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Evaluating macroinvertebrate biological metrics for ecological assessment of streams in northern Portugal

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Abstract A procedure to select the most relevant metrics for assessing the ecological condition of the Douro basin (north Portugal) was developed based upon a set of 184 benthic community metrics. They were grouped into 16 biological categories selected from literature using data collected over 2 years from 54 sites along 31 rivers covering the whole perceived range of human disturbance. Multivariate analyses were carried out to identify the main trends in the macroinvertebrate data, to select reference versus impaired sites, to avoid multicolinearity between metrics, and to identify those that were clearly independent from natural stream typology. Structural metrics, adaptation metrics, and tolerance measures most effectively responded across a range of human influence. We find these attributes to be ecologically sound for monitoring Portugal's lotic ecosystems and providing information relevant to the Water Framework Directive, which asserts that the definition of water quality depends on its "ecological status", independent of the actual or potential uses of those waters.

Keywords Attributes · Traits · Benthic fauna · Ecological condition · Biomonitoring · Aquatic ecosystems

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Introduction

Over the past century, increasing stress on ecosystems has resulted in persistent efforts to find a universal indicator capable of evaluating river quality to use as a tool able to detect impacts on biological resources. Important biological components have been measured and several indices have been developed in the light of these studies. However, none of the attributes used up until now contain a sufficient array of responses capable of distinguishing between different types of impacts (Dolédec et al. 1999). Freshwater biomonitoring has focused on changes in community structure and composition (distribution and abundance). Multimetric approaches integrate several descriptors of the sampled assemblage (Karr 1991; Niemi and McDonald 2004), which can synthesize and interpret large amounts of information and cover effects of multiple stressors (Bonada et al. 2006, 2007; Karr et al. 1986; Lillie et al. 2003). Barbour et al. (1995) and Davis et al. (1996) have also verified that, for a broad range of human impacts on aquatic ecosystems, combined multiple metrics provide an effective way of assessing water resources. Multimetric techniques have been criticized due to the loss of ecological information caused by aggregating metrics into a unique index value (Norris 1995; Suter 1993). Other techniques that rely on multivariate statistical analyses have also been worldwide developed (Wright 1995; Cortes et al. 1998; Oliveira and Cortes 2006; Peeters 2001). Multivariate approaches are based upon statistical relationships between fauna and selected environmental characteristics to discern patterns in community composition. Furthermore, the multivariate analyses provide a statistical objective method for grouping sites with similar macroinvertebrates communities (Reynoldson et al. 1997). Examples of complex but intensively used systems include the River Invertebrate Prediction and Classification System (Wright 1995) used in UK and its derivative, the Australian River Assessment Scheme models (Simpson and Norris 2000) used in Australia and Benthic Assessment of Sediment (Reynoldson et al. 1995) used in parts of Canada.

In recent years, there has been a return to basic ecology, using species traits and functional groups in ecological studies (e.g., Lenat 1993; Palmer et al. 1996; Poff and Allan 1995; Statzner et al. 1994, 1997; Pont et al. 2006; Tomanova et al. 2006). Besides recording species loss or reduction as a result of stress conditions, species traits can pinpoint life history characteristics, more sensitive to disturbance (Phillips 2004). As Poff (1997) states, “traits presumably represent functional relationships with important environmental selective forces, such as stream flooding or drying, local shear stress, temperature extremes, and human pollution”. Reinforcing this idea, Bonada et al. (2007) found that studies at larger spatial scales and higher biological organizational levels using biological traits can anticipate impacts of climate change.

Based upon these findings, a logical step forward would be to test, for the purpose of bioassessment alongside conventional metrics, new attributes (traits of reproduction, life cycle, growth, locomotion, dispersion, etc.) that represent adaptive responses to environmental factors. This study aims to exploit the strengths of both multimetric and multivariate procedures for assessing the suitability of metrics in evaluating the ecological status of water courses. This integrated approach has been used by authors such as Zamora-Munoz and Alba-Tercedor (1996) and Cortes et al. (2002b) namely a multivariate approach to classify sites a priori and establish reference conditions based on environmental variables followed by a multimetric method, based upon a wide range of community characteristics, to then select core metrics by comparing community structure at reference sites and impaired sites.

A nationwide surface water quality monitoring is coordinated by the National Institute of Water (INAG). Until the implementation of Water Framework Directive (WFD; Directive 2000/60/CE—European Commission 2000), the national network consisted of 109 sampling sites, primarily in large rivers (24 river systems). The most important reservoirs whose main use is domestic water supply are included in the program as well. Water samples were taken at monthly intervals and analyzed only for general chemical and physical variables, organic pollution indicators, nutrients, and some heavy metals. The biomonitoring programs that have been carried out almost always are restricted to biological indexes developed for other countries such as Iberian Biological Monitoring Working Party (BMWP’; Alba-Tercedor 2000) and Belgian Biotic Index (BBI; De Pauw and Vanhooren 1983).

Nowadays, under WFD, Portugal is required to develop programs to evaluate physical, chemical, and biological integrity and to adopt water quality standards to restore and maintain that integrity. The WFD is the most important new European legislation concerning aquatic resource management to emerge for decades. The aim of the WFD is long-term sustainable water management based on a high level of protection of the aquatic environment (inland and coastal waters) and prevents further deterioration through better land management. Successful implementation of the WFD will go a long way to protecting and enhancing the quality of all stages of the water cycle in a sustainable manner. Current European Water Policy addresses the increasing awareness and participation of citizens, stakeholders, and other involved parties in decision making. The WFD is innovative since it asserts that the definition of water quality depends on its "ecological status" independent of the actual or potential uses of those waters. Ecological status derives from the assessment of surface water bodies, defined by the global expression of the structure and function of selected biological communities, taking into account geographical and climatic factors as well as the physical and chemical descriptors, including those resulting from human activities. This approach takes into consideration the overlapping unequal and dynamic influence of the three key components (physical, chemical, and biological integrity), similar to the pattern established after Yoder (1995; from Barbour and Yoder 2000). The proposed methodology aims to provide tools that contribute to the implementation of the WFD in defining monitoring programs.

Materials and methods

Study sites and sample collection

Data were collected from the Portuguese section of the Douro catchment and covered all the major tributaries (Fig. 1). The main river of this catchment (river Douro) flows generally westward across Spain and northern Portugal to the Atlantic Ocean at Foz do Douro. It has extensive large traffic in its Portuguese section and is fully regulated for hydropower purposes. Tributaries are small and flow into canyons to enter the larger river. The main impacts of the study area are the

