Environmental Research 156 (2017) 485–493

Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Environmental stressors as a driver of the trait composition of benthic macroinvertebrate assemblages in polluted Iberian rivers

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ARTICLE INFO

Keywords:
Macroinvertebrate traits
Pesticides
Urban pollution
Multiple stressors
Aquatic environment

ABSTRACT

We used the trait composition of macroinvertebrate communities to identify the effects of pesticides and multiple stressors associated with urban land use at different sites of four rivers in Spain. Several physical and chemical stressors (high metal pollution, nutrients, elevated temperature and flow alterations) affected the urban sites. The occurrence of multiple stressors influenced aquatic assemblages at 50% of the sites. We hypothesized that the trait composition of macroinvertebrate assemblages would reflect the strategies that the assemblages used to cope with the respective environmental stressors. We used RLQ and fourth corner analysis to address the relationship between stressors and the trait composition of benthic macroinvertebrates. We found a statistically significant relationship between the trait composition and the exposure of assemblages to environmental stressors. The first RLQ dimension, which explained most of the variability, clearly separated sites according to the stressors. Urban-related stressors selected taxa that were mainly plurivoltine and fed on deposits. In contrast, pesticide impacted sites selected taxa with high levels of egg protection (better egg survival), indicating a potentially higher risk for egg mortality. Moreover, the trait diversity of assemblages at urban sites was low compared to that observed in pesticide impacted sites, suggesting the homogenization of assemblages in urban areas.

1. Introduction

River ecosystems are impacted by a variety of anthropogenic stressors (Vörösmarty et al., 2010) and changes in the taxonomic and functional diversity of local species are expected on the global scale (Olden et al., 2004). However, the successful quantification of the relationship between the occurrence of particular stressors and biological indicators across large geographical areas remains challenging. In addition, an increasing number of stressors are co-occurring and impact the biota simultaneously (Navarro-Ortega et al., 2015). Therefore, it is of utmost importance to disentangle the effects of co-occurring stressors, in order to determine which stressor should be given priority in river basin management. The growing human population and resulting land use changes from natural to urban and agricultural have increased pressure on river ecosystems. Agriculture and urbanization are recognized as being amongst the main causes of stream impairment (Paul and Meyer, 2001). Water and habitat quality are often degraded in the streams draining agricultural land (Allan, 2004) due to the increased input of pesticides, sediments and nutrients, as well as hydrological alterations due to water abstraction (Elbrecht et al., 2016; Tilman et al., 2002). Effects of pesticides on sensitive species have been observed in streams (e.g. Liess and Von Der Ohe, 2005; Schäfer et al., 2007) using trait based SPEAR index. In a recent study by Malaj et al. (2014), the scale of the problem was revealed, since it was estimated that organic pollutants, among which pesticides were the major contributors to the risk, threaten the health of freshwater ecosystems across the whole of Europe. Furthermore, in streams draining urban land, consistent ecological degradation also occurs (Walsh et al., 2005). Increasing run-off from impervious surfaces (i.e., asphalt, concrete or stone), input of storm water from piped drainage systems (Walsh et al., 2005) and wastewater discharges (Paul and Meyer, 2001) can cause drastic changes in urban streams. The symptoms generally associated with urbanization include "flashy" hydrograph, changes in channel morphology, high concentrations of

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http://dx.doi.org/10.1016/j.envres.2017.03.054
Received 18 January 2017; Received in revised form 18 March 2017; Accepted 29 March 2017
Available online 26 April 2017
0013-9351/

metals, nutrients and organic toxicants and elevated water temperature. These modifications generally result in the decline of sensitive species (Wenger et al., 2009) and changes in ecosystem processes such as nutrient uptake (Paul and Meyer, 2001).

Stream macroinvertebrates have long been used as indicators for water quality assessment (Rosenberg and Resh, 1992). However, natural variability and confounding factors can mask the effect of a particular stressor (Schäfer et al., 2007), especially over large geographical area. To overcome this problem, more attention has been given to the use of the biological traits of taxa such as generation time, body size, body form and dispersal ability (Statzner et al., 2005; Tachet et al., 2010; Usseglio-Polatera et al., 2000). These characteristics may be used to help interpret changes in assemblages across environmental gradients and to improve the robustness of traditional stream biomonitoring (Dolédec and Statzner, 2008). According to the habitat template theory (Southwood, 1977), the spatial and temporal characteristics of the habitat provide a framework against which species have evolved characteristic life-history strategies to maximize their fitness and survival (Poff, 1997; Townsend and Hildrew, 1994). Life-history strategies include different combinations of traits that represent the solution to a given ecological problem (Verberk et al., 2008). The use of multiple traits, described through multiple trait categories or states, has successfully discriminated between different stressors (Dolédec and Statzner, 2008; Dolédec et al., 1999; Mondy and Usseglio-Polatera, 2013). Multiple-trait based approaches have shown promise for biomonitoring because most stressors should affect only certain trait categories (Statzner et al., 2001, 2004, 2005), which can be useful for discriminating among multiple stressors. Furthermore, unlike species composition, which changes along geographical and downstream gradients, some traits are thought to vary little across temporal and spatial scales, which makes them useful for large-scale studies (Statzner et al., 2001, 2004, 2005).

In this study, we used invertebrate traits to discriminate between the different types of human impacts in several basins of the Iberian Peninsula. We selected 16 sampling sites from four Mediterranean river basins with known human pressures (pesticides, multiple urban stressors and mixed). We further selected species traits that were thought to specifically respond to these stressors. The aim was to test the ability of a multiple trait-based approach to show that traits were not randomly distributed across assemblages in studied rivers and that different trait combinations responded to specific conditions in relation to the environment (urban vs. pesticide impacted).

