Effects of anthropogenic pollution and hydrological variation on macroinvertebrates in Mediterranean rivers: a case-study in the upper Tagus river basin (Spain)

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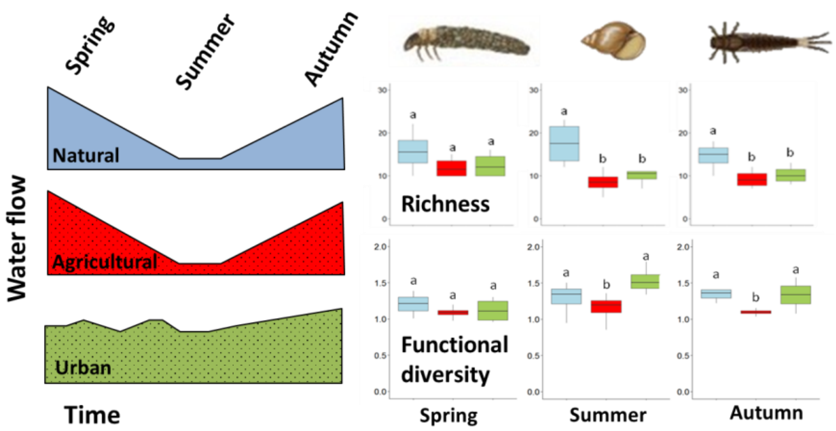
Graphical abstract



Highlights

- We assessed the effect of varying flow conditions and pollution on invertebrates.
- Stronger functional diversity response to pollution during low flow periods.
- Traits representative of pollution and low-flow conditions have been identified.
- Late summer monitoring is recommended to account for maximum ecological disturbance.

Abstract

Seasonal hydrological variation and chemical pollution represent two main drivers of freshwater biodiversity change in Mediterranean rivers. We investigated to what extent low flow conditions can modify the effects of chemical pollution on macroinvertebrate communities. To that purpose, we selected twelve sampling sites in the upper Tagus river basin (central Spain) having different sources of chemical pollution and levels of seasonal hydrological variation. The sites were classified as natural (high flow variation, low chemical impact), agricultural (high flow variation, high agricultural chemical inputs) and urban (limited flow variation, high urban chemical inputs). In these sites, we measured daily water discharge, nutrients, and contaminant concentrations, and we sampled benthic macroinvertebrates during spring, summer and autumn. Significant differences related to toxic pressure and nutrient concentrations were observed between the three groups of sites. Seasonal patterns were found for some water quality parameters (e.g. nitrites, ammonia, suspended solids, metal toxicity), particularly in agricultural sites. Taxonomic and functional richness were slightly lower in the polluted sites (agricultural and urban), particularly during low flow periods (summer and early autumn). Functional diversity was significantly lower in sites with seasonal flow variation (agricultural sites) as compared to the more constant ones (urban sites). The frequency of traits such as large size, asexual reproduction, aquatic passive dispersion and the production of cocoons increased in response to pollution during low flow periods. This study shows that the impacts of anthropogenic chemical pollution on taxonomic and functional characteristics of macroinvertebrate communities seem to be larger during low flow periods. Therefore, further studies and monitoring campaigns assessing the effects of chemical pollution within these periods are recommended.

Keywords: Pollution, hydrological variation, biological traits, invertebrates, functional diversity

1. Introduction

Most Mediterranean rivers are characterized by marked seasonal hydrological variations, undergoing low flows or even drying completely during summer. Climate change and increasing water demands are key drivers contributing to hydrological fluctuations in these ecosystems (Barceló and Sabater, 2010; EEA, 2012; Lobanova et al., 2017), and generally tend to exacerbate water scarcity (Gashit and Resh, 1999; IPCC, 2014; Lobanova et al., 2016). Besides hydrological alterations, several studies show that Mediterranean rivers are severely impacted by chemical contaminants, whose fate and exposure are affected by seasonal flow variations (Petrovic et al., 2011; López-Doval et al., 2013). Low flows lessen the dilution capacity of chemical discharges and alter contaminant degradation patterns due to water temperature fluctuations and changes in organic matter concentrations or light penetration (Ademollo et al., 2011; López-Doval et al., 2013; Rice and Westerhoff, 2017; Arenas-Sánchez et al., 2019a).

Benthic macroinvertebrates are key components of lotic ecosystems, comprising species with a high variability in terms of environmental tolerance and habitat preference (Bonada et al., 2006; Boix et al., 2010). Therefore, they are considered good integrative indicators of chemical and physical disturbances and are widely used to evaluate the ecological status of rivers (Resh and Rosenberg, 1993; Boix et al., 2010; Feio et al., 2015). Hydrological variability is one of the most important factors influencing freshwater macroinvertebrate communities, especially in Mediterranean rivers (Bonada et al., 2007a; Bonada and Resh, 2013; Prat et al., 2014). Periods of low flow involve a reduction in habitat availability (Lahr, 1997; Acuña et al., 2005; Verdonschot et al., 2015) and generally result in increased water temperatures, oxygen depletion, and high nutrient and suspended solid concentrations, which influence species diversity (Stanley et al., 1997; Acuña et al., 2005). Moreover, chemical pollution emitted from agriculture and urbanized areas can lead to toxic effects, and jointly contribute to taxonomic and functional homogenization of macroinvertebrate communities (Manfrin et al., 2013; Ortiz et al., 2005; Sabater et al., 2016; Parreira-de Castro et al., 2018), limiting their ecological functions and their ability to respond to additional stressors (Clavel et al., 2011).

Some studies in the Mediterranean region have shown that the combination of chemical pollution and hydrological stress may reduce macroinvertebrate species richness and the abundance of vulnerable taxa such as Plecoptera, Trichoptera and some Ephemeroptera (Bollmohr and Schulz, 2009; Kalogianni et al., 2017; Karaouzas et al., 2018). However, the number of studies scrutinizing the impact of chemical pollution and hydrological stress on aquatic invertebrates remains limited, with a focus on assessing chemical impacts on communities living in temporary rivers close to or during complete desiccation events (Arenas-Sánchez et al., 2016; Soria et al., 2019).

