Evaluación de los impactos antrópicos en los ríos mediterráneos: bioindicadores, biomarcadores e índices de calidad biótica

Nicole Colin Muñoz

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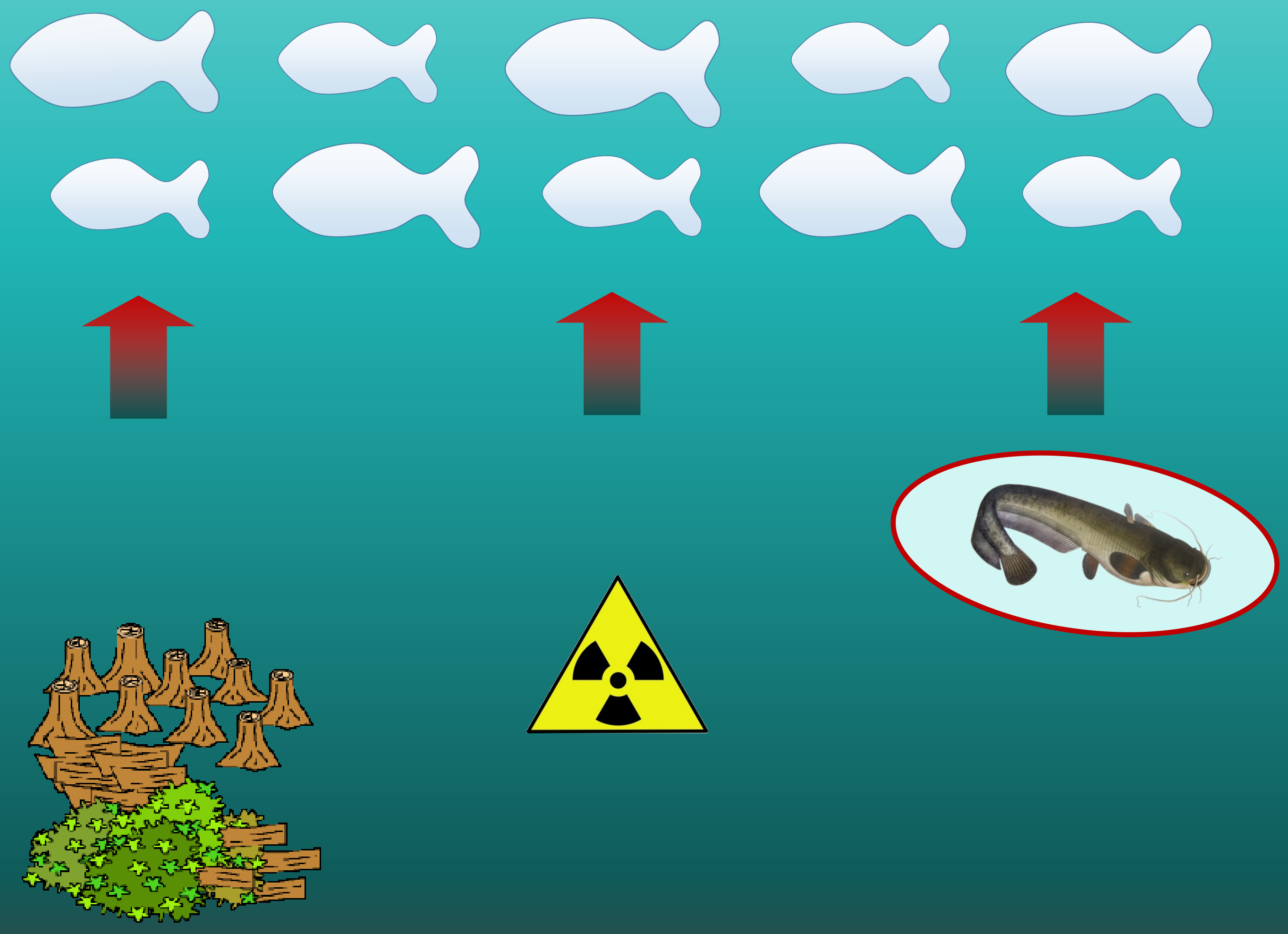

Tesis Doctoral

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Departamento de Biología Evolutiva, Ecología y Ciencias Ambientales
Programa de Doctorado de Biodiversidad H0G01

Evaluación de los impactos antrópicos en los ríos mediterráneos: bioindicadores, biomarcadores e índices de calidad biótica

Memoria presentada por **Nicole Colin Muñoz** para optar por el grado de doctor
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A mi amada hija Sofía y,

a los ríos del mundo por su conservación,
en especial el Ripoll y mi querido San Pedro

Agradecimientos

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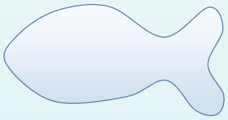
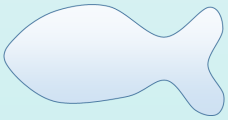
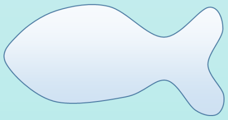






INTRODUCCIÓN

Solo una pequeña parte del agua dulce disponible a nivel mundial se encuentra en sistemas lóticos (0.006%). Esto los convierte en sistemas sujetos a una elevada presión antrópica que amenaza a su rica biodiversidad (Malmqvist & Rundle 2002, Dudgeon & Strayer 2010). Se estima que el porcentaje de especies de vertebrados y decápodos fluviales en algún nivel de riesgo de extinción ya alcanza el 60 % (Collen et al. 2014). Los impactos antrópicos sobre los ecosistemas fluviales ocurren desde muy antiguo con el establecimiento de los primeros asentamientos humanos a lo largo de los grandes ejes fluviales y, desde entonces, éstos no han hecho más que aumentar, especialmente tras la revolución industrial y la intensificación de la agricultura (Vörösmarty et al. 2010). La gama de usos que hacen las poblaciones humanas de los ríos es inmensa, ya sea para la obtención de agua y generación de electricidad vía presas, como medio de eliminación de aguas residuales o para recreo y transporte (Malmqvist & Rundle 2002). Es esperable que las poblaciones humanas dupliquen en 2025 (Vörösmarty et al. 2010). Esto, unido a alteración de los regímenes hidrológicos derivados del cambio climático, hará de los ríos unos ecosistemas más amenazados, especialmente en zonas semiáridas como la cuenca Mediterránea (Petrovic et al. 2012, Davis et al. 2015).

Fruto de la presión antrópica los ríos experimentan importantes alteraciones, incluyendo contaminación, alteraciones hidromorfológicas y, no menos importante, el creciente establecimiento de especies exóticas invasoras (Vörösmarty et al. 2004, Dudgeon & Strayer 2010, Stedera et al. 2010, Fig. 1). En menor o mayor grado todos estos impactos ponen en riesgo la biodiversidad de los sistemas fluviales, pero también el aprovechamiento de un agua de calidad por parte de las poblaciones humanas a un bajo coste (Vörösmarty et al. 2000, Moyle & Mount 2007). De hecho, las aguas residuales urbanas e industriales aún son la principal causa de degradación de los ríos en países desarrollados pese al incremento en la inversión en plantas de tratamiento (Klein 2013). Tampoco son un problema menor la presencia de vertidos ilegales y la contaminación difusa proveniente de zonas agrícolas adyacentes (Klein 2013). Todos estos focos de contaminación suponen una grave amenaza puesto que los ríos son sistemas lineales con capacidad para transmitir los efectos aguas arriba y abajo del vertido (Neal et al. 2008). No obstante, las consecuencias ecológicas de estos impactos en cascada a lo largo de los ejes fluviales todavía no están muy estudiados (Fig A), especialmente en organismos muy móviles como los peces (Flecker et al. 2010).

Nuestra capacidad para predecir los impactos de la contaminación sobre los sistemas acuáticos es limitada fruto de la diversidad de compuestos y sus complejas interacciones. Entre los múltiples contaminantes, los compuestos de nitrógeno y fósforo están entre los más comunes en los efluentes de aguas residuales (Carpenter et al. 2011). A bajas concentraciones estos nutrientes incrementan la productividad del sistema y no constituyen un problema, pero a concentraciones elevadas pueden ser un impacto de primera magnitud, ya sea a nivel de especies concretas (Camargo et al. 2006; Smallbone et al. 2016) o del ecosistema (Smith & Schindler 2009). No obstante en la compleja amalgama de compuestos presentes en las aguas residuales también existen compuestos refractarios a la degradación biológica (ej. pesticidas, metales). Entre éstos, también se incluyen los contaminantes emergentes (ej. fármacos) para los cuales los efectos de las interacciones sobre la fauna acuática son todavía más desconocidos (Barceló & Petrovic 2008, Blasco & Valls 2008, Muñoz et al. 2009).

Junto con la naturaleza química de los contaminantes, las consecuencias ecológicas de sus impactos vienen determinadas por sus interacciones con estresores de tipo natural tales como los de ciclos de avenidas y estiaje típico de los ríos mediterráneos (Petrovic et al. 2012). Cuando el caudal se reduce, la concentración de los contaminantes aumenta dado el bajo poder de dilución del río y, por consiguiente, es esperable que su impacto sea mayor sobre la biota (Petrovic et al. 2012). En los ríos mediterráneos, este problema tiene mayor relevan-

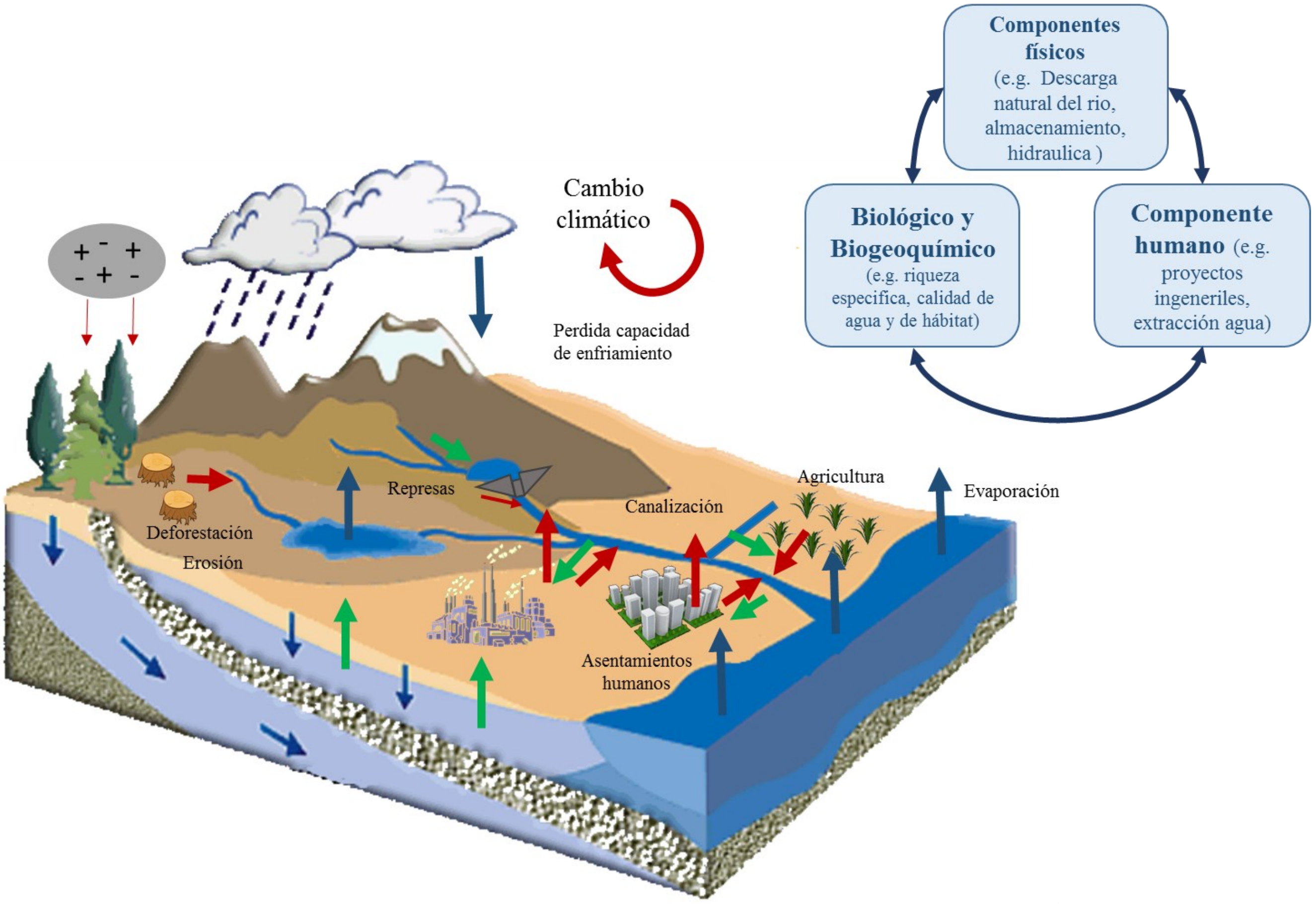


Figura 1. Ciclo del agua y principales alteraciones de origen antrópico.

Los componentes físicos, los cuales son factores naturales como las precipitaciones y la descarga natural de los ríos y el componente humano ligado a las actividades antrópicas. Extraído y modificado de Vörösmarty et al. 2004.

cia, al coincidir la época de bajo caudal con temperaturas elevadas lo cual podría intensificar aún más si cabe los efectos de la contaminación en ectotermos (Chalifour & Juneau 2011, Chandra et al. 2012 , Maceda-Veiga et al. 2015).

Bioindicadores e índices de calidad biótica

Los ecólogos llevan mucho tiempo interesados en los mecanismos que estructuran las comunidades, siendo los de la respuesta al estrés uno de los clásicos (Kolkwitz & Marsson 1909, Odum 1985, Rapport et al. 1985, Gaston & Blackburn 2000, DeWitt & Scheiner 2004). El interés ha sido biogeográfico y ecológico, pero también con el objetivo de desarrollar herramientas que permitan evaluar la salud de los ecosistemas. El origen del uso de la estructura de las comunidades de organismos acuáticos como indicadores -también llamados bioindicadores o especies centinelas- se remonta al sistema de saprobios (Kolkwitz & Marsson 1909). Su principal objetivo fue detectar contaminación orgánica en ríos utilizando como bioindicadores a bacterias u otros microbios. El

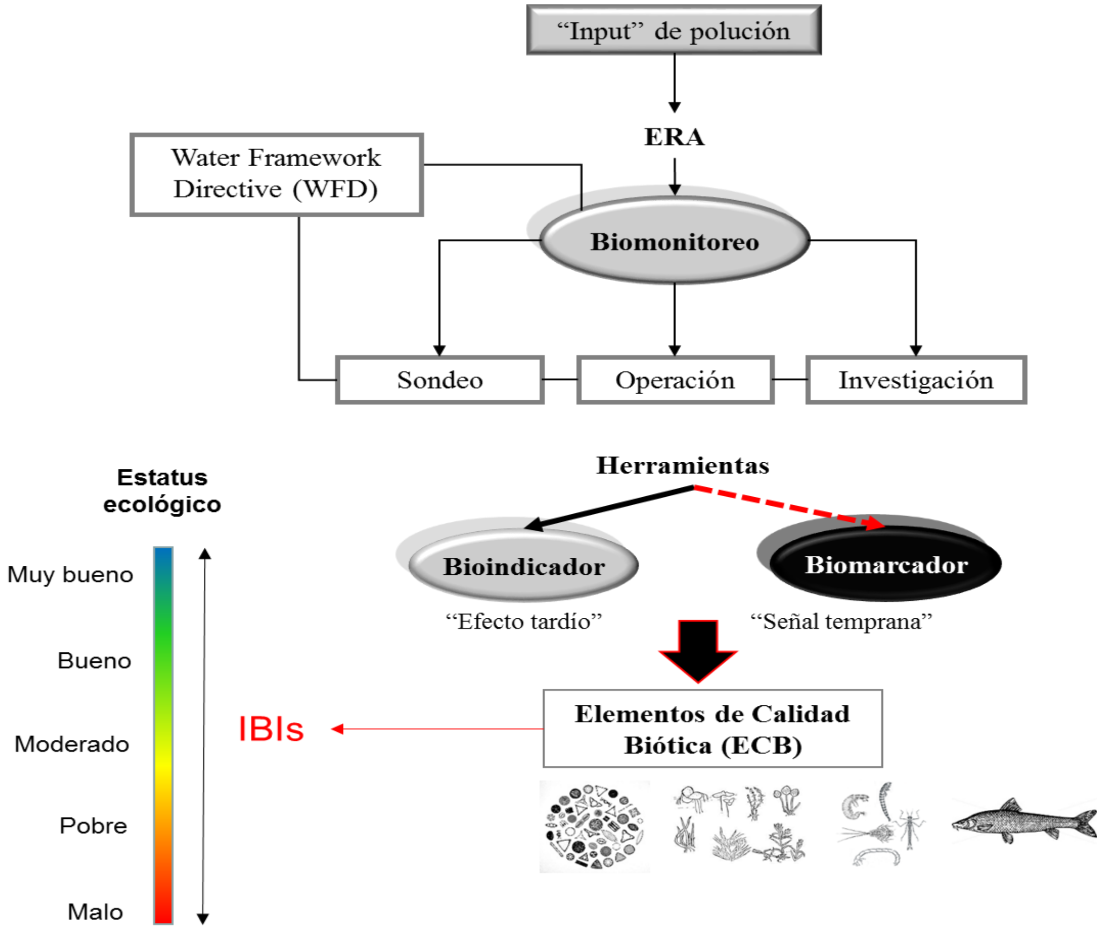


Figura 2. Propuesta de monitoreo de la Directiva Marco del Agua para la Evaluación de Riesgo Ambiental (ERA).

Considera tres fases Sondeo, Operación e Investigación. Las herramienta actual utilizada son los bioindicadores (respuesta tardía) y en negro se señalan los biomarcadores como herramienta potencial para determinar una señal temprana de alerta, sin embargo aún no están considerados dentro del plan de monitoreo Europeo.

sistema de saprobios tuvo su apogeo en los años 50 y 60 (Pantle & Bunck 1955, Woodiwiss 1964, Tuffery & Verneaux 1968) pero ha quedado en la actualidad relegado, a excepción de las algas (ver abajo), al monitoreo de plantas de tratamiento de aguas residuales (Salvado et al. 1995, Pérez-Uz et al. 2010, Canals et al. 2015). A estos métodos basados en microorganismos -principalmente bacterias, algas, protistas y hongos- les siguieron otros utilizando a las comunidades de peces, plantas acuáticas, insectos acuáticos y otros macroinvertebrados como bioindicadores (e.g. Hynes 1960, Karr 1981, Armitage 1983) cuyo uso está más extendido en la actualidad en los programas de biomonitoreo de ríos (Bonada et al. 2006, Friberg et al. 2011).

Junto con la incorporación de nuevos taxones, estos índices –llamados de calidad biótica - han ido diversificando el número de rasgos de la estructura de la comunidad (métricas) que se utilizan en el diagnóstico ambiental, lo cual permite

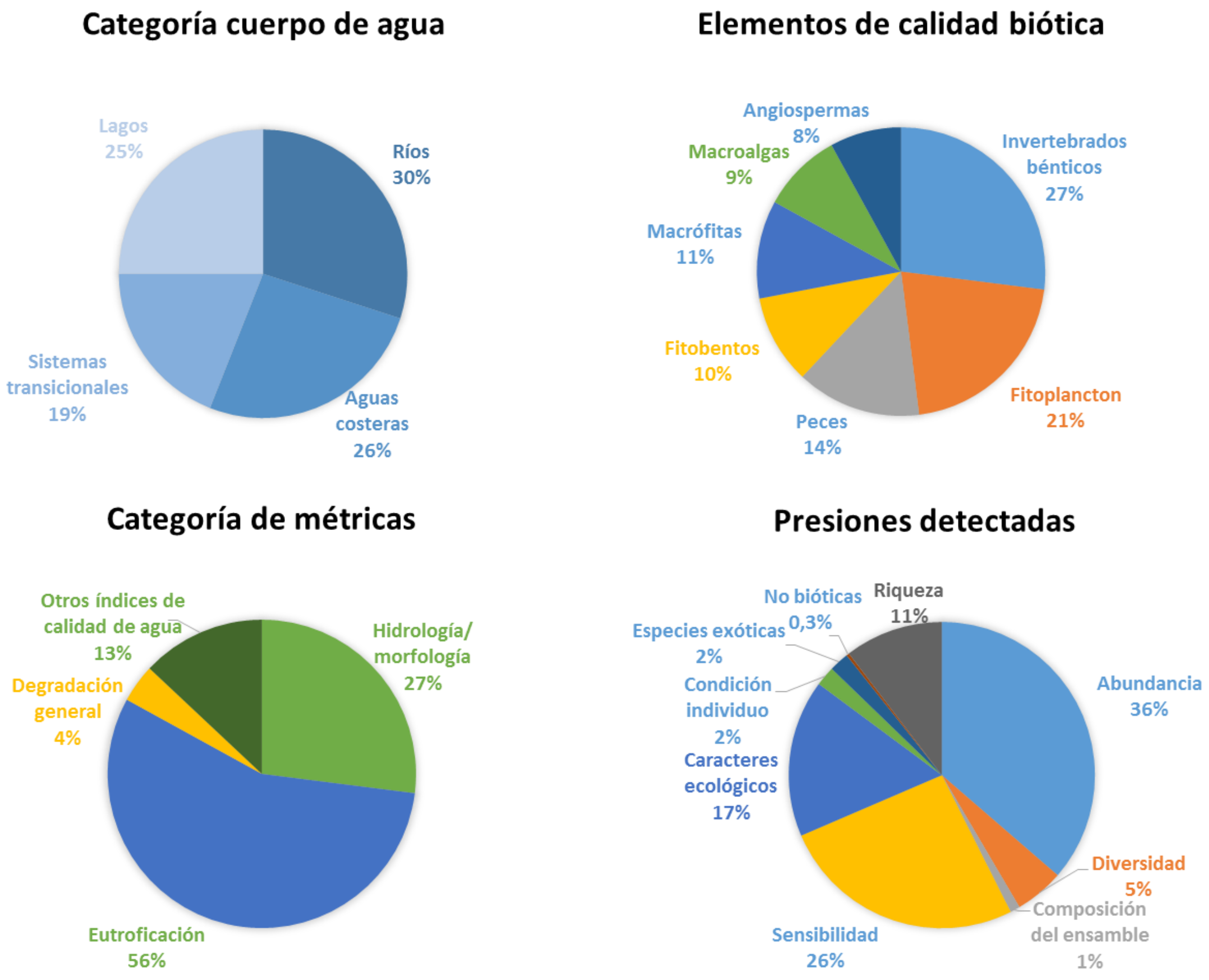


Figura 3. Elementos de calidad biótica.

Frecuencia relativa de las características seleccionadas para métodos de evaluación en sistemas acuáticos europeos. Extraído de Birk et at. 2012 obtener una visión cada vez más holística de las consecuencias de los impactos antrópicos sobre los ríos (Patrick 1949, Prat & Munné 2014). Fruto de tal sofisticación, algunos autores prefieren utilizar el término genérico de índices de integridad biótica. Término introducido por primera vez por Karr (1981) que define a la integridad biótica como "la capacidad de mantener una comunidad adaptada, integrada y balanceada, con una composición, diversidad y organización funcional comparable con el hábitat natural". Otros, sin embargo, prefieren seguir reconociendo el origen de los actuales programas de biomonitoreo al sistema de saprobios y utilizar el término genérico de índices de calidad biótica. Sea cual sea la acepción escogida, lo cierto es que la visión más holística –y por ende con mayor probabilidad de reflejar la integridad ecológica del sistema- se obtiene tras evaluar el efecto de los estresores sobre varios grupos de organismos que tengan diferentes estrategias de vida, incluyendo a los de ciclo de vida corto y largo (Jonhson & Hering 2009, Hering et al. 2006, Jonhson et al. 2006; Marzin et al. 2012).

Sin duda, el paso más importante en la adopción de los bioindicadores en los programas de evaluación ambiental en los ríos europeos fue la entrada en vigor de la Directiva Marco del Agua (DMA) en el año 2000 (Comisión Europea 2000). Esta di-

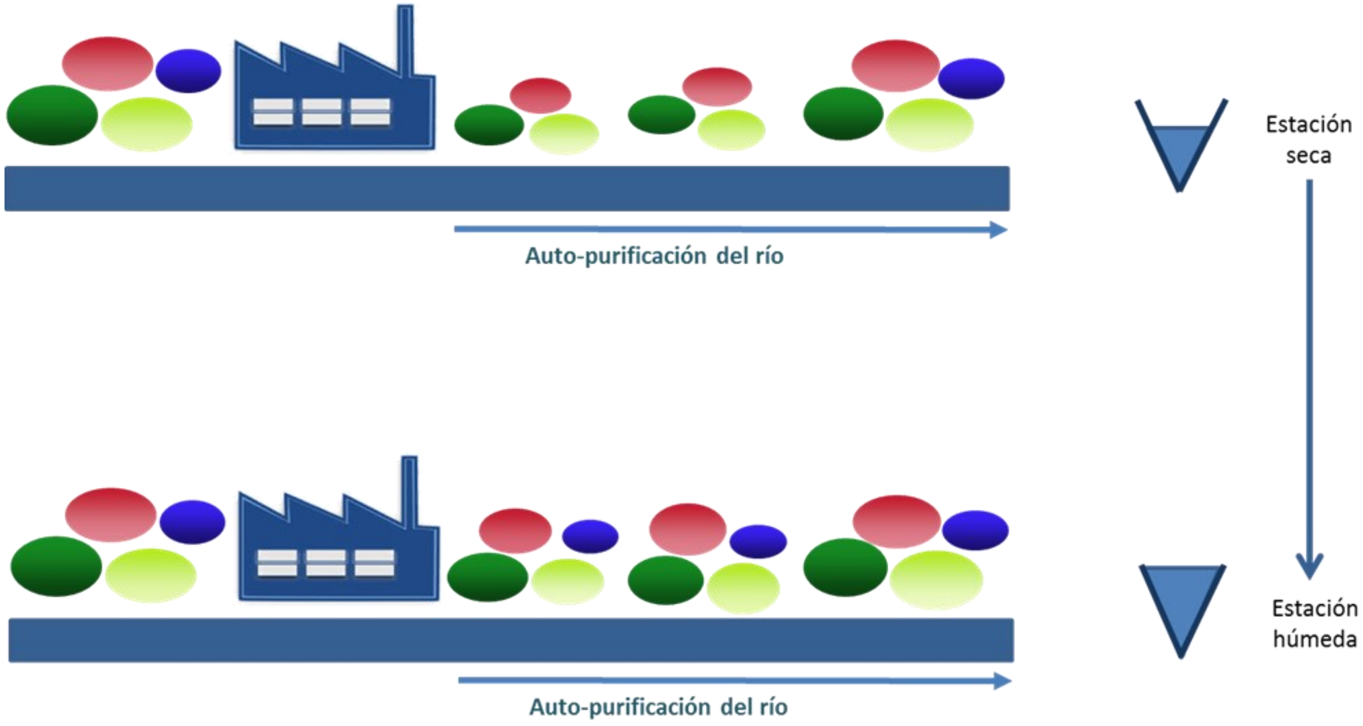


Figura 4. Aproximación multi-taxa de comunidades biológicas

Escenario antes y después de un "input" de contaminación en un tramo de un río en época seca y húmeda. La alteración es mayor y la recuperación más lenta en la estación de bajo caudal, en cambio en la de alto caudal incrementa la capacidad auto-depurativa del río y suavizando el efecto negativo en las comunidades.

rectiva obliga a todos los estados miembros a determinar el estado ecológico de sus aguas a través de bioindicadores a los cuales les acuña el término de elementos de calidad biótica. En su origen la legislación marcaba como fecha límite el 2015 para alcanzar el buen estado ecológico de las aguas comunitarias, aunque se extendió hasta el 2027 ante la imposibilidad de cumplirlo. Dicho estado ecológico debe determinarse en función de parámetros hidromorfológicos, de calidad química del agua y de calidad biológica en base a la estructura de las comunidades. Esto ha dado lugar a la creación de más de 300 índices de calidad biótica debido a la necesidad de crear protocolos específicos para acomodar las particularidades de la fauna y flora de las diferentes tipologías fluviales (Birk et al. 2012, Prat & Munné 2014, Fig.2). El poder diagnóstico de estos índices radica en su capacidad para diferenciar el impacto antrópico del estrés natural. Para ello, se compara la estructura de las comunidades acuáticas de los tramos prueba con los de referencia y, en función de la sensibilidad de los organismos a los impactos antrópicos, se asigna un nivel de calidad (muy bueno, bueno, moderado, pobre y malo). Así pues, los resultados de estos índices permiten hacer una radiografía bastante completa de las consecuencias de los impactos antrópicos sobre las comunidades acuáticas, además de identificar al grupo de organismos más afectado. Esto, junto con su simplicidad, justifica que su uso esté tan extendido. De acuerdo con la DMA se recomienda el uso de índices multimétricos en lugar de los unimétricos, además del uso de múltiples taxones (Fig. 3), lo cual acercaría más los resultados obtenidos por los actuales programas de monitorización a la definición de integridad biótica de Karr (1981).

Como se vislumbra del párrafo anterior uno de los problemas principales del uso de los índices de calidad biótica es su especificidad regional (por tipología fluvial) a la par que su poca capacidad para predecir impactos (Fridberg et al. 2011). A la dificultad de encontrar condiciones de referencia en ríos muy impactados como los mediterráneos (Stoddard et al. 2006, Sánchez-Montoya et al. 2009, Feio et al. 2013), se le suma que las condiciones de referencia pueden modificarse debido al cambio global. Recordemos que la mayoría de los índices de calidad biótica basan su diagnóstico en comparar un tramo prueba con una fotografía puntual de las condiciones de referencia. Otra de las críticas más pujantes es que estos índices están muy desconectados de la teoría ecológica (Friberg et al. 2011). Esto, además de dificultar la comparación entre sistemas, no permite continuar desarrollando teoría ecológica a través de un mejor conocimiento de los mecanismos de respuesta de las comunidades acuáticas al estrés.

Índices de diversidad funcional como metodología complementaria a los índices de calidad biótica

Los ICBs tienen una larga tradición en Europa y, dada la gran inversión de dinero y esfuerzo que se ha realizado en los últimos 15 años en su diseño, no es esperable que haya cambios a corto plazo. No obstante, como ecólogos, no debemos desfallecer en el intento de buscar métodos de diagnóstico mucho más informativos que a la par permitan el avance de la ecología. Uno de los métodos más prometedores de la ecología moderna es el basado en los rasgos biológicos y ecológicos de las especies que recibe en la literatura anglosajona el nombre de “trait-based ecolo-

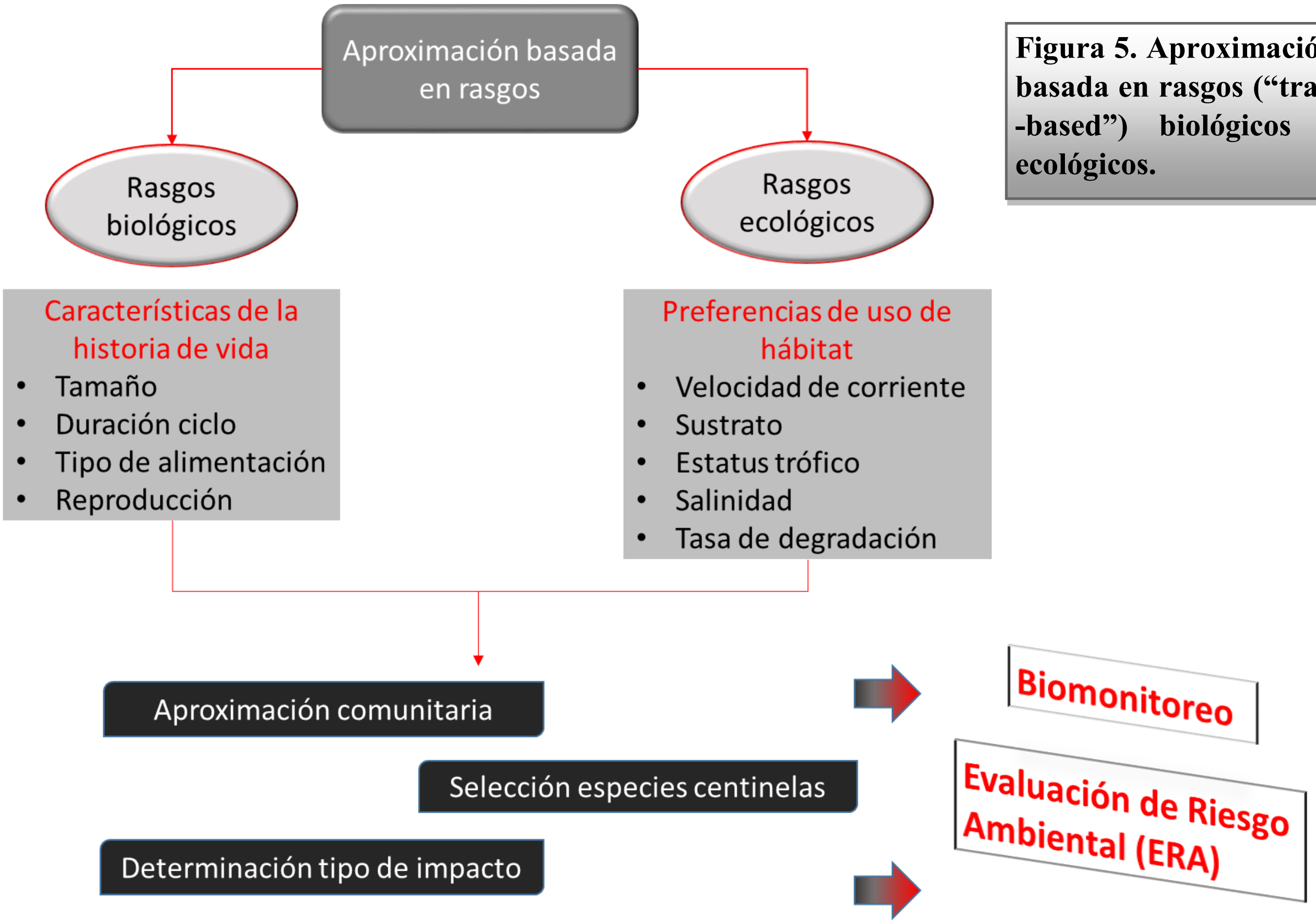


Figura 5. Aproximación basada en rasgos ("trait -based") biológicos y ecológicos.

gy” (fig. 4) (Liess et al. 2008, Liess & Beketov 2011, Van den Brink et al. 2011). Conociendo los factores bióticos y abióticos que determinan la estructura de rasgos de las comunidades podemos responder preguntas ecológicas, biogeográficas y ecotoxicológicas. Así pues, una comunidad dominada por especies de ciclos biológicos rápidos es indicadora de ambientes inestables, como lo es de ambientes salinos una comunidad compuesta por especies eurihalinas.

La ecología basada en rasgos tiene sus orígenes en Kryzhanovskii (1949), quién agrupó a peces utilizando una serie de caracteres eco-morfológicos. Años más tarde, empezó a utilizarse el concepto de gremios (del inglés “guild”) para agrupar especies que habitan un mismo lugar y que explotan recursos similares (Root 1967); concepto que después recibiría el nombre de grupo funcional (Cummins 1973). No fue hasta Poff (1997), con la incorporación del concepto de filtro ambiental (Odum 1985), que no se utilizaron los grupos funcionales como una pieza fundamental para entender los mecanismos de ensamblaje de las comunidades de peces. Según la teoría de filtros ambientales, el entorno “selecciona” a las especies en función de sus rasgos, dejando sólo aquellas con la combinación de rasgos más favorable para vivir en un ambiente dado (Keddy et al. 1992, Poff 1997). Estos filtros pueden ser debido a procesos ambientales a gran escala (biogeográficos) y locales (microclima), con el resultado de patrones de diferenciación taxonómica y funcional espacio-temporales característicos

(Frimpong & Angermeier 2010, Webb et al. 2010).

