Ecology of
FRESHWATER FISH

Assessing freshwater fish sensitivity to different sources of perturbation in a Mediterranean basin.

Journal:	*Ecology of Freshwater Fish*
Manuscript ID:	EFF-08-0075.R4
Manuscript Type:	Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Hermoso, Virgilio; University of Huelva, Biología Ambiental y Salud Pública Blanco-Garrido, Francisco; University of Huelva, Biología Ambiental y Salud Pública Clavero, Miguel; Centre Tecnològic Forestal de Catalunya, Grup d□Ecologia del Paisatge, Àrea de Biodiversitat; Departament de Ciències Ambientals, Universitat de Girona Prenda, José; University of Huelva, Biología Ambiental y Salud Pública; Universidad de Huelva, Biología Ambiental
Keywords:	Bioindicators, bioassessment, human impairment, tolerance, Water Framework Directive

Assessing freshwater fish sensitivity to different sources of perturbation in a Mediterranean basin.

Virgilio Hermoso[1], Miguel Clavero[2,3], Francisco Blanco-Garrido[1,4] and José Prenda[1].

[1] Departamento de Biología Ambiental y Salud Pública, Universidad de Huelva. Avda. Andalucía s/n, 21071 Huelva, Spain.

[2] Grup d'Ecologia del Paisatge, Àrea de Biodiversitat, Centre Tecnològic Forestal de Catalunya. Carretera vella de Sant Llorenç de Morunys km 2, 25280 Solsona, Spain.

[3] Departament de Ciències Ambientals, Universitat de Girona. Campus de Montilivi, 17071 Girona, Spain.

[4] Mediodes, Consultoría Ambiental y Paisajismo S.L. Bulevar Louis Pasteur, 1 Blq. 2-1-1. 29010 Malaga, Spain.

Corresponding author:

Virgilio Hermoso

E-mail: virgilio.hermoso@gmail.com

Tlf: +34 959219895

Fax: +34 959219876

Running headline: Assessing freshwater fish sensitivity

KEYWORDS: Bioindicators, bioassessment, human impairment, tolerance, Water Framework Directive.

Abstract

The accuracy of bioassessment programs is highly limited by the precision of the systems used to derive sensitivity-tolerance values for the organisms used as indicators. We provide quantitative support to the objective evaluation of freshwater fish species sensitivity to different sources of disturbance, accounting for co-variation issues not only between perturbations-natural gradients (especially river size), but also between different perturbations. With this aim we performed two different Principal Component Analyses , i) on a general environmental matrix to obtain a perturbation gradient independent of river size effects, and ii) on human impairment related variables to extract independent synthetic perturbation gradients. Then we checked each species responses to those gradients to assess their sensitivity-tolerance values through an available-used chi-squared analysis in the first approach and through a t-test/ANCOVA analysis in the second one. In this way we obtained sensitivity-tolerance values which could be included in future bioassessment tools, enabling effective evaluations.

Introduction

Freshwater ecosystems are submitted to an accelerated rate of transformation due to the intensive human use they suffer (Vitousek, 1994; Collares-Pereira & Cowx, 2004; Prenda et al,. 2006). This implies a critical threat to a substantive quote of the global biodiversity they hold (Abell, 2002). As an example, only freshwater fish comprise one-fourth of all living vertebrate species (Abell, 2002) and recent assessments suggest that over 30% of them are seriously threatened (World Conservation Union, 2000). Thus, there is an urgent need to assess the ecological status of freshwater ecosystems and determine how they are being affected by human transformations (Revenga & Kura, 2003). Many international laws such as the Clean Water Act in the US or the European Water Framework Directive (WFD) (European Commision, 2000) try to address this problem by requiring protection and restoration of the biological integrity as part of water quality standards. The WFD endorses the application of such principles through the development of bioassessment programs using four different biotic indicators (diatoms, macrophytes, macroinvertebrates and fish). The adequate implementation of such principles requires an adequate knowledge of tolerance limits for those organisms used as bioindicators.

Tolerance to impairment refers to the degree to which an organism can withstand stressors related to human disturbance (Yuan, 2004). Therefore more tolerant organisms can withstand more disturbed environments, but this does not necessarily imply that they could continue to survive in a broader range of conditions, as Shelford´s law of tolerance suggests (Shelford, 1911). The designation of tolerance values is based on interpreting characteristics of different taxon-environment relationships, in a manner similar to that used in studies of ecological niches (Yuan, 2004). Different procedures are being used to estimate sensitivity-tolerance values for aquatic organisms, including expert judgment (Oberdorff et al., 2002), empirical analysis (Meador and Carlisle, 2007, Carlisle et al., 2007, Whittier et al., 2007) or modelling approaches (Armitage et al., 1995; Yuan 2004, Cao & Hawkins, 2005). Then these values are incorporated into biotic indices which mainly compare the expected community composition in the absence of human perturbation with that observed, following the Reference Condition Approach (Wright et al., 1984; Reynoldson et al., 1997).

However, species' diagnostic power is infra-used when using simplified sensitivity values in two categories (tolerant/intolerant) (Oberdorff et al., 2002; Pont et al., 2006, Ferreira et al., 2007). Moreover, most previous studies (usually centered on macroinvertebrates) have defined sensitivity-tolerance with respect to a single source of perturbation (Armitage et al., 1983; Lenat, 1993). A finer knowledge on species sensitivity-tolerance to particular sources of perturbation would allow managers to face more accurate diagnostics of potential causes of impairment (Norton et al., 2000, Yuan, 2004, Meador & Carlisle, 2007) and to tackle efficient corrective programs.

An essential issue to be considered when interpreting species' sensitivity-tolerance is the effect of co-variation issues among different types of perturbation (Meador & Carlisle, 2007) and along natural gradients (Yuan, 2004). River size (or longitudinal gradient) has been pointed out as a key factor explaining the ecology of freshwater ecosystems (Vannote et al., 1980; Pringle, 2001) and structuring fish community composition (Angermeier & Schlosser, 1989; Matthews, 1998; Magalhães et al., 2002). The effect of natural gradients on sensitivity-tolerance values is expected to increase as species' (or any other taxonomic level) home range extent does, since the larger the home range, the broader environmental conditions they occupy. This is of special interest for freshwater fishes, which usually display medium-large spatial domains (e.g. Koster & Crook, 2008).

Mediterranean freshwater fish species have evolved in harsh environments (e.g. facing severe droughts and floods) and have generally developed short lifespans, generalist habitat use, opportunistic feeding strategies, high fecundity and early sexual maturity (e. g. Velasco et al., 1990; Vila-Gispert & Moreno-Amich, 2002). All these ecological characteristics may be a problem in the assessment of their sensitivity-tolerance and may impose serious limitations to the development of effective tools to assess the ecological status of Mediterranean rivers.

In this work, we mainly aim to estimate species' sensitivity-tolerance to human and biotic disturbances facing the question of co-variation and trying to derive sensitivity-tolerance values to make reliable diagnostics of human impairment in a Mediterranean basin. These results could then be integrated in bioassessment programs through biotic indices ensuring objective evaluations.

Methods

Study area

The Guadiana River basin is located in the South-Western Iberian Peninsula draining a total area of 67,039 km^2 to the Atlantic Ocean. It features a typical Mediterranean climate, with high intra and inter-annual discharge variation, with severe and unpredictable floods between autumn and spring and persistent summer droughts (Gasith & Resh, 1999). Mean air temperature ranges from 13 to 18.1 ºC, with a strong intra-annual variation in extreme temperatures. Mean annual precipitation ranges from 350 to 1200 mm (with a mean of 450 mm).