2. Materials and methods

2.1. Study area

The study area included four river basins located across the Mediterranean part of the Iberian Peninsula: the Ebro and Llobregat in the North-East, Júcar in the East and Guadalquivir in the South of the Peninsula (Fig. 1). A total of 16 sites were selected: four sites in the Ebro basin (coded E1, E2, E3 and E5), five sites in the Llobregat basin (L3, L4, L5, L6 and L7), five sites in the Júcar basin (J1, J2, J4, J5 and J6) and two sites in the Guadalquivir basin (G1 and G4). Each site receives a variety of diffuse and point source inputs depending on catchment land use (Fig. S1, Table S1, in Supplementary data). Some of the sites are located in urban areas; the other sites are located in areas where a high risk of pesticide toxicity has previously been reported (De Castro-Català et al., 2016; Kuzmanović et al., 2015a; López-Doval et al., 2012). The data used in this study were gathered within the SCARCE-CONSOLIDER project (Navarro-Ortega et al., 2012) in which the sampling for chemical and biological analyses was performed during the autumn of 2010.

2.2. Physical and chemical data

Organic pollutants were measured using analytical techniques based on gas chromatography-tandem mass spectrometry and liquid chromatography-tandem and hybrid mass spectrometry (Masiá et al., 2013; Osorio et al., 2014). To assess the toxic risk at each sampling site, toxic units (TU) were calculated using the measured concentrations of the compound (MEC) and respective acute toxicity data (EC50) for *Daphnia sp*. The sums of toxic units for each of the compound families ($TU_{pesticides}$ and TU_{metals} in Table 1) were calculated as the risk estimate posed by different groups of toxicants. The major contributors to the pesticide toxicity risk were insecticides (e.g., chlorpyriphos or chlorfenvinphos) whereas copper was the main contributor to the metal toxicity risk. More details on measurements of the chemical compounds and risk assessment associated with our study can be found in Kuzmanović et al. (2015a, 2015b). Other physical and chemical variables included average sediment particle size (Phimoy in Table 1) and variance (Phivar) at the Phi scale [range from −8 (boulder) to > 10 (colloid)], flow variations (expressed as a 3-month coefficient of variation (CV) prior to sampling), average precipitation (3-month average), water temperature (T), dissolved oxygen (O_2), dissolved organic carbon (DOC), conductivity, nutrients ($N\text{-}NO_3$, and $P\text{-}PO_4$), percentage of organic matter in sediment (OM) and the altitude of sampling sites. The OM content, toxic units and nutrient data were log-transformed prior to analysis. The catchment land use types were estimated from Corine Land Cover (2006) using Arc Map 10.1 software and the variable that synthesized naturalness was calculated as the weighted mean of three categories (Urban, Agricultural, Natural) arbitrarily weighted by a coefficient of 1, 5 and 100, respectively (LU in Table 1; see Supplementary material). Further details on chemical and physical data measurements are available in Sabater et al. (2016).

2.3. Site classification

We determined which stressors were present at sampling sites (Fig. 1) and according to the dominant stressor, sites were classified into three groups (pesticide impacted, urban and mixed). The pesticide impacted sites (E1, E5, J1, J2, J4, J5, J6, G1) where those where acute risk was posed by pesticides (logTU > −1, (Fig. S2, Kuzmanović et al., 2015b)). Sites classified as urban (L3, L4, L5, L6, L7, and G4) were those impacted by other stressors (e.g., metals, nutrients, elevated temperature, low oxygen level, Fig. 1) which were all highly correlated to urban land use (Table S2, Supplementary). At urban sites, the risk of pesticide toxicity was below acute levels. Finally, two sites were classified as mixed (E2 and E3) because they were affected both by pesticides and multiple stressors related to urban land use. Whether an environmental variable can be considered a stressor was evaluated on the basis of thresholds derived from legislation or the literature (Table S3).

2.4. Macroinvertebrate sampling

At each site, five sediment samples were randomly collected using a polyvinyl sand corer (24 cm^2 area). Each sample was sieved through a 500-µm mesh and fixed with 4% formaldehyde. Macroinvertebrates were sorted, counted and identified in the laboratory under a dissecting microscope (Leica Stereomicroscope). *Chironomidae* were identified at the genus level, while almost all other taxa were identified at the species level (list of taxa available in Supplementary material). Abundances were referred to on the basis of sediment surface area (De Castro-Català et al., 2015).

2.5. Biological traits

Traits were derived from a European database compiled by Tachet et al. (2010) and completed for Mediterranean taxa by Bonada et al. (Bonada and Dolédec, 2011; Bonada et al., 2007). In this database, the

