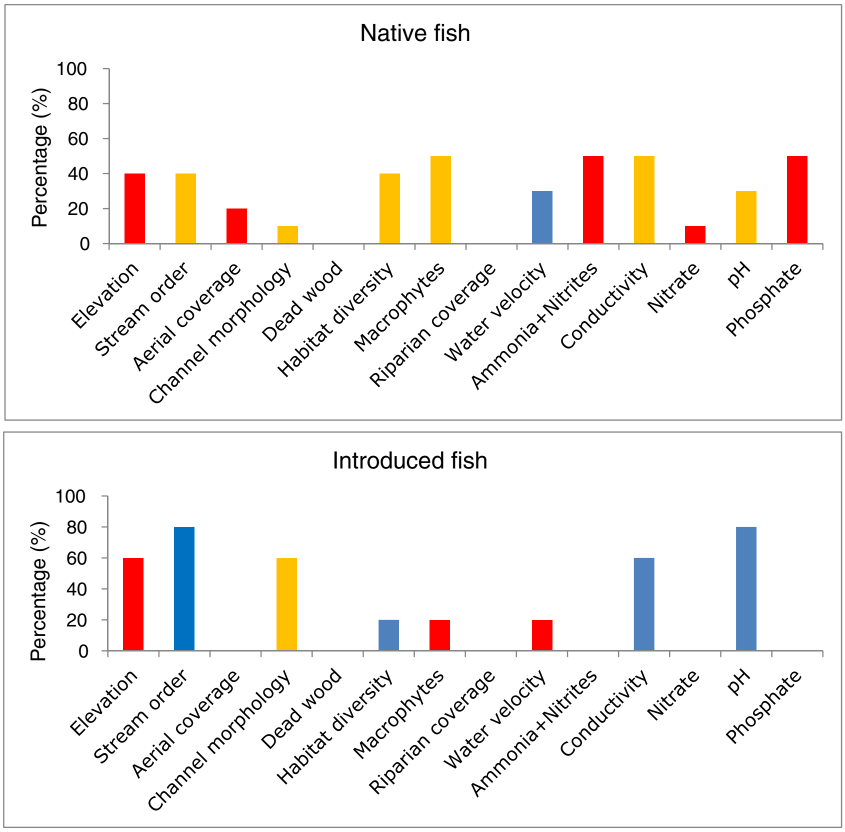

Fig. 2. Percentage of generalised linear mixed models (from Table 4) in which each predictor was retained as having a significant effect (positive: blue, negative: red, and both: orange) on indicators of conservation value related to native and introduced (non-native + translocated) fish species in river reaches of NE Spain. See Appendix S2 for predictor value ranges. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the river blenny (*Salaria fluviatilis*) is not listed as threatened in the IUCN but if left unprotected in Spain, the conservation of the highly threatened mussel *Margaritifera auricularia* can be seriously compromised. This blenny is the unique living host for the larvae of *M. auricularia*, a species that is only found in the Ebro basin (Araujo et al., 2001; Lopes-Lima et al., in press).

Although protected areas did not favour all indicators of threatened status in our study area, they had higher native and less introduced richness and abundance than unprotected areas. We are not aware of eradication or control campaigns for introduced fish in protected areas of NE Spain, therefore our results are likely to be attributed to the fact that protected areas restrict activities such as angling, which is a major pathway of fish introductions (Marr et al., 2010; Maceda-Veiga, 2013). Low human disturbance may also explain why protected areas had more native fish species. However, focal reach protection often does not guarantee good conditions for aquatic taxa, as upstream and downstream insults can jeopardise conservation goals (Nel et al., 2007; Linke et al., 2008). As in other Mediterranean-climate areas (Hermoso et al., 2013; Moyle, 2014), tributaries acted in our study as major refugia for native fish. We thus urge their protection to arrest the decline in native fish species observed in NE Spain (Maceda-Veiga et al., 2010) and especially for 38% of native fish species with a focal distribution. These protected areas could also act as green corridors for many terrestrial species (Baschak and Brown, 1995).

4.2. Management actions should also focus on improving water quality and restoring the natural hydrological regime

Besides the creation of fluvial reserves, we argue that the protection of aquatic fauna requires improving water chemical status, as reported in other Mediterranean regions (Petrovic et al., 2011; Moyle, 2014). Our results show that salinization (Cañedo-Argüelles et al., 2013) and nutrient pollution (e.g. nitrite, nitrate, phosphate) (Camargo and Alonso, 2006; Johnson et al., 2010) are two of the major threats to native fish. However, their interaction has to be considered in management practices. Salinity can alleviate nitrite toxicity to freshwater taxa (Alonso and Camargo, 2008; Noga, 2011) and be anti-parasitic, as reported in aquaculture (Noga, 2011; Maceda-Veiga and Cable, 2014). Likewise, nitrate can protect fish against some monogenean infections (Smallbone et al., 2016). Thus, it may happen that a partial removal of pollutants can worsen the status of a species, and highlights the urgent need of studies examining the context-dependence of effects of pollutant interactions on wild fish populations (Hamilton et al., 2015; Colin et al., 2016). This is particularly important given the complex mixtures of pollutants occurring in rivers (Petrovic et al., 2011; Kuzmanović et al., 2015; Hukari et al., 2016), including water quality hazards such as phosphates for which there is limited insight into their direct toxicity to aquatic taxa.

Poor water and habitat quality were also related in our study to the proliferation of introduced fish species. In particular, they were mostly found in reaches with low water velocity and altered channel morphology, including embankments and weirs, supporting the notion that the natural hydrology of Mediterranean rivers protects native fish (Marchetti et al., 2004; Kiernan et al., 2012). Restoring river connectivity is of major importance to allow migratory species (e.g. *A. anguilla*, *Luciobarbus graellsii*) access to fluvial reserves, although they may transport toxicants and diseases from downstream areas (see Flecker et al., 2010). However, restoring the natural hydrological regime may not control the spread of introduced species, as translocated native species, which represent 26% of fish introductions in our study, have evolved under the Mediterranean climate. Further, non-native species can occur in natural Mediterranean streams (Moyle, 2014; Closs et al., 2015), even though most species, including the worst invaders *Micropterus salmoides*, *Cyprinus carpio* and *Gambusia holbrooki*, perform

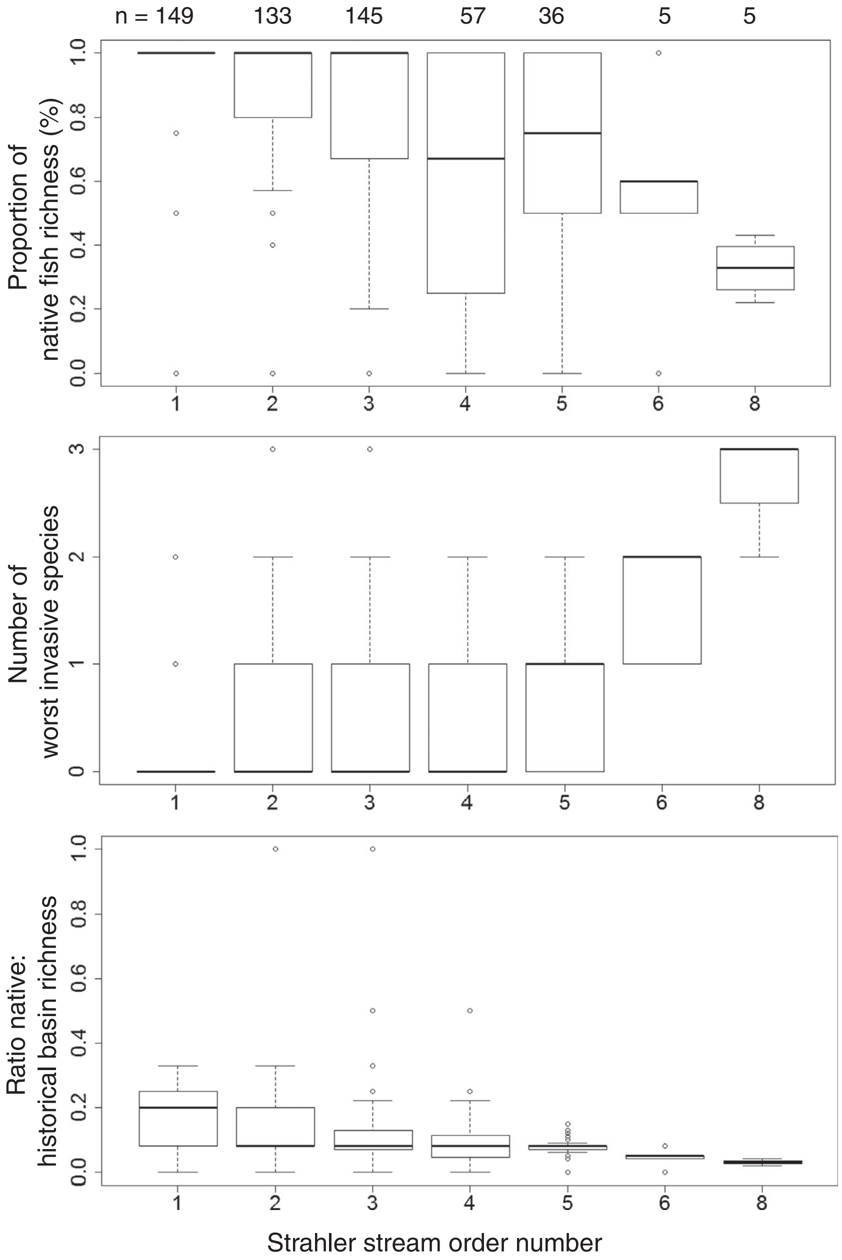

Fig. 3. Relationships between river size as defined by the Strahler river order number and the degree of nativeness of the fish community, including the number of world worst invasive fish species in river reaches of NE Spain (see methods for further details). The number of sampling sites per stream order is shown on the top, and details on fish species composition according to stream order are provided in Appendix S3.

better in low water velocities (Marr et al., 2010; Doadrio et al., 2011). Nonetheless, it is worth noting that their eradication may not be desirable, as long-term introduced species can be playing a key role in recipient communities (Schlaepfer et al., 2011).

Since *C. carpio* and *P. clarkii* were common non-native species in our study area, and profoundly alter aquatic ecosystems (e.g. bioturbation, macrophyte removal) (Gherardi, 2006; Shin-ichiro et al., 2009), it is likely that some of the associations found between introduced species and river conditions are partly explained by their activity. Whether induced by non-native species or not, according to our partitioning analysis of dissimilarity, environmental degradation seem to cause the loss of sensitive species in fish communities, as reported in aquatic invertebrates (Gutiérrez-Cánovas et al., 2013). In our study, the relationship between the nestedness-resultant dissimilarity and water and habitat degradation was poorer (8–14%) than that (31–51%) reported by Gutiérrez-Cánovas et al. (2013). However, our results support previous data on aquatic organisms showing a poor relationship between beta diversity (or related measures) and environmental conditions (Beisner et al., 2006; Heino et al., 2015). Although the reasons are not fully understood, the low explanatory power could be related to the presence of rare species (i.e. numerous absences in the site-by-site species matrix) (see Heino et al., 2015). However, it may also be attributed to the fact that aquatic ecosystems are highly dynamic and a single snapshot sampling of biota and abiotic conditions fails to reveal strong community-environmental relationships (Beisner et al., 2006; Erős et al., 2012; Heino et al., 2015).

The loss of sensitive species does not necessarily mean that the fish community was dominated by non-native species, as taxa sensitive to poor water and habitat quality exist among native and non-native species (Kennard et al., 2005; Maceda-Veiga and de Sostoa, 2011; Segurado et al., 2011). In-depth knowledge of the physiological response of fish to multiple stressors (Maceda-Veiga et al., 2015; Colin et al., 2016) can

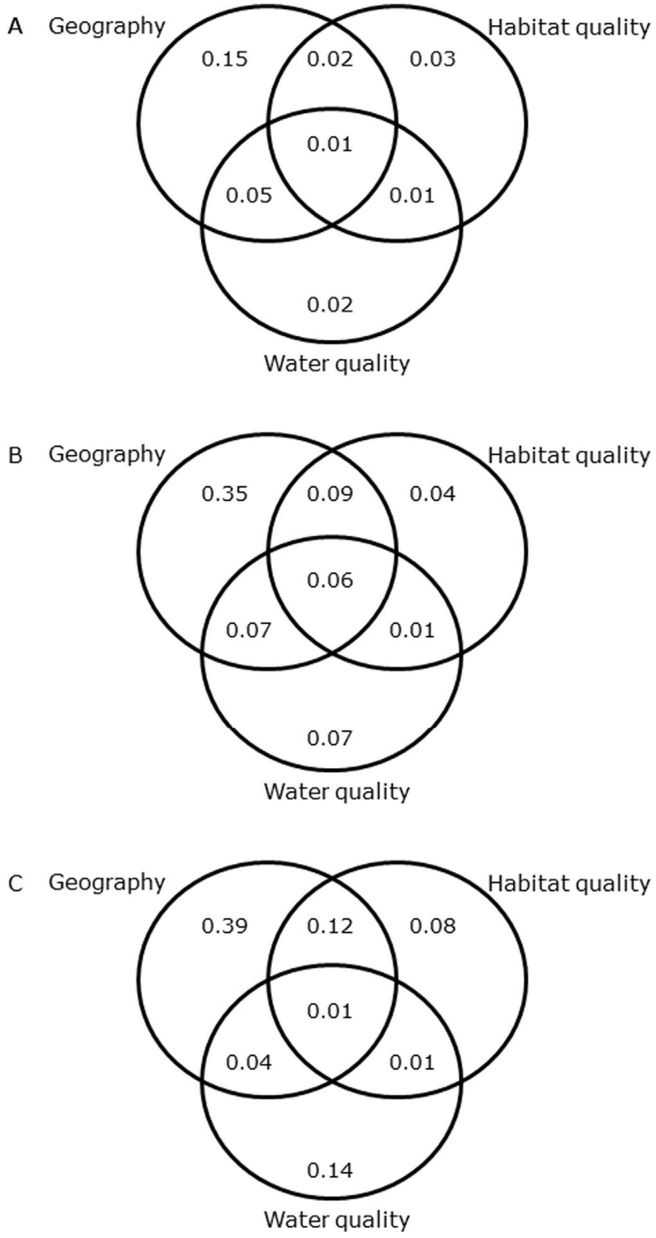

Fig. 4. Venn diagrams showing the unique and shared fractions of variation in fish communities of NE Spain (adjusted R^2) explained by geography, habitat and water quality, for (A) total variation in species composition, and for the two components of total dissimilarity: (B) spatial turnover and (C) nestedness-resultant dissimilarity.

then recommend special protection to particular species, coupled to tributaries as general native fish refugees. In our study, however, the largest fraction of variation in species composition was related to geography, supporting that each basin is a biogeographic unit and has its own history of biological invasions (Doadrio, 1988; Leprieur et al., 2008b). Thus, fine-scale studies in each basin are needed to fully test the potential of tributaries as fluvial reserves, including indicators of taxonomic, functional and phylogenetic diversity that consider interspecific relationships (Strecker et al., 2011; Guareschi et al., 2015; Valiente-Banuet et al., 2015).

5. Conclusions

Our study shows that fish fauna in Mediterranean rivers is at risk by multiple stressors. Different indicators of conservation value are related to different sets of stressors, but restoring water quality and natural flow regimes were identified as management priorities. It will help conserve riverine aquatic diversity and ensure, at a lower cost, the quality of freshwater resources upon which human populations depend on (Vörösmarty et al., 2010; Green et al., 2015). As a complementary action, we propose careful monitoring and focal removal of introduced species in tributaries as current native fish diversity refugees (see also Hermoso et al., 2013). The efficacy of common fish catching methods (electrofishing) is also higher in small than in large rivers (Bohlin et al., 1989). However, the design of fluvial reserves is complex and requires the selection of multiple protected zones with different management regimes (Linke et al., 2008, 2012; Hermoso et al., 2016). Nonetheless, we believe that the design of new protected areas should not change the focus on the management of hydrological regimes and sewage discharges at the basin scale, as this is the most effective way to conserve fluvial diversity. In this regard, our study suggests the need of establishing safe thresholds of pollutant mixtures for the native fauna, especially under forecast climate conditions, and the use of genetic tools to reveal taxonomic gaps.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.09.097.

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Trends in biomarkers, biotic indices, and fish population size revealed contrasting long-term effects of recycled water on the ecological status of a Mediterranean river

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ABSTRACT

Recycled water is important for maintaining river flow in semi-arid regions. However, it has ecological risk, as suggested by comparison of habitat and white and red blood cell count in two wild fish species (*Barbus meridionalis* and *Squalius laietanus*) before and after an input of recycled water in Ripoll River (NE Spain) in 2009. Due to the lack of normal ranges for blood variables in wild fish, we surveyed seasonally the same river reaches in 2013 to test if blood alterations from 2009 compromised the viability of the fish populations. By examining other indicators of river health in baseline and polluted sites (fish abundance, mass-length relationships, and community indices in fish, diatoms and invertebrates), we tested for the superior utility of blood tests in biomonitoring. The comparison of water quality and scores of diatoms and invertebrate indices between polluted and reference sites showed that polluted sites improved from 2009 to 2013. The abundance of *B. meridionalis* also increased in polluted sites, but that of *S. laietanus* declined in 2013 compared to 2009. These results contrast with results of blood analyses in 2009, which suggested that *B. meridionalis* was more seriously affected by pollution than *S. laietanus*. The fish index did not reveal the risk of recycled water to fish health, whereas fish mass-length relationships suggested that *S. laietanus* individuals in 2013 had a better body condition in polluted than in reference sites. Given that the two fish species had opposite results in reference sites, and that the physical habitat was more suitable for *B. meridionalis* in polluted sites in 2013 than was for *S. laietanus*, trends in population size are not only explained by pollution. The role of phenology is suggested by peaks in blood disorders during the breeding season. However, more long-term studies combining indicators of river health at the individual and community scales are needed to fully assess the ecological risk of recycled water in this river. These studies will also help to develop blood tests as reliable health indicators in wild fish populations.