Although it is not an overpopulated area (28 hab/km^2), the landscape has been deeply transformed during the last century by agricultural activities. Almost a half of the basin (49.1%) is currently under agriculture uses (30.6% occupied with intensive agriculture as irrigated lands and 18.5% occupied with extensive agriculture, like olive groves or fruit trees). As a consequence, about 11,000 hm^3 of water is retained in 88 large reservoirs (>1 hm^3) and more than 200 small ones (<1 hm^3) for water supply. Other common human perturbations are related to river channel modifications such as river channelization and degradation and even completely depletion of the riparian forest.

Characterization of fish community and habitat

Fish community was characterized in 241 localities through the whole basin, using electrofishing during spring in 2002, 2005 and 2006. Sampling was conducted once at each location without block-nets along 100 m long stretches, covering all habitats available at this scale. This sampling effort has been proved to be sufficient to capture most species present, except for large rivers, as Filipe et al. (2004) suggest on a previous study in the same area. However, large rivers were not a major problem since no more than 2% of sites were non-wadable. Alternative methodological approaches similar to that used in other European countries for these kinds of environments (Kestemont & Goffaux, 2002) were followed at those sites. All fish were identified to species level when possible and then returned to the water. Given the difficulties to correctly identify young of the year individuals they were not included in the analysis. Only native species were tested for their sensitivity, as exotics presences are highly dependent on human introductions. The use of multiple-year data may reduce the effects of non-representative years and allows tackling more realistic studies, especially in highly-variable environments such as the Mediterranean ones (Gasith & Resh, 1999). This approach has been used elsewhere in this kind of study (e.g., Yuan, 2004; Carlisle et al., 2007). Moreover, presence-absence data has been proved to be more inter/intra annual stable than abundance data in this environment (Magalhães et al., 2007).

Sampling sites were proportionally located along six different river types identified by the Spanish Ministry of Environment (Ministerio de Medio Ambiente, 2005). These river types grouped streams with similar environmental conditions (climate, geology, geography) and arose from the application of one of the classification systems (B) proposed in the WFD (European Commission, 2003). In this way we ensured a correct characterization of both fish and habitat in the basin gathering a wide range of biotic and environmental conditions.

Habitat was characterised through 38 environmental variables, covering three different spatial scales: site, reach and basin. Two approaches were used in this characterization: *in situ* measures, which described micro and mesohabitat characteristics at each locality, and remote GIS measures used to record variables from digital maps (Table 1). *In situ* variables (except water quality measures) were recorded from transects located every 20 meters within the surveyed river stretch (9-21 measures/reach). Then mean values were used for the analysis. Climatic variables were extracted from the Digital Climatic Map of the Iberian Peninsula (Ninyerola et al., 2005) which was built on long temporal series (15-50 years long). Thus, we assumed our data to represent a mean climatic year in the area. For land-cover data, we used a digital map provided by the Guadiana basin´s management authority (Confederación Hidrográfica del Guadiana) which represented the situation at 2003. Potential changes in land uses were ruled out in the short period of time in which the study was carried out. All these environmental metrics could be split in two categories: a) variables that described the natural habitat variability in the basin and b) descriptors of human perturbations (Table 1). All variables were checked for normality and transformed when necessary prior to analysis (arcsine for land uses variables -expressed as %- and log (x+1) for the remaining).

Evaluation of species´ sensitivity to human impairment.

We assessed species´ sensitivity through two different approaches: i) checking the responses of each species' occurrences to a general perturbation gradient, including all the set of environmental variables, as it has traditionally been done (Armitage et al., 1983; Lenat, 1993), and ii) exploring the partial responses of each species' occurrence to a set of independent perturbations (human and biotic). Additionally, we compared our results with other two commonly used approaches to assess species sensitivity (see below). We discarded from the analysis all native species with very low prevalence (<5%) (*Anguilla anguilla*, *Alosa alosa*, *Gobio lozanoi* and *Luciobarbus guiraonis*), due to the difficulty to differentiate their presences-absences from a random distribution. Although other authors have pointed out their value for bioassessment (Cao et al., 1998), this is not the aim of this work and their sensitivity-tolerance would have low interpretable value and it may have immediate negative consequences on bioassessment as Van Sickle et al. (2007) recently pointed out. Thus we finally considered 10 species in the analysis (listed in Table 3).

Definition of Environmental, Human Impairment and Fish Community gradients.

To face the general problem of co-variation in the assessment of species´ sensitivity, we carried out a set of multivariate analysis to extract independent synthetic perturbation gradients. In a first approach a Principal Component Analysis (PCA) was applied to the environmental variables x sites matrix (Table 1), to account for natural variability in the analysis. A varimax rotated PCA was used in this case to clarify the sense of the extracted gradients (Table 2). The first two PCs accounted for the 42.7% of the original variance of our data. The PC1 was mainly related to variables describing land uses at the basin and reach scales, chemical perturbations and the conservation status of the riparian forest (Table 2). This gradient was also related to some climate variables such as mean air temperature and rainfall as well as altitude. All the variables describing river size scored highly in the PC2 (Table 2). Thus, two independent gradients were identified: a perturbation-climatic gradient in PC1 and a longitudinal natural gradient in PC2. Given the orthogonal nature of these two PCs the species´ response to the general perturbation-climatic gradient could be tested discarding the effect of river size, as Kennard et al. (2005) suggested.

A second PCA was carried out exclusively on human impairment variables (Table 1) to obtain a set of synthetic perturbation variables. This would allow in depth studies of species´ responses to specific stressors considering co-variation issues among perturbations and perturbations-longitudinal gradient. The first 6 PCs extracted from this PCA with eigenvalues>1 (McGarigal et al., 2000) explained more than two thirds of the original variance (68.6%). Each of them was related to a particular source of human impairment such as the portion of the basin in natural condition (with low agriculture or urban uses levels) (PC1_Nat); phosphorous enrichment, probably related to urban waste water (PC2_Phs); effects of downstream river regulation (PC3_Dwn); increase in nitrates concentration due to agriculture fertilizers (PC4_Nta); other effects related to agriculture at the reach scale (PC5_Agr) and upstream river regulation (PC6_Ups). Only basin naturalness and effects of downstream river regulation gradients were significantly related to the longitudinal natural gradient described above (Pearson´s $r=0.3$, $p<0.001$, and $r=0.5$, $p<0.001$, for basin naturalness and downstream river regulation respectively).

As freshwater fish communities tend to vary along longitudinal gradients -upstream-downstream- in this environment (Magalhães et al., 2002), their spatial location within this natural gradient must be accounted for in species sensitivity-tolerance analyses. A Correspondence Analysis (CA) was performed in a species´ presence-absence x sites matrix to identify the main patterns of variation in fish community composition within the study area. The first dimension (DIM 1 which accounted for 21.9% of the fish community variance) was strongly correlated to the longitudinal gradient (Pearson´s r=0.61, p>0.001), showing a clear spatial change in fish community composition through the longitudinal gradient. Thus, some species tended to appear predominantly in headwaters while others occurred mainly in low or medium stretches.

Species´ sensitivity to general human disturbances.

The general perturbation gradient (PC1) was split into five equivalent portions to evaluate the hypothesis of non-randomness of species distribution along it. The intensity with which the species used each portion (measured as the number of localities where each species was present) was compared to its availability (measured as the total number of localities within each portion). The null hypothesis of random association between the amount of habitat available and used was tested through a Chi-square test (Prenda et al., 1997; Morán-López et al., 2005). If rejected, a partitioned Chi square test was conducted to determine those portions that contributed to the statistical significance, i.e., in which perturbation class the species was over- or under-represented. An overuse of low impacted portions and an under-use of degraded ones would be expected for sensitive species, the opposite pattern for tolerant species, while insensitive species should exhibit a random use of the whole perturbation gradient. Then an index of species sensitivity (Available/used index) was built as the difference in over/under use of both extremes of the perturbation gradient (considering over/under use as the difference between used-available sites in Fig 2). Positive values (the species overused the less degraded portion and rejected or disappeared from the degraded portion) indicate sensitive species while negative values (the species under-used the less degraded portion and/or overused the degraded one) are related to tolerant species.