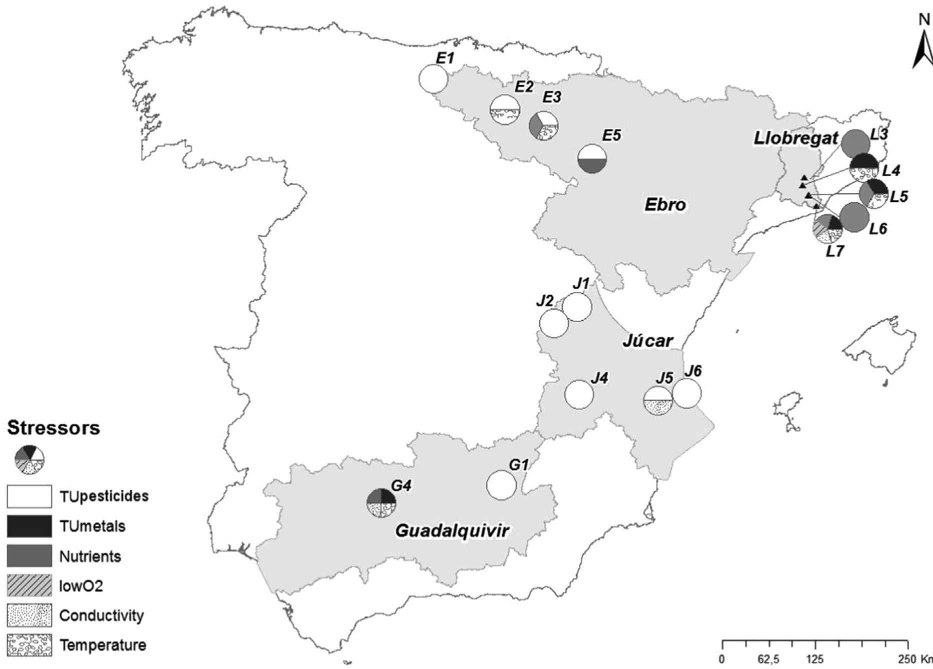

Fig. 1. Potential stressors at sampling sites including toxic units of pesticides ($TU_{pesticides}$), toxic units of metals (TU_{metals}), nutrients, low oxygen levels (lowO2), conductivity and temperature.

Table 1
Mean and range (in parenthesis) of physical and chemical data (n = 16).

Variable	Value
Altitude (m)	379 (5–1180)
LU (%)	40 (4.3–95)
CV (%)	45 (3–112)
Phivar (φ)	2.8 (0.5–5.3)
Phimoy (φ)	1.3 (−1 to 3.2)
O_2 (mg/L)	9.4 (5.9–11.2)
T (°C)	16 (6–28)
pH	8 (7.5–8.2)
Conductivity (μS/cm)	805 (162–1372)
DOC (mg C/L)	4.9 (2.1–10.2)
$N\text{-}NO_3$ (mg /L)	1.9 (0.1–9.2)
$N\text{-}NH_4$ (mg/L)	0.3 (0.1–1.2)
$P\text{-}PO_4$ (mg/L)	0.1 (0–0.6)
$TU_{pesticides}$	−0.8 (−1.4 to −0.1)
TU_{metals}	−1.7 (−3.3 to −0.7)
OM (%)	4.2 (1.1–13.2)
Precipitation (mm)	599 (100–1600)

affinity of each taxon for a given trait state or category is quantified by a score with a value of 0 if there is no affinity of the taxon for the trait state or category, 1 if low affinity, 2 if medium affinity and ≥3 if strong affinity (i.e., fuzzy coding approach; Chevenet et al., 1994). Trait information at the genus or species level was used when data was available, otherwise subfamily or family level information was used (for *Chironomidae, Oligochaetae* and *Enchytraeidae*, respectively). However, if taxonomic levels higher than species are used, the trait structure of assemblages is generally conserved (Dolédec et al., 2000; Gayraud et al., 2003). The initial trait table contained the affinities of the taxa, collected as described above, 39 trait categories distributed in 8 traits (Table S5, Supplementary). Trait-affinity scores were further treated as frequency distributions, i.e., they were rescaled to sum to 1 for a given taxon and a given trait (e.g., Gayraud et al., 2003; Schmera et al., 2015). We selected eight biological characteristics expected to respond to stressors in the studied basins. We selected the frequency affinities of taxa for small size (< 5 mm), short lifespan of adults (< 1 year), plurivoltinism (> 1 generation per year), and predation as separate traits. Egg protection was assessed by adding the frequency affinities of taxa for ovoviviparity, clutches in vegetation and terrestrial clutches. "Deposit feeding" combined the frequency affinities of taxa for absorption through tegument and deposit feeding *sensu stricto*. For each taxon, dispersal ability was assessed using an index similar to that proposed by Bonada et al. (2012), which uses the four dispersal categories (aquatic passive, aquatic active, aerial passive and aerial active) of the Tachet et al. (2010) database. Aquatic passive was weighted by 1, aquatic active by 5, aerial passive by 10 and aerial active by 20. The dispersal ability of each taxon was thus computed as the weighted mean across the dispersal categories. Finally, we computed the food diversity ingested by each taxon as a Simpson index ($1-\sum p_i^2$, with p_i as the proportion of a given food item).

2.6. Data analysis

The relationship between macroinvertebrate traits and environmental variables was investigated by RLQ (Dolédec et al., 1996) and fourth-corner analyses (Legendre et al., 1997). RLQ is an extension of the co-inertia analysis (Dolédec and Chessel, 1994) that allows relating three tables: a trait table (named **Q**), an environmental table (**R**) and a species abundance table (**L**) that is used as the link between **Q** and **R**. RLQ provides the simultaneous ordination of species, their traits and the environmental variables. It has been successfully applied in ecological studies dealing with birds (Hausner et al., 2003), beetles (Ribera et al., 2001), freshwater macroinvertebrates (Díaz et al., 2008) and aquatic plants (Baattrup-Pedersen et al., 2016). As recommended by authors, the three tables were analyzed separately prior to RLQ analysis. Correspondence analysis (CA) was performed on the abundance table and principal components analysis (PCA) on the trait and environmental tables, respectively. In brief, RLQ summarizes the multivariate structures by searching for the linear combinations of traits and environmental variables (describing stress) on which sites and taxa are projected, providing new site and taxa scores that are the most covariant. These new scores must be compared to those from the separate analyses of each table to assess how much of their variability is taken into account by the RLQ analysis and to evaluate the strength of the relationship between traits and stressors. The overall significance of this relationship was further assessed via a global Monte-Carlo test using 99,999 random permutations of the table rows of **R** (sites; model 2; Dray et al., 2014) and of the table rows of **Q** (species; model 4; Dray et al., 2014). Fourth-corner analysis was used to find significant bivariate relationship between single trait and environmental variable. Furthermore, combination of RLQ and fourth-corner analysis was used to evaluate the significance of associations between traits and combination of environmental variables (identified by RLQ) and environmental variables and combination of traits (identified by RLQ, see Dray et al. (2014) for further details). The significance of relationship was assessed using Pearson r correlation coefficient (for details, see Dray et al. (2014)). In these two latter approaches, the false-discovery-rate adjustment method was used to correct *P*-values according to bias due to multiple-test comparisons. Statistical analyses and graphical outputs were computed with the ade4, vegan and corrplot packages implemented in the R software (Core Team, 2015; Chessel et al., 2004; Dray and Dufour, 2007; Dray et al., 2007; Oksanen et al., 2016).