In Europe, the ecological status assessment of rivers and other surface water bodies is conducted following the recommendations provided by the Water Framework Directive (WFD, Directive 2000/60/EC; EC, 2003), which requires the monitoring of phytoplankton, macroalgae, fish and benthic macroinvertebrates. According to the WFD, the monitoring of macroinvertebrates in surface waters should be performed once or twice per year (in summer and/or winter), with sampling times and frequencies varying according to Member State experts' decision. In some Member States, like Spain, the monitoring of benthic macroinvertebrates is generally performed in spring, which is considered the optimal sampling season as it yields maximal taxonomic diversity (MAGRAMA, 2013). Mediterranean rivers subjected to severe hydrological fluctuations and chemical pollution generally exhibit highest biodiversity declines during low-flow periods (Arenas-Sánchez el al., 2016; Karaouzas et al., 2018), which generally occur during the summer season or shortly afterwards. Therefore, it is expected that monitoring of macroinvertebrates during spring does not capture the worst-case conditions resulting from the combination of these two stress factors.

According to the WFD, benthic macroinvertebrate assessments should be performed based on the taxonomic identification of the monitored individuals. Nevertheless, several studies show that monitoring or data analyses based on biological traits can complementarily be used to provide information on the mechanistic response of aquatic invertebrate assemblages to environmental constraints (Statzner and Bêche, 2010; Piló et al., 2016, Rico et al. 2016; Kuzmanović et al., 2017), and have been recommended to form part of future regulatory guidelines for the ecological status assessment of surface waters (Baattrup-Pedersen et al., 2017; Berger et al. 2018). Based on the available literature, we identified traits that are expected to correspond to an increase or decrease in tolerance of aquatic invertebrates to each of these two stress factors (Table 1). For example, the tolerance of aquatic invertebrates to chemical pollution may be decreased for organisms that have gills or small sizes, due to their potential increased chemical uptake (Paul and Meyer, 2001; Rico et al. 2015). On the other hand, the ability to reproduce using terrestrial eggs, or to perform diapause or produce cocoons, can contribute to the high tolerance of invertebrates to harsh environments characteristic of low-flow conditions, by increasing their isolation capacity or delaying their development (Williams 2005; Bonada et al. 2007b).

The main objective of this study was to rate how and to what extent varying hydrological conditions of Mediterranean rivers modify the effects of anthropogenic chemical pollution on benthic macroinvertebrate communities. For that purpose, we evaluated the taxonomic and functional responses of macroinvertebrate communities in the upper Tagus river basin (central Spain), considering permanent rivers subjected to different magnitudes of hydrological variation and chemical pollution. Our hypotheses were that pollution is expected to reduce taxonomic and functional diversity of macroinvertebrate communities, and that such impacts may be larger during low-flow periods as a result of lower dilution potential and altered physico-chemical conditions. Additionally, based on the expected responses described in Table 1, we also aimed at identifying biological traits that could be indicators of hydrological stress conditions and/or pollution status.

2. Materials and methods

2.1. Study area and site classification

The Tagus River basin is representative of Mediterranean climate, with high temperatures and pronounced summer droughts affecting the majority of surface waters. Twelve sites were selected in the upper part of this basin based on different magnitudes of annual hydrological variation and sources of anthropogenic pollution (Figure 1). Hydrological variation categories were established based on daily flow data provided by the Tagus River Basin Authority (Figure A1 in Appendix A), which were measured with flow gauges placed at each sampling site or slightly upstream. Sites were defined as severely influenced by hydrological variation when more than 15% of the total number of days in the year had flow values below the 20% quantile of the mean annual flow (Figure A1). The pollution status was established on the basis of land use within the drainage sub-basin of each sampling site. Land use was assessed using the Corine land cover layers (2006) and ArcGIS software, as described in Arenas-Sánchez et al. (2019b). The sites were classified as predominantly (1) natural (forests, grasslands without human alteration), (2) agricultural, and (3) urban (the latter including industrial activities). Agricultural sites were those located in sub-basins with >75% agricultural land use and <1% urban land use, and that were severely affected by hydrological variation (i.e., >15% of the total number of days being dry). Urban sites were those located in drainage sub-basins with >1% of the land use classified as urban, presenting less pronounced hydrological variation, potentially due to the relatively constant wastewater flow contributions from cities. Remaining sites having lower agricultural and urban influence were classified as natural sites and were notably affected by seasonal flow variation (Table A1).

Table 1. Trait categories that are expected to influence the tolerance of freshwater invertebrates to pollution and low flow conditions by means of resistance or recovery strategies (+: high tolerance; -: low tolerance; +/-: high or low tolerance have been observed depending on the dominant response mechanism).

