RECOLONIZATION PROCESS AND FISH ASSEMBLAGE DYNAMICS IN THE GUADIAMAR RIVER (SW SPAIN) AFTER THE AZNALCÓLLAR MINE TOXIC SPILL

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Abstract

The Guadiamar River (SW Iberian Peninsula) received a major toxic spill (6 hm^3) from a tailing pond in 1998 that defaunated 67 km of the main stem. Following early mud removal works, the fish assemblage was annually monitored at four affected sampling sites and one located in the upstream non-affected reach of the Guadiamar River as reference. Fish abundance and assemblage structure were analyzed. Principal response curve (PRC) was applied to assess the recovery trends and to identify the most influential species. A non-metric multidimensional scaling (NMDS) ordination and PERMANOVA were applied to evaluate changes in fish assemblage structure between sites and years. Overall, the affected reaches harboured fish within two years of the spill. Colonists arrived mainly from the upstream and downstream non-affected Guadiamar River reaches and, to a lesser extent, from three lateral tributaries. It is likely that the proximity, connectivity and environmental conditions of non-affected fish sources greatly influenced the recolonization process in each site. The structure of the fish community in the affected sites was initially similar to that in the unaffected reference stretch, but changed dramatically with time and each site followed its own trajectory. Currently, long-term threats such as mining leachates, urban sewage, agricultural pollution and exotic fish species expansion, have probably exceeded the initial spill effect. This highlights the large effect of anthropogenic factors on freshwater ecosystem resilience, and the need to significantly reduce both pollution and exotic species if the affected reach of the Guadiamar River is to recover fully.

Introduction

Fish assemblages have often been used in biological monitoring to reflect the stress applied to an aquatic ecosystem (e.g. Albanese at al., 2009, Kubach *et al.*, 2011). Whenever a disturbance causes partial or total defaunation, subsequent fish responses include initial habitat recolonization and subsequent assemblage recovery (Sheldon and Meffe, 1995). Fish recolonization processes mainly depend on both habitat fragmentation and species traits. Physical or chemical barriers between colonists and the defaunated area may reduce their potential recolonization rates after a disturbance (Kubach *et al*., 2011). This rate is positively related to species abundance, mobility and to a lesser extent, spawning. Thereby, abundant species supply more colonist individuals and may be more likely to settle within reaches because they are better matched to local habitat conditions than species that were previously scarce (Sheldon and Meffe, 1995). After large-scale disturbances, fish often start the recolonization process triggered by floods from non-affected reaches and tributaries within the basin (e.g. Kubach *et al*., 2011).

One of the most harmful anthropogenic aquatic disturbances ever registered in Europe took place in the Guadiamar River, South-western Spain. On 25 April 1998, a tailing pond located in Aznalcóllar (Seville) ruptured, discharging 4 hm^3 of acidic water and 2 m^3 of metallic mud (Aguilar *et al*., 2003). The spill, composed mainly of iron, sulphur and heavy metals, flowed into the Agrio River and reached the Guadiamar River, where over 60 km of the fluvial course were defaunated (Grimalt and Macpherson, 1999). Unfortunately, cleaning and remediation works aggravated the effects of the toxic spill, with major implications for the geomorphological, hydrological and geochemical characteristics of the river (Gallart *et al*. 1999). As a last long term measure, a Recovery Plan (PICOVER) was implemented not only to repair the damaged ecosystems, but aiming to transform the affected area into a green corridor between two well conserved ecosystems: Sierra Morena in the north and Doñana National Park in the south (Arenas *et al*., 2008). Once the restoration tasks were over, the few studies that addressed the recovery of fish assemblages (Fernández-Delgado and Drake, 2008; Pérez-Alejandre; 2009) provided ambiguous early conclusions that considered an ongoing recolonization process that tends to the pre-disturbance conditions.

The purpose of this study was to evaluate the long-term effects of the Aznalcóllar toxic spill on the Guadiamar River fish assemblage. The specific objectives were to: (1) study the recolonization process, pinpointing the main colonist sources, obstacles and dominant species dynamics; and (2) assess whether the fish assemblage in the affected reach can be considered recovered 13 years after the toxic spill.