3. Results

3.1. Separate ordinations

A correspondence analysis (CA) performed on the faunistic table yielded a first and second axis that explained 25.8% and 17.5% of the total variability, respectively. The best possible correlation between taxa and sites equaled 0.65 (square root of the first eigenvalue = 0.42), suggesting a fairly good ordination of taxon composition. A chi-squared test further demonstrated the non-independence between sites and taxa ($P < 0.001$). The first CA axis separated the Júcar river sites (J in Fig. 2B) from the three other rivers. The second CA axis separated pesticide impacted sites from urban sites (Fig. 2A). The Júcar had higher proportions of *Tanytarsus* sp., *Ephemera* sp. and *Potamopyrgus* sp., potentially reflecting coarser sand in the sediment than in the other rivers. The pesticide impacted sites of the Ebro and Guadalquivir rivers had more Oligochaetes (*Lumbriculus* sp., Enchytraeidae, *Limnodrilus* sp.) and chironomids (*Nanocladius* sp., *Stictochironomus* sp. and *Microspectra* sp.), which are commonly found in fine sediments. At the urban sites of the Llobregat (L in Fig. 2B) and Guadalquivir (G in Fig. 2B), *Cryptochironomus* sp., *Polypedilum* sp., *Limnodrilus* sp., *Micronecta* sp., *Potamothrix* sp. and *Caenis* sp. were more abundant.

A PCA performed on the environmental table yielded a first and second axis that explained 44.8% and 16.2% of the total variability, respectively. The first PCA axis separated the sites according to the stressors, i.e., urbanization (left side of the axis; Fig. 3A) vs. pesticides (right side; Fig. 3A). Two sites (E2 and E3) with a mixture of both types of stressors appeared between the above two groups (Fig. 3A). Four basins differed in the stressor present (Fig. 3B). In particular, sites in the Júcar basin were mainly affected by pesticides whereas sites in the Llobregat were mainly affected by urban stressors; the Ebro and Guadalquivir had sites with either one of the stressor types or a combination of both (Fig. 3B). The first PCA axis thus opposed sites with mainly high pesticide toxicity ($TU_{pesticides}$, Table 2) and high dissolved oxygen concentration (O_2) to sites with high values for temperature, dissolved organic carbon (DOC), nutrients (P-PO4, N-NO3), metals (TU_{metals}), precipitation and flow variation (CV) associated with urbanization (Table 2).

Finally, a PCA performed on the trait table yielded a first and second axis that explained 48.1% and 18.2% of the total variance, respectively. The taxa associated with the positive side of the first PCA axis included *Branchiura sp., Limnodrilus sp., Potamotrix sp., Lumbriculus sp.* and *Enchytraeidae,* which are prominently deposit feeders and plurivoltine taxa (Fig. S1, see Supplementary material). The taxa associated with the negative side of the first PCA axis included all other taxa (e.g., *Dicranota sp., Caenis sp., Microspectra sp., Tanytarsus sp., Ephemera sp.*), which prominently protect their eggs, are short-lived and small-sized, and disperse easily (Fig. S3, see Supplementary material). The second PCA axis was positively correlated with predation and food diversity.

3.2. RLQ analysis

The relationship between the trait composition of macroinvertebrate assemblages and environmental stressors was globally significant (Model 2 simulated $P < 0.001$; Model 4 simulated $P < 0.025$). This relationship was mainly summarized by the first RLQ axis, which explained 85.6% of the total cross-variance between the traits and environment, whereas the second axis only contributed to 8.0% (Table 3). Because of this low value, the second axis will not be discussed further.