Trait	Trait Category	Pollution		Low flow	
Size	Small size <1cm	-	Larger surface/volume ratio, high exposure to some toxicants[1]		
Life cycle duration/ No. cycles per year	Short<1year	+	High population internal and external recovery capacity[2]	+	High population internal and external recovery capacity[3]
Reproduction type	Asexual	+	High internal recovery capacity through fast reproduction[4,5]	+	High internal recovery capacity through fast reproduction[3,5]
	Isolated eggs	-	Larger surface/volume ratio, high exposure to toxicants than eggs in clutches[6]		
	Clutches	+			
	Terrestrial/ Vegetation clutches			+	Isolation capacity from harsh environmental conditions [3]
Dispersal	Aerial active		Recolonization of polluted waters from unpolluted areas[7]	+	Recolonization of less dry sites from dried riverbeds or stagnant sites[3]
	Aquatic passive	+			
Substrate relation	Surface swimmer	+	Avoidance of unfavorable conditions	+	Avoidance of unfavorable conditions
	Burrowers	+	Benefit from the deposition of organic matter[8]	+	Benefit from the deposition of organic matter[9]
Resistance forms	Diapause	+	Additional isolation from the environment or stopping development during unfavorable conditions[10]	+	Additional isolation from the environment or stopping development during unfavorable conditions [3,11]
	Resistant eggs	+		+	
	Cocoons	+		+	
Respiration	Gills	-	Higher exposure to toxicants in gill-or tegument breathers due to higher surface/volume ratios and higher chemical uptake differing between compounds[1,7]	+	More specialized structures (i.e. aerial or gills over tegument) are beneficial under low oxygen conditions due to more stagnant waters, high organic matter and high temperature[3,10]
	Tegument	-		-	
	Aerial	+	Oxygen depletion due to high organic matter content favors aerial respiration over gills/tegument[10]. Lower chemical uptake[7]	+	
Food/Feeding type	Predation	-	Large predators are exposed to toxicants by food ingestion[1]	+	Higher intra-specific competition can lead to dominance of predators[3]
	Detritus<1mm	+/-	Benefit from deposition of detritus associated with organic pollution[1,12], but polluted sediments favors chemical uptake[12]	+	Benefit from deposition of fine detritus under low flows[13]
	Macrophytes			+	More abundant macrophyte (shredders) and periphyton biomass (scrapers)[3]
	Microphytes			+	

1: Dolédec and Statzner (2008); 2: Townsend and Hildrew (1994); 3: Bonada et al. (2007b); 4: Dolédec et al. (2006); 5: Lange et al. (2014); 6: Díaz et al. (2008); 7: Rico et al. (2015); 8: Berger et al. (2018) ; 9: Usseglio-Polatera et al. (2000); 10: Statzner and Bêche (2010); 11: Williams (2005); 12: Piló et al. (2016) ; 13: Feio and Dolédec (2012).

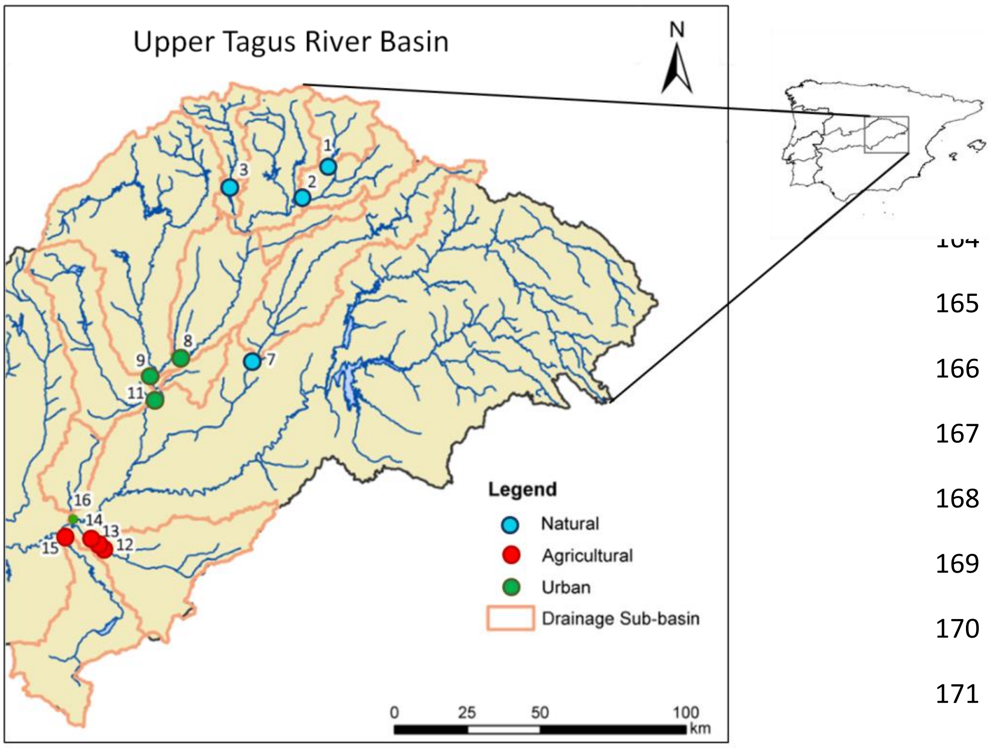


Figure 1. Map showing the study area (upper Tagus river basin) and selected sampling sites. Sites are marked with different colors according to their established category.

2.2. Sampling and analysis of abiotic parameters

Three sampling campaigns were carried out at each sampling site in 2016: spring (April 11-14), summer (July 11-14) and autumn (November 21-24). Each of these sampling campaigns aimed at covering three representative stages of the hydrological cycle of Mediterranean rivers, namely: (1) base flow conditions, which corresponds to the period with highest precipitation, (2) contraction phase during summer dry periods, and (3) expansion phase, after the first autumn rainfall events.

Water temperature, dissolved oxygen (DO), electrical conductivity (EC) and total suspended solids (TSS) were measured in the middle section of the river transect with a portable multiparameter probe (HANNA Instruments, USA, model HI98194). At each site and sampling date, flow values (m^3/s) were obtained from the daily series monitored by the Tagus River Basin Authority at the nearest flow gauge. Substrate composition was recorded as the percentage of stones and blocks, gravel and pebbles, sand, clay and fine inorganic material, macrophytes, algae, plant debris and mud (Table A2). A Principal Component Analysis (PCA) performed on the substrate proportions and a one-way ANOVA on the PCA scores of the first-two axes, indicated a minor influence of substrate for separating sites (see detailed description in Appendix A and Figure A2), so substrate composition was not considered further in the analyses. Water samples were taken for the analysis of nutrients (NO_2^-, NO_3^-, NH_4^+, PO_4^{-3} and total P), dissolved organic carbon (DOC), metals (Mn, Fe, Cu, Zn, Cd, Pb, Hg) and 52 organic contaminants: 20 pesticides and 32 point source contaminants (PSC). PSCs included 24 pharmaceuticals (9 of them antibiotics), 4 estrogens and steroids, and 3 alkaloids and other stimulants (see Rico et al. (2019) and Arenas-Sánchez et al. (2019b) for further details on the sampling and analysis of nutrients and water contaminants).