1. Introduction

The inputs of effluents from sewage treatment plants (STPs) are a major threat to aquatic biodiversity, especially in arid and semi-arid regions (Prat and Munné, 2000). Mediterranean rivers are a prime example, as their natural low dilution ability intensifies the effects of pollution on the biota (Arenas-Sánchez et al., 2016; Petrovic et al., 2011). This situation worsens with water overabstraction, which is expected to increase further under forecast climate and human population growth models (Mekonnen and Hoekstra, 2016; Vörösmarty et al., 2010). However, recycled water can aid in maintaining river flow in a context of water scarcity (Halaburka et al., 2013; Vörösmarty et al., 2010). Nonetheless, the long-term effects of recycled water on wild vertebrates are still poorly studied in Mediterranean rivers.

Fish are a major component of freshwater biodiversity and among the most threatened taxa worldwide (Arthington et al., 2016). As well as conservation interest per se, fish are pivotal in aquatic food-webs. Fish transfer energy along rivers (Flecker et al., 2010), and act as host for the larvae of endangered unionid mussels (Lopes-Lima et al., 2016). In Mediterranean rivers, the conservation concern of fish extirpation is

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particularly acute because only one or two fish species often control the community from the top-down (e.g. Sostoa et al., 1990). Official monitoring schemes appraise the ecological status of rivers in Europe and North-America using indices of biotic quality (see Karr, 1981; Aparicio et al., 2011; Lyons, 2012). At best, these indices detect impairment when fish populations sharply decline or after a species extirpation, and this is often too late to take conservation actions and prevent local extinction. Biotic indices based on diatoms and invertebrates are more sensitive than fish indices to subtle changes in water quality (Barbour et al., 1999), but they may not correlate well with fish metrics (e.g. Colin et al., 2016a). Thus, the development of alternative monitoring tools that detect early signs of disease in fish is a priority in Mediterranean rivers (Colin et al., 2016b).

Peripheral blood tests (PBTs) are promising cheap, non-destructive tools to assess fish health, as occur in domestic animals and human medicine (Maceda-Veiga et al., 2015). Only a drop is needed to obtain a full cell profile from a blood smear, which makes it minimally invasive and suitable for small fish (e.g. Filby et al., 2010). By examining red blood cell morphology, it is possible to assess DNA damage through determining the frequency of abnormal cells (Bolognesi and Hayashi, 2011; Pacheco and Santos, 2002), and disorders in red blood cell synthesis via counting the relative abundance of dividing and immature cells (Maceda-Veiga et al., 2015). Relative white blood cell (WBC) count is used as an indicator of stress and/or infection (Correa et al., 2016; Davis et al., 2008). Blood parasites can also be directly detected in blood smears (Maceda-Veiga et al., 2015).

Despite the preceding advantages, PBTs are one of the least used indicators to assess fish health, especially in Mediterranean rivers (Colin et al., 2016b). Only the detection of micronuclei in red blood cells has a wider acceptance, including in ecosystems other than Mediterranean rivers and in wild taxa other than fish (e.g. Barata et al., 2010; Bolognesi and Hayashi, 2011; Pacheco and Santos, 2002;). This is likely to be attributed to the long-tradition of this assay in ecotoxicology, coupled to the possibility of high-throughput automated analysis (e.g. Barata et al., 2010). However, some fish species do not form micronuclei (e.g. Maceda-Veiga et al., 2013), and thus counting all types of red cell abnormalities is advisable (Pacheco and Santos, 2002; Maceda-Veiga et al., 2015). Another major limitation for the use of PBTs is that the normal range of blood variables is unknown for many wild fish (Maceda-Veiga et al., 2015). This questions how blood alterations scale up at the population level; a concern shared with other biomarker approaches, including body condition indices (Colin et al., 2016b). In order to increase the ecological relevance of biomarkers, more research into their natural variability and their integrated response at the population scale is necessary.

The Ripoll River fauna has been degraded in downstream reaches because of a legacy of heavy industrial pollution (Prat and Rieradevall, 2006). After the construction of STPs in 2003 the water quality of this river improved considerably, but its ecological status was still far from good (Sostoa, 2006; Tecnoambiente, 2012). In 2009, two native fish species from this river, the Mediterranean barbel (*Barbus meridionalis*) and the Ebro chub (*Squalius laietanus*), had severe blood cell alterations, and metal concentrations in their tissues were above legal thresholds after the STPs (Maceda-Veiga et al., 2013). However, the risk of chronic pollution to fish populations was difficult to assess due to a possible long-term adaptation (see Biagianti-Risbourg et al., 2013).

Here, we explored the ecological effects of recycled water in Ripoll River by examining variations in scores of fish, invertebrates, and diatom-based indices of biotic quality, and fish abundance from 2002 to 2013. If fish population viability was compromised by the blood disorders from 2009, we expected to find a reduction in fish abundance after the STPs in 2013. If blood tests can be used as indicators of river health, we expected to find similar blood alterations in fish from 2013 as we did in 2009 (Maceda-Veiga et al., 2013), paralleling changes in scores of diatom and invertebrate indices. Finally, we examined blood cell alterations seasonally in 2013 to test whether pollution effects were acute for fish during summer due to warm temperature and low water flow.

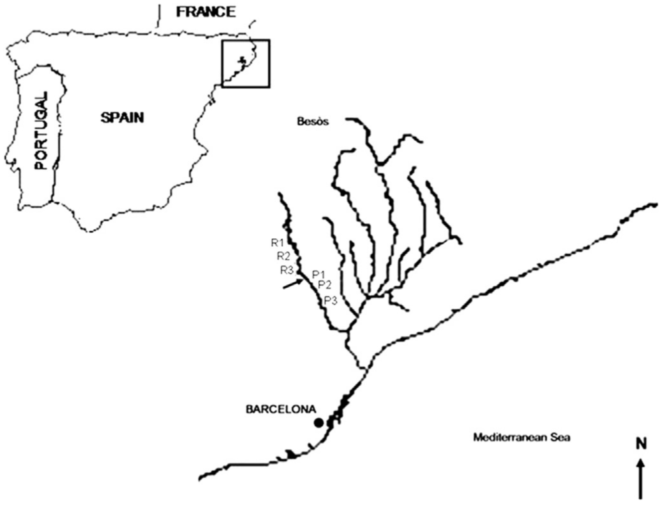

Fig. 1. Location of the three upstream and downstream reaches surveyed in Ripoll River to examine the impact of treated sewage discharges (arrow) on wild native fish populations over the period 2002–2013.

2. Materials and methods

2.1. Study area and general sampling design

The Ripoll is a 39.5 km river that travels north to south the calcareous region of Vallès Occidental, flowing into the river Besòs close to Barcelona. This river typifies well the hydrological regime of Mediterranean rivers, alternating torrential floods with prolonged droughts in autumn and summer, respectively. Water abstraction to satisfy human demand reduces further water flow during drought. To assess the ecological risk of recycled water in this river, we surveyed three reference sites upstream (R1, R2, R3) and three tested sites (P1, P2, P3) downstream the discharge site of three major urban and industrial STPs (Fig. 1). These STPs treat more than 38,000 m^3/day of sewage following a physico-chemical and biological treatment with removal of nitrogen and phosphorous. In relation to raw water, effluents have a 27.5-fold reduction in Biochemical Oxygen Demand-5 (BOD_5), a 10.4-fold decrease in general BOD, a 25-fold reduction in the level of Suspended Solids, and a 5.27-fold decrease in Total Nitrogen level (STP technical report).

Fish were surveyed in April 2012, July 2012, November 2012 and February 2013 for fish abundance, biometry, and blood data. For comparison, we used data from our summer fish surveys in 2002 (fish abundance and biometry; Sostoa et al.) and in 2009 (fish abundance, biometry and blood; Maceda-Veiga et al., 2010). Previous studies confirm the good ecological status of the three reference sites (e.g. Prat and Munné, 2000; Sostoa, 2006; Tecnoambiente, 2012). A heavy pollution black spot and a weir between sites R3 and P1 (see Colin et al., 2016a) guarantee that fish do not move from tested (P1, P2, P3) to reference sites (R1, R2, R3).

2.2. Fish surveys and focal species

Fish were sampled by single pass electrofishing using a portable unit which generated up to 200 V and 3 A pulsed DC in an upstream direction, covering the whole wetted width of the 100-m long reach surveyed at each sampling site following an international standardised fish sampling method (CEN EN 14962, 2005; CEN EN 14011). Our focus was two native species, the Ebro chub (*Squalius laietanus*) and the Mediterranean barbel (*Barbus meridionalis*) belonging to *Cyprinidae*, which is the most common fish family in European rivers (Kottelat and Freyhof, 2007). They also typify well the status of freshwater fish in Mediterranean rivers. The distribution of these two species has markedly declined in recent decades, and the barbel is included in Annexes II and V of the EU Habitats Directive and in Appendix III of the Bern Convention (Crivelli, 2006). Further, *S. laietanus* is a pelagic, omnivorous fish, and *B. meridionalis* is benthic and mostly invertivorous, which allowed us to assess the differential effect of recycled water on species with different ecological strategies. The breeding season of *B. meridionalis* ranges from May to June and in *S. laietanus* from April to May (Sostoa et al., 1990).

2.3. Blood analyses, gross external examination and biometric data

Fish were identified to species level, counted, and a random set of individuals of the two fish species (n = 50 fish/site/species when possible) measured (fork length, mm) and weighed to the nearest mg. A subsample of these fish (n = 10 fish/site/species) of similar size (*B. meridionalis* mean ± S.E. = 90 ± 0.5 mm and *S. laietanus* = 112 ± 0.5 mm) was used for the blood analyses (see below). Due to the lack of sexual dimorphism, fish were not sexed but sexual maturity is at 44 mm for *B. meridionalis* and at 50 mm for *S. laietanus* (Casals, 2005).

All measured and weighed fish were visually inspected to detect deformities, ulcers and other macroscopic signs of disease. Fish were anaesthetised with a buffered MS222® solution (0.02% Tricaine methane-sulfonate, Sigma) to reduce stress. Fish weight and length data were used to calculate the Scaled Mass Index (SMI) (Peig and Green, 2009), which has been recently validated as fish body condition index (Maceda-Veiga et al., 2014). Changes in animal's body condition can affect its fitness, but a bad condition cannot be assumed until specific indicators of disease are used (Peig and Green, 2009).

A 1 mL heparinised syringe for human insulin was used to withdraw 0.2 mL of blood from the caudal vein of each fish individual. A blood smear was immediately made, air-dried in the field, and then stained with Diff-Quick® in the laboratory upon arrival. The identification and relative counts of all blood cell types followed Maceda-Veiga et al. (2015). Briefly, we quantified the relative count of all types of white blood cells (out of 100 WBCs) to assess general fish stress and signs of infection (see Davis et al., 2008). To specifically test whether there was risk of DNA damage, the frequency of abnormal red blood cells (RBCs) was recorded (out of 1000 RBCs). Abnormalities were scored as micronuclei, lobed, kidney-shaped, fragmented nuclei, and RBCs with basophilic stippling. The same procedure was used for the frequency of senescent, immature, and dividing RBCs. These parameters indicated cell lyses or disorders in RBC synthesis, but the release of immature cells is also associated with general stress (Fänge and Nilsson, 1985). RBC counts were expressed out of 1000 RBCs × 100 to facilitate interpretation.

All experimental procedures followed the guidelines provided by the Animal Welfare Committee (CEEA 510/14), and were approved by the Government of Catalonia (DAAM 7992). Fish captures were standardized to CPUE (fish abundance/time in h x sampled water volume in m^3). We converted CPUEs from Maceda-Veiga et al. (2012) to this scale. We had controlled for differences in sampling surface area but not in water volume. After sampling, all fish were released to the capture site.

2.4. Changes in water and physical habitat quality between reference and polluted sites

Differences in river water conditions across the study area were assessed using 10 water quality variables, and two indices of biotic quality based on diatoms and invertebrates. Unique to biotic indicators is their ability to integrate the impacts of complex mixtures of measured and unmeasured contaminants in water and sediment over time, thereby providing a better picture than water chemistry alone of the river chemical status (see Barbour et al., 1999; Colin et al., 2016b).

Water quality was measured prior to each fish survey using the multi-parametric digital probe YSI® 553 MPS for pH, temperature (°C), conductivity (µS/cm) and dissolved oxygen (mg/l), and the colorimetric test kit VISOCOLOR® (MACHEREY-NAGEL GmbH & Co. KG., Dueren, Germany) for carbonate (°KH), and general water hardness (°GH), and for ammonium (NH_4^+, mg/l), nitrite (NO_2^-, mg/l), nitrate (NO_3^-, mg/l) and phosphate (PO_4^{3-}-P, mg/l) concentrations. These variables provide an overall picture of major water quality stressors to the biota, including nutrient pollution, acidification, and salinity (e.g. Johnston et al., 2015; Kaushal et al., 2005; Smallbone et al., 2016). Our water quality analyses were combined with monthly records of pH, dissolved oxygen, conductivity, ammonium, nitrate, and phosphate from the Catalan Water Agency (ACA 2017), and the median was calculated when possible (see statistical analyses).

Scores of the Iberian Monitoring Working Party for macroinvertebrates (IBMWP, Alba-Tercedor et al., 2002) and the *Indice de Polluo-Sensibilité* for diatoms (IPS, Coste, 1982) were downloaded from technical reports and a local website providing long-term data of biomonitoring studies in our region (http://www.ub.edu/barcelonarius/visor/). For comparison with the results of blood analyses, we calculated the Index of Biotic Integrity for Catalonia (IBICAT2b) using fish as bioindicators (García-Berthou et al., 2015). This index determines the status of fish populations using traits at the population and community levels, including the relative abundance of fish for each trophic guild.

Last, the indices of riparian quality QBR (Munné et al., 2003) and hydro-morphology IHF (Pardo et al., 2004) were calculated to determine whether differences in physical habitat quality between reference and polluted sites could mask the inferences of water quality from fish and macroinvertebrate data. QBR ranks 0–100 based on the nativeness of riparian trees, the connectivity between riparian areas and adjacent woodlands, and the riparian area cover (Munné et al., 2003). The IHF also ranks from 0 to 100 but focuses on the microhabitat structure (e.g. type of substrate) (Pardo et al., 2004).

2.5. Statistical analyses

All analyses were conducted in R (R Development Core Team, 2016) using the libraries *stats*, *car*, and *lmer4*. Generalised linear mixed models (the function *glmer*) were used to examine changes in the frequency of white (neutrophils and monocytes) and red blood cells (abnormal, immature, senescent, and dividing) between reference and polluted sites across seasons using a binomial error distribution. Site was included as random factor to control for multiple measurements for each unit (site). Fish length was introduced as covariate in the models for blood variables to account for potential differences in fish sensitivity to pollution with age. Given that more than 500,000 cells were examined, and various observers were required to speed the process, observer was included as factor in the models. However, it was deleted due to the lack of significance. To show the observer effect, we explored coefficients of variation among observers for all blood variables in reference and polluted sites. Relationships among water properties and blood cells were examined using Kendall's tau coefficients. Data from water agencies was only available for one sampling site in the polluted and clean river reaches throughout the study, as it was data on fish in 2002. Thus, we only used descriptive statistics to show variation in CPUEs, biotic indices, and physical and water quality across years. Statistical threshold was established at $P \leq 0.05$.

3. Results

3.1. Changes in water and physical habitat quality over space and time

Water quality in Ripoll River reduced after the STPs effluents in all sampling occasions (Table 1). Nonetheless, the chemical status of polluted sites improved notably in 2013 when compared to 2002 and 2009, as shown by a decrease in the values of conductivity, ammonium,

Table 1
Median and standard error (when possible, see methods) of water properties, physical habitat structure, and four indices of biotic quality based on riparian cover (QBR), macroinvertebrates (IBMWP), diatoms (IPS), and fish (IBICAT2b) measured in Ripoll River over the study period (2002–2013).