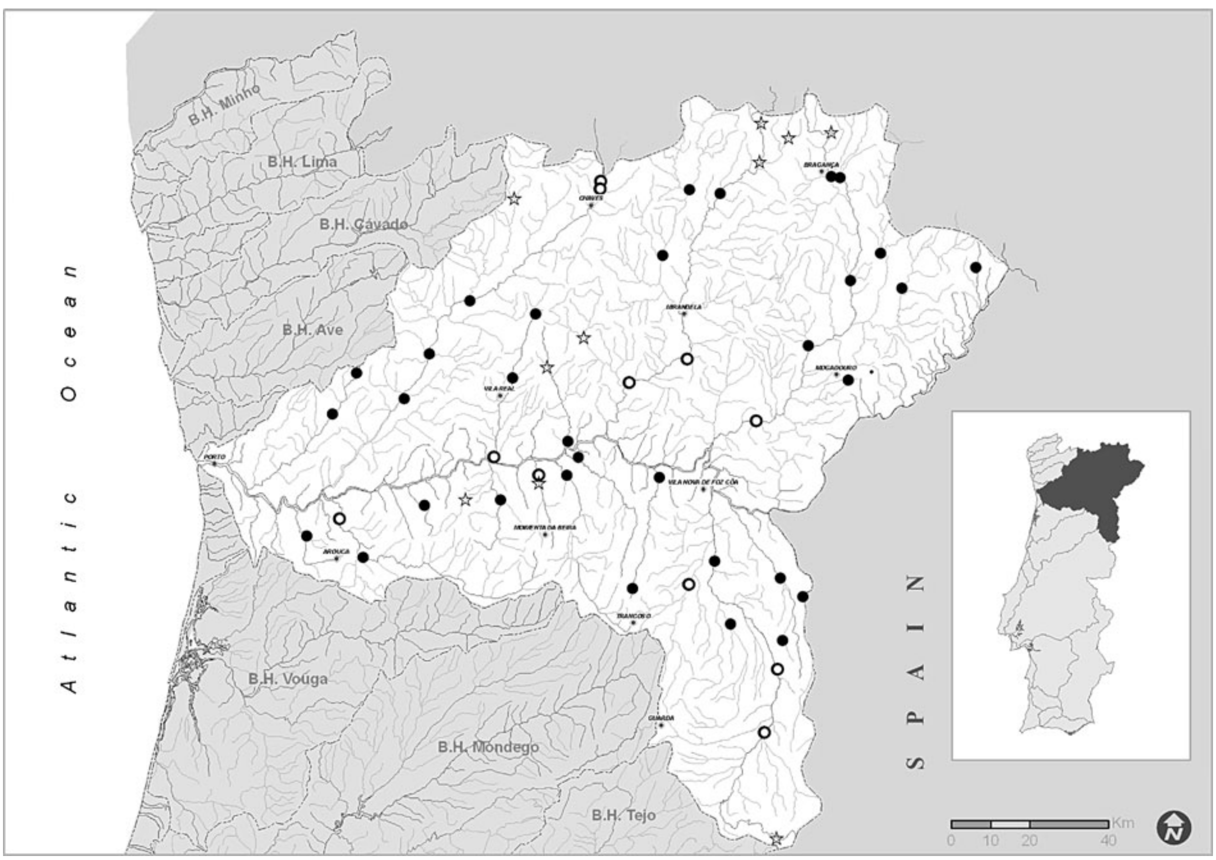


Fig. 1 Location of the Douro basin in north Portugal, showing sampled sites. Reference (*star*) and highly impaired sites (*unfilled circle*) are illustrated. The *full circles* are sites with fair impairment

strong urban pressure in the coastal strip creating environmental problems with very negative effects in the running water. The remaining area is dominated by agroforestry and low population density. The main characteristics of reference and impacted sites are summarized in Table 1.

Benthic macroinvertebrates were sampled in summer 1997 and 2000 at 54 sites from 31 rivers

Table 1 Main characteristics of studied sites of the Douro basin (reference and impacted sites)

	Method	Reference sites mean (min–max)	Impaired sites mean (min–max)
Altitude (m)	Measured from 1:25000 topographic maps	535 (121–823)	310 (76–653)
Mean catchment slope	Van Haveren (1986) method	0.42 (0.20–0.74)	0.52 (0.28–0.85)
Stream order	Strahler system measured in 1:250,000 topographic maps	3 (2–4)	4 (3–6)
Mean wetted channel width (m)	Distance from bank to bank at a transect representative of the stream width in the sampled area using a tape measure	6.6 (2.6–14.6)	20.2 (6.8–80)
Mean water depth (m)	Measured at 3 transects over the sampled reach using a graduated stick	0.45 (0.17–0.60)	0.74 (0.35–1.45)
Distance from source (km)	Distance from source (headwater) to the sampling site measured along the river from 1:250,000 topographic maps	28 (10–63)	71 (40–110)
Mean daily air temperature (°C)	Average air temperature measured during the period 1931–1960 from 1:1,000,000 Atlas do Ambiente. It was used the maximum value of each class	12.3 (10–15)	14.2 (12–16)
Mean annual precipitation (mm)	Average annual precipitation measured during the period 1931–1960 from 1:1,000,000 Atlas do Ambiente. It was used class midpoint value	1035 (650–1300)	745 (550–1400)
Anthropogenic perturbation—anthP	Each perturbation factor in the neighboring area to the reach (e.g., agriculture, urban zone, road beds, pasture, inert extraction, parking lots, deforestation, reforestation, waste, clear cutting,...) received the weight of 1 or 2 if it was present less or more than 10%, respectively. The final score was obtained by the sum of all values of the both slopes	2.7 (0–8)	5.9 (0–15)
Riparian canopy cover—%ripC	Percentage of wetted bed shaded by riparian vegetation, measured with a spherical densiometer	68 (36–96)	8 (0–45)

Table 1 (continued)

	Method	Reference sites mean (min–max)	Impaired sites mean (min–max)
SAV	It was used 6 categories (0 = no trees;...; 5 = continuous arboreal vegetation) weighted by scores: 5 (present in more than 33% of the area) or 3 (less than 33% of the area is occupied by that category). Evaluation of both banks. The final score was obtained using the following formula: $\mathrm{SAV} = \sum \frac{\sum \frac{\mathrm{lb_i} \times \mathrm{p_j}}{n} + \sum \frac{\mathrm{rb_i} \times \mathrm{p_j}}{n}}{2}$ where n = number of the observed classes for each bank; lb_i = class in the left ban; rb_i = class in the right bank; $p_j = 4$ weight	20.8 (10–25)	11.2 (1.5–25)
QBR	Index developed by Munné et al. (2003)	85 (60–100)	60 (20–90)
UA	Impact of urbanization on river. Classes 1 to 5: 1 = ≤1% urban land; 2 = low impact (≤10% urban land); 3 = moderate impact (10–20% urban land); 4 = strong impact (20–40% urban land); 5 = Severe impact (≥40% urban land)	1 (1–2)	2 (1–4)
SLS	Deviation from natural stream bottom as a result of deposition. Classes 1 to 5 with a score of 5 representing maximum deviation and 1 the minimum alteration	1 (1–2)	2 (2–3)
Affluent load generated by human population ($\mathrm{t\ year}^{-1}$)	BOD—amount of biochemical degradable substances (chemical stressor) that flow into the stream; TSS—amount of sediments that come into the stream and remain in suspension in water column. N_{tot} and P_{tot}—amount of total nitrogen and total phosphorous that comes into the stream, respectively. Firstly was calculated the generated load: total number of inhabitants for drainage basin multiplied by capitation—g/inhab day (coefficient used in the Water National Plan, 2001). Secondly, the resulting value was multiplied by 1 minus the proportion of removed domestic load by treatment stations of residual waters (the appropriate conversion coefficients were used)		
BOD_5		45.4 (7.5–139.3)	750.7 (86.7–1624)
TSS		64.8 (10.7–198.7)	1070.7 (124–2315)
N_{tot}		9.1 (1.5–27.8)	149.8 (27.3–324.1)
P_{tot}		2.8 (0.5–8.5)	45.8 (5.3–98.9)
Conductivity ($\mu\mathrm{S\ cm}^{-1}$)	Measured in the field using WTW LF 330	56 (21–130)	132 (56–363)

distributed along the whole range of perceived environmental conditions. To assess anthropogenic impacts, attributes describing riparian vegetation, water quality, and instream variables were recorded at each site (some parameters are described in Tables 1 and 5). Composite multihabitat samples were taken at each site using a hand net (350 μm mesh size). Each habitat (e.g., riffle, pool, edge, vegetation) was sampled in proportion to its representation. Hand-net contents were store in sample containers and refrigerated, once in the laboratory macroinvertebrates were sorted live and preserved. All organisms were removed, counted, and identified using standard keys developed for the Iberian Peninsula, usually to species level of taxonomic resolution, with the exceptions of Hydracarina (order), Diptera (family, subfamily, or tribe for Chironomidae), Oligochaeta (family), and Coleoptera (genus or species).