Toxicity of pollutant mixtures for invertebrates in the collected samples was evaluated using Toxic Units (TU). The TU approach relies on the summation of the individual toxic pressures exerted by each compound to a given standard test species assuming concentration addition (Sprague, 1971; Liess and Von Der Ohe, 2005). In this study, they were calculated as the sum of the ratio between the measured environmental concentration of each chemical and the corresponding EC50-48 for *Daphnia magna* (using immobility as endpoint). TUs were calculated separately for metals, pesticides and PSCs. EC50 values were derived from laboratory experiments, or from QSARs (Quantitative Structure-Activity Relationships) when experimental data were not available. Further details on the selection of toxicity values and the TU calculations are provided in Appendix A.

2.3. Macroinvertebrate data

2.3.1. Sampling and identification

Three macroinvertebrate samples were collected at each sampling site per sampling campaign. Samples were taken using a Surber net from the left, middle and right sections of the river, trying to cover the main available habitats. The outlining frame was placed on the river bottom with the net pointing downstream and all substrate within the frame was rubbed or stirred at a depth of 5 to 10 cm for 2 minutes to collect invertebrates. Organisms were transferred to a plastic container and preserved with 70% ethanol until further identification in the laboratory.

Taxonomic identification was performed based on Tachet et al. (2010). Due to damage or lost features of some of the preserved organisms, identification at genus level was not possible for all taxa. Consequently, to keep consistency, taxonomic classification was done at the family level. Chironomidae was one of the most abundant families. To adjust the weight of this family in the analysis, we considered identifying five subfamilies or tribes (Orthocladiinae, Tanypodinae, Diamesinae, Tanytarsini, Chironomini). Since identification for this group was not always possible at the same taxonomic level, a compensative adjustment for the coarser taxonomic resolution was performed according to the method described in Appendix A. Macroinvertebrate samples collected at each site were pooled together and abundances were ln(x+1) transformed to reduce the impact of dominant taxa and to approximate a normal distribution of the data.

2.3.2. Biological traits

Information on ten biological traits (see Table A6 for full set of categories analyzed) for the invertebrate taxa identified in this study were extracted from Tachet et al. (2010). In this database, the affinity of each taxon to the different trait categories is quantified using a "fuzzy" coding approach (Chevenet et al., 1994). This method gives an affinity score per taxon and trait category ranging from "0" (no affinity) to X (X, the strongest affinity; with X varying from 3 to 5 depending on the trait). This way of coding is described elsewhere (e.g. Bournaud et al., 1992; Chevenet et al., 1994), and allows for considering the varying nature of trait sources, and the within-family or within-genus trait variation. Affinity scores were standardized so that their sum for a given taxon and a given trait equaled one, yielding trait category profiles for each taxon (see e.g. Gayraud et al., 2003).

Matching of our invertebrate monitoring dataset to the Tachet et al. (2010) database was done at the family level, and trait category profiles were averaged across genera. Since some trait differences are expected between Mediterranean and non-Mediterranean taxa (Bonada and Dolédec, 2011), family trait averages were calculated only considering the Mediterranean genera identified by Bonada and Dolédec (2011). In this way, each genus was given a weight proportional to the number of Iberian species recorded in the Freshwater Ecology database (https://www.freshwaterecology.info) and the Global Biodiversity Information Facility database (GBIF; https://www.gbif.org). When a genus had no identified or recorded species, a minimum value of one was given. For generalist taxa such as Diptera and Oligochaeta, no Mediterranean genera could be identified in most cases, so average values for all genera included in Tachet et al. (2010) were used.

2.4. Data analyses

2.4.1. Abiotic parameters

Abiotic parameters were individually assessed for normality using the Shapiro-Wilk test. The type of transformation giving the best fit (S-W statistic close to 1, p-value>0.05) for each variable was selected for further analyses. PCAs were performed on hydrological, physical and chemical, and contaminant (i.e., TUs) parameters considering all seasons together to evaluate overall differences between site groups (natural, agricultural, urban), and comparing the three sampling seasons within groups of sites to assess temporal differences. Statistical differences between seasons or between groups of sites were assessed by a Monte-Carlo test with 999 permutations on the variance of environmental parameters explained by groups. Finally, a one-way ANOVA was applied to each variable to account for significant differences between groups of sites, and to test for seasonal differences within each group of sites.

2.5.2. Structural and functional indices

The effects of chemical pollution and hydrological variation on the structural and functional characteristics of the macroinvertebrate community were evaluated in terms of taxonomic and trait-based richness and diversity indices. In addition, we calculated the Iberian Biological Monitoring

Working Party (IBMWP) index (Alba-Tercedor et al., 2004), which is commonly used for assessing the biological status of surface waters in Spain according to the WFD. We evaluated taxonomic richness and diversity based on the total number of taxonomic entries (i.e., families) and the Simpson index, respectively. Functional richness was calculated as the amount of niche or functional space occupied by the trait categories of all taxa in the community (Villéger et al., 2008). Functional diversity was assessed using the Rao quadratic entropy (RaoQ, Champely and Chessel, 2002), which sums the trait distances of any pair of taxa weighted by their relative abundance. For these indices, significant differences between the three groups of sites were tested by one-way ANOVAs followed by a pairwise t-test performed at each season.