	2002		2009		2012/2013	
	Reference	Polluted	Reference	Polluted	Reference	Polluted
Water properties						
Temperature (°C)	22 ± 1.9	24.45 ± 2.65	21.3 ± 4.20	26.66 ± 2.05	21.00 ± 1.85	26.54 ± 1.61
Dissolved oxygen (mg/l)	7.3	12.9	11.65 ± 1.11	9.75 ± 0.78	10.8	12.8
Ammonium (mg/l)	0.04 ± 0.01	0.21 ± 0.10	0.00 ± 0.04	0.2 ± 0.86	0.00 ± 0.01	0.01 ± 0.32
Nitrite (mg/l)	0.02 ± 0.01	0.45 ± 0.10	0.04 ± 0.02	0.47 ± 0.15	0.02 ± 0.01	0.10 ± 0.08
Nitrate (mg/l)	2.00 ± 1.33	9.40 ± 1.91	5.10 ± 1.40	13.45 ± 2.29	3.00 ± 1.01	8.00 ± 3.87
Phosphates (mg/l)	0.09 ± 0.14	1.65 ± 0.35	0.2 ± 0.14	2.4 ± 0.63	0.2 ± 0.10	1.00 ± 0.42
Conductivity (μS/cm)	679 ± 43	2300 ± 482	572 ± 124	1782 ± 132	535 ± 45	1492 ± 162
pH	8.1	8.5	8.20 ± 1.64	8.2 ± 0.65	7.80 ± 0.12	8.00 ± 0.13
General hardness (°dGH)	20	20	19	22	22 ± 0.93	24 ± 1.56
Carbonate hardness (°dKH)	14	15	15	15	15 ± 0.92	15 ± 0.89
Physical habitat						
Runs (%)	20	25	20	25	20	25
Riffles (%)	30	70	30	70	30	70
Pools (%)	50	5	50	5	50	5
Water flow (m^3/s)	nm	nm	nm	8.13	nm	5.19
Qualitative river flow (0–10)	6	6	7	6	5	5
Indices of biotic quality						
QBR	G(80)	VP(5)	G(80)	P(25)	G(75)	VP(10)
IBMWP	VG(105)	P(26)	VG(137)	P(22)	VG(165)	M(51)
IPS	(G)14	(P)5	(G)15	(P)7	(G)14.7	(P)7.2
IBICAT	M(2.6)	VP(0)	M(2.6)	M(3)	VG(4.7)	VG(4.75)

IBQ = Very good (VG), good (G), moderate (M), poor (P) and very poor (VP) ecological status.
nm = not measured.

and nitrite. In 2013, the concentration of phosphates in polluted sites was still high compared to the reference sites, but the concentration of dissolved oxygen was higher (Table 1). An increase in the scores of macroinvertebrate and diatom indices paralleled the improvement in water quality in polluted sites from 2002 to 2013 (Table 1). The good ecological status of the reference sites was evident in all water properties and biotic indices throughout the entire study period (Table 1). However, the fish index was the least sensitive to variations in water quality over space and time (Table 1).

The Fluvial Habitat Index (IHF) score was above 40 in all sites, indicating that changes in physical habitat quality had a little effect on the scores of biotic indices. Nonetheless, the predominant habitats were pools in reference sites, and riffles in polluted reaches, and a marked decline in river flow occurred in 2013 (Table 1). The riparian index QBR showed that the riparian cover was better preserved in reference sites than polluted sites.

3.2. Changes in fish abundance, fish size, and body condition over space and time

We captured up to six fish species in Ripoll River from 2002 to 2013, but *B. meridionalis* and *S. laietanus* were the only native species found at both reference and polluted sites (Table 2). Trends in CPUEs varied with species and river status over time, but with a consistent increase for the two native species from 2002 to 2009. All non-native fish species showed a tendency to decrease in CPUEs from 2002 to 2013 apart from *L. gibbosus* that increased in reference sites in 2013 (Table 2). In this year, *B. meridionalis* CPUE was higher in tested than in reference sites. By contrast, *S. laietanus* increased in CPUE in reference sites but reduced at polluted sites when compared to 2009 (Table 2). No fish species was captured in polluted sites in 2002 (Table 2).

The size structure of the two native fish populations showed a similar trend in reference sites, with larger fish in 2002 and 2013 than in 2009 (Table 2). In polluted sites, median size of *S. laietanus* individuals increased from 2009 to 2012, whereas *B. meridionalis* size remained stable. From 2002–2013 median values of SMI in *B. meridionalis* were also rather stable in polluted sites, but declined for *S. laietanus* (Table 2). A decrease in SMI was found for the two native species in reference sites from 2009 to 2012 (Table 2). No gross signs of disease were detected in all the fish examined.

3.3. Effects of water quality, season, fish size and observer on blood variables in 2013

WBCs and RBCs differed between polluted and reference sites (Table 3). In polluted sites, the frequency of neutrophils and monocytes markedly increased in *S. laietanus* and *B. meridionalis*, respectively, compared to reference sites (Table S1). Eosinophils and basophils were not detected in all sampling occasions. Since WBC readings are relative counts, variations in lymphocytes, the predominant WBC type, are not shown, i.e. their proportion reduces with an increase in other WBC types.

The frequency of abnormal RBCs increased in *S. laietanus* in polluted sites, whereas no clear effect was found for *B. meridionalis* (Table S1; but see lobed RBCs in Table S2). For *S. laietanus*, the frequency of kidney shaped, lobed, and basophilic stippling made the largest contribution to variation in abnormal RBCs between polluted and reference sites, but no major differences were detected in the micronuclei frequency. The senescent RBC frequency did not significantly vary with river status in *B. meridionalis*, whereas it notably decreased in *S. laietanus* in polluted sites (Table S2).

The effect of river status on the frequency of immature RBCs depended on season. In spring and summer, both fish species had higher immature RBC frequencies in reference sites (Fig. 2). However, in autumn and winter, this tendency was less apparent in polluted sites, particularly for *S. laietanus* due to marked increase in immature RBCs (Fig. 2). Season had main effects on the frequency of rest of cell types (Table 3), which mostly peaked in winter or spring (Figs. 2 and 3) apart from senescent RBCs that did it in summer (Fig. 2). Blood readings varied with observer, but no major differences were found in central

Table 2
Median and standard error of captures per unit of effort (CPUEs) of the two native and four non-native fish species found in Ripoll River over the study period (2002–2013). For 2012 only data from summer was used to be results across years comparable. Changes in fish size and body condition based on the Scaled Mass Index were also shown for the two native fish species.

	2002		2009		2012/2013	
	Reference	Polluted	Reference	Polluted	Reference	Polluted
Barbus meridionalis						
CPUEs	0.46	0.00	1.96 ± 1.52	5.85 ± 2.11	1.58 ± 0.79	8.30 ± 3.49
Fork length (mm)	116 ± 3.5	nc	88 ± 3.2	103 ± 2.5	117 ± 2.8	105 ± 1.5
Scaled Mass Index (g)	14.38 ± 0.44	nc	13.70 ± 0.14	13.07 ± 0.16	12.13 ± 0.27	13.12 ± 0.12
Squalius laietanus						
CPUEs	0.48	0.00	0.61 ± 1.84	7.58 ± 1.94	0.78 ± 0.28	3.97 ± 2.86
Fork length (mm)	149.5 ± 5.7	nc	112 ± 3.7	110 ± 2.9	138 ± 2.4	129 ± 10.5
Scaled Mass Index (g)	20.25 ± 0.23	nc	21.17 ± 0.19	22.06 ± 0.33	19.24 ± 0.49	21.47 ± 0.30
Non-native fish species						
CPUEs *Cyprinus carpio*	0.04	0.00	0.00	0.00 ± 0.05	0.00	0.00 ± 0.02
CPUEs *Micropterus salmoides*	0.00	0.00	0.00 ± 0.01	0.00	0.00	0.00
CPUEs *Lepomis gibbosus*	0.72	0.00	0.11 ± 0.26	0.00	0.41 ± 0.04	0.00
CPUEs *Gambusia holbrooki*	0.82	0.00	0.00	0.00 ± 0.23	0.00	0.00

nc = not captured.

Table 3
Results of GLMMs on the effects of pollution, season, and fish size on the frequency of white and red blood cells in the two native fish species in Ripoll River. Site was included as random term in all models. The interaction between season and pollution was only included in the models when its effect was significant at $P \leq 0.05$.

	Barbus meridionalis		*Squalius laietanus*	
Blood cell types	Chi	P value	Chi	P value
Neutrophils				
Pollution	0.11	0.74	5.77	**0.016**
Season	10.94	**0.012**	55.94	< **0.001**
Fish size	2.52	0.11	11.78	< **0.001**
Monocytes				
Pollution	12.33	< **0.001**	2.29	0.13
Season	52.43	< **0.001**	30.66	< **0.001**
Fish size	6.98	**0.008**	0.93	0.33
Abnormal RBCs				
Pollution	3.83	**0.05**	17.96	< **0.001**
Season	91.11	< **0.001**	76.96	< **0.001**
Fish size	3.82	**0.05**	1.25	0.26
Senescent RBCs				
Pollution	0.19	0.66	10.48	**0.001**
Season	8.77	**0.03**	19.47	< **0.001**
Fish size	4.32	**0.04**	2.34	0.13
Immature RBCs				
Pollution	3.11	0.08	1.17	0.28
Season	34.1	< **0.001**	89.66	< **0.001**
Fish size	0.97	0.33	0.07	0.79
Pollution × Season	8.51	**0.037**	27.79	< **0.001**
Diving RBCs				
Pollution	2.09	0.15	5.45	**0.01**
Season	2.22	0.53	6.25	0.14
Fish size	0.09	0.75	0.03	0.86

tendency. The lowest coefficient of variation was for neutrophils and immature RBCs, and the largest was for monocytes and dividing RBCs. Polluted sites generally had larger coefficients of variation in blood readings than reference sites (Table S1). Blood readings also differed with fish size, with contrasting effects between species (Table 3). In *S. laietanus*, most blood variables had a negative relationship with fish size, whereas the relationship was mostly positive for *B. meridionalis*.

3.4. Interlinking fish health indicators and their association with water quality

Most fish health indicators were not strongly correlated to each other (Table S3). Only a weak but significant relationship was found between abnormal RBCs and the condition index SMI. At least one cell type was significantly related to indicators of poor water quality (Table 4). *Squalius laietanus* was the species with the strongest correlations between blood variables and water properties. A positive relationship was found between the values of monocytes and ammonium (Table 4). The abnormal RBC frequency was positively related to conductivity and phosphate values. In contrast, the nitrite level was negatively associated with the senescent RBC frequency (Table 4). For *B. meridionalis*, only a significant but negative relationship was found between the immature RBC frequency and the levels of nitrite and nitrate (Table 4). For temperature, there was a negative relationship with the neutrophil frequency in *B. meridionalis*, but a positive effect was observed in immature RBCs for *S. laietanus* (Table 4).

4. Discussion

This study is the first in showing the long-term effects of blood disorders on wild native fish from Mediterranean rivers. Blood analyses in 2009 suggested that *B. meridionalis* was more seriously affected by pollution than *S. laietanus* in Ripoll River (Maceda-Veiga et al., 2013; 2010). However, the same study design has indicated the opposite in 2013. Water analyses and diatom and invertebrate indices show an improvement in the river status from 2009 to 2013. The abundance of *B. meridionalis* also increased in polluted sites between 2009 and 2013, whereas that of *S. laietanus* declined in 2013 compared to 2009. Since abundances of these fish species showed the opposite tendency in reference sites, differences in population size cannot be fully explained by the input of recycled water. The regional fish index of river quality did not detect pollution effects.

Peripheral blood tests (PBTs) were a superior alternative to the fish index IBICAT2b to assess the status of Ripoll River, in compliance with the EU's Water Framework Directive (EU Commission, 2001). The low diagnostic power of fish biotic indices in Mediterranean rivers is attributed to their low fish diversity, which reduces the number of traits available to make diagnostics (e.g. Magalhães et al., 2008; Aparicio et al., 2011). Despite this, fish indices are used widely by water agencies, and biomarkers are mostly used in research (Magalhães et al., 2008; Colin et al., 2016b). A major concern that comes from our study is that the fish index IBICAT2b can mask biotic and abiotic causes of impairment in rivers. Whereas all indicators agreed with the good status of reference sites from 2002 to 2009, IBICAT2b scored low because of the presence of non-native species (see also Benejam et al., 2008). When reference sites lacked of them in 2013, a good status was reached, even though native fish abundance had not notably improved

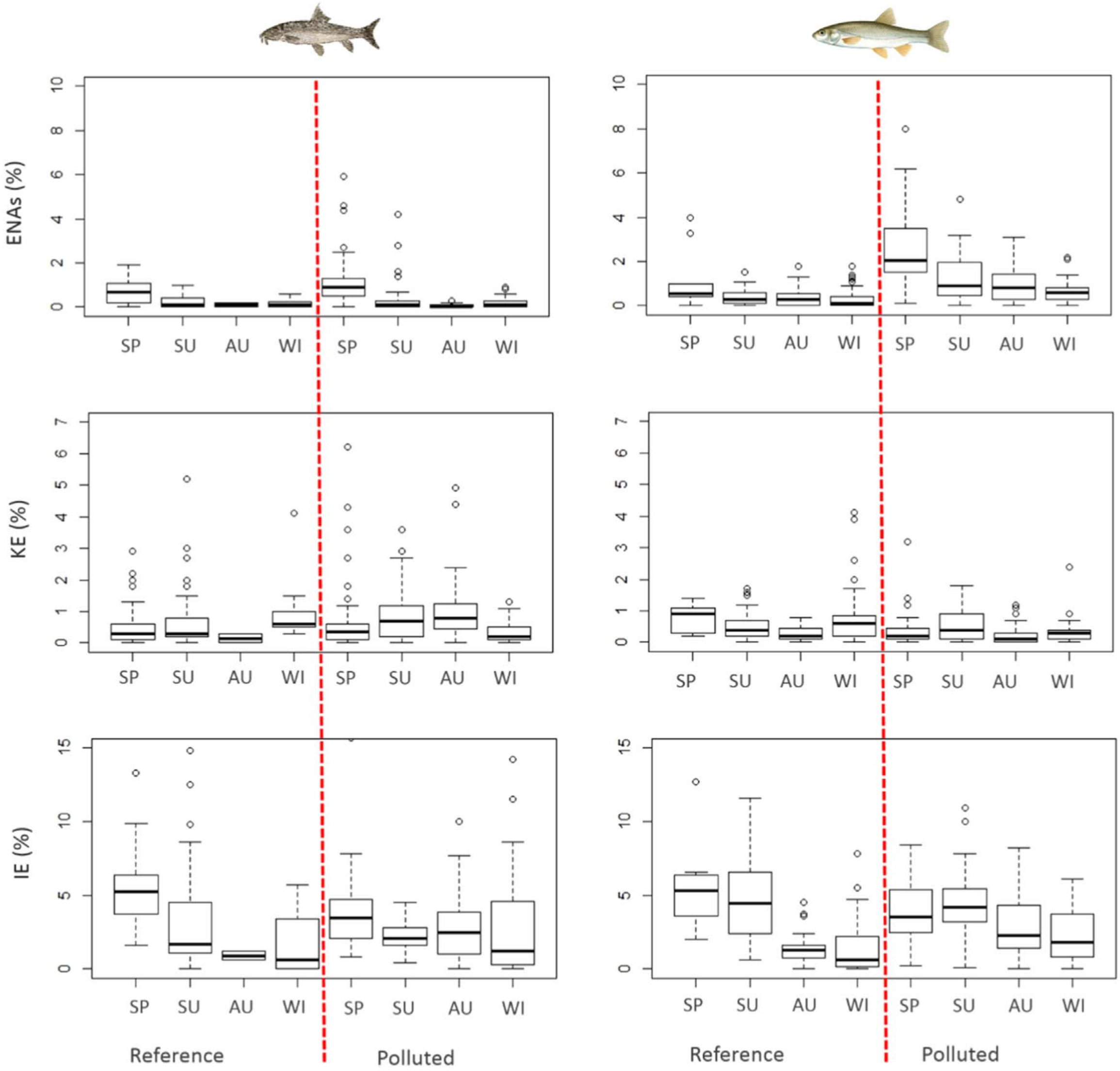

Fig. 2. Changes in the frequency of abnormal (ENAs), senescent (KE) and immature red blood cells (IE) in *B. meridionalis* and *S. laietanus* from reference and polluted reaches of Ripoll River along four seasons (spring, SP; summer, SU; autumn, AU; winter, WI) during the period 2012/2013.

from previous years.

The fish index IBICAT2b also performed worse than diatom and macroinvertebrate indices at determining the polluted status of river reaches downstream the STPs. Fish indices could be seen as less suited than diatoms and invertebrates to detect pollution sources (Benejam et al., 2008; Colin et al., 2016b). However, fish integrate other impacts than water quality, including hydro-morphological alterations, and over larger temporal scales than diatoms and invertebrates because of the longer life-span of fish (Barbour et al., 1999). Therefore, lag-responses can occur in the populations we examined, but the conservation status of many fish species in Mediterranean rivers (Smith and Darwall, 2006) requires anticipating lethal effects at the individual level.

The SMI body condition index showed that the two fish species were better nourished in polluted than were in reference sites. The most plausible explanation for this result is a better food quality in polluted sites due to eutrophy (i.e. high primary production), and the generalist diet of *B. meridionalis* and *S. laietanus* (e.g. Mas-Martí et al., 2010). However, the large median size of *S. laietanus* individuals in polluted sites compared to the reference sites suggests that the recruitment of *S. laietanus* may have declined in polluted sites in 2013. Less recruitment should have implied a reduction in the relative weight of gonads detectable in the SMI body condition index calculated using total fish weight. This may not have occurred because our surveys in 2013 were carried out just after (*B. meridionalis,* May-June) and later (*S. laietanus*, April-May) the breeding season of these fish species (Sostoa et al., 1990).

Our study in 2013 showed similar blood alterations in *S. laietanus* in polluted sites to *B. meridionalis* in 2009 (e.g. peak in neutrophils, abnormal red cells) (Maceda-Veiga et al., 2013), but the observed increase in *B. meridionalis* abundance from 2009 to 2013 suggests that pollution did not compromise the viability of the population. However, in reference sites, the abundance of *B. meridionalis* reduced from 2009 to 2013, indicating that factors other than pollution may have affected the physiological response of the fish. Polluted sites had a higher percentage of riffles and less of pools than reference sites, and *B. meridionalis* often occurs in sites with faster water velocities (mean = 0.90 m/s) than *S. laietanus* (mean = 0.69 m/s) (Maceda-Veiga et al., 2017). Polluted sites had a lower water depth than reference sites, further supporting a poor performance of the pelagic *S. laietanus* in relation to the benthic *B. meridionalis*. However, the mechanisms behind variations in fish abundance are likely to be highly complex (e.g. Matthews and Marsh-Matthews, 2003; Magalhães et al., 2007). For instance, the dramatic water flow reduction in 2013 may have accentuated the stress response on pelagic over benthic fish.