Identification of potential benthic metrics

A total of 184 macroinvertebrate assemblage attributes were selected for testing as potential metrics. These attributes included 16 biological categories ("Electronic supplementary material") ranging from those traditionally tested and integrated in multimetric indices (richness, composition and dominance measures, tolerance and intolerance descriptors, and biotic indices) to species traits. A broader set of invertebrate traits were studied (respiration, resistance forms, locomotion, and substrate relation—habit/behavior, habitat preference, forms to avoid the drift, functional feeding groups, life cycle characteristics, aquatic stages, reproduction metrics, dispersal measures, and maximal size) since that can be applied to larger temporal and spatial scales which may vary widely between ecoregions (Charvet et al. 2000; Statzner et al. 2005; Bonada et al. 2006). Selected metrics were compiled from a wide variety of works including Kerans and Karr (1994), Resh (1994), Statzner et al. (1994), Barbour et al. (1995, 1996, 1999), Fore et al. (1996), and Kashian and Burton (2000). Benthic macroinvertebrate traits were based on Charvet et al. (2000), Dolédec et al. (1999), and Usseglio-Polatera et al. (2000).

Functional feeding groups and habit/behavior were assigned according to the primary category documented by Tachet et al. (2002) and data bases supplied by "Usseglio-Polatera in 2005" and complemented with information based upon Merritt and Cummins (1996) and Barbour et al. (1999). Categorization according to sensitivity to pollution was based upon Alba-Tercedor and Sanchez-Ortega (1988), Cortes (1992), and Monzón (1996). Three biological indices were applied: the BMWP', BBI, and Family-Level Biotic Index (FBI; adapted from Hilsenhoff 1988). Family tolerance scores for the FBI index were used in agreement with Alba-Tercedor (2000), since they are adapted for the Iberian fauna. Reproduction metrics, resistance forms, mechanisms to avoid drift, dispersal measures, and maximal size were divided into classes (see "Electronic supplementary material") and calculated based on information described mainly in the last update of data bases supplied by "Usseglio-Polatera in 2005". Respiratory physiology and habitat preference macroinvertebrate classifications were based upon Hynes (1979), Richoux (1982), Margalef (1983), Faessel (1985), Askew (1988), Chinery (1992), Wetzel (1993), Fitter and Manuel (1994), Nieser et al. (1994), and Vieira-Lanero (2000). Other publications such as Thorp and Covich (1991) and online papers found via online literature searches were used to complement existing data or provide missing data. When conflicting natural history information for the same taxon occurred, we considered both sources of information. So, the two more important designations (except for life cycle duration that was considered the maximum of three designations) were used. Data were divided by the total number of designations.

All the above information used for assemblage characterization was supplemented and in some cases modified by specific information relating to local fauna and information found in literature and online sites. When ambiguities concerning a particular attribute could not be resolved, data were discarded. Whenever possible, metrics were defined at species level. Some of them included higher taxonomic levels (see "Electronic supplementary material"). Metrics were expressed in quantitative terms, most of them either as relative proportions or number of taxa.

Data analysis

Identification of reference and impaired sites

Definition of human disturbance categories Disturbed and reference (or minimally disturbed) sites were identified, firstly, based on a criterion to assess the human impact of European rivers (FAME Consortium 2004) linked to the implementation of the WFD and in the principles of REFCOND (2003) and, secondly, by their physicochemical characteristics, for a consistent and independent split. By using the first criterion, sites were classified according to anthropogenic disturbance by quantifying ten stressors that covered from local impacts (observed in situ) to the ones at the catchment level determined from geographical information system, to assess impacts in the river basin upstream. The list of descriptors included land use intensification, urban area, structure of riparian layer (including invasive plants), river connectivity, sediment load, hydrological modifications (water abstraction and flow regulation), symptoms of acidification or toxicity, morphological condition, symptoms of eutrophication, and invasive plant or animal species. Each variable was allocated to one of five classes according to the magnitude of the stressor under evaluation (1 corresponds to high status = reference conditions: only minor, negligible alterations, and 5 indicates a bad status: severe impact). This procedure allowed to define the reference sites (a) according to their total score, derived from ranking the scores of environmental degradation, and (b) when none of the above variables occurred in the two "worst" classes.

Finally, collected physical and chemical data were compared with the national Water Institute (INAG) water quality classification scheme which uses 27 parameters that describe principal nutrients and micropollutants, to classify water quality according to uses. Thus, a site was considered unimpaired if it belonged to class A, impaired if it belonged to classes B or C and highly impaired if it belonged to classes D or E.

Species ordination The relation of the macroinvertebrate communities with the previous classification of sites produced by the environmental variables was assessed by multivariate ordinations of species of taxa abundance structure (log $(x + 1)$ transformed) for each year, using non-metric multidimensional scaling analysis (NMDS) based on similarity scores (Bray–Curtis coefficient) using PRIMER v 5.2.2 (Clarke and Gorley 2001). Separation of reference and the most degraded sites was further refined by integrating the 1997 and 2000 data and checking for interannual variability. Reference sites with interannual consistency along disturbance categories were retained. A similarity percentages (SIMPER) analyses (SIMPER-species contributions) was done to quantify the degree of differences found between years or quality categories, as well as species that contribute more to these differences. This analysis although not perform formal test of hypotheses provides a list of species in order of their percentage contribution to dissimilarities (Bray–Curtis dissimilarity) between groups or similarities within groups (Clarke and Warwick 2001). Metrics-derived values were then compared between reference and impaired sites defined above.

Evaluation of metrics

Biological patterns and human disturbance The optimal combination of potential metrics for discriminating between reference and degraded sites was determined using detrended correspondence analysis (DCA; Hill and Gauch 1980). This analysis evaluates the ecological sensitivity and response direction of all potential benthic attributes to increasing human stress for each year, extracting the most relevant biological patterns, and then relating them to patterns induced by human activity. DCA provides a more precise representation of environmental gradients than principal component analysis, since it arranges the data in such a way to avoid the compression of the ordination axes and quadratic distortion (Hill 1979). The data were log $(x + 1)$ transformed with the exception of metrics expressed in percentage. These metrics were not transformed since they were normally distributed (Shapiro–Wilk test for normality).

Assessment of metrics Pearson correlation coefficients of the DCA axis that best reflected the disturbance gradient and test metrics were derived; only significant correlations ($p \leq 0.05$) were retained. The positive or negative signs of the Pearson correlation coefficients indicated the direction of the candidate metric (i.e., increasing or decreasing in response to perturbation). When signals of the two coefficients (year 1997 and year 2000) were not coincident, the response was considered as variable and eliminated, unless we knew the cause of variation. The DCA was computed using the package CANOCO version 4.0 (Ter Braak and Smilauer 1998). Metrics responses that were not in accordance with literature were also discarded.

Candidate metrics were further tested for ecological consistency. First, a coefficient of variation (CV) was calculated for each metric in the group of all reference sites that presented interannual consistency, as a measure of within-site variability. CVs were calculated from the multiyear reference data, once this combination had absorbed the interannual variability verified previously. Low CV is an important condition for determining the suitability of a metric in detecting anthropogenic impacts. Kashian and Burton (2000) state that CVs $> 50\%$ are ineffective in detecting between impaired and unimpaired conditions. Based on this criterion, only candidate metrics with CVs $< 50\%$ were retained for further analyses.

Based on the approach advocated by Barbour et al. (1996), metrics with many zero or low values in the reference sites were assessed to avoid nondetection of lower values. Metrics that followed this pattern were also eliminated from the subsequent tests ("Electronic supplementary material").