2.5.3. Relationship between taxa distribution and their traits

Co-inertia analysis (Dolédec and Chessel, 1994; Dray et al., 2003) was performed to assess the correlation between taxa distribution and trait data from each season, and to evaluate the contribution of seasons to the differences between sites. A significant correlation would be the sign that trait selection operates according to site and season. Before co-inertia analysis, taxonomic and trait profiles of taxa were analyzed using Fuzzy Correspondence Analysis (Chevenet et al., 1994). In these analyses, taxa were given the same weight (1/*n* being *n* the number of taxa), so that the influence of highly abundant taxa was reduced (see Chevenet et al., 1994). Rare taxa with less than two organisms in only one sampling site per season were not included in the analysis. We used the *RV*-coefficient (Robert and Escoufier, 1976) to measure the correlation between trait data and taxa distribution and we assessed the significance of the *RV*-coefficient using 999 random Monte-Carlo permutations of the taxa distribution table. The amount of random values higher than our observed *RV*-coefficient gave us a simulated p-value.

To test for differences between groups of sites with different pollution level and hydrological variation, one-way ANOVAs followed by t-tests were performed on the site scores of the first-two co-inertia axes. Besides, taxa with scores between the highest absolute values and 75% of the lowest (absolute) value, along the two axes, were selected as those having the largest contribution to the separation of sites. Finally, to assess trait-specific responses to pollution, hydrological variation, and especially to pollution under low flow conditions, only trait categories whose contribution summed 90% of the variance along the first two co-inertia axes (when significant) were considered in each season. Their distribution along the co-inertia axes was assessed, determining whether they were more prevalent in polluted or natural sites. Only trait categories showing an increase in the relative contribution to the variance of the first two co-inertia axes in summer and/or autumn (as potentially delayed low flow period) were considered for further statistical analysis. We computed trait category profiles by multiplying the relative abundance of taxa per site and frequency of trait categories of taxa. One-way ANOVA followed by t-test was applied to each trait category profile in natural, agricultural and urban sites in each season. These final tests allowed evaluating the seasonal variation on the differential occurrence of those categories in each group of sites, as signs of response to hydrological variation (i.e. frequency increase in natural sites during low flow periods) or enhanced pollution effect in agricultural and/or urban sites.

All statistical analyses were performed in the R environment (R Development Core Team, 2019), using the ade4 (Dray and Dufour, 2007), vegan (Oksanen et al., 2016) and FD (Laliberté et al., 2014) packages. Due to the large number of tests performed, corrections for multiple testing were applied (i.e., false discovery rate).

3. Results

3.1. Spatial and temporal variation of abiotic parameters

The first two axes of the PCA performed on the abiotic parameters accounted for 43% and 21% of the total variance, respectively. Along the first PCA axis, $TU_{Pesticides}$, total N, N-NO_2, P-PO_4 and TU_{Metals}, were the most contributive parameters separating urban from natural sites (Figure 2, Table 2). Other parameters with less influence on that axis were DOC and N-NH_3. Along the second PCA axis, higher N-NO_3, TSS, conductivity, pH values and lower flow isolated agricultural sites (Figure 2, Table 2). A

Monte-Carlo permutation test demonstrated that the three groups of sites were significantly different (simulated p-value = 0.001, R^2 = 0.478).

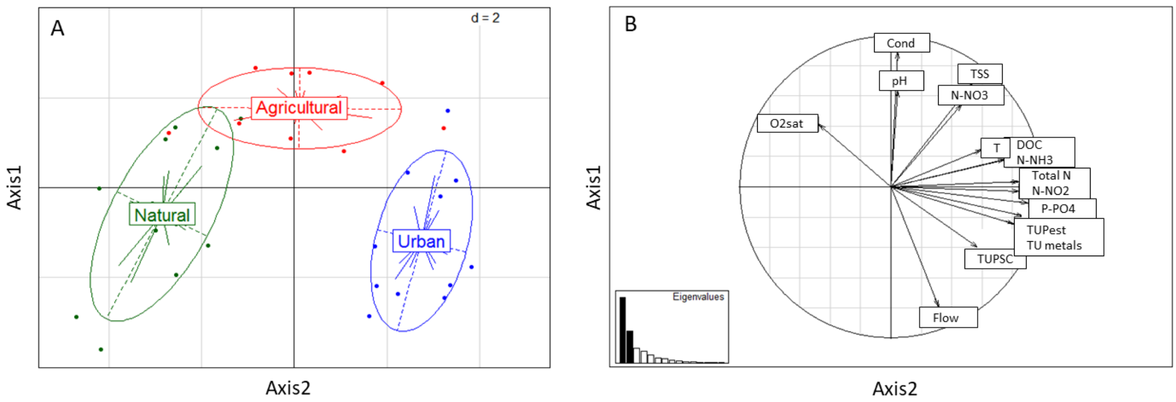


Figure 2. Results of a PCA performed on the abiotic parameters measured in the 12 sampling sites in spring, summer and autumn. A) Distribution of the sampling sites (dots) and groups of sites (ellipses) along the first-two axes (noted Axis1 and Axis2). B) Correlation circle showing the abiotic parameters loadings along the first two PCA axes. Insert gives the diagram of eigenvalues (maximum: 6.5).

In addition, the PCA and Monte-Carlo tests performed on abiotic parameters within groups of sites showed that the sampling season significantly influenced the parameter values in natural (simulated p-value: 0.026, R^2: 0.32) and agricultural (simulated p-value: 0.002, R^2: 0.40) sites, but only marginally in urban sites (simulated p-value: 0.075, R^2: 0.27). Marked differences occurred between spring and summer samples, particularly in agricultural sites (Figure A3). Water flow was noticeably lower in all groups of sites in summer, but this seasonal difference was not significant due to the variability among sites (Table A3). N-NO_2 and N-NH_3 concentrations increased in summer in all groups of sites, with higher values in polluted sites, especially in the urban ones (Table 2, Table A3). Overall, oxygen saturation was lower in summer and autumn, with values below 70-80% in some samples taken in agricultural sites; whereas in urban sites this parameter was regularly maintained below 70% in all samples. Suspended solids (TSS) showed similar values for agricultural and urban sites in spring; whereas they increased in all sites during summer, becoming slightly higher in agricultural sites (Table A3). Water temperature was expectedly up to 7˚C higher in summer in all groups of sites but was not especially higher in sites with lower flows (Table A3). TU_{PSC} did not show a clear seasonal variation, while $TU_{Pesticides}$ and TU_{Metals} showed significant seasonal variation in agricultural sites, with higher values in spring and summer, respectively (Table 2, Table A3).