The pelagic *S. laietanus* had high frequency of abnormal red blood cells in polluted sites compared to the benthic *B. meridionalis*, and this

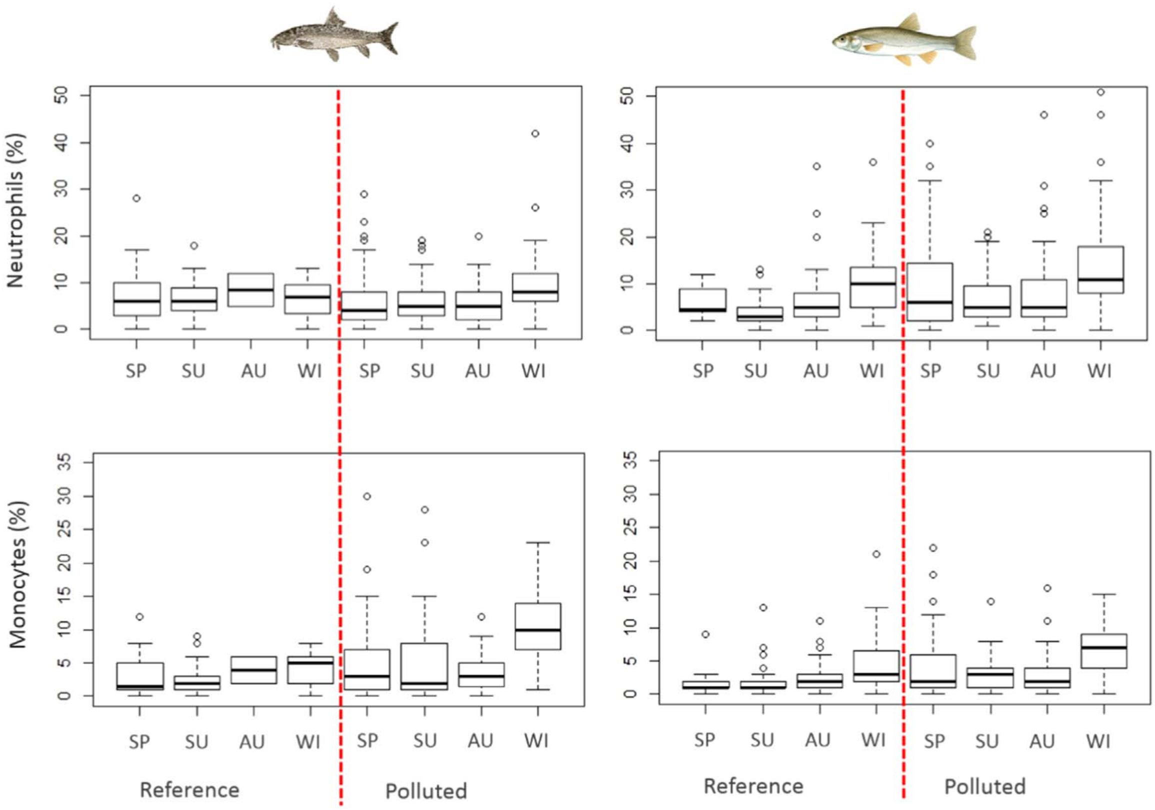

Fig. 3. Changes in the frequency of neutrophils and monocytes in *B. meridionalis* and *S. laietanus* from reference and polluted reaches of Ripoll River along four seasons (spring, SP; summer, SU; autumn, AU; winter, WI) during the period 2012/2013.

Table 4
Kendall tau correlation coefficients among the frequency of white (neutrophils and monocytes) and red blood cells (immature erythrocytes, IE; senescent erythrocytes, KE; and abnormalities, ENAs) and indicators of water quality status measured in Ripoll river in all four seasons during 2012 (n = 24 sampling occasions). In bold coefficients significant at P ≤ 0.05.

	Neutrophils	Monocytes	IE	KE	ENAs
Barbus meridionalis					
Temperature	**−0.347**	−0.349	0.307	−0.060	0.294
Conductivity	0.201	0.144	−0.137	−0.137	−0.130
pH	−0.155	−0.007	−0.006	−0.155	0.253
General hardness	0.131	0.242	−0.230	−0.304	−0.206
Ammonium	0.167	0.342	−0.081	−0.128	−0.108
Nitrite	−0.034	0.344	**−0.355**	0.021	−0.236
Nitrate	0.014	0.334	**−0.393**	0.022	−0.234
Phosphate	−0.260	0.072	−0.123	−0.028	−0.021
Carbonate hardness	0.140	0.194	−0.308	−0.146	−0.323
Squalius laietanus					
Temperature	−0.057	−0.210	**0.468**	0.065	**0.322**
Conductivity	0.066	0.101	0.164	−0.167	**0.358**
pH	0.151	0.191	0.190	0.119	0.248
General hardness	0.128	0.151	−0.009	0.125	−0.014
Ammonium	0.177	**0.450**	0.110	−0.095	0.108
Nitrite	0.069	0.290	−0.099	**−0.401**	0.269
Nitrate	0.141	0.299	−0.125	−0.248	0.186
Phosphate	0.092	0.196	0.281	−0.326	**0.384**
Carbonate hardness	0.097	0.125	**−0.324**	−0.147	−0.174

dysfunction indicates DNA damage (Ayllon and García-Vazquez, 2000; Pacheco and Santos, 2002). This result seems to disagree with the relative high tolerance of *S. laietanus* to poor water quality compared to *B. meridionalis* reported by other methods (e.g. Maceda-Veiga and de Sostoa, 2011; Segurado et al., 2011). However, we found that *B. meridionalis* had, on average, a lower frequency of immature red cells than *S. laietanus* in polluted than in reference sites, and blood cell synthesis inhibition can reduce the formation of abnormal cells (Pacheco and Santos, 2002; Cazenave et al., 2014). In *S. laietanus*, the high percentage of abnormal cells can be also explained by less cytolitic effects (see Pacheco and Santos, 2002). However, this was an unlikely causal factor because *S. laietanus* had a high frequency of senescent cells in polluted compared to reference sites.

In summer, we expected to find more blood alterations than in other seasons because pollutants concentrate during low water flow conditions, potentially intensifying their toxicity (Petrovic et al., 2011). The higher percentage of blood alterations observed in spring suggests that the breeding season was more stressful for the fish than summer. Whereas acute stress can cause a release of immature red blood cells into the peripheral blood (see Fänge and Nilsson, 1985), a high percentage of these cells may also suggest the need to increase oxygen transport due to a high activity (i.e. breeding). This reasoning illustrates the difficulties of diagnosing diseases in wild fish due to the lack of normal ranges of blood variables (Maceda-Veiga et al., 2015). Another constraint may be the subjectivism in the identification of cell types manually by colour and shape (Maceda-Veiga et al., 2015). However, this was of minor importance for reliable indicators of pollution effects in our study, such as the abnormal cell frequency which had the lowest coefficient of variation among observers.

5. Conclusions

Recycled water is important for maintaining river flow in Mediterranean climate areas. However, results of fish blood analyses and diatom and invertebrate indices suggest that current sewage treatments threaten the biota in Ripoll River. These results contrast with the fish index IBICAT2b that showed no ecological impact, and the SMI which indicated that the fish had a better body condition in polluted than in reference sites. Contrasting results are expected because the response of these metrics vary, from days (blood samples) to years (community structure). Chronic pollution may have also caused effects at other levels not assessed in our study (e.g. behaviour, trophic ecology, and genetic structure). Therefore, more long-term studies combining specific (e.g. enzymes related to biotransformation pathways) and non-specific biomarkers (e.g. blood tests) are needed to fully assess the ecological risk of recycled water in this river. Biomarkers should be included in official monitoring schemes, and blood tests are a promising non-destructive tool to monitor the health status of wild fish populations.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.07.048.

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The presence of non-native species is not associated with native fish sensitivity to water pollution in greatly hydrologically altered rivers

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HIGHLIGHTS

- Rivers are subject to multiple stressors, which may interact in complex or unexpected ways.
- We explored whether non-native species modulate native fish species' response to water pollution.
- Invasions, water quality or their interaction were weak predictors in our models.
- Interactions among stressors may be less common or pervasive than some reports suggest.
- Restoring hydro-morphology in rivers most probably is the most urgent management task.

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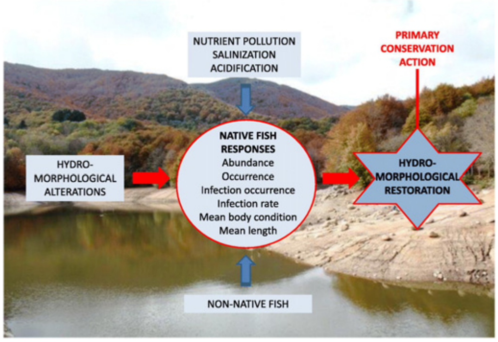
GRAPHICAL ABSTRACT



ABSTRACT

There is a risk of 'ecological surprises' if multiple potentially interacting stressors are managed individually, which is a question attracting significant current interest. Habitat degradation and species introductions are major threats to global biodiversity, and riverine fish are among the most threatened taxa in the world. Our interest was whether the presence of non-native species can affect native fish sensitivity to water quality deterioration in a large region in northeastern Spain (99,700 km^2, 15 catchments, 530 sites). We used a 'base model' with geographical and hydro-morphological variables, which are the major shaping factors in rivers. We tested whether water pollution, non-native species, or their interaction provided an improved understanding of patterns of distributions and health measurements of the twelve most common native species. There was little evidence that variation in native species abundance, where they occurred, the presence of diseases and changes in mean fish length or body condition was affected by water deterioration, the presence of non-native species, or their interaction. The disease rate and occurrence of native species might be affected, to a minor degree, by water quality changes and the presence of non-native species. Environmental conditions between sites with and without non-native fish differed in the condition of riparian areas and in water quality. Based on presence-absence data and changes in abundances through weighted average equations we also derived potential safe levels of salinization, nutrient pollution, and pH for the native fish. Overall, additive effects of stressors prevail over interactions, and the restoration of natural hydro-morphology in rivers is likely to be the most effective management approach to improving the prospects for the native fish fauna.

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1. Introduction

Human activities have increased the diversity and magnitude of stressors affecting ecosystems worldwide (Dirzo et al., 2014; Ceballos et al., 2015), and rivers are a prime example (Vörösmarty et al., 2010). A stressor is a biotic and abiotic fluctuation, usually beyond the normal range of values for that process at a given site and time, that induces a biological response (Barrett et al., 1976; Côté et al., 2016). Fluvial organisms evolved with natural stressors such as the upstream-downstream gradient (Vannote, 1980) and seasonal changes in water flow (Lake, 2003). The biota may be exposed to the combined effect of multiple anthropogenic stressors (Piggott et al., 2015; Jackson et al., 2016). The disruption of natural hydrological regimes has been related to the decline of native species (Marchetti and Moyle, 2001; Wenger et al., 2011), and the establishment of non-native species (Catford et al., 2011; Kiernan et al., 2012), one of the most pernicious forms of ecological change (Vilà and Hulme, 2017). However, the ways in which these factors interact with other stressors, such as reduced water quality, are poorly understood (Colin et al., 2016a; Jackson et al., 2016).

There is a pressing need to erect, and to test, a general ecological theory of the influence of multiple stressors (Vinebrooke et al., 2004; Lindenmayer et al., 2010; Piggott et al., 2015). While experimental testing of the effects of multiple stressors is important, manipulations rarely can be conducted at the spatial and temporal scales at which the biota experiences the stressors (Mac Nally, 1997). Therefore, observational data often are employed to identify the potential effects of stressors both singly and in combination (e.g. Schinegger et al., 2016; Gieswein et al., 2017). Studies examining how non-native species and abiotic stressors affect native fauna have major management applications alongside their importance in a theoretical sense (Lindenmayer et al., 2010; Côté et al., 2016). For instance, if the presence of non-native species does not add substantially to the effects of hydrology on natives, this suggests that the removal of non-native species may be less effective than restoring rivers' natural hydrology for biodiversity conservation. Further, if the presence of non-native species exacerbates the effects of toxicity of water hazards to natives, then current water quality standards that ignore the influence of non-native species (e.g. Adams, 2003) may underestimate the actual extent of adverse effects.

Fish are pivotal in aquatic food-webs and have high socioeconomic value due to their importance for food and recreational angling (Ormerod, 2003). Fish probably are affected in complex ways by water pollution, the introduction of non-native species, and by hydrological alterations in rivers. For instance, the water pollution stress can intensify during drought and reduce the quantity and quality of prey (e.g. benthic invertebrates, Boix et al., 2010; Colin et al., 2016b) upon which fish feed (e.g. De Sostoa, 1990). The activities (e.g. bioturbation) of some non-native species, such as the common carp (*Cyprinus carpio*), can further degrade water quality and the whole aquatic community (Shin-Ichiro et al., 2009; Maceda-Veiga et al., 2017b). Non-native species may be more tolerant than native species to human impacts (e.g. Marchetti et al., 2004), so human-altered conditions might advantage non-natives relative to native species. Alongside other impacts (Cucherousset and Olden, 2011), non-native species cause trophic competition and injuries to natives, which may lead to infections (e.g. *Saprolegnia*) (Noga, 2011). Furthermore, non-native fish can introduce novel parasites into the recipient communities, such as has occurred with the Asiatic anchor worm (*Lernaea cyprinacea* Linnaeus, 1758) in some European rivers (Sánchez-Hernández, 2017).

There is observational evidence that pathogens completing their cycle in one host often proliferate if there is nutrient pollution (Johnson et al., 2010), one of the most common water effects in rivers from sewage discharges (Rissman and Carpenter, 2015). Nutrients may indirectly benefit parasites because the associated increased productivity can increase host abundance or quality (Johnson et al., 2010). Nutrients can also increase pathogen virulence (e.g. *Aspergillus*, Olutiola and Cole, 1977). However, at high concentrations, nutrients and other widespread pollutants, such as salinity (Williams, 1987) can act adversely on parasites (Noga, 2011; Smallbone et al., 2016). The effects of pollutants may depend on context and may act as immune-suppressors (Biagianti-Risbourg et al., 2013). However, the combined effects of biotic and abiotic stressors may be difficult to detect because responses to pollution exposure (Biagianti-Risbourg et al., 2013) and species invasions (Parker et al., 1999) may be lagged. Ideally, studies of the impacts of abiotic and biotic stressors in a single snapshot of time should include indicators of the health status of fish populations (Colin et al., 2016a), from weeks and months (e.g. body condition, and presence of ectoparasites) to years (e.g. size population structure and abundance).

Here, we asked whether non-native species modulate the effects of water pollution on native fish in the hydrologically altered Mediterranean rivers of northeastern Spain. Many fish in Mediterranean rivers are highly threatened (Smith and Darwall, 2006) and it is crucial to evaluate how these multiple stressors affect their persistence. These rivers are exemplars of the stressor combinations (e.g. riparian removal, water abstraction, pollution, invasions) occurring in other Mediterranean rivers in Europe and elsewhere (Hooke, 2006; Marchetti and Moyle, 2001; Tockner et al., 2009), and make Spanish rivers among the most degraded in Europe (Tockner et al., 2009). We focused on fish because fish are affected by water degradation, hydro-morphological alterations or the release of non-native species (e.g. Boix et al., 2010; Colin et al., 2016b; Maceda-Veiga et al., 2017b). We expanded upon this knowledge by exploring in detail whether there is evidence for interactive effects of these stressors for twelve native European species. These include many species belonging to Cyprinidae (the dominant fish family in Europe) and species with a wide European distribution, such as the European eel (*Anguilla anguilla* Linnaeus, 1758) and brown trout (*Salmo trutta* Linnaeus, 1758) (Kottelat and Freyhof, 2007).

We tested whether the presence of non-native species, including some of the world's worst aquatic invaders, such as the largemouth blackbass (*Micropterus salmoides* Lacèpede, 1802) and common carp (*Cyprinus carpio* Linnaeus, 1758), act as a potential confounding factor for establishing safe water-quality standards for native fish in regions highly prone to biological invasions (Leprieur et al., 2008), including northeastern Spain (Maceda-Veiga et al., 2017a). Reduced fish abundance and smaller fish with poorer body condition were expected if the river conditions were beyond their tolerance thresholds because physiological stress typically causes size-selective mortality and alterations in growth rate (Snieszko, 1974; Bonga, 1997; Pankhurst and Van Der Kraak, 1997). Given that nutrient pollution and salinity can alter infectious disease dynamics (Johnson et al., 2010; Noga, 2011; Smallbone et al., 2016), we expected this to be reflected in fish infected with *L. cyprinacea* or *Saprolegnia*. Last, the potential safe thresholds of nutrient pollution, salinization, and pH for the fish were estimated from the river conditions at which capture rates were highest for the twelve native fish species.

