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Title: Disturbance accumulation hampers fish assemblage recovery long after the worst mining spill in the Iberian Peninsula

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Summary

The influence of environmental variables on native and exotic fish species richness and diversity was analyzed eight years after one of the most environmentally harmful toxic spills worldwide. Environment-diversity relationships were addressed at different scales, and values were also compared with those of six similar basins that were not affected by the spill, with the aim of determining whether this disturbance was still exerting an influence on the fish assemblage. Results showed higher native species richness in environments with low human influence, no reservoirs upstream, a large drainage area and coarse substrate reaches. For native fish, variables at both the catchment and site scales were equally relevant. Exotic fish were mainly favored by site-scale factors such as valley width downstream from the reservoir, where the alteration of the river channel and accumulated disturbances give them an advantage versus natives. Overall, eight years

after the accident, richness and diversity of the Guadiamar fish assemblage seemed more affected by anthropogenic impacts than by the long-term influence of the toxic spill. This work highlights that the potentially synergic effects of anthropogenic factors must be taken into account when monitoring the long-term effects of pollution events.

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Introduction

On April 25th 1998 one of the largest tailing dam failures in Europe (Rico et al., 2008) occurred when a 50-meter breach opened in the tailing pond dike of the Aznalcóllar mine (SW Spain). This breach caused the release of about 4 hm^3 of acidic water with dissolved metallic compounds and 2 hm^3 of mud, mainly composed by floated pyrite (Aguilar et al., 2003). To stop the spill from reaching downstream Doñana National Park several dams were constructed (López-Pamo et al., 1999). Nevertheless, 67km of the Guadiamar River's main channel (a tributary of the Guadalquivir River) were polluted by toxic spill, composed mainly by Fe, S and several heavy metals (Aguilar et al. 2003). Unfortunately, the coarse mechanical removal of contaminants from the stream and flood plain aggravated the effects of the toxic spill, with major implications for the geomorphological characteristics of the river (Gallart et al., 1999). After these cleanup operations in the affected area, a Recovery Plan (PICOVER) was implemented, aimed at repairing the damaged ecosystems and transforming the affected area into a green corridor between two well conserved ecosystems: Sierra Morena in the north and Doñana National Park in the south (Márquez-Ferrando et al., 2009).

In spite of these efforts, it is difficult to assess whether restoration actions were successful for the recovery of the fish community of the Guadiamar River, since very few studies addressed this issue in the years following the spill (but see Fernández-Delgado and Drake 2008). Recently, some studies have focused on the spill effects on fish somatic condition (De Miguel et al. 2013), described fish composition (De Miguel et al. 2014) or addressed population dynamics and recolonization processes (De Miguel et al. 2015). However, to date no studies have analyzed the influence of environmental variables and the potential residual effects of the spill on fish diversity.

Fish assemblage composition changes over time and space (Magalhaes et al., 2002), and fish have both local and catchment mobility (Pinto et al., 2006). Therefore, assemblage characteristics and structure will be determined by a wide range of biotic and abiotic processes that act at different scales. It is thus important to carry out analyses at both the catchment and site scales in order to identify the spatial scale at which the most important variables for fish assemblages are acting (Morán-López et al, 2012).

In this study, the main aims were 1) to identify the main environmental variables that determine both native and exotic fish species richness and diversity in the Guadiamar River basin at different scales, and 2) to assess whether the toxic spill can still be considered influential for fish richness and diversity eight years after the accident.

Materials and Methods

Study area

The Guadiamar River basin is located in the South-western Iberian Peninsula at latitude 37º 10’ to 37º 45’ N and longitude 6º 10’ to 6º 25’ W, near the Guadalquivir River mouth in the Atlantic Ocean (Figure 1). The basin area is 1.325 km^2, and altitude ranges from 4 to 544 m.a.s.l. Climate is sub-humid Mediterranean with oceanic influences, with average temperatures between 9ºC in winter and 29ºC in summer. Mean annual rainfall is 624 mm, oscillating between 754 mm in the source and 543 mm in the mouth. Rain falls abundantly in autumn, winter and spring and is almost completely absent in summer. This severe drought causes the drying of most small streams or the creation of isolated pools of different sizes (Gasith and Resh, 1999). The basin shows a geological transition linked to river section type. The upper section (near the source) lies within the Sierra Morena mountain range, where forestry and livestock ranching land uses are predominant; the middle section is under agricultural and urban land uses; and the lower section forms a plain that precedes the marshland at the mouth. The hydrological network is interrupted by two small reservoirs that collect less than 4 hm^3 in the source area and one large reservoir (20 hm^3) in the Agrio River. This reservoir was built in 1977 to supply mining industry needs, and it is located slightly upstream from the spill point (Borja et al. 2001) (Figure 1).