Obtaining a subset of independent metrics A preliminary canonical correspondence analysis (CCA) with forward selection (stepwise procedure) was performed, for both datasets (metrics and taxa abundance) containing the 2 years of samplings (1997 and 2000 combined), to eliminate metrics that did not make any significant contribution to explaining biological variation derived from disturbance. Only metrics with $p \leq 0.05$ were selected for the next step. In order to distinguish between natural typological variability from perturbation-induced changes, typological variables such as distance from source, stream width, altitude, stream order, zonation (according to Illies and Botosaneanu 1963), catchment area upstream of the site, and total stream length were considered as covariables in this CCA. The resulting variation inflation factors (VIFs) were assessed. Metrics with high VIFs (>20) values imply redundancy (multicolinearity) with other metrics, a phenomenon that should be avoided since no unique contribution is made to the regression equation (Ter Braak and Smilauer 1998). Consequently, the metric with the highest VIF was excluded and the CCA procedure was repeated until all metrics exhibiting multicolinearity were excluded. The environmental and biological variables (metrics) were previously standardized $[(x - \bar{x}) \div \mathrm{sd}\,(x)]$ to achieve comparable scales and the macroinvertebrates abundance were transformed by $\log\,(x + 1)$. The package CANOCO version 4.0 was used for the CCA analyses.

Establishing the relationship between metrics and human disturbance gradient Following the metrics selection procedure using CCA, Pearson correlations were derived between variables describing human disturbance and the preselected metrics, ensuring that only metrics that were good indicators of human impacts were retained. Variables used to test metrics were conductivity (cond), riparian canopy cover (%ripC), anthropogenic perturbation (anthP), structure of arboreal vegetation (SAV), riparian habitat quality (QBR index; Munné et al. 2003), urbanization area (UA; categories 1–5), sediment load segment (SLS; categories 1–5), dissolved oxygen (O_2), turbidity, upstream dams (UD; categories 1–5), and effluent load generated by human population: biochemical oxygen demand (BOD_5), total suspended solids (TSS), total nitrogen (N_{tot}), and total phosphorous (P_{tot}). These variables and another ones (water temperature, substratum size, mean water depth and velocity, stream width, and stream flow) were measured simultaneously with invertebrates sampling. Finally, benthic attributes that best discriminated between the reference and degraded sites were illustrated by box-and-whisker plots. This method, described by Barbour et al. (1996), rejects metrics if the interquartile range of impaired or reference sites overlaps with the median of the other. Results from the 2 years were compared and only metrics that detected a gradient of human impact in both years were accepted as core metrics with potential to be part of a biological indicator system.

Results

Selection of reference and impaired sites

NMDS based on species/site data of 1997 (2D stress value = 0.23, 3D stress value 0.17) and data from 2000 (2D stress value = 0.19, 3D stress value = 0.13) differentiated between undisturbed and highly impaired sites (Fig. 2a, b). That is, identified sites in the reference and highly impaired groups (ten sites in each category) for each year were basically the same (Fig. 2). The distinction between these two subsets of sites was confirmed with SIMPER tests performed in the multiyear NMDS ordination. NMDS analysis displayed a gradient of disturbance with the sites distributed along a gradient of human impacts of increasing magnitude. Average dissimilarity between 1997 and 2000 was 77.09%, making clear the existence of a strong interannual variability (Fig. 3; Table 2). A total of 174 species (43% of the total taxa)

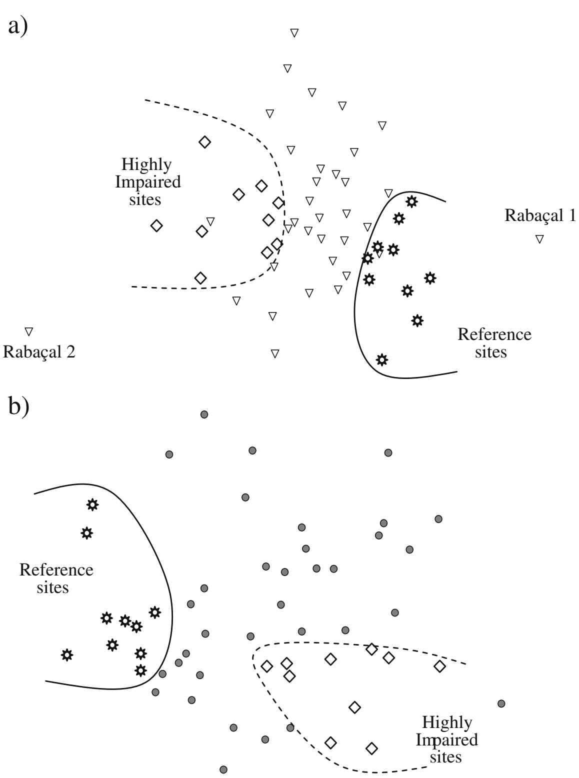


Fig. 2 NMDS ordination plot of sites **a** sampled in 1997. Outliers Rabaçal 1 and Rabaçal 2 were excluded from the impaired and reference sites, respectively, because they had low abundance and diversity probably caused by sampling difficulties. **b** Sampled in 2000. The *curved lines* represent an imaginary separation between the reference and impaired sites from the remaining ones. *Identical symbols* for reference and impaired sites were used to illustrate the same places

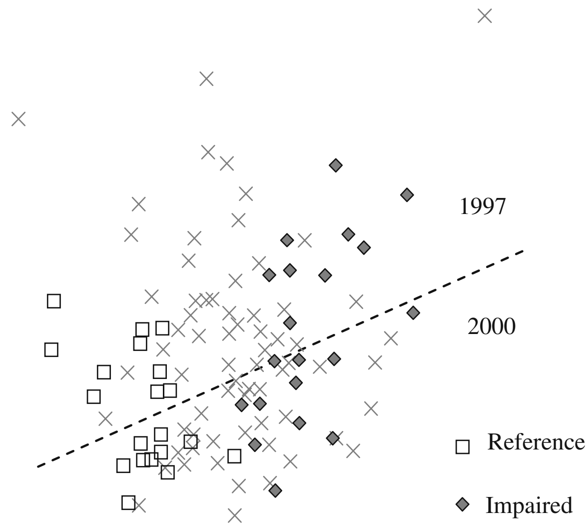


Fig. 3 NMDS ordination plot of multiyear data representing the variability between years and the separation between reference and impaired sites. The *dot line* represents an imaginary separation among years 1997/2000

Table 2 Percentage breakdown of average dissimilarity between groups of years (1997 against 2000) and groups of impaired versus reference sites in Douro catchment, using SIMPER analysis

Factors	Groups	Average similarity (%)	Average dissimilarity (%)
Years	1997	25.07	77.09
	2000	33.30	
Ref/Imp	Reference	34.24	79.89
	Impaired	29.43	

Table 3 Average contribution of species principally responsible for intragroup similarities within each year of sampling