2. Materials and methods

2.1. Study area

We gathered environmental and fish data from our own surveys performed in northeastern Spain from 2002 to 2009 (e.g. Figuerola et al., 2012; Maceda-Veiga et al., 2017a). This data set consisted of 530 sampled sites that involved all Catalonian catchments from the Muga to Riudecanyes basins, and the whole River Ebro and part of the Garonne basin (Fig. 1). Our data set accounted for most river typologies in this region in terms of hydrological alterations, riparian characteristics, geology, water quality, river size and water flow. Most of the rivers sampled are small and have a typical Mediterranean hydrological regime, with deep droughts in summer and torrential floods in autumn. However, large rivers peak in flow in spring from snowmelt. We surveyed in low-flow conditions because this is when fish populations

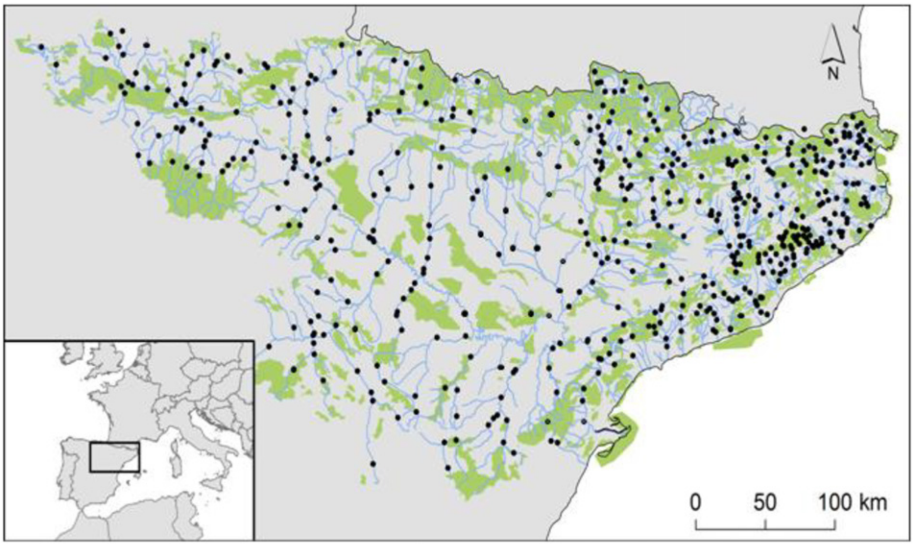

Fig. 1. Location of the 530 sampling sites surveyed in northeastern Spain with protected areas highlighted in green. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

are more stable and can be sampled most effectively using electrofishing (see below). River mouths were not surveyed because they cannot be properly sampled with electrofishing.

2.2. Fish surveys

An international standardized fish sampling method was employed (CEN standards EN 14962 and EN 14011) in accordance with the European Water Framework Directive (EC 2000). Fish were sampled by a single-pass electrofishing using a portable unit which generated up to 200 V and 3 A pulsed D.C in an upstream direction, covering the whole wetted width of the 100-m long reaches surveyed at each location (Benejam et al., 2012; Maceda-Veiga et al., 2017a). The location of each sampling site was selected based on representativeness, and included a variety of habitat types (pools, rifles and runs) as appropriate for each site. The same equipment was used among sites to avoid bias in fish captures (Benejam et al., 2012), and the fish captured were kept in buckets provided with air pumps until the end of the survey. Fish captures were expressed as captures per unit of effort (CPUE, fish caught divided by fishing time in minutes and the area surveyed in m^2). A pilot study in our study area comparing estimates of species richness and abundance between single and four pass electrofishing showed that estimates from single pass were reasonably high with 80–100% of the species detected and 60–90% of the individuals captured (A. Sostoa, *unpublished data*).

Fish were identified to species and a random set of individuals (≥40) from each species was measured (total length, mm) and weighed (mg) after being anaesthetized in a buffered MS222® solution (0.02%, Tricaine methane-sulfonate, Sigma®). The uncertain taxonomical status of four taxa (*Barbatula* spp., *Phoxinus* spp., *Gobio* spp., and *Carassius* spp.) was due to the description of new species after we did our surveys and the presence of several morphological cryptic species of genus *Carassius* (Freyhoff J. pers. comm.). A straightforward change in the nomenclature of the former is complicated because these taxa are subject to inter-basin transfers by anglers (e.g. Maceda-Veiga et al., 2017a). Fish species were defined as: (1) introduced, if they did not occur historically in a basin and in the Iberian Peninsula (IP); or (2) translocated, if their presence is the result of an introduction from another basin within the studied basins where they are native (Table 1). Introduced and translocated species were grouped as non-native (Copp et al., 2005).

2.2.1. Fish body condition and parasite surveys

Body condition is a key variable for fisheries management because it can act as a proxy for predicting future fish growth and reproductive success (e.g. Marshall et al., 1988; Bentley and Schindler, 2013). The Scaled Mass Index (SMI) was used as an index of body condition (BCI) using the method of Peig and Green (2009), and was calculated as: $SMI = W_i(L_0/L_i)^{b_{SMA}}$, where W_i and L_i are the weight and length of each individual respectively, L_0 is a suitable length against which the BCI values were standardized, and b_{SMA} is the scaling exponent, (i.e. the slope of a standardized major axis regression of the mass-length relationship). For L_0 we used the median for each fish species (see Table S1).

Measured fish were inspected visually for infections of the water mold *Saprolegnia* and the anchor worm *L. cyprinacea*. We focused on these two parasites because they are well-known pathogens causing high mortality in fish, especially *Saprolegnia* (Noga, 2011), and are the most frequently recorded external macro-parasitic infections in the region (e.g. Gutiérrez-Galindo and Lacasa-Millán, 2005; AMV pers. observ.). These parasites are easy to detect visually (see Noga, 2011), which facilitates logistics in extensive fish surveys. Destructive sampling was not possible given the conservation status of many native species. All fish were released alive in each site at the end of the survey.

2.3. Descriptors of river environmental conditions

We characterized each sampling site with 13 variables related to geography, water and habitat quality. As geographical features, we recorded the basin name and elevation (m.a.s.l.) in each site using Google Earth®. Elevation was used as a surrogate for the position of the sampling site in the river, and summarise the role of natural spatial gradients in fish indicators, as previously validated in this region (Maceda-Veiga et al., 2013; Murphy et al., 2013). We also calculated the Strahler stream order number (Strahler, 1964) using a map (1:50000) as a measure of river size. Rivers were ranked from a small, first order tributary to the largest main river based on a hierarchy of tributaries.

Prior to each fish survey, we measured seven water-quality variables in situ. A digital multiparametric YSI® sonde was used for temperature (°C), conductivity (μS/cm) and pH, and the colourimetric test kit VISOCOLOR® for ammonium ($mg \cdot l^{-1}$), nitrite ($mg \cdot l^{-1}$), nitrate ($mg \cdot l^{-1}$) and phosphate-P ($mg \cdot l^{-1}$) concentrations. These variables

Table 1
Occurrence (%) and status (endemism, native and introduced) of fish species in rivers from northeastern Spain. Acronyms are provided for the 12 most frequently recorded native species (>4.53) included in the analyses as response variables (see methods). (^{t}) indicates translocated native species in some catchments of NE Spain, and (^{w}) indicates species listed as world's worst invasive species according to the Invasive Species Specialist group (http://www.issg.org/).

Scientific name	Acronyms	Occurrence	Status in Spain
Achondrostoma arcasii (Steindachner, 1966)	ACAR	4.72	Endemism
Anguilla anguilla (Linnaeus, 1758)	ANAN	11.51	Native
Barbatula spp.[a]	BASP	12	Native^t
Barbus haasi (Mertens 1925)	BAHA	26.06	Endemism
Barbus meridionalis (Risso, 1827)	BAME	15.66	Native
Cobitis calderoni (Bacescu, 1962)	–	2.45	Endemism
Cobitis palludica (de Buen, 1930)	–	0.19	Endemism
Cottus hispaniolensis (Bacescu & Mester, 1964)	–	0.57	Native
Gobio spp.[a]	GOSP	26.91	Native^t
Luciobarbus graellsii (Steindachner, 1866)	LUGR	28.11	Endemism^t
Parachondrostoma miegii (Steindachner, 1866)	PAMY	21.89	Endemism^t
Phoxinus spp.[a]	PHSP	30.94	Native^t
Salaria fluviatilis (Asso, 1801)	SAFL	4.53	Native
Salmo trutta (Linnaeus, 1758)	SATR	34.34	Native^t
Squalius laietanus (Doadrio, Kottelat & de Sostoa, 2007)	SQLA	16.23	Native
Gasterosteus aculeatus (Linnaeus, 1758)	–	0.94	Native
Alburnus alburnus (Linnaeus, 1758)	–	9.06	Introduced
Ameirus melas (Rafinesque, 1820)	–	0.75	Introduced
Carassius spp.[a]	–	1.51	Introduced
Cyprinus carpio (Linnaeus, 1758)	–	14.91	Introduced^w
Esox lucius (Linnaeus, 1758)	–	0.38	Introduced
Gambusia holbrooki (Girard, 1859)	–	2.45	Introduced^w
Lepomis gibbosus (Linnaeus, 1758)	–	4.15	Introduced
Micropterus salmoides (Lacèpede, 1802)	–	1.32	Introduced^w
Oncorhynchus mykiss (Walbaum, 1792)	–	1.89	Introduced
Pseudorasbora parva (Temminck & Schlegel, 1846)	–	0.19	Introduced
Rutilus rutilus (Linnaeus, 1758)	–	0.38	Introduced
Sander lucioperca (Linnaeus, 1758)	–	0.38	Introduced
Scardinius erythrophthalmus (Linnaeus, 1758)	–	3.4	Introduced
Silurus glanis (Linnaeus, 1758)	–	1.89	Introduced

[a] Taxa pending of genetic studies to confirm species identify in some catchments of NE Spain.

provide an overview of major water quality stressors affecting directly (e.g. direct toxic effects) or indirectly (e.g. alterations in infectious disease dynamics, food availability, and disruption of biogeochemical cycles) the fish fauna, such as nutrient pollution (Snieszko, 1974; Camargo and Alonso, 2006; Johnson et al., 2010), acidity (Ormerod et al., 1987; Pye et al., 2012), and alterations in the overall ionic composition of water (e.g. heavy metals, sulphates, chlorides), as defined by conductivity (hereafter salinity, Williams, 1987; Kefford et al., 2002). Peaks in salinity and nutrient levels occur in rivers after discharge of sewage effluents from industries (e.g. a textile industry in Colin et al., 2016b) or urban waste (e.g. Maceda-Veiga et al., 2010). Sewage discharges are typically a complex matrix of pollutants (e.g. oil products, drugs, heavy metals, pesticides, nutrients, and chlorides), so that our water-quality variables are likely to be good indicators of general, chronic water pollution events. This reasoning also applies to diffuse pollution from adjacent agricultural areas because runoff brings fertilizers and many ions to rivers that alter electrical conductivity and nutrient concentrations (Rissman and Carpenter, 2015). The degree of silting was estimated as the % of the streambed covered by sediment <1 mm (Townsend et al., 2008). Silting is caused by heavy eutrophication, runoff, and by the release of detritus from sewage treatment plants from overflow (e.g. Quevauviller et al., 2007; Townsend et al., 2008; Rissman and Carpenter, 2015).

We used an integrated measure of habitat diversity derived from the US Rapid Bioassessment Protocol (Barbour et al., 1999) to characterize physical habitat quality. This measure ranks the proportion of riffles, tables and pools, and the presence of permanent refugees for fish (e.g. caves), from 0 to 10. We computed the riparian vegetation quality index QBR (Munné et al., 2003) as an integrated measure of river morphology and riparian quality used widely by water agencies in Spain. QBR ranks the total riparian cover (if there is riparian forest and it is connected with adjacent natural forest areas), cover structure (% of native tree species, shrubs and helophytes, and how these are distributed along the riparian area), cover quality (presence of non-native trees), and river channel naturalness (e.g. presence of weirs, walls) on a scale from 0 to 100 (see details in Munné et al., 2003). We calculated mean current speed ($m \cdot s^{-1}$) in each sampling site from three values measured along transects set perpendicular to the water flow at 20-m intervals. Last, the percentage of dead wood (>1 cm diameter) was estimated in each 100-m river reach because this is a major determinant of habitat complexity and river productivity (Gregory et al., 2003).

2.4. Data analyses

2.4.1. Defining the response variables and groups of predictors

We explored whether the presence of non-native species can affect the sensitivity of native fish species to water-quality stressors by using six response variables: (1) species occurrence; (2) species abundance if present; (3) disease occurrence; (4) disease rate where disease was detected; (5) log(fish length); and (6) log(body condition). Individual measures of length and body condition (defined by the SMI) were averaged for fish species and site. Disease rate was defined as the number of infected fish from number inspected. Models for binary variables (1 and 3) used the full data-set ($N = 530$ sampling sites) to identify the determinants of the presence of fish or disease. Then, within the pool of sites where infected fish ($N = 170$) or any of the twelve native fish species were detected ($N = 451$), we explored how variation in fish abundance (2), disease rate (4), mean fish size (5) and mean SMI (6) was affected by the same predictors, namely geographical and hydro-morphological variables (*hv*), water quality stressors (*wq*), and the presence of non-native fish species (*ispp*) (see below).

2.4.2. Modelling the response of native fish species as function of environmental stressors and the presence of non-native species

All predictors were included in the models individually (see details in Table S2), but are collectively referred to as *hv*, *wq*, and *ispp* for brevity. Temperature was excluded because of its high correlation with elevation (Spearman's $\rho > 0.70$). All environmental predictors were standardized (0 mean, unit variance) using the function *scale* in R to aid model fitting and to have variation among disparate variables on a comparable scale (i.e. in units of standard deviations). Values for fish length and SMI were log-normally distributed, so these data were log-transformed prior to the modelling techniques. Fish predictors (i.e. abundances of introduced piscivores, translocated native piscivores, and of all non-native fish species) were transformed to P/A to improve model fitting because of the highly skewed distribution.

We used generalized linear mixed models (the function *glmer* in R) for each of the six native fish response variables as function of the five predictor combinations, differing only in the error structure most appropriate for the response and hence the link function T (e.g. Bernoulli/logit for occurrence, binomial/logit for rates, and normal/identity for log(fish length)). Our base model included geography and hydro-morphological descriptors (*hv*), and the other four models added water quality variables (*wq*), non-native fish species (*ispp*), and the interaction between $wq * ispp$: (1) $T(\text{response}) = \alpha + hv + \rho$; (2) $T(\text{response}) = \alpha + hv + wq + \rho$; (3) $T(\text{response}) = \alpha + hv + ispp + \rho$; (4) $T(\text{response}) = \alpha + hv + wq + ispp + \rho$; (5) $T(\text{response}) = \alpha + hv + wq + ispp + wq * ispp + \rho$. All models were multi-species models (all twelve fish species were analysed together). Fish species, basin, and year (collectively referred to as $\rho_{.}$) were included in all models as random effects to be able to account for potential systematic differences among basins, years, and native species.

If non-native species modulated the effects of water quality variables, then we expected that model (5) would be a substantially better fit than model (4). The first three models for each response were fitted for completeness. Model parsimony was tested using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC), and models 4 and 5 were explicitly assessed with regard to the importance or the inclusion of the interaction term $wq^{*}ispp$. If a 'best' model was identified (ΔAIC or ΔBIC > 2), we assessed model fit by correlating observed and fitted values, which were obtained by using the function *predictSE.merMod* in the R. Models were validated through diagnostic plots of model residuals following Zuur et al. (2009). AIC and BIC were both assessed because these penalize complex models to different degrees (Burnham and Anderson, 2002).

2.4.3. Native fish species: 'preferred' river conditions

Three analyses were done to provide information on the potential preferred conditions for the native fish species. Differences in geographic, hydro-morphological, and water-quality variables between invaded and non-invaded sites first were tested using Mann-Whitney *U* tests. The same method was used to compare environmental conditions between sites with and without fish (whether native or non-native) to identify the existence of pollution 'black spots'. Statistical significance for *U* tests was $P < 0.05$. Last, we estimated the potential preferred water conditions for the twelve native fish species collectively based on changes in their abundances using weighted-average equations. Here, $WA = (Y_1X_1 + Y_2X_2 + \ldots + Y_nX_n) / (Y_1 + Y_2 + \ldots + Y_n)$, where Y_i is the relative abundance of the twelve native fish species in sites (from 1 to N), and X_i is the corresponding water-quality variable. WA was estimated for each water variable (ammonium, nitrite, nitrate, phosphate-P, salinity, and pH), and the result indicated the water conditions where total fish captures were higher on average. All statistical analyses were performed using the R package (R Core Team, 2016) and the libraries *lme4* (Bates et al., 2015) and *AICcmodavg* (Mazerolle, 2016).

3. Results

We captured 14 non-native and 16 native fish species, of which six (*Barbatula* spp.; *Gobio* spp.; the Ebro barbel, *Luciobarbus graellsii* Steindachner, 1866; the Ebro nase, *Parachondrostoma miegii* Steindachner, 1866; *Phoxinus* spp.; and *S. trutta*) were translocated native species in some catchments (Table 1). The most frequently recorded non-native species (>3.4% of sampling sites) were *C. carpio*, the bleak (*Alburnus alburnus* Linnaeus, 1758), the pumpkinseed (*Lepomis gibbosus* Linnaeus, 1758), and the rudd (*Scardinius erythrophthalmus* Linnaeus, 1758) (Table 1). Four native species were very rare (Table 1) so we focused on analyses for the other twelve. The twelve includes species mostly found >500 m of elevation (e.g. *S. trutta* and *Barbatula* spp.), and typical representatives of medium, lowland reaches (e.g. *A. anguilla* and the Ebro chub *Squalius laietanus* Doadrio, Kottelat & de Sostoa, 2007) at different water velocities (Fig. S1). Native and non-native fish species were captured in a wide range of geographic, hydro-morphological, and water conditions (Table S3).