Species´ sensitivity has been assessed through many different indices and approaches (Cao & Hawkins, 2005; Lenat, 1993). We applied two of these indices focused on the evaluation of species´ sensitivity to general perturbation gradients, such as the Tolerance Value (TV) from Knapp et al. (2005) and the RD/TD index of Hawkins et al. (2000), to check for parallelism with our results. TV is based on observed vs expected presences (O/E) in test sites derived from RIVPACS models. TV values larger than 1 identify tolerant species while those TV<1 indicate sensitive taxa. We used the outcomes from an Assessment by Nearest Neighbour Analysis (ANNA) model (Linke et al., 2005) instead of RIVPACS given its higher performance on our data set (Hermoso et al., in press). This method was initially developed for predicting the occurrence of macroinvertebrates in South-west Australia. In ANNA, sites are treated as a continuum avoiding artificial classifications, and predictions are derived from the environmentally most-similar reference sites. The ANNA model finds the set of most environmentally-similar reference sites for each target site, and predicts its community composition based on the community composition of those nearest neighbours (Linke et al., 2005). Our ANNA model used the nearest 6 reference sites to predict species occurrences (see Linke et al., 2005 and Hermoso et al., in press for more details about ANNA models). The RD/TD index, which we adapted to be used with presence-absence data, measures species' tolerances as the relationship between the proportional difference in mean taxon abundance between reference and test sites. Our index was assessed as the ratio RP/TP, where RP and TP resemble RD and TD in Hawkins` index (Hawkins et al., 2000). Here RP and TP are the proportion of reference -R-and test -T- sites where the species occurred respectively. In this modified index, tolerant species would show values close to 0 (the species was present in a reduced proportion of reference sites while was present in a high proportion of test sites), while sensitive species would get higher values over 1 (the species was present in a high proportion of reference sites while present in a reduced proportion of test sites). Reference sites were selected from the original data set as the less affected by human perturbation (low urban or agricultural land uses at the basin and reach scale -500 m around the sampling point-, bank and channel structure in natural condition, a naturalized riparian forest and exotic species accounting for less than 5% of total fish abundance). We considered 70 reference sites which were located along the whole longitudinal gradient, though not homogeneously distributed. Finally, we looked for potential influences of the natural longitudinal gradient on sensitivity-tolerance values. With this aim each species´ scores in all the three indices were correlated to their loadings in the DIM1. No significant correlation would be expected if the indices were completely independent of river size effects.

Species´ sensitivity to specific sources of human and biotic disturbance.

We explored the relationship between species´ presence-absence and the set of independent perturbation gradients defining the main sources of human disturbances. We also used two additional measures of biotic perturbation dealing with the degree of exotic fish dominance in the community (percentage of both total exotic abundance and species richness). None of these biotic perturbation measures was highly influenced by the longitudinal gradient (Pearson´s r<|0.2| for both the percentage of total exotic abundance and species richness) and any other human perturbation (Pearson´s r<0.17 for all possible perturbation gradients-biotic variables combinations). Therefore, they did not introduce any additional source of co-variation in the analysis. We used percentages instead of original data (total exotic abundance and species richness) to avoid the effect of local abundance and species richness in the analysis.

We tested the effects of species presence-absence on each independent perturbation gradient, aiming to identify significant differences in the perturbation gradients between occupied and unoccupied sites. When perturbations were independent from the longitudinal gradient we used t-test to compare occupied and unoccupied sites. Whenever we detected a significant relationship between perturbations and the longitudinal gradient we used ANCOVA models to test for these differences. We used the longitudinal gradient as covariate, testing the influence of each target species presence-absence of each species (factor) on each perturbation gradient (dependent variable). We ran ANCOVA analysis using a two-step procedure: i) first, we tested the homogeneity of slopes assumption through the significance of the interaction term (presence-absence × longitudinal gradient), in case of significance we kept this complete model; ii) When the interaction was not statistically significant, it was deleted from the models, and standard ANCOVA analyses were run.

The ratio between the ranges of each perturbation gradient in which the species was present/absent was used as a measure of their sensitivity in this case. All of them were tested for their relationship with the longitudinal gradient in the same way as it was previously done with the general perturbation gradient.

Results

Species´ sensitivity to general human disturbances.

The analysis of available/used through the 5 equivalent segments of the general perturbation-climatic gradient pointed out the sensitivity of each species to this gradient (Fig. 2 and Fig. 3). Some species showed a clear sensitivity, over-using the best preserved portions, avoiding (or disappearing from) the perturbed portions and showing the highest values for the Availability/used Index hence (*Luciobarbus sclateri*, *Pseudochondrostoma willkommii* and *Anaecypris hispanica*). Other species showed an intermediate sensitivity as they used the best preserved portions as they were available and under-used only the worst portions with intermediate values for the index (*Luciobarbus microcephalus*, *Luciobarbus comizo* and *Salaria fluviatilis*). Insensitive species were characterized by a general use as available (*Iberocypris alburnoides*, *Cobitis paludica*) or erratic patterns in the use of the whole gradient (*Squalius pyrenaicus* and *Iberochondrostoma lemmingii*) (Table 3).

The two alternative tested indices (RP/TP and TV) were highly correlated (Pearson´s r=0.78, p=0.008) showing similar patterns in species tolerance, but not concordant with our previous results (Fig. 3). Additionally each species sensitivity scores were related to their respective loading within the CA ordination gradient (Table 3). Thus, the species present at lower reaches tended to show higher tolerance values (*L. comizo* and *S. fluviatilis*) than the species present in headwaters-middle reaches (*S. pyrenaicus*, *I. lemmingii*, *L. sclateri* and *A. hispanica*) (Fig. 3). This effect was not detected in the Available/used Index (Table 3).

Species´ sensitivity to specific sources of human and biotic disturbance.

The t-test/ANCOVA analysis allowed to deep on each species´ sensitivity to particular sources of human perturbation at a finer scale. These results are summarized in Figure 4. As expected, the most sensitive species to the general perturbation gradient showed the strongest responses to some synthetic disturbance variables (*L. sclateri* to nutrient enrichment due to both P and N, *P. willkommii* to P enrichment, the effects of agriculture at the reach scale and upstream river regulation; *A. hispanica* mainly responded to the basin naturalness status and the enrichment in nitrates). However, this refined approach showed responses even for those species labeled above as insensitive. *S. pyrenaicus* showed significant responses to nitrates enrichment and upstream river regulation, *I. lemmingii* to upstream river regulation and *I. alburnoides* to the surrounding agriculture stress gradient and upstream river regulation. In the same way, stronger responses were found for intermediate sensitive species as *S. fluviatilis*, *L. comizo* and *L. microcephalus* to surrounding agriculture. No species was found to be sensitive to downstream river regulation after accounting for the effect of river size. All the species, except *L. comizo* and *S. fluviatilis,* showed a significant response to the biological degradation. Additionally, no significant effect of river size was found in these sensitivity-tolerance values (Table 3).