1997		2000	
Species	Contribution (%)	Species	Contribution (%)
Chironomini gen. sp.	14.81	Chironomini gen. sp.	11.11
Hydracarina	11.55	*Caenis luctuosa*	7.99
Tanytarsini gen. sp.	7.46	Tanytarsini gen. sp.	7.91
Tanypodinae gen. sp.	6.96	Tanypodinae gen. sp.	6.59
Orthocladiinae gen. sp.	5.33	Hydracarina	5.49
Caenis luctuosa	4.49	Orthocladiinae gen. sp.	5.44
Lumbriculidae gen. sp.	3.50	Diptera pupae	3.43
Dryops sp.	2.71	*Aquarius najas*	3.32
Physella acuta	2.17	*Hydropsyche* sp. 2	2.60
Mystacides azurea	1.97	Simuliidae gen. sp.	2.42
Calamoceras marsupus	1.80	Lumbriculidae gen. sp.	2.15
Oulimnius sp.	1.79	*Baetis fuscatus*	1.77
Laccophilus sp.	1.67	*Mystacides azurea*	1.72
Diptera pupae	1.66	Gerridae juvenil	1.64
Gomphus pulchelus	1.59	*Ecdyonurus* gr. *venosus*	1.53
Lumbricidae gen. sp.	1.54	*Leuctra aurita*	1.41
Aquarius najas	1.32	*Choroterpes picteti*	1.32
Gerris sp. 1	1.23	*Ancylus fluviatilis*	1.23
Tubificidae gen. sp.	1.18	*Laccophilus* sp.	1.22
Hydropsyche sp. 2	1.11	*Cloeon* gr.*simile*	1.21
Atyaephyra desmaresti	1.09	*Baetis rhodani*	1.13
Platycnemis latipes/acutipennis	1.07	Hydroptilidae gen. sp.	1.12
Leuctra fusca	1.02	*Oulimnius* sp.	1.10
Boyeria irene	0.95	*Cloeon* gr. *dipterum*	1.01
Euleuctra geniculata	0.78	*Ephemerella ignita*	0.94
Atrichops sp.	0.77	*Baetis* sp.	0.88
Baetis rhodani	0.69	*Habrophlebia eldae*	0.86
Onychogomphus uncatus	0.68	*Boyeria irene*	0.81
Erpobdella monostriata	0.60	*Rhyacophila* sp.	0.77
Coenagrion puella	0.57	*Polycentropus flavomaculatus*	0.75
Calopteryx virgo	0.54	*Naucoris maculatus maculatus*	0.73
Stenelmis canaliculata	0.53	*Leuctra geniculata*	0.72
Ecdyonurus gr.*venosus*	0.49	*Hydrometra stagnorum*	0.72
Corixidae gen. sp.	0.49	Enchytraeidae gen. sp.	0.72
Atherix sp.	0.47	*Erpobdella monostriata*	0.64
Anacaena sp.	0.46	*Gerris lateralis*	0.61
Simuliidae gen. sp.	0.42	*Physa fontinalis*	0.61
Leuctra sp. 1	0.42	*Micronecta scholtzi*	0.55
Haliplus sp.	0.42	*Calamoceras marsupus*	0.52
Naucoris maculatus maculatus	0.42	*Platycnemis latipes/acutipennis*	0.50
Hydrochus sp.	0.40	*Onycogomphus uncatus*	0.47
Nepa cinerea	0.39	Tubificidae gen. sp.	0.46
Platycnemis cf. *latipes*	0.35	*Pseudocentroptilum pennulatum*	0.42
Potamopyrgus jenkinsi	0.34	*Leuctra* sp. 1	0.41
		Epeorus silvicola	0.39
		Dryops sp.	0.37
		Allogamus ligonifer	0.31
		Pyrrhosoma nymphula	0.31

Table 4 Average contribution of species principally responsible for intragroup similarities within reference and highly impaired sites

Reference			Impaired		
Species	Contribution (%)	Abundance class	Species	Contribution (%)	Abundance class
Chironomini gen. sp.	6.66	−	Chironomini gen. sp.	18.94	+
Hydracarina	6.59	−	*Caenis luctuosa*	15.76	+
Tanytarsini gen. sp.	5.63	−	Hydracarina	12.38	+
Orthocladiinae gen. sp.	4.93	−	Tanypodinae gen. sp.	6.68	+
Tanypodinae gen. sp.	4.37	−	Orthocladiinae gen. sp.	5.87	+
Calamoceras marsupus	3.77	+	Tanytarsini gen. sp.	5.84	+
Oulimnius sp.	2.99	+	*Atyaephyra desmaresti*	4.46	+
Habrophlebia eldae	2.88	+	Diptera pupae	2.98	+
Aquarius najas	2.81	+	Lumbriculidae gen. sp.	2.91	+
Leuctra sp. 1	2.78	+	*Physella acuta*	1.69	+
Ecdyonurus gr. *venosus*	2.48	+	*Aquarius najas*	1.45	−
Polycentropus sp. 1	2.45	+	*Hydropsyche sp.* 3	1.36	+
Dryops sp.	2.36	−	*Coenagrion puella*	1.34	+
Onycogomphus uncatus	2.36	+	Mystacides azurea	1.17	+
Atherix sp.	2.29	+	*Choroterpes picteti*	1.04	+
Hydropsyche sp. 2	2.25	+	*Micronecta scholtzi*	0.96	+
Leuctra geniculata	2.18	+	Ecdyonurus gr. venosus	0.87	−
Baetis rhodani	2.08	−	Platycnemis latipes/acutipennis	0.81	+
Boyeria irene	1.98	+	Gomphus pulchelus	0.81	+
Caenis luctuosa	1.84	−	Simuliidae gen. sp.	0.77	+
Hydraena sp.	1.83	−	*Laccophilus* sp.	0.76	+
Leuctra aurita	1.70	+	Gerris sp.	0.74	+
Simuliidae gen. sp.	1.52	−	*Cloeon* gr. *simile*	0.67	+
Sericostoma sp. 1	1.50	+			
Rhyacophila sp. 1	1.43	+			
Diptera pupae	1.42	−			
Elmis sp.	1.32	−			
Orectochilus villosus	1.18	−			
Ancylus fluviatilis	1.17	−			
Mystacides azurea	1.13	−			
Ephemerella ignita	1.12	−			
Polycentropus flavomaculatus	1.00	−			
Allogamus ligonifer	0.99	+			
Lumbriculidae gen. sp.	0.93	−			
Epeorus silvicola	0.80	+			
Dixidae gen. sp.	0.72	−			
Baetis sp. 2	0.63	+			
Aphelocheirus occidentalis	0.63	+			
Calopteryx virgo	0.59	+			
Limnius sp.	0.58	+			
Leuctra fusca	0.54	+			
Protonemura meyeri	0.52	+			
Gerridae gen. sp.	0.43	−			
Baetis fuscatus	0.40	−			
Dupophilus brevis	0.40	+			

The signals + (increase) or − (decrease) in column “Abundance class” indicate which species are more or less abundant in reference or impaired sites accounted for 90% of the dissimilarity between reference and impaired sites, and 74 (18% of the total of species) contributed for 66% of the dissimilarity. SIMPER results of reference versus impaired sites (Fig. 3; Table 2) correspond with the pattern observed in the previous analyses. A value of 79.89% dissimilarity between the most and the least disturbed sites confirms that these reference and impaired sites groups are truly distinct despite strong interannual variation. These observations correspond with our initial findings, based on groups established using physicochemical data.

Principal representative macroinvertebrate taxa of the reference and impaired groups (to a cumulative percentage of 90%) are presented in Tables 3 and 4. Reference sites are typified by intolerant species belonging to Trichoptera, Ephemeroptera, Plecoptera, Coleoptera, and Heteroptera such as *Calamoceras marsupus* (Brauer), *Sericostoma* sp. (Latreille), *Allogamus ligonifer* (McLachlan), *Habrophlebia eldae* (Jacob and Sarton), *Ecdyonurus* gr. *venosus* (Fabricius), *Leuctra aurita* (Navas), *Protonemura meyeri* (Pictet), *Oulimnius* sp. (Gozis), *Elmis* sp. (Latreille), *Limnius* sp. (Illiger), *Dupophilus brevis* (Mulsant), *Aquarius najas* (Degeer), and *Aphelocheirus occidentalis* (Nieser and Millán; Table 4). Disturbed sites are characterized almost exclusively by tolerant species from across several orders such as Diptera, Ephemeroptera, Trichoptera, and Heteroptera Table 4. On the other hand, the species that most contributed to dissimilarities between reference and impaired sites were associated to intolerant species (e.g., *Leuctra* sp., *Sericostoma* sp., *C. marsupus*, *A. najas*, *Brachycentrus subnubilus*). Only 15% of the total of the species data (65 species) had contributed with 2/3 to the dissimilarities among sites.