3.1. Modelling the six native fish responses as function of environmental stressors and the presence of non-native fish species

Generalized linear mixed models revealed that the base model including geographical and hydro-morphological features was the most parsimonious among the five tested for explaining variation in native fish abundance, the presence of the ectoparasites *L. cyprinacea* or *Saprolegnia*, and changes in mean fish size or SMI (Table 2, Table S4). Water quality, non-native species or their interaction did not add substantially to this base model (Table 2). We assessed model fit for the fish responses by correlating observed values with those predicted using the *predictSE.merMod* function. The R^2 values were close to 0, suggesting that even though the base models were the most parsimonious, these were poor at explaining variation in those responses (Table 2). For native fish species occurrence, the AIC and BIC criteria selected different combinations of predictors. The additive model including geographical and hydro-morphological features plus water quality variables and the presence of non-native species was the *best* model using AIC, but the base model was superior when using BIC for assessment. So, there was no overall *best* model for native fish species occurrence, but the interactive model was poorer than the purely additive one (Table 2).

For variation in disease rate among infected fish with *L. cyprinacea* or *Saprolegnia*, the AIC and BIC criteria consistently identified the model including the additive effects of all our set of predictors plus the interaction between water quality variables and the presence of non-native species as the *best* model (Table 2). However, overall model fit again was poor with the correlation between the observed values and the predicted values being $R^2 = 0.045$ (95% CI $= 0.012, 0.094$). Therefore, even though the full-interaction model for disease rate if there was disease was substantially superior to its rivals, the model was poor in its own right.

3.2. River environmental conditions that potentially favour the native fish species

Generalized linear mixed models showed limited evidence that the presence of non-native fish affect markedly native fish tolerance to salinization, nutrient pollution, pH, and silting. Then, our next step was to explore the river environmental conditions that are likely to favour the presence and abundance of the twelve native fish species. The comparison of environmental descriptors between invaded and non-invaded sites revealed that non-natives had a positive association with low elevation and low scores of the habitat index QBR, and high values of salinity, pH, ammonium, nitrite, and silt (Tables S3 and S5). Similar analyses between fish and fish-less river reaches highlighted the existence of extreme environmental conditions (e.g. salinity >4108 μS/cm, Table S3) in which no fish species (native or non-native) was captured. Based on the results of weighted average equations using the abundance of all twelve native fish species combined, the river conditions that are likely to benefit these fish species are: ammonium $= 0.07\ \text{mg}\cdot\text{l}^{-1}$, nitrite $= 0.03\ \text{mg}\cdot\text{l}^{-1}$, nitrate $= 2.94\ \text{mg}\cdot\text{l}^{-1}$, phosphate-P $= 0.25\ \text{mg}\cdot\text{l}^{-1}$, pH $= 7.9$, and salinity $= 644$ μS/cm. These values are rather similar to the median values for these water quality variables obtained by the pair-wise comparison of sites with and without native fish species (Table S5). However, there can be variability in water quality requirements among native fish species (Fig. 2).

4. Discussion

Our study is a first in showing that, with multiple lines of evidence, the sensitivity of 12 native European fish species to water pollution is not associated with the presence of non-native species, a major driver of global ecological change (Cucherousset and Olden, 2011; Vilà and Hulme, 2017). These results emerge from modelling the combined effects of alterations in the river hydro-morphology, water quality, and the presence of non-native fish species. Overall, the most parsimonious models only included the river hydromorphology. This indicates that the restoration of water velocity, riparian area, and physical habitat structure is likely to be a more effective management action for the native fish than removal of non-native species and improving water quality. Our results also suggest that additive effects of environmental degradation and non-native species occurrence on native fish prevail over interactions, which potentially simplifies management actions.

Interactions among stressors are at the core of many discussions on the reasons for current global biodiversity crisis (Paine et al., 1998; Folt

Table 2
Fit statistics for generalized linear mixed models assessed by AIC and BIC values exploring the additive and interactive effects of water quality degradation (*wq*) and the presence of non-native fish species (*ispp*) on six native fish responses in rivers under strong hydro-morphological pressure (*hv*). All models include basin, year and native fish species as random terms (ρ). All random terms and predictors within each category (*hv*, *wq*, and *ispp*) were included in models individually (see Table S2 for details of values for best models). An assessment of the adequacy of the *best* model was made using R^2 between observed and fitted values (see Methods).

	Models					Conclusion	Best model overall fit
Native fish responses	$\alpha+hv+\rho.$	$\alpha+hv+wq+\rho.$	$\alpha+hv+ispp+\rho.$	$\alpha+hv+wq+ispp+\rho.$	$\alpha+hv+wq+ispp+wq*ispp+\rho.$	'Best' model Interaction vs non-interaction	R^2 (95% conf. interval)
Occurrence[a]							
AIC	5183	5175	5173	5165	5178	No clear candidate	–
BIC	5250	5281	5259	5291	5423	Interaction model worse	
Abundance|occurred[b]							
AIC	4348	4371	4363	4384	4453	$\alpha+hv+\rho.$	0.003(0.016, 0.057)
BIC	4404	4456	4433	4485	4645	Interaction model worse	
log(Fish length)							
AIC	1046	1088	1064	1106	1225	$\alpha+hv+\rho.$	$-0.005(-0.018, <10^{-6})$
BIC	1099	1168	1130	1201	1405	Interaction model worse	
log(Scaled Mass Index)							
AIC	1094	1139	1119	1164	1286	$\alpha+hv+\rho.$	$-0.002(-0.012, 0.001)$
BIC	1146	1220	1185	1258	1466	Interaction model worse	
Disease occurrence[a]							
AIC	1290	1284	1294	1288	1295	$\alpha+hv+\rho.$	0.005(<0.001, 0.017)
BIC	1340	1364	1359	1384	1480	Interaction model worse	
Disease rate|diseased[a]							
AIC	2589	2481	2588	2477	2291	$\alpha+hv+wq+ispp+wq*ispp+\rho.$	0.045(0.012, 0.094)
BIC	2628	2543	2639	2551	2435	Interaction model much better	

[a] Presence and absence data.
[b] Excluded sites without fishes and sites in which no fish were infected, respectively.

et al., 1999; Piggott et al., 2015; Côté et al., 2016), but little is known of such effects for freshwater fish (Jackson et al., 2016; Schinegger et al., 2016). Our study addressed for the first time the interactive effects of non-native species and water pollution on six indicators of the health status of native fish, and supports that the risks of interactive effects may have been overestimated (Côté et al., 2016). Overall, our models identified hydro-morphological alteration as a major stressor in rivers of northeastern Spain, in agreement with a study examining diversity patterns (Maceda-Veiga et al., 2017a). Miranda et al. (2005) found that the decline of the Southern Iberian spined-loach (*Cobitis paludica* de Buen, 1930), which is currently rare in our study area (Table 1), was related to the construction of hydrological impoundments. Therefore, hydro-morphological restoration would seem to be the primary management action to benefit native fish in northeastern Spain, as recommended for many rivers worldwide (Marchetti and Moyle, 2001; Schmutz et al., 2016). In particular, we suggest creating natural flow regimes with median water velocities of 0.4 $\mathrm{m \cdot s/^{-1}}$, which is suggested by the characteristics of sites at which native fish species were found (Table S1).

Hydro-morphological restoration can provide many benefits for native fish (Schmutz et al., 2016), including the control of non-native species and water pollution. Most non-native fish introduced in Mediterranean rivers appear to prefer slow water speeds (Marr et al., 2013), even though they often thrive in similar values than natives, as shown in our study. Higher water flow increases the dilution power of rivers and hence a river's ability to degrade pollutants can be partially improved (e.g. Colin et al., 2016b), especially if combined with riparian restoration (Osborne and Kovacic, 1993). However, many physico-chemical and biological variables of rivers affect biodegradation (e.g. temperature, solar irradiation, biofilm biomass), coupled to the different chemical and physical properties of the pollutants themselves (Alexander, 1999; Gurr and Reinhard, 2006). Although water pollution and non-native species can adversely affect native fish (Cucherousset and Olden, 2011; Colin et al., 2016a), we found little evidence for effects on the twelve native fish species. The absence of a signal might be due to rivers being very dynamic and a single snapshot sampling of biota and abiotic conditions may not be able to reveal strong relationships (Heino et al., 2015), especially in fish due to their high mobility (e.g. Maceda-Veiga et al., 2017a).

Extreme concentrations of ammonium, a well-known tracer of untreated wastewater (Quevauviller et al., 2007) that is highly toxic in its unionized form at basic pH (Emerson et al., 1975), were quite frequent (Fig. 2). The maximum values of nitrite and conductivity (i.e. salinity) detected provide evidence further that some pollution 'black spots' remain after investment in sewage treatment plants in recent decades (Munné et al., 2012), as has been reported in spatially restricted studies (Maceda-Veiga et al., 2010; Colin et al., 2016b). Moreover, extensive environmental chemical surveys (López-Serna et al., 2012; Kuzmanović et al., 2015) indicate that other toxicants that we did not measure (e.g. oils, metals, pesticides, and antibiotics) have been detected in these rivers, the effects of some of which are currently unknown (see emerging pollutants in Petrovic et al., 2008). The existence of 'black spots' caused only by unmeasured toxicants might have obscured our environmental-fish relationships. However, this seems to be unlikely because the entry of these compounds to rivers is mainly through urban and industrial sewage discharges that typically also affect nutrient concentrations and river salinity (e.g. a textile industry in Colin et al., 2016b).

Nutrient pollution by nitrate and phosphate is a growing major threat to aquatic ecosystems (Smith and Schindler, 2009; Rissman and Carpenter, 2015). However, native fish from Mediterranean rivers probably can acclimate more easily to nitrogen and phosphorous pollution than to those just listed (but see Camargo and Alonso, 2006; Smallbone et al., 2016). As a by-product of metabolism, concentrations of nitrogen and phosphorous can increase in the isolated pools of small rivers in which fish congregate during drought (Magoulick and Kobza, 2003). Salinization may also do so but can protect fish against nitrogen toxicity (Wedemeyer and Yasutake, 1978; Noga, 2011) and some infections, including *Saprolegnia* (Mifsud and Rowland, 2008; Noga, 2011). Based on the median values at which we found any of the twelve fish species, nitrite, and ammonium should be about 0 at pH 8 due to high toxicity (see details in Emerson et al., 1975). For

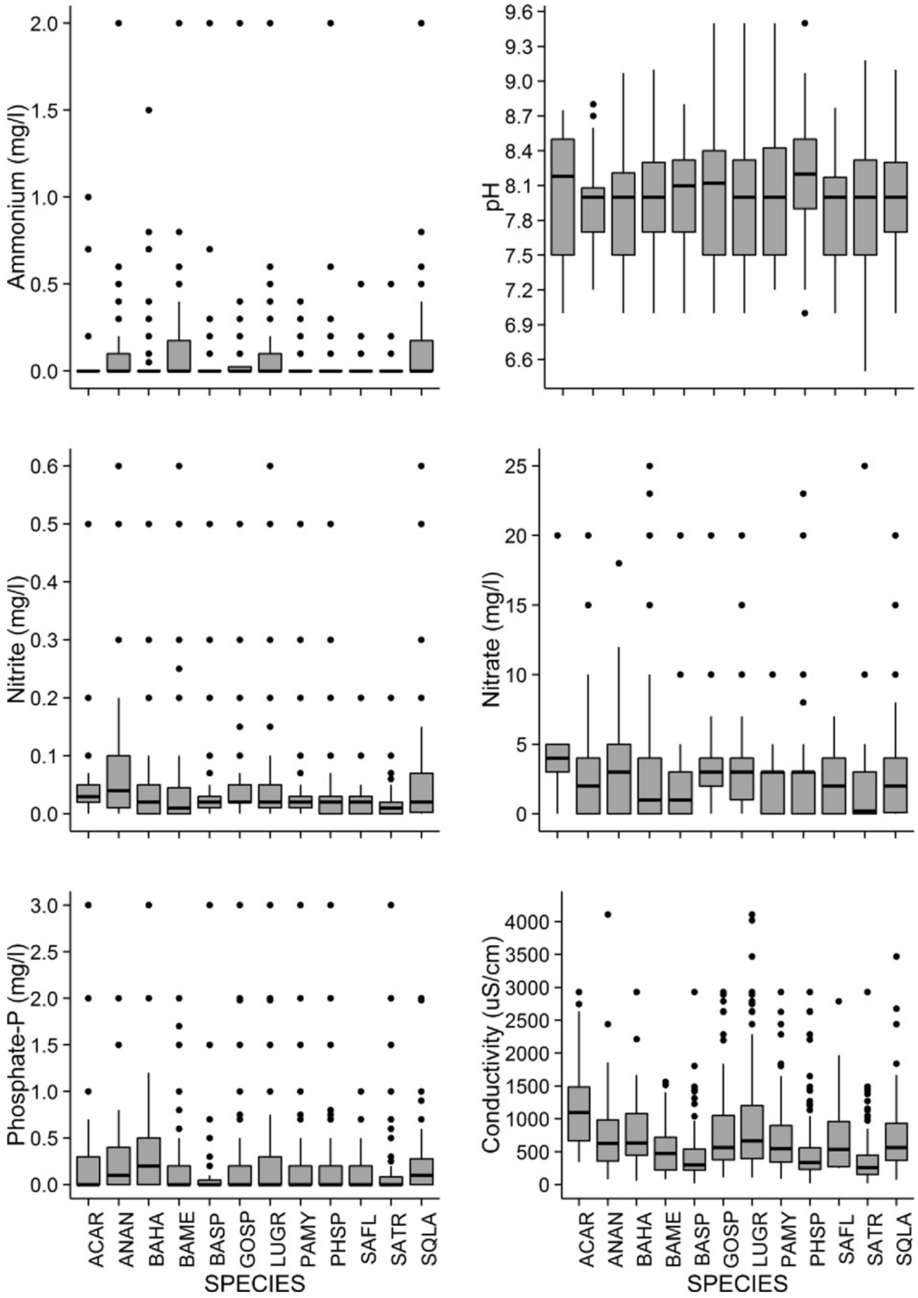

Fig. 2. Changes in water properties in river reaches of northeastern Spain at which the twelve native fish species were found (ACAR, *Achondrostoma arcasii*; ANAN, *Anguilla anguilla*, BAHA, *Barbus haasi*; BAME, *Barbus meridionalis*; BASP, *Barbatula* spp.; GOSP, *Gobio* spp.; LUGR, *Luciobarbus graellsii*; PAMY, *Parachondrostoma miegii*; PHSP, *Phoxinus* spp.; SAFL, *Salaria fluviatilis*; SATR, *Salmo trutta*; and SQLA, *Squalius laietanus*). Each box corresponds to 25th and 75th percentiles; the dark line inside each box represents the median; whiskers show the minima and maxima except for outliers shown as filled circles.

salinity, nitrate and phosphate-P, 'optimal' values seem to be ~560 μS/cm, <2 mg NO_3^- l^{-1}, <0.1 mg PO_4^{3-}-P l^{-1} (see Table S3). These results were mostly consistent with the weighted average approach using fish abundances, but we present here the lowest values for all these water-quality variables in line with the precautionary principle. There is evidence of their direct toxicity to fish and their negative effects at the ecosystem level (Camargo and Alonso, 2006; Johnson et al., 2010; Noga, 2011; Kefford et al., 2002). However, we caution for potential differences in tolerance among native species (see Fig. 2 and Maceda-Veiga and de Sostoa, 2011).

Although we did not find that non-native species modulate significantly the sensitivity of native fish to water pollution, non-native species mostly were found in river reaches with a higher percentage of silt, greater salinity, and higher concentrations of ammonium and nitrite, than were natives. This suggests that non-native species are likely to be more tolerant than natives, being the introduced *C. carpio* probably the most tolerant. This species profoundly alter the environment while bioturbing (Shin-Ichiro et al., 2009). However, 30% of the non-native species in our study were native species usually transferred between basins by anglers, so that some of these natives may fit within the tolerance category of some introduced (see Maceda-Veiga and de Sostoa, 2011). Nonetheless, even introduced species can perish when exposed to extreme values of nutrient pollution and salinity (e.g. >4108 μS/cm).

Non-native species may carry pathogens that spread among native species, such as the *L. cyprinacea*. We found four more native fish hosts (*A. anguilla*; the Mediterranean barbel *Barbus meridionalis* Risso, 1827; *Phoxinus* spp.; and the river blenny *Salaria fluviatilis* Asso, 1801;

Table S3) to add to the list of Sánchez-Hernández (2017). Although there was little evidence in our data, poor water quality and non-native fish occurrence may have enhanced disease rate on natives, possibly due to a higher immune-suppression. Lentic habitats favoured by human activity may increase further the transmission of parasites that only use one host to complete their life-cycle. An outbreak of the white spot disease (*Ichthyophthirius multifiliis*) on the Iberian redfin barbel (*Barbus haasi* Mertens 1925) occurred in a Mediterranean stream during a multi-seasonal drought (Maceda-Veiga et al., 2009).

Due to highly skewed distributions, we had to use presence/absence data for non-native species rather than abundances, and this may have limited the explanatory power of models. None of the common data transformations that we considered helped to make the abundance data for non-natives normal or uniform, so that the use of presence/absence was the best statistical option. However, the effects of trophic competition and predation depend on abundances so more subtle nuances might arise if the data were restricted to only those sites at which non-natives were caught. Adults of many non-native fish (e.g. *M. salmoides*; the Wels catfish, *Silurus glanis* Linnaeus, 1758; and the pike-perch, *Sander lucioperca* Linnaeus, 1758) are piscivorous and their juveniles, and the rest of introduced species (e.g. *A. alburnus*, *L. gibbosus*, *S. erythrophthalmus*), are invertivorous or omnivorous like most native fish in Mediterranean rivers, including northeastern Spain (De Sostoa, 1990).