The first axis accounted for 97% of the variability of the environ-

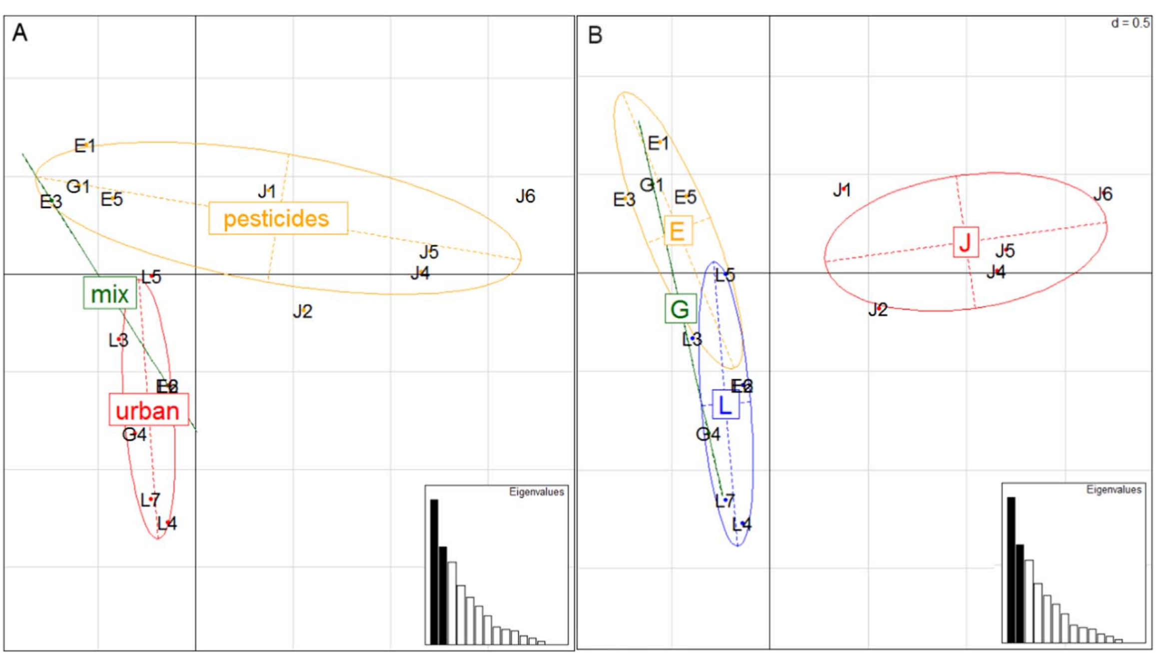

Fig. 2. Results of a CA performed on fauna abundance showing the site ordination (A) grouped by dominant stressor and (B) by river basin (J-Júcar, E-Ebro, G-Guadalquivir, L- Llobregat). Inset represents the diagram of eigenvalues.

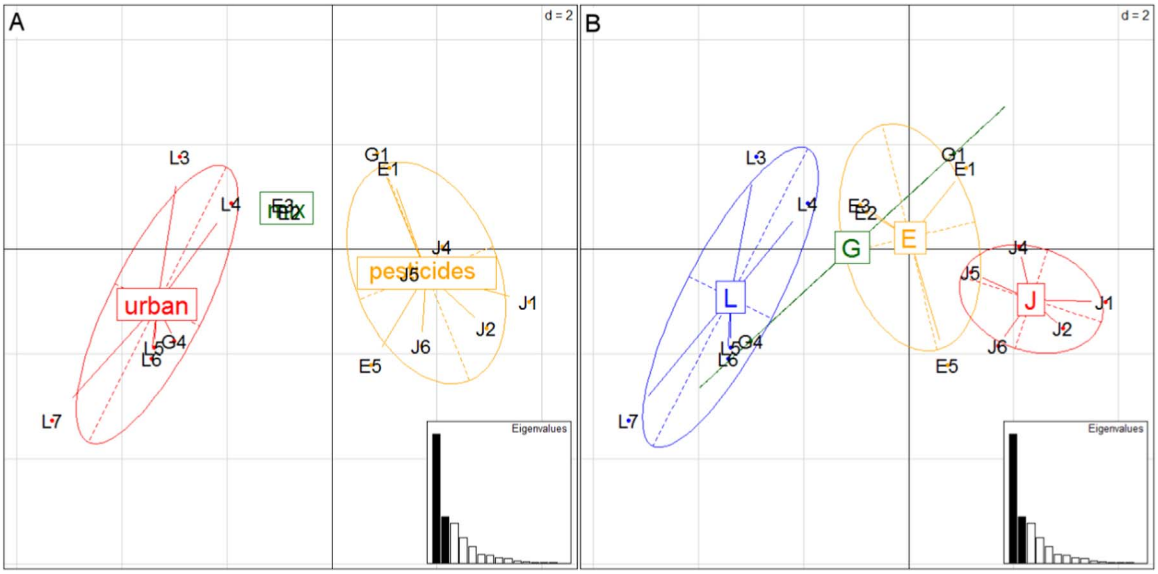

Fig. 3. Results of a PCA performed on the environmental table showing sites (A) grouped by dominant stressors (B) by river basin (J-Júcar, E-Ebro, G-Guadalquivir, L-Llobregat). Inset represents the diagram of eigenvalues.

Table 2
PCA loadings for first two components.

Variable	Comp1	Comp2
LU	0.3	−0.2
N-NO$_3$	−0.9	0.0
P-PO$_4$	−0.9	−0.3
O$_2$	0.7	0.1
DOC	−0.9	−0.1
Conductivity	−0.4	−0.7
Temperature	−0.9	−0.2
pH	0.3	0.3
TU$_{pesticides}$	0.7	−0.2
TU$_{metals}$	−0.9	0.2
OM	−0.3	0.9
Phimoy	−0.4	0.8
Phivar	−0.3	0.4
CV	−0.8	−0.0
Precipitation	−0.7	0.0

Table 3
Summary of the RLQ analysis.