Material and Methods

Study area

The Guadiamar River basin is located in the South-western Iberian Peninsula covering an area of 1.880 km^2 (Figure 1). The upper section flows through the western Sierra Morena, with typical xeric Mediterranean forests. Thereupon, the river crosses a predominantly agricultural area on sedimentary hills and, finally, the southern end turns into a channelized marsh stretch that flows into the Guadalquivir river mouth within the Doñana National Park (Borja *et al*., 2001). From a hydrological point of view the Guadiamar is a typical Mediterranean river, with a severe summer drought, annual average temperature above 10 ºC and annual average rainfall of 600 mm often causing floods (Aguilar *et al*., 2003). The main river network in the basin consist of the Guadiamar River main stem and its most important tributaries, such as the Agrio River, the Ardachón stream, the Alcarayón stream, the De la Cigüeña stream and the Majaberraque stream (Figure 1). This Agrio River, located in the boundary between the upper and middle section of the basin, was the first watercourse to receive the spill and hence, it flowed to the Guadiamar River mouth into the Doñana National Park (Fernández-Delgado and Drake, 2008; Figure 1).

The Guadiamar River network is disrupted by several physical and chemical barriers. Some of these disturbances represent an important interruption to fish movement and therefore, an obstacle for recolonization processes. The Agrio reservoir in the Agrio River is the largest transversal obstacle in the watershed. Nevertheless, two other major barriers located in the Guadiamar River main stem were likely a direct obstacle to fish recolonization from downstream sources. Both are ancient mill weirs, the first (height = 2 m) is placed 2 km downstream of the lowest sampling site (E5) in the longitudinal design and upstream, the second (height = 1.5 m) is located between E5 and E4, at 3 km and 4 km from these points, respectively. Moreover, three major chemical barriers may also hamper the recolonization process. Specifically, leachates from Aznalcóllar mines to the Agrio River in the upper section (Arambarri *et al*., 1996) and two major untreated sewage inputs, one towards the lower section of the Alcarayón stream in the middle section and the other to the channelized De la Cigüeña stream in the lower section (Fernández-Delgado *et al*. 2014).

Sampling design

Fish assemblage was monitored at five sampling sites located in the Guadiamar River main stem (longitudinal sampling design). Due to the need for quick information after the spill, four sites were selected according to accessibility and trying to maximise coverage of the affected fluvial reach. Unfortunately, the hazardous nature of the toxic spill and rapid decomposition of fish impeded collection or identification of dead fishes within the study area, unlike the downstream marshland, where 37.4 t of dead fish mixed with mud were identified, including carps (75%), mullets (10%), barbels (6%), eels (4%) and other species (5%) (Del Valls and Blasco, 2005). On the other hand, the closest pre-disturbance survey was carried out in 1996-1997 and it provided only species presence/absence data from several locations within the affected reach (Doadrio, 1996 and 2001). Thus, given this scarce previous information, an additional fifth sampling site was established 6 km upstream from the affected reach to represent non-affected assemblage conditions in the context of the mining spill, hereafter referred to as reference site (E1 in Figure 1). Downstream, within the affected reach, the four original sampling sites were named E2, E3, E4 and E5 (Figure 1). The first site affected by the spill (E2) was located at the confluence with the Agrio and Guadiamar rivers, whereas E3, E4 and E5 were situated 9 km, 19 km and 26 km, downstream of this confluence, respectively (monitoring stretch: 32 Km from E1 to E5, Figure 1). For our objective of evaluating fish assemblage recovery processes, we assumed that all the affected sampling sites (E2, E3, E4 and E5) began the recovery from the same state of disturbance.

Fish were sampled once a year at each sampling site at the time of low annual flow (July-August) for nine years. Because of safety restrictions and cleaning works after the spill, the first sampling was carried out in 1999, and monitoring was uninterrupted until 2006. Additional funds allowed a final sampling effort in 2011. Altogether, 45 surveys were conducted in this longitudinal sampling design. Monitoring at the five sampling sites took place in stretches with low-flow conditions (runs or pools); water width and depth of sampling stretches averaged 15 m and 2 m, respectively; clay and sand were the predominant substrate, with some gravel and a few boulders. At site level, fish were caught using two passive sampling methods: (i) setting ten minnow-traps (0.5 m length, 0.03 m diameter entrance), distributed only in the bank of pools, for roughly 18 hours; and (ii) one multi-mesh gillnet (30 m long and 1.5 m deep) placed transversely running from the bank of pools, with mesh sizes ranging from 10 mm to 200 mm, soaking time approximately 18 hours.