As with all correlative studies, we cannot definitely show cause-effect relationships, so that our suggestions need testing with specific experiments. It is possible that our fish-environment relationships are distorted because native fish species occur in far from their optimal conditions. More work can shed light on this by examining how multiple stressors affect direct measures of fitness (e.g. fecundity, survivorship). Nonetheless, the results of the present study builds on previous ones (e.g. Boix et al., 2010; Maceda-Veiga et al., 2010, 2017a) examining other facets of fish communities and populations (e.g. community structure, physiological alterations), including different data, to provide evidence that there is urgent need to remedy some of the hydro-morphological alterations and pollution spills occurring in this region. These results do not exclude the benefits that focal removal of non-native species could have for the native fish in reaches of high conservation value, such as tributaries (Maceda-Veiga et al., 2017a).

5. Conclusions

The risk of 'ecological surprises' from the diversity and magnitude of stressors affecting ecosystems is a strong theme in the ecological literature (Paine et al., 1998; Lindenmayer et al., 2010; Piggott et al., 2015; Jackson et al., 2016), and has important management implications. Our results suggest that there is little if any evidence for the importance of interactions among water-quality and non-native species as stressors, so that managing stressors independently probably is the most appropriate course of action and is likely to have major conservation benefits (Côté et al., 2016). We provide information for potential safe water quality values for twelve native fish. However, water quality and non-native species occurrence did not add substantially to our models. Therefore, we suggest that restoring natural hydro-morphology in rivers is likely to be the most effective management approach to improving the prospects for this native fish fauna.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.07.010.

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Environmental stressors and the transmission of a globally invasive parasite in riverine fish: additive vs interactive effects

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Abstract

The increased rate of outbreaks of infectious diseases in ecosystems is among the most dramatic consequences of global change, particularly when outbreaks affect highly threatened, important taxa, such as freshwater fish. However, the links between disease–inducing epizootics and widespread environmental stressors, including nutrient pollution and salinization, in freshwater organisms are largely unexplored. Ours is the first study to examine additive and interactive effects of pervasive environmental stressors on a riverine host–parasite system along large–scale environmental gradients. We explored the host and environmental factors that singly, or in combination, are likely to influence the spread of a worldwide invasive parasite, the anchor worm *Lernaea cyprinacea*, in an extensive region of north–eastern Spain (99,700 km^2, 15 river catchments, *N* = 530 sites). Smaller fish species had higher probabilities of infection, while lower altitudes, river salinity and nutrient pollution were associated with higher probabilities of infection in 19 endemic and widely distributed fish species. We found no evidence that interactive effects among major riverine stressors affected the occurrence of *L. cyprinacea* in fish despite the current emphasis on the importance of accounting for interactions among stressors in biodiversity management; our results suggest that parasite management might be effective on a stressor–by–stressor basis. Given that nutrient pollution and salinization were two of the major factors contributing to increased occurrence of *L. cyprinacea*, the improvement of wastewater treatment and agricultural practices, and the restoration of riparian areas probably would reduce the occurrence of this widespread parasite among native fish.

Keywords: alien species; epizootics; eutrophication; generalized linear mixed models; hierarchical partitioning; host–parasite interactions; multiple stressors; salinization

Introduction

Infectious diseases are emerging at unprecedented rates and scales, raising concerns among conservationists, ecologists, and public health agencies (Jones *et al*., 2008; Smith *et al*., 2014). Although the causes of the emergence of many diseases are uncertain, human actions probably contribute substantially to many epidemics (Budria & Candolin, 2014; Rogalski *et al*., 2017). Human activities, such as intensive agriculture (Ponisio *et al*., 2016), fishing (Kaiser *et al*., 2015), and dam construction (Poff *et al*., 2007), are homogenizing aquatic ecosystems around the world, and disease outbreaks often arise when ecosystems become simplified (Elton, 1958). Ecosystems are altered further by the accumulation of alien species, which continues unabated (Seebens *et al*., 2017). Given that the global human footprint is expected to increase even further (Hoekstra & Wiedmann, 2014), understanding how human impacts affect infection dynamics is a priority for disease ecology under global change.

Global change factors consist of a variety of biotic and abiotic alterations induced by human activities, which can interact in complex ways (see Piggott *et al*., 2015; Côté *et al*., 2016). Whereas 'native' host–parasite interactions have a long co–evolutionary history, this is not the case for novel pathogenic interactions, if say, native host vulnerability to alien parasites is higher than it is to native parasites (Dunn & Hatcher, 2015). Other global change processes, such as aquatic nutrient pollution (Sinha *et al*., 2017), can further increase host vulnerability if such processes increase the virulence of a disease (Vega Thurber *et al*., 2014). However, the impacts of pollution on host-parasite relationships are difficult to predict due to the dependence on context (Marcogliese & Pietrock, 2011; Budria *et al*., 2017). While high concentrations of pollutants, including nutrients, can kill both parasites and hosts (Vidal–Martínez *et al*., 2010; Smallbone *et al*., 2016), nutrients also may improve host body-condition and facilitate hosts to better cope with the effects of pollutants and parasites (Marcogliese & Pietrock, 2011; Budria *et al*., 2017). Nutrient pollution also is expected to promote the transmission of horizontally transmitted parasites (e.g. ectoparasites) because it increases host population density (McKenzie & Townsend, 2007). External parasites are often more sensitive to environmental fluctuations in aquatic systems than are internal parasites (Overstreet, 1993). Riverine ecosystems are among the most dynamic and human–affected ecosystems in the world (Williamson *et al.*, 2008), yet the ways in which multiple natural and anthropogenic factors affect interactions and epidemiology in host–parasite systems remain little studied (Blasco–Costa *et al*., 2013; Hofmann *et al*., 2016).

A globally important parasite is the Asiatic anchor worm *Lernaea cyprinacea* Linnaeus 1758, which is a parasitic copepod introduced widely into temperate and tropical regions with carp *Cyprinus carpio* Linnaeus, 1758 (Hoffman, 1999; Hassan *et al*., 2008; Welicky *et al*., 2017). The anchor worm is capable of infecting fish and amphibians (Hoffman, 1999; Kupferberg *et al*., 2009), and high mortalities have been reported for fish suffering from anchor worm infections (Noga, 2011; Raissy *et al*., 2013). The anchor worm is expected to spread further with global warming (Bulow *et al*., 1979; Sánchez–Hernández, 2017) and when fish are forced into high concentrations during drought (Medeiros & Maltchik, 1999; but see Welicky *et al*., 2017). Infections by parasitic copepods, including *L. cyprinacea*, are influenced by fish size (Poulin *et al*., 1991), water–quality stressors, such as nutrient enrichment (Palm, 2011) and salinity, which can kill the parasites (Noga, 2011). However, rivers experience other stressors, such as alterations in river morphology and riparian areas, which can reduce body condition in fish hosts (Maceda–Veiga *et al*., 2014), potentially increasing fishes' susceptibility to disease. On the other hand, interactions among stressors may be advantageous to the fish if, say, salinization reduces nutrient toxicity (Sampaio *et al*., 2002; Noga, 2011). Therefore, the effects of combinations of environmental stressors may not be predictable from the established individual effects of stressors; i.e., the effects of stressors may not be additive (Williams et al., 2007; Hiers *et al*., 2016).

Determining the spatial location of disease outbreaks, the individuals that are most vulnerable, and the environmental conditions that promote infection, are central thinking in disease ecology (Stephens *et al*., 2016). A major limitation in understanding parasitic infections is that most surveys are limited spatially (Stephens *et al*., 2016), which hinders the identification of clear relationships between disease and large–scale environmental gradients. While the effects of multiple factors on parasite transmission are testable experimentally (e.g. Lenihan *et al*., 1999; Studer & Poulin, 2013), manipulations rarely can be conducted at the spatial and temporal scales at which biota actually experience the stressors (Mac Nally, 1997). Smaller scale experiments may not represent well patterns of spatial and temporal variation in stressors and hence the consequent biotic effects (Englund & Cooper, 2003). Experiments exploring the effects of multiple factors also require many animals as experimental units, which is ethically unacceptable for studies on vertebrates, especially threatened species, under animal welfare legislation (e.g. EU Directive 2010/63/UE5). Therefore, the use of extensive field surveys typically is used to identify the factors that, alone or in combination, are harmful to biota (e.g. Dahm *et al*., 2013; Gieswein *et al*., 2017; Radinger *et al*., 2017), including the causes of parasite transmission.

Here, we explored the host and environmental factors that singly, or in combination, might influence the spread of the alien parasite *L. cyprinacea* in 19 fish species in an extensive area of northeastern Spain (99,700 km^2, 15 catchments). These rivers are subject to major environmental stressors that are common in rivers worldwide (e.g. impeded water flows, riparian removal, nutrient pollution, and salinization) (Tockner *et al*., 2009; Moyle, 2014), and such rivers are highly prone to biological invasions (Leprieur *et al*., 2008), including by *L. cyprinacea* (Sánchez–Hernández, 2017). The wide range of orographic and river conditions in this Spanish region (Sabater *et al*., 2009; Maceda–Veiga *et al*., 2013; 2017a) allowed us to explore how combinations of different stressors might affect a host–parasite system along upstream–downstream gradients. Fish species include endemic taxa (e.g. *Barbus haasi* Mertens 1925, *Parachondrostoma miegii* Steindachner, 1866), widely distributed European native species (e.g. *Anguilla anguilla* Linnaeus, 1758, *Salmo trutta* Linnaeus, 1758), and worldwide invasive fish species (e.g. *Cyprinus carpio* Linnaeus, 1758, *Gambusia holbrooki* Girard, 1859) (Froese & Pauly, 2017). Alien fish often replace native fish in degraded rivers (Maceda–Veiga *et al*., 2017a), but the anchor worm has low host–specificity (Hoffman, 1999; Noga, 2011), and so, can exploit most fish hosts.

Anchor worm infections were expected to be more prevalent in lowland rivers because the copepod is a warm–water species, and infections may be more limited by the riverine environment than by host availability given the parasite's lack of host specificity. We expected the probability of parasite occurrence to be the highest in degraded rivers because pollution often induces immunosuppression in fish and promotes disease (but see Biagianti–Risbourg *et al*., 2013; Smallbone *et al*., 2016). We explored the occurrence of interactions among some of the major natural and anthropogenic stressors that may govern parasite dynamics. Given that such stressor combinations are present around the world, the identification of these interactions will help to project the distribution of the parasite beyond our study area. Moreover, our work will inform whether the management of river stressors to control diseases can be on a stressor–by–stressor basis or needs to consider their interactive effects on the parasite.

Materials and methods

Study area

We gathered data on river environmental conditions and fish from surveys performed in northeastern Spain from 2002 to 2009 (e.g. Figuerola *et al*., 2012; Maceda–Veiga *et al*., 2017a,b). This data set consisted of 530 sampled sites that involved all Catalonian catchments from the Muga to Riudecanyes basins, and the whole River Ebro and part of the Garonne basin (Fig. 1). We surveyed most river typologies in this region for hydrological alterations, riparian characteristics, geology, water quality, river size and elevation. Most of these rivers are small and have a typical Mediterranean hydrological regime, with droughts in summer and potentially torrential floods in autumn. Large rivers peak in flow in spring from snowmelt. We surveyed in low–flow conditions when fish populations are more stable and can be sampled most effectively using electrofishing (see below). Water temperatures ranged from 9–31ºC, depending on elevation, and both native and alien fish species were present in a wide range of geographic, hydro-morphological, and water conditions (see Maceda-Veiga *et al*., 2017 for further details). The conservation status of riparian areas ranges from relatively well–preserved riparian forests to grasslands, extensive agriculture areas, and weedy areas (Sabater *et al*., 2009), including stands of the globally invasive *Arundo donax* (Maceda–Veiga *et al*., 2016).

Fish surveys

We used an international standardized fish sampling method (CEN standards EN 14962 and EN 14011), in accordance with the European Water Framework Directive (EU Directive 2000/60/EC). Fish were sampled by single–pass electrofishing using a portable unit that generated up to 200V and 3 A pulsed DC in an upstream direction, covering the whole wetted width of the 100–m long reaches at each location (e.g. Maceda–Veiga *et al*., 2017a). We selected the location of each sampling site based on representativeness, and included a variety of habitat types (pools, rifles and runs). The same fishing equipment was used at all sites to avoid potential bias in fish captures. Fish captures were expressed as captures per unit of effort (CPUE, fish caught divided by fishing time in minutes and the area surveyed in m^2). A pilot study in our study area, comparing estimates of richness and abundance between single– and four–pass electrofishing, showed that estimates from single pass were reasonably high with 80–

100 % of the species detected and 60–90 % of the individuals captured (A. Sostoa, *unpublished data*).

Fish were identified to species, counted, and a random set of individuals (≥40 if possible) from each species in each sampling site was measured (total length, mm) and weighed (mg) after being anaesthetized in a buffered MS222® solution (0.02%, Tricaine methane–sulfonate, Sigma®). The entire external surface of measured fish was inspected visually for infections of *L. cyprinacea*. This copepod is easy to detect visually (see Noga, 2011), which facilitates logistics in extensive fish surveys. All fish were allowed to recover in buckets provided with air pumps and released at the site of capture. The procedures used in this study were approved by the University of Barcelona Ethical Committee (C.E.E.A. 510/14) and fish captures were authorized by the Autonomous Government of Catalonia (AP/003).

Taxonomic status of four fish taxa (*Barbatula* spp., *Phoxinus* spp., *Gobio* spp., and *Carassius* spp.) was uncertain due to the description of new species after we completed our surveys and the presence of morphologically cryptic species of the genus *Carassius*. A straightforward change in the nomenclature of the former is complicated because these taxa are subject to inter–basin transfers by anglers (e.g. Doadrio *et al*., 2011). The native status of fish was defined as follows (Table 1): (a) native, if the fish historically occurred in the basin where captured according to regional faunal lists (Doadrio *et al*., 2011), and (b) alien, if the fish species did not historically occur in the basin of capture or elsewhere in the Iberian Peninsula (IP). Each fish species was assigned to a trophic guild (mostly 'omnivorous' or 'invertivorous'), a swimming guild ('benthic' or 'water column') and a migratory-behaviour guild ('almost sedentary' or 'migratory') based on regional fish atlases (de Sostoa *et al*., 1990; Doadrio *et al*., 2011), scientific articles (e.g. Benejam *et al*., 2010; Colin *et al*., 2016) and our experience because the ecology of some of these species is still poorly reported in the literature. In our study area, only the European eel (*A. anguilla*) migrates between marine and freshwater habitats, while the other migratory species (e.g. *S. trutta*, *P. miegii*, *Luciobarbus graellsii* Steindachner, 1866) typically perform within-river upstream migrations for spawning (Doadrio *et al*., 2011).

Environmental stressors

We used 12 variables related to geography, water quality and habitat quality to explore which river environmental conditions might be associated with the spread of *L.*

cyprinaea (Table S2). We recorded the basin name and elevation (m.a.s.l.) using Google Earth®. Elevation was used as a surrogate for the position of the sampling site in the river, and to represent natural spatial factors affecting aquatic organisms (e.g. Maceda–Veiga *et al*., 2017a). We calculated the Strahler stream order number (Strahler, 1964) using a map (1:50000) as a measure of river size. Rivers were ranked from small, first–order tributaries to the largest main river based on a hierarchy of tributaries.

Prior to conducting each fish survey, we measured seven water–quality variables *in situ*. A digital multiparametric YSI® probe was used for temperature (ºC), conductivity (µS/cm) and pH, and the colorimetric test kit VISOCOLOR® for ammonium (mg/l), nitrite (mg/l), nitrate (mg/l) and phosphate–P (mg/l) concentrations. These water variables measure nutrient pollution, acidity, and changes in the overall ionic composition of water (i.e. salinity), which are major stressors affecting freshwater organisms directly (e.g. toxic effects) and indirectly (e.g. alterations in disease dynamics, food availability, and biogeochemical cycles) (e.g. Johnson *et al*., 2010; Noga, 2011; Cañedo–Argüelles *et al*., 2016). Many toxicants in sewage from industries and urban and agricultural areas (e.g. heavy metals, pesticides, and drugs) alter nutrients, pH and conductivity in rivers (e.g. textile industry in Colin *et al*., 2016), so that these variables are used as general indicators of chronic water pollution. We estimated silting as the % of the streambed covered by sediment < 1 mm (Townsend *et al*., 2008). Silting is associated with high levels of eutrophication, agricultural runoff, and the release of detritus from sewage treatment plant outflows.

River hydro–morphology and the quality of riparian areas were characterized with the index QBR (Munné *et al*., 2003), which is an integrated measure widely used by water agencies in Spain. These factors can affect host–parasite relationships either directly by altering ecosystem productivity (Acuña & Tockner, 2010), or indirectly by altering a river's ability to degrade pollutants (Gurr & Reinhard, 2006). QBR ranks the total riparian cover (the presence of riparian areas and their connectivity among them and with adjacent woodlands), cover structure (percentage of riparian trees, shrubs and emergent aquatic plants), cover quality (presence of alien riparian species), and river channel naturalness (e.g. presence of weirs, channels, and walls) on a scale from 0 to 100. Last, we calculated the mean current speed (m/s) in each sampling site from three values measured along transects set perpendicular to the water flow at 20–m intervals.