Total inertia: 2.226					
Eigenvalues:					
	Ax1	Ax2			
	1.907	0.179			
Projected inertia (%):					
	Ax1	Ax2			
	85.662	8.017			
Cumulative projected inertia (%):					
	Ax1	Ax2			
	85.660	93.680			
Eigenvalues decomposition:					
	eig	covariance	sdR	sdQ	correlation
eig1	**1.907**	1.381	2.550	1.649	0.328
eig2	0.179	0.422	1.290	1.514	0.216
Inertia & coinertia R:					
	inertia	max	ratio		
eig1	6.505	6.721	0.968		
eig1 + 2	8.169	9.145	0.893		
Inertia & coinertia Q:					
	inertia	max	ratio		
eig1	2.718	3.851	0.706		
eig1 + 2	5.011	5.308	0.944		
Correlation L:					
	correlation	max	ratio		
eig1	0.328	0.647	0.508		
eig2	0.216	0.531	0.407		

mental table and 70% of the variance of the trait table. In addition, the new set of site and species scores had a correlation of 0.33 along the first RLQ axis, which was 51% of the best possible correlation (i.e., obtained from the separate CA of the fauna abundance table; Table 3). In accordance with the high proportion of variability of the environmental table taken into account by RLQ (Table 3), the ordination of sites along the first RLQ axis was similar to that obtained from the separate PCA of environmental variables, which differentiated the urban from the pesticide impacted sites (Fig. 4A). The first RLQ axis also partly incorporated differences in the sampling location (river basin) and the natural longitudinal variability of the sites (Fig. 4B; explained variance = 0.56; $P < 0.005$), since most urban sites were situated along the lower parts of rivers, especially in Llobregat and pesticide impacted sites mainly along the upper and middle parts of the rivers. In contrast, unlike the separate CA of fauna in which sites in the Júcar basin were isolated from the other pesticides-impacted sites (Fig. 2A), the first RLQ axis grouped together all the sites impacted by pesticides (Fig. 4A–D), thus taking into account the variability of sites expressed along the second CA axis. Egg protection was the prominent trait of the taxa at pesticide impacted sites (Fig. 4E and F). In fact, most of the traits dominated at those sites; whereas the prominent traits in assemblages at urban sites included plurivoltinism and deposit feeding, suggesting higher trait diversity at the pesticide impacted sites than at the urban sites (Fig. 4A and E).

3.3. Fourth-corner test

We were unable to find a significant bivariate trait–environmental variable association after applying the *P*-value adjustment. This result suggests that a combination of stressors, rather than a single stressor, was acting on a combination of traits. Therefore, we further investigated the relationship between individual traits and the first RLQ environmental axis (combination of environmental variables) and individual environmental variables and the first RLQ trait axis (combination of traits). Plurivoltinism and deposit feeding were positively associated with the first RLQ environmental axis whereas egg protection was negatively associated with the first RLQ environmental axis (Fig. 5A). The environmental variables significantly positively asso-