Amén de estos factores naturales biogeográficos y climáticos, la fuerte presión antrópica a la que están sometidos los ecosistemas acuáticos actúa de filtro ambiental adicional (Menezes et al. 2010). Todavía son bastante desconocidos los patrones funcionales de respuesta de las comunidades acuáticas al estrés (Heino 2013), con un gran debate en torno a cuáles son los rasgos más informativos y sobre la mejor manera de integrar dicha información, incluyendo los llamados índices de diversidad funcional (Botta-Dukat 2005, Villeger et al. 2008, Mouillot et al. 2013, Gagic et al. 2015). Asimismo, la introducción de especies exóticas, o la substitución de especies nativas por exóticas, añade un nivel más de complejidad a la hora de interpretar los cambios en la estructura funcional de las comunidades acuáticas (Olden et al. 2006, Laughlin et al. 2011, Villéger et al. 2011). Podría darse el caso que no existan cambios funcionales debido a impactos antrópicos porque la estructura funcional de las comunidades haya incorporado nuevos rasgos -por adaptación

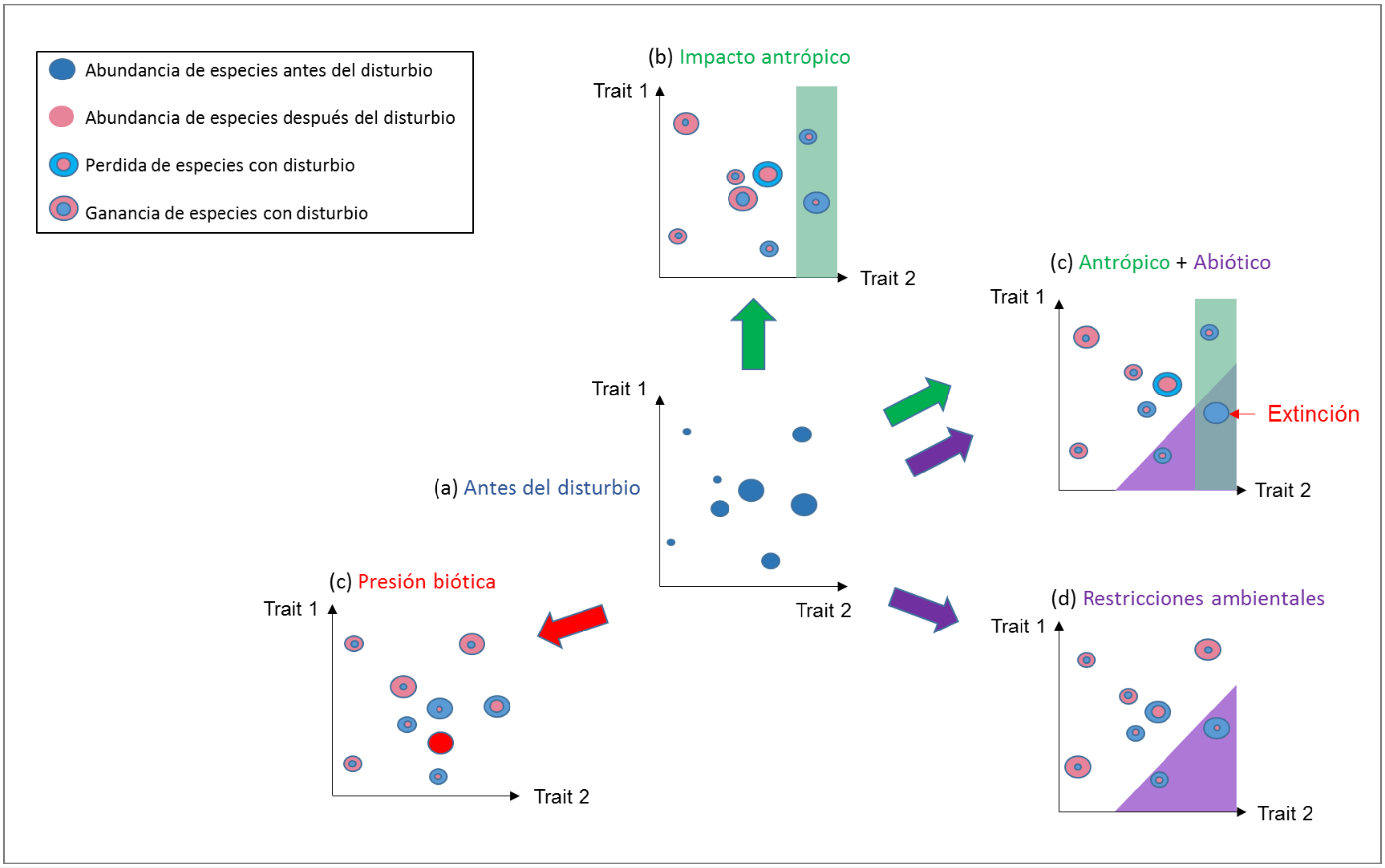


Figura 6. Cambio potencial en la estructura funcional de una comunidad de especies después de tres tipos de disturbio

a) Espacio funcional definido por dos rasgos ("traits") donde ocho especies son incluidas. B) Impacto antrópico reduce las poblaciones con un alto valor para el "trait" 1. c) Presión biótica a través de la presencia de especies introducidas (círculo rojo), reducción de las poblaciones de especies nativas más cercanas en el especio funcional por competencia o predación. d) Filtros por restricciones ambientales con un alto valor en el "trait" 1 y bajo valor para el 2. e) Impacto aditivo en el espacio funcional induce a la extinción de una especie (Extraido: Mouillot et al. 2013).

local o introducción de especies- que las hagan aptas para vivir en estos nuevos ambientes creados por el hombre (Fig. 5). Es por ello que no parece que tenga mucho sentido realizar análisis funcionales exclusivamente para las especies nativas (Erős 2007, Moulliot et al. 2013). Los patrones de respuesta del ecosistema deben interpretarse en conjunto y, en el caso de los ríos, las especies introducidas representan un porcentaje nada desdeñable.

A la complejidad ecológica de desentrañar los mecanismos de respuesta funcional de las comunidades sometidas a múltiples agentes estresores bióticos y abióticos, se le añade el debate social sobre si las especies exóticas deben o no conservarse (Schlaepfer et al. 2012, Kumschick et al. 2015). Es cierto que sólo sabemos los impactos de un porcentaje relativamente pequeño en relación al número de especies que se establecen, y que existe un sesgo en la literatura hacía los estudios que demuestran efectos negativos (Schlaepfer et al. 2012). No obstante, esto no debe relajar la preocupación sobre la introducción de especies exóticas, especialmente a la tasa actual, al tener consecuencias a menudo impredecibles o con efectos a largo plazo (Parker et al. 1999, Simberloff et al. 2013).

Hacia los biomarcadores no letales como herramientas de diagnóstico

Uno de los principales problemas de hacer diagnósticos a nivel de comunidad es que cuando "el cambio" tiene lugar puede ser demasiado tarde para establecer medidas de conservación. Puede decirse que los cambios en la composición de las comunidades se explican por dos mecanismos básicos: el reemplazamiento y la pérdida de especies (Baselga 2010). Así pues, cuando un índice de calidad biótica o uno de diversidad funcional detecta impacto, ya se ha producido, en el mejor de los casos, una drástica bajada de efectivos poblacionales. Esto no tiene mayor importancia para organismos cosmopolitas o especies localmente muy abundantes, pero sí para comunidades pobres en especies y seriamente amenazadas como las de peces en los ríos mediterráneos (Maceda-Veiga 2013). Es en estos sistemas donde adquiere una especial relevancia el desarrollo de métodos de diagnóstico que sirvan de alerta temprana a nivel de individuo antes que los efectos sean visibles en otros niveles de organización biológica.

Fruto de estos problemas nacieron nuevas herramientas de diagnóstico a nivel de individuo utilizando los llamados biomarcadores (Hagger et al. 2006, 2010, Sanchez & Porter 2009, Martínez-Haro et al. 2015, Planelló et al. 2015, Prat & Munné et al. 2014). Su origen se encuentra en medicina humana, y entre las múltiples definiciones existentes (Peackall 1992, Adams 2002, Lam 2009, Janz et al. 2013, ver más detalles en el capítulo 3), sirven para cuantificar la respuesta fisiológica y comportamental de los organismos al estrés. Cumplen, por tanto, el ser un sistema de alerta temprana y se convierten en una herramienta ideal en conservación. Ahora bien, como en el caso de la diversidad funcional, sigue existiendo el problema del número más adecuado de biomarcadores (rasgos) a utilizar. No obstante, tienen la ventaja que existe un conocimiento más profundo sobre su especificidad diagnóstica, así que se puede afinar mejor la causa del impacto ecológico y sus posibles consecuencias a nivel de población. Algunos autores serían partidarios de la incorporación de los biomarcadores como un rasgo más en las aproximaciones de rasgos funcionales. No obstante, sorprende que la Directiva Marco del Agua todavía no los haya adoptado como herramientas de diagnóstico oficiales (Fig. 6). Por tanto, existe la necesidad urgente de mostrar su valor diagnóstico en sistemas tan vulnerables como los ríos mediterráneos y, en particular, en su ictiofauna, además de comparar su valor diagnóstico en relación a las metodologías tradicionales. Desde un punto de vista de restauración, detectar impactos incipientes, también reduce el coste e incrementa las garantías de recuperación.

A la versatilidad de los biomarcadores para detectar la presencia de tóxicos y sus efectos en la fauna acuática, es de recibo mencionar que su uso entraña problemas de conservación y éticos al requerir su aplicación el sacrificio de animales. La mayoría de pruebas diagnósticas en ecotoxicología tradicional se basan en determinar alteraciones enzimáticas en hígado, lesiones tisulares en éste u otros órganos diana tales como riñón o branquia, entre otros, y todos estos procedimientos invasivos no son compatibles con la conservación de especies amenazadas. Además, la entrada en vigor de normativas cada vez más restrictivas en el uso de animales en investigación (ej. Orden Ministerial ECC/566/2015) hace imprescindible desarrollar nuevos procedimientos de diagnóstico no letal que reduzcan el sacrificio de animales en los proyectos de investigación.

A semejanza de la medicina humana y la veterinaria de animales domésticos, los análisis de sangre tienen un gran potencial como herramientas de diagnóstico en animales salvajes incluyendo a los peces continentales (Maceda-Veiga et al. 2015). El poder diagnóstico de una gota de sangre fue demostrado en los trabajos pioneros en ciprínidos nativos de ríos mediterráneos que además mostró una buena correlación con procedimientos histopatológicos clásicos (Maceda-Veiga et al. 2010, 2013). No obstante, la ausencia de valores de referencia de los parámetros sanguíneos para peces salvajes sigue mermando la posibilidad de emitir diagnósticos certeros en ausencia de biomarcadores clásicos. En este sentido puede ayudar establecer la variabilidad natural de los parámetros sanguíneos a lo largo del año, y relacionar alteraciones hematológicas con cambios poblacionales. A fin de cuentas, la situación ideal es que las alteraciones en los niveles de biomarcadores nos permitan predecir las alteraciones que la presencia de un tóxico puede producir a nivel de población. Todos los organismos tienen una cierta capacidad para tolerar impactos que incluso pueden acabar en aclimatación sin que se produzca un cambio a mayores niveles de organización. Por tanto, no sólo es importante la capacidad de los biomarcadores para detectar impacto, si no su relevancia ecológica, es decir, su capacidad para predecir cambios más allá de efectos individuales.

Los ríos mediterráneos del noreste de España como modelo de estudio

Los ríos del noreste de España tipifican muy bien la larga historia de impactos antrópicos acontecidos en otros ríos mediterráneos del mundo (Elvira 1995, Sabater et al. 2009, Moyle 2014). Estos ecosistemas tan vulnerables se encuentran en cinco regiones del mundo, entre los 32º-40º N y S del ecuador: costa de California, Chile central, la región del cabo en Sudáfrica, el suroeste de

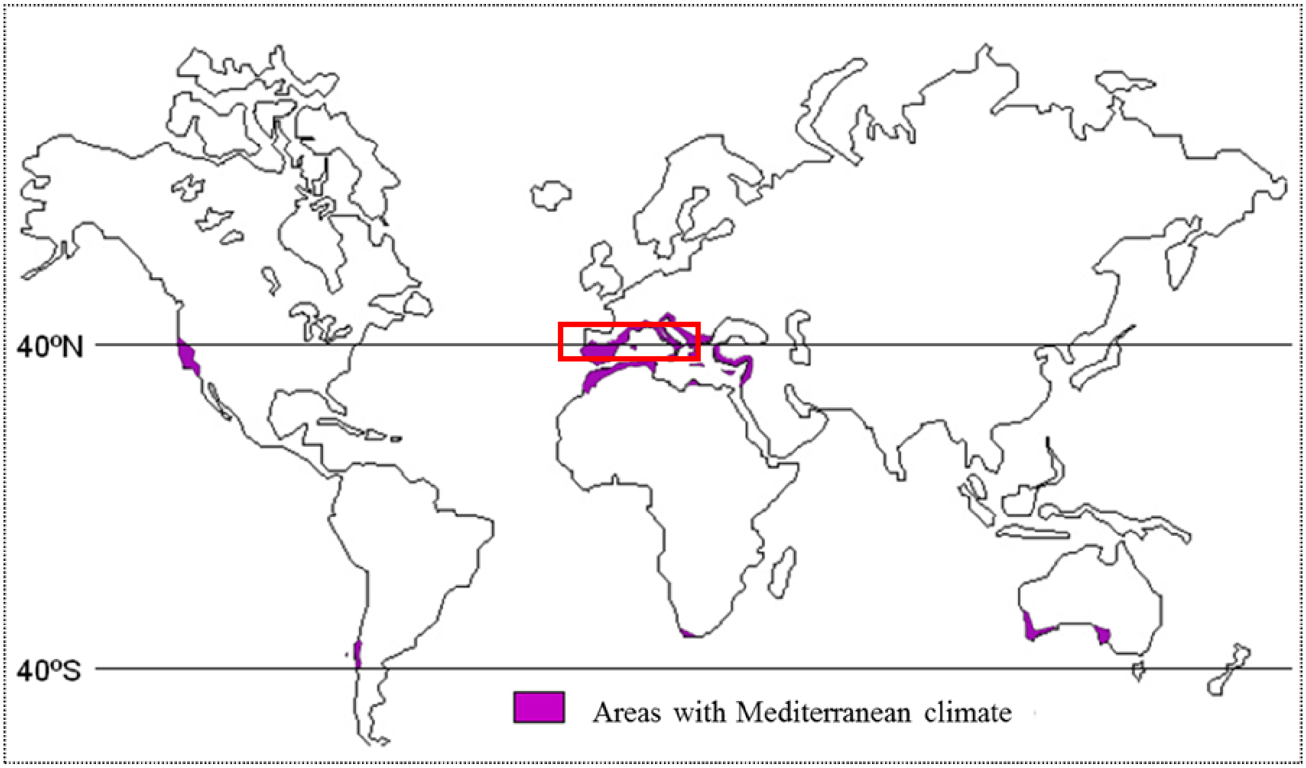


Figura 8. Mapa de áreas con clima Mediterráneo en el mundo, con un rectángulo se señala el área de ríos mediterráneos europeos.

Australia y la cuenca mediterránea europea (Fig. 7). La biota de los ríos mediterráneos españoles comparte con estas áreas geográficas su elevada endemicidad (Myers 2000, Doadrio et al. 2011), así como la marcada regresión que ha sufrido las poblaciones de peces nativos fruto de la elevada presión antrópica (Elvira 1995, Aparicio et al. 2000; Maceda-Veiga et al. 2010). Estos ecosistemas resultan un sistema ideal para estudiar las interacciones entre impactos antrópicos y estresores naturales, debido a los marcados pulsos de estiaje y avenidas que caracterizan su régimen hidrológico (Bonada & Resh 2013). A esto se le une la compleja orografía de esta región, con tramos fluviales a más de 1500 metros de altitud hasta el nivel del mar, y una diversidad de usos del suelo que crea largos gradientes de los más diversos impactos ambientales (Sabater et al. 2009). Además, el noreste español es un punto caliente en lo que a peces introducidos se refiere (Leprieur et al. 2008).

En los últimos años han empezado a proliferar en la literatura científica estudios que resaltan la necesidad de examinar los efectos combinados de estresores sobre los sistemas acuáticos continentales (e.g. Ormerod et al. 2010, Stendera et al. 2012; Jackson et al. 2016). Los ríos mediterráneos no pueden ser refractarios a esta necesidad y, en especial, su amenazada ictiofauna. Aunque pueda parecer sorprendente, todavía no existen trabajos específicos que determinen el efecto modulador del estiaje mediterráneo sobre poblaciones de peces nativos expuestos a vertidos de aguas residuales. Éstos son todavía la principal causa de degradación ambiental y, teniendo en cuenta que son varios los trabajos que resaltan el fuerte papel estructurador del estiaje (e.j. Lake 2000, Matthews & Marsh-Matthews 2003, Magalhães et al. 2007), las consecuencias de su efecto combinado podrían ser nefastas para la biota acuática.

Otro aspecto importante a destacar del noreste peninsular de cara a esta tesis es que existe una larga tradición en el uso de los índices de calidad biótica y monitoreo ambiental. Esto amén de permitir analizar respuestas a escala de comunidad a lo largo de gradientes ambientales de diversa índole, también ayuda a seleccionar tramos de estudio concretos que sirvan de caso estudio para testar el potencial de nuevas herramientas de diagnóstico ambiental.

Objetivos

Objetivo General

Esta tesis tiene como objetivo general explorar los efectos estructurales y funcionales de la contaminación y otros estresores naturales y de origen antrópico sobre los organismos de los ríos mediterráneos utilizando indicadores de diferentes niveles de organización biológica. Al combinar nuevas herramientas de diagnóstico con métodos tradicionales, la presente tesis analiza los puntos fuertes y débiles de los actuales procedimientos de monitoreo ambiental. Además, dado que su principal modelo de estudio son los peces, esta tesis contribuye a un mejor conocimiento sobre las causas de su marcada regresión.

Objetivos Específicos

Para llevar a cabo el objetivo principal, esta tesis se ha organizado en cuatro capítulos que tratan diferentes escalas y aproximaciones de diagnóstico ambiental y que se corresponden con cada uno de los objetivos específicos.

Objetivo específico 1

Evaluar el impacto de un vertido de aguas residuales industriales sobre un río mediterráneo utilizando índices de calidad biótica basados en diatomeas, macrófitos, macroinvertebrados y peces de acuerdo con la Directiva Marco del Agua. Junto con testar la utilidad de estos índices para detectar impacto ecológico en vertidos industriales, este capítulo examinará la capacidad auto-depuradora del río aguas abajo, identificará al taxón más vulnerable y testará la hipótesis que el impacto ecológico es más severo en verano (Capítulo 1).

Objetivo específico 2

Analizar los cambios en la estructura funcional de las comunidades de peces a lo largo de gradientes de degradación ambiental en múltiples cuencas del noreste español utilizando los llamados índices de diversidad funcional basados en la ecología de rasgos (“trait-based ecology”). Estos resultados se compararán con los índices de calidad biótica tradicionales con el objetivo de valorar su uso como nuevas herramientas de diagnóstico ambiental (Capítulo 2).

Objetivo específico 3

Además de incrementar el poder diagnóstico de las aproximaciones a escala de comunidad/población, la presente tesis busca mejorar nuestra capacidad para detectar efectos sub-letales a escala de individuo (biomarcadores). Con este fin, el presente capítulo identifica las tendencias en el uso de biomarcadores en peces y macroinvertebrados de ríos mediterráneos para poder sugerir mejoras que incrementen su valor diagnóstico en el marco de la ecología teórica y que los hagan compatibles con la conservación de especies amenazadas (Capítulo 3).

Objetivo específico 4

Tras poner de manifiesto el capítulo 3 la necesidad de incrementar el uso de biomarcadores no letales en peces, el presente capítulo profundizará en el valor diagnóstico de los análisis de sangre periférica. Se determinará la variabilidad natural de los parámetros hematológicos a lo largo de un año y se relacionarán los cambios acontecidos con las tendencias de abundancia en las poblaciones de peces estudiadas a lo largo de un gradiente de contaminación. De nuevo, a fin de valorar su potencial diagnóstico, se compararán estos resultados con los índices de calidad biótica de uso tradicional (Capítulo 4).

INFORME DEL DIRECTOR

La doctoranda **Nicole Colin Muñoz** presenta su tesis doctoral titulada "**Evaluación de los impactos antrópicos en los ríos mediterráneos: bioindicadores, biomarcadores e índices de calidad biótica**".
El director de tesis, el **Dr. Adolfo de Sostoa Fernández** y el co-director **Dr. Alberto Maceda Veiga**, informan que la presente tesis doctoral consta de cuatro trabajos científicos a modo de capítulos. Los capítulos están publicados, enviados o en evaluación para envío a revistas científicas internacionales reconocidas en el Science Citation Index (SCI). A continuación se detalla la contribución del doctorando en cada uno de ellos, así como el factor de impacto según el Thomson Institute for Scientific Information:

CAPITULO 1

Colin, N., Maceda-Veiga, A., Flor-Arnau, N., Mora, J., Fortuño, P., Vieira, C., Prat, N., Cambra, J., De Sostoa, A., 2016. Ecological impact and recovery of a Mediterranean river after receiving the effluent from a textile dyeing industry. Ecotoxicology and Environmental Safety 132: 295-303. http://dx.doi.org/10.1016/j.ecoenv.2016.06.017

Factor de impacto (2016): 3.13

El doctorando ha participado en el trabajo de campo para la obtención de los datos, el análisis de los datos y la redacción científica.

CAPITULO 2

Colin, N., Villéger, S., Wilkes, M., De Sostoa, A., Maceda-Veiga, A. Functional diversity measures revealed impacts of human activites on species-poor freshwater fish assemblages.

Pendiente de envío para ser sometido a Ecological Application

Factor de impacto (2016): 4.55

El doctorando ha participado en el análisis de los datos y en la redacción científica.

CAPITULO 3

Colin, N., Porte, C., Fernandes, D., Barata, C., Padrós, F., Carrassón, M., Monroy, M., Cano-Rocabayera, O., De Sostoa, A., Piña, B., Maceda-Veiga, A. 2016. Ecological relevance of biomarkers in monitoring studies of macro-invertebrates and fish in Mediterranean rivers. The Science of the Total Environment 540: 307. http://dx.doi.org/10.1016/j.scitotenv.2015.06.099.

Factor de impacto (2016): 3.976

El doctorando ha participado en la obtención de la información a través de la revisión bibliográfica, el análisis de los datos y la redacción científica.

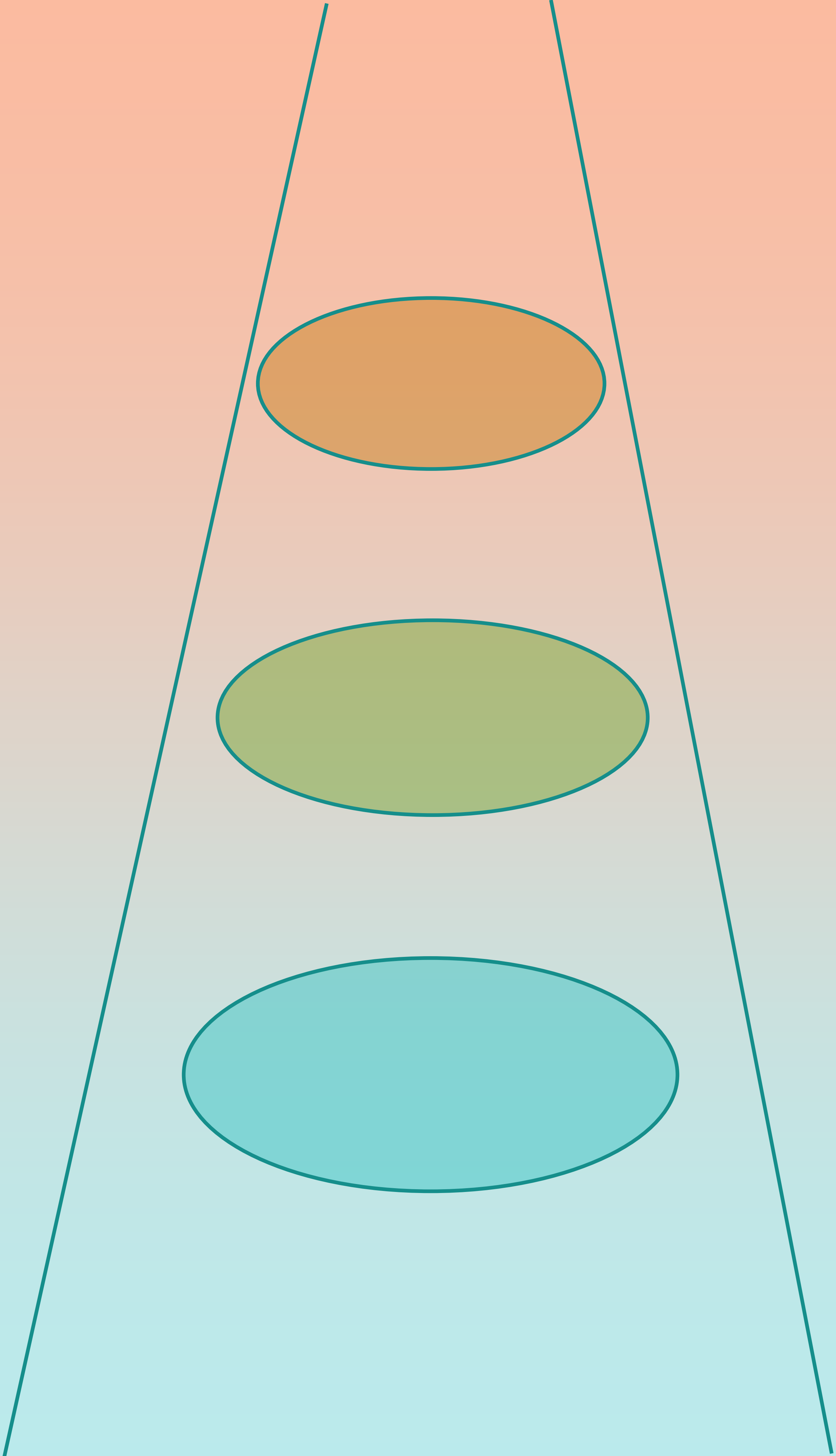
CAPITULO 4
Colin, N., Maceda-Veiga, A., Monroy, M., Ortega-Ribera, M., Llorente, M., De Sostoa, A.
Trends in biomarkers, biotic indices and abundances revealed contrasting long-term effects of sewage discharges on native fish populations in a Mediterranean river.
Enviado para ser sometido a Aquatic Toxicology
Factor de impacto (2016): 3.557
El doctorando ha participado en el trabajo de campo para la obtención de los datos, el análisis de los datos y la redacción científica.

Los coautores participantes en los artículos que componen esta tesis no han utilizado ni implícita ni explícitamente ninguno de estos trabajos para la elaboración de otras tesis doctorales.

Adolfo de Sostoa Fernández

Alberto Maceda Veiga

Barcelona, 8 de Marzo de 2017.



CAPÍTULO 1

Impacto ecológico y recuperación de un río mediterráneo después de recibir un efluente de aguas residuales de origen industrial

Resumen

El sector textil es uno de los más importantes a nivel mundial, siendo responsable de hasta un 20% de todos los problemas de contaminación derivados de la actividad industrial. Pese a esto, nuestro conocimiento sobre cómo los ecosistemas fluviales responden y se recuperan de este impacto es escaso. En este estudio, examinamos, desde el verano de 2012 hasta la primavera de 2013, la calidad del agua y el estado ecológico aguas arriba y 1,5 km aguas abajo de la entrada de una planta de tratamiento de aguas residuales de una industria textil en el río Ripoll, NE de España. Para determinar el estado ecológico utilizamos cambios en la diversidad taxonómica y en 10 índices de calidad biótica basados en diatomeas, macrófitos, macroinvertebrados y peces. Los resultados mostraron un deterioro considerable de la calidad del agua y de todas las comunidades biológicas en la entrada del efluente de la depuradora. Sin embargo, se produjo una notable mejoría 1.5 km aguas abajo. La severidad del impacto varió entre los taxones y las estaciones del año, siendo los peces el taxón más afectado y la primavera la estación del año con el mejor estado ecológico. Existió una fuerte correlación entre las variables de calidad del agua y muchos índices de calidad bióticos, lo que denota que estamos ante un problema de contaminación crónica que afecta a múltiples niveles tróficos. Por consiguiente, este estudio sugiere que es urgente mejorar el tratamiento de las aguas residuales de esta industria a fin de preservar la integridad ecológica del río Ripoll y, en especial, su ictiofauna. Asimismo, nuestro estudio demuestra el valor diagnóstico de los índices de calidad biótica basados en diatomeas, macroinvertebrados y peces, tal y como establece la Directiva Marco del Agua Europea.

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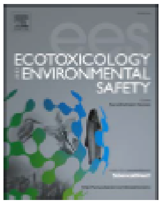

Ecological impact and recovery of a Mediterranean river after receiving the effluent from a textile dyeing industry

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ABSTRACT

The textile industry is one of the largest sectors globally, representing up to 20% of industrial water pollution. However, there is limited insight into how fluvial ecosystems respond and recover from this impact. From summer 2012 to spring 2013, we examined water quality and ecological status upstream and 1.5 km downstream the input of a textile industry wastewater treatment plant (WWTP) in Ripoll River, NE Spain. The ecological status was determined via diversity measures and 10 biotic indices based on diatoms, macrophytes, macroinvertebrates and fish. Our results showed that the WWTP severely deteriorated water quality and biological communities at the discharge site, but that they improved at 1.5 km downstream. Severity also varied across taxa and seasons, being fish the most affected taxa and spring the season with the best ecological status. The strong correlation amongst water quality variables and many biotic indices across taxa indicated that this is a chronic pollution event affecting multiple trophic levels. Thus, this study suggests that there is an urgent need to invest in wastewater treatment in this industry to preserve the ecological integrity of Ripoll River and especially its fish fauna. Likewise, it illustrates the diagnostic power of biotic indices based on diatoms, macroinvertebrates and fish, as driven by the European Water Framework Directive.

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1. Introduction

The textile industry is one of the largest sectors globally, representing up to 20% of total industrial water pollution (Keenan et al., 2004; Zaffalon, 2010). Effluents from textile industries contain many pollutants including heavy metals, dyes, acids, and flame retardants that do not naturally degrade and are discharged into surface waters after a conventional wastewater treatment (WWTP) (Zaffalon, 2010). In semi-arid regions, such as the Mediterranean area, the environmental risk is particularly acute because the low dilution ability of rivers intensifies pollution stress, especially in the drought season (Petrovic et al., 2011). Seasonal changes in temperature also modulate the toxic action of pollutants on the aquatic biota, including algae (Chalifour and Juneau, 2011), plants (Chawla et al., 1991), invertebrates (Maceda-Veiga et al., 2015a), and fish (Chandra et al., 2012). Thus, inter-seasonal studies examining how aquatic communities respond to the effluents from textile industries are central to fully determine their ecological impact.

Driven by the international legislation such as the EU's Water Framework Directive (2000/60/EC), biological indices (BI) are widely used in official monitoring schemes to appraise the ecological impact of anthropogenic insults, including sewage discharges, on aquatic communities (Barbour et al., 1999; Bonada et al., 2006; Friberg et al., 2011). These procedures based their diagnostics in comparing community structure attributes (metrics) of sentinel taxa, mostly diatoms, macroinvertebrates, and fish, in tested sites against those expected in reference conditions (Karr, 1981; Munné and Prat, 2009; Hering et al., 2010; Birk et al., 2012; Feio et al., 2014). Despite criticisms and caveats (Bonada et al., 2006; Friberg et al., 2011), BIs continued popularity can be explained by their simplicity (Dallas, 1997; Chessman et al., 1997). A major concern is when BIs developed initially to detect organic

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pollution are used as generic water quality indicators (but see Sabater, (2000), Carafa et al. (2011) and Munné and Prat (2011)). Further, the use of multiple taxa may be seen as an unjustified increase in monitoring costs, as relatively few studies have compared the diagnostic value of BIs using more than two taxa in Europe (Hering et al., 2006), including Mediterranean rivers (e.g. Blanco et al., 2007; Benejam et al., 2008; Marzin et al., 2012). As conservation budgets are increasingly constrained, there is an urgent need to gain insight into how multiple BIs across taxa respond to different combinations of stressors; this will also help resource managers to identify the most vulnerable taxa at an age when freshwater diversity is declining at unprecedented rates (Strayer and Dudgeon, 2010).

Most research into the ecological impact of textile industry waste is based on bioassays (e.g. Graça et al., 2002; Sharma et al., 2007; Tigini et al., 2011). There is also some evidence of the effects of textile effluents on particular taxa in the field (Giorgi and Malacalza, 2002; Gómez et al., 2008) but, as far as we are aware, the entire structure of the aquatic community has not been specifically investigated. In this study, we examine in detail the combined effects of season and the effluent from a textile dyeing industry WWTP on water quality and the structural integrity of the aquatic community along 1.5 km in a Mediterranean river. While chemical surveys only identify a fraction of environmental pollutants, biological indicators assess the overall effects of measured and unmeasured pollutants interacting with natural and other anthropogenic stressors in the complexity of natural systems (Munné and Prat, 2009; Friberg et al., 2011). In this regard, we used diversity measures and scores of 10 BIs based on diatoms, macrophytes, macroinvertebrates and fish to test three hypothesis. First, we expected that proximity to the discharge site, particularly in low flow conditions, would intensify the impact of the effluent on the river, as defined by poor water quality, low diversity of aquatic taxa and low BI scores. Second, if all indicators have the same diagnostic value, we expected them to show similar patterns. Finally, as rivers can self-depurate (e.g. Cerqueira et al., 2008), we predicted an increase in the water quality and ecological status of the river downstream the discharge site.

2. Materials and methods

2.1. Study area

This study was carried out along a 1.5 km reach in the Ripoll River, Spain, exposed to the effluent of a textile dyeing industry built in 1960s (41°37′27″N, 2°04′22″E, Fig. 1). To assess its ecological and environmental impact, we sampled seasonally water quality and four sentinel taxa at the same time in one upstream sampling site (reference site, R1) and three downstream sites (P1, P2 and P3) in July 2012 (summer), November 2012 (autumn), February 2013 (winter) and early June 2013 (spring). Previous studies examining water quality and ecological status confirm the reference status of R1 (e.g. Prat and Rieradevall, 2006). Our study area is unique in enabling us to assess the effects of a textile industrial effluent on the aquatic biota, as a nearby urban area discharge their waste water into a sewage treatment plant downstream our study area. This area has calcareous geology and a typical Mediterranean climate, with torrential floods in autumn and spring. Water abstraction for industry and human consumption reduces further river water flow, ranging from 0.005 m^3/s to 2.8 m^3/s in the nearest downstream town to our study area over the last 10 years (ACA, 2016). Whilst data on the volume of the industrial effluent was not available to us, it represents a large fraction of river water flow, especially during drought, as occurs in many Mediterranean rivers (Prat and Munné, 2000). The substrate was mainly composed of cobbles, and no physical barriers exist between the discharge site and our last downstream sampling site. Riparian area is dominated by evergreen oak trees (*Quercus ilex*) and Aleppo pines (*Pinus halepensis*), with patches of giant reed (*Arundo donax*), wild blackberry (*Rubus ulmifolius*) and small crops. The fish fauna consisted of two native species, the Mediterranean barbel (*Barbus meridionalis*) and the Ebro chub (*Squalius laietanus*), and two exotic species, the common carp (*Cyprinus carpio*) and the pumpkinseed (*Lepomis gibbosus*).

2.2. Water and physical habitat quality

Prior to survey the aquatic community, we analysed water quality in each sampling site using a multi-parametric digital

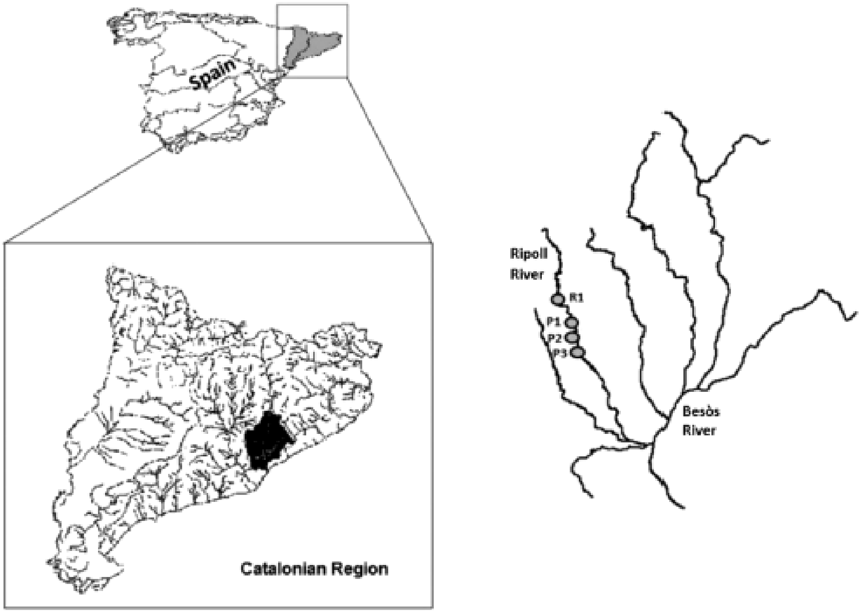

Fig. 1. Location of sites sampled from summer 2012 to spring 2013 in NE Spain. Upstream (R1) and downstream sites (P1, P2 and P3) of the discharge site of the effluent from a textile industry wastewater treatment plant were evenly distributed along a 1.5 km river reach.

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; 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. These parameters provide an overview of some of the most important water quality stressors affecting the aquatic biota, including nutrient pollution (Camargo and Alonso, 2006; Smallbone et al., 2016), acidity (Ormerod et al., 1987; Pye et al., 2012), and alterations in the overall ionic composition of water, as defined by water hardness and conductivity (Williams, 1987; Kefford et al., 2012).

After the survey of biological indicators, we calculated the average width (m), depth (m) and current velocity (m/s) in each sampling site based on three values measured along transects set perpendicular to the water flow at 20 m intervals. Current velocity and river section (width × depth) was used to calculate river flow (l/s). Additionally, we used the Fluvial Habitat Index (IHF, Pardo et al., 2004) to determine the potential confounding effect of changes in physical habitat structure between reference and tested sites on the response of biological indices to water pollution. Briefly, this index uses data on substrate, current velocity, depth, shadow, habitat diversity and aquatic vegetation to evaluate habitat suitability for the aquatic fauna. Scores above 40 indicate that physical habitat structure play a minor role in the results of biological indices, whereas scores below 40 suggests that biological indices can underestimate the water quality status due to poor habitat quality.