Discussion

In this study we provide quantitative support to the evaluation of fish species sensitivity-tolerance to human disturbances in a Mediterranean basin. A deep knowledge on species sensitivity is fundamental for a successful diagnostic of human disturbances affecting a target area and the ability to undertake effective remedial programs to face the present severe situation of freshwater ecosystems, and the correct implementation of the WFD exigencies.

The ability to develop accurate bioassessment programs based on bioindicators is highly limited by the precision of the systems used to derive sensitivity-tolerance values for the target taxa. Although many efforts have been focused on the evaluation of macroinvertebrate sensitivity to different human perturbations (Armitage et al., 1987; Yuan, 2004; Carlisle et al., 2007), little attention has been devoted to freshwater fish (Meador & Carlisle, 2007, Whittier at el., 2007) and there is no information concerning to European freshwater fish species. Instead of empirical-derived values of species tolerance or sensitivity, expert judgment has traditionally been applied in freshwater fish based bioassessment programs (Karr, 1981; Oberdorff et al., 2002; Pont et al., 2006). The expert judgment relies on subjective appreciations that have proved to be unreliable in previous studies (Lenat, 1993). Therefore, the empirical approach we followed in this study ensures an objective evaluation of freshwater fish sensitivity-tolerance and suggests an important contribution to the future implementation of fish-based bioassessment programs.

Presence-absence data is the basis of some of the more widespread an applied bioassessment methods all around the world, as RIVPACS or AUSRIVAS (Wright et al., 1993; Norris, 1996). It could be expected that these methods are insensitive to many perturbations, because individual populations of some species can suffer a considerable degradation before becoming locally extinct. However, at the assemblage level, presence-absence data appears to be sufficiently robust to allow the detection of reasonably subtle differences among sites (Hawkins et al., 2000). Only slightly differences have been reported in taxa tolerance values when using presence-absence data instead of abundance data (Yuan, 2004). Population densities are submitted to a greater seasonal and annual variation rates than presences-absences, especially in harsh environments such as the Mediterranean (Magalhães et al., 2007). Moreover, this simpler approach has additional advantages vs abundance-based ones, since the characterization of taxa's abundance is largely more complex and difficult to standardize than the evaluation of species presences-absences. Sampling accuracy could then be reflected not only in bioassessment results, but also in sensitivity values (Lenat, 1993). Additionally, many efforts have been focused on the study of the optimal sampling effort to adequately characterize the presence of species in Mediterranean environments (Filipe et al., 2004) rather than abundance. The WFD requires the use of abundance data, as well as presence-absence, in the implementation of bioassessment programs. However, if presence-absence data allows reasonably accurate assessments, we could face all the drawbacks previously emphasized when using abundance data and discard them from future bioassessment tools.

In a first step we tackled the need to account for natural environmental gradients when analyzing species´ sensitivity-tolerance. The broad range of environmental (natural and perturbation) variables gathered in this study addresses potential shortcomings associated to inaccurate sampling designs. However there are a number of human perturbations which were not included in this study and should be considered in further approaches. We applied an available/used analysis through a broad perturbation-climatic gradient free from stream size effects, which has been described as a key factor determining species´ occurrences in freshwater systems (Vanote et al., 1980; Pringle, 2001; Magalhães et al., 2002). Given that perturbations are not homogeneously distributed along the natural longitudinal gradient, sensitivity-tolerance values can be confounded by the portion of the longitudinal gradient where each species occurred preferentially. However we were not able to completely discard natural effects from the perturbation gradient, since it also included some climatic and altitude variables. Given the clear spatial pattern that all these natural variables followed in the basin (from the southwest portion with the lowest altitudes and the highest mean rainfall and air temperatures to the northeast portion with the highest altitudes and the lowest mean precipitations and air temperature) the current gradient could also be reflecting a spatial gradient in perturbations. This only implied major problems for the evaluation of *Luciobarbus sclateri* whose natural distribution is restricted to the southwest portion of the basin, while the remaining species appeared homogeneously distributed throughout the whole basin. Thus, a more specific study focused on this species should be highly recommended to better portray the spatial limitation of this broad study. Most of the remaining species showed to be sensitive to this gradient at different intensities. However, this result was not concordant with the outcomes from two alternative methods. A clear relationship between the sensitivity values derived for each species and their pattern of spatial distribution within the longitudinal gradient pointed out the influence of river size on species´ sensitivity values in both alternative methods. Different causes may be responsible for these results. The exclusion of natural covariates had been previously reported as an important cause of differences in sensitivity measures in certain taxa (Yuan, 2004). We did not remove the effect of river size in Hawkins´ RP/TP index, since although reference localities were defined along the whole longitudinal gradient, they were not homogeneously distributed. They were mainly located in headwaters-middle reaches, while few of them were found in low reaches. As a consequence, species occurring in low reaches tended to have over-estimated tolerance values, whereas species which mainly occurred in headwaters showed under-estimated tolerance values. Although downstream habitats are known to be naturally more stressful and species occurring there tend to show larger physiological flexibility, and tolerance hence, than species occurring in upstream reaches (Mathews, 1998), this pattern is not so clear in Mediterranean rivers, where headwater reaches become harsh environments for freshwater fish during summer droughts (Magalhães et al., 2002). Thus, natural adaptive strategies can be discarded as the main reason of the results showed by this index. In the second method that we tested, Cao & Hawkins computed species tolerance through the ratio O/E presences in perturbed sites in their TV index. River size effect was *a priori* accounted for in this approach as predictive models included variables describing the longitudinal gradient, but this hypothesis was not supported by our results. In this case the effect of predictive models inaccuracies may be the cause of such result, although the ANNA model was which best fitted the data in previous studies in the same study area (Hermoso et al., in press). Therefore, the effect of river size must be considered when evaluating species sensitivity to human perturbation and special care must be taken if predictive models are used.

In most studies, sensitivity has been defined with respect to a single gradient of anthropogenic disturbance (Hilsenhoff, 1987; Armitage et al., 1983) lacking the evaluation of species´ sensitivities to particular sources of human perturbation. Given the urgent need of more accurate diagnostics of human impairment, the use of these kinds of studies would be greatly enhanced if they were focused on specific sensitivities to a wider number of independent perturbations (Norton et al., 2000, Meador & Carlisle, 2007, Whittier, et al., 2007). In this complex scenario an additional factor such as co-variation among different sources of perturbation must be considered (Meador & Carlisle, 2007). We tackled this issue through the generation of a number of synthetic independent variables representing the most significant measured perturbations within the study area. Although PCA is a common procedure used to identify the main sources of variation in ecological studies and to define synthetic variables in sensitivity-tolerance studies (Yuan, 2004, Whittier et al., 2007), special care must be taken when interpreting the results (artificial variables are used to test species´ responses instead of original perturbations). We also avoided river size effects using a longitudinal gradient as covariate in the analysis when necessary. In this finer analysis clearer responses of species to particular sources of human impairment were found, even for species previously classified as low sensitive or insensitive. Thus, through more detailed approaches like ANCOVA analysis or GAM models used in similar studies (Yuan, 2004) the need of more in depth information on species sensitivity for an accurate site specific diagnostic on human disturbances can be addressed.

Significant effects were found for almost every synthetic perturbation gradient at least on one species. *A. hispanica* showed the strongest response to the degree of land and riparian transformation at the basin and reach scales, appearing predominantly in the most natural sites. *L. sclateri* was the most sensitive species to changes in water quality (nutrient enrichment), *P. willkommii* to upstream river regulation and *S. fluviatilis* to effects derived from agriculture at the reach scale (possibly an increased rate of sedimentation according to the ecological requirements of this species). Slighter responses were found for the remaining species which ensures the capability of future fish-based bioassessment tools to detect the main environmental impairment causes and make accurate diagnostics. Additionally all the species except *L. comizo* and *S. fluviatilis* responded to the degree of biotic impairment. However, none of the tested species showed a clear response to downstream river regulation. Due to the poor conservation status of large-migratory species, such as *A. anguilla* or *A. alosa*, they could not be included in this study. These species would have showed clearer responses to this former perturbation.