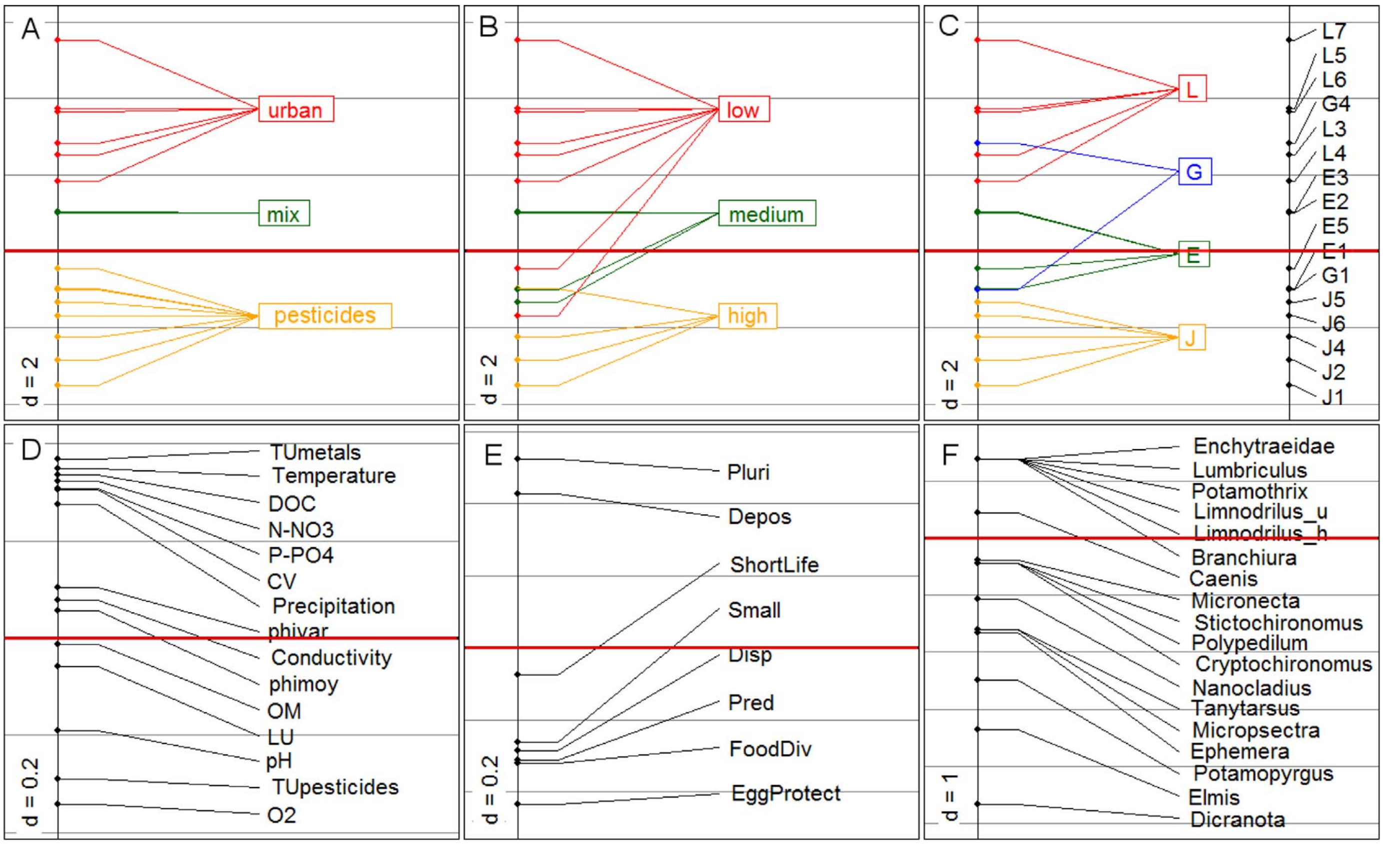

Fig. 4. Sites grouped by (A) the dominant stressors, (B) the altitude (C) river basins (J-Júcar, E-Ebro, G-Guadalquivir, L-Llobregat), (D) environmental variables (phivar-sediment particle size variance, phimoy-average sediment particle size, LU- variable that synthesized naturalness, CV- flow variations) (E) traits (pluri-plurivoltinism, depos-deposit feeding, disp-dispersal ability, pred-predation, food div-food diversity) and (F) taxon scores along the first RLQ axis. The red horizontal line corresponds to zero at the first axis, it separates negative (up) from positive (down) score. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

ciated with the first RLQ trait axis were nutrients (N-NO3, P-PO4), DOC, temperature, TU_{metals}, CV, while oxygen contents (O_2) and $TU_{pesticides}$ were negatively related to the first RLQ trait axis (Fig. 5B). In summary, there was a positive association between the environmental variables describing stress associated with urban sites (N-NO3, P-PO4, DOC, temperature and TU_{metals}) and plurivoltinism and deposit feeding whereas $TU_{pesticides}$ was associated with egg protection even if the oxygen content (O_2) was higher.

4. Discussion

Our analyses allowed us to assess the importance of different

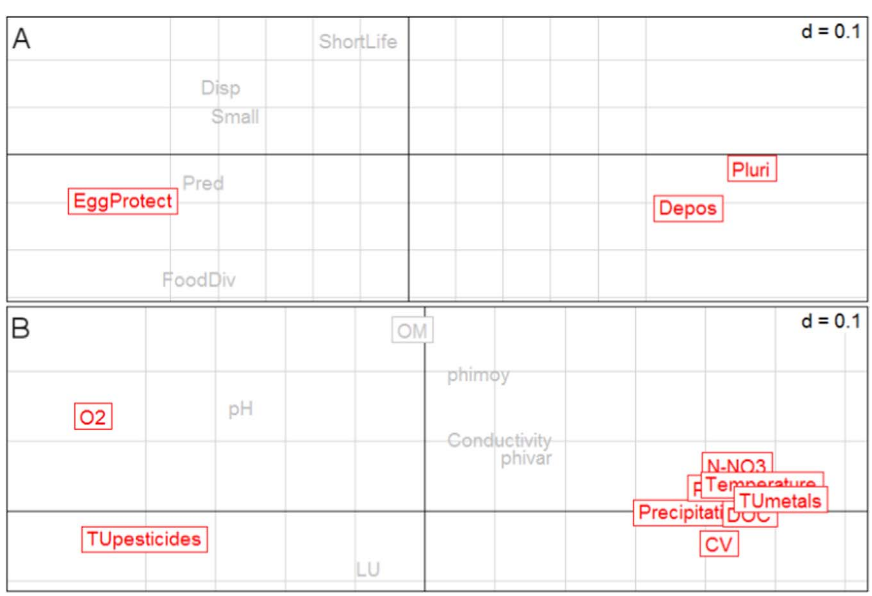

Fig. 5. Significant relationships (*P*-adjusted < 0.05 in red) represented along with the first-two RLQ axes (for readability) (A) between the RLQ environmental axes and individual traits and (B) between the RLQ trait axes and individual environmental variables (*P*-adjusted < 0.1 in red). Non-significant relationships are labeled in light grey. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

stressor (i.e., pesticides and multiple urban stressors) as drivers of the trait composition of macroinvertebrate assemblages of the selected rivers, confirming our first hypothesis that traits were not randomly distributed across assemblages. We observed that the gradients of urban stressors and pesticide toxicity pointed in opposite directions. Sites with more intense urbanization were less impacted by pesticides and sites with more pesticide pollution were less impacted by the various stressors associated with urbanization. The latter gradient was associated with altitude because lowland areas are generally more densely populated in comparison to higher altitude areas, as observed elsewhere (e.g., Dolédec et al., 2006; Rico et al., 2016).

The sites characterizing the extremes of this gradient differed in species assemblages and environmental conditions. Therefore, we expected such differences to be reflected in the life-history strategies of invertebrates (Verberk et al., 2008). Indeed, as hypothesized (H2), the trait composition of assemblages at sites impacted by pesticides differed from that at urban sites, suggesting that different trait combinations respond to specific conditions in contrasting environments (urban vs. pesticide polluted). However, it is not excluded that pesticides are also affecting the trait composition at urban sites. But their concentration were at the sublethal levels so their influence at those sites is expected to be less pronounced compared to the sites where concentrations were high enough to cause acute effects.