2.3. *Biological surveys and indicators of ecological status*

We used taxonomic richness and diversity, as defined by the Shannon-Wiener index (Moreno, 2001), and 10 biological indices based on diatoms, macrophytes, macroinvertebrates and fish as widely used indicators of the structural integrity of aquatic communities (Barbour et al., 1999; Bonada et al., 2006; Munné and Prat, 2009). Details on the surveys are outlined below and the computation of each biotic index is described in the references provided.

2.3.1. *Diatoms*

Diatom sampling, preparation and counting followed CEN standards (CEN 2003, 2004) and the specific recommendations of local water authorities for the use of diatoms as biological indicators in Mediterranean rivers. Three medium-size cobbles (20 × 20 cm) were collected from the river bottom in a well-lit riffle section. Cobbles with filamentous algae and soft sediment were discarded. The cobbles were scraped with a knife to detach the algal community (3 cm^2) and samples were rinsed in 5 ml of river water and preserved in 4% formaldehyde until analysis. To clean organic matter and carbonate salts from diatom frustules, diatoms were digested in 33% hot hydrogen peroxide over 12 h, the supernatant was poured and the pellet was then digested in 2 ml of 35% hydrochloric acid. Slides were mounted using Naphrax® (Brunel Microscopes Ltd., Chippenham, Wiltshire, UK), and up to 400 valves were counted per slide under the light microscope Zeiss JENEVAL at x1000 magnification. Taxa were identified at the species level following mainly Krammer and Lange-Bertalot (1991,1997) and Lange-Bertalot (2001). As biotic indices, we used three multimetric indices, Specific Polluosensitivity Index (IPS, Coste 1982), the Diatom Biologic Index (IBD, Prygiel and Coste, 2000) and the index of the European Economic Community (CEE, Descy and Coste, 1991), using the software OMNIDIA® version 5.3 (Lecointe et al., 1993, 1999).

2.3.2. *Aquatic macrophytes and riparian cover*

As there is no international standardised protocol to use macrophytes as biological indicators, we used the procedure developed by the Spanish Government. A 100-m long reach was selected and the representatives of all submerged and emerged macrophytes (spermatophytes, bryophytes, pteridophytes and macroalgae) were identified to species level in-situ or in the laboratory. The percentage of cover, as proxy of the abundance of each species, was also recorded in each sampling site. As biotic indices, we used three multimetric indices: Macrophyte Biological Index for French Rivers (IBMR, Haury et al., 2006), the Index of Macrophytes (IM, Suárez et al., 2005), and the Index of Fluvial Macrophytes (IMF, Flor-Arnau et al., 2015). To specifically characterise the quality of the riparian vegetation cover, we calculated the QBR index (from the Catalan 'Qualitat del Bosc de Ribera'; Munné et al., 2003) only once through the study period. Briefly, it ranks 0 (poor) to 100 (good) the status of the riparian vegetation considering total vegetation cover, structure and quality, and it downweights for the presence of exotic plant species.

2.3.3. *Macroinvertebrates*

Benthic macroinvertebrates were collected in each sampling site using a Surber sampler of 0.1023 m^2 and 250 μm mesh size following the protocol MIQU (from the Catalan protocol 'MacroInvertebrats QUantitatiu'; Núñez and Prat, 2009). This protocol requires sampling all river microhabitats, differentiating between dominant (occupying >5% of the sample area) and marginal habitats (≤5%). In each sampling site, 8 Surber samples were taken from dominant habitats and 4 from marginal habitats following the criterion proposed by Mondy et al. (2012), and the representativeness of each microhabitat in our sampling sites. All samples from the same site were pooled in a single sample and preserved in 4% formaldehyde. In the laboratory, animals were sorted under a stereoscope, identified to the level necessary to calculate the different biotic indices (family level for all groups of macroinvertebrates with the exception of Hydracarina, nematodes and oligochaetes that were identified at the order level) and counted. Taxa were identified following Tachet et al. (2002), Pace et al. (2014). As biotic indices, we calculated the unimetric Index of the Iberian Biomonitoring Working Party (IBMWP, Alba-Tercedor et al., 2002), the Multimetric Index for Mediterranean streams (IMMiT, Munné and Prat, 2009) and the unimetric Ephemeroptera-Plecoptera-Trichoptera index (EPT, Munné and Prat, 2009).

2.3.4. *Fish*

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 reach surveyed at each sampling site following an international standardised fish sampling method (CEN standards EN 14962 and EN 14011). All microhabitats were sampled as driven by the European Water Framework Directive. The same crew and equipment was used across sites and seasons to avoid bias in fish captures (see Benejam et al. (2012)). Fish were identified to species level, counted, and released in each sampling site. Fish captures were standardised to captures per unit of effort prior to calculate the multimetric index IBICAT2b (García-Berthou et al., 2015). Field work on fish was approved by the Regional Government of Catalonia.

2.4. *Statistical analyses*

The degree of congruence among biological indicators and among environmental variables was assessed using Spearman's rank correlation coefficients, and one variable of each strongly correlated pair was selected ($|r| \geq 0.7$). The same analysis was then used to examine relationships between the least correlated biotic measures and water quality variables. Significance was reached at $p < 0.05$. All statistical analyses were performed in R (R Development Core Team, 2014).

3. Results

As expected, the lowest water flow was observed in Ripoll River in summer and winter and consequently, differences in conductivity and nitrogenous and phosphorous content between tested and reference sites were higher in these seasons (Table 1). In contrast, these water quality parameters markedly reduced in spring when the river water flow increased (Table 1). A slightly increase in water temperature also occurred in site P1 throughout the entire study, and in pH in most tested sites compared to the reference site, particularly in winter and spring (Table 1). No clear differences in overall water quality were observed downstream site P1. However, the tested site P3 had the lowest values of conductivity, ammonium and nitrite in some seasons (Table 1). Most indicators of water quality were significantly correlated, with positive associations between conductivity ammonium, nitrite, nitrate and phosphate, and negative between dissolved oxygen, temperature, conductivity and nitrite (Table 2).

The results of biological indicators mirrored those of water quality variables, showing a marked decreased in the ecological status of Ripoll River after the input of the industry (Table 3 and Fig. 2). However, differences in river conditions across seasons between tested and reference sites were more marked using biotic indicators. As both physical habitat structure and water quality can affect aquatic communities, it was necessary to test for the potential effect of the former on the results of biological indicators. Since the Fluvial Habitat Index scores were above 50 in all sampling sites, our results can be mostly attributed to alterations in water quality. While the major causal factor to changes in water quality was the industry, non-point source pollution from adjacent agricultural areas could have also occurred in tested sites. In fact, the quality of the riparian vegetation, as defined by the QBR index, was higher in the reference (90) than in the tested sites (25–55) due to the presence of small crops and exotic plants.

Up to 45, 34, 40 and 4 representatives of diatoms, macrophytes, macroinvertebrates and fish were recorded in the Ripoll River (Table 3). After the textile industry, a marked decrease in overall taxonomic richness occurred, being diatoms the exception (Table 3). The impact was particularly acute for fish, as no species was found in sites P1 and P2, and the site P3 was dominated by exotic species and one of the two native species (*Squalius laietanus*). Biotic indices (BIs) also detected well the impact of the textile industry, with the exception of most macrophyte indices (Fig. 2). Most BIs across taxa showed that the Ripoll River had a poorer ecological status in sites P1 and P2 than in the reference site R1. Some BIs (e.g. IBICAT2b, IPS) also indicated that river ecological status improved downstream, but with a marked variability across seasons (Fig. 2). In particular, macrophyte and macroinvertebrate indices showed the lowest scores in tested sites in winter, while diatom indices did so in summer (Fig. 2). In contrast, diatom and most macroinvertebrate indices mostly indicated that the ecological status of Ripoll River increased in spring (Fig. 2). In the reference site, scores of most BIs remained stable through the study with the exception of the IM macrophyte index and the EPT and IBMWP macroinvertebrate indices (Fig. 2).

Most BIs developed for the same taxa were strongly correlated ($r \geq 0.7$) with the exception of those of macrophytes (Table 4). The IMF and IM macrophyte indices were, however, strongly correlated with all three macroinvertebrate indices, but poorly related to the three diatom indices and the fish index (Table 4). The latter only correlated strongly with the IPS diatom index and the EPT macroinvertebrate index (Table 4). While our chemical data detected a small fraction of the pollutants released by the textile industry in snapshot of time, highly significant relationships were found between all BIs and at least one water quality variable, particularly conductivity and nutrient pollution (Table 5), suggesting a chronic pollution event. In contrast, diversity measures across taxa were mostly poorly related to water quality measures (Table 5).

4. Discussion

Our study shows that the discharge of the effluent from a textile dyeing industry waste water treatment plant (WWTP) sharply worsened the water quality and ecological status of a Mediterranean river. Although the impact was evident in diversity measures and biotic indices of all four taxa examined, fish were the most affected and macrophytes the least. However, our results also showed that river conditions in spring alleviated the ecological impact, and that the river status largely improved at 1.5 km downstream the discharge site in most seasons.

As expected, biotic indicators reflected better the impact of the WWTP across seasons than water quality variables. At best, chemical monitoring provides an exhaustive list of contaminants (Petrovic et al., 2011). However, these analyses are often expensive and cannot inform about the combined effects of complex pollutant mixtures and natural stressors (e.g. seasonal changes in temperature and water flow) on the biota (Petrovic et al., 2011; Friberg et al., 2011; Birk et al., 2012). Unique to biological indicators is also their ability to integrate impacts across ecosystem

Table 1
Physical and chemical water properties measured before (R1) and after the discharge site of a textile industry effluent (P1, P2, and P3) in the Ripoll River across all four seasons (SU: summer, AU: autumn, WI: winter and SP: spring). Values in bold indicate that water quality was poor according to the standards of the Catalan Water Agency.

	R1				P1				P2				P3			
	SU	AU	WI	SP	SU	AU	WI	SP	SU	AU	WI	SP	SU	AU	WI	SP
Temperature (°C)	18.6	9.7	7.9	16.1	24.1	14.6	15.5	17.5	23.1	13.7	11.1	18.5	19.5	12.3	11.9	18.8
Dissolved oxygen (mg/L)	7.0	11.0	10.2	10.0	6.7	8.6	7.5	10.4	6.5	9.1	8.2	9.4	7.2	9.3	9.2	8.5
Flow (l/s)	14.0	83.4	14.3	149.9	30.1	32.0	14.4	201.2	17.1	120.3	21.0	265.6	65.0	139.0	80.3	249.4
pH	7.5	7.7	7.5	7.8	7.7	7.7	8.3	8.0	7.5	7.8	8.2	8.0	7.5	7.6	7.7	8.0
Total hardness (°dH)	21	22	20	21	26	29	31	21	29	25	29	20	24	20	26	22
Carbonate hardness (°dH)	25	19	16	18	19	23	38	18	16	20	34	18	22	27	22	19
Conductivity (μS/cm)	628	469	442	543	**4053**	**1853**	**4465**	586	**5199**	**1073**	**3250**	612	**1654**	**1243**	**1954**	768
Ammonium (mg/L)	<0.2	<0.2	<0.2	<0.2	**1.0**	<0.2	**0.8**	0.1	0.6	<0.2	**3.0**	<0.2	<0.2	<0.2	**3.0**	<0.2
Nitrite (mg/L)	<0.02	<0.02	0.02	0.03	1.0	0.4	0.1	0.02	1.0	0.3	0.1	0.03	0.03	0.05	0.3	0.07
Nitrate (mg/L)	<1.0	4.0	2.0	5.0	**35.0**	9.0	3.0	5.0	**60**	7.0	5.0	5.0	3.0	5.0	8.0	5.0
Phosphate (mg/L)	<0.2	0.3	0.1	<0.2	**1.0**	**0.9**	0.1	**0.5**	**6.0**	**0.7**	0.3	<0.2	**1.0**	0.2	**0.7**	0.5

Table 2
Spearman rank correlation coefficients (r) between the 11 environmental variables with data of all four seasons combined.

	1	2	3	4	5	6	7	8	9	10
1. Dissolved oxygen										
2. Temperature	−0.64**									
3. Conductivity	−0.78**	0.33								
4. Flow	0.53*	0.07	−0.38							
5. Nitrite	−0.56*	0.25	0.83**	−0.13						
6. Nitrate	0.22	0.24	0.51*	0.23	0.83**					
7. Ammonium	−0.35	0.03	0.67**	−0.31	0.52*	0.39				
8. pH	0.01	−0.31	0.35	0.07	0.53*	0.66**	0.34			
9. Phosphate	−0.44	0.34	0.57*	−0.07	0.65**	0.64**	0.32	0.44		
10. Carbonate hardness	−0.33	−0.20	0.45	−0.27	0.14	−0.19	0.19	0.17	−0.07	
11. General hardness	−0.59*	0.12	0.82**	−0.42	0.72**	0.43	0.62**	0.30	0.57*	0.40

** $p \le 0.01$.
* $p \le 0.05$.

Table 3
Changes in taxonomic richness and diversity (Shannon index) of the four sentinel taxa (diatoms, macrophytes, macroinvertebrates, and fish) used as bioindicators to assess the ecological status of Ripoll River before (R1) and after the discharge site of a textile industry effluent (P1, P2, and P3) across all four seasons (SU: summer, AU: autumn, WI: winter and SP: spring).

	R1				P1				P2				P3			
Taxonomic richness	SU	AU	WI	SP	SU	AU	WI	SP	SU	AU	WI	SP	SU	AU	WI	SP
Diatoms	28	21	15	40	42	35	40	45	45	31	37	33	45	35	39	38
Macrophytes	34	21	12	25	20	8	13	17	20	18	8	16	21	19	6	13
Macroinvertebrates	35	28	23	25	29	12	13	22	24	14	12	12	40	18	12	13
Fish	3	3	3	2	0	0	0	0	0	0	0	0	2	4	2	3
Taxonomic diversity																
Diatoms	0.9	1.0	0.9	1.2	0.7	1.1	1.1	0.9	0.7	1.0	1.0	1.2	1.0	1.2	0.9	1.1
Macrophytes	1.5	1.3	1.0	1.3	1.2	1.0	0.5	1.0	1.2	1.2	0.8	1.1	1.3	1.2	0.7	1.0
Macroinvertebrates	1.9	1.9	2.0	1.4	1.8	1.2	1.2	2.0	2.0	1.3	0.8	1.6	2.1	1.6	1.0	1.5
Fish	0.1	0.3	0.3	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1

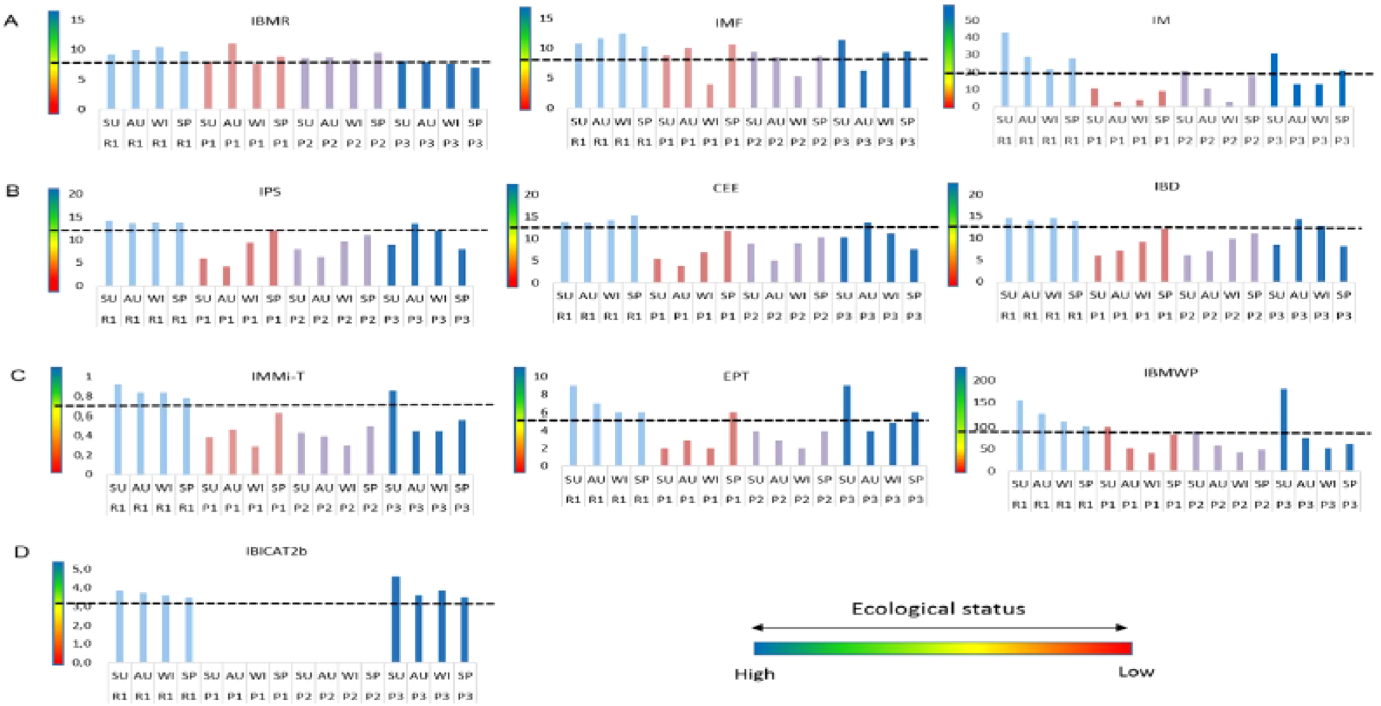

Fig. 2. Scores of 10 biotic indices based on macrophytes (A), diatoms (B), macroinvertebrates (C) and fish (D) used to assess the ecological status of Ripoll River before (R1: light blue) and after the discharge site of an industrial textile dyeing industry (P1: red, P2: violet and P3: blue) from summer 2012 to spring 2013. Broken lines indicate the threshold above which the ecological status of this water body mass is 'good' according to the Catalan Water Agency. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 4
Spearman rank correlation coefficients (r) between the 10 biotic indices calculated in the current study with data of all four seasons combined.

		Macrophytes					Diatoms					Macroinvertebrates					Fish	
		1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2
Macrophytes	1. IMF																	
	2. IM	0.69**																
	3. IBMR	0.56*	0.20															
	4. Diversity	0.74**	0.56*	0.31														
	5. Richness	0.44	0.66**	0.29	0.58*													
Diatoms	1. IPS	0.39	0.54*	0.28	0.32	0.24												
	2. IBD	0.38	0.44	0.38	0.28	0.11	0.96**											
	3. CEE	0.52*	0.63**	0.32	0.44	0.34	0.96**	0.90*										
	4. Diversity	−0.27	−0.35	−0.54*	−0.20	−0.28	−0.37	−0.38	−0.37									
	5. Richness	−0.02	−0.21	−0.27	0.19	0.10	−0.46	−0.55*	−0.55*	0.55								
Macroinvertebrates	1. IMMi-T	0.81**	0.81**	0.32	0.64**	0.52*	0.39	0.31	0.54*	−0.26	−0.16							
	2. BMWP	0.89**	0.79**	0.49	0.83**	0.78**	0.58*	0.58*	0.67**	−0.30	0.03	0.76**						
	3. EPT	0.84**	0.85**	0.26	0.58*	0.51*	0.60*	0.56*	0.68**	−0.11	−0.09	0.94**	0.76**					
	4. Diversity	0.47	0.55*	0.17	0.60*	0.50*	0.46	0.36	0.60*	−0.43	0.08	0.37	0.66**	0.42				
	5. Richness	0.66**	0.68**	0.26	0.82**	0.86**	0.21	0.11	0.36	−0.26	0.15	0.59*	0.95**	0.58*	0.60*			
Fish	1. IBICAT2b	0.56*	0.56*	−0.04	0.56*	0.30	0.75**	0.61*	0.62*	−0.04	−0.22	0.69**	0.62	0.78**	0.39	0.44		
	2. Diversity	0.14	0.25	−0.14	0.21	0.41	−0.19	−0.36	−0.07	−0.06	0.52*	0.12	0.27	0.16	0.27	0.36	−0.22	
	3. Richness	0.46	0.67**	−0.02	0.38	0.86**	0.63**	0.69**	0.66**	−0.32	−0.43	0.61	0.53	0.67**	0.52	0.34	0.83**	−0.19

** $p \leq 0.01$.
* $p \leq 0.05$.

compartments (e.g. water-sediment) depending on the ecology of the sentinel taxa (Barbour et al., 1999; Bonada et al., 2006). For instance, many unmeasured pollutants in our study that typically occur in textile industry waste, such as heavy metals (Zaffalon, 2010), could have been trapped in the sediment (Monroy et al., 2014; Roig et al., 2015). This can explain why the impact was acute to organisms that live or feed on the river bottom, supporting previous data on diatoms (Sabater, 2000), benthic macroinvertebrates (Munné and Prat, 2009) and the fish *Barbus meridionalis* (Maceda-Veiga et al., 2013).

A striking result of the study is that no fish occurred in the most polluted sites in any season, which is particularly worrisome because the distribution area of *B. meridionalis* and *Squalius laietanus* has sharply declined in recent decades (Maceda-Veiga et al., 2010). The former also has unique genetic traits in this river (Machordom et al., 1990) and is included in the Annexes III and V of the EU's Habitats Directive (Crivelli, 2006). Many pollutants can explain their absence (e.g. Keenan et al., 2004; Colin et al., 2016), but a peak in ammonium concentration is likely to be a major causal factor in our study, as it is highly toxic to fish at high pH and temperature values (Camargo and Alonso, 2006). In spring, however, water quality largely improved in the most upstream tested sites and fish were neither recorded. Whilst our water analyses showed a snapshot of time, this can be explained by the long-term heavy pollution of these reaches, as both long-lived taxa such as fish (years) and short-lived taxa such as diatoms and macroinvertebrates (weeks, months) (Barbour et al., 1999) were severely affected. However, the absence of fish can also be related to a lower recolonization ability, as the home range of a sister species to the captured *B. meridionalis*, the Iberian red-fin barbel (*Barbus haasi*), is less than 100 m (Aparicio and de Sostoa, 1999). Chemical cues may also have limited further upstream fish movements (see Flecker (2010)), including the highly mobile *S. laietanus* (Sostoa de, 1990). Finally, water quality can also explain why relatively tolerant fish species, *S. laietanus* and the common carp (*Cyprinus carpio*) (Maceda-Veiga and De Sostoa, 2011), dominated the fish community in the most downstream site, as all sampling sites were within the distribution range of *B. meridionalis* (Sostoa de, 1990).

According to biotic indices, the impact of the WWTP was acute for diatoms in summer and for macrophytes and macroinvertebrates in winter. As expected, in these seasons, low water flow increased pollution levels and hence their effects on the biota. Variation in taxa response across seasons is likely to be attributed to their phenology; for instance, total richness of macroinvertebrates and aquatic plants naturally reduces in winter. Our results also showed that the fish index score was rather stable in the last sampling site despite water quality varied across seasons. In fish species-poor systems, such as Mediterranean streams, the diagnostic power of fish indices is limited by the reduced number of metrics at the community level (Moyle and Randall, 1998; Magalhães et al., 2008). However, this could improve via the use of more metrics at the individual level, such as body mass-length relationships (Maceda-Veiga et al., 2014), blood analyses (Maceda-Veiga et al., 2015b) and population size structure (Figuerola et al., 2012; but see Murphy et al. (2013)). This may also apply to most macrophyte indices used in this study as they performed worse than those based on diatoms and macroinvertebrates. However, since macrophyte richness was relatively high, and the IM performed reasonably well, their diagnostic ability could improve with new metrics at the community level.

In our study, diversity measures and biotic indices performed well in revealing the impact of the WWTP, supporting the notion that both are useful indicators of ecological impairment (Mason, 1996; Munné and Prat, 2009). However, we caution for the use of diversity measures as stand-alone metrics, because pollution

Table 5
Spearman rank correlation coefficients (r) between the least correlated water quality measures (from Table 2) and biotic indicators (from Table 4) measured in the Ripoll River with data of all four seasons combined.

	Temperature	Conductivity	Flow	Nitrate	pH	Carbonate hardness
IBMR	−0.29	-0.59^{**}	−0.06	−0.13	−0.22	−0.41
IPS	−0.40	-0.65^{**}	−0.02	-0.65^{**}	−0.14	−0.09
IMMiT	0.03	-0.75^{**}	0.09	-0.58^{**}	−0.46	−0.33
Diatom diversity	0.16	0.35	0.27	0.19	0.38	0.31
Diatoms richness	0.61^{**}	0.47	0.16	0.53^{*}	−0.03	−0.14
Macroinvertebrate diversity	0.10	−0.23	−0.18	−0.01	-0.64^{**}	−0.45
Fish diversity	0.87^{**}	0.08	0.19	0.27	−0.11	0.03

** $p \leq 0.01$.
* $p \leq 0.05$.

effects on each taxa can go in opposite directions, as reported in our study and elsewhere (Mason, 1996). Many biotic indices across taxa were also strongly correlated to each other, including uni- and multimetric indices of macroinvertebrates such as the EPT and IMMi-T. This does not necessarily mean that all taxa give the same diagnostic picture, since they respond to different combinations of stressors; for instance, the impact of hydromorphological disturbances is often more severe on fish and macrophytes than on diatoms and macroinvertebrates (Marzin et al., 2012). Thus, values of pair-wise correlations among biotic indices can vary depending on the stressor gradient examined (e.g. Sabater, 2000; Damásio et al., 2007; Benejam et al., 2008). Likewise, multimetric indices typically provide a more integrated assessment of the aquatic community than uni-metric indices (Karr, 1981; Bonada et al., 2006; Munné and Prat, 2009), even though their scores were similar in our study. In fact, European Commission suggests the use of multimetric indices that respond to multiple environmental insults and show a low variability in the reference data (Heiskanen et al., 2004), as occurs with the macroinvertebrate IMMi-T, the fish IBICAT2b and all diatom indices in our study.

Despite textile industry waste has many pollutants refractive to biological degradation (Zaffalon, 2010), our study showed that the ecological status of the river improved downstream. However, this study cannot fully assess the recovery of the aquatic biota, as effects may be visible at other levels of biological organisation than those examined in this study (e.g. ecosystem metabolism, biochemical pathways) (Damásio et al., 2007; Colin et al., 2016). Likewise, pollutants can accumulate in tissues of aquatic organisms such as fish (Maceda-Veiga et al., 2013; Monroy et al., 2014) and so be transferred to predators such the European otter (*Lutra lutra*, Ruiz-Olmo et al., 2001) that has been recently recorded close to our study area. Although the textile industry was the major causal factor to our results, this study cannot fully rule out the effects that other stressors such as run-off from adjacent crops (Moss, 2008) and biological invasions, including the invasive plant *Arundo donax* (Maceda-Veiga et al., 2016), may have had on the aquatic community. Thus, studies using more specific indicators of chemical pollution (see biomarkers in Colin et al. (2016)) are needed to fully understand how organisms respond to industrial effluents in this Mediterranean river. As biomarkers have a phylogenetic component, their use can also help to increase the predictive ability of current biomonitoring schemes across study areas.

5. Conclusion

This study shows that a Mediterranean river largely recovered from the impact of a textile dyeing industry at 1.5 km downstream. Diversity measures and biotic indices in diatoms, macroinvertebrates and fish were identified as the most sensitive indicators of biological impairment. However, the perfect tool to determine ecosystem health does not exist, and it is the combination of indicators of impairment at different levels of biological organisation (e.g. individual, community, and ecosystem) that will give us the best diagnostic picture (Friberg et al., 2011; Colin et al., 2016). In conclusion, this study suggests that there is an urgent need to invest in wastewater treatment in this industry to preserve the ecological integrity of Ripoll River and especially the connectivity between native fish populations that were the most affected taxa.

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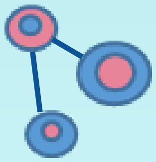

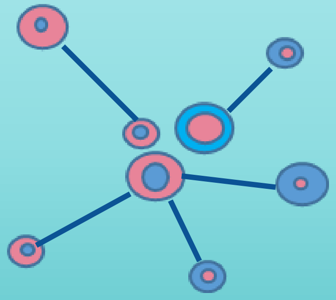

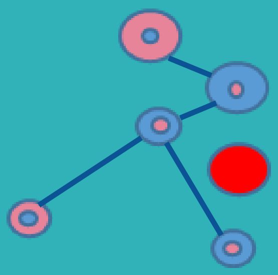

CAPÍTULO 2

Medidas de diversidad funcional reflejan impactos de la actividad humana en sistemas de baja riqueza de especies

Resumen

La ecología basada en rasgos se ha desarrollado durante décadas con el objetivo de inferir alteraciones en procesos ecológicos a partir de la estructura funcional de las comunidades. Sin embargo, todavía existe un gran desconocimiento sobre cómo la actividad humana afecta a la diversidad de rasgos en sistemas de baja riqueza taxonómica. En este estudio se utilizó una extensa base de datos (99.700 km2, n = 387 localidades) a fin de analizar cómo la riqueza total de especies y tres índices de diversidad funcional (especialidad funcional, originalidad y entropía) basados en comunidades de peces varían en función de los efectos combinados de estresores naturales y antrópicos en 15 cuencas del NE de España. Los análisis de partición de la varianza y de GLMM demostraron de forma consistente que los índices de diversidad funcional explicaron más varianza que la riqueza taxonómica. La altitud pareció intensificar el efecto de la degradación física del hábitat sobre la estructura funcional de las comunidades de peces. La biomasa de peces exóticos, además, redujo notablemente las combinaciones de rasgos únicos de las comunidades. Sin embargo, la alteración de la calidad del agua jugó un papel menos importante en la variación de los tres índices funcionales, y las características geográficas fueron las variables más importantes para la variación en riqueza total. La comparación de los índices de diversidad funcional con los indicadores tradicionales del estado de salud de los ríos en esta región puso de manifiesto el fuerte papel estructurador de las introducciones de peces en estas comunidades. Además, este estudio comparativo sugiere que los índices funcionales son mejores como indicadores de impacto ambiental que el tradicional índice de calidad biótica basado en peces, desarrollado a partir de la entrada en vigor de la Directiva Marco del Agua Europea. En resumen, las actividades humanas están configurando la estructura funcional de las asociaciones de peces en los ríos mediterráneos con posibles ramificaciones para los servicios ecosistémicos que éstos sustentan. La adopción de los índices de diversidad funcional en los protocolos de biomonitorización podría ayudarnos a comprender y predecir mejor los efectos de las perturbaciones antrópicas en los amenazados ríos mediterráneos.

Functional diversity measures revealed impacts of human activites on species-poor freshwater fish assemblages

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Abstract

Trait-based ecology has been developed for decades to infer changes in ecosystem processes based on the functional structure of communities. However, we still have limited insight into how human activities affect trait diversity in species poor systems. In this study we used an extensive data-set (99,700 km^2, n=430 sites) to examine how taxonomic richness and three functional diversity (FD) facets (functional specialization, originality and entropy) of fish assemblages respond to the combined effects of natural and anthropogenic disturbances in 15 catchments in NE Spain. Variation partitioning and GLMM approaches consistently showed that functional diversity indices explained more variance than total fish richness. Altitude appeared to intensify the effect of physical habitat degradation on the functional structure of fish assemblages. Non-native fish biomass also seemed to reduce unique trait combinations in fish assemblages. However, changes in water quality play a limited role in variation in all three FD facets, and geographical features were only highlighted as major driver for taxonomic richness. The comparison of FD facets with traditional indicators of ecosystem health further supports the strong impact of fish introductions in the community structure of fish in these Mediterranean rivers. Further, it showed that FD indices perform better than a fish index of biotic integrity developed in this region to appraise environmental degradation, in compliance with the EU's Water Framework Directive. On a corollary, human activities are shaping the functional structure of fish assemblages in Mediterranean rivers with potential ramifications for the ecosystem services they support. The adoption of FD facets in biomonitoring can help us to better understand and predict the effects of anthropogenic disturbances in the vulnerable Mediterranean rivers.

Keyword: trait-based ecology, functional ecology, Mediterranean rivers, multiple stressors, exotic fish, fish community, habitat alterations.

1. Introduction

Biodiversity loss is occurring at unprecedented rates on Earth and freshwater ecosystems are probably among the most affected (Strayer and Dudgeon, 2010, Vörösmarty et al., 2010; Tittensor et al. 2014). Major threats to freshwater biodiversity include hydromorphological alterations, water pollution, fisheries, and the release of non-native species (Maybeck, 2003, Vörösmarty et al., 2010). Besides conservation issues *per se*, alterations in freshwater diversity imperil important human resources such as food and drinking water (Vörösmarty et al., 2010). Several international initiatives such as the International Decade for Action – 'Water for Life' (Dudgeon et al., 2007) and the EU's Water Framework directive (EU Commission, 2000) aim to promote river conservation. However, a better understanding of how freshwater animal communities respond to the combined effects of natural and anthropogenic stressors is needed to develop more effective management strategies (Ormerod et al., 2010).

The loss of sensitive species is a well-known response of aquatic communities to stress (Kolkwitz and Marsoon, 1909), and the basis of the myriad of taxonomic-based procedures developed, hereafter referred to as indices of biotic quality (IBQ) to assess the ecological status of rivers (Karr, 1981; Birk et al., 2012). Despite being widely adopted by official monitoring schemes, many criticisms have arisen from their use (reviewed by Friberg et al. 2011; Jackson et al. 2016). For instance, climate change may compromise their diagnostic ability by shifting the reference conditions against which tested sites must be compared (Barbour et al., 2010). Most importantly, the extensive use of IBQ may have limited our ability to develop theory on how communities respond to stress. Ecological niche theory states that habitat acts as 'filter' selecting those species with the best set of traits for a given condition (Chase and Leibold, 2003). Thus, geographically distant communities under similar stressors are expected to have different species but similar trait combinations (e.g. Bonada et al., 2007).

Towards predicting the response of communities, functional ecology has been developing for the last decades (Statzner et al., 2001; Petchey and Gaston 2006), with development of functional diversity indices based on functional traits, i.e. attributes of organisms that are link to their response to environment or their role in ecosystem processes (FD; Petchey and Gaston, 2002; Mouillot et al., 2013; Maire et al. 2015). It is widely recognised that FD measures are a superior alternative to taxonomic-based approaches to detect effect of human impacts (e.g. Hooper et al., 2005; Villéger et al., 2010; Gagic et al., 2015). However, its advantages over IBQ have not been specifically investigated. IBQ often use community traits in making diagnostics, but resultant score does not explicitly account for functional diversity.

In this study we assess how freshwater fish assemblies in NE Spain respond to multiple stressors. This region has a long-history of anthropogenic disturbances (e.g. droughts, water pollution, physical habitat degradation, and introduction of non-native species (Mas-Marti et al. 2010; Figuerola et al., 2012; Maceda-Veiga et al., 2017), and provides a unique opportunity to assess how these shape the functional structure of fish assemblages. Although the life-history of fish species is adapted to the typical flood-drought regime of Mediterranean rivers (e.g. Vinyoles et al. 2010; Doadrio et al. 2011), fish diversity could be affected by the interactive effects of anthropogenic disturbances. The low fish species richness in Mediterranean rivers (often < 4 species, e.g. Maceda-Veiga et al. 2010) contrasts with the higher richness in other European rivers and may limit the performance of FD indices to detect the effects of stressors.

The objectives of our study are: i) to assess the response of taxonomic richness and three FD facets (functional specialization, originality and entropy) to the combined effects of natural and anthropogenic stressors, and ii) to compare the diagnostic ability of FD indices with traditional IBQ widely used in NE Spain. We expect that indices considering functional identity of species will provide a better description of the effects of human activities on fish assemblages than species richness, since community-habitat relationships should be mediated via functional traits (e.g., Suding et al. 2008). If FD indices have potential to become new monitoring tools in species-poor systems, we also expect them to perform better than the current fish index developed in this region to appraise ecosystem health.

2. Materials and methods

2.1. Study area

The study area is located in NE Spain and comprises 15 catchments, including the complete Ebro River and part of the Garonne basin (Fig 1). Except the Garonne, all rivers travel east from the Cantabrian, Pyrenean or low mountains to the sea. Overall, the selected river basins drain an extensive area of up to 99,700 km^2 and typify well the natural and anthropogenic stressors experienced by other Mediterranean rivers in terms of geology, physical habitat structure and water properties (Sabater et al., 2009). The north-east of Spain is also a hotspot of fish introductions worldwide (see Leprieur et al. 2008). Most of these rivers are small and follow a typical Mediterranean hydrological regime, with severe droughts in summer and 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 can be properly sampled using electrofishing (see below). These conditions are also likely to intensify the effects of anthropogenic stressors on aquatic organisms (Petrovic et al., 2011).