Tolerance values are commonly simplified to ordinal scales for being used within biotic indices in bioassessment tools (Yuan, 2004; Carlisle et al., 2007). As Whittier and Hughes (1998) noted there is a considerable variability in the number of tolerance classes ranging from one to ten in published literature. Additionally there is not a standardize criteria for selecting the limits in tolerance values between classes. For that reason we suggest avoiding these classifications and using quantitative measures of species´ sensitivity-tolerance values. Further studies are needed to introduce this continuum concept on biotic indices used in bioassessment programs.

Acknowledgements

The fieldwork was funded by the Confederación Hidrográfica del Guadiana through the project "Diseño y Explotación de la Red de Control Biológico de la Cuenca del Río Guadiana" and the Ministry of Science and Technology (REN2002-03513, CGL2005-02699). V. Hermoso holds a pre-doctoral fellowship financed by the Spanish Ministry of Education (AP-2004-1414). We also thank two anonymous reviewers for their constructive comments and Anna Jones for the English review.

References

Abell, R. 2002. Conservation Biology for the biodiversity crisis: a freshwater follow-up. Conservation Biology 16: 1435-1437.

Angermeier P.L. & Schlosser, I.J.. 1989. Species-area relationships for stream fishes. Ecology 70: 1450-1462.

Armitage, P.D., Moss, D., Wright, J.F. & Furse, M.T. 1983. The performance of a new biological water quality score system based on macroinvertebrates over a wide range of unpolluted running-water sites. Water Research 17: 333-347.

Armitage, P.D., Gunn, R..J.M., Furse M.T.,. Wright, J.F & Moss, D. 1987. The use of prediction to assess macroinvertebrate response to river regulation. Hydrobiologia 144: 25-32.

Cao, Y. & Hawkins, C.P. 2005. Simulating biological impairment to evaluate the accuracy of ecological indicators. Journal of Applied Ecology 42: 954-965.

Cao, Y., Williams, D.D. & Williams, N.E. 1998. How important are rare species in aquatic community ecology and bioassessment? Limnology and Oceanography 43: 1403-1409.

Carlisle, D.M, Meador, M.R., Moulton II, S.R. & Ruhl, P.M. 2007. Estimation and application of indicator values for common macroinvertebrate genera and families of the United States. Ecological Indicator 7: 22-33.

European Commicion. 2003 Common Implementation Strategy for the Water Framework Directive (2000/60/EC). Identification of Water Bodies, January 2003.

European Commision. 2000. Directive 2000/60/EC of the European Council and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy. Official Journal of the European Communities, L327 1-72.

Ferreira, M.T., Caiola, N., Casals, F., Oliveira, J.M. & De Sostoa, A. 2007. Assessing perturbation of river fish communities in the Iberian Ecoregion. Fisheries Management and Ecology 14: 519-530.

Filipe, A.F., Marques, T.A., Seabra, S., Tiago, P., Ribeiro, F., Moreira Da Costa, L., Cowx, I.G. & Collares-Pereira, M.J. 2004. Selection of priority areas for fish conservation in Guadiana River Basin, Iberian Peninsula. Conservation Biology 18: 189-200.

Gasith, A. & Resh, V.H. 1999. Streams in Mediterranean climate regions: abiotic influences and biotic responses to predictable seasonal events. Annual Review of Ecology and Systematics 30: 51-81.

Hawkins, C.P., Norris, R.H., Hogue, J.N. & Feminella, J.W. 2000. Development and evaluation of predictive models for measuring the biological integrity of streams. Ecological Applications 10: 1456-1477.

Hermoso, V., Linke, S. & Prenda J. (in press). Identifying priority sites for the conservation of freshwater fish biodiversity in a Mediterranean basin with high degree of threatened endemics. Hydrobiologia.

Hilsenhoff, W.L. 1987. An improved biotic index of organic stream pollution. The Great Lakes Entomologist 20: 31-37.

Karr, J.R. 1981. Assessment of biotic integrity using fish communities. Fisheries **6**: 21-27.

Kennard, M.J., Arthington, A.H., Pusey, B.J. & Harch, B.D. 2005. Are alien fish a reliable indicator of river health? Freshwater Biology 50: 174-183.

Knapp, R.A., Hawkins, C.P., Ladau, J. & Mcclory, J.G. 2005. Fauna of Yosemite National Park lakes low resistance but high resilience to fish introductions. Ecological Applications 15: 835-847.

Koster, W.M. & Crook, D.A. 2008. Diurnal and nocturnal movements of river blackfish (Gadopsis marmoratus) in a south-eastern Australian upland stream. Ecology of freshwater fish 17: 146-154.

Lenat, D.R. 1993. A biotic index for the southeastern United States: derivation and list of tolerance values, with criteria for assigning water-quality ratings. Journal of the North American Benthological Society 12: 279-290.

Linke, S., Norris, R.H ., Faith, D.P. & Stockwell D. 2005. ANNA: A new prediction method for bioassessment programs. Freshwater Biology 50: 147-158.

Magalhães M.F., Batalha, D.C. & Collares-Pereira, M.J. 2002. Gradients in stream fish assemblages across a Mediterranean landscape: contributions of environmental factors and spatial structure. Freshwater Biology 47: 1015-1031.

Magalhães, M.F., Beja, P., Schlosser, I.J. & Collares-Pereira. M.J. 2007. Effects of multi-year droughts on fish assemblages of seasonally drying Mediterranean streams. Freshwater Biology 52: 1494-1510.

Matthews W.A. 1998. Patterns in freshwater fish ecology. Chapman & Hall: New York.

McGarigal K, Cushman, S. & Sttaford, S. 2000. Multivariate statistics for wildlife and ecology research. Springer: New York.

Meador, M.R. & Carlisle, D.M. 2007. Quantifying tolerance indicator values for common stream fish species of the United States. Ecological Indicators 7: 329-338.

Ministerio de Medio Ambiente. (2005) Análisis de las características de las demarcaciones. Caracterización de los tipos de ríos y lagos, June 2005.

Morán-López, R., Pérez-Bote, J.L., Da Silva Rubio, E. & Corbacho Amado, C. 2005. Summer habitat relationships of barbells in south-west Spain. Journal of Fish Biology 67: 66-82.

Munné, A., Prat, N., Solá, C., Bonada, N. & Rieradevall, M. 2003. A simple field method for assesing the ecological quality of riparian habitat in rivers and streams: QBR index. Aquatic Conservation: Marine and Freshwater Ecosystems 13: 147-163.

Ninyerola, M., Pons, X. & Roure, J.M. 2005. Atlas Climático Digital de la Península Ibérica. Metodología y aplicaciones en bioclimatología y geobotánica. ISBN 932860-8-7. Universidad Autónoma de Barcelona, Bellaterra.

Norris, R.H. 1996. Predicting water quality using reference conditions and associated communities. In R. C. Bailey, R. H. Norris and T. B. Reynoldson (eds.). Study design and data analysis in benthic macroinvertebrates assessments of freshwater ecosystems using a reference site approach. Technical workshop, North American Benthological Society, Kalispell, Montana, USA. Pp 32-52

Norton, S.B., Cormier, S.M., Smith, M. & Jones, R.C. 2000. Can biological assessments discriminate among type of stress? A case study for the eastern cornbelt plains ecoregion. Environment Toxicology Chemistry 19: 1113-1119.