The taxonomic composition differed greatly among river basins, in particular separating pesticide-impacted sites in the Júcar from the other sites in the other rivers impacted by the same stressor, suggesting that presence of similar stressors may not result in similar taxonomic composition. However, we observed a similar trait composition of macroinvertebrate assemblages at sites influenced by the same stressors in different rivers. For example, the trait composition of urban sites in the Llobregat (L3-7) (NE of Iberian Peninsula) was similar to that at the urban sites of the southernmost river, the Guadalquivir (G4). Besides, the trait composition of pesticide-impacted sites in the Eastern peninsula in the Júcar basin (J1-6) was similar to that observed at sites in the North-East in the Ebro (E1, E5) and in the South in the Guadalquivir (G1). Furthermore, sites including multiple urban stressors and pesticides (E2, E3) were positioned between these two extremes along the first RLQ axis, indicating their partial similarities to both urban and pesticide-impacted sites. The higher consistency of responses obtained from trait composition in comparison to taxonomic composition has previously been reported, and arises because traits are expressed in many species and trait composition can be compared among regions that differ in their taxonomic composition (Horrigan and Baird, 2008; Statzner et al., 2001). However, given that first RLQ axis also partly incorporated differences in the sampling location (i.e., most of the urban sites were located in Llobregat and most of pesticide-impacted sites were located in Júcar) we cannot exclude completely the influence of sampling location and some unmeasured variables, especially hydromporhological alterations, to our results. Hydromprphological alterations might have the influence on the macroinvetebrate trait composition due to the simplification and changes of their habitat by e.g., water abstractions, flow regulations and morphological alterations such as straightening and canalization. Besides, it would be ideal to include unimpacted or minimally impacted sites in this kind of study to have a reference sites for comparison with impacted sites. This would allow us to observe the deviations from the natural community composition in the presence of stressors. However, in our study even sites with high percentage of natural land were not free of stressors. This was surprising, since even the sites with very small areas of artificial land upstream had concentrations of pesticides at the ecologically relevant levels (e.g., J1 and L4).

Several studies have already described the influence of different types of stressors on the structure of benthic macroinvertebrate assemblages in Iberian rivers. These stressors have included pharmaceuticals (De Castro-Català et al., 2015), metals and pesticides (Kuzmanović et al., 2015b; López-Doval et al., 2012), as well as multiple co-occurring stressors (Sabater et al., 2016). Here, we investigated the influence of different stressors on the functional trait structure of assemblages. Assemblages characterized by several species traits (e.g., predators or having small size and dispersal ability) dominated at pesticides-impacted sites. Egg protection was prominent, which indicate the higher risk for egg mortality possibly caused by high pesticide toxicity. In contrast, at urban sites species were mainly plurivoltine, which indicates resilience potential (Southwood, 1977; Townsend and Hildrew, 1994). Plurivoltinism enable species to recover after disturbance events such as periodic exposure to toxicants or "flashy" hydrology (i.e. frequent larger flow events) that are characteristic of urban streams (Walsh et al., 2005), but also after natural disturbances such as high flow variability that are common in Mediterranean rivers (Bonada et al., 2007). The prominence of plurivoltine species, which increased with urbanization and flow irregularity, may suggest a confounding effect between stressors and natural flow variability. Moreover, as Mediterranean rivers have a naturally low flow during the summer and an associated lower dilution capacity, a higher exposure of species to toxicants can be expected (Arenas-Sánchez et al., 2016; Petrovic et al., 2011). This interaction between stressors and natural factors may lead to more severe effects than in more temperate rivers. Moreover, deposit feeding was significantly related to urbanization, indicating a possible response to hydrological disturbances (Feio and Dolédec, 2012) or nutrients, similarly to what was observed for the marine environment (Grall and Chauvaud, 2002). Finally, urbanization was associated with a decrease in predator abundance. This could be related to heavy metal pollution due to higher exposure of the taxa from the "top of the food chain" to the metals due to the biomagnification (Dolédec and Statzner, 2008).

Since we were unable to find a single trait–environmental variable association we assumed that a combination of stressors was affecting the trait composition of assemblages, especially at urban sites where several stressors had significant effects on trait composition. Similarly, we found that several physical and chemical stressors were simultaneously influencing the invertebrate assemblages of the Iberian rivers and explaining the high proportion of taxonomic variability, indicating the shared effect of multiple stressors (Sabater et al., 2016). Finally, urbanization and its related stressors seem to have an important effect on the trait homogenization of assemblages since only two out of eight traits showed prominence at urban sites. Functional trait homogenization at the most impaired sites may be an expression of the loss of functional diversity due to the combined occurrence of habitat simplification and the presence of contaminants. This phenomenon may impair the functionality, stability, resilience and resistance of ecosystems by reducing species-specific responses to environmental changes (Olden et al., 2004; Stachowicz et al., 2002). As a side effect, trait homogenization of assemblages within the whole region may increase its vulnerability to large-scale events such as climate change (Olden et al., 2004). Our study thus complements others that have recently attempted to assess the effect of different stressors on functional homogenization (Mondy and Usseglio-Polatera, 2014; Olden et al., 2004).

5. Conclusions

RLQ analyses coupled with fourth-corner permutation tests proved a powerful tool to reveal the difference in the trait composition of macroinvertebrate assemblages impacted by different types of stressors. Urbanization seems to have a somewhat stronger impact on trait composition than pesticides, since different life strategies were limited at urban sites which indicated trait homogenization. Our results suggested that macroinvertebrate assemblages at sites influenced by similar stressors in different rivers across the Iberian Peninsula may have a similar trait composition, despite their difference in taxonomic composition. This latter finding confirms that multiple trait-based approaches may usefully complement taxonomic approaches in large-scale studies. However, a larger study including more sampling sites and preferably minimally impacted sites would be necessary to exclude the potential influence of natural variability and give more support to our findings.

Acknowledgments

This study has been supported by the EU FP7 project GLOBAQUA [Grant Agreement No. 603629], the NET-Scarce project [Redes de Excelencia CTM2015-69780-REDC] and by the Generalitat de Catalunya [Consolidated Research Groups: 2014 SGR 418 – Water and Soil Quality Unit and 2014 SGR 291 – ICRA]. MK acknowledges AGAUR fellowship of the Generalitat de Catalunya. Special thanks to Laura Armendáriz for the identification of the *Oligochaeta*.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envres.2017.03.054.

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