Table 2. Mean (± standard deviation) parameter values for each group of sites, and ANOVA p-values (P) indicating statistical differences between seasons. -: not significant.

	Natural		**Agricultural**		**Urban**	
	Mean±SD	**P**	**Mean±SD**	**P**	**Mean±SD**	**P**
Flow ($m^3 s^{-1}$)	1.24±0.83	-	0.15±0.13	-	8.76±7.18	-
Temperature (˚C)	12.0±4.3	0.001	13.9±5.1	<0.001	16.7±5.91	<0.001
pH	7.98 ±0.84	-	8.10±0.53	0.053	7.70±0.68	-
Conductivity (µS cm^{-1})	1871±1718	-	5040±287	-	1383±869	-
TSS (mg L^{-1})	24.2±31.3	-	113±114	<0.001	57.2±63.1	-
O2sat (%)	84.3±10.2	0.043	81.7±20.6	-	66.9±13.5	-
DOC (mg L^{-1})	2.94±1.42	-	6.03±1.33	-	6.76±1.35	-
N-NH_3 (mg L^{-1})	0.01±0.02	0.043	0.08±0.18	-	0.19±0.26	0.069
N-NO_2 (mg L^{-1})	0.006±0.006	0.001	0.05±0.07	0.067	0.42±0.46	0.069
N-NO_3 (mg L^{-1})	1.65±1.3	-	5.53±2.87	-	3.61±2.16	-
Total N (mg L^{-1})	1.7±1.32	-	6.24±2.80	-	10.2±3.65	-
P-PO_4 (mg L^{-1})	0.005±0.004	-	0.06±0.07	-	0.34±0.28	-
TU_{Metals}	0.08±0.05	-	0.15±0.08	0.019	0.51±0.30	-
$TU_{Pesticides}$	1E-05±2E-05	-	1E-04±1E-04	0.009	2E-03±1E-03	-
TU_{PSC}	6E-05±7E-05	-	3E-04±4E-04	-	4E-04±3E-04	-
TU_{Total}	7E-05±7E-05	-	4E-04±4E-04	-	2E-03±1E-03	-

3.2. Impact of chemical pollution and hydrological variation on invertebrate communities

3.2.1. Structural and functional indices

Taxonomic and functional richness, functional diversity and the IBMWP (Iberian Biological Monitoring Working Party) index showed significant (ANOVA p-value<0.05) or marginally significant (ANOVA 0.05≤p-value<0.10) higher values in natural than in agricultural and urban sites (Figure 3; Table A4). These differences were larger in summer and autumn for most indices, with generally higher values in summer for natural sites as compared to spring. Moreover, in summer, agricultural sites showed lower values in taxonomic and functional richness compared to the urban sites. Such trend was also observed, to a lower extent, in autumn (Figure 3, Table A4). Functional diversity was consistently low in agricultural sites but increased in urban sites in summer and autumn (Figure 3). The IBMWP index showed significant differences related to the three sample groups but did not show significant seasonal differences within each sample group. Taxonomic diversity did not show clear differences among groups (Table A4; Figure A4).

3.2.2. Taxonomic and trait response to pollution and hydrological variation

3.2.2.1 Relationship between taxa distribution and their traits

The co-inertia analysis performed at each season showed that the taxa distribution and their traits were significantly correlated, indicating a non-random distribution of traits according to sites on each season (Table A5; Figure 4). The percentage of variance explained by the first co-inertia axis ranged from 26% to 30%, and from 18% to 24% for the second co-inertia axis, so that about 50% of the total variability was considered by the first two axes in each season (Table A5).

Along the first co-inertia axis, polluted sites (agricultural and urban) were separated from less natural sites at each season, with a more significant difference between sites in summer compared to the other seasons (Table 3). Furthermore, agricultural sites were significantly distinguished from urban sites on the first co-inertia axis in summer, with the latter sites showing the largest differences with natural sites (Figure 4, Table 3). In autumn, polluted sites (agricultural and urban) were significantly separated along the second co-inertia axis (Table 3).

Table 3. Results from the one-way ANOVA and pairwise t-test (p-values) performed on the site scores corresponding to the first and second co-inertia axis (A: natural; B: agricultural; C: urban). n.s.: not significant; NA: not assessed due to the non-significant differences in the ANOVA.

	Co-inertia Axis1				**Co-inertia Axis2**			
	ANOVA	**Pairwise t-test**			**ANOVA**	**Pairwise t-test**		
		A-B	**A-C**	**B-C**		**A-B**	**A-C**	**B-C**
Spring	0.040	n.s.[1]	0.015	n.s.	n.s.	NA	NA	NA
Summer	0.001	0.020	<0.001	0.010	n.s.	NA	NA	NA
Autumn	0.006	0.004	0.005	n.s.	0.030	n.s.	n.s.	0.010

[1]0.1>p-value>0.05

Overall, Hirudinea (Glossiphoniidae, Erpobdellidae), Gastropoda (Physidae), Oligochaeta (Lumbriculidae, Enchytraeidae, Tubificidae), Diptera (Psychodidae, Tipulidae, Tanitarsini, Chironomini) showed higher relative abundance in sites affected by pollution (agricultural and urban); while Plecoptera (Leuctridae, Capniidae), Ephemeroptera (Potamanthidae, or Heptageniidae), Trichoptera (Rhyacophilidae) and Bivalvia (Sphaeriidae) were more abundant in more natural sites (Figure 4B). The number of taxa with a large contribution to the differences between groups of sites along each co-inertia axis was higher in summer and autumn (i.e. taxa with scores above the threshold; Figure 4B). In these two seasons, Odonata (Aeshnidae) and Coleoptera (Elmidae) or Diptera (Tipulidae or Athericidae, especially in autumn) were more prominent in natural sites. Caenidae (Ephemeroptera) were strongly associated with agricultural sites (Figure 4B). This taxon, together with other taxa such as Lymnaeidae (Gastropoda) or Psychodidae (Diptera), contributed to the significant differences between polluted and natural sites in summer and autumn, as well as increased abundances of pollution tolerant taxa in urban sites such as Glossiphoniidae or Erpobdellidae (Figure 4B; Table B1).