Metric selection

A consistent pattern of disturbance in the ordination space was displayed by DCA analyses for each year, i.e., sites were distributed in the same order along the disturbance gradient. The Pearson coefficients of first axes correlations with all potential untransformed metrics showed a positive

Table 5 Pearson correlations between selected metrics (indicated on "Electronic supplementary material") through CCA analyses and variables of human pressure

	fP	fH	fEPT	%5TD	%br	%diap	fSwi	fCling	%Cling	%exR	%preR	gShr	sfil	fGath	%Int	IBMWP	FBI	%Hem	%CluV	%aqP
cond (μS cm^{-1})	−0.43	−0.12	−0.48	0.30	0.01	−0.08	−0.09	−0.26	0.09	−0.30	−0.20	−0.46	−0.31	−0.38	−0.44	−0.44	−0.49	−0.26	−0.31	0.20
%ripC	0.39	0.10	0.41	−0.15	−0.11	−0.17	0.31	0.37	0.22	0.34	0.02	0.52	0.25	0.38	0.53	0.49	0.38	−0.20	0.20	0.10
anthP	−0.25	−0.11	−0.35	0.39	−0.17	−0.12	−0.26	−0.32	−0.01	−0.28	−0.14	−0.21	−0.31	−0.13	−0.42	−0.30	−0.40	−0.33	−0.37	0.07
SAV	0.26	0.07	0.29	−0.14	−0.21	0.05	0.09	0.17	0.03	0.24	−0.01	0.35	0.09	0.25	0.29	0.34	0.17	−0.23	0.27	0.14
QBR	0.48	0.10	0.48	−0.32	0.03	0.01	0.16	0.43	0.07	0.40	0.18	0.37	0.35	0.14	0.55	0.48	0.45	0.17	0.31	0.02
UA	0.05	−0.12	−0.10	0.27	−0.38	−0.09	−0.18	−0.06	−0.01	−0.03	0.03	−0.02	−0.06	0.01	−0.18	−0.03	−0.20	−0.32	−0.05	0.29
SLS	−0.50	−0.02	−0.53	0.34	−0.13	0.14	−0.08	−0.52	−0.07	−0.43	−0.13	−0.27	−0.45	−0.26	−0.48	−0.51	−0.44	−0.22	−0.29	0.07
O_2 (mg/L)	−0.08	−0.13	−0.10	0.14	0.03	0.00	−0.21	−0.13	0.19	0.04	0.06	−0.27	0.10	−0.24	−0.05	−0.24	−0.10	−0.11	−0.24	0.23
Turbidity	0.56	−0.02	0.58	−0.15	−0.10	−0.18	−0.02	0.38	0.08	0.30	0.29	0.53	0.35	0.44	0.43	0.56	0.31	−0.22	0.05	0.22
UD	−0.15	0.05	−0.23	0.17	−0.15	−0.01	−0.02	−0.00	0.07	−0.02	−0.07	−0.21	−0.08	−0.20	−0.15	−0.12	−0.19	−0.13	−0.03	0.22
BOD_5 (t year^{-1})	−0.24	−0.33	−0.34	0.19	0.00	0.12	−0.45	−0.47	−0.05	−0.11	0.18	−0.44	−0.11	−0.22	−0.27	−0.42	−0.23	−0.10	−0.23	0.29
TSS (t year^{-1})	−0.24	−0.33	−0.34	0.19	0.00	0.12	−0.45	−0.47	−0.05	−0.11	0.18	−0.44	−0.11	−0.22	−0.27	−0.42	−0.23	−0.10	−0.23	0.29
N_{tot} (t year^{-1})	−0.24	−0.33	−0.34	0.19	0.00	0.12	−0.45	−0.47	−0.05	−0.11	0.18	−0.44	−0.11	−0.22	−0.27	−0.42	−0.23	−0.10	−0.23	0.29
P_{tot} (t year^{-1})	−0.24	−0.33	−0.34	0.19	0.00	0.12	−0.45	−0.47	−0.05	−0.11	0.18	−0.44	−0.11	−0.22	−0.27	−0.42	−0.23	−0.10	−0.23	0.29

Marked correlations are significant at $p < 0.05$ ($N = 54$)

association between the obtained metric score and increased perturbation and vice versa.

From the original group of 184 metrics, a subset of 78 attributes met the criteria of low reference site within-site variability (low CVs). Inspection of zeros and low values in reference sites further refined this selection ("Electronic supplementary material").

CCA analysis, including typological covariables following forward selection, resulted in 32 metrics that made a significant contribution ($p < 0.05$) and were independent of natural variability. Examination of VIFs of the successive CCAs analyses removed another 12 attributes.

The Pearson correlation coefficients for the 20 retained metrics (Table 5) showed that only % diap and % Cling showed no association with the selected disturbance variables (Table 5). Despite this, we retained all 20 metrics for further analysis, since they may have responded to variables that were not considered in this study. Examination of the boxplots revealed 12 the 20 retained candidate metrics to have little or no overlap between most disturbed and reference sites, satisfying the last metric selection criteria (Fig. 4). These included three structural metrics (fP, fEPT, and %5TD), seven adaptation metrics (fSwi, fCling, %exR, gShr, sFil, and fGath), and three metrics that

Fig. 4 Box plots of macroinvertebrate metrics selected by CCA for reference and impaired sites, obtained separately for 1997 and 2000. *Boxes* are interquartile ranges (25%ile and 75%ile), *range bars* show maximum and minimum of nonoutliers, *small squares* are medians, and *dots* are outliers