Statistical analyses

Analyses were conducted in R (R Core Team, 2016) using the packages and functions outlined below. We constructed binomial generalized linear mixed models using *L. cyprinacea* presence (0 or 1) on each individual fish as the response variable to determine which environmental and host predictors are important for this infection. There were random factors (collectively referred to as $\rho_.$) for year, species, basin, and site, which accounted for multiple measurements for a unit (e.g. multiple conspecifics for the same site) and potential systematic differences among components of these factors. All predictors (Table S1) were standardized (0 mean, unit variance) so that all predictors were on comparable scales (i.e. each predictor is expressed in units of its standard deviation) after continuous variables were log-transformed to reduce the influence of extreme values. Predictors were collectively referred to as *host* (fish host variables), *geo* (geography), *wv* (water velocity), *wq* (water quality), and *phq* (physical habitat quality) for brevity (Table S1). However, all predictors within each set of predictors were included in the models as individual variables. Temperature was excluded because of its high correlation with elevation (Spearman's $\rho > 0.70$). Nutrient concentrations were aggregated based on their toxic severity (Camargo & Alonso, 2006; Noga, 2011), such as ammonium and nitrite (TN) and nitrate and phosphate–P (NP).

Following Grueber *et al*. (2011), we built a 'global' model using the function *glmer* in the *lme4* package (Bates *et al*., 2015). This model included all nine environmental predictors, five host predictors, and the random factors for year, species, basin and sampling site. We then constructed nested models (i.e. models with and without a given predictor) to explore whether parasite transmission was strongly associated with fish host abundance, fish body size, fish trophic guild, fish migratory behaviour and fish swimming behaviour (Table S2). Host abundance in a given site was the total combined abundance of all fish species recorded as hosts for *L. cyprinacea* in our region (e.g. Sánchez–Hernández, 2017; Maceda–Veiga *et al*., 2017). We used individual length as a measure of fish body size but outcomes were similar using fish body mass (results not shown).

Using the *best* model (see below for a description of how this was determined) with selected host predictors as a baseline, we built nested models to explore the potential additive effects of all four sets of environmental predictors (*geo*, *wv*, *wq*, and *phq*) to the probability (Pr) of *L. cyprinacea* infection (Table S3). We then used the resultant *best* model to test for additive effects (+) of *geo*, *wv*, *wq*, and *phq* compared with five models including interactions (*) among these four sets of stressors. These models explored whether: (1) the effects of poor habitat and water quality interact with elevation ($\mathrm{Pr} = \alpha + host + geo + \mathrm{elevation} * wv + \mathrm{elevation} * wq + \mathrm{elevation} * phq + \rho$).; (2) changes in water velocity interact with water or habitat quality degradation ($\mathrm{Pr} = \alpha + host + geo + wv * wq + wv * phq + \rho$).; (3) water and physical habitat quality interact together ($\mathrm{Pr} = \alpha + host + geo + wv + wq * phq + \rho$).; (4) salinization interacts with nutrient toxicity ($\mathrm{Pr} = \alpha + host + geo + wv + \mathrm{salinity} * \mathrm{TN} + \mathrm{salinity} * \mathrm{NP} + \mathrm{pH} + phq + \rho$).; and (5) pH interacts with nutrient pollution ($\mathrm{Pr} = \alpha + host + geo + wv + \mathrm{pH} + \mathrm{pH} * \mathrm{TN} + \mathrm{pH} * \mathrm{NP} + phq + \rho$). Although the number of stressor combinations is large, we only sought to compare additive and interactive effects among some of most prevalent stressors, which is a crucial first step in multiple-stressors studies (Piggott *et al*., 2015).

Best models were identified using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC), which penalize model complexity to different degrees and hence are recommended for use in combination (see Fabozzi *et al*., 2014 for further details). Top–ranked models (ΔAIC or ΔBIC ≤ 2.0) were considered the *best* descriptors of the data from the candidate set and the likelihood of non-additive effects was determined by whether the *best* model included just additive or additive *and* interactive terms. For example, if interactive models had greater values of AIC or BIC than the corresponding additive models, then the stressors most probably are operating independently of each other and the likelihood of them having interactive effects on the probability of *L. cyprinacea* occurrence would be low. That is, the added model complexity of interactive models does not provide sufficient improvement in model fit to justify the inference that interactions are important. The absolute adequacy of the *best* models for predicting the presence of *L. cyprinacea* was assessed using the function *auc* (Area Under Curve, designated as 'AUC' in Tables) in the *pROC* package (Robin *et al*., 2011). The *model.avg* function (Burnham & Anderson, 2002) was then used to calculate average parameter estimates from the final *best* models, and indicated the importance of each set of predictors (host attributes, *geo, wv, wq,* or *phq*) on a scale from 0 to 1.

The relative importance of the host and environmental descriptors selected by the AIC-BIC approach in explaining variation in the occurrence of *L. cyprinacea* was compared by using hierarchical partitioning analysis of deviance (HP; function *hier.part* Walsh & Mac Nally, 2002). The function *rand.hp* was used to assess the significance of HP models using a randomization test for hierarchical partitioning analysis based on the upper 0.95 confidence limit. While causality cannot be determined in correlational studies, HP partitions the independent contributions of each predictor to variation in the response variable, and distinguishes these from the joint contribution with other predictors. We note that predictors are always correlated, even when the most highly correlated are excluded (Spearman's $\rho > 0.70$), which can bias regression estimates of GLMMs (see details in Mac Nally, 2002). The strength of GLMMs is that they can handle random effects, which is not possible in HP analysis, so we used averaged fish size per sampling site to avoid pseudo-replication. The importance of the environmental descriptors selected by the AIC-BIC approach was further assessed by comparing data between sites with and without infected fish using Mann-Whitney tests with $\alpha = 0.05$.

Results

A total of 30 932 fish individuals from three orders and seven families was captured in the 15 Mediterranean catchments. Infection was seen in all fish species, apart from the alien *G. holbrooki* and the natives *Cobitis calderoni* Bacescu, 1962 and *Achondrostoma arcasii* Steindachner, 1966 (Table 1). The highest prevalence of infection was in five native cyprinids (*B. haasi*, *Gobio* spp., *L. graellsii*, *P. miegii*, and *Squalius laietanus* Doadrio, Kottelat & de Sostoa, 2007) and in two alien species, the cyprinid *C. carpio* and the centrarchid *Lepomis gibbosus* Linnaeus, 1758 (Table 1).

Among host predictors, generalized linear mixed models (GLMM) revealed that fish length was more influential than total fish abundance or fish species' ecology (trophic guild, swimming and migratory behaviour) in accounting for the presence of *L. cyprinacea*, after having controlled for potential systematic differences among years, species, basins, and sites (see B5 in Table 2). This was an adequate model based on auc, and indicated that smaller fish were more vulnerable to *L. cyprinacea* infection than larger ones (Table 3). However, model averaging showed that water properties were as influential in accounting for infection as fish length, followed by geographical features, physical habitat quality, and water speed (see predictor importance in Table 3). The regression estimates suggested that salinity and nutrient pollution (i.e. nitrate and phosphate–P) made the largest contribution to variation in the probability of *L. cyprinacea* occurrence, with an overall positive effect (Table 3; Fig. 2). The relationship between elevation and the probability of *L. cyprinacea* occurrence elevation was negative (Table 3; Fig. 2).

The hierarchical partitioning analysis of deviance examined the independent contribution (in % of explained deviance) of the predictors identified by the GLMM analyses (fish size, salinity, nutrient pollution and pH) to variation in the occurrence of *L. cyprinacea*. The occurrence of *L. cyprinacea* was significantly associated with fish length (53.3% of independently explained deviance), although the collective effect of the set of environmental predictors was also significant and explained almost as much independent variation (46.7%). Of the latter, variation in *L. cyprinacea* occurrence was about twice as strongly related to salinity (17.3%) than to the other three environmental predictors (elevation 9.2%, pH 9.3% and nutrients 11.0%), but all four environmental predictors were significantly related to the occurrence of *L. cyprinacea* (assessed by the HP randomization test). There were significant differences in elevation (Mann Whitney U $W = 3523$; $P < 0.05$), salinity ($W = 1877$; $P < 0.05$), nutrient pollution ($W = 1922$; $P < 0.05$) but not in pH ($W = 2299$; $P > 0.05$) between sites with infected and non-infected fish. Univariate tests did not include the effects of variation in other predictors of the multivariate models (i.e. generalized linear mixed models and hierarchical partitioning) and large differences detected in the GLMMs or HP may be visually subtle on a stressor-by-stressor basis (Fig. 2).

GLMM showed that the base additive model, including geographical, hydro–morphological features, water properties and fish length, was more parsimonious (i.e. had the lowest AIC and BIC values) than the five interactive models (see AM in Table 4), so that including interactive effects among environmental stressors in the base additive model did not markedly improve its fit.

Discussion

Our study is the first to examine additive and interactive effects of widespread water and physical habitat stressors on a riverine host–parasite system. The presence of a widespread alien parasite, the anchor worm *L. cyprinacea*, was explained mostly by fish–host size, additive combinations of altitude, water degradation and, to a minor degree, hydro–morphological alterations. Smaller fish and fish in lowlands experienced a higher rate of infection. Contrary to our expectations, the presence of the parasite was positively related to salinity, which is a widespread pollutant (Cañedo–Argüelles *et al*., 2016) with anti–parasitic properties (Noga, 2011). We found that nutrient concentrations in rivers passing through extensive agricultural areas, such as in north–eastern Spain, promoted infection rates of *L. cyprinacea*. Given that freshwater fish are in marked decline around the world (Closs *et al.*, 2016), and that this parasite is a host generalist (Hoffman, 1999), our results can contribute information to management actions to reduce the spread of *L. cyprinacea* among riverine fish.

Whereas interactions among stressors are at the core of many discussions of the underlying reasons for the current biodiversity crisis (Paine *et al.,* 1998; Piggott *et al.,* 2015), little is known for freshwater fish (Jackson *et al.,* 2016; Schinegger *et al.,* 2016). We found no evidence that interactive effects among major riverine stressors were related to the occurrence of *L. cyprinacea* in fish. These results support the notion that the risk of interactive effects may have been overstated (Côté *et al*., 2016; Maceda–Veiga *et al*., 2017), and suggest that the individual management of stressors probably is the most appropriate course of action to prevent the expansion of this highly invasive parasite. Although the global decline of freshwater fish is due to many factors (Closs *et al.,* 2016), there is growing concern about the role of infectious diseases (e.g. Gozlan *et al*., 2005). We detected the highest prevalence of *L. cyprinacea* in native fish species (e.g. *P. miegii, S. laietanus*) that have experienced significant declines in northeastern Spain (Maceda–Veiga *et al.,* 2010). However, the lowest prevalence was in widespread, alien fish species, such as *G. holbrooki* and *A. alburnus* (Froese & Pauly, 2017), excepting *C. carpio*, which was the probable source of *L. cyprinacea* (Hoffman, 1999; Hassan *et al*., 2008).

After controlling for the inter–specific variability in the presence of *L. cyprinacea*, we found that smaller fish had a higher probability of infection. This outcome differs from the work of Poulin *et al*. (1991), who found, in controlled exposure trials in clean laboratory water, that infection rates by another species of parasitic copepod increased with fish size. The contrasting patterns could be due to pollution–induced immunosuppression in our systems, which may affect juveniles more than adult fish (see Biagianti–Risbourg *et al*., 2013). Differences in the microhabitat used by each life stage also may explain the results; juveniles generally occupy slow–flowing waters (Ribeiro *et al*., 2013), which facilitate the spread of *L. cyprinacea* (Medeiros & Maltchik, 1999). High spatial concentrations of fish were expected to promote the transmission of *L. cyprinacea* (Hoffman, 1999; Noga, 2011), but we did not find evidence for such an effect. Given that there were large differences in prevalence among fish species (Table 1), our host–parasite relationships may be obscured by differences in the taxonomic composition of fish communities depending on the riverine environment (e.g. Maceda–Veiga *et al*., 2017). Ecological traits of the fish hosts (e.g.

migratory behaviour, trophic ecology) also seemed to have played a limited role in explaining the prevalence of *L. cyprinacea*, possibly because we lack detailed information on the ecology of these species, such as differences in diet and habitat use (e.g. home-range), a major determinant of infection rate (Knudsen *et al*., 1996; MacColl, 2009). We found that the occurrence of *L. cyprinacea* was almost equally related to environmental conditions than to fish–host factors. Other potential parasite reservoirs, such as frogs, for which we have no data, may have affected the prevalence of *L. cyprinacea* (see Kupferberg *et al*., 2009).

Our results suggest that the spread of *L. cyprinacea* might be limited by high altitude and hence low water temperatures (Hoffman, 1999; Noga, 2011). However, even species that prefer cold water, such as the strictly freshwater populations of *S. trutta* in the Mediterranean (Doadrio *et al*., 2011), were infected, raising concerns about the spread of *L. cyprinacea* through entire vulnerable faunas from climate warming (Sánchez–Hernández, 2017), although our results suggest that improving water chemistry could mitigate that effect. Our findings indicated that a decrease in nitrate and phosphate concentrations probably would reduce the spread of *L. cyprinacea*, a conclusion that differed from those of Palm (2011), who suggested that nutrients negatively affected crustacean parasites. Sewage discharges from urban areas and intensive farming are the major reason for cultural eutrophication in many aquatic ecosystems (Smith & Schindler, 2009; Rissman & Carpenter, 2015). Given that human pressures on riverine ecosystems continue to grow (Vörösmarty *et al*., 2010; Sinha *et al*., 2017), it is crucial to make these by–products of human activity less environmentally damaging by, say, optimizing agricultural fertilizer application (Basso *et al*., 2016) and by regenerating riparian vegetation that acts as ‘green filters’ (Naiman & Decamps, 1997).

Improvement in sewage treatment and nutrient retention in riparian areas and reductions in water abstraction would reduce river salinity (e.g. Basin Salinity Management Plant, 2015; Tal, 2017), for which we found a positive association on the presence of *L. cyprinacea*, although aquacultural practices posit that salinity kills freshwater ectoparasites (Noga, 2011). We used conductivity as a proxy for salinity, which is widely done (Cañedo–Argüelles *et al*., 2016), but conductivity is altered by common ions of clean sea water (e.g. chlorides, sodium) and by industrial discharges (e.g. metal pollution, Colin *et al*., 2016). Therefore, conductivity might have been a general indicator of many pollutants in our study rather than a measure of salinity *per* *se*. Our results support the notion that the effects of pollution on host–parasite relationships are context–dependent (Marcogliese & Pietrock, 2011). The probability of *L. cyprinacea* occurrence increased with nitrate and phosphate concentrations, although no infected fish were found in waters with concentrations of between 15–25 mg/l. Although the direct toxicity of nutrients to *L. cyprinacea* remains to be determined, there may be a lethal effect, which was shown experimentally for the impact of nitrate on the guppy–*Gyrodactylus* fish–parasite relationship (Smallbone *et al*., 2016). pH was an important contributing factor to explaining variation in the probability of *L. cyprinacea* occurrence in our models, but it was not significant in Mann-Whitney U tests. Univariate tests do not account for the relative effects of covariates, i.e. pH effects on biota are likely to have been conditioned by the additive effects of nutrients such as ammonia (Emerson *et al*., 1975). However, simple bivariate plots (see Fig. 2) may be more valuable for managers to take action on particular stressors than when the differences are shown as linear combinations of predictors in models.

Hydro–morphological alterations and riparian zone characteristics appeared to be of little importance in explaining the variation in the probability of *L. cyprinacea* occurrence, notwithstanding that such alterations are known stressors for riverine biota (Poff *et al*., 2007; Maceda–Veiga *et al*., 2017). The absence of a signal might be due to rivers being very dynamic and that snapshot samples of biota and abiotic conditions may not necessarily reveal strong relationships (Heino *et al*., 2015; Maceda–Veiga *et al*., 2017). However, water velocity is likely to vary as much as nutrient concentrations and salinity, and these two factors had discernible effects on the occurrence of *L. cyprinacea*. A plausible explanation for the lack of association with river hydro–morphology is that slow water velocity is only critical during the attachment stage of the parasite, and we found mostly mature *L. cyprinacea* individuals with egg clutches (A. M.V., personal observation). Therefore, although infection was not clearly related to water velocity in our study, there may be negative relationships at an early stage of the infection, for which we have no data. Similarly, riparian forests are composed of native tree species with large differences in the decomposition rates of leaves, which influences food availability (Naiman & Decamps, 1997) and hence fish growth and body condition. Given that the index of riparian quality used for the current study (i.e. QBR) did not discriminate well among native tree species, it is likely that detailed data on the taxonomic composition of riparian areas might have provided better discrimination between riparian quality and the probability of *L. cyprinacea* infection.

Despite the control of parasitic infections being highly complex in natural waters, our study provides guidance at scales relevant to resource managers. Nitrate, phosphate and high conductivity are still major widespread stressors to European rivers up to 10 years after the enforcement of the EU's Water Framework Directive (EC, 2000). These results suggest that although more than 70% of the human population in northern and central Europe is connected to a wastewater treatment plant that implements tertiary treatment for removal of nutrients, this is an urgent need for such treatment in southern European countries, where this percentage is just 50% in Spain and as little as 13% in Malta (Environmental Protection Agency, 2013). Although these countries are relatively small territories, the figures are very important at the EU scale considering the Mediterranean area, including Spain, is a global hotspot of biodiversity (Myers *et al*., 2000). As with all correlative study, many factors can affect our results, including secondary infections (e.g. bacteria, viruses, fungi) promoted by *L. cyprinacea* that kill the fish host rapidly due to their short life cycles (Noga, 2011) and so, may have underestimated our environment–parasite relationships. Improving water quality has long been advocated for its multiple benefits in biodiversity conservation, including the advantages rivers provided to society (Hering *et al*., 2015; Liu *et al*., 2017; Zhou *et al*., 2017). Our study shows that another benefit would be the control of the spread of a widespread alien parasite among native fish.

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