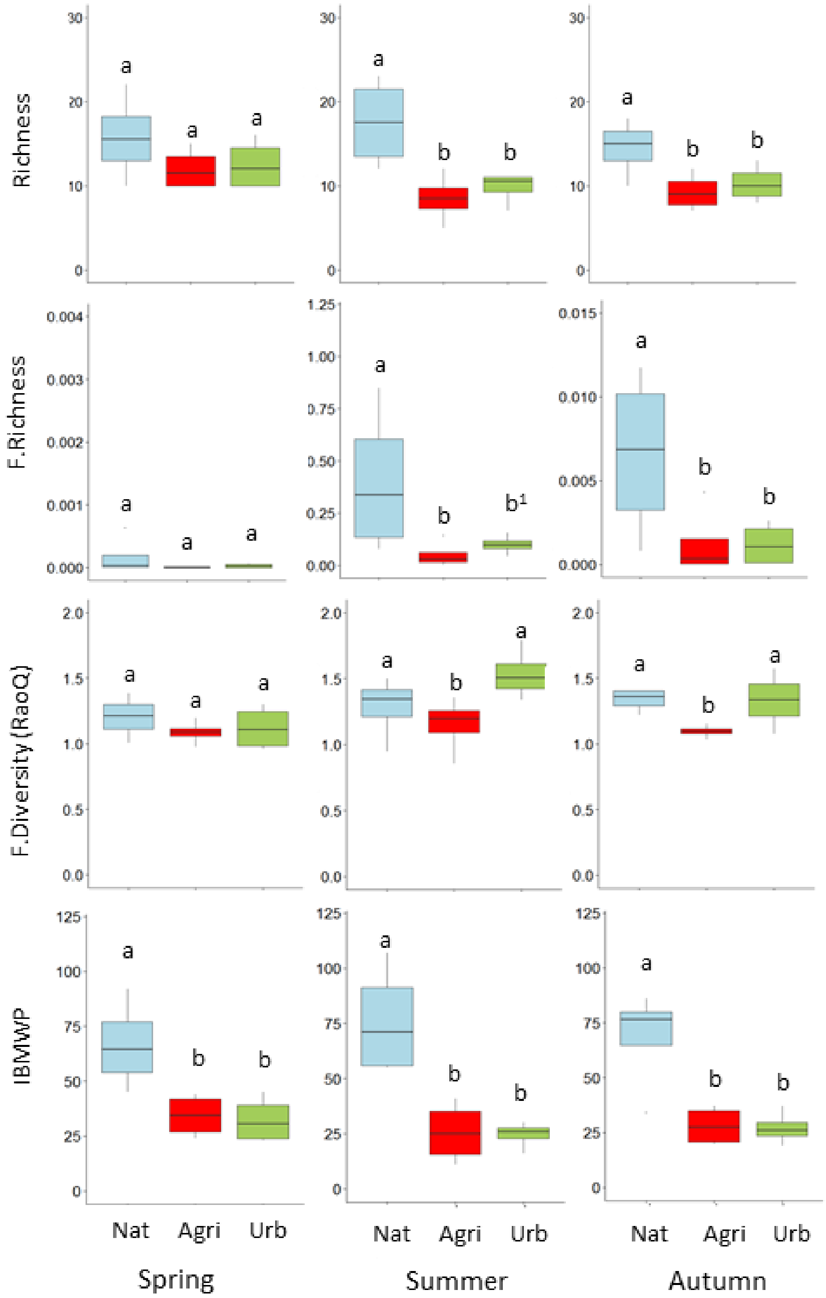


Figure 3. Taxonomic and functional richness, functional diversity (RaoQ) and Iberian Biological Monitoring Working Party (IBMWP) values in the natural (Nat), agricultural (Agri) and urban (Urb) sites in spring, summer and autumn. Lowercase letters indicate significant differences (pairwise t-test, p-value<0.05) between groups of sites within each season. [1]0.1>p-value>0.05.

3.2.2.2. Trait distribution in the sampled communities

The main traits responding to pollution included, by decreasing order of importance, reproduction type, resistance forms, number of cycles per year and respiration, in all sampling seasons (Figure 4C; Table A6). Altogether, these traits explained up to 60% of the total explained variance along the first co-inertia axis in spring (Table A6). In summer and autumn, the role of these traits contributing to differences between groups of sites on the first-two co-inertia axes was slightly reduced, and the contribution of other traits such as dispersal strategies, feeding habits and substrate relation increased (Table A6).

Several trait categories showed significant differences between groups of sites (ANOVA p-value<0.05) in summer and/or autumn (Figure 5, Table A7), and notable changes in profiles between seasons (see Figure A5 for significant categories with no notable temporal changes). Large size, asexual reproduction, aquatic passive dispersion and the use of cocoons as resistance form showed no significant differences between groups in spring, but had a significantly higher frequency in urban sites than in natural sites in summer. In agricultural sites, the frequency of those traits was lower than in urban sites, but tended to be higher than in natural sites, while in autumn differences between most of these trait frequencies tended to decrease again (Figure 5). These trait categories can be classified as having a response to the co-occurrence of pollution and hydrological variation, with a most prominent response in urban sites.