Oberdorff, T., Pont, D., Hugueny, B. & Porcher, J.P. 2002. Development and validation of a fish-based index for the assessment of "river health" in France. Freshwater Biology 47: 1720-1734.

Pont, D., Hugueny, B., Beier, U., Goffaux, D., Melcher, A., Noble, R., Rogers, C., Roset, N. & Schmutz, S. 2006. Assessing river biotic condition at a continental scale: a European approach using functional metrics and fish assemblages. Journal of Applied Ecology 43: 70-80.

Prenda, J., Clavero, M., Blanco-Garrido, F., Menor, A. & Hermoso, V. 2006. Threats to the conservation to biotic integrity in Iberian fluvial ecosystems. Limnetica 25: 377-388.

Prenda, J., Armitage, P.D. & Grayston, A. 1997. Habitat use by the fish assemblage of two chalk streams. Journal of Fish Biology 51: 64-79.

Pringle, C.M. 2001. Hydrologic connectivity and the management of biological reserves: a global perspective. Ecological Applications **11**: 981-998.

Revenga, C. & Kura, Y. 2003. Status and trends of biodiversity of inland water ecosystems. Technical series number 11. Secretariat of the Convention on Biological Diversity, Montreal, Quebec, Canada.

Reynoldson, T. B., Norris, R.H., Resh, V.H., Day, K.E. & Rosenberg, D.M. 1997. The reference condition: a comparison of multimetric and multivariate approaches to assess water-quality impairment using benthic macroinvertebrates. Journal of the North American Benthological Society 16: 833-852.

Shelford, V.E. 1911. Ecological succession I. Stream fishes and the method of physiografic analysis. Biological Bulletin 21: 9-35.

Van Sickle, J., Larsen, D.P. & Hawkins, C.P. 2007. Exclusion of rare taxa affects performance of O/E index in bioassessment. Journal of the North American Benthological Society 26: 319-331.

Vanote, R. L., Minshall, G.W., Cummins, K.W., Sedell, J.R. & Cushing, C.E. 1980. The river continuum concept. Canadian Journal of Fisheries and Aquatic Science 37: 130-137.

Vitousek, P. M. 1994. Beyond global warming: ecology and global change. Ecology **75**: 1871-1876.

Whittier, T.R. & Hughes, R.M. 1998. Evaluation of fish species tolerances to environmental stressors in lakes in the Northeastern United States. North American Journal of Fisheries Management 18: 236-252.

Whittier, T.R., Hughes, R.M., Lomnicky, G.,. Peck, D.V & Herlihy, A.T. 2007. Fish and amphibian tolerance values and an assemblage tolerance index (ATI) for western USA streams and rivers. Transactions of the American Fisheries Society 136: 254-271.

World Conservation Union (IUCN). 2000. The 2000 IUCN red list of threatened species. IUCN, Gland, Switzerland. http://www.redlist.org.

Wright, J.F., Moss, D., Armitage, P.D. & Furse, M.T. 1984. A preliminary classification of running-water sites in Great Britain based onmacroinvertebrates species and the prediction of community type using environmental data. Freshwater Biology 14: 221-256.

Wright, J.F., Furse, M.T. & Armitage, P.D. 1993. RIVPACS: a technique for evaluating the biological water quality of rivers in the UK. European Water Pollution Control 3: 15-25.

Yuan, L.L. 2004. Assigning macroinvertebrate tolerance classifications using generalized additive models. Freshwater Biology 49: 662-677.

Table 1. Environmental variables used to characterize the sampled sites. * Denotes human potentially perturbed variables and used to describe perturbation gradients.

Scale	Variable	Method	Code	Mean	Range
Site	Water depth (cm)	*In situ*	DEP	42.8	7.0-200
	Shelter availability (m^2 of shelter/river width)	*In situ*	SHE	5.6	0.0-60.6
	Elevation (m)[1]	GIS	ELE	384.1	7.1-974.9
	Relative position (dist. to the most headwater point/total length of the stream)[2]	GIS	POR	0.47	0.04-1.00
	Stream order (Strahler)[2]	GIS	ORD	2.1	1.0-6.0
	Distance to headwater (km)[2]	GIS	HED	68.1	3.6-1,036.1
	Distance to Guadiana River (km)[2]	GIS	GUA	58.2	0.0-196.0
	River width (m) *	*In situ*	WID	10.8	1.4-123.0-1.4
	Substrate coarseness (Wentworth scale) *	*In situ*	SUS	5.3	1.0-9.0-1.0
	Riparian Quality Index (QBR, Munné et al., 2003) *	*In situ*	QBR	61.8	0-100-0
	NH_4^+ (mg/L) *	*In situ*	AMO	1.38	0.02-51.60
	NO_2^- (mg/L) *	*In situ*	NTI	0.10	0.01-2.00
	NO_3^- (mg/L) *	*In situ*	NTA	4.09	0.50-55.90
	PO_5^{3-} (mg/L) *	*In situ*	PHS	1.00	0.05-23.20
	SO_4^{2-} (mg/L *	*In situ*	SLF	110.1	10.0-2380.0
	Cl^- (mg/L) *	*In situ*	CLR	56.1	2.0-834.0
	Water temperature (ºC) *	*In situ*	WTE	20.5	9.4-32.6
	Conductivity (µS/cm) *	*In situ*	CND	624.7	38.0-3230.0
	pH *	*In situ*	PH	7.84	2.21-10.63
	Annual precipitation (mm/m^2)[3]	GIS	PRE	593.1	370.2-1114.5
	Solar radiation (10 KJ/m^2*day*µm)[3]	GIS	RAD	2033.9	1646-2227
	Average annual air temperature (ºC)[3]	GIS	ATEM	15.85	13.0-18.0
	Distance to the nearest reservoir upstream (km)[2] *	GIS	DUP	41.1	0.0-196.0
	Distance to the nearest reservoir downstream (km)[2] *	GIS	DWN	25.9	0.2-115.8
Reach (500 m)	Slope ($^0/_{00}$)[1]	GIS	SLO	5.92	0.00-58.03
	Sinuosity[2]	GIS	SIN	1.23	1.00-2.79
	Land uses[4]				
	Urban/Industrial (%) *	GIS	RUI	1.0	0.0-36.0
	Intensive agriculture (%) *	GIS	RIA	29.0	0.0-100.0
	Extensive agriculture (%) *	GIS	REA	7.0	0.0-100.0
	Natural (%) *	GIS	RNA	63.0	0.0-100.0
Basin	Basin area (Drainage surface in each site, 10^3 km^2)[1]	GIS	ARE	260.1	0.9-5919.1
	Gravelius index (Area/Perimeter)(m)[1]	GIS	GRA	1.68	1.14-2.68
	Land uses[4]				
	Urban/Industrial (%)*	GIS	BUI	0.4	0.0-6.7
	Intensive agriculture (%)*	GIS	BIA	22.5	0.0-97.0
	Extensive agriculture (%)*	GIS	BEA	11.0	0.0-89.1-0.0
	Natural (%)*	GIS	BNA	65.8	0.9-100.0
	Reservoir (%)*	GIS	BRS	0.32	0.0-21.2
	Population density (Hab/Km^2)[5]*	GIS	POP	21.0	0.0-459.3

Data sources
1 Digital Elevation Model 1:100.000. Confederación Hidrográfica del Guadiana.
2 Stream network provided by the Confederación Hidrográfica del Guadiana.
3 Atlas Climático Digital de la Península Ibérica (Ninyerola et al., 2005). Available at http://opengis.uab.es/wms/iberia/index.htm (May 2006).
4 CORINE Land-Cover 1:100.000. Confederación Hidrográfica del Guadiana.
5 Instituto Nacional de Estadística, available at www.ine.es (May 2006).

