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

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ABSTRACT

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

## 1. Introduction

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

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

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

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

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

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

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

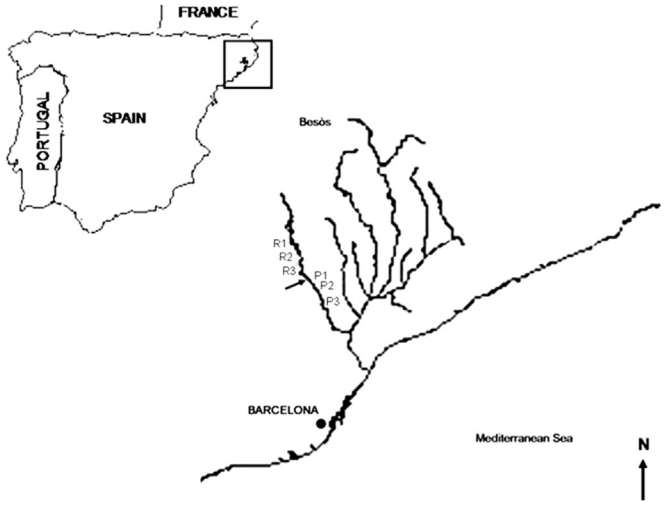


**Fig. 1.** Location of the three upstream and downstream reaches surveyed in Ripoll River to examine the impact of treated sewage discharges (arrow) on wild native fish populations over the period 2002–2013.

## 2. Materials and methods

### 2.1. Study area and general sampling design

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

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

### 2.2. Fish surveys and focal species

Fish were sampled by single pass electrofishing using a portable unit which generated up to 200 V and 3 A pulsed DC in an upstream direction, covering the whole wetted width of the 100-m long reach surveyed at each sampling site following an international standardised fish sampling method (CEN EN 14962, 2005; CEN EN 14011). Our focus was two native species, the Ebro chub (*Squalius laietanus*) and the Mediterranean barbel (*Barbus meridionalis*) belonging to *Cyprinidae*,