In addition, the most important tributaries that flow into the Guadiamar River main stem (Figure 1) and a Guadiamar stretch, just downstream of the river-marsh transition (Doñana marshland), were sampled and considered as non-affected fish sources after the spill. In these non-affected sources, fish were sampled twice, in 2003 and 2006, and only information about species richness was obtained. Electrofishing following the CEN standard protocol (CEN, 2003) was the sampling gear used in the tributaries, whereas the same multi-mesh gillnet and minnow-traps described above, were also used in the Doñana marshland sampling site.

Data analyses

In surveys carried out from 1999 onwards, fish abundance was estimated using catch per unit effort (CPUE), standardizing total species catch with both passive sampling methods to 24 hours.

Sampling site E1 (reference site) was considered representative of non-affected fish assemblage conditions, so a principal response curve (PRC) was used to test differences between the affected sites and the reference site through time. The PRC approach constitutes a multivariate method, based on redundancy analyses, which describes changes in assemblage response over time in relation to a control (Van den Brink *et al*., 2003). The principal component is plotted against time, giving a PRC of the fish assemblage for each sampling site. A quantitative interpretation of the effects at species level is possible by scoring the species weight, according to each species accounting for the deviances. PRC were performed considering fish abundance at the species level. Monte Carlo permutations tests commonly carried out to test the significance of the axis (Van den Brink *et al*., 2003) could not be performed because of lack of sampling replicates in the same year.

Non-metric multidimensional scaling (NMDS) ordination was used, after CPUE log(x+10) transformation, to extract spatio-temporal patterns in fish assemblage structure (Kruskal and Wish, 1978). NMDS is a general ordination procedure recommended for non-normal or questionably distributed data and calculates ranked ecological distances (McCune and Grace, 2002), providing a relative measure of proportional similarity in fish assemblage structure (Kubach *et al*., 2011). NMDS estimates distances between samples out of a derived “sample by sample” matrix. This matrix is obtained by transforming the original matrix using a dissimilarity measure. NMDS is not restricted to Euclidean distance measure but any dissimilarity measure can be used, which can also relax the requirement of normality of data (Van den Brink *et al*., 2003). We used the Bray–Curtis dissimilarity distance to compute the resemblance matrix among sites. In this study, distances between reference site data and those from the affected reach were used to detect fish community recovery trends.

The statistical significance of differences in fish assemblages between years was tested using a semi-parametric permutational multivariate analysis of variance using the Bray-Curtis distance matrices (henceforth PERMANOVA). One PERMANOVA was performed per site, species abundances acted as the dependent variables, and both axes (time and site) were factors, so axes weight in each case was also assessed. Abundance values from E4 in 2005 were not included because during this year the sampling site was confined to an isolated pool where fish abundance (mainly *Luciobarbus sclateri*) was overestimated.

All statistical analyses were performed using R version 2.12.1 (R Development Core Team, 2012) and its package 'vegan' (Oksanen *et al*., 2011).

Results

Fish assemblage composition

A total of 6243 fish representing 13 species (7 native and 6 exotics) were caught during the whole monitoring period of the longitudinal sampling sites (Table 1). The dominant family was Cyprinidae, which accounted for 46.1% of the total species richness within the monitored stretch, followed by Centrarchidae and Mugilidae.

There were some differences in the fish species found in the affected reach respect to the pre-disturbance assemblage data from 1996 (Table 1). Three native species (*Anguilla anguilla*, *Iberochondrostoma lemmingii* and *Squalius pyrenaicus*) previously caught were not captured during surveys after the spill; however, five new exotics were detected.