Table 2. Set of multivariate analysis used to define Environmental, Human Impairment and Fish Community gradients. Only loadings >0.6 (when possible) are shown. Variable codes in Table 1.

Aim	Technique	Variables	Extracted gradients	% expl. var. (Eigenvalue)	Negative extreme	Positive extreme	Denomination
Extract a general human perturbation gradient free of river size effects	PCA	All listed in Table 1	PC1	20.9 (8.14)	**RNA** (-0.77) **BNA** (-0.72) **ATEM** (-0.71) **PRE** (-0.62) **QBR** (-0.60)	**BIA** (0.78) **ELE** (0.63) **RIA** (0.62) **SLF** (0.60)	***General perturbation-climatic gradient***
			PC2	13.8 (5.39)		**HED** (0.89) **ARE** (0.87) **ORD** (0.83) **POR** (0.77) **WID** (0.76) **SUS** (0.67)	***Longitudinal natural gradient***
Obtain patterns in fish community distribution	CA	Species´ presence-absence	DIM1	21.9			***Biotic gradient***
Identify independent and relevant human perturbation gradients	PCA	Human impairment related in Table 1	PC1_Nat	28.9 (5.48)	**BNA** (-0.81) **RNA** (-0.75) **QBR** (-0.61)	**BIA** (0.67) **POP** (0.64) **BUI** (0.61)	***Basin naturalness***
			PC2_Phs	12.0 (2.29)	**PHS** (-0.70)		***P Enrichment***
			PC3_Dwn	8.6 (1.63)	**DWN** (-0.83)	**BRS** (0.81)	***Downstream river regulation***
			PC4_Nta	7.2 (1.37)	**NTA** (-0.48)		***N Enrichment***
			PC5_Agr	6.2 (1.17)	**REA** (-0.57)		***Surrounding agriculture***
			PC6_Ups	5.7 (1.08)	**UPS** (-0.55)		***Upstream river regulation***

Table 3. Species´ sensitivity values. The Pearson correlation coefficients between species´ sensitivities and their location within the longitudinal gradient (DIM1, see Table 2) are also shown. *** denotes $p<0.001$ and ** $p<0.01$. The first three indices correspond to the study of species´ responses to a general perturbation gradient. The second group of indices shows the species´ sensitivities to specific sources of perturbation.

Species	RP/TP¶	TV§	Avail/used	B. Natural	P Enrich	N Enrich	Agricult	Upst. regulation	Exotic Abund	Exotic S
Anaecypris hispanica	3.7	0.7	0.44	0.3	0.9	0.9	0.5	0.4	0.2	0.2
Cobitis palúdica	1.3	0.4	0.01	1.1	0.9	1.3	0.8	0.7	1.0	0.9
Iberocypris alburnoides	2.0	0.4	0.03	0.8	0.9	1.0	0.6	0.7	1.0	0.8
Iberochondrostoma lemmingii	3.0	0.2	-0.04	0.8	0.6	1.0	0.6	0.6	0.8	0.6
Luciobarbus comizo	0.6	7.5	0.09	0.5	0.8	0.7	0.5	0.7	1.0	0.7
Luciobarbus microcephalus	1.4	1.6	0.09	0.8	0.3	1.0	0.6	0.6	1.0	0.6
Luciobarbus sclateri	3.8	1.4	0.31	0.7	0.5	0.2	0.4	0.5	0.9	0.8
Pseudochondrostoma willkommii	3.5	0.9	0.24	0.6	0.5	1.0	0.6	0.6	1.0	0.6
Salaria fluviatilis	1.2	3.5	0.11	0.4	0.8	0.4	0.4	0.7	1.0	0.6
Squalius pyrenaicus	3.1	0.2	0.04	0.7	0.9	1.0	0.6	0.7	1.0	0.8
Pearson´s r	**0.84*****	**0.77****	0.08	0.41	0.50	0.52	0.37	0.25	0.03	0.36

¶ Hawkins et al (2000)
§ Knapp et al. (2005)

Figure 1. Guadiana River basin and location of sampling sites.

Figure 2. Analysis of preference for the five equivalent portions in which the general perturbation gradient was split. The available number of sites is represented in white columns and the adjusted number of used in black columns. The Chi-squared statistic and its associated p value are also given. Significant differences were interpreted as overuse (up arrow) or under-use (down arrow).

Figure 3. Scores of three indices used to evaluate species tolerance to the general perturbation gradient A). TV measures the O/E relationship in test sites for each species (predictions were derived from ANNA models); RP/TP is the ratio proportion of reference/ test sites where each species were present. The Availability/use Index measures the difference in over/under use of the best and worst portions (1 and 5 respectively) of the general perturbation gradient pointed out in Fig. 2. Relationship between tolerance values and scores of each species in the first axis of the Correspondence Analysis (DIM1) ordination is showed in B). Both, the indices and CA were carried out in the same data matrix (n=241 sites).

Figure 4. Mean ± SE for species presence (white dots) and absence (black dots) at the 6 independent synthetic perturbation gradients and the biotic perturbation variables. * Denotes significant differences found in the ANCOVA or t-test analysis when avoiding the effect of river size ($p<0.05$).

