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

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Running headline: Assessing freshwater fish sensitivity

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; Reynolds 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 °C, 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
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 (PCI) 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 \( \frac{RP}{TP} \), 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 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 et al., 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 and 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
categorization 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. willkommmii* 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


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

<table>
<thead>
<tr>
<th>Scale</th>
<th>Variable Description</th>
<th>Method</th>
<th>Code</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site</td>
<td>Water depth (cm)</td>
<td>In situ</td>
<td>DEP</td>
<td>42.8</td>
<td>7.0-200</td>
</tr>
<tr>
<td></td>
<td>Shelter availability (m² of shelter/river width)</td>
<td>In situ</td>
<td>SHE</td>
<td>5.6</td>
<td>0.0-60.6</td>
</tr>
<tr>
<td></td>
<td>Elevation (m)</td>
<td>GIS</td>
<td>ELE</td>
<td>384.1</td>
<td>71-974.9</td>
</tr>
<tr>
<td></td>
<td>Relative position (dist. to the most headwater point/total length of the stream)</td>
<td>GIS</td>
<td>POR</td>
<td>0.47</td>
<td>0.04-1.00</td>
</tr>
<tr>
<td></td>
<td>Stream order (Strahler)</td>
<td>GIS</td>
<td>ORD</td>
<td>2.1</td>
<td>1.0-6.0</td>
</tr>
<tr>
<td></td>
<td>Distance to headwater (km)</td>
<td>GIS</td>
<td>HED</td>
<td>68.1</td>
<td>3.6-1036.1</td>
</tr>
<tr>
<td></td>
<td>Distance to Guadiana River (km)</td>
<td>GIS</td>
<td>GUA</td>
<td>58.2</td>
<td>0.0-196.0</td>
</tr>
<tr>
<td></td>
<td>River width (m)</td>
<td>In situ</td>
<td>WID</td>
<td>10.8</td>
<td>1.4-123.0</td>
</tr>
<tr>
<td></td>
<td>Substrate coarseness (Wentworth scale)</td>
<td>In situ</td>
<td>SUS</td>
<td>5.3</td>
<td>1.0-9.0</td>
</tr>
<tr>
<td></td>
<td>Riparian Quality Index (QBR, Munné et al., 2003)</td>
<td>In situ</td>
<td>AMO</td>
<td>1.38</td>
<td>0.02-51.6</td>
</tr>
<tr>
<td></td>
<td>NH₄⁺ (mg/L)</td>
<td>In situ</td>
<td>NTA</td>
<td>0.60</td>
<td>0.01-2.00</td>
</tr>
<tr>
<td></td>
<td>NO₂⁻ (mg/L)</td>
<td>In situ</td>
<td>NTA</td>
<td>0.60</td>
<td>0.01-2.00</td>
</tr>
<tr>
<td></td>
<td>NO₃⁻ (mg/L)</td>
<td>In situ</td>
<td>PHS</td>
<td>0.60</td>
<td>0.05-53.0</td>
</tr>
<tr>
<td></td>
<td>PO₄³⁻ (mg/L)</td>
<td>In situ</td>
<td>SLF</td>
<td>0.60</td>
<td>10.0-2380.0</td>
</tr>
<tr>
<td></td>
<td>Cl⁻ (mg/L)</td>
<td>In situ</td>
<td>CLR</td>
<td>0.60</td>
<td>2.0-834.0</td>
</tr>
<tr>
<td></td>
<td>Water temperature (ºC)</td>
<td>In situ</td>
<td>WTE</td>
<td>20.5</td>
<td>9.4-32.6</td>
</tr>
<tr>
<td></td>
<td>Conductivity (µS/cm)</td>
<td>In situ</td>
<td>CND</td>
<td>624.7</td>
<td>38.0-3230.0</td>
</tr>
<tr>
<td></td>
<td>pH</td>
<td>In situ</td>
<td>PH</td>
<td>7.84</td>
<td>2.21-10.6</td>
</tr>
<tr>
<td>Reach (500 m)</td>
<td>Annual precipitation (mm/m²)</td>
<td>GIS</td>
<td>PRE</td>
<td>593.1</td>
<td>370.2-1114.5</td>
</tr>
<tr>
<td></td>
<td>Solar radiation (10 KJ/m²<em>day</em>µm)</td>
<td>GIS</td>
<td>RAD</td>
<td>2033.9</td>
<td>1646-2227</td>
</tr>
<tr>
<td></td>
<td>Average annual air temperature (ºC)</td>
<td>GIS</td>
<td>ATEM</td>
<td>15.85</td>
<td>13.0-18.0</td>
</tr>
<tr>
<td></td>
<td>Distance to the nearest reservoir upstream (km)²</td>
<td>GIS</td>
<td>DUP</td>
<td>41.1</td>
<td>0.0-196.0</td>
</tr>
<tr>
<td></td>
<td>Distance to the nearest reservoir downstream (km)²</td>
<td>GIS</td>
<td>DWN</td>
<td>25.9</td>
<td>0.2-115.8</td>
</tr>
<tr>
<td>Basin</td>
<td>Basin area (Drainage surface in each site, 10³ km²)</td>
<td>GIS</td>
<td>ARE</td>
<td>260.1</td>
<td>0.9-5919.1</td>
</tr>
<tr>
<td></td>
<td>Gravelius index (Area/Perimeter)(m)</td>
<td>GIS</td>
<td>GRA</td>
<td>1.08</td>
<td>1.14-2.68</td>
</tr>
<tr>
<td></td>
<td>Urban/Industrial (%)</td>
<td>GIS</td>
<td>BUI</td>
<td>0.4</td>
<td>0.0-6.7</td>
</tr>
<tr>
<td></td>
<td>Intensive agriculture (%)</td>
<td>GIS</td>
<td>BIA</td>
<td>22.5</td>
<td>0.0-97.0</td>
</tr>
<tr>
<td></td>
<td>Extensive agriculture (%)</td>
<td>GIS</td>
<td>BEA</td>
<td>11.0</td>
<td>0.0-89.1-0.0</td>
</tr>
<tr>
<td></td>
<td>Natural (%)</td>
<td>GIS</td>
<td>BNA</td>
<td>65.8</td>
<td>0.9-100.0</td>
</tr>
<tr>
<td></td>
<td>Reservoir (%)</td>
<td>GIS</td>
<td>BRS</td>
<td>0.32</td>
<td>0.0-21.2</td>
</tr>
<tr>
<td></td>
<td>Population density (Hab/Km³)</td>
<td>GIS</td>
<td>POP</td>
<td>21.0</td>
<td>0.0-459.3</td>
</tr>
</tbody>
</table>