Fish abundance

During the monitoring period, two species were present in all sites every year: one native, *L. sclateri*, accounting for 50% on average (range 30%-73%) of all CPUEs collected, and one exotic, *L. gibbosus*, accounting for 16% on average (range 3%-31%). *L. sclateri* was the dominant species, except in the reference site (E1), where it was often codominant with *Pseudochondrostoma willkommii* (36% of total captures). This last species was considerably less abundant in E2, and absent in the rest of the monitoring stretch. Although *S. alburnoides* complex was present in every sampling site, it was the least abundant native species, accounting for just over 3% of all individuals collected. It occurred in the reference site but was almost absent in the affected reach. Among the exotic species, the second most dominant was *A. alburnus*, accounting for 12% (range 4%-26%) of all individuals collected on average, but absent in the reference site. *Gambusia holbrooki* and *Micropterus salmoides* accounted for 9% on average (range 6%-13%) and 10% (range 4%-17%), respectively. *M. salmoides* was present in all sampling sites, whereas *G. holbrooki* was caught only in the affected reach. No other species accounted for more than 3% of all individuals collected at any sampling site, nevertheless, all species have also been taken into account for assemblage structure analyses.

During this study, at least three different phases could be distinguished for fish abundance trends in the affected sites. First, early spill removal works resulted in an increase from the lowest initial values (1999) to a maximum in the second year after the spill (2000), reaching similar abundance values between the reference and the affected sites (Figure 2). However, between 2001 and 2004, there was a stable phase for both native and exotic species in most sampling sites, with a slight increase for natives and decrease for exotics. The third phase is characterized by a fluctuating trend that sampling sites underwent from 2005 onwards, when most sampling sites had higher different trends in native and exotic species abundance. Moreover, the last sampling in 2011 showed how exotic species abundance mightily increased in the affected reach and decreased to a minimum in the reference site, resulting in higher values for exotic species in the affected reach than in the reference site at the end of the study period. On the contrary, native species abundance in the reference site remained above that in the affected reach.

Assemblage structure dynamics

River channel conditions after the spill triggered large differences between the affected sites (E2, E3, E4 and E5) and the non-affected upstream reference site (E1). This divergence started to decrease after two years (Figure 3). Then, between 2002 and 2004, assemblages from the affected sites maintained a similar structure to that of the reference site. However, from 2005 all assemblages started to diverge, becoming very different by the end of the study period. These assemblage trends were more influenced by some species than others. PRC identified *A. alburnus, L. gibbosus, P. willkommii* and *L. sclateri* as the species with greatest weight on assemblage structure (Figure 3). As previously mentioned, lack of sampling replicates made the quantification of the species' influence by PRC impossible. PERMANOVAs were used to test this influence.

NMDS ordination (Figure 4) revealed a similar spatio-temporal recovery pattern of fish assemblage structure to that displayed by PRC. Along Axis 1, the position of the reference site showed relatively little variability across time. All samples from the reference site occupied a localized area towards the negative end of this axis, indicating relative stability in assemblage structure. In 1999, affected sites were in the opposite end of Axis 1 and in the positive part of Axis 2. From 2000 to 2004, the affected sites increased in similarity with respect to the reference assemblage on Axis 1. E2 reached the reference site area in 2001 and then maintained a close resemblance for 3 more years. However, from 2005, affected sites tended to diverge from the reference assemblage again. This trend did not derive towards the initial dissimilar starting point at the positive ends of both axes, but it is directed towards the negative end of Axis 2, where no sites appeared before (Figure 4).

PERMANOVA revealed no significant differences between years in E1 ($F_{(1,8)}$= 1.187; p = 0.345). However, these differences were significant for E2 ($F_{(1,8)}$= 4.4854; p = 0.008), E4 ($F_{(1,8)}$= 3.2358; p = 0.015) and marginally significant ($p < 0.1$) for E3 ($F_{(1,8)}$=2.0664; p = 0.091) and E5 ($F_{(1,8)}$= 3.2667; p = 0.056). In the PERMANOVA with site, sample and site-year interaction, site accounted for 31% (p = 0.001) of the variance explained by the model; year accounted for 10% (p = 0.001); and site-year interaction accounted for 10% (p = 0.032). Thus, the model explained 52 % of the variance.