Sampling protocol

The sampling period was divided into three campaigns to maintain the same capture method in all sites: winter (2006) for the low flow streams and spring and summer (2007) for the high flow courses that were not wadeable during the rainy period (winter). A Geographic Information System (ArcGIS v. 9.2) was used to divide the Guadiamar River network into hydrological fragments, which were defined as the stretch between each junction of streams. One sampling site was located per 10km or per hydrological fragment, ensuring that the whole perennial stream network (except the marshland) was covered. This resulted in a total of 22 sampling sites.

Fish sampling was carried out by one-single-pass electrofishing (220 V, 2-5 A, C.C.). Battery or small engine backpack modality was used when stream was < 1 m deep and <5 m wide; while a large engine on the riverbank was used when the stream was between 1 and 1,4 m deep and wider than 5 m. The length of the sampling site was 100 m in streams

< 5 m wide, and for wider streams, the length was 20 times the width. Captured fish were identified to species and kept submerged in an appropriate container during sampling.

Habitat data were collected at two different scales: catchment and site. Site variables were mainly collected in situ before fishing. Catchment scale variables were obtained using ArcGIS 9.2® and data layers were either freely available or provided by the Regional Environment Agency (Consejería de Medio Ambiente y Ordenación del Territorio, 2015). Nevertheless, some site variables such as distances to source, mouth and reservoirs, and abiotic variables such as vegetation or valley width, altitude and stream order, were calculated using GIS. Water quality data were provided by the Regional Environmental Agency, and ran in a scale of 1-4 1 (low organic and inorganic pollution) – 4 (high organic and inorganic pollution) (Consejería de Medio Ambiente y Ordenación del Territorio, 2015). The bank stability Index was calculated at both catchment and site scale based on slope, vegetation cover, height and substrate of the river bank (Fernández-Delgado et al., 2014). Thereby, a total of 62 environmental variables thought to be relevant for fish species richness and diversity were recorded at each sampling site (Appendix 1). In several cases, variables were summarized by means of PCAs (see Statistical analyses section and Appendix 1).

Statistical analyses

In order to identify the main factors that determine native species richness (S) and diversity (H, Shannon's diversity index; Shannon and Weaver, 1949) in the Guadiamar River basin General linear models were used. Predictor variables were considered at two different scales: catchment and site (Table 1). First, a general model was created using all variables at both scales, including their interactions. This model would reveal the scale at which the most important factors for fish diversity were acting. Second, two further models were derived, one for each scale, in order to find out which were the most relevant drivers at that particular scale.

A similar approach was used for exotic species. However, in this case models were not computed for diversity (H) because several sampling sites had none or just one exotic species, resulting in a large number of sites with zero values. This large proportion of zeroes would result in a weak model, so only species richness (S) was modeled for exotic species.

A priori variable selection was carried out due to the large number of predictor variables (see Appendix 1), their collinearity and ecological redundancy, and parsimony considerations. First, some groups of related variables that implied ecological redundancy were summarized by means of PCAs. In all cases, the Kaiser (1960) criterion (eigenvalue >1) was used to define the principal components to be chosen as the final variables:

PCA 1 – Habitat characteristics at the site scale ("Habitat" hereafter). The main axis represents a gradient from pools and fine material (negative end) to riffles and coarser substrates (positive end).

PCA 2 – Factors affecting bank stability at the site scale ("Stability" hereafter). The principal component represented a gradient from lowest (negative end) to highest (positive end) risk of erosion.

PCA 3 – Land uses at the site scale ("Site uses" hereafter). The main axis represented a gradient from greater human impact (urban and agricultural areas at the positive end) to less humanized uses (native forests at the negative end).

PCA 4 – Land uses at the catchment scale ("Catchment uses" hereafter). This main axis represented a gradient from greater human impact (urban and agricultural areas at the negative end) to less humanized uses (native forests at the positive end).

The second step to reduce the number of variables was to test for collinearity between the remaining ones (Appendix 1) using Pearson´s correlations. Whenever the correlation coefficient between two variables was greater than 0.75, one of the variables was chosen for the regression models.