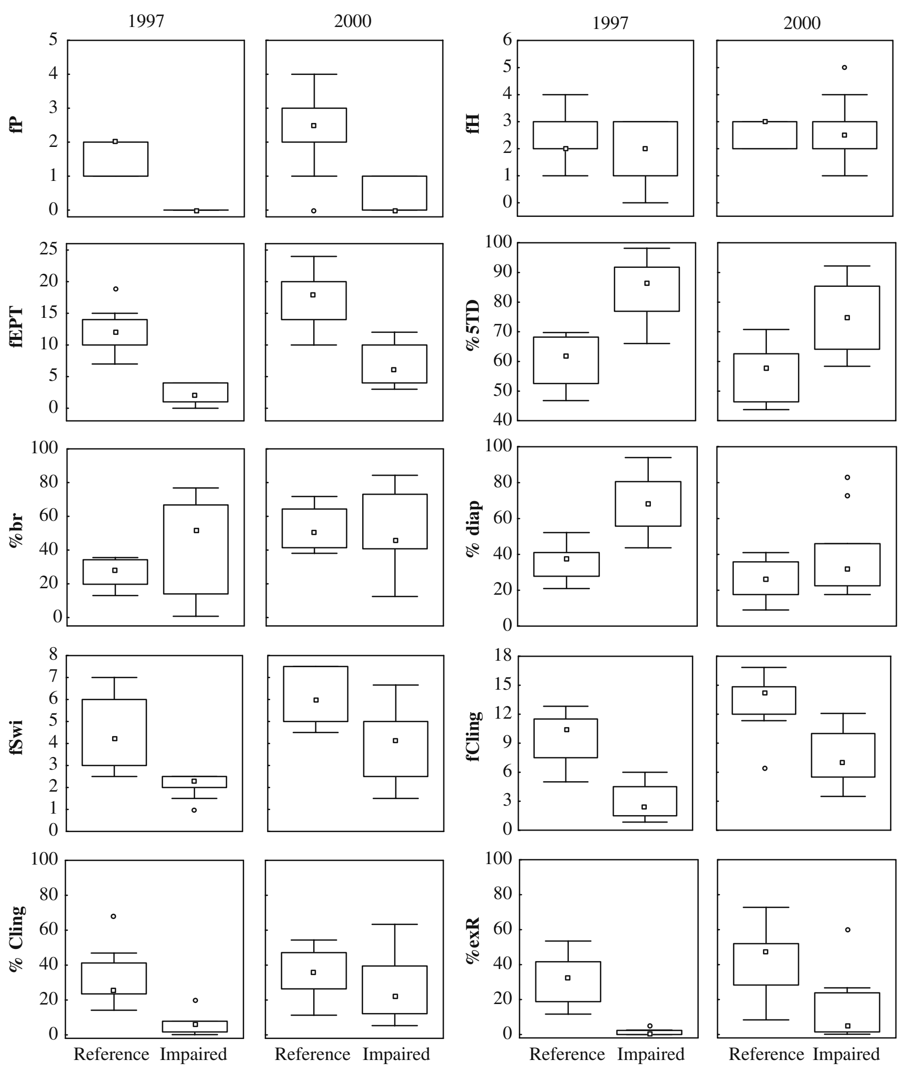
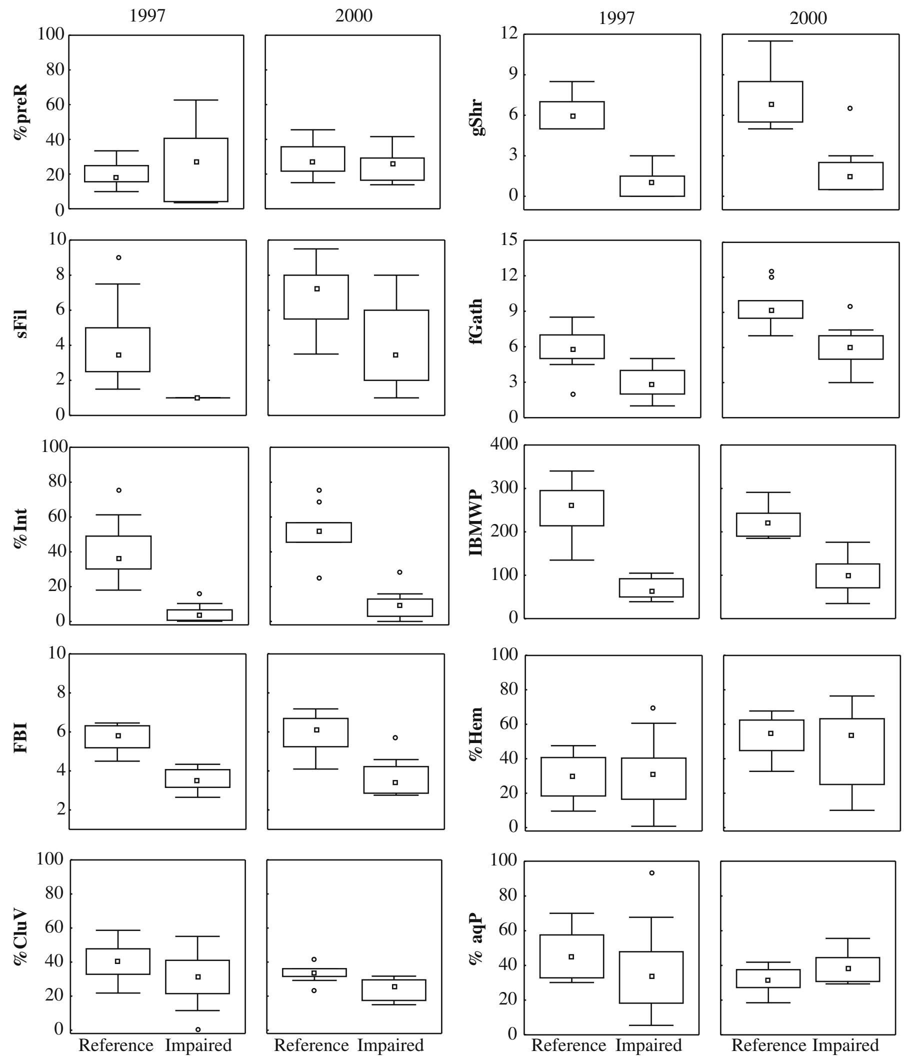


Fig. 4 (continued)



measure the tolerance level (%Int and two indices: IBMWP and FBI).

Discussion

To identify human impacts on aquatic assemblages, measurements of the individual, community coupled with landscape level scales are required (Butcher et al. 2003) ensuring that when metrics are organized and selected systematically within a regional framework, multimetric indices effectively measure changes along perceived disturbance gradients (Karr and Chu 1999). This study included a large variety of point and nonpoint-source disturbances, including agriculture, pasture, deforestation and afforestation, physical alterations of riparian and instream habitats, road building, impoundments, urban areas, and mining. Ecological condition was described by a large array of metrics (functional characteristics, i.e., traits, and attributes derived from structural and condition aspects of communities) in order to identify which best assess impairment. In particular, we wanted to test the possibility of integrating biological attributes into an assessment system.

In this study, it was verified an interannual variability (Table 2; Fig. 3). These differences between years (1997 versus 2000) could be explained by the weather conditions over the analyzed period that corresponded to extreme events, such as droughts and floods. The year of 1997 was one of the fifth hotter years of the last 150 years, and conversely, the winter of 2000 was the third rainiest of the last 30 years. Temporally, heterogeneous environment is an event which communities are naturally dependent (Poof and Ward 1989; Hildrew and Giller 1994). According to Minshall (1988), changes in flow, light, temperature, resource supply, and many other variables can occur over time scales, with varying degrees of predictability Poff (1992). However, in spite of the observed temporal changes in macroinvertebrate communities as a result of natural environmental variability, differences in spatial distribution of sampling sites across each year were not manifested for both years. Observing NMDS ordination and SIMPER analyses (Fig. 3; Table 2), the same environmental gradient structuring the characteristics of sites and the species occurring in them becomes clear. As a result, we can conclude that the dynamic nature of both environmental conditions and community structure in lotic environments do not have major effects on the sensitivity of the species and consequently in the sensitivity of this method.

Our results showed that 12 attributes successfully distinguished between the reference and impaired sites and also emphasized the value of combining conventional metrics (abundance and/or richness of species, biotic indices, and tolerance measures) with biological traits (habit/behavior measures, habitat preference, and trophic groups) for reliable lotic biomonitoring. Fifty percent of the selected metrics were traits related to locomotion (2), preference of species in terms of current velocity (1), and functional feeding groups (3). Similar to other studies (Dolédec et al. 1999; Poff 1997), our results confirm that, in the catchment context, species traits can be used to assess stress magnitude in running waters and have considerable potential as a benchmark for biomonitoring and improving the traditional biomonitoring approach. Rodgers et al. (1979) stressed that the integration of structural/compositional and functional metrics provides the best mean of assessing impairment. Phillips (2004) notes that, rather than simply record loss or reduction in numbers of species as a result of a disturbance, use of species traits allows us to identify the most sensitive life history characteristics. In an attempt to use the functional characteristics of lotic macroinvertebrates for biomonitoring, Charvet et al. (1998) and Dolédec et al. (1999) found that the use of a large variety of biological traits was more reliable than community structure in detecting stream pollution in terms of species composition and abundances. Also, Charvet et al. (2000) concluded that community structure based on biological traits, unlike taxonomic composition, was relatively stable across large environmental gradients (geology, altitude, geographical coordinates, stream order, and slope) enabling the use of these descriptors for biomonitoring over large geographic regions. According to Phillips (2004), another benefit of using complementary species traits is their stability through space and time, unlike species composition. Vieira et al. (2006) refer as well that once some functional traits may not be constrained by taxonomy, they can be applicable at multiple spatial scales (local, regional, and continental) and can provide a consistent method for assessing community responses to environmental gradients. Thus, it becomes evident that assessment protocols based solely only on conventional attributes may not be sufficiently sensitive to distinguish impairment, whereas trait-based variables can detect problems that may otherwise be overlooked.