Fish recolonization sources

Sampling of non-affected tributaries and Doñana marshland area identified fish assemblages that were a likely source of colonizing individuals after the spill removal works (Table 1; Figure 5). The largest native species assemblage was found in the upstream Guadiamar River main stem (Table 1). This source supplied six native species, *L. sclateri, P. willkommii, S. alburnoides, S. pyrenaicus, Cobitis paludica* and *I. lemmingii*, together with two exotics, *L. gibbosus* and *M. salmoides*. On the other hand, the largest exotic species assemblage was detected downstream in the Doñana marshland sampling site (Table 1; Figure 5). Regarding the tributaries, Agrio River and Ardachón stream were potentially the largest lateral contributors, providing native species such as *L. sclateri*, *S. alburnoides* and *S. pyrenaicus*, together with the exotic *G. holbrooki* and *L. gibbosus* (Table 1). Downstream, *C. paludica* was the only species caught in the Alcarayón stream, and Majaberraque stream was the last tributary holding likely colonists, in this case *G. holbooki* (Figure 5).

Discussion

Guadiamar River fish assemblages at the different sampling sites evolved in different ways throughout the 13 years following the spill. Several barriers hampered recolonization from tributaries; however, this process was carried out and is still underway.

The PRC and NMDS analyses, based on fish abundance, offered both overall and specific approaches to explain the observed patterns. First, the early spill effect and subsequent cleaning works, especially the withdrawal of vast amounts of soil in the summer of 1999 that cut and dried several main stem reaches (Arenas *et al*., 2008), impeded fish establishment in the affected reach until (E2-E5) 2000 (two years after the spill). From that year, fish assemblage structure in affected sites tended towards that of the reference site (E1), where native species were dominant and exotics were scarce (Table 1, Figures 3 and 4). The increase in assemblage similarity was especially relevant in E2, which was the nearest sampling site to the reference. Thus, between 2001 and 2004 (three-six years after the spill), fish assemblage structure in affected sites stabilized, with slight increases or decreases in similarity, depending on the sampling year, to that of the reference site (Figure 2). These first signs of recovery were similar to several studies where a defaunated river stretch, experimentally or by accident, was considered. Thus, Albanese *et al*. (2009) concluded that most fish populations recovered 2 years after defaunation and only species with low movement rates took longer. Ensign and Leftwich (1997) mention a time lapse of 1 year to overall assemblage recovery, but 2-3 years or longer were needed for certain species or specific age structures to reach previous conditions. And probably, the most similar study to our case, investigating the effects of an oil spill placed the time of recovery in fish assemblage structure at 4.3 years after the spill (Kubach *et al*., 2011). Interestingly, from 2005 onwards, fish assemblage structure in the affected reach diverged from that of the reference site again (Figures 3 and 4). This year was the driest in the sampling period (SAIH, 2012) and native species, better adapted than exotics to drought (Ribeiro and Collares-Pereira, 2010), were favoured in those upstream reaches where flow was mightily reduced (E1 and E2, Figure 2). However, exotic species thrived in the affected reach because flood shortage enhanced the lentic nature and stable flow of this area (Clavero and Hermoso, 2011). In subsequent years, native species decreased in the upstream sites (E1 and E2) because of both downstream displacement by floods and recovery of interactions with exotics (Ribeiro and Leunda, 2012). Nevertheless, at the end of the sampling period, native species abundance returned to average values for each sampling site. On the other hand, exotic species abundance recovered in upstream sites (E1 and E2) and both, floods that displaced individual downstream and upstream migration from Doñana marshland, increased the abundance of exotics in the affected reach at the end of the sampling period (Figure 2).

Regarding recolonization sources, the unaffected upstream and downstream Guadiamar River main stem seemed to be the most relevant fish source (Figure 5). Areas upstream from the spill provided mainly native species from a low disturbance area where natural conditions still remain. Introduced centrarchids present upstream, were occasionally displaced with floods. Potential colonists from downstream sources may be mainly migratory native and exotic species present in the highly human-modified marshland. Lateral sources from tributaries contributed to recolonization to a lesser extent because of accumulation of urban sewage, water collection and diffuse agricultural pollution, that largely reduced water quality (Fernández-Delgado and Drake, 2008) and caused fish assemblage to become poorer or absent as the tributaries go downstream. Nevertheless, floods enhance fish drift (Harvey, 1987) and dilute pollution (Cánovas *et al.*, 2010), so upstream fish may be able to reach the tributary mouth and swim into the Guadiamar River main stem. After such pulse events, Ardachón stream could be considered as the third main fish source due to the highest richness species value among the tributaries (Table 1, Figure 5). Alcarayón and Majaberraque streams may have only a slight contribution to recolonization, but in a monospecific and antagonistic way. The first could be the source of a native species (*C. paludica*) while the second of an exotic one (*G. holbrooki*) (Figure 5).