The final regression models were applied to a total of 22 cases (n=22) and a maximum of 6 predictor variables, since a larger number of variables would lead to a Type 2 error (Field, 2005). The final variable list in each case included those that showed a high correlation with the relevant dependent variable and low collinearity with the other selected variables. A list of all the variables included in the 8 models is presented in Table 1.

The best models supported by the data were selected using the Akaike Information Criterion (Burham and Anderson, 2002). This allowed us to decide which model explained the most variance whilst being most parsimonious (Johnson and Omland, 2004). Variance partitioning of the significant variables selected for the general model was performed to identify the most important scale in each case.

Since the spill point is located 5 km downstream from the main reservoir in the Agrio River (Agrio reservoir hereafter), there is an almost complete overlap between both influences on fish. In order to clarify the effect of each factor, species richness and diversity values downstream from the Agrio reservoir were compared with values downstream of 6 reservoirs from similar watersheds within the Guadalquivir river basin. These watersheds had been sampled by the same research group, with the same methods, between 2006 and 2009 (Fernández-Delgado et al., 2014). The statistical comparison was carried out using ANOVA and post-hoc t-tests, applying the Holm p-value adjustment method.

All analyses were performed using R version 2.12 and packages: vegan, hier.part, gtools and asbio (R Development Core Team, 2012).

Results

Fish assemblage

A total of 13 fish species were found in the study area, 8 native and 5 exotic (Table 2). Both native species richness and diversity reached maximum values (6 and 1,60, respectively) in sampling site 7, located upstream from the affected area; while the maximum value for exotic species richness (4) was located in sampling site 22, the one furthest downstream from the affected area (Table 2).

Native species

a) General models

Significant models were obtained for native species richness (S-na) and diversity (H-na), which accounted for 70% (R^2 = 0.70) and 52% (R^2 = 0.52) of the variance, respectively. The main factors included in the best models were similar for S-na and H-na (Table 1). These models identified “Catchment uses” and “Number of reservoirs upstream” as the most influential factors. Variance partitioning using *hierpart* showed that they accounted for 33% and 28% of the explained variance, respectively, in the case of S-na; and 46% and 31%, respectively, in the case of H-na. A positive relationship was found between the main axis of PCA4 (“Catchment uses”) and both dependent variables, and this means that higher native fish richness and diversity are found in natural forest areas with respect to those with agricultural or urban land uses. In contrast, a negative relationship with “Number of reservoirs upstream” was found, which indicates that the more reservoirs upstream from a site, the lower the native fish richness and diversity. “Drainage area” and “Habitat” were a second group. These variables accounted for 20% and 19% of the explained variance, respectively, for S-na. For H-na, “Drainage area” explained 23% of the variance. Most likely, the positive relationship observed between S-na and H-na and

"Drainage area" simply reflects the species-area relationship that occurs as you go downstream: drainage area increases and so does the number of species found. The positive relationship found between Axis 1 of PCA1 ("Habitat") and S-na reflects that S-na is higher in reaches with coarser substrate and clearer water.

Variance partitioning applied to the significant variables at both site and catchment scale accounted for a similar overall proportion of the variance (0.51 and 0.55, respectively) for S-na, and also for H-na (0.45 and 0.55, respectively). This means that both scales are equally important for native fish richness and diversity, so further models were developed including only variables measured at each scale.

b) Catchment models

As expected given the general results, where only variables at the catchment scale were considered, significant models were obtained for native species richness (S-na_C) and diversity (H-na_C) at the catchment scale, which accounted for 23% ($R^2 = 0.23$) and 11% ($R^2 = 0.11$) of the variance, respectively (Table 1). Again, the main axis of PCA4 ("Catchment uses") was selected as the main driver for S-na_C and H-na_C. This positive relationship suggests that, at a wide scale, native fish richness and diversity are higher in areas where land uses are more natural.