Data sources
1 Digital Elevation Model 1:100.000. Confederación Hidrográfica del Guadiana.
2 Stream network provided by the Confederación Hidrográfica del Guadiana.
4 CORINE Land-Cover 1:100.000. Confederación Hidrográfica del Guadiana.
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.

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

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

§ 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

- **L. sclateri**
  - Chi^2 = 133.6
  - p < 0.001

- **P. willkommii**
  - Chi^2 = 102.7
  - p < 0.001

- **L. microcephalus**
  - Chi^2 = 33.6
  - p < 0.001

- **I. lemmingii**
  - Chi^2 = 8.6
  - p < 0.001

- **L. comizo**
  - Chi^2 = 33.6
  - p < 0.001

- **C. paludica**
  - Chi^2 = 49.7
  - p < 0.001

- **I. alburnoides**
  - Chi^2 = 8.0
  - p < 0.01

- **S. fluviatilis**
  - Chi^2 = 33.5
  - p < 0.001

- **S. pyrenaicus**
  - Chi^2 = 25.5
  - p < 0.001

- **A. hispanica**
  - Chi^2 = 25.1
  - p < 0.001

N sites

Chi^2 = 133.6
p < 0.001

Chi^2 = 102.7
p < 0.001

Chi^2 = 33.6
p < 0.001

Chi^2 = 8.6
p < 0.001

Chi^2 = 8.0
p < 0.01

Chi^2 = 33.5
p < 0.001

Chi^2 = 25.5
p < 0.001

Chi^2 = 25.1
p < 0.001

Low Impairment High

PC1
FIGURE 3. Hermoso et al.
FIGURE 4. Hermoso et al.