Fish fauna includes endemic cyprinids (e.g. Iberian red-fin barbel, *Barbus haasi*; the Ebro nase *Parachondrostoma miegii*), amphidromous species such as the European eel (*Anguilla anguilla*)

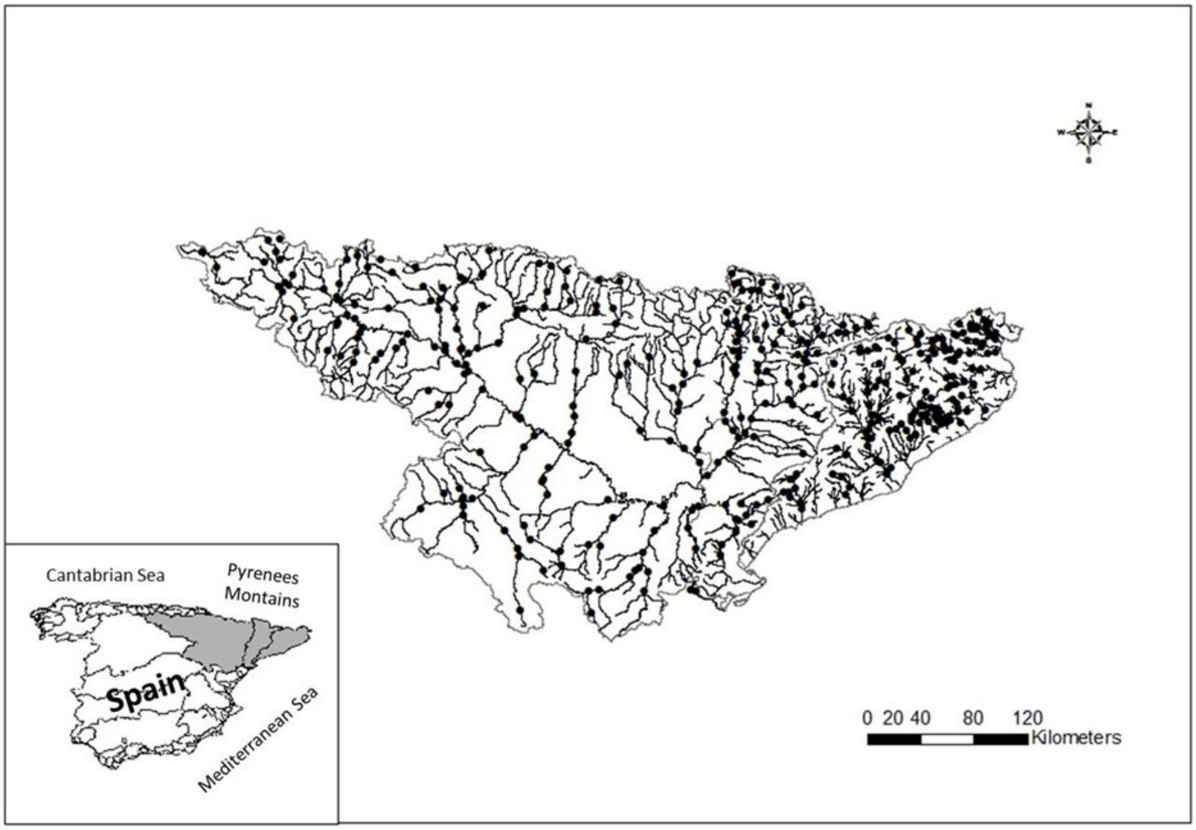

Figure 1. Location of Catalonia Basins included whole Ebro Basin, in northeaster Spain (box). 380 samples sites (black points) for fish sampling, habitat and water quality variables.

and many non-native species (~50%) that have been introduced for the two last centuries, including some of the world's worst invasive species (e.g. the common carp *Cyprinus carpio*, the eastern mosquito fish *Gambusia holbrooki*) (Maceda-Veiga et al., 2017). Fish species mostly found in river mouths such as mullets (*Liza* spp., *Chelon labrosus* and *Mugil cephalus*) were excluded from the analysis, as they play a minor ecological role at the basin scale. Riparian vegetation include native and non-native coniferous and deciduous trees (e.g. *Alnus glutinosa*, *Platanus hispanica*, *Coryllus avellana*), and invasive herbs such as *Arundo donax* (e.g. Maceda-Veiga et al., 2016).

2.2. Fish surveys

We assembled fish data from 430 surveys performed in NE Spain from 2002 to 2008 (e.g. Maceda-Veiga and de Sostoa, 2011; Maceda-Veiga et al. 2014). Fish were captured following an international standardised fish sampling method (CEN standards EN 14962 and EN 14011), in compliance with the EU's 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. We covered the whole wetted width of the 100-m long reaches surveyed in each site, which include a variety of habitat types (pools, rifles and runs) (see Maceda-Veiga et al. 2017 for further details). Fish were then identified to species level, counted and a representative set of individuals of each species (50 individuals when possible) weighted to the nearest mg. Fish were anaesthetised with a buffered MS222® solution (Tricaine methane-sulfonate, Sigma) to reduce stress. All fish were released in each site when recovered. Fish abundance and biomass were standardised to account for sampling effort.

2.2. Functional characterization of fish

To describe the functional identity of each fish species, we selected a set of 9 complementary traits that are related to key biological functions such as food acquisition, locomotion and reproduction (Table 1) (Olden et al. 2006, Villéger et al., 2013, Buisson et al., 2013). Traits were coded using continuous or ordinal variable. We used fish Atlases (Kottelat and Freyhof, 2007; Doadrio et al., 2011), electronic databases (http://www.fishbase.org), the scientific literature, and our own expertise to provide a functional description of native and non-native fish species (Table 1). Ordinal traits were assigned a single state based on a majority of evidence rule according to adult preferences following Olden et al. (2006). The lack of an in-depth ecological knowledge of some fish species precluded the use of more traits. We also acknowledge that species traits can differ among populations (Ackerly and Cornwell, 2007), but we lack of this specific information for the present study.

2.3. Measuring functional diversity

Functional diversity indices (FD) of fish assemblages were computed in a multidimensional space (Villéger et al. 2008, Mouillot et al. 2013, Maire et al. 2015). Briefly, we first computed pair-wise functional distances between species according to their traits values using the Gower distance (Gower, 1966). Secondly, we applied a

Table 1. Trait type, function, biological traits and categories for fishes considered in the present study.

Trait type	Function	Trait	Categories	Categorical value
Categorical	**Reproduction**	Reproductive strategy	Fractional Spawner	1
			Up the river	2
			Migratory	3
			Parental care	4
			Ovoviviparous	5
			Spawns several substrate	6
		Fecundity (egg number)	<2000	1
			2000-10000	2
			>10000	3
	Trophic interaction	Trophic guilds	Herbivore-detritivore	1
			Omnivore	2
			General invertivore	3
			Surface/water column invertivore	4
			Benthic invertivore	5
			Piscivore	6
	Habitat use	Vertical position	Benthic	1
			Benthopelagic	2
			No Benthic	3
		Velocity preference	Fast	1
			Moderate	2
			Slow-None	3
			General	4
Continuous	**Locomotion and food acquisition**	Shape factor	Describe ratio	
		Swimming factor	Describe ratio	
	Survival	Average weight		
		Maximal age		

principal coordinated analysis (PCoA) to the resultant matrix to build the multidimensional Euclidean space (Villéger et al., 2008). Thirdly, we selected the first three PC axes (mSD: 0.012) as the most representative functional space of our data-set (Maire et al., 2015). Finally, we computed FD indices based on position of species in the functional space (Villéger et al. 2008).

We selected three FD indices that assess three complementary facets of FD (Mouillot et al. 2013) that can be calculated even with less than 3 species, namely functional entropy (FEnt), functional specialization (FSpe), and functional originality (FOri). More precisely, FEnt was calculated such that 1/(1-Q), where Q is Rao's quadratic entropy computed as the biomass-weighted sum of pairwise functional distance among species within the community (Ricotta and Szeidl, 2009). FEnt increases when species with the highest biomass are functionally distinct (Mouillot et al., 2013). FSpe was calculated as the biomass-weighted mean distance in the functional space to the average value of all the species present at the regional scale (Bellwood et al. 2006). It reaches high values when the largest biomass belongs to species with extreme traits values. Finally, we calculated FOri as the biomass-weighted mean distance to the nearest species within the functional space (Mouillot et al., 2013). It increases when species with unique trait combinations have the largest biomass in the community assembly.

2.5. Statistical analyses

All analyses were computed in R (R Development Core Team, 2013) using the libraries stats, MASS, lme4 (Bates et al. 2016), psych (Revelle 2016), and hier.part (Walsh and Mac Nally 2015). As reported in our previous study (e.g. Maceda-Veiga et al. 2014), Spearman rho coefficient was used to remove redundant variables (r > 0.7) from the combined set of 24 environmental variables. A principal component analysis (PCA) was then applied to summarise variation in the remnant 11 variables. The 'varimax' rotation was used to facilitate the interpretation of axes, and the resultant stressor gradients related to anthropogenic impact were included in models as predictors. Continuous variables were log-transformed and percentages were arc-sine square -root transformed to improve linearity.

Generalised linear mixed models (GLMMs) were used to examine relationships among diversity measures (Total richness, FEnt, FSpe, and FOri), natural features, and the gradients of anthropogenic impact. Basin was included as random term to control for potential autocorrelation due to biogeographic factors at the basin level. Altitude was also included in the models to account for the role of natural geographical gradients in the analysis. As anthropogenic stressors, we additionally included in models the ratio between non-native fish and total fish biomass to explore the relative contribution of non-native fish to the variation in the functional diversity of fish assemblages. Prior to the GLMM analyses, FSpe and FOri were standardised to values between zero (minimum) and one (maximum). As 1<FEnt<2 we subtracted one from FEnt values to express the index as a proportion. Binomial error distributions were used for proportional data (FSpe, FOri, and FEnt), and Poisson errors were used for taxon richness. Models were validated via visually inspecting diagnostic plots of residuals.

To further test the robustness of our results, we used a hierarchical partitioning (HP) analysis ('hier.part' function in R) using the error distributions validated in the GLMM approach. HP models deal with collinearity among predictors (e.g. between altitude and habitat degradation, see Murphy et al., 2013), which even in small amounts can bias regression parameters (Freckleton, 2011). Whilst causality cannot be determined in observational studies, HP decomposes the variation of dependent variables in unique and joined fractions of a set of predictors (Walsh and Mac Nally, 2011). We assessed the significance of HP models using a randomization test for hierarchical partitioning analysis (function 'rand.hp'). As many regressors can generate rounding errors in HP models, we validated their outputs by changing the order of predictors, as recommended by Walsh and Mac Nally (2011).

To assess the potential improvement provided by the incorporation of FD indices in environmental monitoring, we used an independent data-set (see above). Spearman rank correlations were used to examine relationships among FD indices, traditional IBIs and general indicators of river status, such as total nutrient concentration, conductivity, the habitat quality index RBA, and the predominance of non-native species in the fish assemblage in richness and biomass. Significance in HP analysis was based on the upper 0.95 confidence interval and at P<0.05 in the remaining statistical procedures.

Table 2. Loadings for axes 1 and 2 according to PCA built with water physico-chemical variables and habitat quality features measured in rivers from north-eastern Spain. Bold values are considered high ≥ 0.4 (Maceda-Veiga et al.

Environmental Variables	PC1	PC2
Habitat structure	-0.24	0.67
Riparian coverage	-0.09	0.79
Channel conservation	-0.05	0.80
pH	0.03	0.02
Temperature	0.27	-0.28
Ammonium	0.80	-0.08
Nitrite	0.79	-0.13
Nitrate	0.76	-0.09
Phosphates	0.49	-0.23
Conductivity	0.43	-0.31
Macrophytes	-0.06	0.04

3. Results

3.1. Taxonomic and functional diversity of fish assemblages

A total of 29 fish taxa were recorded (for a full list see Annex) belonging to 9 orders and 12 families, including 15 species of *Cyprinidae*. Fifteen taxa were native and fourteen non-natives. Maximum species richness in a site was of 15 (seven native and eight non-natives, Fig 3). Across the study area indices of functional specialisation (FSpe) and functional originality (FOri) reached their maximum value (1), whereas the functional entropy index (FEnt) ranged from 1 to 1.8 (Fig 4). Taxon richness was significantly correlated to all FD indices although FEnt (r=0.54; P<0.01), and to lower extent with FSpe (r=0.24; P<0.01) and FOri (r=0.26; P<0.01).

3.2. Anthropogenic stressor gradients

Two first axes of PCA computed with water quality and physical habitat properties (PC1 and PC2), explained 42.88 % of variance (Table 2). PC1 accounted for 29.6% of the variance and was mainly driven by indicators of water quality (ammonium, nitrite, nitrate, and phosphates). PC2 explained 13.8% of variance and was mainly related to hydromorphological alterations (riparian coverage, habitat structure, and channel morphology). Other PC gradients produced by the PCA (PC3 and PC4) were not used as anthropogenic stressors given the variables selected (e.g. temperature and macrophytes), even though both explained a reasonable amount of variance (11.18% and 10.84%, respectively).

3.3. Relative contribution of natural and anthropogenic stressors to diversity measures

Since human activities tend to concentrate

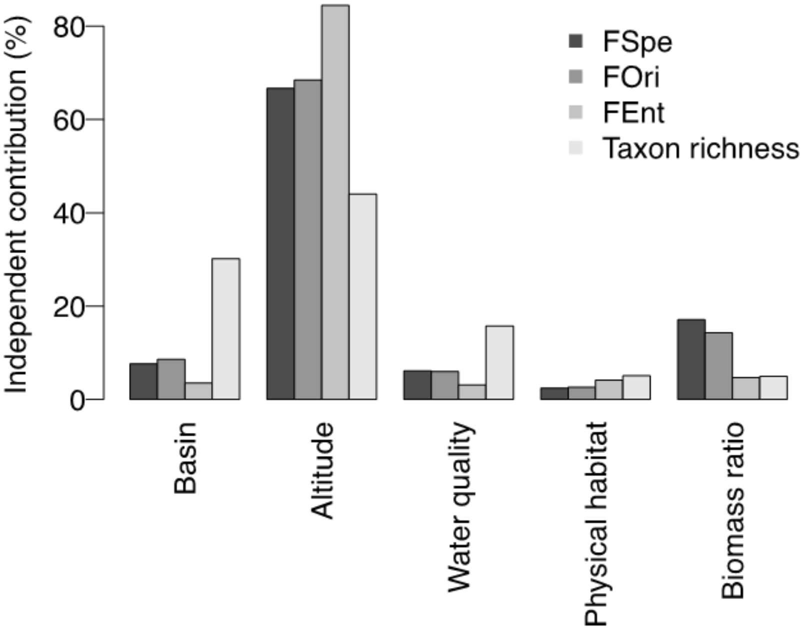

Figure 2. Independent contribution (%) of the five predictors used in the generalised linear models to the explained variation of the hierarchical partitioning models performed on functional diversity measures (FSpe, FOri, FEnt) and total fish species richness. All predictors were significant at the 95% confidence interval based on a randomized permutation test (rand.hp function).

in lowlands, it was necessary to disentangle the relative effect of altitude and anthropogenic impacts (PC1, PC2 and non-native species biomass ratio) on fish diversity. Hierarchical partitioning (HP) models revealed that altitude made the largest independent contribution to the variation in all four fish diversity measures (Fig 2). All three functional diversity (FD) indices followed the trend observed for taxon richness with altitude (Figs 3 and 4). In upstrem sites above than 1000 m, FD indices and richness values were very low. As altitude declined below 500 m FD indices increased rapidly. However, species richness peaked at around 500-600 m, with a less gradual trend (Fig 3). The number of non-native taxa reduced with increasing altitude (Fig 4), with only rainbow trout (*Oncorhynchus mykiss*) found above than 600 m.

Anthropogenic stressors also explained a significant unique fraction of variation in functional diversity indices (Fig 2). However, changes in taxon richness were mainly attributed to natural factors, namely altitude and basin (Fig 2). These results were mostly concordant with the GLMM approach, which showed that basin played a minor role in explaining all three functional diversity indices. All had random effect variances for basin very close to zero, whereas it increased for taxon richness (Table 4). However, the two modeling techniques ranked the contribution of altitude and anthropogenic stressors to variation in functional diversity measures in a different order (Fig 2, Table 3). FSpe and FOri responded to variations in altitude and non-native biomass ratio in the same way, but FOri was not related to PC2 (Table 3). PC1 was not retained as a major an-

Table 3. Generalized linear mixed model results for fixed effects. P values <0.05 in bold.

	Estimate	**SE**	*z*	***p***
Taxon richness				
(Intercept)	2.540	0.234	10.876	**1.50E-27**
log(altitude + 1)	-0.294	0.038	-7.829	**4.94E-15**
Physical habitat	0.733	0.218	3.361	**0.0008**
Water quality	-0.184	0.131	-1.407	0.1594
Non-native biomass ratio	0.148	0.102	1.446	0.1481
log(altitude + 1)*Physical habitat	-0.128	0.036	-3.565	**0.0004**
log(altitude + 1)*Physico-chemical	0.022	0.027	0.817	0.4141
FSpe				
(Intercept)	6.640	1.432	4.636	**3.55E-06**
log(altitude + 1)	-1.742	0.282	-6.177	**6.52E-10**
Physical habitat	3.691	1.443	2.559	**0.0105**
Water quality	-0.692	0.719	-0.962	0.3362
Non-native biomass ratio	3.072	0.546	5.625	**1.86E-08**
log(altitude + 1)*Physical habitat	-0.700	0.278	-2.518	**0.0118**
log(altitude + 1)*Physico-chemical	0.164	0.160	1.026	0.3048
FOri				
(Intercept)	9.147	1.840	4.971	**6.65E-07**
log(altitude + 1)	-2.189	0.364	-6.017	**1.78E-09**
Physical habitat	1.970	1.681	1.172	0.2413
Water quality	-0.720	0.723	-0.996	0.3190
Non-native biomass ratio	-3.423	1.279	-2.677	**0.0074**
log(altitude + 1)*Physical habitat	-0.400	0.341	-1.175	0.2399
log(altitude + 1)*Physico-chemical	0.145	0.176	0.826	0.4088
FEnt				
(Intercept)	4.529	1.472	3.077	**0.002**
log(altitude + 1)	-1.478	0.298	-4.958	**7.11E-07**
Physical habitat	5.808	2.111	2.752	**0.006**
Water quality	-0.991	0.987	-1.005	0.315
Non-native biomass ratio	0.343	0.973	0.353	0.724
log(altitude + 1)*Physical habitat	-0.981	0.406	-2.416	**0.016**
log(altitude + 1)*Physico-chemical	0.186	0.227	0.820	0.412

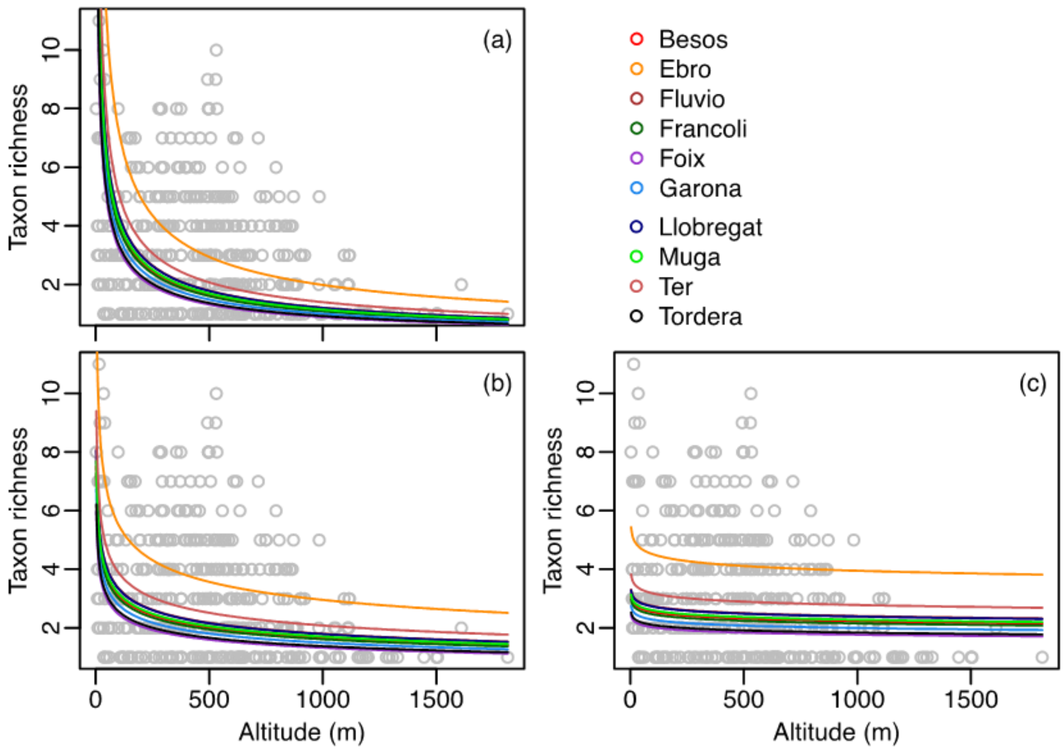

Figure 3. Relationship (mean fitted values from GLMM) between taxon richness and altitude (with basin as random effect) at three levels of physical habitat quality (PC2): PC2 = maximum (a); PC2 = median (b); and PC2 = minimum (c).

thropogenic stressor for any of the four fish diversity measures (Table 3, Fig 2).

Interactive effects among altitude and anthropogenic stressors revealed that altitude modulated the effect of habitat degradation on fish diversity. As habitat quality reduced, from the maximum (Fig 3a), to the median (Fig 3b) and the minimum (Fig 3c) values on PC2, the tendency for taxon richness to decline with altitude was attenuated. At low altitudes (<300 m) FEnt was high in sites with well-preserved habitats but where habitat alterations were extreme, the index took minimal values regardless of altitude. For FSpe decreased with increasing altitude and increased with increasing non-native species biomass (Fig 4). In contrast to FSpe, FOri reduced with increasing biomass of non-native species (Fig 4d).

Table 4. Generalized linear mixed model results for random effect.

(Basin)	Variance	SD
Taxon richness	0.0736	0.2712
FSpe	2.51E-10	1.58E-05
FOri	3.74E-08	1.93E-04
FEnt	2.54E-10	1.59E-05

3.4. Correlations among water and habitat quality indicators, fish diversity measures and indices of biotic quality

At least one indicator of environmental degradation was significantly correlated, either with a functional diversity measure or an index of biotic quality (Table 5). In particular, diatom (IPS) and macroinvertebrate-based (IBMWP) indices had a highly negative relationship with conductivity and total nutrient content. Conversely, total nutrient content was positively related to FSpe, FOri, and the alternative fish metric TIV for water quality (Table 5). A positive relationship was also found for the habitat quality index (RBA) with the IPS index and the TIV metric for habitat quality. In contrast, the RBA index was negatively correlated with FSpe and FOri (Table 5). All water and habitat indicators were poorly related to total fish richness, the fish index IBICAT, and the functional index FEnt (Table 5).

The strongest correlations were found among all three functional diversity indices (FSpe, FOri, and FEnt) and non-native fish measures (Table 5). The latter were also positively associated with total native richness but to a lesser extent. A weak but negative relationship was also observed for the IBICAT index and non-native fish measures (Table 5). In contrast, a strong negative relationship was found among non-native fish measures, the IPS and IBMWP indices, and the fish metrics TIV (Table 5).

4. Discussion

Our study shows the superior alternative of three functional diversity indices (specialisation, FSpe; originality, FOri; and redundancy; FEnt) to fish richness and a fish Index of Biotic Quality widely used in NE Spain to determine river's ecological status. As well as detecting impairment, our results illustrate the potential of functional diversity measures to identify the mechanisms behind the changes observed in a fish assemblage (e.g. loss of generalists). However, this study also highlights that non-native fish and environmental degradation can both affect functional diversity, making the establishment of cause-effect relationships difficult. Although non-native fish can be numerically dominant in NE Spain (>50%), they seem not to have buffered the response of fish assemblages to environmental degradation.

4.1. Relative contribution of natural and anthropogenic stressors to diversity measures

Altitude was found to make the largest independent contribution to total fish richness and functional diversity indices. The most plausible explanation for this result is that surface area increases downstream and hence the resources available (Angermeier and Schlosser, 1989; Lomolino, 2000). However, in lowland, non-invaded sites, we found a marked increase in FOri and FSpe from 40% to 100% of originality and specialisation. While suggesting that the ecological role of species diversifies downstream, these results indicated that some traits become dominant, as reported by Karadimou et al., 2016. In our study, particular traits (e.g. amphidromous reproductive strategy, omnivorous diet, slow water velocity preference) were more common in downstream (e.g. *Anguilla anguilla*) than in upstream fish species (e.g. *Barbus meridionalis*) (de Sostoa et al., 1990). Unique trait combinations also dominated in biomass in invaded, low land sites. Like elsewhere in the Mediterranean area (Marr et al., 2010), non-native fish in NE Spain are large predators (e.g. *Silurus glanis*, up to Kg) with life-histories typical of species from permanent, low water velocities (de Sostoa et al., 1999; Ribeiro et al., 2008). By contrast, native species evolved in fast waters with

Table 5. Spearman rank correlation coefficients between functional diversity measures (FSpe and FOri), habitat quality indices (RBA), proportion of exotic species in biomass and richness, the indices of biotic integrity using fish (IBICAT, TIV_RBA, TIV_WATER), diatoms (IPS) and macroinvertebrates (IBMPW) as bioindicators and tolerance indicator values of fish communities to water and habitat deterioration used in this region (see methods).

	Functional diversity measures and IBIs								
Environmental stressors	FSpe	FOri	FEnt	Richness	IBICAT	IBMWP	IPS	TIV_RBA	TIV_WATER
Conductivity	0.197	0.124	-0.060	-0.059	-0.104	-0.481***	-0.396**	-0.498**	0.306
Total Nitrogen	0.436**	0.427**	0.160	-0.001	0.112	-0.576***	-0.684***	-0.398*	0.439*
RBA	-0.469***	-0.454**	-0.060	-0.072	-0.017	0.357	0.466***	0.401*	-0.531**
Non-native biomass	0.609***	0.666***	0.445**	0.332*	-0.282*	-0.440**	-0.592***	-0.804***	0.900**
Non-native ratio	0.525***	0.575***	0.325*	0.305*	-0.297*	-0.584***	-0.688***	-0.762***	0.793***

Note: Significance codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0

marked seasonality, and tend to have wider trophic spectra and multiple spawning events (e.g. Herrera et al., 1992; de Sostoa et al., 1999; Vinyoles et al., 2010).

In our study, the relative contribution of non-native fish and physical habitat quality to all three diversity measures was index and model-specific. Incongruence between traditional linear models (LMs) and hierarchical partitioning (HP) analyses are likely to be attributed to collinearity among predictors (Walsh and Mac Nally, 2011). Non-native fish tend to concentrate in heavily altered river reaches (e.g. embankments, weirds) (Marchetti et al., 2004; Maceda-Veiga et al., 2017), which limits the ability of traditional statistical methods to disentangle the weight of these two factors. This can explain why LMs and HPs ranked predictors in a different order, and highlights the power of variation partitioning analyses to elucidate the causes behind the observed patterns (see also Buisson et al., 2008; Murphy et al., 2013). The perceived relationship affects management; the removal of non-native species requires of different actions than restoring habitat quality. Nonetheless, habitat improvements might solve both issues, as rivers with natural flow regimes are often the least invaded (Poff et al., 1997; Marchetti and Moyle, 2001; Bernardo et al., 2003; but see Propst et al. 2008).

The functional indices FSpe and FOri were found in our study to perform better in detecting anthropogenic impacts than total fish richness and FEnt, which were mostly driven by natural factors. Geographical features including basin are major shaping forces of the taxonomic composition of communities (Richards et al., 1996; Williams et al., 2003; Maceda-Veiga et al., 2017). Although FSpe and FOri indices were found in our study to offset this major drawback of taxonomic-based approaches, ecology cannot be disconnected from taxonomy. A good knowledge of species is required to assign traits properly (e.g. Sánchez-Hernández et al., 2011; Rodríguez-Lozano et al., 2016). In our study, FSpe and FOri indices also showed a differential response to anthropogenic impacts, supporting the notion that multiple indices provide complementary information in studies of functional diversity (Mouillot et al., 2013). In particular, we found that FOri reduced when non-native fish dominated in biomass, whereas FSpe increased in these conditions and also depended on physical habitat quality.

As FOri decreases in assemblages with a less original combination of traits (see Mouillot et al., 2013), our results are compatible with a high functional redundancy in invaded sites. The decrease in FOri was simultaneously driven by an increase in FSpe, indicating an increase in species with extreme trait combinations (Mouillot et al., 2013). However, we caution for the use of functional redundancy as a desirable community property (see Rosenfeld, 2002; Joner et al. 2011; Bruno et al., 2016). As pointed out by Brandl et al. (2016), a limited resolution of trait-based approaches may mask the loss of functionally unique (native) species (i.e. increasing FOri) with potential ramifications for ecosystem functioning, in addition to the conservation concern of species loss. This problem is likely to be acute in naturally species-poor and heavily invaded systems such as in fish assemblages from Mediterranean rivers. In these systems one or two native fish species (see Maceda-Veiga et al., 2010) often top-down control the aquatic community, and so all are likely to be functionally unique. Therefore, the door is opened to consider functional redundancy just an artefact of our coarse approximation of organisms' ecological role (Loreau, 2004). Likewise, the presence of non-native fish species may not be necessarily bad, as mammals and waterbirds can feed on non-native fish (Maceda-Veiga et al., 2017).

4.2. Comparison of the diagnostic power of fish diversity measures and indices of biotic quality

Riverine taxa integrate the combined effects of stressors over variable spatio-temporal scales based on their different life-history (Barbour et al., 1999). Thus, biotic indices may not correlate with fluctuating variables such as water chemistry. However, these associations can inform about their diagnostic ability, especially in chronically polluted systems such as Mediterranean rivers. In our study, the diatom and invertebrate-based indices had the strongest correlations with water quality, especially with total nutrient load. These results support that these indices were originally developed to detect organic pollution, even though they are now used to assess many pollution sources (e.g. Munné and Prat, 2009; Juttner et al., 2012; Colin et al. 2016). Interestingly, a strong negative relationship was also found in our study between these two indices and non-native fish measures. This relationship can be attributed to the fact that non-native species tend to occur in degraded sites, but also that they can be the direct cause of degradation (e.g. Shin-Ichiro et al., 2009). Nonetheless, we still have limited insight into how biological invasions affect current biomonitoring schemes.

The confounding effect of non-native fish in biomonitoring tools was acute for fish-based measures, particularly for IBICAT. It did not significantly respond to water and habitat variables, and was markedly affected by non-native fish occurrence (see also Benejam et al. 2009). In our study, fish richness was also poorly related to environmental degradation and mostly driven by non-native fish measures. Conversely, FSpe and FOri responded to both, even though signs of their relationships with abiotic and biotic stressors often differed from our large-scale data-set. This highlights the assemblage-dependence of functional analyses upon the functional space occupied by each fauna (Mouillot et al. 2011). Finally, our study also showed that invaded sites were dominated by relatively tolerant species to poor water and habitat quality according to TIV scores developed by Maceda-Veiga and de Sostoa (2011). These results support the prevailing assumption in literature that non-native fish are highly tolerant species (Ribeiro et al. 2008; Hermoso et al. 2011; but see Kennard et al. 2005; Maceda-Veiga and de Sostoa, 2011), but can also suggest that native species living there have wider tolerance ranges.

5. Conclusions

Our study provides evidence of the suitability of FSpe and FOri indices as new diagnostic tools in species-poor systems. They were responsive to physical habitat and biotic degradation, even though the direction of the change was found to be index specific. Changes in water quality play a limited role in variation in all three

FD facets, and taxonomic richness was only mostly driven by geographical features. The comparison of FD facets with traditional indicators of ecosystem health supports that fish introductions play a major role in the structure of fish assemblages in these rivers. Further, it showed that FD indices perform better than a fish index developed in this region to assess environmental degradation, as driven by the EU's Water Framework Directive. In conclusion, the use of FD measures in fish will help us to better understand and predict the impact of anthropogenic disturbances in Mediterranean rivers.

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CAPÍTULO 3

Relevancia ecológica de los biomarcadores en los estudios de monitoreo de macroinvertebrados y peces en ríos mediterráneos

Resumen

Los ríos mediterráneos son probablemente uno de los ecosistemas más singulares y amenazados del mundo debido a su elevada endemicidad y a una larga historia de impactos antrópicos. La conservación de la biodiversidad tiene una importancia ecológica *per se*. Además, también garantiza la conservación de los beneficios que los ecosistemas acuáticos proporcionan a la sociedad y determina la capacidad de estos ecosistemas para resistir la presión antrópica, incluidos los efectos del cambio climático. Centrándonos en estudios de macroinvertebrados y peces, este trabajo de síntesis revisa los usos dados hasta la fecha a los biomarcadores (respuestas estresantes y enzimáticas, disruptores endocrinos, trazadores tróficos, metabolitos energéticos y biliares, indicadores genotóxicos, alteraciones histopatológicas y comportamentales, análisis genéticos y sofisticadas técnicas omicas) para determinar los efectos de la actividad humana sobre la biodiversidad de los ríos mediterráneos europeos. Además, discutimos cómo la relevancia ecológica de los estudios de biomarcadores podría incrementar a través de una selección más cuidadosa de las especies centinelas, en función de sus características ecológicas y, en especial, de su posición en las redes tróficas de los ríos mediterráneos. Este trabajo también ofrece algunas sugerencias que permitirían incrementar el realismo ecológico de los experimentos, además de analizar los datos de manera que se obtengan resultados más rigurosos y fáciles de entender por los gestores. Nosotros abogamos por analizar el estado de salud de múltiples especies clave para el funcionamiento del ecosistema, y de esta manera disminuir la probabilidad que efectos sub-letales severos estén afectando al ecosistema. En resumidas cuentas, este trabajo de síntesis resalta que los estudios de biomarcadores detectan efectos sub-letales en los individuos que pueden dar lugar a efectos severos a mayores niveles de complejidad biológica. Por consiguiente, la Directiva Marco del Agua Europea debiera incorporar los biomarcadores como herramientas oficiales en el diagnóstico del estado de salud de los ríos europeos.

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Ecological relevance of biomarkers in monitoring studies of macro-invertebrates and fish in Mediterranean rivers

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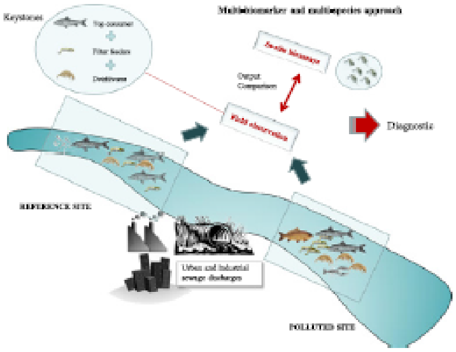
HIGHLIGHTS

- Biomarkers can assess the effects of multiple stressors on Mediterranean rivers.
- Biomarker studies need to increase the ecological realism.
- Multi-species and multi-biomarkers can help predict ecosystem impairments.
- A proposal to select biomarkers in Mediterranean rivers is outlined.
- Biomarkers should be incorporated to the EU's Water Framework Directive.

GRAPHICAL ABSTRACT



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ABSTRACT

Mediterranean rivers are probably one of the most singular and endangered ecosystems worldwide due to the presence of many endemic species and a long history of anthropogenic impacts. Besides a conservation value per se, biodiversity is related to the services that ecosystems provide to society and the ability of these to cope with stressors, including climate change. Using macro-invertebrates and fish as sentinel organisms, this overview presents a synthesis of the state of the art in the application of biomarkers (stress and enzymatic responses, endocrine disruptors, trophic tracers, energy and bile metabolites, genotoxic indicators, histopathological and behavioural alterations, and genetic and cutting edge omic markers) to determine the causes and effects of anthropogenic stressors on the biodiversity of European Mediterranean rivers. We also discuss how a careful selection of sentinel species according to their ecological traits and the food-web structure of Mediterranean rivers could increase the ecological relevance of biomarker responses. Further, we provide suggestions to better harmonise ecological realism with experimental design in biomarker studies, including statistical analyses, which may also deliver a more comprehensible message to managers and policy makers. By keeping on the safe side the health status of populations of multiple-species in a community, we advocate to increase the resilience of fluvial ecosystems to face present and forecasted stressors. In conclusion, this review provides evidence that multi-

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biomarker approaches detect early signs of impairment in populations, and supports their incorporation in the standardised procedures of the Water Frame Work Directive to better appraise the status of European water bodies.

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1. Introduction

Mediterranean rivers are one of the most vulnerable ecosystems worldwide due to a long-history of anthropogenic impacts, including habitat degradation and species introductions (Bonada and Resh, 2013). The conservation concern is particularly great in these systems because of their high percent of endemic species (Maceda-Veiga, 2013; Quiñones and Moyle, 2015), and the ramifications that biodiversity loss can have for ecosystem function and support services (Hooper et al., 2005). Water pollution is still one of the major threats to Mediterranean rivers despite a marked investment in sewage treatment plants in recent decades (Prat and Munné, 2000; Sabater et al., 2009). This is attributed, in part, to the reduced dilution ability of these rivers due to climate and water abstractions (Prat and Munné, 2000). Other pollution sources such as agricultural run-off and some illegal spills further increase the environmental concern of pollutants (López-Doval et al., 2013; Sabater et al., 2009). In some Mediterranean rivers, the risk posed by chemicals to the biota is particularly worrisome due to a long list of regulated and emerging pollutants, including pharmaceuticals and personal care products (Gorga et al., 2015; Von der Ohe et al., 2011). Since this risk may increase under forecasted global change scenarios (Petrovic et al., 2011; Vörösmarty et al., 2010), understanding how pollutants affect the biota of Mediterranean rivers is central to adapt policies and develop effective management strategies.