c) Site models

Site-scale models were significant for both native species richness (S-na_S) and diversity (H-na_S), accounting for 53% ($R^2 = 0.53$) and 35% ($R^2 = 0.35$) of the variance, respectively (Table 1). The main axis of PCA1 ("Habitat") was identified as the most influential factor for S-na_S and variance partitioning showed that it accounted for 45% of the explained variance, whereas it was not selected in the case of H-na_S. This positive relationship between the main axis of PCA1 (“Habitat”) and S-na_S reinforces the same trend described for the General model (native richness is higher in those reaches with coarser substrate and clearer water). “Distance to source” however, was the most important factor for H-na_S and the second most important for S-na_S. According to variance partitioning, this factor accounted for 54% and 23% of the explained variance, respectively. This positive relationship between “Distance to source” and H-na_S and Sna_S, reflects a similar explanation to that suggested for “Drainage area” in the General model, showing the species-area relationship that occurs as you go downstream: distance to source increases and so does the number of species found. “Number of reservoirs upstream” was selected as the second most important factor for H-na_S and the third for S-na_S, accounting for 46% and 21% of the explained variance, respectively. This negative relationship concurs with that observed in the General model and reinforces the idea that the more reservoirs upstream from a site, the lower the native fish richness and diversity. The last variable selected by the model at the site scale was the main axis of PCA3 (“Site uses”), which accounted for 11% of the explained variance for S-na_S. In this axis human impact is located at the positive end, so this negative relationship shows how, at the site scale, native fish richness is lower in areas where land uses are more humanized, the same trend as that observed in the General and catchment-scale models.

Exotic species

a) General model

A significant model was found for S-ex (Table 1). “Mean channel width” was the most influential variable, accounting for 42% of the variance (R^2 = 0.42). This positive relationship suggests that exotic fish richness in the Guadiamar River is greater in the wider valleys of the lower sections of the river, away from the narrow valleys near the source. The final model included only this variable, measured at the site scale, which suggests that this is the most important scale for exotic species richness. Therefore, only a more detailed site model was computed for exotic species richness.

b) Site model

The model for exotic species richness at the site scale (Sex_S) was significant and explained 53% of the variance (R^2 = 0.53) (Table 1). As in the General model, "Mean valley width" was identified as the most influential variable, followed by "River length covered by reservoirs upstream" (RLCRU, Table 1) in this case, accounting for 55% and 45% of the explained variance, respectively. The positive relationship between "Mean valley width" and exotic species richness confirms the results of the General model: an increase in the number of exotic species as the channel becomes wider further away from the source. The other positive relationship ("River length covered by reservoirs upstream") shows how exotic species are linked to reservoirs upstream.

Spill Effect

The ANOVA that compared species richness in the reach downstream from the Agrio reservoir vs. the six selected reservoirs in similar watersheds yielded a significant result ($F_{(6,19)}$= 5.465, p = 0.002). The post-hoc t-tests revealed significant differences between the Guadiamar reach and reaches downstream from three reservoirs (Cala, Pintado and Rumblar, t = 3.67, p = 0.020; t = 3.72, p = 0.020; and t = - 0.90, p = 0.040; respectively), whereas there were no differences with four others (Huesna, Montoro, Rumblar and Fernandina) (Table 3). On the other hand, no significant differences were found ($F_{(6,19)}$= 2.384, p = 0.069) between native species diversity downstream from the Agrio reservoir and any of the other six selected reaches. Similarly, no significant differences were found neither for exotic species richness ($F_{(6,19)}$= 1.126, p = 0.384) nor diversity ($F_{(6,19)}$= 0.917, p = 0.504) (Table 3).

Discussion

Results revealed the main environmental variables that influence the Guadiamar River fish assemblage 8 years after the mining accident. Richness and diversity followed similar trends for native species, and differences were found at the site and catchment scales for both native and exotic species. It was difficult to determine whether there is still an influence from the spill on these parameters, since there were very few significant differences between Guadiamar data and data from other watersheds, and the Agrio reservoir exerted a confounding effect.

There was a strong influence of a catchment-scale factor such as land use on the native species assemblage of this basin. In agreement with other authors (e.g., Corbacho and Sánchez, 2001; He et al., 2010), natural areas present higher native species richness and diversity than those with some human impact (agricultural or urban land uses). This is probably because the life cycle requirements of the fish species considered are not fulfilled in areas with increasing denaturalization of environmental conditions (Hughes et al., 2010). Deforestation at the catchment scale and elimination of local riparian vegetation due to agricultural practices decreases shelter availability in riverbanks and increases erosion and water turbidity (Aguiar and Ferreira, 2005). Furthermore, urban land uses raise the organic load through sewage discharges, thus reducing the concentration of oxygen in the water (Ferreira et al., 2005). The extent of these effects will determine the presence or absence of certain species and therefore, affect the overall diversity of the fish assemblage.

At the site scale, the number of reservoirs upstream acts as the other main influence for native richness and diversity, representing a pivotal point for fish distribution in the basin under study. According to variance partitioning, upstream reservoirs are even more important than the well-known species-area relationship trend of higher richness with greater drainage area (McArthur and Wilson, 1967; Sheldon, 1988), which is evident near the Agrio reservoir (Figure 1). At this point, however, the trend is reversed and native species richness decreases dramatically downstream from the dam. This decline is probably due to the artificial conditions of the reach immediately downstream from the reservoir (R.J. De Miguel, personal observation), where there is an absence of necessary habitat elements and fish may be suffering from heavy predation pressure from exotic species after dam release periods (Clavero and Hermoso, 2011).

