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De Miguel, RJ, Galvez-Bravo, L, Oliva-Paterna, FJ and Fernandez-Delgado, C (2016) Disturbance accumulation hampers fish assemblage recovery long after the worst mining spill in the Iberian Peninsula. JOURNAL OF APPLIED ICHTHYOLOGY. 32 (1). pp. 180-189. ISSN 0175-8659

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

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13

14 Summary

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

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

30

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33

34 Introduction

35 On April 25th 1998 one of the largest tailing dam failures in Europe (Rico et al., 2008)
36 occurred when a 50-meter breach opened in the tailing pond dike of the Aznalcóllar mine
37 (SW Spain). This breach caused the release of about 4 hm³ of acidic water with dissolved
38 metallic compounds and 2 hm³ of mud, mainly composed by floated pyrite (Aguilar et al.,
39 2003). To stop the spill from reaching downstream Doñana National Park several dams
40 were constructed (López-Pamo et al., 1999). Nevertheless, 67km of the Guadiamar
41 River's main channel (a tributary of the Guadalquivir River) were polluted by toxic spill,
42 composed mainly by Fe, S and several heavy metals (Aguilar et al. 2003). Unfortunately,
43 the coarse mechanical removal of contaminants from the stream and flood plain
44 aggravated the effects of the toxic spill, with major implications for the geomorphological
45 characteristics of the river (Gallart et al., 1999). After these cleanup operations in the
46 affected area, a Recovery Plan (PICOVER) was implemented, aimed at repairing the
47 damaged ecosystems and transforming the affected area into a green corridor between
48 two well conserved ecosystems: Sierra Morena in the north and Doñana National Park in
49 the south (Márquez-Ferrando et al., 2009).

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

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

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

68

69 Materials and Methods

70 Study area

71 The Guadianar River basin is located in the South-western Iberian Peninsula at latitude
72 37° 10' to 37° 45' N and longitude 6° 10' to 6° 25' W, near the Guadalquivir River mouth
73 in the Atlantic Ocean (Figure 1). The basin area is 1.325 km², and altitude ranges from 4
74 to 544 m.a.s.l. Climate is sub-humid Mediterranean with oceanic influences, with average

75 temperatures between 9°C in winter and 29°C in summer. Mean annual rainfall is 624
76 mm, oscillating between 754 mm in the source and 543 mm in the mouth. Rain falls
77 abundantly in autumn, winter and spring and is almost completely absent in summer. This
78 severe drought causes the drying of most small streams or the creation of isolated pools of
79 different sizes (Gasith and Resh, 1999). The basin shows a geological transition linked to
80 river section type. The upper section (near the source) lies within the Sierra Morena
81 mountain range, where forestry and livestock ranching land uses are predominant; the
82 middle section is under agricultural and urban land uses; and the lower section forms a
83 plain that precedes the marshland at the mouth. The hydrological network is interrupted
84 by two small reservoirs that collect less than 4 hm³ in the source area and one large
85 reservoir (20 hm³) in the Agrio River. This reservoir was built in 1977 to supply mining
86 industry needs, and it is located slightly upstream from the spill point (Borja et al. 2001)
87 (Figure 1).

88 Sampling protocol

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

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

101 < 5 m wide, and for wider streams, the length was 20 times the width. Captured fish were
102 identified to species and kept submerged in an appropriate container during sampling.

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

118 Statistical analyses

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

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

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

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

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

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

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

149 The second step to reduce the number of variables was to test for collinearity between the
150 remaining ones (Appendix 1) using Pearson’s correlations. Whenever the correlation

151 coefficient between two variables was greater than 0.75, one of the variables was chosen
152 for the regression models.

153 The final regression models were applied to a total of 22 cases (n=22) and a maximum of
154 6 predictor variables, since a larger number of variables would lead to a Type 2 error
155 (Field, 2005). The final variable list in each case included those that showed a high
156 correlation with the relevant dependent variable and low collinearity with the other
157 selected variables. A list of all the variables included in the 8 models is presented in Table
158 1.

159 The best models supported by the data were selected using the Akaike Information
160 Criterion (Burham and Anderson, 2002). This allowed us to decide which model
161 explained the most variance whilst being most parsimonious (Johnson and Omland,
162 2004). Variance partitioning of the significant variables selected for the general model
163 was performed to identify the most important scale in each case.

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

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

175

176 Results

177 Fish assemblage

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

183

184 Native species

185 a) General models

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

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

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

210

211 b) Catchment models

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

219

220 c) Site models

221 Site-scale models were significant for both native species richness (S-na_S) and diversity
222 (H-na_S), accounting for 53% ($R^2 = 0.53$) and 35% ($R^2 = 0.35$) of the variance,
223 respectively (Table 1). The main axis of PCA1 (“Habitat”) was identified as the most
224 influential factor for S-na_S and variance partitioning showed that it accounted for 45%
225 of the explained variance, whereas it was not selected in the case of H-na_S. This positive