The use of sentinel species (bio-indicators) has been traditionally used in studies of bio-monitoring, including environmental risk assessment (Friberg et al., 2011). Whilst chemical surveys only identify a fraction of environmental pollutants, often those included in 'priority lists', the use of bio-indicators enables assessing the overall effects of measured and unmeasured pollutants interacting with natural and other anthropogenic stressors in the complexity of natural systems (Birk et al., 2012). Driven by the requirements of international legislation, such as the EU's Water Framework, different taxonomic groups (e.g. algae, macrophytes, macro-invertebrates and fish) are used to determine the ecological status of water bodies across member states (Birk et al., 2012; Friberg et al., 2011). By virtue of their different life-span bio-indicators track environmental variations at variable temporal scales, ranging from weeks (invertebrates) to years (fish) (Barbour et al., 1995; Muñoz et al., 2012). Also, since bio-indicators differ in ecological traits such trophic position, they can inform if pollutants have permeated upwards the food-webs (bio-accumulation and bio-magnification risks) (Monroy et al., 2014). Besides a direct interest for policy-making, these foregoing traits enable using bio-indicators as an umbrella for protecting other taxa (Ormerod et al., 2010); a feature particularly relevant in monitoring heavily human-disturbed but ecologically unique systems like Mediterranean rivers (Bonada and Resh, 2013).

Traditionally, bio-monitoring of running waters has been made with community-based measures, named multi-metric indices (MMIs), being macro-invertebrates and fish the dominant taxa (Friberg et al., 2011). Since first attempted by Karr (1981), many MMIs have been developed worldwide with more than 300 available in Europe (Birk et al., 2012). These procedures based their diagnostics in comparing community features (metrics) between tested sites in relation to those predicted or observed in a set of reference sites (Dallas, 2013; Hawkins et al., 2010). Despite their relatively easy applicability, a major caveat is that MMIs do not identify causes of impairment (Muñoz et al., 2012), and even eclipse biotic and abiotic impacts such as habitat degradation and introduced species (Benejam et al., 2009; Maceda-Veiga et al., 2014). Also, MMIs typically fail to detect subtle effects at the individual level (Damásio et al., 2007; Muñoz et al., 2012), and then when detrimental effects are visible at the community level it can be too late to undertake conservation measures and to prevent local extinctions (Clements and Rohr, 2009). In species poor systems, such as the fish communities of Mediterranean rivers, the diagnostic ability of MMIs is particularly limited given the reduced set of metrics available (Aparicio et al., 2011; Figuerola et al., 2012). Thus, practitioners need more specific, sensitive and complementary diagnostic procedures, especially because Mediterranean rivers harbour markedly threatened fish (Quiñones and Moyle, 2015), arthropods (Pedraza-Lara et al., 2010) and molluscs species (Lopes-Lima et al., 2014).

Biomarkers, defined here as any measurable molecular, cellular, histological, physiological or behavioural response (Depledge, 1993; Fig. 1), have been adopted as 'early-warning signals' of individuals' imbalance (Galloway, 2006; Wu et al., 2005). Often on a stressor-specific basis, biomarkers have improved our mechanistic understanding of how multiple stressors affect aquatic biota (e.g. Faria et al., 2010a; Kidd et al., 2007). However, the ecological relevance of biomarkers – linkage between responses from (sub-) individual to population and ecosystem levels – is often unclear (Friberg et al., 2011). This may explain why the use of biomarkers is still not extended in bio-monitoring schemes (Amiard-Triquet et al., 2012). This critical review aims to increase the ecological relevance of biomarker approaches in European Mediterranean rivers via three aspects: selection of bio-indicator species of macro-invertebrates and fish according to their ecological traits and the food-web structure of Mediterranean rivers, selection of biomarkers according to its specificity and ecological relevance, and selection of appropriate experimental designs and data treatments to increase the diagnostic ability and ecological relevance of biomarker outputs. Finally, we outline future research lines that may contribute to a more systematic adoption of biomarker approaches in the bio-monitoring schemes of European Mediterranean rivers.

2. Macro-invertebrates and fish as bio-indicator species

Several traits justify the use of macro-invertebrates and fish as sentinels in bio-monitoring, including their relatively well-known taxonomy, functional role and the goods they provide to society (e.g. fisheries) (Friberg et al., 2011). However, this perspective is not exempt of a debate on whether ecosystems or particular taxa should be the target in bio-monitoring and conservation (e.g. Friberg et al., 2011; Lindenmayer et al., 2007). Despite criticisms and caveats, it is well-established that the loss of keystone species results in multi-trophic impacts influencing ecosystem processes (Rodríguez-Lozano et al., 2015; Rohr et al., 2006). However, we are still far from fully understanding the biodiversity-ecosystem functioning relationship and predict the consequences of changes in species assemblies (Ings et al., 2009). Thus, the precautionary principle recommends species conservation regardless of its functional role. This is essential nowadays given that species loss occurs at alarming rates (Dudgeon, 2013), and recent evidence suggests that the biodiversity level required to sustain freshwater ecosystem functions may have been underestimated in a changing environment (Perkins et al., 2015). Clearly, assessing and restoring whole-ecosystem processes can provide multiple benefits, including species conservation (Lindenmayer et al., 2007). However, policy-makers also need to show society visible pieces of ecosystem health, and by virtue of their charismatic nature macro-invertebrates and fish meet this requirement.

Albeit challenging, in-deep knowledge of the community structure and species ecology can help select *the best* set of bio-indicators and hence increase the ecological relevance of bio-monitoring outputs

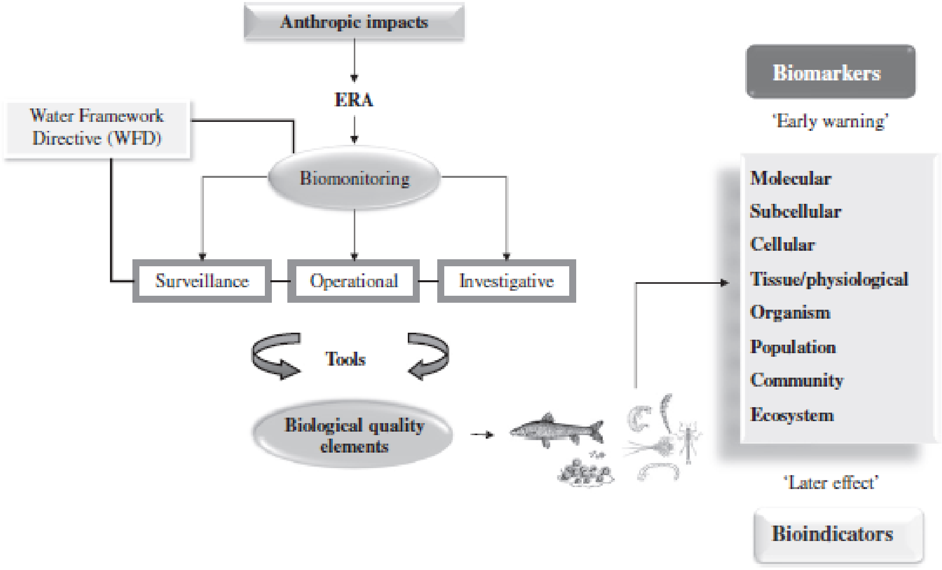

Fig. 1. Applications of biomarker responses within the scheme of the EU's Water Framework Directive for the assessment of the status of water bodies across member states using biological quality elements (bio-indicators).

(Clements and Rohr, 2009; Ebenman and Jonsson, 2005). For instance, one fish species top-down control aquatic communities in small Mediterranean streams (e.g. Figuerola et al., 2012), and experimentally induced-changes in these food-webs suggest that fish extirpation can markedly alter ecosystem function (Rodríguez-Lozano et al., 2015). Also, in Mediterranean rivers, mussels and crayfish can be locally abundant, and as ecosystem engineers strongly alter nutrient-cycling and the physical habitat structure (Gherardi, 2007; Sousa et al., 2009). Other invertebrates involved in detritivorous pathways, such as gammarids and larvae of certain insects, can also markedly influence the processing of dead organic matter in rivers (Wallace and Webster, 1996). Thus, any stressor that compromises the population viability of any of these taxa is more prone to potentially affect the whole ecosystem (Amiard-Triquet et al., 2012). Such effects at ecosystem level may magnify when 'tightly' related keystones are affected, such as the native fish and mussels' larvae (Order Unionida) host-parasite relationship (Lopes-Lima et al., 2014). However, differentiating health disorder from compensatory responses is a major challenge in studies at cellular and biochemical level. Nonetheless, as for human medicine, the use of a set of biomarkers with a different sensitivity and specificity can help diagnose the causes of ecological impairment (see biomarker section).

Besides the use of ecological traits, data on the relative biomass (more ecologically relevant than abundance) and distributional range of a species can be used to further support its ecological relevance in a study area. Since exotic species are widespread in European Mediterranean rivers (e.g. Hermoso and Clavero, 2011; Maceda-Veiga et al., 2010; Sousa et al., 2009), the question is whether they can be used as bio-indicators (Dallas, 2013). One may argue that the 'wide tolerance' of exotic species is advantageous because they can be used as sentinels in subtle and acute pollution events, including sites where natives are extirpated (Dallas, 2013). However, all species like living in good conditions and examples of relatively sensitive or tolerant species are found amongst native and introduced species (Cairns, 1986; Maceda-Veiga et al., 2014). In Mediterranean rivers many ecotoxicological studies conducted in freshwater species have focused on relatively tolerant exotic species, such as the common carp (*Cyprinus carpio*), the red-swamp crayfish (*Procambarus clarkii*) and the zebra mussel (*Dreissena polymorpha*) (Fig. 2 and Supplementary material 2). This can be explained because exotic species are generally of no conservation value, although may have underestimated the effects of pollution on natives. A fruitful research area but poorly explored is to compare the relative tolerance of native and/or exotic species to stressors using biomarkers (e.g. Faria et al., 2010a,b; Lavado et al., 2004; Maceda-Veiga et al., 2013). Besides refining current tolerance classifications (e.g. Bonada et al., 2004; Maceda-Veiga and de Sostoa, 2011), these studies could help identify species according to their vulnerability to stressors and/or invasive traits (Gallagher et al., 2015; Lennox et al., 2015). For example, Faria et al. (2010b) found that transplanted native naiads were less vulnerable to mercury pollution than two exotic freshwater bivalve species (*D. polymorpha* and *Corbicula fluminea*).

3. Biomarker types: target system, sensitivity and ecological relevance

The need for rapid and sensitive tools to reveal sub-lethal effects in aquatic organisms has led to a long list of biomarkers. In European Mediterranean rivers, biomarker studies are mostly based on lethal sampling procedures using the liver in fish and the whole body in macro-invertebrates as targets (Fig. 3). According to Hopkin (1993), an ideal biomarker measure should meet the '5 Rs' rule, namely: relevance, reliability, robustness, responsiveness, and reproducibility. However, most biomarkers show a negative relationship between ecological relevance, and sensitivity and predictability (Hopkin, 1993). Also, the number of biomarkers used is often limited by logistic and financial constraints. In an attempt to facilitate this selection, the sections below give an overview of the most relevant biomarkers for macro-invertebrates and fish of European Mediterranean rivers based our experience and classified according to the target system involved and their sensitivity and ecological relevance.

3.1. Stress responses

Exposure to challenging conditions triggers a cascade of metabolic alterations in individuals induced by the release of stress hormones into body fluids (Romero, 2004). Such cascading response allows individuals to cope with stressors and recover homeostasis at the expense of temporary suppressing energy costly activities (e.g. reproduction) (Romero, 2004). However, when this ability is exceeded, stress reduces seriously individuals' fitness and even survival (Romero, 2004). By far the most adopted stress biomarker in fish is cortisol (Wikelski a Cooke, 2006), whereas hypoglycaemic and catecholamine hormones are the choice for crustaceans and bivalves, respectively (Lacoste et al., 2001). However, collecting baseline levels of these hormones in wildlife is extremely difficult because they peak shortly after handling. This has

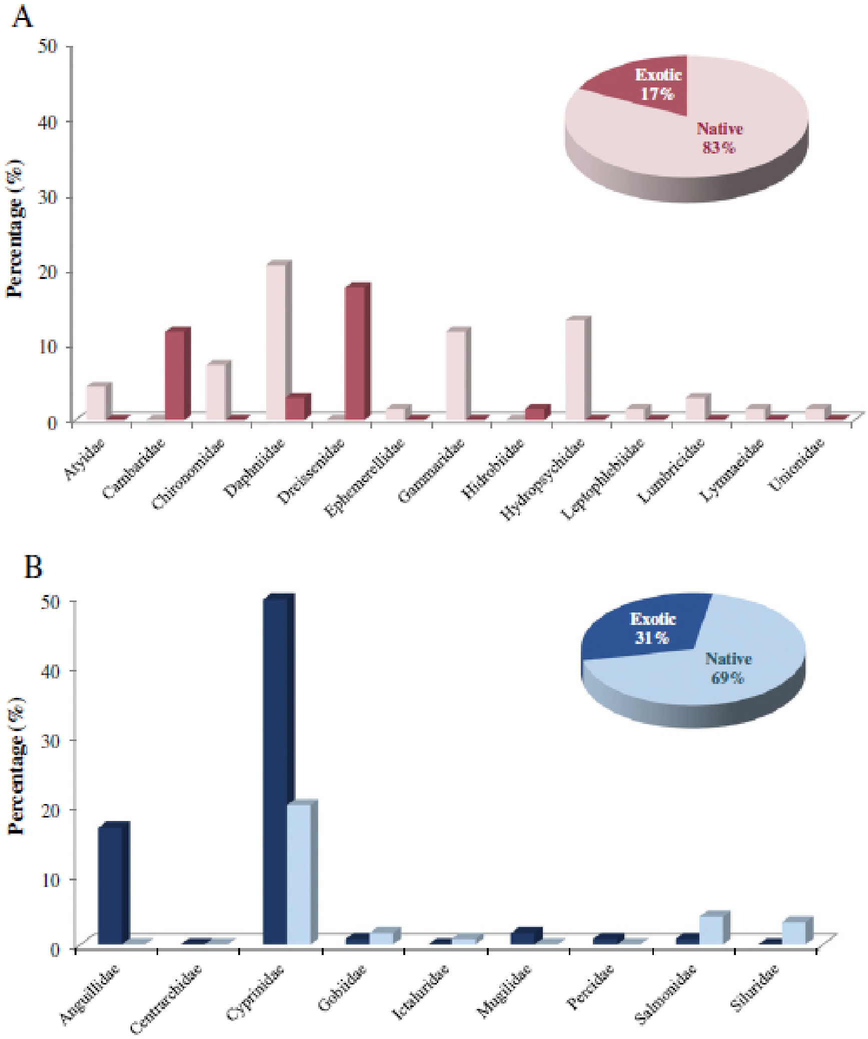

Fig. 2. Percentage of families of macro-invertebrates (A) and fish (B) of European Mediterranean rivers in which biomarkers were used. Note that Dreissenidae and Cyprinidae families dominate biomarker studies in macro-invertebrates and fish, respectively.

led to the adoption of the ratio heterophil (or neutrophil): lymphocytes as an indirect stress measure in wild vertebrates (see Davis et al., 2008). Whilst such ratio can mask general stress response and disease, the examination of the relative counts of all white cell types can aid in differentiating the two responses (Davis et al., 2008; Maceda-Veiga et al., 2015). Specifically, a stress response generally causes increased percent of immature erythrocytes and reduced total number of lymphocytes and eosinophils along with a peak in neutrophils (see Maceda-Veiga et al., 2015). A similar approach for macro-invertebrates is not available. Nonetheless, immunoassays including white blood cells proliferation and macrophage burst and phagocytic activity can be used either in macro-invertebrates (Galloway and Depledge, 2001) or fish (Zelikoff et al., 2000), and also help disentangle stress from immune responses.

The stress-fitness relationship is a pivotal topic in ecology but such relationship is still far from being clear (Fairhurst et al., 2013; Wikelski and Cooke, 2006). Fitness has been positively and negatively related to stress measures in several wild species but as far as we are aware, none of them have focused on wild free-living fish or macro-invertebrates of European Mediterranean rivers. A study in north-eastern Spain suggests that leukocyte profiles could be a suitable stress indicator for native cyprinids exposed to sewage discharges (Maceda-Veiga et al., 2013). However, it is unknown how long the stress response lasts and if it may affect individuals' fitness. Thus, the potential of stress measures as early signals of health disorders with high ecological relevance is still embryonic in macro-invertebrates and fish of this area. Besides linking stress measures in body fluids with other biomarkers of health imbalance, the use of less dynamic tissues (e.g. carapace, shell, fin, scales) should be explored as a more long-term integrative measure of stress in these taxa.

3.2. Enzymatic and oxidative stress responses

An impressive array of enzymes, metabolites and biotransformation pathways are involved in the different mechanisms of defence used by organisms to cope with chemical stress. Such mechanisms participate in the detoxification and removal of pollutants, but also in the neutralisation of metabolites or molecular species (e.g. reactive oxygen species, ROS) generated by their presence (Di Giulio et al., 1995). Since biochemical mechanisms are induced shortly after toxicant exposure, these responses can be considered as 'early signals' of disease (Van der Oost et al., 2003).

A sensitive but unspecific enzymatic probe to assess pollution effects is to measure the 7-Ethoxyresorufin O-deethylase (EROD) activity of the cytochrome CYP1A (Van der Oost et al., 2003). This plays a key role in the biotransformation of many pollutants, including polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins, petroleum products and drugs. In Spanish Mediterranean rivers, over 10-fold increased EROD activity was detected in common carps (*C. carpio*) exposed to effluents from sewage treatment plants (Fernandes et al., 2002; Lavado et al., 2006). These authors also reported that EROD

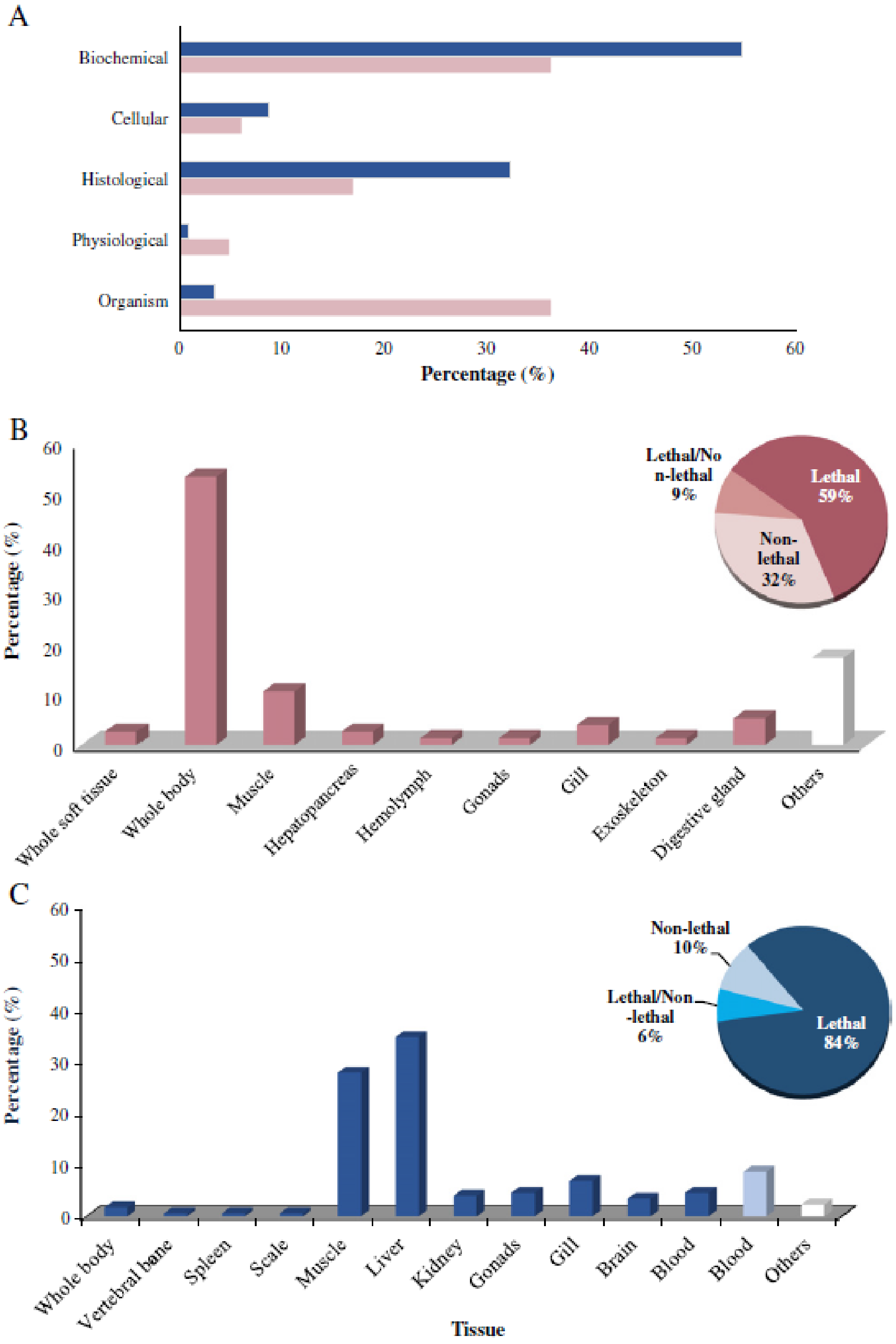

Fig. 3. Percentage of biomarker types used to assess the status of European Mediterranean rivers using macro-invertebrates and fish as bio-indicators (A). Note that the type of target tissues is also shown for macro-invertebrates (B) and fish (C).

activity was related to the levels of PAHs and organo-chlorinated compounds. Other primary defences against chemicals (e.g. metals, organo-chlorine compounds) and physical stressors (e.g. ultraviolet radiation) are anti-oxidant enzymes, proteins such as metallothioneins, metabolites such as glutathione (GSH), and phase II enzymes such as glutathione-S-transferase (GST). In particular, the enzymes superoxide dismutase (SOD; converts O_2 to H_2O_2), catalase (CAT; reduces H_2O_2 to H_2O), glutathione peroxidase (GPx; detoxifies H_2O_2 or organic hydro-peroxides produced by lipid peroxidation), glutathione reductase (GR, converts oxidised glutathione to its reduced form), glutathione S transferase (GST, is a phase II enzymes that conjugates pollutant metabolites and hydro-peroxides with glutathione) and levels of reduced glutathione that are used by cells to maintain their oxidative status are the biomarkers of choice to appraise oxidative stress (Di Giulio et al., 1995). Besides chemicals and radiations, factors such as oxygen partial pressure, temperature and salinity, and animal diet (e.g. carotenes, vitamins), also influence the animals' sensitivity to oxidative stress (Livingstone, 2003). Thus, any factor that disrupts the balance between antioxidant/pro-oxidant systems of organisms can lead to health disorders due to enzyme inactivation, protein degradation, DNA damage and lipid peroxidation (Livingstone, 2003; Peña-Llopis et al., 2003). This lack of specificity has been advantageous to better assess the status of aquatic communities in Mediterranean rivers exposed to multiple stressors (Barata et al., 2005; Damásio et al., 2010; Prat et al., 2013; Puértolas et al., 2010).

Other unspecific and sensitive enzymatic responses affected by pollutants are digestive enzymes (e.g. cellulase, glucosidase, and amilase) (Dedourge-Geffard et al., 2009; Palais et al., 2012). For example, changes in these enzymatic responses preceded mortality (<7 days later) in zebra mussels (*D. polymorpha*) or amphipods (*Gammarus fossarum*) exposed to metals in laboratory (Dedourge-Geffard et al., 2009) and field conditions (Palais et al., 2012). Also, these enzymatic activities were found to be affected before the target sentinel experienced growth and body condition alterations (Hyne and Maher, 2003). Together with general disorders in body systems, chemicals can cause major alterations in particular animals' functions via the impairment of specific enzymes. For instance, the normal behaviour and muscular function of animals depends on acetylcholinesterase (AChE) enzymes, which are inhibited by neurotoxic pollutants such as organophosphorus and carbamate pesticides, hydrocarbons and metals (Barata et al., 2004; Forget et al., 1999). Their inhibition result in a build-up of acetylcholine, which causes a prolonged over-stimulation of the nerve/muscle fibres and consequently, negative effects on locomotor activities (Almeida et al., 2010; Brewer et al., 2001). However, the use of this and other enzymatic activities as biomarkers needs to consider environmental (e.g. pH, temperature, water conductivity) and biotic factors (e.g. diet, life-history stage) that are well-known determinants of biochemical reactions.

In European Mediterranean rivers, there is growing evidence that macro-invertebrates and fish experience oxidative stress and neurotoxic effects, yet no study exists on digestive enzymes. Alterations of antioxidant enzymes (e.g. SOD, CAT, GST, GSH) plus increased levels of lipid peroxidation, often measured as thiobarbituric acid reactive equivalent species, were detected in zebra mussels (*D. polymorpha*) and red-swamp crayfish (*P. clarkii*) exposed to chlorine-alkali industry residues in the Ebro River (Faria et al., 2010a,b). Such signs of oxidative stress were also related to DNA damage and the bioaccumulation of organochlorides (e.g. PCBs, hexachlorobenzene (HCB), and dichlorodiphenyltrichloroethane (DDT)) and metals (e.g. mercury and cadmium) (Faria et al., 2010a,b). Also, in Mediterranean rivers nearby industrial, urban or agricultural areas, the larvae of the wild caddisfly *Hydropsyche exocellata* and transplanted cladocerans (*Daphnia magna*) had altered levels of the antioxidant enzymes CAT and GST compared to baseline sites (Barata et al., 2007; Damásio et al., 2008). Elsewhere in the Ebro River basin, flame retardants bioaccumulation (polybrominated diphenyl ethers) was associated with hepatic GPx and GST activity in the barbels *Luciobarbus graellsii* collected downstream an industrial area (Raldúa et al., 2008). These barbels also experienced reduced brain AChE. In the field, inhibition of AChE activity was also observed in individuals of *D. magna* exposed to pesticide residues, confirming the diagnostic value of AChE for detecting pesticide pollution (Barata et al., 2007; Damásio et al., 2008). As for other vertebrates, some enzymatic responses can unequivocally diagnose disorders in particular organs of fish (e.g. peak in transaminase enzymes is related to liver pathology) (Maceda-Veiga et al., 2015), but these enzymes have not yet been used in species of European Mediterranean rivers.

3.3. Endocrine disruption

A number of both natural and/or man-made chemicals with widespread distribution in European Mediterranean rivers may act as endocrine disruptors (ECs), affecting the growth, development and reproduction of aquatic wildlife. These compounds include natural and synthetic hormones (e.g. contraceptives) and many synthesised chemicals that reportedly act unintentionally as ECs (e.g. organochlorine pesticides, polychlorinated biphenyls (PCBs), organotin compounds, polycyclic aromatic hydrocarbons (PAHs), and alkyl-phenols) (Colborn et al., 1993). Typically, ECs interfere with oestrogen or androgen receptors and thus act as agonists or antagonists of endogenous hormones (Colborn et al., 1993). However, growing evidence indicates that ECs may also modulate the activity/expression of steroidogenic enzymes (Fernandes and Porte, 2013). Since some ECs exert biologic activity at low concentrations, the environmental concern may even be exacerbated in Mediterranean rivers by the increase in pollutant levels due to the low water flow.

Induction of vitellogenin (Vtg), the precursor molecule of yolk proteins, in juveniles or males of oviparous fish species is a well-established biomarker of the effect of xenoestrogenic contaminants (WHO/IPCS, 2002). Vtg synthesis, in female fish, is regulated by estradiol (E2) circulating levels in plasma. However, in males, usually this protein is not synthesised because of the very low, or even undetectable, plasmatic levels of E2. Nonetheless, when males are exposed to E2-like substances, they can synthesise Vtg to the same degree as females, and the diversion of vital proteins or lipids to Vtg formation in male fish can have negative effects. In two tributaries of the Llobregat River, elevated plasmatic levels of Vtg were detected in up to 50% of male carps (*C. carpio*) exposed to discharges of sewage treatment plants (STP) (Solé et al., 2002). Such estrogenic effect was related to marked alterations in gonads (intersex and testicular atrophy), and was also strongly correlated ($r \geq 0.80$) with high levels of nonylphenol in water (up to 600 μg/L) and sediment (up to 645 μg/kg) (Petrovic et al., 2002; Solé et al., 2002). A highly positive relationship was also found between the plasmatic levels of Vtg in these carps and those of estriol ($r = 0.78$) and estrone ($r = 0.94$) in water (Petrovic et al., 2002). Few years later, downstream the discharge site of the Zaragoza's STP Lavado et al. (2004) also reported signs of estrogenic disorders in male carps, including marked histological alterations in gonads, low plasmatic levels of testosterone and gonado-somatic index, and high plasmatic levels of Vtg. These studies provide the first evidence of endocrine disruption in carps from Mediterranean rivers. However, none of these studies have provide evidence that high levels of Vtg in males or the existence of intersexes affects reproduction in the studied species.

3.4. Indicators of energy metabolism and trophic tracers

Abiotic (e.g. metals, pesticides) and biotic (e.g. parasites, species with agonistic behaviour) stressors can alter the energy budget of sentinel species (Barata et al., 2004). This can occur via impairing energy intake (e.g. reduced feeding rates, poor food quality) and/or increasing energy demands to cope with metabolic disorders caused by these stressors. When energetic levels are strongly altered, animals can reduce growth, longevity, fecundity and increase disease susceptibility (Amiard-Triquet et al., 2012). Thus, measures of feeding acquisition, growth and/or energy budget are biomarkers with high ecological relevance but low specificity, being energy intake the most sensitive and earliest indicator of energy imbalance (Barata et al., 2004). In European Mediterranean rivers, little is known about how stressors, including introduced species, affect the energy budget, growth and/or the trophic ecology of native macro-invertebrates and fish (e.g. Alcaraz et al., 2008; Carballo et al., 2005). Typically, research into energy acquisition in this area has been focused on fish using traditional diet analyses and/or body condition indices as proxy measure of energy reserves (e.g. Benejam et al., 2009; Maceda-Veiga et al., 2014).

According to previous data in other aquatic species and regions (Sokolova, 2013), the adenylate energy is a promising biomarker of energy reserves in macro-invertebrate and fish of European Mediterranean rivers. Similarly, particular biomarkers (heart rate, oxygen consumption and plasma lactate) raise as promising measures of metabolic activity for the species of this area, based on previous data on marine mussels (e.g. Sarà and de Pirro, 2011) and European (e.g. Guimarães et al., 2009) and North American freshwater fish species (e.g. Cooke et al., 2004). Also in North America, measures of aerobic metabolism have been employed to assess the vulnerability of species to climate change (Gallagher et al., 2015). In European Mediterranean rivers, such measures have also been used but only in an ecotoxicological context and using blood cells. Maceda-Veiga et al. (2013) showed reduced haematocrit (HCT) and haemoglobin (Hb) levels in the Mediterranean barbel (*Barbus meridionalis*) compared to the Ebro chub (*Squalius laietanus*) in a polluted river in Spain. Besides direct toxicity, these results may suggest that warming and pollution are more likely to cause severe effects on *B. meridionalis* than on *S. laietanus* since HCT and Hb are related to oxygen transport (Maceda-Veiga et al., 2015). The validation of this hypothesis, however, requires of direct measures of oxygen consumption, which could also provide useful insights into how global warming affects other fish species and macro-invertebrates in European Mediterranean rivers.

Besides examining individual effects, biomarkers related to animal nutrition enable the use of solid ecological principles to predict multi-trophic impacts. Specifically, three theories (biological stoichiometry, the metabolic theory, and the geometric nutritional ecology) try to explain how food-webs are structured based on the balance of multiple chemical elements between individuals and the environment (Allen and Gillooly, 2009; Urabe et al., 2010). A practical example is a study developed in Mexico by Capps and Flecker (2013) that used the elemental composition and excretion rates of the invasive catfish *Pterygoplichthys* spp. to assess its effect on river biogeochemistry. Other measures of elemental composition are stable isotopes, which are used to determine animal's trophic position and diet composition (Jardine et al., 2003) but also to track nitrogen pollution (Schlacher et al., 2007). The rationale is that a consumer incorporates the isotopic composition of its prey in a predictive way in relation to an ecosystem baseline (Jardine et al., 2003). However, growing evidence indicates that isotopic assimilation (discrimination factors) can vary between species and their tissues (Bond and Diamond, 2011; Cano-Rocabayera et al., 2015). Since such knowledge is still very limited for many species in European Mediterranean rivers, these tracers should only be used after an accurate calculation of discrimination factors and a solid ecological knowledge of the target species (Bond and Diamond, 2011).

Other complementary trophic tracers to stable isotopes with similar requirements are fatty acids (Napolitano, 1999) but have not yet been used in European Mediterranean rivers. Comparing the nutritional composition (i.e. nutrient quality) of macro-invertebrates and fish between polluted and baseline sites, and the energy fluxes using food-web tracers, could increase our knowledge of how complex chemical mixtures interacting with natural and other anthropogenic stressors affect the functioning of these ecosystems. However, gathering this data is too labour intensive to be practical in current routine monitoring schemes, although more feasible than observing species interactions directly. Nonetheless, the use of trophic tracers in case-studies can shed light on the architecture of ecological networks in Mediterranean rivers and how resilient this is to multiple stressors. Inherent to these studies is also to improve our understanding of associations between ecosystem functions and species traits. For instance, fine trophic studies can increase the accuracy of models using functional traits as trophic guilds in use (e.g. herbivore, omnivore, and insectivore) are probably a too coarse classification of species diets. Two sympatric Mediterranean barbels of the genus *Luciobarbus* typify this situation, they would be grouped into the same trophic guild (omnivory) based on gut content analyses, even though these also reveal that diet overlap is low (Pires et al., 2001). This is not to say that a degree of simplification of reality is not necessary to see the wood of the trees in the complexity of natural systems. Rather it highlights that detailed case-studies are needed to refine species trait categories and better understand food-webs, even though such studies seem to be underappreciated in the 'quality standards' of current academic careers.

3.5. Bile metabolites

Bile is a well-known body fluid in vertebrates for its digestive function, as well as to serve as excretion route for bilirubin (a by-product of recycled erythrocytes) and metabolites generated by the liver during the detoxification processes. In fish, elevated levels of biotransformation products (e.g. metabolites of non-persistent compounds) in bile may also be considered as biomarkers. In general, exposure to contaminants that are easily metabolised such as PAH, phenols (e.g. alkyl-phenols) and aromatic amines amongst others cannot be assessed by simply measuring their tissue residues, as the rapid metabolism and elimination of these pollutants by fish result in low residual concentrations in liver and muscle tissues. PAHs typify this type of xenobiotics and hence the determination of PAH metabolites in bile can serve as biomarker of exposure (Martínez-Gómez et al., 2013). Laboratory and field studies have both demonstrated that the presence of PAH metabolites in bile faithfully reflect levels of recent exposure, and several analytical methods have been applied either for the screening or the identification and quantification of PAH metabolites (Beyer et al., 2010). In European Rivers, these biomarkers have been used in the Mediterranean barbel (*B. meridionalis*) to detect exposure to both pyrogenic and petrogenic PAHs (Damásio et al., 2007).

Alkylphenols (octyl- and nonylphenol) are also detected in fish bile (Bizarro et al., 2014; Lavado et al., 2004). Thus, very high levels of nonylphenol (NP) (168 μg/g) were reported in the Ebro barbel (*L. graellsii*) from the Cinca River, a tributary of the Ebro River. Also common carps (*C. carpio*) sampled in the Ebro River, in the vicinity of an urban STP receiving sewage generated by 460,000 inhabitants, and in areas affected by chlorine industry wastes, had average NP values in bile of 17–20 μg/g (Lavado et al., 2004). These concentrations were higher than those measured in fish from coastal and estuarine areas (Bizarro et al., 2014; Fernandes and Porte, 2013; Martínez-Gómez et al., 2013), and were associated with marked alterations in the endocrine system of male carps (see the endocrine disruption section and Lavado et al. (2004)). Recently, the polycyclic musk galaxolide, bisphenol A, estradiol, ethynylestradiol and some pesticides have been detected in the bile of the thick-lip grey mullet (*Chelon labrosus*) captured in estuaries of the Basque Country (Bizarro et al., 2014), a common species in the mouths of many European Mediterranean rivers (Kottelat and Freyhof, 2007). These and other studies generally confirm the feasibility, sensitivity and relevance of bile metabolites as biomarkers of exposure; however, factors such as species metabolism, feeding status, season and water temperature must be carefully considered when applying these biomarkers in studies of environmental risk assessment.

Finally, the development of new analytical techniques (liquid chromatography-high resolution mass spectrometry (LC–MS/MS) and gas chromatography–mass spectrometry (GC–MS/MS)) will increase the repertoire of bile metabolites biomarkers for bio-monitoring. This excellent source of 'new' biomarkers will better appraise the effects of pollutants that are easily metabolised, but also the effects of contamination and other stressors on endogenous metabolism. Nonetheless, it is worth mentioning that some of these metabolites can already be determined using the available technology but their potential has not yet been examined in bio-monitoring.