In addition, and in agreement with Ferreira et al. (2007), the analysis shows how at the site scale native species prefer coarser substrates and fast-flowing water, typical of natural areas. A coarse substrate implies the absence of fine material overload from agricultural erosion, reservoir deposits upstream or urban pollution (Doadrio, 2001). Fast flowing water is characteristic of less-disturbed source areas, while calm waters are found in higher proportion in the middle and lower sections of the river. In these lower sections there is often greater habitat degradation and an accumulation of exotic species, resulting in an unsuitable environment for native species (Ferreira et al., 2007).

Regarding exotic species in the Guadiamar River basin, results confirm that there is a greater number of exotics towards the mouth of the river (Moyle and Light, 1996; Kopp et al., 2009). This is probably due to greater anthropogenic impacts as the river reaches its lower section (Sheldon, 1988), and the accumulation of exotic individuals from upstream reservoirs plus those going upstream from the mouth (Clavero et al., 2004). Moreover, in the Guadiamar River basin, initial habitat degradation after the spill favored the rapid colonization of exotic species (Olias et al., 2005). Toxic mud removal works inevitably caused the elimination of important natural elements for native species such as riparian vegetation or rocky shelters. This left an altered area where exotic species, generalists and better adapted to degraded zones (Corbacho and Sánchez, 2001), have established more successfully than natives.

Exotic species establishment in the Guadiamar River basin is a consequence of fishermen and government introductions for sport fishing (Fernández-Delgado, 2003). The reservoir therefore becomes a source of exotic species, but their dispersal is not homogeneous along the river course. Downstream colonization is more effective than upstream, since individuals barely go upriver towards the source streams. This asymmetrical movement may have a twofold explanation. First, the exotic species in the Guadiamar River basin possess either a flattened body adapted to lentic ecosystems, such as centrarchids and cyprinids, or a small size, such as the eastern mosquitofish *G. holbrooki*. Both body shapes have not evolved to be efficient in dealing with upstream colonization of the turbulent streams that fill the reservoir (Bernardo et al., 2003), while the fusiform native species find no problems to overcome these currents and even use upstream areas as spawning sites (Nikolsky, 1963; Herrera and Fernández-Delgado, 1992). The second cause may also be related to the adaptation of exotic species to the stable conditions of the water bodies where they originally inhabit (Elvira and Almodóvar, 2001). These stable conditions can be found in reservoirs and their regulated downstream tailwaters, but reaches immediately upstream suffer large fluctuations with strong flows during rainy periods and drought during summer, so they are inappropriate environments for exotics (Magalhaes et al., 2002).

Unfortunately, the attempt to discern between spill and reservoir effects did not yield a clear result, but suggests a combination of events. The observed native species richness and diversity depletion caused by reservoirs in other river basins, similar to Guadiamar, provides a range of values, and those observed in the Guadiamar River fit within that range. Therefore, the current potential effects of spill remnants are not strong enough to cause abnormal fish species richness and diversity values. The same result is obtained for exotic species, which suggests that the set of factors that promote exotic species richness in the Guadiamar River basin are equal to those found in other similar river basins, not affected by the spill. This may be because the habitat recovery works have minimized the spill effect and the reach originally affected is now exposed to the same impacts that it suffered before this event.

In summary, the native species of the Guadiamar River basin are favored by environments with low human or reservoir influence, a large drainage area and coarse substrate reaches where flow sweeps along fine materials. Therefore, both catchment and site scale approaches must be taken into account when relevant factors for native species are addressed. On the contrary, exotic species thrive mainly due to site-scale factors downstream from the reservoir, where increasing valley width entails accumulated disturbances as the river flows towards the mouth that may give them an advantage versus natives. Eight years after the spill, it is difficult to determine whether there is still exerts an effect on fish species richness and diversity. Its influence does not seem greater than that of other human disturbances acting on this watershed and on the other biogeographically similar watersheds considered. Currently, the Agrio reservoir seems to be the main disruptor of natural fish diversity in the Guadiamar River basin. This work highlights that studies that aim to assess or monitor similar accidents should take into account the previous and current impacts of other anthropogenic factors, such as upstream reservoirs or humanized land uses.

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