When considering the relevance of barriers, mining leachates in the Agrio River were likely the most harmful for recolonization. Although the Agrio reservoir may be restraining downstream fish displacement from the upstream tributaries to the affected reach, fish from Los Frailes stream, that connects onto the Agrio River downstream from the dam, were also absent near the confluence with the Guadiamar River (E2) (Figure 5). This fish absence may be because the Agrio River crosses the mining area in this stretch, and becomes contaminated by acid mine drainage (Olías *et al.*, 2006). This mining pollution is previous to the April 1998 spill (Arambarri *et al.*, 1996) and it has not been adequately addressed yet. A second considerable chemical barrier was urban sewage that fills the De la Cigüeña stream, which may have stopped upstream fish from reaching the affected reach (Fernández-Delgado and Drake, 2008). On the contrary, the two mills in the main stem lower section did not represent a significant enough obstacle to prevent upstream fish recolonization because catadromous species (*Liza ramada* and *Mugil cephalus*), whose only source could be the downstream marshland, were present upstream from the mills (E4, Table 1) during the study period (Figure 5).

Most species underwent an initial rise in abundance because a continuous flow was restored after the cessation of the main cleaning works. However, most of these species maintained a low abundance in the affected reach during the sampling period. Only *L. sclateri, P. willkommmii* and *L. gibbosus* maintained stable populations through the entire sampling period. These three species together with *A. alburnus* were identified by the PRC as the species with greater weight on assemblage structure (Figure 3). Consequently, the overall fish assemblage recovery process in the affected reach must be addressed taking into account the dynamics of these four species that stood out in the fish assemblage patterns. The southern Iberian barbel, *L. sclateri,* was the dominant species in both the affected and non-affected reaches of the Guadiamar River. This native potadromous species is endowed with a high capacity for dispersal and notoriously resistant to pollution that other native species are not able to face (Encina *et al*., 2006). Consequently, these characteristics identified *L. sclateri* as the best colonist of the affected reach. Southern straight-mouth nase, *P. willkommii,* was the co-dominant species in the upstream non-affected reach of the Guadiamar River, together with *L. sclateri*. However, it was almost absent in the affected area (Table 1).This native potadromous species' feeding habits consist on scraping algae or macroinvertebrates fixed to the stony riverbed (Bellido *et al*., 1989). Since the affected reach lacks many of those macroinvertebrates (Solà, 2004) and both anthropic pollution and sediment accumulation are still increasing (Carrascal *et al*., 2008), *P. willkommii* will rarely recolonize the affected reach as long as this trend is not changed. On the other hand, *L. gibbosus* was the exotic species most abundant in the upstream non-affected reach, so in the first flood that connected the Guadiamar River main stem, mainly larvae should have been one of the most displaced downstream towards the affected reach (Harvey, 1987). The absence of predators in this defaunated stretch enabled most larvae of this species reached the next age-group (Harvey, 1991). Nevertheless, this *L. gibbosus* demographic explosion decreased to a low but stable level in the affected reach throughout the following years. This decrease may be due to feeding habits turn to the polluted riverbed (García-Berthou and Moreno-Amich, 2000) and intraspecific predation that previous individuals experience reaching maturity

(Harvey, 1991). *A. alburnus* however, was not present in the Guadiamar River until the last sampling period (2011, Table 1), but during this year it shared exotic co-dominance with *L. gibbosus* in the affected reach. This species not only depends on reservoirs where has been introduced, in this case, through the Guadalquivir River basin (Vinyoles *et al*., 2007), but also on upstream tributaries (Hladík and Kubecka, 2003), where it finds shallow riffles adequate for multiple spawning (Kottelat and Feyhof, 2007). This ability for upstream migration together with the absence in the rest of the Guadiamar basin until 2009 (Fernández-Delgado *et al*., 2014), suggest a hypothesis on colonization not from the Agrio reservoir, but from the downstream Guadalquivir water bodies.