3.6. Genotoxic effects

Radiations (e.g. ultraviolet) and many chemicals (e.g. metals, pesticides) can alter DNA integrity of macro-invertebrates and fish (Bolognesi and Hayashi, 2011; Guilherme et al., 2014). The consequences range from tumour formation to alterations in growth, fecundity and longevity of organisms (Theodorakis et al., 2000). There are several methods to assess DNA damage but by far the most adopted are the single-cell gel electrophoresis assay (also known as the comet assay) (Collins, 2004; Frenzilli et al., 2009) and the micronuclei (MN) and nuclear abnormalities in blood cells (ENAs) tests (Bolognesi and Hayashi, 2011; Guilherme et al., 2014). The major difference between the comet assay and MN/ENAs tests is that DNA damage detected in the former can be subject to a repair process, whereas MN/ENAs tests signals chromosome breakage or loss and mitotic spindle apparatus dysfunction, which are hardly reparable lesions (Collins, 2004; Maceda-Veiga et al., 2015). We recommend counting both MN and ENAs for the benefit of diagnostic accuracy since it provides a more complete picture of genotoxic effects on individuals (Maceda-Veiga et al., 2015). Although these tests/assays cannot identify the genotoxic agent, certain erythrocyte abnormalities appear to be mostly related to particular pollutants (e.g. basophilic stippling and lead pollution) (Maceda-Veiga et al., 2015). Further, improved versions of the comet assay can make it more specific toward a particular kind of DNA lesion (e.g. oxidative stress; Collins, 2004) but, as far as we are aware, these are still poorly used in bio-monitoring.

Regarding the ecological relevance of genotoxicity assays, DNA damage in the germ cells is more likely to propagate across generations even though somatic cells are equally useful for the assay (Devaux et al., 2011; Boettcher et al., 2010; Santos et al., 2013). Most genotoxic assays in invertebrates have been performed on whole-body or somatic cells, with cladocerans (*Daphnia* spp.) and bivalves (e.g. *D. polymorpha*, *C. fluminea* and *Unio pictorum*) being the most common sentinel taxa (Frenzilli et al., 2009). In strictly freshwater fish, most genotoxic assays have focused on blood cells, mainly erythrocytes, of a wide range of species of European Mediterranean rivers, including chubs (*Squalius* spp.), minnows (*Phoxinus* spp.) and barbels (*Barbus* spp.) (Supplementary material 4). A major drawback of whole-body approaches is that they limit our ability to generate mechanistic understanding of genotoxic differential effects between tissues. More studies comparing genotoxic effects between somatic and germ cells are needed, coupled to further research into the relationship between genotoxic and life-history data to increase the ecological relevance of genotoxic assays. In any case, the absence of increased ENA/MN frequency should be interpreted with care, because it may reflect multi-level impairment of ENA expression rather than the absence of genotoxic compounds or the ability of organisms to cope with DNA damaging agents (Bolognesi and Hayashi, 2011). Further, these results could reflect the adaptation of populations to pollution. Albeit advantageous, it can encompass a loss of genetic variability that may result in other negative effects on the target population (see genetics section).

The use of germ cells in genotoxic assays raises ethical and conservation concerns, especially when endangered species are involved. However, crayfish eggs or early larvae stages of freshwater mussels (see Lima et al., 2006) can be non-lethally sampled and perhaps could be used as surrogate of gonads in genotoxicity assays on species of European Mediterranean rivers. In fish, it is possible to collect either sperm or oocytes from mature individuals during the breeding season to perform genotoxicity assays. Zhou et al. (2006) found a positive relationship between DNA strand breaks measured by the comet assay in spermatozoa of male carps (*C. carpio*) and reduced hatching rate and sperm mobility at more than 50 μM of duroquinone. Likewise, spermatozoa DNA damage in salmonids was positively associated with increased embryo abnormality rates and delayed growth (Devaux et al., 2011). This link between spermatozoa DNA damage and progeny developmental effects was also reported for the three-spined stickleback (Santos et al., 2013). Thus, sperm rises as non-lethal alternative to gonad tissue in genotoxicity assays and with high ecological relevance. Nonetheless, studies comparing genotoxic effects on sperm and blood cells are needed, because unlike sperm the latter can be sampled in Mediterranean freshwater fish species in all seasons.

3.7. Histopathological alterations

Sub-lethal exposure to chemical pollutants can induce in macroinvertebrates and fish a number of lesions in tissues and organs with potential to be used as biomarkers in bio-monitoring (Faria et al., 2014; Van der Oost et al., 2003). Some of these disease signs are macroscopic (e.g. tumours, skeletal malformations, fin erosion, increased volume of target organs) and can be easily quantified via gross visual examination (Au, 2004) and tissue-whole body mass indices (e.g. hepato-somatic index) (Lavado et al., 2004). Others, however, are alterations in cells and whole tissue architecture that are only detected via histopathology (Au, 2004; Rautenberg et al., 2015). Traditionally, histopathology has been a descriptive science with certain subjectivism in the selection, processing, and examination of the target tissues. However, guidelines are now available to score and report histopathological data (Bernet et al., 1999; Dietrich and Krieger, 2009), even following well-established international procedures for certain organs (mainly liver and gonads). Nonetheless, these directives have been applied to laboratory freshwater species or marine species that are the focus of international monitoring schemes. In European Mediterranean rivers, most histopathological research is based on occasional surveys of wild crustaceans, bivalves and fish in reservoirs and estuaries (Supplementary material 3 and 4), and the relatively few examples in running waters are on fish (e.g. Lavado et al., 2004; Raldúa et al., 2008).

Chemical pollution has been associated with a wide range of histocytological alterations (e.g. inflammatory processes, necrosis, testis-ova, vascular disorders, neoplastic cells), reflecting the severity and duration of pollution events (Au, 2004; Dietrich and Krieger, 2009). Positive correlations between pollutant levels (e.g. pharmaceuticals, pesticides) and histological alterations (e.g. neoplastic lesions, testis-ova) are reported for several species both in field and laboratory conditions, further supporting the use of histopathological biomarkers as faithfully indicators of environmental pollution (e.g. Dietrich and Krieger, 2009; Rautenberg et al., 2015). Other advantages of histopathology are the easy collection, processing and storage of samples and the possibility to assess many body systems from the same individual (Handy et al., 2002; Van der Oost et al., 2003). Also, histopathological biomarkers (e.g. testis-ova) can identify the pollution type via its mode of action (e.g. endocrine disruption), although they cannot reveal toxic identity (Bernet et al., 1999; Dietrich and Krieger, 2009). Advanced histopathological techniques such as in-situ hybridization and immunohistochemistry can refine these diagnostics, but they are only available for laboratory models such as zebrafish (*Danio rerio*) (Thienpont et al., 2011).

The poor histological knowledge of native species can explain why histopathological studies in European Mediterranean rivers have mostly examined laboratory and aquaculture species (e.g. *D. rerio*, *Oncorhynchus mykiss*) or widespread exotic species (e.g. *C. carpio*, *C. fluminea*), due to the availability of reference material (e.g. Pinkney et al., 2009; Rautenberg et al., 2015). Comparing individuals between tested and baseline sites may not be a safe option, because pristine sites do not really exist in European rivers, and then misdiagnosis can occur without in-deep anatomical, histological and physiological knowledge of the target species. For instance, liver fatty deposition has been proposed as sensitive histopathological biomarker in the three spined stickleback (*Gasterosteus acculeatus*) (Handy et al., 2002). However, the degree of fat in the liver varies naturally depending on the species and individual status, and then cannot be unequivocally associated with health disorders, unless the physiology of the target species is well-known. Similarly, the size and frequency of melano-macrophage aggregates are related to pollution and infection diseases as well as natural processes (aging) (Agius and Roberts, 2003). Thus, these and other general histological alterations (e.g. circulatory disturbances, epithelium thickness and lifting, presence of cellular debris) are not pathological biomarkers in their own right. Nonetheless, they together with changes in metabolic enzymes or blood cell profiles can identify health disorders, as demonstrated for wild cyprinids exposed to flame retardants (Raldúa et al., 2008) and sewage discharges (Koca et al., 2008; Maceda-Veiga et al., 2013) in European Mediterranean rivers.

Whilst diagnosis in histopathology is based on the exhaustive examination of slides to record multiple disease evidence, we acknowledge that routine monitoring schemes need of easy recognisable alterations that ideally indicate alone a pathological condition. Particular alterations associated with pollution events (e.g. focal necrosis, neoplasm, atrophy, and pyknosis) typifies this situation, ranking as severe tissue lesions in guidelines for scoring histological alterations (Bernet et al., 1999). Since these alterations are likely to compromise animal life, they can be considered histopathological biomarkers with high ecological relevance compared to those mentioned above. Such relevance can be even higher if the target tissue is gonad due to its direct link with population maintenance. The presence of testis-ova and changes in gonad maturity stages are two common histopathological biomarkers of endocrine disruption used in fish of European Mediterranean rivers (Lavado et al., 2004). The latter, however, requires good knowledge of the reproductive cycle of the target species, especially in these rivers where individuals within a population show multi-spawning and hence asynchrony in gonadal maturation along the breeding season (e.g. Vinyoles et al., 2010).

Besides examining particular target tissues (mostly liver), a whole body approach gives a more integrative picture of pollutant effects even though this approach is still scarcely used in ecotoxicology (Fig. 5). In European Mediterranean rivers, most native and introduced adult fish species are medium size cyprinids (Kottelat and Freyhof, 2007) and need to be dissected for histological processing. In contrast, juveniles (<3 cm fork length) can be embedded in blocks as a whole like laboratory fish species (Dietrich and Krieger, 2009). Other benefits of using juveniles compared to adults are their higher sensitivity to toxicants (with exceptions, see Stubblefield et al., 1999), and the fact that their extraction may have a lower impact on populations due to their low reproductive value and higher natural mortality. Also, electrofishing is needed to capture adults, whereas a dip net can be used to collect juveniles within the aquatic vegetation of a margins (AS and AMV personal experience). Thus, we propose histological studies on juveniles as a practical and sensitive diagnostic tool to fully assess the risk posed by complex chemical mixtures to the biota of European Mediterranean rivers. Native small size adult fish cannot be used for lethal tests in this area because are mostly listed as endangered or near-threatened in legislation, including *G. acculeatus* in Spain (Doadrio et al., 2011).

3.8. Behavioural traits

Multiple stressors (e.g. pollution, temperature, infections, reduced water flow, and predation) can alter the behaviour of macro-invertebrates and fish and potentially cause far reaching impacts on ecosystems. Movements of migratory species, such as the European eel (*Anguilla anguilla*) and cyprinids of the genus *Luciobarbus*, influence the energy transfer along European rivers, perhaps as nicely demonstrated for salmonids in the Pacific (Gende et al., 2002). Such migrations are also essential for the dispersion of the native mussels that use these species as host for their larvae (see Lopes-Lima et al., 2014). Further, behavioural traits (e.g. differences between species in predator avoidance, agonistic behaviours, and foraging rates) can directly affect the structure of aquatic communities. When intermittent Mediterranean rivers disconnect and the biota remains in isolated pools, these traits related to the competitive abilities of animals might be crucial to explain the community structure of these rivers after drought. Given this link between behaviour and ecosystem/community responses, behavioural biomarkers are considered of high ecological relevance (Amiard-Triquet, 2009).

Other advantages of behavioural biomarkers are that they are cheap, non-lethal and relatively easy to quantify using modern image analysis software, such as video tracking of locomotor patterns (e.g. Macedo-Sousa et al., 2007). Also, behavioural biomarkers are unspecific, thus enabling the evaluation of the ecological risk of toxicants with unknown mode of action (Amiard-Triquet, 2009). Further, these biomarkers can be very sensitive given that avoidance behaviour (escape, valves closure in bivalves) generally occurs at low pollutant levels to avoid harm (Cooper and Bidwell, 2006). However, in fish and invertebrates, chemo-receptors can also be affected by toxicants even at an early-life stage, thus limiting the use of this biomarker as 'early signal' of disease. For instance, if embryos of fathead minnows (*Pimephales promelas*) are exposed to copper, a reduced ability to respond to chemical alarm stimuli occurs when adults (Carreau and Pyle, 2005). Olfactory impairments can, however, also lead to other behaviour alterations due to the role of chemo-signals in animal communication (e.g. sexual recognition and location of breeding grounds) (Wyatt, 2014). Toxicants can also affect other sensory systems (light receptors and mechano-receptors) involved in these and other vital functions, such as feeding, locomotion and defence (agonistic behaviours) (Amiard-Triquet, 2009).

Together with sensory systems, other body systems can be affected by multiple-stressors, including toxicants, and induce behavioural alterations. For instance, reduced swimming velocity is related to alterations of cholinesterase activity (ChE) in several fish species exposed to organophosphate or carbamate pesticides (e.g. Almeida et al., 2010). The former also impairs the burrowing ability of the Asian clam (*C. fluminea*) (Cooper and Bidwell, 2006). Moreover, changes in ventilation frequency occur in aquatic organisms exposed to elevated temperatures and chemicals that impair oxygen transport (e.g. nitrites) or alter ion gill exchange (e.g. metals) (Cooper and Bidwell, 2006; Macedo-Sousa et al., 2008). Further, some pollutants can induce alterations in hormone levels that may impair migration, for instance thyroid hormones appear to play a key role in the colonization of river habitats by elves of the European eel (*A. anguilla*) (Imbert et al., 2008). In contrast, environmental levels of urban-use pesticides appear to attract salmons (*Oncorhynchus kisutch*) although these impair their response to food stimuli (Tierney et al., 2011). Alterations of feeding behaviour are also used as biomarker in ecotoxicology (see energy metabolism section). Thus, behavioural biomarkers can integrate health disorders in different body systems that are likely to affect individual fitness and survival (Amiard-Triquet, 2009).

In European Mediterranean rivers, behavioural biomarkers are poorly used compared to traditional ecotoxicological approaches, especially in fish (Fig. 3). Also, such behavioural data for macro-invertebrates and fish found in these rivers was only recorded in laboratory conditions (Supplementary material 3 and 4). This is likely to be attributed to the logistic constraints of field studies to measure typical behavioural endpoints (e.g. locomotion, ventilation rate). Nonetheless, marking techniques allow free-living fish and crayfish to be tracked and determine movement and migration patterns under variable environmental conditions. We also advocate to integrate this information in studies using food-web tracers (see SIA in energy metabolism section) to explore how animal movement may alter energy pathways in rivers. Most importantly, tagging allows follow-up the life-history traits of each individual (e.g. growth, weight) in multi-biomarker approaches, which will enable us to better appraise the ecological relevance of biomarkers. Practitioners should, however, bear in mind that behavioural biomarkers may vary during the day (circadian-rhythms), although this is still poorly studied even in laboratory ecotoxicological assays (e.g. Macedo-Sousa et al., 2007).

Other useful behavioural biomarkers are traits related to individuals' personality, in particular measures of boldness and shyness. These traits are related, for instance, to the ability of individuals to innovate (learning) and use new habitats and food sources, and to their willingness to approach predators (Réale et al., 2007). Thus, studies on individuals' personality can greatly increase our understanding of ecological trade-offs (Réale et al., 2007) and invasion processes (Carrete et al., 2012). For instance, personality can help explain why some species are more prone to become successful invaders and how their invasibility may be affected by a changing environment. Also, these behavioural biomarkers allow us to test if the vulnerability of native species to introduced species is influenced by alterations in their personality under different environmental (e.g. presence of pharmaceuticals, warming, reduced water flow) and biological scenarios (e.g. aquatic community assembly including parasites). Therefore, it is clear that these studies can help to better identify vulnerable native species and successful invaders according to their different personalities. However, in macro-invertebrates and fish of European Mediterranean rivers, these behavioural biomarkers have only been applied to one non-model native fish species and in a reproductive context (see Fabre et al., 2014). Thus, the potential of traits related to personality for eco-toxicological and invasive studies in this region remains largely unexplored.

3.9. Genetics and the omics Era

Traditional genetic biomarkers (e.g. microsatellites, mitochondrial cytochrome) can inform about how the evolutionary history of populations influences their response to stressors. Yet they are still poorly used in ecotoxicological studies (Amiard-Triquet et al., 2012 and Supplementary material 3 and 4). Low within-population genetic diversity, for instance, is related to a lower adaptive capacity of populations to cope with novel stressors. Also, when a spill occurs, a high between-population genetic diversity (gene flow) can explain the differential sensitivity of individuals in a population, coupled to suggest a possible greater ecological risk due to the movement of individuals between polluted and clean sites. Thus, the identification of resident and immigrants in a population is central to determine conservation units and also to know the river area to which the evaluated toxic effects refer to (Amiard-Triquet et al., 2012). This is markedly important when bio-indicators are highly mobile species and/or introduced species, including native species often translocated between European Mediterranean rivers such as the brown trout (*Salmo trutta*) (Maceda-Veiga, 2013). The unknown invasion history of an introduced population (e.g. single or multiple introductions) can lead to misleading conclusions on its response to stressors, although this is still poorly studied even in terrestrial animal invaders (Novo et al., 2015).

Apart from explaining within- and between-populations variability, genetic biomarkers improve our mechanistic understanding of how stressors affect individuals (Piña and Barata, 2011). This is the domain of omic techniques, a modern research area that tries to understand how expressed genes (transcriptomics), proteins (proteomics) and metabolites (metabolomics) are altered by stressors. These three disciplines can provide a more compressive picture of the toxicity mechanisms on whole body systems and may lead to the development of predictive models to estimate the susceptibility of individuals to stressors based on their genotype. The main limitation for the use of these techniques in environmental approaches has historically been our poor physiological and genomic knowledge of environmentally-relevant species (Piña and Barata, 2011). This is particularly acute for microarray analyses that help determine, for instance, if pollution has altered a metabolic pathway via the simultaneous quantification of the set of genes involved (Piña and Barata, 2011). Nonetheless, in poorly known wild species (at the genome level), these analyses can provide useful insights into metabolic disorders if a relatively large set of reliable transcriptomes is available. For instance, using published sequence tags from several *Dreissena* species, Navarro et al. (2011) quantified the gene-expression profile of *D. polymorpha* exposed to metals and other pollutants in the low Ebro River. The expression of some of these genes was also correlated with body condition index and lipid content, confirming the implications of these genes on physiological disorders in this species. Current advances in high-throughput sequencing techniques applied to both model and non-model species will greatly contribute to the generalization of omic approaches to environmental issues.

Other uses of omic techniques in European Mediterranean rivers were to assess the consistency of gene expression profiles between life-stages and tissues sampled. In *C. carpio* collected in the Ebro River, Navarro et al. (2009) found that the expressions of kidney metallothioneins (MT-I and MT-II) mirrored the mercury concentration of muscle, liver and kidney, but that the same genes expressed in the liver reportedly were not related to mercury pollution. Navarro et al. (2011) also reported that the profile of 9 transcriptomes in adults and pediveliger larvae of *D. polymorpha* was rather similar after an exposure to mercury, cadmium and copper. RNA is currently quantified by modern Real-Time PCRs that enable the detection of as few as 10 to 100 RNA molecules. This allows the screening of many individuals and genes, and ultimately aids in linking exposure and disease, as well as in understanding how results of eco-toxicological assays can relate to wild populations and human health. It also allows using small, dispensable body parts (e.g. scales and blood) to monitor biological responses to pollutants, like cyp1a induction (the genetic counterpart of the classical EROD assay) upon exposure to dioxin-like substances (Olivares et al., 2010).

Besides changes in the DNA code itself, other factors (inheritable and not) can determine the relationship between genes and the environment (Head et al., 2012). This is the domain of epigenetics that studies the effects of chemical marks on top of the DNA code (e.g. DNA methylation, histone modification) on gene expression (Head et al., 2012). DNA methylation is probably one of the best understood mechanisms of epigenetic inheritance and may explain how multiple stressors, including chemicals, can have lasting and even multigenerational effects on health in wildlife (Head, 2014). Such chemical marker also seems to be suitable for epigenetic studies using blood as non-lethally sampled tissues (Head, 2014). As far as we are aware, epigenetics have not yet been used in wild macro-invertebrates and fish of European Mediterranean rivers. Arguably, there will be a long pathway until epigenetics and the preceding cutting-edge techniques can be a reliable and powerful diagnostic tool for wildlife. This also applies to DNA pyrosequencing for the examination of the symbiotic bacteria of macro-invertebrates and fish, which are related nutrient assimilation and immune response (e.g. Sullam et al., 2012), and perhaps could be coined next-generation biomarkers. Since descriptive studies still dominate literature, a key step forward will be when more studies examine how all these cutting-edge biomarkers respond to multiple stressors and their health implications including fitness costs.

4. Experimental approaches: from mechanistic understanding to ecological relevance

Besides selecting bio-indicators whose population dynamics are more likely to trigger whole-ecosystem responses, the ecological relevance of biomarker outputs depend on the experimental approach. In European Mediterranean rivers, biomarker studies have been used in laboratory or field-based conditions but relatively few have combined both (Fig. 4). Considering the physiological knowledge of wild aquatic species in this area is scanty, and biomarkers can show inter-species, population and tissue variability in sensitivity, recovery and induction (Wu et al., 2005). Biomarker-stressor relationships should be fully tested in new systems. A two-step process of validation consists of using laboratory tests to determine cause-effect relationships between stressors and biomarkers followed by field studies to test for the strength of these relationships under a variable degree of complexity in natural systems. Only when a solid knowledge of biomarker responses is reached, and the understanding of the target system is sufficient, we can truly appraise the magnitude of health disorders in individuals and correctly assign risk for ecosystems using modelling approaches (Lombardo et al., 2015; Veltman et al., 2014).

Following general research principles, the interpretation of biomarker responses requires of controls. In the field, the standard practice is to compare tested sites in relation to a baseline site, even though the variability of natural systems may recommend several reference sites (Dallas, 2013; Sanchez et al., 2010). However, it can be difficult to determine reference conditions due to widespread habitat degradation (Dallas, 2013). At worst, hatchery-reared individuals of the same species can be used as proxy of baseline biomarker data. However, better approaches are to use wild animals in-situ assays (see Liber et al., 2007) or bring some wild individuals to the laboratory and keep them

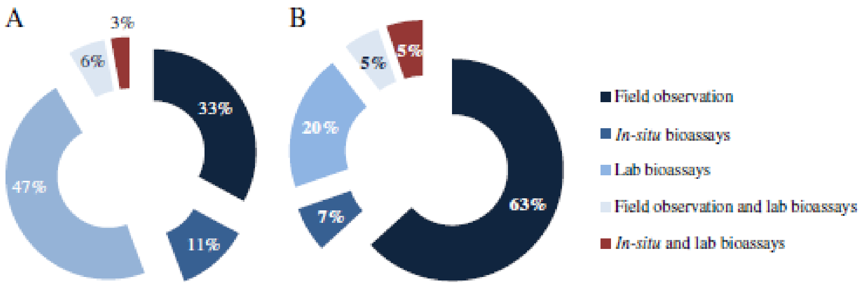

Fig. 4. Percentage of experimental design types used in biomarker studies on macro-invertebrates (A) and fish (B) of European Mediterranean rivers. Notice that in-situ bioassays and field observations in free-living species dominate biomarker studies in macro-invertebrates and fish, respectively.

in clean water before collecting baseline data (e.g. Aborgiba et al., 2016). These two approaches account for the genetic diversity of wild populations, thus increasing the ecological realism (see genetics section). Further, the use of wild species eliminates the risk of exotic species release into natural systems. Most standard test species are exotics (e.g. mummichog *Fundulus heteroclitus*), and there is some evidence of the establishment of feral populations (Gisbert and López, 2007). If natives are the target, experimental procedures must be compatible with their conservation status, coupled to meet the animal welfare standards of any experimental study in vertebrates (EU. Directive 2010/63/EU).

Either for policy-making or mechanistic understanding, practitioners using biomarkers aim to assess how biological responses respond to stressors. Whilst the causal link is established in the laboratory and hence field studies can only provide correlative evidence, statistics can help reduce *noise* in the later. For instance, variation partitioning (VP) analyses (Borcard et al., 1992) can disentangle the colinearity between variables and explore their independent contribution to the variation in biomarker data (Mac Nally, 2002). Also, general and generalised linear models (GLM, Crawley, 1993) enable using different slopes of relationships between stressors and biomarkers, what explains their use as complementary approach to VP in ecological studies (e.g. Murphy et al., 2013; Maceda-Veiga et al., 2014). Besides GLM and VP, other multivariate techniques commonly used in ecology (see Dray et al., 2012; Ter Braak and Šmilauer, 2014) enable exploring linear and non-linear relationships between biomarkers and explanatory variables (e.g. organism size and sex, season, parasites, a set of environmental variables). Most practitioners, however, do not accommodate the statistical properties of the data at hand and also focus on simple designs (i.e. comparison between tested and baseline sites). This limits our ability to generate novel hypothesis for experimental studies and to test for the strength of biomarker responses under the complexity of natural systems (Baird et al., 1996; Van der Vliet and Ritz, 2013).

Generally, biomarker studies also use a small set of individuals and sites (Supplementary material 3 and 4), which makes difficult to robustly assess the health status of populations and validate biomarker responses globally. Often, yield individuals in a population contribute disproportionate to the mean trend in biomarker responses and/or population persistence (e.g. reproductive value is related to size). Consequently, examining the shape of biomarker responses (e.g. mean, variance, maximum values, and coefficient of variations) will provide a more comprehensive picture of stressor effects on populations (Baird et al., 1996; Devin et al., 2014). Statistical analyses such as PCA create linear combinations of measures maximising the variance explained (Ter Braak and Šmilauer, 2014). However, these synthetic gradients may not be ideal for management because the real contribution of each biomarker cannot be appraised and then future studies need to use the same set of biomarkers, which increases monitoring costs and complexity (Vaughan and Ormerod, 2005). Arguably, tests of differences in biomarker responses between groups are more intuitive compared to ordination methods (e.g. PCA, CCA, RDA), and then increase the accessibility of biomarker outputs to managers and policy makers. Finally, we suggest that the ecological risk of chemicals in Mediterranean rivers will be better assessed if bio-indicators are sampled in the drought season because it is when they are likely to be more vulnerable to anthropogenic stressors. Biomarker results could then be combined with demographic studies (e.g. Maltby et al., 2001) and species sensitive distribution models (e.g. Solomon and Takacs, 2002) to further increase their ecological relevance. As highlighted by Garcia et al. (2014), this would also add a degree of realism to spatial projections of climate change exposure that consider all species to be equally affected (no matter its physiology) by climate change-induced threats.

5. Where biomarker approaches should be going? Our personal view

Biomarkers aim to give complementary information to measures of community structure and function in monitoring studies to better appraise how stressors affect ecosystems (e.g. Puértolas et al., 2010; Damásio et al., 2011a; Friberg et al., 2011). This final goal is shared by ecology and ecotoxicology sciences but unlike the former, ecotoxicology is still firmly rooted in developing mechanistic understanding of stressor effects on a reduced number of individuals of a single target species in a snapshot of space and time. This is not to say that we necessarily need to assess how each pollutant affects biomarker responses of all macro-invertebrate and fish species present in a river under variable conditions, including the presence of other pollutants. Rather we need to measure a practical and cost-effective number of biomarkers on a set of bio-indicators that indicate overall and specific types of human impacts. Such responses of biomarkers can be initially validated in the multi-stressor context of a given basin (Fig. 5). This meet the legislation (e.g. EU's River Basin Management Plans) and also ecological criteria, given that each basin is a functional and geographic unit in relation to other basins. All native species within a basin have a common biogeographical and pollutant history and ecological processes between rivers stretches are strongly linked (river continuum concept, Vannote et al., 1980). Nonetheless, the feasibility of using the same diagnostic toolkit across basins or with as few adaptations as possible is desirable for the benefit of practicality.

Our proposal to validate a relevant set of biomarkers with ecological relevance and potential to be applied across basins follows a 6 tier approach: 1) chemical scenarios are designed using data on a catchment with extensive chemical surveys to determine the concentrations and pollutant types classified according to the mode of action (e.g. endocrine disruption, neurotoxic, beta-blockers), 2) each chemical scenario is associated with a target aquatic community since the structure of aquatic communities and pollution impacts varies along the upstream-downstream gradient, being lowland reaches more disturbed and diverse than headwaters, 3) a set of potential keystone species with different ecological roles (e.g. top consumer, organic matter processers) is selected within each target community (Fig. 5), 4) general and specific biomarkers (targeting the modes of action of pollutants) are applied together to the keystones chosen to appraise the effects of each chemical scenario in individual assays for each keystone, 5) a set of baseline and impacted sites distributed across rivers within the catchment

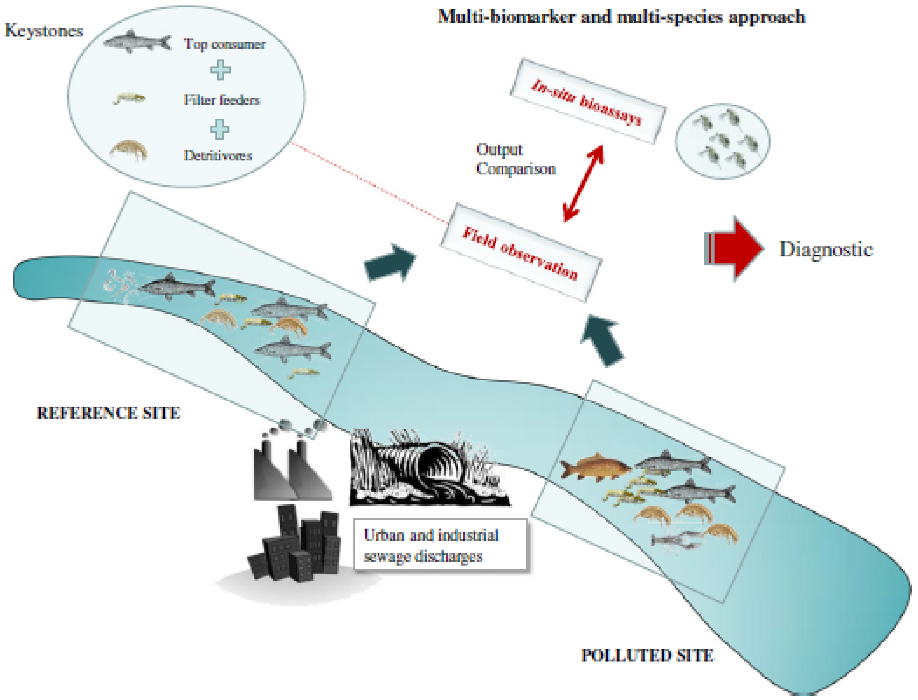

Fig. 5. Step-wise procedure for the validation of a set of biomarkers with ecological relevance and potential to be applied to macro-invertebrates and fish across European Mediterranean rivers. We propose to compare biomarker outputs from different keystones (the top consumer + representatives of two additional trophic guilds) in an aquatic community between tested and reference sites. These sites should be in the same river but in heavily impacted systems different rivers could be used if they have similar geographical features (e.g. altitude, latitude) as these can influence species' life-history traits (e.g. Benejam et al., 2009). The relationship between these results and those obtained from in-situ bioassays with a model organism is also recommended for the benefit of simplicity in future monitoring studies.

where the different chemical and biological scenarios were drawn is used to validate the biomarker responses in real conditions (ideally between different rivers with similar geographical features and keystone species use), 6) a longitudinal study (e.g. two consecutive years) should also be performed to appraise the ecological relevance of any 'early signal' of disease (i.e. biomarker outputs) observed in the populations of the selected keystone species.

The potential widespread use of a set of biomarkers validated via the preceding proposal lies on the fact that some of the selected keystones are widespread or replaced with closely related species in other catchments. Since biomarker responses have a phylogenetic component, sister species (e.g. species of the same genus) are likely to show similar physiological responses. The Mediterranean barbel (*B. meridionalis*) and the Iberian red-fin barbel (*B. haasi*) typify this situation in north-eastern Spain, because are top consumers with similar ecological and biological traits and their distributional ranges do not overlap (Doadrio et al., 2011). Regarding the pollution types, different rivers are likely to have common mixtures, given that some chemicals are of widespread use (e.g. pharmaceuticals), and catchments can be affected by similar impact types (e.g. urban effluents, agricultural waste) (e.g. Gorga et al., 2015; Sabater et al., 2009). This raises the possibility to predict pollutant types in a river stretch in relation to changes in humans' behaviours (e.g. most common drugs prescribed by doctors) and the impact types present along a catchment (e.g. agriculture and nitrate pollution). If there is sufficient knowledge of the sensitivity of different keystones to a variety of chemical cocktails, these models may also be a valuable and adaptive management tool to provide sound indication of which stretches are at high ecological risk and require careful in-situ bio-monitoring. This vibrant research area can increase the predictive power of bio-monitoring tools, but accuracy may be compromised by many confounding factors such as the genetic background of populations and their acclimation to pollution.

A major requisite of bio-monitoring procedures is simplicity and although the same set of biomarkers can be used across rivers, the possible use of different keystones as bio-indicators increases complexity. In an attempt to reduce complexity, within the above mentioned experimental design the effects of the preceding chemical scenarios can also be appraised on caged laboratory (e.g. the cladoceran *D. magna*) or local models (e.g. the snail *Physella acuta* and the larvae of the caddisfly *H. exocellata*) (Damásio et al., 2011b; De Castro-Català et al., 2013). If the relationship between biomarker responses in wild and caged bio-indicators is predictable, the replacement of free-living keystones with these caged models can occur for the benefit of simplicity and species conservation. This is particularly important for keystones with conservation concern, such as some fish species which are also subjected to the restricted regulations of any experimental study on vertebrates. The differential response of invertebrates and fish to chemicals, however, does not allow the extrapolation of the effects on one species to another, although some metabolic pathways are highly preserved across taxa. Thus, comparing biomarker responses between lethal and non-lethally alternatives (e.g. scales, fin, blood, faeces, cell cultures, reproductive fluids, sexual behaviour and other individual traits) should broaden the scope of current eco-toxicological studies to make them progressively better aligned with conservation and animal welfare standards.

Whilst biomarker responses can be difficult to interpret, the integration of several biomarker measures can unequivocally sign disease, especially when histopathological and behavioural biomarkers are used. Convincing arguments are important because governments expend large annual budgets in bio-monitoring, and ecological impacts can have serious legal consequences. Probably, this implies that novel biomarkers will only replace well-established toxicological procedures such as histopathology in the long run, and that biomarkers measured on a single bio-indicator cannot provide a solid evidence of ecological impact. In this regard, biomarker responses are less well-studied in macro-invertebrates than in fish, which may limit further their diagnostic ability. Also, in compliance with the EU's REACH legislation, assessing the effect of emerging pollutants may require the development new biomarkers on macro-invertebrates and fish. In the latter, we argue that determining biomarker responses on adults using non-lethal alternatives plus the histopathological assessment of juveniles could provide a good picture of the effects of chemical mixtures on a population. This will have little effects on its viability but high ecological relevance (see histopathological section). Although zebrafish embryos or introduced species inhabiting with natives are already used as bio-indicators, these outputs require the validation on natives to appraise the risk of misleading conclusions. For endangered species, we propose that surplus individuals from captive breeding programmes can be used in eco-toxicological assays (e.g. Varó et al., 2008).

Comparative studies using indicators at multiple levels of biological organisation have been used in European Mediterranean rivers to compare the diagnostic ability of biomarkers in relation to traditional approaches. In rivers exposed to chronic pollution, biomarkers detected impairment where traditional community-based approaches failed (e.g. Damásio et al., 2007; Puértolas et al., 2010). In highly polluted rivers, such as the Anoia River in north-eastern Spain, community-based metrics detected impairment and biomarkers refined the diagnostic indicating that endocrine disruptors were a major threat to fish fauna (Fernandes et al., 2002: Solé et al., 2002). Also, biomarkers have consistently highlighted the ecological risk of a stretch in the Ebro River using macro-invertebrates (Faria et al., 2010a), fish (Navarro et al., 2009) and birds as bio-indicators (Barata et al., 2010). Thus, growing evidence indicates that biomarkers are powerful allies to detect ecological risk and its causes, supporting their use in routine monitoring schemes. A final prove of their diagnostic value will be when long-term studies examine the effects of multi-stressors on rivers at multiple levels of biological organisation and ecosystem services, which is indeed the goal of ongoing EU-funded projects such as GLOBAQUA (www.globaqua-project.eu). As highlighted by Forbes et al. (2006) and Wu et al. (2005), we also want to emphasize here that correlations in a snapshot of time between biomarkers and responses at higher levels of biological organisation do not imply that biomarkers are predictors of ecological change. Other benefit of using biomarkers in bio-monitoring is to develop a cross-disciplinary framework that can increase our knowledge of how pollutants affect wildlife and human populations, as promoted by the One Health Initiative (http://www.onehealthinitiative.com/). In fact, the use of biomarkers in bio-monitoring originated from human medicine (Galloway, 2006).