Over the last decade, the use of species traits has become more important in assessing stream system disturbance (Metcalfe 1989; Townsend and Hildrew 1994; Dolédec et al. 1996, 1999; Mabry et al. 2000; Ribera et al. 2001; Statzner et al. 2001a; Bady et al. 2005; Dziock et al. 2006). However, only a very small group of traits has been studied in combination with taxonomic indicators to produce macroinvertebrate based indices for biocriteria and assessment (Barbour et al. 1996; Fore et al. 1996; Kerans and Karr 1994). This paper has presented a heuristic framework that seeks, through a vast group of conventional metrics and species traits, to choose the best combinations of attributes for multiscale and cumulative disturbance effects with a view to integrate this approach into future monitoring systems. There is, however, a lack of information for many endemic invertebrate species concerning individual autecology (morphological and life history) and we support Poff's (1997) comments that more research is needed to quantify species traits in stream organisms in order to obtain important biological insights and better understanding of species-environmental relations. For this reason, future studies should focus on the documentation of clear relationships between biological traits and different human impacts on macroinvertebrates of running waters.

Our examination of all selected metrics has shown that "conventional", well-tested, and used water quality assessment metrics such as the biotic indices IBMWP and FBI make a highly significant contribution to this work. These indices are based on the detection of organic pollution, based on a community's response to high organic loading and decreased dissolved oxygen levels. Despite of some selected metrics have been developed solely for assessing organic pollution, many also can be used to define the tolerance of species to other human impacts. For example, species richness of Ephemeroptera, Plecoptera, and Trichoptera groups decreases not only with an increase of nutrient concentration (Barbour et al. 1996; Bratton et al. 1980; Fitzpatrick et al. 2001) but also with an increase of contaminants, heavy metals, thermal pollution (Lillie et al. 2003), flow disruption (Fitzpatrick et al. 2001; Lillie et al. 2003), sediment input (Lenat 1984; Meban 2001; Quinn and Hickey 1990), acid rain (Peterson and Van Eeckhaute 1992), and acid mine drainage (Zarger et al. 1986). Plecoptera (fP) are among the most sensitive indicator organisms and can indicate impairment resulting from low dissolved oxygen or siltation (Chirhart 2003). Measures of tolerance, such as %Int, indicate sensitivity of the assemblage and component species to various types of perturbation (Hilsenhoff 1987). According to Chirhart (2003), the presence of moderate numbers of intolerant taxa is an indicator of good aquatic health. Trophic and functional benthic structure measures (e.g., gShr, sFil, and fGath) are also useful since they are influenced by nutrient enrichment (Fitzpatrick et al. 2001; Quinn and Hickey 1990), disturbance of riparian corridors (Cummins 1973; Cummins et al. 1989), and impoundment and regularization (Cortes et al. 2002a; Voelz and Ward 1991). Concerning dominance measures and despite the difficulty of providing guidelines for interpreting a community structure because of the existence of a myriad of natural communities (Lillie et al. 2003), results have shown that the percentage of a dominant organism increases with increasing perturbation (Barbour et al. 1996; Chirhart 2003) notwithstanding they are not particularly sensitive to moderate amounts of organic or toxic loadings (Plafkin et al. 1989). The percent contribution of dominant taxon metric (%5TD) proved to be a good indicator in our study area. Regarding habitat preference, Vieira et al. (2004) verified that rheophily and microhabitat preferences may be indicative of hydrological disturbance after a wildfire. Rheophilous taxa has been also associated to small rivers without organic pollution (Usseglio-Polatera et al. 2000) confirming, this way, the ability of metrics like %exR in discriminating impairment.

It was difficult to establish a cause–effect relationship between traits that describe habitat/behavior (fSwi, fCling) and environmental variables due to the scarcity of information. However, Charvet et al. (1998) mention that mobility via swimming may be sensitive to chemical contamination, and Chirhart (2003) highlights that a lack of clinger taxa can indicate siltation or substrate embeddedness as a result of erosion.

From all tested functional traits, only trophic-based and locomotion traits could distinguished between reference and disturbed sites. Previous studies from temperate zone frequently found that life-history-related traits (e.g., number of descendants per reproductive cycle, number of reproductive cycles/individual, life duration of adults) responded best to a range of anthropogenic stressors and those traits related to feeding strategies, body shape, and respiration generally were more weakly related to perturbations (Dolédec et al. 1999, 2006). A possible explanation for the absence of response in our study to life-history-related traits is that it is related to ecoregions. Portugal, inserted in the biogeographic region of Iberian Peninsula according to Illies (1978) and WFD (Annex XI), is a region with very different characteristics from those where the referred studies took place. In spite of trait-based approaches rely on evolutionary responses to environmental selective forces across broad geographical gradients (Dolédec and Statzner 2008), Dolédec et al. (1999) refer that this type of traits can be constrained by geographic latitude and alerts that the future studies have to show over which geographic range such a trait may serve in biomonitoring. Moreover, our rivers are much less impacted than the studied large rivers in mid-Europe that have been largely altered by human activities. This could be another answer once the diverse expected communities has different life-history strategies and functional responses to major environmental characteristics that in our study did not reveal to be responsive to perturbation.

From the 12 retained metrics, 11 need to be identified to family or genus levels and only one needs the species-level resolution (sFil). This last one is a trait related to feeding strategies. In agreement with Dolédec et al. (1998, 2000) and Gayraud et al. (2003), trait-based approaches using higher levels of taxonomic identification (for example, genus and family) may adequately describe trait occurrence and may be more time efficient. Moulton et al. (2000) also refers that trait-based metrics also may be robust to taxonomic ambiguities, which can influence how taxonomically based metrics respond to environmental gradients. Thus and according to these authors, we think that the best basis for assemblage description must be one of these two taxonomic levels or both, genus and family levels.

Consequent evaluations suggest that the selected metrics are good indicators of water quality in the Douro catchment since they describe most of the variation verified in invertebrate assemblages and distinguish between different environmental conditions. The present set of metrics has the advantage of integrating traits with "conventional" metrics, which makes them potentially able to assess multiscale effects, knowing that the human impacts acting at higher scales are successively modified (filtered) by local variables (Frissell et al. 1986; Poff 1997). Thus, this approach provides a starting point for the selection of metrics that can be reliably applied to larger geographical areas beyond the river basin. Following screening and validation across a gradient of human influence, these metrics could be used in a refined index to better characterize community responses to general environmental degradation. Additionally, this information can be a baseline for developing strategies for the biomonitoring of aquatic ecosystem health and provide information relevant to WFD, more specifically, with regard to its application in Portugal. Thus, the set of traits selected in this work could be used also to define assemblage types and determine expected biological conditions at reference sites for biomonitoring programs in a similar way to the ones that has been used at other European countries (Dolédec et al. 1999, 2000; Charvet et al. 2000; Statzner et al. 2001b; Dziock 2006; Henle et al. 2006; Dolédec and Statzner 2008).

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