In summary, most fish species recolonized the affected reach within two years of the spill, after the main cleaning works ceased and the first large flood took place. This recolonization process came mainly from the upstream and downstream non-affected Guadiamar River reaches, and to a lesser extent from three lateral tributaries. Our results suggest that differences in the proximity and connectivity of non-affected fish sources greatly influenced the recolonization process in each site mainly in the early recovery phase. The structure of the fish assemblage at the affected reach was initially similar to that in the unaffected reference stretch. However, in the last sampling dates, the fish assemblage in the affected reach became more dissimilar from the upstream non-affected reach of the Guadiamar River. At the end of the study period, the upstream non-affected reach of the Guadiamar River held a fish assemblage abundant in native species, while exotics were most abundant in the affected reach. This result is consistent with other fish assemblage changes after severe fish kill events (Winston *et al*., 1991; Cambray, 2003; Dextrase and Mandrak, 2004; Badino and Bona, 2007). However, poor previous information cannot prove whether these differences began as a result of the spill or if it was an on-going process. According to our results, currently long-term threats such as mining leachates, urban sewage, agricultural pollution and exotic fish species expansion, have exceeded the initial spill effect, and this highlights the great effect of anthropogenic factors on freshwater ecosystem resilience. Therefore, in spite of the large effort invested in the recovery of the affected area, from the ichthyological point of view, the affected reach of the Guadiamar River will not recover unless both pollution and exotic species are seriously reduced.

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Table 1. List of the fish species caught and locations within the Guadiamar River basin during the pre-disturbed sampling in 1996, affected reach monitoring (1999-2011) and the non-affected parts of the studied river system (2003 and 2007).

Species	Pre-disturbance Doadrio (1996)	Longitudinal sampling sites 1999	2000	2001-2006	2011	Non-affected fish sources
Natives						
Anguilla anguilla	(+)					M
Atherina boyeri	(+)				E2	M
Cobitis paludica	(+)	E1	E1	E1,E2,E3	E1,E3	Gup, AG, AR, AL
Pseudochondrostoma willkommii	(+)	E1	E1,E2	E1,E2,E4	E1,E2	Gup, AG
Iberochondrostoma lemmingii	(+)					Gup, AG
Luciobarbus sclateri	(+)	E1	E1,E2,E3,E4	E1,E2,E3,E4,E5	E1,E2,E3,E4,E5	Gup, M, AG, AR, DC
Squalius alburnoides complex	(+)	E1	E1,E2,E4	E1,E2,E3,E4,E5	E1	Gup, AG, AR
Squalius pyrenaicus	(+)					Gup, AG, AR
Liza ramada	(+)			E4,E5	E4	M
Mugil cephalus	(+)			E5		M
Exotics						
Alburnus alburnus	( )				E1,E2,E3,E4,E5	M
Carassius gibelio	( )	E1	E1	E3,E4,E5		M, AG
Cyprinus carpio	(+)	E1	E1,E3,E4	E1,E2,E3,E4,E5	E3,E5	M
Gambusia holbrooki	( )		E3,E4,E5	E2,E4,E5	E2,E3	M, AG, AR, MA
Lepomis gibbosus	( )	E1	E1,E2,E3,E4	E1,E2,E3,E4,E5	E1,E2,E3,E4,E5	Gup, M, AG, AR
Micropterus salmoides	( )	E1	E1,E2	E1,E3,E4,E5		Gup, AG

Longitudinal sampling sites: non-affected (E1), affected (E2, E3, E4 and E5); pre-disturbance data only in the affected reach (E2-E5), (+) present and ( ) absent; non-affected fish sources (Gup: upper Guadiamar, M: Doñana marshland sampling site, AG: Agrio River, AR: Ardachón stream, AL: Alcarayón stream, MA: Majaberraque stream and DC: De La Cigüeña stream).