6. Conclusions

Our review supports that biomarkers are central to detect early pernicious effects on populations before a system may collapse into a state from which recovery is difficult, highly costly or impossible (extinctions) (e.g. Kidd et al., 2007). Chronic pollution is one of the most extended and dangerous ecological threats because it causes abrupt shifts in ecosystems with unpredictable ecological, social and economic consequences. Such effects are likely to be acute in Mediterranean rivers due to their high degree of endemicity and the long-history of anthropogenic impacts that may limit their ability to cope with future stressors. We advocate accounting for this uncertainty by keeping on the safe side the health status of aquatic communities as it may increase ecosystem resilience. Thus, immediate actions should be applied to reduce ecological risk in systems where health disorders affect multiple species and particularly keystones. Biomarkers also seem basic to refine modern ecological approaches based on species traits and to assess the effectiveness of restoration practices. Yet this is still poorly studied even with conventional community-based measures. In conclusion, the use of biomarkers plus measures of community structure and ecosystem function can help to better understand and predict the ecological consequences of multiple stressors on Mediterranean rivers. This will require the revision of the current protocols established by the EU's Water Framework Directive to appraise the status of water bodies across member states.

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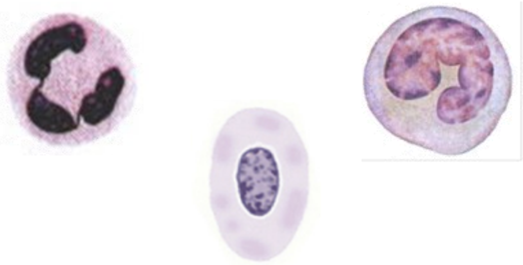

CAPÍTULO 4

La tendencia en biomarcadores, índices bióticos y abundancias revela efectos contrastados a largo plazo de las descargas de aguas residuales en las poblaciones de peces de los ríos mediterráneos

Resumen

El agua de depuradora desempeñará un papel cada vez más importante en el mantenimiento del caudal de los ríos en regiones áridas y semiáridas. Sin embargo, todavía desconocemos cuáles podrían ser sus efectos sobre la vida silvestre a largo plazo. En este estudio examinamos cambios en la abundancia de peces, en las puntuaciones de los índices de calidad biótica calculados en diferentes taxones (diatomeas, invertebrados y peces) y en biomarcadores (análisis de sangre y índice de condición corporal a escala) con el objetivo de evaluar el impacto ecológico de las descargas de aguas residuales tratadas en el río Ripoll, NE España, en el período 2002-2013. A fin de testar la hipótesis que el estiaje incrementa la toxicidad de los contaminantes, los biomarcadores sanguíneos fueron muestreados en los peces nativos *Barbus meridionalis* y *Squalius laietanus* a lo largo de las cuatro estaciones del año, entre 2012 y 2013. *Barbus meridionalis* mostró un claro incremento en su abundancia en tramos contaminados durante todo el período de estudio, mientras que la de *S. laietanus* se redujo notablemente en 2013. Ambas especies mostraron cierta buena condición física en los tramos contaminados pero con síntomas de estrés (neutrofilia, monocitosis y/o liberación de eritrocitos inmaduros). Además, *S. laietanus* presentó valores más altos de biomarcadores específicos de presencia de contaminantes (% de células anormales) que *B. meridionalis*, principalmente en primavera. Esto sugiere que *S. laietanus* fue la especie más sensible a la contaminación y que el periodo reproductor fue más estresante para los peces que el verano. No obstante, el hecho que las características hidromorfológicas fueran diferentes entre tramos de referencia y contaminados, y que esto diera lugar a tendencias opuestas en las abundancias de las dos especies en los primeros, sugiere que las variaciones en el caudal pueden cambiar la respuesta de especies pelágicas y bentopelágicas al estrés por contaminación. Los índices de diatomeas e invertebrados captaron mejor las variaciones en la calidad del agua que el índice de peces. En resumidas cuentas, el agua de depuradora presenta riesgo genotóxico para las poblaciones de peces y aumenta el peligro de causar impactos en toda la red trófica del río Ripoll. Es necesario mejorar el tratamiento de las aguas residuales en los ríos mediterráneos en vistas a los envites del cambio climático. En este sentido, los análisis de sangre se presentan como un método económico para estudiar cómo estas mejoras afectan a la salud de los peces.

Trends in biomarkers, biotic indices and abundances revealed contrasting long-term effects of sewage discharges on native fish populations in a Mediterranean river

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Abstract

Recycled water will play an increasing role in maintaining river flow in arid and semi-arid regions. However, its long-term effects on wildlife populations are still poorly studied. In this study we used trends in fish abundance, indices of biotic quality across taxa (diatoms, invertebrates and fish), and biomarkers (blood tests and scaled body condition index) to assess the ecological impact of treated sewage discharges in Ripoll River (NE Spain) over the period 2002-2013. To test the hypothesis that drought would increase the pollution stress, blood markers were sampled seasonally in the native fish *Barbus meridionalis* and *Squalius laietanus* in 2013. *Barbus meridionalis* consistently increased in abundance in polluted sites over the entire study period, but *S. laietanus* markedly declined in 2013. Both species had better condition under pollution but with signs of stress (neutrophilia, monocytosis, and/or release of immature cells). Further, *S. laietanus* had higher values of specific pollutant biomarkers (% abnormal cells) than *B. meridionalis* and mostly in spring, suggesting that the former was the most sensitive species and the breeding season more stressful than summer. Nonetheless, contrasting hydro-morphological features and trends in fish abundance in reference sites suggests that changes in water level may shift the response of pelagic and benthopelagic species under similar pollution stress. Diatom and invertebrate indices captured better variations in water quality than the fish index. In conclusion, recycled water has genotoxic risk for fish populations and potential to cause multi-trophic impacts in Ripoll River. Better sewage management strategies are needed in Mediterranean rivers under water scarcity, and blood tests are a cheap way to monitor their consequences for fish health.

Keywords: conservation physiology, water scarcity, fish blood, hydrology, recycled water.

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 good example, as their natural low dilution ability intensifies the ecological risk of the myriad of toxicants released by STPs (Petrovic *et al*., 2011; Arenas-Sánchez *et al*., 2016). This situation worsens with water overabstraction, which is expected to increase further under forecast climate and human population growth models (Vörösmarty *et al*., 2010; Mekonnen and Hoekstra, 2016). However, recycled water can aid in maintaining river flow in a context of water scarcity (Vörösmarty *et al*., 2010; Halaburka *et al*., 2013). Nonetheless, the long-term effects of effluents from STPs on wild vertebrates are still poorly studied in Mediterranean rivers.

Fish are a major component of freshwater biodiversity and one of the most threatened taxa worldwide (Arthington *et al*., 2016). As well as conservation interest *per se*, fish play a pivotal role in aquatic food-webs, either as top predators or as prey for high order consumers (e.g. Ruiz-Olmo *et al*., 2001). Further, 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).

The conservation concern of fish extirpation is particularly acute in Mediterranean rivers where only one or two species often top-down control the aquatic community (e.g. Maceda-Veiga *et al*., 2010). Traditionally, biomonitoring schemes have appraised the ecological status of fish communities using the so-called indices of biotic integrity (see Karr, 1981, Aparicio *et al*., 2011; Lyons, 2012). At best, these indices detect impairment when populations sharply decline or after a species extirpation, and this may be too late to take conservation actions. Thus, the development of alternative monitoring tools that detect early signs of disease in fish populations is a priority in Mediterranean rivers (Colin *et al*., 2016a).

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 little invasive and even suitable for small fish (e.g. Filby *et al*., 2010). By examining red blood cell morphology, it is possible to detect 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 count is used as an indicator of stress and/or infection (Davis *et al*., 2008). Furthermore, blood parasites can 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*., 2016a). Only the detection of micronuclei in red blood cells has a wider acceptance, including in ecosystems other than Mediterranean rivers and in wildlife 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 counting them automatically. However, the formation of micronuclei may not be so common across taxa (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 raises the question of how blood alterations scale up at the population level; a concern shared with other biomarker approaches, including body condition indices (Colin *et al*., 2016a). In order to increase the ecological relevance of biomarkers, then more research into their natural variability and their integrated response at the population scale is necessary.

The Ripoll River has been long-term defaunated in downstream reaches due to untreated domestic and industrial sewage discharges (Prat and Rieradevall, 2000). Water quality improved considerably after the construction of STPs in 2002 and hence aquatic fauna (Ecoprogress, 2008; Sostoa *et al*., 2006). In 2009, native fish species were found in this river after STPs, even though they had marked alterations in blood cells and liver tissue (Maceda-Veiga *et al*., 2013). In order to assess the population consequences of these alterations, the same river reaches were re-sampled seasonally in 2012/2013. If blood disorders are likely to have long-term lethal effects, we should find evidence when comparing fish abundance among years. If blood variables have potential to become a new biomonitoring tool, we still expect to find differences among polluted and reference sites in 2012/2013. Finally, we predict pollution stress to be acute for fish during the drought season due to warm temperature and low water flow.

2. Materials and methods

2.1. Study area

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 summer and winter. Water abstraction for industry and human consumption reduces further river water flow, ranging from 0.005 m^3/s to 2.8 m^3/s in a town of our study area over the last 10 years (ACA 2016a). To assess the health status of its fish populations over space and time, we sampled three sampling sites upstream (R1, R2 and R3) and downstream (P1, P2 and P3) the discharge site of three major urban and industrial STPs (Figure 1). Fish surveys were carried out in April 2012, July 2012, November 2012 and February 2013, but only in summer in 2002 and 2009. STPs treat more than 38.000 m^3/day of sewage with ~80% efficiency following a biological treatment with removal of nitrogen and phosphorous. We are not aware of major differences in water treatment over the entire study period.

Previous studies in this region confirm the good ecological status of reference sites, and the impacted status of downstream sites (e.g. Prat and Munné, 2000; Ecoprogress, 2008; Sostoa *et al.*, 2006). Since physical barriers impede fish movements between reference and polluted reaches, our study area provides a unique opportunity to assess the long-term impact of sewage discharges on fish populations of Mediterranean rivers. Due to the heavy pollution in upstream reaches (see Colin *et al.*, 2016b), recolonization of impacted sites from upstream reaches is unlikely. However, fish ladders were built in these reaches after our study in 2009 (Maceda-Veiga *et al.*, 2013), which is used here for comparison. The ladders seemed not to work properly in 2011 (authors observation), but they may have enabled fish to migrate from further downstream sites.

2.2. Fish surveys and focal species

Fish were sampled by a single pass electrofishing using a portable unit which generated up to 200V and 3 A pulsed D.C 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 standards EN 14962 and EN 14011). We focused this study on two native fish species, the Ebro chub (*Squalius laietanus*) and the Mediterranean barbel (*Barbus meridionalis*) belonging to *Cyprinidae*, which is the most frequent fish family in European rivers (Kottelat and Freyhof, 2007). They also typify well the status of freshwater fish in the Mediterranean region. The distribution of these two species have markedly declined in recent decades (Maceda-Veiga *et al.*, 2010), and the barbel is included in the Annexes II and V of the EU's Habitats Directive and in the Appendix III of the Bern Convention (Crivelli, 2006). In addition, *S. laietanus* is a benthopelagic, omnivorous fish, and *B. meridionalis* is a benthic, mostly invertivorous species, which enabled us to assess the differential effect of sewage discharges on fish species with different ecological strategies. The breeding season of *B. meridionalis* ranges from May to June and in *S. laietanus* from April to May (de Sostoa et al. 1990).

2.3. Blood analyses, gross external examination and biometric data

Fish were identified to species level, counted and a representative set of individuals of the two fish species (50 individuals when possible) measured (fork length, mm) and weighted to the nearest mg. All these fish were visually inspected to detect deformities, ulcers or other macroscopic signs of disease. Fish were anaesthetised with a buffered MS222® solution (Tricaine methane-sulfonate, Sigma) to reduce stress. Fish weight and length data was used to calculate the Scaled Mass Index (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 testing it with specific indicators of disease (Peig and Green, 2009).

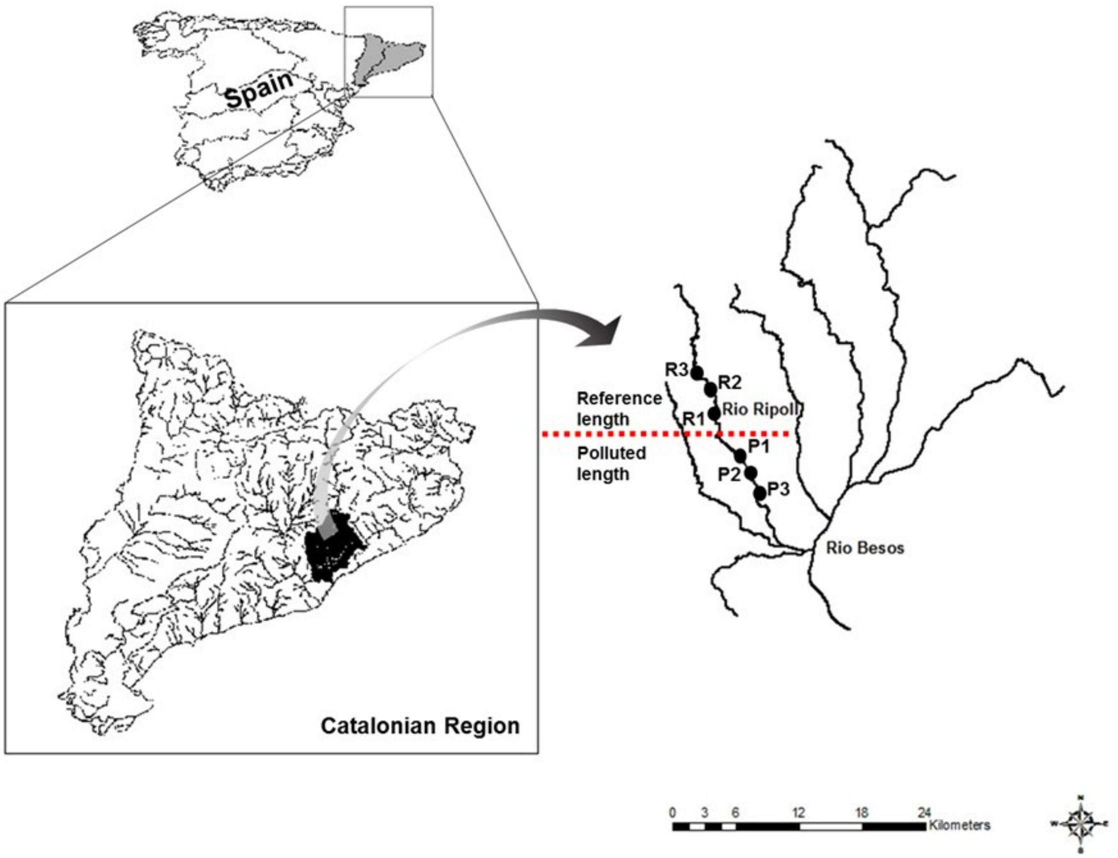

Figure 1. Location of sites sampled from spring 2012 to winter 2013 in Ripoll river, Catalonia region, NE Spain. Upstream (R1, R2, R3) and downstream sites (P1, P2 and P3). Red line indicate limit of polluted length.

Blood analyses followed Maceda-Veiga *et al.* (2015). 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 (n=10 fish/ site/species). A blood smear was then made and air -dried in the field, and stained with Diff-Quick® in the laboratory upon arrival (less than 8 h). To determine fish stress and the presence of infectious diseases, the relative abundance of all types of white blood cells was determined based on 100 white blood cells. To specifically assess the presence of compounds with capacity to cause DNA damage, the frequency of abnormal red blood cells (also known as ENA assay) was recorded based on 1000 red blood cells. Alterations were scored as micronuclei, lobed, kidney-shaped, fragmented nuclei and cells with basophilic stippling. The same procedure was used to determine the frequency of senescent (dead), immature, and dividing red blood cells (see Figure 3 in Maceda-Veiga *et al*., 2015 for cell pictures). These parameters revealed cell lyses or disorders in blood cell synthesis, even though the release of immature cells can also be related to general stress (Fänge and Nilsson, 1985). Results were expressed as (abnormalities/1000 RBCs)*100.

All experimental procedures followed the guidelines provided by the named Animal Welfare Committee (CEEA 510/14), and were approved by the Regional Government of Catalonia (DAAM 7992). Fish captures were standardized to CPUE (fish abundance/time in h x sampled water volume in m^3). We also converted CPUEs from Maceda-Veiga *et al*., (2010) to this scale. We had controlled for differences in sampling area but not in water volume. All fish were released in each sampling site.

2.4. Physical habitat and water quality

Prior to each fish survey, we analysed water quality in each sampling site using a multi-parametric digital probe YSI® 553 MPS for pH, temperature (ºC), conductivity (µS/cm) and dissolved oxygen (mg/l), and the colorimetric test kit VISOCOLOR® (MACHEREY-NAGEL GmbH & Co. KG., Dueren, Germany) for carbonate (ºKH), and general water hardness (ºGH), 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 stressors to the freshwater biota, including nutrient pollution, acidification, and salinization (e.g. Kaushal *et al*., 2005; Johnston et al., 2015; Smallbone et al., 2016). We combined our analyses with monthly water chemical analysis provided by the Catalan Water Agency and a local website (http://www.ub.edu/barcelonarius/visor/), and calculated the median to provide a better picture of variations in water quality over the study period.

To further evaluate changes in river chemical status between reference and impact sites from 2002 to 2012, we gathered the scores of biotic indices based on macroinvertebrates (IBMWP, Alba-Tercedor et al., 2002) and diatoms (IPS, Coste 1982) from technical reports and the stated website. Unique to biotic indicators is their ability to integrate the impacts of complex mixtures of contaminants in water and sediment over time, thereby providing a better picture than water chemistry alone of the river status (see Barbour *et al*., 1999; Colin *et al*., 2016b). We also calculated the fish index IBICAT2b (García-Berthou e*t al.* 2015) to compare with the blood analysis approach. Finally, to describe physical habitat quality, we estimated the percentage of runs, rifles and pools in each site. Additionally, we calculated the QBR (Munné *et al*., 2003) and IHF indices (Pardo *et al*., 2004); two widely used procedures in Spain to appraise the status of the riparian cover and the physical habitat structure of rivers.

2.5. Statistical analyses

All analyses were computed in R (R Development Core Team, 2013) using the libraries stats, car, and lmer4. Generalised linear mixed models (GLMMs) 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 term to avoid pseudoreplication. Fish length was introduced in models for blood variables as covariate to account for differences in fish sensitivity with age. Given that more than 500.000 cells were examined, and various observers were required to speed the process up, observer was also included in the models. However, it was deleted due to the lack of significance. To show the observer effect, we provided coefficients of variation among observers for all

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 (ºC)	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 (ºdGH)	20	20	19	22	22±0.93	24±1.56
Carbonate hardness (ºdKH)	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
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	14	5	15	7	14.7	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 blood variables in reference and impacted 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 each river status throughout the entire study, as it was data on fish in 2002. Thus, we only used descriptive statistics to show variation in CPUEs, biotic indices, and environmental properties across years. Statistical threshold was established at P≤0.05.

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	nd	88±3.2	103±2.5	117±2.8	105±1.5
Scaled Mass Index (g)	14.38±0.44	nd	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	nd	112±3.7	110±2.9	138±2.4	129±10.5
Scaled Mass Index (g)	20.25±0.23	nd	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

3. Results

3.1. Changes in physical habitat structure and water properties over space and time

As expected, water quality decreased in a consistent manner after the input of effluents from sewage treatment plants over the study period (2002-2013) (Table 1). However, polluted sites notably improved in 2013 when compared to 2002 and 2009, as shown by a decrease in conductivity, ammonium and nitrite values. A concomitant increase in dissolved oxygen concentration was also found in polluted sites, together with signs of eutrophication, as denoted by the high levels of phosphates (Table 1). A recovery of macroinvertebrate and diatom assemblies paralleled the improvement in water quality, as shown by an increase in scores of IPS and IBMWP indices in polluted sites over time (Table 1). Water properties and all biotic indices confirmed the good status of reference sites throughout the entire study (Table 1). Nonetheless, the fish index IBICAT2b was the least sensitive to the variations in water properties over space and time (Table 1).

The Fluvial Habitat Index (IHF) score was above 40 in all sites, indicating that changes in physical habitat structure had a little effect on IBMWP and IPS scores. Nonetheless, pools were the predominant habitat in reference sites and riffles in polluted reaches, and river flow markedly declined in 2013 (Table 1). Likewise, the QBR index indicated that the riparian cover was better developed in reference than in polluted sites.

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≤0.05.

	Barbus meridionalis			*Squalius laietanus*		
Blood cell types	Chi	df	P value	Chi	df	P value
Neutrophils						
Pollution	0.11	1	0.74	5.77	1	**0.016**
Season	10.94	3	**0.012**	55.94	3	**<0.001**
Fish size	2.52	1	0.11	11.78	1	**<0.001**
Monocytes						
Pollution	12.33	1	**<0.001**	2.29	1	0.13
Season	52.43	3	**<0.001**	30.66	3	**<0.001**
Fish size	6.98	1	**0.008**	0.93	1	0.33
Abnormal RBCs						
Pollution	3.83	1	**0.05**	17.96	1	**<0.001**
Season	91.11	3	**<0.001**	76.96	3	**<0.001**
Fish size	3.82	1	**0.05**	1.25	1	0.26
Senescent RBCs						
Pollution	0.19	1	0.66	10.48	1	**0.001**
Season	8.77	3	**0.03**	19.47	3	**<0.001**
Fish size	4.32	1	**0.04**	2.34	1	0.13
Immature RBCs						
Pollution	3.11	1	0.08	1.17	1	0.28
Season	34.1	3	**<0.001**	89.66	3	**<0.001**
Fish size	0.97	1	0.33	0.07	1	0.79
Pollution x Season	8.51	3	**0.037**	27.79	3	**<0.001**
Diving RBCs						
Pollution	2.09	1	0.15	5.45	1	**0.01**
Season	2.22	3	0.53	6.25	3	0.14
Fish size	0.09	1	0.75	0.03	1	0.86

Table 4

Median and standard error of white and red blood cell variables in the two native fish species of Ripoll river plus the coefficient of variation of blood readings among observers. Effect size indicates the magnitude of change in blood variables comparing median values in reference and polluted reaches. In bold effect sizes significant at $P \leq 0.05$ (from Table 3).

	Barbus meridionalis					*Squalius laietanus*				
	Reference		Polluted			Reference		Polluted		
	Median±S.E.	CV observ.	Median±S.E.	CV observ.	Effect size	Median±S.E.	CV observ.	Median±S.E.	CV observ.	Effect size
White blood cells										
Neutrophils	6.00±0.47	0.63	5.00±0.46	0.69	-1.20	5.00±0.55	1.12	6.00±0.79	0.64	**+1.20**
Monocytes	2.00±0.26	1.44	4.00±0.41	0.87	**+2.00**	2.00±0.28	0.87	3.00±0.38	1.09	+1.50
Red blood cells										
Abnormal	0.20±0.05	0.83	0.20±0.06	0.76	**+1.25**	0.20±0.05	0.69	0.90±0.13	0.81	**+4.50**
Immature	3.40±0.32	0.54	2.40±0.18	0.62	**-1.40**	1.60±0.27	0.60	3.20±0.15	0.59	**+2.00**
Senescent	0.40±0.09	0.91	0.45±0.07	1.68	+0.89	0.40±0.06	1.29	0.20±0.03	3.19	**-2.00**
Division	0.00±0.00	2.30	0.00±0.00	5.97	0.00	0.00±0.00	1.68	0.00±0.01	2.71	0.00

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

Up to six fish species were found in Ripoll River over the study period (2002-2013), being the Mediterranean barbel (*B. meridionalis*) and the Ebro chub (*S. laietanus*) the only native species (Table 2). Trends in CPUEs varied with species and river status over time, but with a consistent increase for the two native species between 2002 and 2009. Most non-native fish species showed a tendency to decrease in CPUEs over the entire study period, but *L. gibbosus* increased in reference sites in 2013 (Table 2). In this year, *B. meridionalis* also peaked in CPUE in polluted reaches but markedly declined in reference sites. The opposite trend was found for *S. laietanus*, with a slight increase in reference reaches, and a strong decline under pollution 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 shape in reference sites, with larger fish in 2002 and 2012/2013 than in 2009 (Table 2). In polluted sites, median size of *S. laietanus* increased between 2009 and 2012, whereas that of *B. meridionalis* remained stable. Median values of SMI in *B. meridionalis* were also rather stable in polluted reaches over time, but tended to decline for *S. laietanus* (Table 2). In reference conditions, the two native species showed a marked decrease in median values of SMI between 2009 and 2012 (Table 2). No macroscopic signs of disease (either lesions or macroparasites) and blood parasites were observed in the fish examined.

3.3. Effects of pollution, season, fish size and observer on blood variables

The effect of pollution on white (WBCs) and red blood cell (RBCs) profiles was still evident in 2013 (Table 3). Whereas *S. laietanus* markedly increased the neutrophil frequency under pollution, *B. meridionalis* did so with monocyte number (Table 4). No differences occurred in other types of WBC. Eosinophils and basophils were not found, and lymphocytes were the predominant WBC type. Thus, changes in the frequency of monocytes or neutrophils resulted in the opposite effect on relative lymphocyte count. For RBCs, the frequency of abnormal cells increased in *S. laietanus* under pollution, whereas no clear effect was found for *B. meridionalis* (Table 4; but see lobed RBCs in Appendix S1). The frequency of kidney shaped, lobed, and basophilic stippling made the largest contribution to variation in abnormal RBCs between polluted and reference sites. No major differences were observed in micronuclei occurrence. The senescent cell frequency also did not significantly vary with river status in *B. meridionalis*, whereas it notably decreased in *S. laietanus* under pollution (Table 4).

3.4. Interlinking biomarkers and their association with water properties

At least one cell type was significantly related to indicators of poor water quality (Table 5). Squalius laietanus was the species showing the strongest correlations for WBCs and RBCs. A positive relationship was found between monocyte occurrence and ammonium concentration (Table

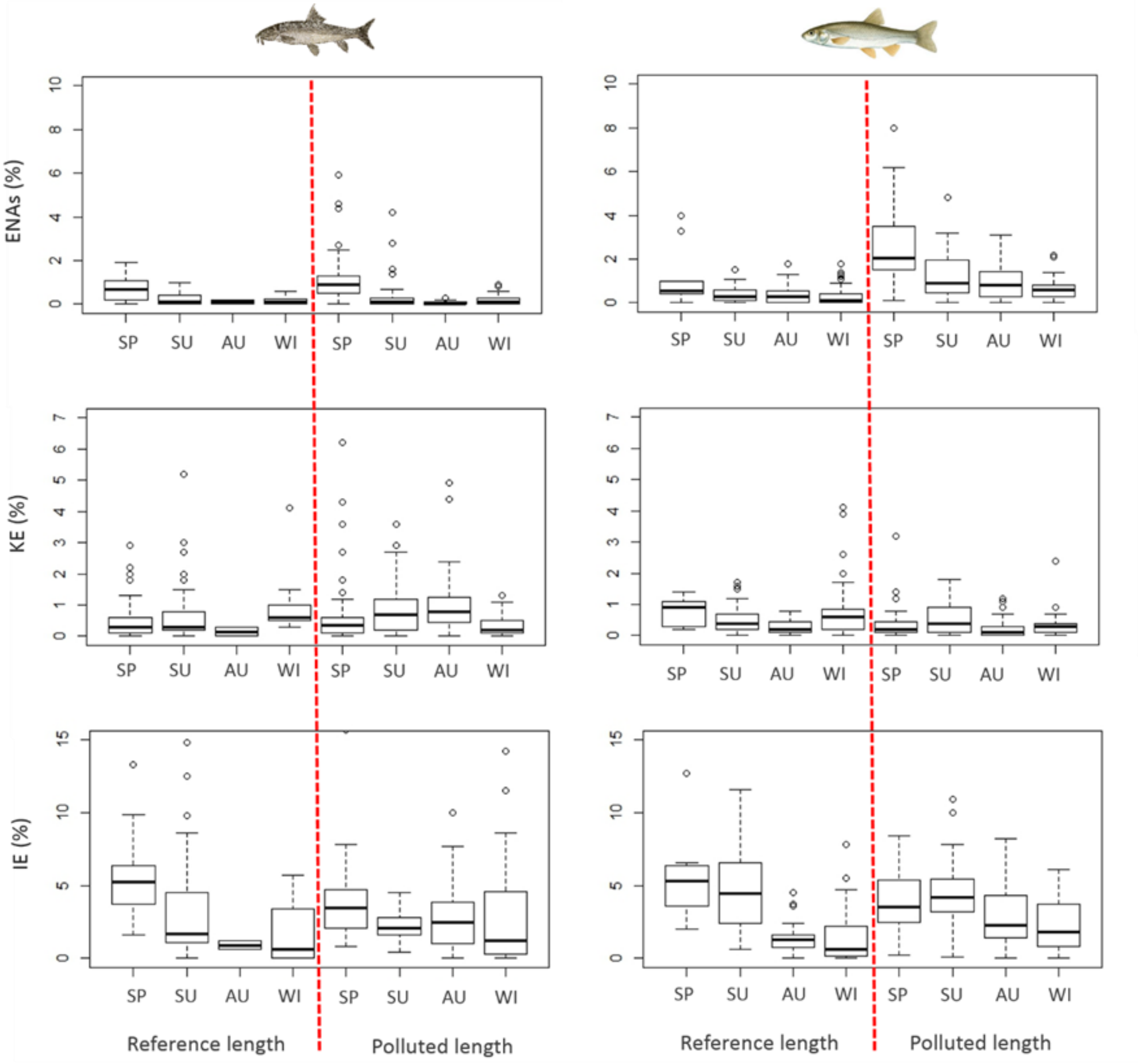

Figure 2. Box plot of mean frequency of erythrocyte abnormal red cells (ENAs), immature red cells (IE) and division red cells in peripheral blood of *B. meridionalis* and *S. laietanus* collected from the Ripoll river, by length river type (reference and polluted) and seasons.

5). The abnormal RBC frequency was also positively associated with conductivity and phosphate values. In contrast, nitrite concentration was negatively related to senescent RBCs (Table 5). For B. meridionalis, only a significant but negative relationship was found between the immature RBC frequency and the levels of nitrite and nitrate (Table 5). Neutrophil occurrence in B. meridionalis also had a negative relationship with temperature, whereas the opposite occurred with immature RBCs in S. laietanus (Table 5). Finally, most biomarkers were not strongly correlated to each other. Only a slight but positive correlation was found between abnormal RBCs and the fish body condition index SMI (Appendix S3).

4. Discussion

The present study shows that the long-term exposure to treated sewage discharges has contrasting effects on populations of two native fish species in a Mediterranean river. Whereas Barbus meridionalis consistently increased in abun-

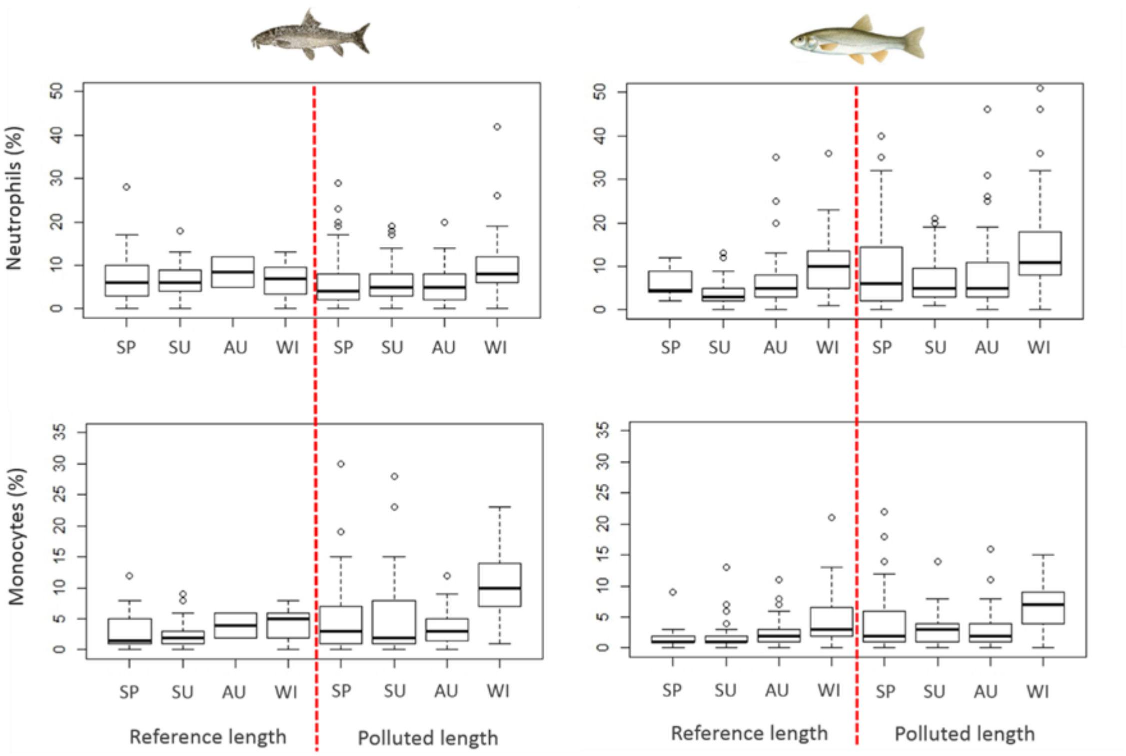

Figure 3. Box plot of mean frequency of leukocyte profile; neutrophil and monocyte, in peripheral blood of *B. meridionalis* and *S. laietanus* collected from the Ripoll river, by length river type (reference and polluted) and seasons.

dance in polluted sites over the study period 2002 -2013, Squalius laietanus markedly declined in 2013. The strength of change in red and white blood profiles indicated that pollution affected more severely S. laietanus than B. meridionalis in 2013. This contrasts with our results in the same study system in 2009 (Maceda-Veiga et al., 2013). We suggested that pollution stress may have intensified in the pelagic S. laietanus over time due to the low water depth of polluted sites. However, specific pollution markers were not higher in summer than in spring, suggesting that the breeding season was more important causal factor than drought. The score of the fish index IBICAT2b did not vary with water quality status.

Our results support that non-destructive blood tests are a superior alternative to the widely used fish indices to assess river status in European rivers, as driven by the EU's Water Framework Directive (EU Commission, 2001). The low diagnostic power of fish-based 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, official monitoring schemes are still firmly rooted in fish indices, and biomarker approaches are mainly used in research (Magalhães et al., 2008; Colin et al., 2016a). A major caveat of the index IBICAT2b is that it masked the cause of impairment in our study. Whereas all indicators agreed with the good status of reference reaches from 2002 to 2009, IBICAT2b scored moderate because of exotic species (see also Benejam *et al*., 2008). When reference sites lacked of them in 2013, a good condition was reached, even though native fish number had not notably improved from previous years. The good condition scored by IBICAT2b in polluted sites further suggests their poor performance as indicator of environmental degradation.

This caveat of the fish index can be offset with the good performance of the diatom and macroinvertebrate indices. They typically encapsulate better slight changes in water quality than those fish-based (Benejam et al., 2008, Colin et al., 2016b). However, the global decline of fish in Mediterranean rivers (Smith and Darwall, 2006) requires of anticipating the lethal effects detected by fish indices to ensure their protection. The differences observed in blood tests between reference and polluted sites in this and our previous study (Maceda-Veiga et al., 2013) make

Table 5

Kendall tau correlation coefficients among the frequency of white (neutrophils and monocytes) and red blood cells (immature erythrocytes, IE; senescent erythrocytes, SE; 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	SE	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

them promising complementary tools in biomonitoring. However, we found similar blood disorders in S. laietanus in our study than in B. meridionalis in 2009 (e.g. peak in neutrophils, abnormal red cells), and the latter increased its abundance under pollution in 2013. Nonetheless, in reference sites, the abundance of B. meridionalis notably reduced in 2013 compared to 2009, suggesting that factors other than water pollution also explain trends in fish populations.

Since drought is a major structuring factor of fish communities (Matthews and Marsh-Matthews, 2003), a lower water depth in polluted sites can explain the observed decrease in S. laietanus abundance compared to reference sites. The low percentage of pools in polluted sites may have increased further the drought stress (Davey and Kelly, 2007), particularly during the drought of 2013. Pools have slower water velocities than riffles, and S. laietanus typically occur in sites with slower water velocities (mean=0.69 m/s) than B. meridionalis (mean=0.90 m/s) (calculated from Maceda-Veiga et al., 2017). Thus, a higher percentage of riffles in the polluted sites from our study may have favoured the benthopelagic B. meridionalis over the pelagic S. laietanus. However, the mechanisms behind variations in fish abundance are likely to be highly complex (e.g. Lake, 2003; Magalhães et al., 2007, Matthews and Marsh-Matthews, 2003). For instance, pelagic fish are easier to capture than benthic fish, and piscivorous birds such as herons were only observed in polluted sites (authors' observation).

Whereas IBMWP revealed major differences in macroinvertebrate composition between polluted and reference sites in 2011, the Scaled Mass Index showed that the two fish species were better nourished in the former. The most plausible explanation for this result is a better food quality in polluted sites because of eutrophy, coupled to a wider trophic spectrum of B. meridionalis and S. laietanus (e.g. Mas-Martí et al., 2010). However, differences in gonad maturation cannot be disentangled from our results. Surveys were carried out in 2012 just after (B. meridionalis, May-June) and later (S. laietanus, April-May) the breeding period of these fish species (de Sostoa et al. 1990).