FIGURE 1. Hermoso et al

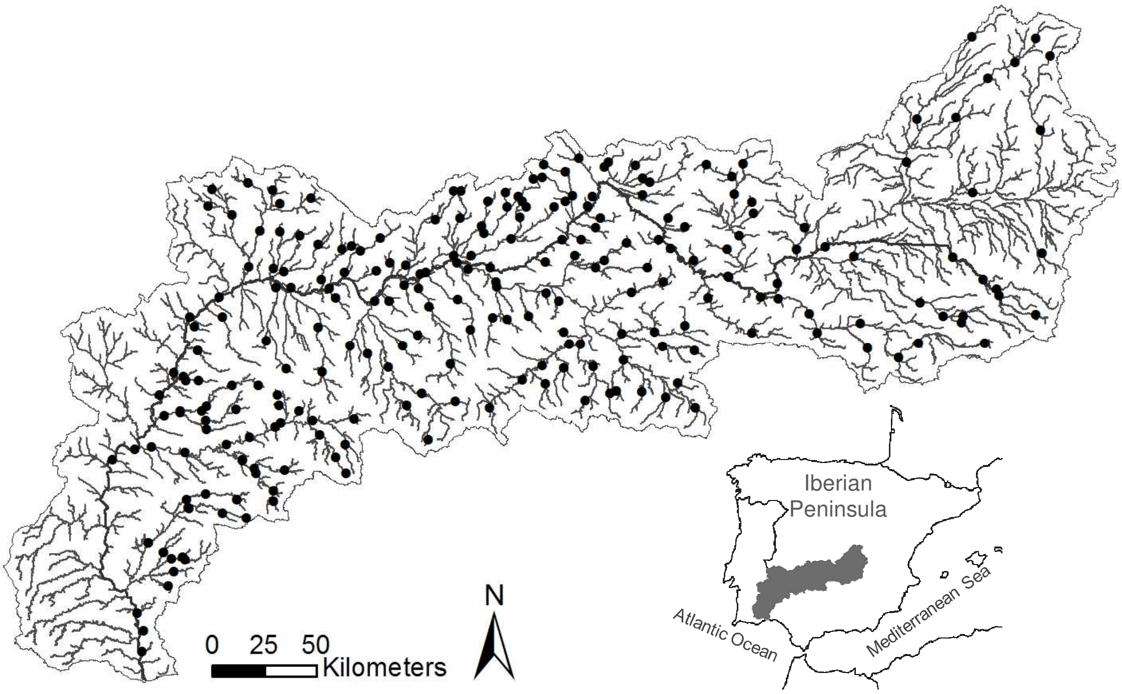

FIGURE 2. Hermoso et al

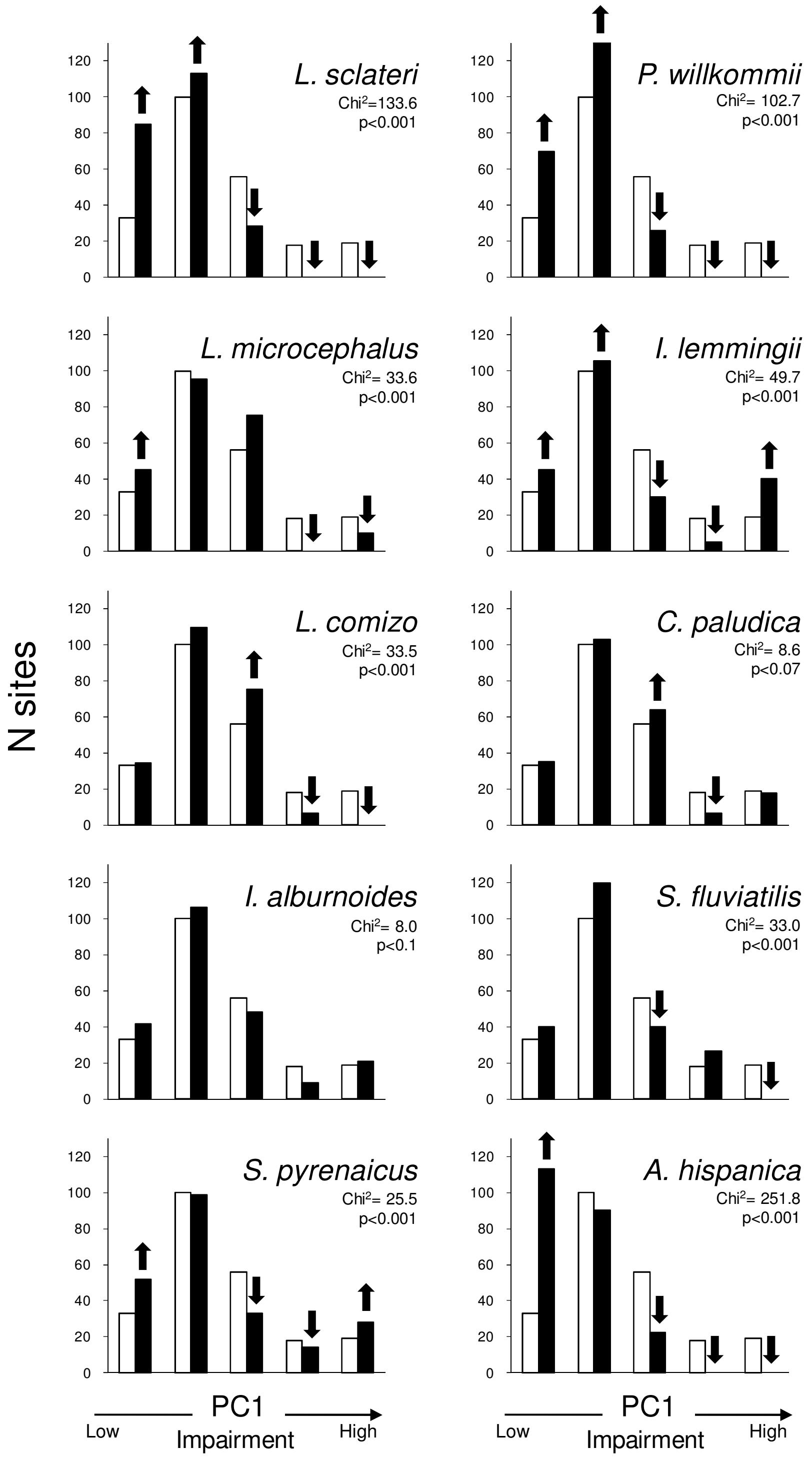

FIGURE 3. Hermoso et al

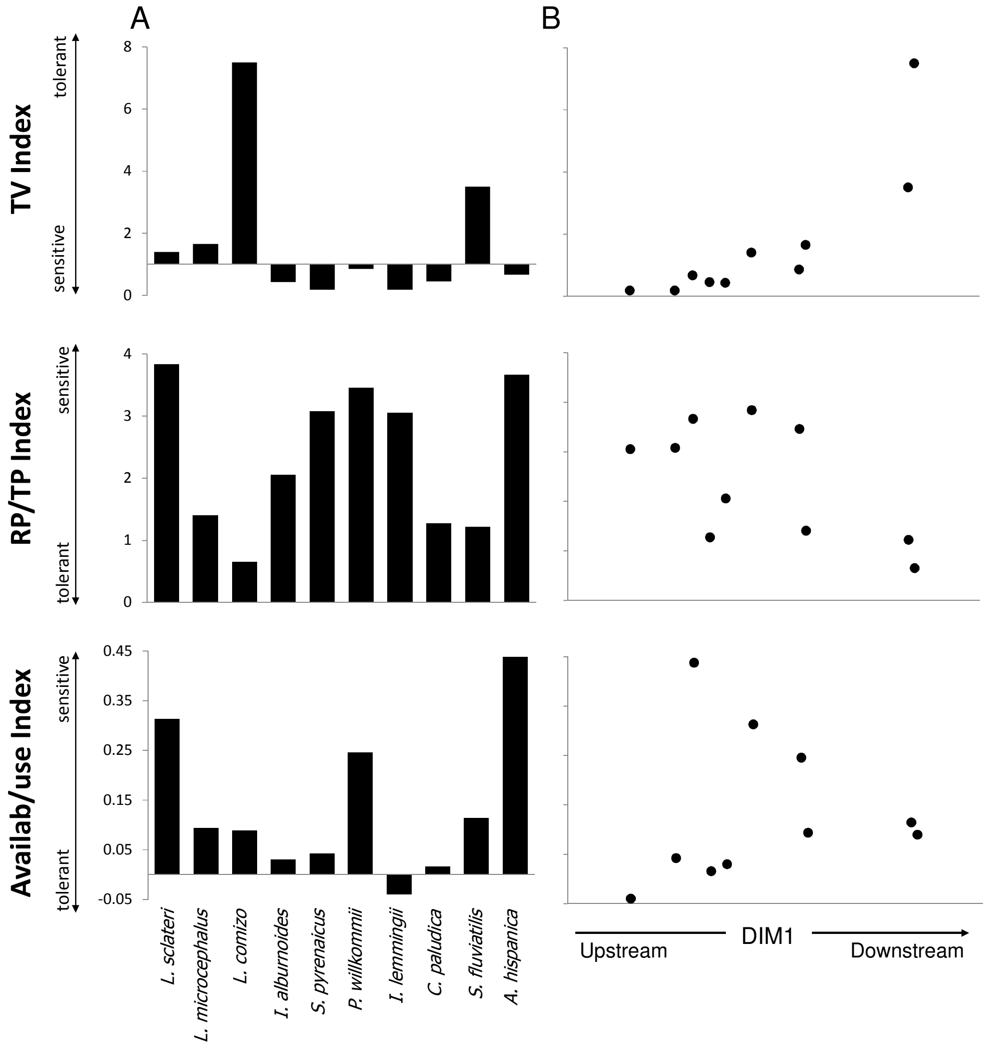

FIGURE 4. Hermoso et al.