226 relationship between the main axis of PCA1 (“Habitat”) and S-na_S reinforces the same
227 trend described for the General model (native richness is higher in those reaches with
228 coarser substrate and clearer water). “Distance to source” however, was the most
229 important factor for H-na_S and the second most important for S-na_S. According to
230 variance partitioning, this factor accounted for 54% and 23% of the explained variance,
231 respectively. This positive relationship between “Distance to source” and H-na_S and
232 Sna_S, reflects a similar explanation to that suggested for “Drainage area” in the General
233 model, showing the species-area relationship that occurs as you go downstream: distance
234 to source increases and so does the number of species found. “Number of reservoirs
235 upstream” was selected as the second most important factor for H-na_S and the third for
236 S-na_S, accounting for 46% and 21% of the explained variance, respectively. This
237 negative relationship concurs with that observed in the General model and reinforces the
238 idea that the more reservoirs upstream from a site, the lower the native fish richness and
239 diversity. The last variable selected by the model at the site scale was the main axis of
240 PCA3 (“Site uses”), which accounted for 11% of the explained variance for S-na_S. In
241 this axis human impact is located at the positive end, so this negative relationship shows
242 how, at the site scale, native fish richness is lower in areas where land uses are more
243 humanized, the same trend as that observed in the General and catchment-scale models.

244

245 Exotic species

246 a) General model

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

252 suggests that this is the most important scale for exotic species richness. Therefore, only a
253 more detailed site model was computed for exotic species richness.

254

255 b) Site model

256 The model for exotic species richness at the site scale (Sex_S) was significant and
257 explained 53% of the variance ($R^2 = 0.53$) (Table 1). As in the General model, “Mean
258 valley width” was identified as the most influential variable, followed by “River length
259 covered by reservoirs upstream” (RLCRU, Table 1) in this case, accounting for 55% and
260 45% of the explained variance, respectively. The positive relationship between “Mean
261 valley width” and exotic species richness confirms the results of the General model: an
262 increase in the number of exotic species as the channel becomes wider further away from
263 the source. The other positive relationship (“River length covered by reservoirs
264 upstream”) shows how exotic species are linked to reservoirs upstream.

265

266 Spill Effect

267 The ANOVA that compared species richness in the reach downstream from the Agrio
268 reservoir vs. the six selected reservoirs in similar watersheds yielded a significant result
269 ($F_{(6,19)} = 5.465$, $p = 0.002$). The post-hoc t-tests revealed significant differences between
270 the Guadiamar reach and reaches downstream from three reservoirs (Cala, Pintado and
271 Rumblar, $t = 3.67$, $p = 0.020$; $t = 3.72$, $p = 0.020$; and $t = -0.90$, $p = 0.040$; respectively),
272 whereas there were no differences with four others (Huesna, Montoro, Rumblar and
273 Fernandina) (Table 3). On the other hand, no significant differences were found ($F_{(6,19)} =$
274 2.384 , $p = 0.069$) between native species diversity downstream from the Agrio reservoir
275 and any of the other six selected reaches. Similarly, no significant differences were found

276 neither for exotic species richness ($F_{(6,19)} = 1.126$, $p = 0.384$) nor diversity ($F_{(6,19)} = 0.917$, p
277 $= 0.504$) (Table 3).

278 Discussion

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

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

299 At the site scale, the number of reservoirs upstream acts as the other main influence for
300 native richness and diversity, representing a pivotal point for fish distribution in the basin
301 under study. According to variance partitioning, upstream reservoirs are even more

302 important than the well-known species-area relationship trend of higher richness with
303 greater drainage area (McArthur and Wilson, 1967; Sheldon, 1988), which is evident near
304 the Agrio reservoir (Figure 1). At this point, however, the trend is reversed and native
305 species richness decreases dramatically downstream from the dam. This decline is
306 probably due to the artificial conditions of the reach immediately downstream from the
307 reservoir (R.J. De Miguel, personal observation), where there is an absence of necessary
308 habitat elements and fish may be suffering from heavy predation pressure from exotic
309 species after dam release periods (Clavero and Hermoso, 2011).

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

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

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

347 Unfortunately, the attempt to discern between spill and reservoir effects did not yield a
348 clear result, but suggests a combination of events. The observed native species richness
349 and diversity depletion caused by reservoirs in other river basins, similar to Guadianar,
350 provides a range of values, and those observed in the Guadianar River fit within that
351 range. Therefore, the current potential effects of spill remnants are not strong enough to
352 cause abnormal fish species richness and diversity values. The same result is obtained for
353 exotic species, which suggests that the set of factors that promote exotic species richness
354 in the Guadianar River basin are equal to those found in other similar river basins, not
355 affected by the spill. This may be because the habitat recovery works have minimized the

356 spill effect and the reach originally affected is now exposed to the same impacts that it
357 suffered before this event.

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

373

374 Acknowledgements

375 This study is part of the projects “Bases para la elaboración de un plan de conservación
376 de los peces continentales autóctonos de Andalucía” and the Guadamar Green Corridor
377 Research Program (PICOVER), both funded by the Andalusian Regional Government.
378 We thank Teresa Saldaña, Antonio Barranco, David Redondo, Manuel Fernández,
379 Enrique Pino, Alejandro Ramiro, Javier Peña and Francisco Aranda for their help both in
380 the field and with GIS.

381

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