More specific pollutant indicators were the frequency of abnormal red blood cells, which are related to DNA damage (Ayllon and García-Vazquez, 2000; Pacheco and Santos, 2002). Squalius laietanus had higher levels than B. meridionalis, suggesting that the former was more sensitive to pollution. This apparently disagrees with tolerance categories reported for these species 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 compared to reference sites,

and blood cell synthesis inhibition can reduce the formation of abnormal cells (Pacheco and Santos, 2002). In *S. laietanus*, its high percentage of abnormal cells could also be explained by a lack of cytolitic effects (see Pacheco and Santos, 2002). However, this was an unlikely causal factor because this species had a high frequency of dead red cells in polluted compared to reference sites.

As well as deleterious effects, peaks in blood cells can be interpreted as an ability of animals to cope with stress (e.g. Nunn *et al*. 2000; Davis *et al*., 2008) in the absence of normal ranges (Maceda-Veiga *et al*., 2015). Further, the observed peak in monocytes in *B. meridionalis* can be related to infection diseases (Davis *et al*., 2008). Although we did not observe signs of disease by naked eye, pathogens tend to proliferate in warm, polluted waters at high fish density. However, the breeding season (spring) seemed to have been more stressful for fish than summer; then the observed increase in blood cell synthesis, as estimated by the percentage of immature cells, can reflect the ability of fish to acclimate to this period of high oxygen demand. Nonetheless, chronic pollution exposure may have caused effects at other levels (e.g. behaviour, gonad alterations, trophic ecology, genetic structure) not assessed in our study that should be examined to fully determine the sensitivity of these fish populations to pollution.

5. Conclusions

Our study highlights the need of combining more population analyses with biomarkers in order to better predict the population consequences of their outputs. Blood tests had higher diagnostic power than a local fish index. Lower coefficients of variation among observers in specific pollution indicators such as the abnormal cell frequency further highlights their diagnostic potential. However, the lack of normal ranges precludes making straightforward diagnostics. That being said, this study shows that recycled water has genotoxic risk and potential to cause multi-trophic impacts in River Ripoll. Thus, a more efficient sewage management strategy under water scarcity is recommended, and blood tests can help to monitor the consequences for fish health.

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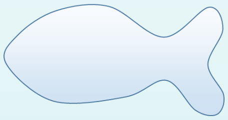
Appendix

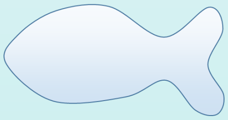
A1. Results of GLMMs on the effects of pollution, season and fish size on each type of RBC abnormality measured in the two native fish species in Ripoll River. Site was included as random term in all models. Pollution effect shows the strength and direction of the correlation, and bold values indicate significance at at $P \leq 0.05$.

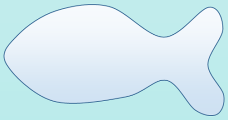
	Barbus meridionalis				*Squalius laietanus*			
Abnormalities	Chi	df	P value	Pollution effect	Chi	df	P value	Pollution effect
Kidney shaped								
Pollution	1.99	1	0.16	ns	25.24	1	**<0.001**	1.09±0.22
Season	71.31	3	**<0.001**		66.24	3	**<0.001**	
Fish size	2.24	1	0.13		2.21	1	0.14	
Lobed								
Pollution	19.12	1	**<0.001**	1.10±0.25	0.39	1	0.53	ns
Season	47.98	3	**<0.001**		37.66	3	**<0.001**	
Fish size	11.03	1	**<0.001**		0.20	1	0.65	
Fragmented nuclei								
Pollution	0.04	1	0.84	ns	0	1	0	ns
Season	0.1	3	0.99		0	3	1	
Fish size	0.71	1	0.39		2.29	1	0.13	
Basophilic stippling								
Pollution	0.91	1	0.34		5.41	1	**0.02**	2.28±0.99
Season	0.09	3	0.99		12.31	3	**0.006**	
Fish size	0.88	1	0.35		0.29	1	0.59	
Micronuclei								
Pollution	0.68	1	0.41		0.19	1	0.66	
Season	2.62	3	0.45		7.09	3	0.07	
Fish size	1.41	1	0.24		1.38	1	0.24	

A2. Kendall tau correlation coefficients among fish blood variables and the body condition index SMI at the individual level for each native fish species in Ripoll River. In bold coefficients significant at P≤0.001.

	ENAs	IE	DE	SE	SMI	Neutrophils
Barbus meridionalis						
Abnormal RBCs						
Inmature RBCs	**0.231****					
Dividing RBCs	-0.071	0.067				
Senescent RBCs	-0.128*	-0.087	0.065			
Scaled Mass Index	**0.186****	0.118*	-0.016	-0.075		
Neutrophils	0.029	0.024	-0.042	-0.068	-0.094	
Monocytes	-0.066	-0.035	0.057	0.014	0.040	**0.204****
Squalius laietanus						
Abnormal RBCs (ENAs)						
Inmature RBCs (IE)	**0.218****					
Dividing RBCs (DE)	0.054	0.099				
Senescent RBCs (SE)	-0.125*	-0.036	-0.008			
Scaled Mass Index	**0.241****	**0.263****	0.069	-0.006		
Neutrophils	0.032	-0.148*	-0.008	-0.055	-0.081	
Monocytes	-0.047	-0.092	0.019	-0.015	0.007	**0.200****







DISCUSIÓN GENERAL

Más de 100 años después del desarrollo del embrión de los actuales índices de calidad biótica (ICB) – el sistema de saprobios – son muchas las voces que claman por la adopción de nuevas estrategias de monitoreo ambiental (Friberg et al. 2011, Woodward et al. 2015). La presente tesis ahonda en esta necesidad (Capítulos 2, 3 y 4) no sin antes reconocer (Capítulo 1) el valor diagnóstico los actuales ICB para detectar el impacto ecológico de estresores ambientales (vertidos industriales) diferentes de para los que fueron creados en origen (contaminación orgánica). Es probable que las agencias de gestión del agua vean con recelo la propuesta de métodos alternativos de monitoreo después de la gran inversión en creación de múltiples ICB adaptados a sus aguas jurisdiccionales. No es objetivo de esta tesis deslegitimar el papel que han tenido los ICB durante todos estos años, ni tampoco ignorar su potencial para subsanar algunas críticas como su bajo poder predictivo. Ahora bien, resulta lícito resaltar que su poder diagnóstico merma en demasía cuando se aplican a comunidades pobres en especies, como las de peces de los ríos mediterráneos ibéricos, y que los intentos para incorporar mejoras tampoco puede decirse que hayan sido muy fructuosos. A este problema se le añade, como muestra el Capítulo 4, que los ICB de peces emiten un diagnóstico confuso al mezclar causas bióticas (invasiones) y abióticas (contaminación), problema que tampoco se ha estudiado en profundidad para el resto de taxones. Y, finalmente, desde un punto de vista de conservación, no resulta deseable que una herramienta de diagnóstico con la finalidad última de proteger la biodiversidad detecte impacto sólo tras un descenso marcado en la abundancia o incluso la desaparición de alguna especie. Es por todo esto, además de mejorar nuestro conocimiento mecanicista sobre cómo el estrés afecta a las comunidades, que esta tesis aboga por el uso de nuevas herramientas de diagnóstico basadas en la diversidad funcional y los biomarcadores.

El uso de bioindicadores y la contaminación en ríos mediterráneos

La presente tesis ilustra en los Capítulos 1 y 4 que los vertidos de aguas residuales, tanto urbanas como industriales, son todavía un problema grave de degradación ambiental en el río Ripoll. Esta situación es compartida con otros ríos mediterráneos donde el bajo caudal merma el éxito de procesos de depuración que se aplican en cuencas del norte de Europa (Prat & Munné 2000). Debe reconocerse que la situación ecológica del río Ripoll ha mejorado en los últimos años. Como se declara en el Capítulo 4, pasó de carecer de peces en los tramos bajos hasta el año 2000 (inmediaciones de la ciudad de Sabadell), a tener poblaciones de barbos (*Barbus meridionalis*) y bagres (*Squalius laietanus*) a partir del año 2006 de acuerdo a nuestros muestreos (Sostoa et al. 2006). Si bien la calidad del agua en estos tramos permite la vida piscícola, no es así aguas arriba donde la presencia de la industria textil "La Satina" es un auténtico punto negro (ver Capítulo 1). Existe una depuradora en dicha empresa, aunque desconocemos qué tipo de tratamiento realiza.

Puede darse el caso que esté infra-dimensionada para el volumen de aguas residuales que trata o, más probablemente, que aplique sólo un tratamiento basado en fangos activos que resulta del todo inadecuado para tratar efluentes industriales. Estos efluentes están formados mayoritariamente por compuestos refractarios a la degradación biológica, y éste es el proceso dominante en las plantas de tratamiento de aguas residuales convencionales (ej. Pérez-Uz et al. 2010, Canals et al. 2013, Ramírez et al. 2014).

Existe la tecnología para mejorar la calidad de los efluentes de depuradora sea cual sea su composición química (ej. Radjenovic et al. 2008, 2009, Becker et al. 2016), el problema es su coste. Sin duda, optimizar el tratamiento de aguas de residuales es uno de los retos más importantes de futuro en los sobreexplotados ríos mediterráneos (Barceló & Petrovic 2008, Brack et al. 2015). Su poder de dilución va a la baja por la excesiva captación de agua y los albores del cambio climático, mientras la cantidad de contaminantes de nueva síntesis que generamos, unidos a los preexistentes, no para de crecer (Verlicchi et al. 2010, Petrovic et al. 2011, López-Serna et a. 2012). Sirve de poco que las agencias del agua crean la figura de "masa de agua muy modificada" para escapar de los estándares de calidad ecológica en ríos muy impactados. Es hacerse trampas al solitario teniendo en cuenta que, ante la escasez de agua, el uso de agua reciclada, junto con la creación de plantas desaladoras para agua de boca, será la norma en regiones con clima árido o semiárido como la cuenca mediterránea (Anderson 2003, Hurlimann & McKay 2007, Dolnicar et al. 2011).

Un factor clave en la adopción de nuevas estrategias de manejo de los efluentes de aguas residuales es el contar con herramientas de monitoreo ambiental eficientes. Entre las múltiples críticas que reciben los ICB está que fueron diseñados básicamente para detectar contaminación orgánica, especialmente los de macroinvertebrados y diatomeas (Friberg et al. 2011). Sin embargo, esta tesis (Capítulo 1) se une a otros trabajos existentes, donde queda demostrada su capacidad para detectar otro tipo de impactos, incluidos los vertidos de aguas residuales industriales (Sabater 2000, Munné & Prat 2009, Carafa et al. 2011). Además, este estudio es, probablemente, uno de los pocos realizados en el área mediterránea, incluyendo la península Ibérica (Hering et al. 2006, Benejam et al. 2008, Marzin et al. 2012), en los que se compara el poder diagnóstico de ICB para múltiples taxones (diatomeas, macrófitos, macroinvertebrados y peces). Dicha comparativa permitió corroborar que son los índices de diatomeas y macroinvertebrados los que mejores captaron la variación en la calidad del agua del tramo estudiado a lo largo de todo el año. Esto se explica por su ciclo de vida más rápido que da lugar a ventanas de respuesta más cortas (Barbour et al. 1999). Por el contrario, los peces tienen un ciclo de vida más largo y sus poblaciones tardan más en recuperarse tras un impacto en ausencia de migración. Así pues, en el Ripoll, aunque la calidad del agua de los tramos más contaminados, en época de estiaje, mejoró con el aumento del caudal del río tras la llegada de las lluvias, los tramos aguas abajo de la industria textil carecieron de peces durante todo el periodo de estudio. Existió la posibilidad de migración, ya que no hay barreras físicas en la cuenca y aparecen poblaciones de peces a 1.5 km de la entrada del efluente de la depuradora. Aunque desconocemos las razones de fondo, a las posibles causas explicadas en el Capítulo 1, quizá podría añadirse que los peces detecten las débiles señales químicas que vienen de los tramos contaminados y decidan no migrar aguas arriba, especialmente el pelágico *S. laietanus*. Sea como fuere, los peces fueron el taxón más afectado por los vertidos de la industria y en las estaciones del año con bajo caudal (invierno y verano), aquellas en los cuales los efectos ecológicos fueron más severos. Se confirma, por tanto, que el estiaje intensifica el estrés por contaminación en la fauna acuática (Petrovic et al. 2011), aunque no parece que sea siempre debido a su efecto sinérgico con el incremento de temperatura como sugieren los trabajos experimentales (ej. Chalifour & Juneau, 2011, Chandra et al. 2012, Maceda-Veiga et al. 2015).

Índices de diversidad funcional como metodología complementaria a los ICB

Si bien los ICB permitieron detectar impacto en los tramos del río Ripoll bajo los efluentes de la industria textil, no puede decirse que mejoraran nuestro conocimiento mecanicista sobre cómo éstos afectaron a la fauna acuática. Los ICB permitieron identificar al taxón más afectado, pero pensemos que una comparación de riqueza de especies hubiera dado el mismo resultado al encontrarse los tramos más contaminados defaunados. Sobre el conocimiento mecanicista, puede asumirse por la teoría ecológica de filtros ambientales (Southwood 1977, 1988, Odum 1985) que los cambios en las puntuaciones de los ICB entre localidades contaminadas y de referencia son debidos a la pérdida de las especies sensibles. Sin embargo, las aproximaciones de ICB no permiten testar a priori hipótesis basadas en la teoría ecológica (Baird et al. 2008, Friberg et al. 2011, Woodward et al. 2015). Además, no permiten comparar la respuesta de las comunidades entre áreas geográficas -aún bajo el mismo clima- al haberse desarrollado más de 200 ICBs para hacer frente a la idiosincrasia de cada región (Birk et al. 2012). Éste es un problema común en las aproximaciones que se basan en caracteres taxonómicos en lugar de funcionales. Aunque sean geográficamente distantes, todos los ríos de clima mediterráneos comparten una serie de filtros ambientales que determinan la biología y ecología de las especies que en ellos habitan (Bonada & Resh 2013). Así pues, los barbos de montaña (*Barbus meridionalis* y *Barbus haasi*) son taxonómicamente diferentes, viven en cuencas separadas, pero son ecológicamente muy similares (de Sostoa et al. 1990). Esto hace presagiar que un monitoreo basado en rasgos funcionales pueda hacer que comprendamos mejor los patrones generales de respuesta de la comunidades a los estresores antrópicos.

Vistas las limitaciones ecológicas de los ICB, en el Capítulo 2 nos planteamos testar una aproximación funcional como herramienta de diagnóstico en las comunidades de peces, de lejos uno de los grupos taxonómicos que ha sufrido una mayor regresión en los ríos mediterráneos (Elvira 1995, Smith & Darwall 2006, Maceda-Veiga 2013). Puede asumirse que rasgos de composición de la comunidad correlacionan con los rasgos funcionales, sin embargo, se ha visto que ambos procedimientos proveen información complementaria (Hladyz et al. 2011, Villeger et al. 2012). Además, cabe pensar que del conjunto de rasgos funcionales que caracterizan a una especie pueden existir rasgos que estén fuertemente correlacionados, pero que otros puedan ser ortogonales (Herrera 2009).

Nuestra experiencia en el Capítulo 2 en más de 10 cuencas del noreste español muestra que los rasgos funcionales tienen un gran potencial como una herramienta de diagnóstico en las comunidades de peces. En particular, mostraron cierta correlación con indicadores de degradación ambiental; uno de los principales impactos de los ríos mediterráneos (Hermoso & Clavero 2011, Moyle 2011, Maceda-Veiga 2013). Además, la correlación entre los índices de diversidad funcional y los indicadores ambientales fue más fuerte que la que mostró el ICB para peces. No obstante, no debe olvidarse que la estructura funcional de la comunidad de peces estuvo mayoritariamente explicada por factores geográficos (ej. cuenca, altitud) como suele ocurrir en los estudios de peces a macroescala (ej. Murphy et al. 2013, Maceda-Veiga et al. 2017).

La existencia de colinearidad entre predictores naturales y de origen antrópico es común en los estudios de macroescala lo que impide establecer relaciones de causa efecto. Sin embargo, a través del análisis de los efectos parciales (ver Mac Nally 2002, Peres-Neto et al. 2006) de los predictores de interés (estresores antrópicos) sobre las variables respuesta (índices de diversidad funcional) es posible hacerse una idea sobre su importancia relativa. En este sentido, hay que destacar la contribución relativa que tuvieron la riqueza de especies exóticas de peces en los análisis de diversidad funcional del Capítulo 2. Esto pudiera plantear la realización de un estudio de diversidad funcional exclusivo para las especies nativas, pero lo cierto es que las especies exóticas juegan un papel cada vez más importante en las comunidades de peces de los ríos mediterráneos (Marr et al. 2010). Sin ir más lejos, en algunas de las cuencas estudiadas, el porcentaje de especies exóticas supera en más del 50% al de nativas (Maceda-Veiga et al. 2017), razón por la cual decidimos analizar la comunidad de peces en su conjunto. Es probable, por tanto, que la señal de la correlación entre los índices de diversidad funcional y las variables ambientales haya sido menor de la esperada para la fauna nativa. La mayoría de especies exóticas de nuestra área de estudio se ven favorecidas por las alteraciones hidromorfológicas tales como la canalización y construcción de azudes (de Sostoa et al. 1990).

Una variable controvertida que se vislumbra de la aplicación de los índices de diversidad funcional es la redundancia funcional (Mouillot et al. 2011). En especial, es preocupante que pueda utilizarse como una manera de evaluar la resistencia de los ecosistemas a los impactos antrópicos. Es decir, se asume que en una comunidad con una elevada redundancia funcional, más de una especie realiza “el mismo rol” en el ecosistema, con lo cual su pérdida no es de esperar que cause “mayores” problemas. En esta definición, ante todo, no debe olvidarse que la conservación del patrimonio natural tiene un valor *per se* y todavía estamos lejos de comprender a ciencia cierta el papel de las especies en los ecosistemas. Razón por la cual los resultados de estos índices de diversidad funcional están condicionados por la matriz de rasgos utilizada (Mouillot et al. 2011, Villéger et al. 2012). Es, por tanto, esperable que, dada la diversidad de rasgos que caracterizan a una especie, otro conjunto de rasgos biológicos y ecológicos cambie los resultados. Además, que un impacto no tenga efectos aparentes sobre la estructura funcional actual del sistema no quiere decir que no hayamos erosionado su respuesta funcional desde un punto de vista evolutivo (Díaz et al.

2013).

Se dice que los rasgos funcionales deben seleccionarse en base a criterios ecológicos ligados a funciones ecológicas concretas (Mouillot et al. 2011, Villeger et al. 2012). Por ejemplo, el tamaño corporal está correlacionado con el metabolismo de los animales (Kleiber 1932) y por ende con su actividad en el ecosistema (Peters 1986, Brown et al. 2004). Los problemas surgen cuando dos especies de tamaño similar tienen, por ejemplo, también una ecología trófica similar. Si el número de rasgos funcionales es reducido – pensemos en una comunidad pobre en especies- es esperable que el potencial diagnóstico de los índices de diversidad funcional también se reduzca. Por tanto, tienen estos índices un problema compartido con los ICB de peces en ríos mediterráneos ibéricos, al que se le suma la dificultad para su cálculo en comunidades pobres (Maire et al. 2015). Una solución para esto, pensando retrospectivamente en nuestro caso, hubiera sido utilizar la identidad de los rasgos funcionales sin encapsularlos en forma de índice. No obstante, existe una discusión abierta sobre si el uso de muchos rasgos mejora los resultados, especialmente cuando todavía tenemos carencias de historia natural.

Un ejemplo de estos problemas de carencia de historia natural es que haya especies que no constituyan grupos funcionales obvios (ej. herbívoros), como ya ha pasado con animales carismáticos marinos como la tortuga verde *Chelonia mydas* (omnívora; Cardona et al. 2010) o el erizo de mar *Arbacia lixula* (omnívoro; Wangesteen et al. 2011). Además, se ha visto en estudios de polinizadores que pocos rasgos bien correlacionados con la función ecológica de interés (tamaño del fruto) la predicen mejor que la simple diversidad de rasgos (Garibaldi et al. 2015). Por tanto, los estudios de diversidad funcional no pueden desligarse de los de historia natural; siendo fundamentales para entender la compleja arquitectura de la biodiversidad, aunque sean menos rentables académicamente dentro de los actuales estándares de "calidad". Otro problema de los estudios de diversidad funcional, que también se aplica a nuestro caso, es que pocas veces se examinan simultáneamente los procesos ecológicos y estructura funcional de las comunidades. La mayoría son inferencias a partir de datos de comunidad y es conocida la estructura dinámica de las redes ecológicas con cierta capacidad amortiguadora (Verhoef & Off 2010, Bascompte & Jordano 2013). Esto lleva implícita una visión sesgada sobre la importancia a nivel de ecosistema de los resultados encontrados, y por consiguiente de los rasgos seleccionados, aunque parece existir consenso en que la riqueza funcional explica más varianza de una comunidad dada que la específica (Gagic et al. 2015).

Uso de Biomarcadores en sistemas mediterráneos

A la necesidad de identificar qué rasgos funcionales se relacionan mejor con los diferentes procesos ecológicos, se le añade la falta de indicadores de causa-efecto. Este hueco podrían ocuparlo los biomarcadores tal y como se detalla en el Capítulo 3. Si bien es cierto que no todos tienen esta propiedad, se cumple con bastantes de ellos (ej. el ensayo de anomalías eritrocitarias, incremento en transaminasas). En el peor de los casos pueden no identificar el agente causal, pero diferenciar entre causas abióticas (calidad del agua) y bióticas (invasiones), y el tipo de contaminación. Por ejemplo, un incremento en transaminasas está relacionado con el daño hepático y un elevado porcentaje de anomalías eritrocitarias con la presencia de compuestos genotóxicos (Maceda-Veiga et al. 2015). En algunos casos, el diagnóstico es bastante certero, por ejemplo, la inhibición de la enzima acetilcolinesterasa está relacionada con la exposición a pesticidas organofosforados (Ranjbar et al. 2002). Si a esto le sumamos que algunas vías metabólicas están muy conservadas en las ramas evolutivas de los vertebrados, es fácil pensar en la capacidad diagnóstica con potencial predictivo de los biomarcadores.

Especies cercanas filogenéticamente es esperable que tengan una respuesta fisiológica similar, aunque nunca debe olvidarse la variabilidad intraespecífica en ecología (West-Eberhard 1989). Así pues, no hace falta escudriñar los patrones de respuesta de todas las especies si no que se trata de identificar aquellos biomarcadores con mayor señal filogenética y poder diagnóstico. Del mismo modo, no se puede pretender estudiar los efectos tóxicos de toda la compleja amalgama de compuestos liberados al medio, incluyendo los contaminantes emergentes (Verlicchi et al. 2010, López-Serna et a. 2012). La identificación de las posibles sinergias y patrones de respuesta comunes por grupos de contaminantes (ej. antibióticos betalactámicos, pesticidas organofosforados, antiinflamatorios esteroideos) es una posible aproximación tal y como se declara en el Capítulo 3. Este capítulo también destaca la importancia de detectar efectos sub-letales en piezas clave para el funcionamiento de los ecosistemas como son los peces. Es bien conocido que la desaparición del depredador en la cúspide de la cadena trófica desencadena efectos severos en cascada a nivel de todo el ecosistema (ej. Gende et al. 2002, Myers et al. 2007, Letnic et al. 2012) y, los peces de río, a escala de masa de agua, ocupan un nivel trófico muy elevado. Así pues, determinar su estado de salud con certeza tiene ramificaciones que van más allá de su conservación tal y como se ha demostrado experimentalmente en ríos mediterráneos (Rodríguez-Lozano et al. 2015).

Uno de los problemas que se puso de manifiesto en la revisión de usos de biomarcadores en ríos mediterráneos (Capítulo 3) es el predominio de muestreos destructivos (letales) y el uso de muchos biomarcadores, a menudo con valor diagnóstico redundante. Tal y como se comentaba para el caso de la diversidad funcional, no está claro que el uso de múltiples biomarcadores mejore el diagnóstico. Mucho menos facilita la interpretación de los resultados, especialmente si se integran su información en forma de gradientes (análisis de componentes principales) como viene siendo al uso. Estos análisis son muy populares en ecología pero no permiten identificar la contribución de cada una de las variables que incluyen, además de asumir una relación lineal entre variables (Tabachnick et al. 2001; Vaughan & Ormerod 2005). Esto implica que para conocer el valor diagnóstico real de cada uno de los biomarcadores sea necesario repetir los estudios con la misma combinación de biomarcadores en otro sistema. Mucho más fácil de interpretar -especialmente para los gestores- resulta, por ejemplo, un análisis de correlaciones por pares para eliminar los biomarcadores que den información redundante y realizar a continuación gráficos de cajas y bigotes para cada uno de los seleccionados por tramos de referencia y contaminados como se vislumbra de las recomendaciones metodológicas realizadas en el Capítulo 3.

Los muestreos destructivos no suelen suponer un problema en los estudios de especies no amenazadas, aunque siempre resulta no deseable desde un punto de vista ético y más con la entrada en vigor de la nueva legislación en bienestar animal (Orden Ministerial ECC/566/2015). En el Capítulo 3 se puso de manifiesto el potencial de los análisis de sangre en peces como herramienta de diagnóstico, evidencia que se suma a los trabajos previos publicados por nuestro equipo (Maceda-Veiga et al. 2010, 2013, 2015). Junto con destacar sus ventajas, todos estos trabajos también hacen énfasis en que el principal problema de este tipo de diagnósticos en peces salvajes es la ausencia de rangos de normalidad. Dicho de otro modo, no existe la posibilidad, como en medicina humana o veterinaria de animales domésticos, de asegurar con el análisis de sangre el estado de salud del animal. Algunos parámetros son más informativos que otros en este aspecto, pero sigue siendo necesario comparar entre localidades de referencia y contaminadas para acercarnos al diagnóstico correcto. Esta incertidumbre puede hacer que alteraciones a priori severas en unos individuos concretos no tengan mayor repercusión en la población de peces en generaciones futuras. Siendo la población la unidad de conservación qué duda cabe que la relevancia ecológica de los resultados baja si no permiten predecir cambios a escala mayor de individuo.

Aproximaciones multi-disciplinares y proyecciones futuras en biomonitoreo

Siendo conscientes de esta problemática nos planteamos el Capítulo 4 de la presente tesis doctoral que tuvo un doble objetivo. Por un lado, el análisis de la variabilidad temporal en los parámetros sanguíneos nos permitió conocer mejor los rangos de normalidad de estos para el barbo de montaña (*B. meridionalis*) y la bagra (*S. laietanus*). Junto con mejorar el potencial diagnóstico de los análisis de sangre para estas dos especies nativas de los ríos mediterráneos, este capítulo también permitió profundizar en la relevancia ecológica de estas analíticas. Es por ello que combinamos datos de análisis de sangre de trabajos previos a esta tesis (2009) y el actual (2012/2013) con series temporales de cambios en la abundancia de las dos especies (2002 -2013). De haber supuesto un problema serio para la viabilidad de la población las alteraciones reportadas en trabajos previos, nos esperábamos un descenso en la abundancia de peces en el siguiente muestreo. Resulta sorprendente, por tanto, que la especie con más alteraciones hematológicas en el año 2009 (*B. meridionalis*) presente un incremento en su abundancia en el 2011/2012, mientras que la especie en situación contraria (*S. laietanus*) haya reducido notablemente su abundancia hoy y en día.

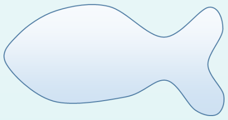
No se han producido mejoras significativas en el proceso de depuración de aguas en el tramo estudiado ni tampoco existe posibilidad de migración de tramos de referencia a contaminados por existencia de barreras físicas. Ahora bien, existen marcadas referencias hidromorfológicas (% de rápidos, tablas y pozas) entre localidades de referencia y contaminadas, y se produjo un descenso de caudal del río en 2010/2011. Puesto que *B. meridionalis* y *S. laietanus* tuvieron tendencias opuestas en cambios de abundancia en los tramos de referencia a las observadas en los contaminados, esto nos hace suponer que las causas no deben buscarse exclusivamente el efecto directo de los tóxicos. Es bien sabido el papel estructurador que tiene el régimen hidrológico sobre las comunidades de peces en ríos mediterráneos y, en especial, el papel de las pozas como refugio durante el estiaje (Lake 2003, Matthews & Marsh-Matthews 2003, Davey & Kelly 2007, Magalhães et al. 2007). Esto unido los diferentes hábitos natatorios de *B. meridionalis* (bentopelágico) y *S. laietanus* (pelágico) nos hace suponer que la disminución de la columna de agua en las localidades contaminadas haya supuesto un estrés adicional muy fuerte para *S. laietanus*. No en vano, sus efectivos poblacionales incrementaron en las localidades de referencia con mayor presencia de pozas que los tramos contaminados mientras los del barbo descendieron bajo estas mismas condiciones hidrológicas.

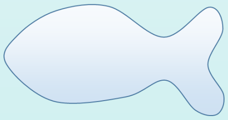
Como ocurre en los estudios observacionales resulta difícil asegurar si la tolerancia relativa de las dos especies a la contaminación cambió con los años y si existen diferencias en su capacidad de aclimatación, algo abordable sólo en condiciones experimentales donde pueda separarse el efecto de la contaminación del hidrológico. Tampoco podemos desligar nuestros resultados de los efectos de la depredación por parte de garzas (F. Ardeidae) que fueron frecuentemente observados en los tramos contaminados (observ. personal). Es de esperar que depreden más sobre peces pelágicos al ser más fáciles de localizar que sobre los bentónicos. En resumidas cuentas, los resultados de este último análisis hematológico parecen contradecir las categorías de tolerancia relativa a calidad de agua pobre asignadas para *B. meridionalis* y *S. laietanus* a través de medias ponderadas de abundancia en estudios a gran escala (Maceda -Veiga & de Sostoa 2011, Segurado et al. 2011). Sin embargo, sin analizar su respuesta a tóxicos concretos y determinar la influencia relativa de los cambios en la columna de agua es difícil asegurar cuál de las dos especies es más tolerante. Ahora bien, todo parece apuntar que para estas dos especies, el periodo reproductor fue todavía más estresante que el estiaje.

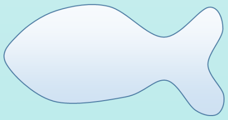
Además de comparar la tolerancia relativa de *B. meridionalis* y *S. laietanus* a un gradiente de degradación de calidad de agua, en el Capítulo 4 también se realiza un estudio comparativo de poder diagnóstico de los estándares ICB con los análisis de sangre. Como ya se puso de manifiesto en el Capítulo 1, los ICB de diatomeas y macroinvertebrados capturaron mejor la variación en la calidad del agua del área de estudio. De hecho, el ICB de peces identificó como impactado los tramos de referencia, nuevamente debido al diagnóstico confuso que realiza de degradación por causas bióticas (presencia de especies exóticas) y bióticas (contaminación). Por tanto, para evaluar el estado de salud del río resultaron más acertados los ICB de otros grupos taxonómicos junto con los análisis de sangre para peces. En especial, porque el ICB de peces no detectó impacto en el tramo contaminado pese a que el resto de indicadores, incluidos los otros ICB, así lo hicieron. En relación a los análisis de sangre, el parámetro más ligado a la contaminación fue el porcentaje total de eritrocitos anómalos a diferencia del porcentaje de micronúcleos que es el que tiene mayor tradición en ecotoxicología (Fenech et al. 2003, Barata et al. 2010). El Capítulo 4 apoya, por tanto, la necesidad de examinar todos los tipos de anomalías eritrocitarias puesto que, aunque tedioso, es muy informativo pudiéndose dar falsos-negativos de efectos genotóxicos con sólo la observación de micronúcleos.

La existencia de indicadores tradicionales (ICB de diatomeas y macroinvertebrados) que detectaran la existencia de riesgo ecológico en los tramos contaminados puede llevar al lector a la disyuntiva sobre la necesidad de tediosos ensayos de sangre en peces. Todavía es pronto para generalizar estos correlatos, sin obviar que es bien sabido que diferentes combinaciones de estresores afectan de manera diferente a los cuatro grupos taxonómicos que se utilizan en biomonitorización (Barbour et al. 1999). Una posible aproximación es realizar un biomonitoreo jerarquizado, donde la obtención de respuesta en unos indicadores lleve a realizar pruebas adicionales, algo así como los diagnósticos médicos. Por ejemplo, si los macroinvertebrados dan señal de mala calidad de agua quizá sea conveniente realizar un estudio de biomarcadores en peces para acabar de valorar el riesgo ecológico. Asimismo, parece claro que, aun usando procedimientos de monitoreo estándares, es necesario desligar la riqueza de especies exóticas de las variables de impacto ambiental. Son problemas diferentes que requieren soluciones distintas e interesa que los gestores tengan información diagnóstica lo más precisa posible.

En la disyuntiva sobre que bioindicador es ignorable no debe olvidarse que los peces son un nodo muy importante para el funcionamiento de los ecosistemas, no sólo desde un punto de vista trófico sino también de relaciones de hospedador-parásito. Por ejemplo, las náyades de agua dulce (F. Unionidae) –muy amenazadas a escala global-dependen de los peces nativos para completar su ciclo biológico al ser sus larvas parásitas obligadas (gloquideos) (Lopes-Lima et al. 2017). Además, como se indicaba en el párrafo precedente, muchas especies de aves y mamíferos (ej. Nutria; Ruiz-Olmo et al. 2001) se alimentan de peces. Por tanto, inferir el estado de salud de las poblaciones de peces a partir de otros grupos taxonómicos es un riesgo que no podemos asumir, en especial dado el reducido número de especies nativas que naturalmente habitan en nuestros ríos y su estado de conservación. Los análisis de sangre suponen un paso más hacia su estandarización como herramienta de diagnóstico tanto en peces salvajes como cautivos. Y, los estudios de tolerancia relativa, otro paso al frente a la hora de identificar a las especies más amenazadas por los impactos antrópicos y a las que suponen un mayor riesgo medioambiental. Una especie sensible muere por los efectos de la contaminación, pero la relativamente tolerante acumula tóxicos a lo largo de su vida y existe mayor probabilidad que pueda transferirlos a toda la red trófica a través de sus depredadores.







CONCLUSIONES

1. En pleno siglo XXI los vertidos de aguas residuales todavía causan graves impactos ecológicos en los ríos mediterráneos. Mejorar el tratamiento de aguas residuales junto con garantizar los caudales ambientales es el gran reto de futuro, especialmente en los albores del cambio climático que intensificará los efectos de la contaminación.

2. Los índices de calidad biótica han demostrado ser útiles para detectar impactos ecológicos, pero en comunidades pobres en especies, como en las de peces de ríos mediterráneos ibéricos, su poder diagnóstico se reduce considerablemente. Esto unido a su baja capacidad para identificar las causas del impacto y detectar efectos sub-letales aboga por el uso de metodologías alternativas.

3. Los diagnósticos basados en cambios en los rasgos biológicos y ecológicos de los individuos cumplen con este requisito. Además, estos estudios permiten dilucidar las leyes que gobiernan la respuesta de las comunidades ante múltiples estresores. Éste es un aspecto básico, tanto para testar hipótesis ecológicas, como para mejorar nuestra capacidad predictiva sobre cómo afectará el cambio global al funcionamiento de los ríos mediterráneos.

4. Los rasgos biológicos y ecológicos de las especies son variados y diferentes selecciones podrían dar lugar a resultados contrapuestos. Todo apunta a que la clave no está en la cantidad de rasgos utilizados si no en su calidad. Es necesario, por tanto, que futuros estudios analicen simultáneamente la estructura funcional de las comunidades y varios procesos ecológicos a fin de identificar mejor los rasgos con mayor relevancia ecológica.

5. Junto con los rasgos a nivel de especie, el estudio de biomarcadores sanguíneos augura su potencial como metodología no destructiva o letal en el uso rasgos biológicos a escala individual. Esto debiera incrementar el poder diagnóstico de los estudios de diversidad funcional en comunidades de peces de baja diversidad. Además, posibilita testar la señal filogenética de los biomarcadores sanguíneos que es clave para inferir la respuesta de especies en diferentes ramas evolutivas. No en vano, especies hermanas se espera que sean fisiológicamente más similares.

6. Entre los biomarcadores sanguíneos, la señal más clara con el gradiente de contaminación se obtuvo en el recuento de las anomalías totales eritrocitarias, aunque fueron necesarios el resto de parámetros para determinar mejor la existencia de daños genotóxicos. No obstante, se confirmó el riesgo de obtener falsos negativos si se utiliza sólo la aproximación tradicional en ecotoxicología del ensayo de micronúcleos.

7. La ausencia de rangos de normalidad en los estudios de biomarcadores en animales salvajes dificulta su valor diagnóstico. Es por ello que se considera importante que futuros estudios relacionen respuestas a escala individual con cambios en tendencias poblacionales, solo así podremos estar seguros de su relevancia ecológica.

8. Aunque nuestros trabajos mostraron que la comunidad de macroinvertebrados y diatomeas es más sensible que la de peces a la variación en la calidad del agua, esto no reduce su valor bioindicador. Sólo implica que es necesario determinar patrones de respuesta antes que se desestructuren las comunidades. Esto es clave no sólo para conservar los peces si no también a toda la fauna acuática amenazada y los servicios ecosistémicos que dependen de ellos.

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