The use of gills or tegument as respiration mechanism, and the feeding type (deposit and macrophyte feeders) showed significant differences between groups in all sampling seasons, therefore showing a clearer response to pollution (Figure 5). The relative frequency of tegument-based respiration and the relative frequency of deposit feeders increased in polluted sites, with higher values in urban sites as compared to the agricultural ones, especially in summer. On the other hand, the proportion of gill breathers and macrophyte feeders was lower in polluted sites as compared to the natural ones. Within the polluted sites, the urban sites presented lower values than the agricultural ones, particularly during summer (Figure 5).

4. Discussion

4.1. Abiotic parameters

Observed seasonal trends of abiotic parameters can be interpreted as an indication of potential stressors and increased risk to aquatic communities during low flow periods. We found higher N-NO_2 and N-NH_3 concentrations in summer, especially in urban sites, which may be a result of lower river dilution capacity and of the influence of higher temperatures on the level of un-dissociated ammonia (N-NH_3). This also supports the fact that flowing waters close to urban areas were most likely sustained by wastewater effluents (treated or untreated) during low flow periods (Rice and Westerhoff, 2017). Lower oxygen levels in agricultural sites in summer and autumn may also indicate an increase in organic matter concentration related to the ecosystem contraction and accelerated respiration rates associated with temperature increase, as well as lowered oxygen solubility (Carere et al., 2011). A more evident effect of ecosystems contraction and turbulence processes associated with rapid temperature inversions under low flows was observed for suspended solids (TSS) in agricultural sites in summer, as also described by Williams (2005). Increased water temperature in all groups of sites in summer means it is not possible to consider this variable as an individual stressor, but potential combined effects with other stressors such as contaminants are likely (Holmstrup et al., 2010; Arenas-Sánchez et al., 2019a). TU_{PSC} did not show a clear seasonal variation, suggesting that there is a relatively constant emission of PSCs in the evaluated rivers (Arenas-Sánchez et al. 2019b; Rico et al. 2019). The seasonal variation of $TU_{Pesticides}$ in agricultural sites was most likely related to seasonal pesticide application patterns, with the highest concentrations and toxic pressure found related to herbicides in spring (see Rico et al., 2019; Arenas-Sánchez et al., 2019b). Yet, it should be noted that herbicide toxicity values were well below acute and chronic toxicity thresholds for invertebrates (i.e. 0.001 and 0.1, respectively). TU_{Metals} also showed a clear seasonality. In this case, the increase in toxic pressure in summer can be related to the low water flow during the ecosystems' contraction period. Therefore, our study shows that a series of environmental conditions that are most likely associated to flow reduction, together with higher toxic metal pressure, are more recurrent in agricultural sites compared to natural or urban sites. Although having higher and permanently flowing waters, urban sites also presented a potential increased risk in summer due to lowered dilution potential.

4.2. Structural and functional indices

The stronger detrimental effects of pollution during low flow conditions were indicated by several taxonomic and functional indices. The most pronounced differences between natural, and agricultural and urban groups of sites, were observed in summer and in autumn, with the lowest values found in agricultural sites. It should be noted that during the study year, the annual minimum flow was generally reached in late summer-early autumn, slightly before our autumn sampling campaign (see Figure A1). This may explain the large community impacts observed within this season, and is in line with the study by Karaouzas et al (2018), who showed that invertebrate communities show maximum responses to water stress (based on variables such as discharge or mean duration of low spills) within 45 days after the water contraction phase.

Functional diversity tended to increase in urban sites in summer and autumn as compared to natural and agricultural sites. This suggests that the diversity of trait categories has more uniform patterns under stable polluted environments, as previously found by Parreira-de Castro et al. (2017) and Mor et al. (2019). However, Clavel et al. (2011) also describe that generalist taxa present in these polluted environments tend to be less complementary than specialist taxa in niche-rich environments, which leads to lower ecosystem productivity and resilience (Fynke and Snyder, 2008). Functional richness showed very low values in all groups of sites in spring as compared to summer and autumn, which can be interpreted as a sign of functional redundancy during spring and the need for ecological specialization during low flow periods. On the other hand, increased values for several indexes in natural sites during summer may be related with the fact that initial phases of ecosystem contraction or flow reduction can lead to higher habitat heterogeneity, density and diversity values (Acuña et al. 2005; Datry et al., 2016; Arenas-Sánchez et al. 2016). Taxonomic diversity did not show clear differences among site groups. This can be related to the fact that the monitored communities were dominated by few taxa with high abundances in each group of sites (Table B1). Finally, our study showed that the IBMWP index allowed us to distinguish differences between site groups but was not sensitive to structural community changes related to seasonal pollution variability between sites and/or hydrological periods.

4.3. Invertebrate communities and their traits in response to pollution and hydrological variation

We found a significant correlation between the abundance of taxa and their traits, and significant differences between groups of sites and seasons. This indicates that a selection of trait categories operates according to varying chemical pollution and flow conditions. The differences between groups of sites increased in summer and autumn (as well as the number of taxa with a large contribution to it; Figure 4B), which is an additional indication of the influence of flow conditions and chemical pollution on the monitored macroinvertebrate communities.

The majority of taxonomic responses observed in our analysis correspond to tolerant or sensitive taxa to stress identified elsewhere. For example, the increase in Odonata, Coleoptera and Diptera abundances observed in natural sites under hydrological stress conditions (i.e., summer and autumn) has already been shown by several authors (Williams, 2005; Bonada et al., 2007a; Skoulikidis et al., 2011). On the other hand, Caenidae and several Diptera taxa have shown a relatively high tolerance to pollution and hydrological stress in other studies performed in the Mediterranean region (Sabater et al., 2016; Kalogianni et al., 2017), which is in line with the observed taxonomic descriptions of our agricultural sites in summer and autumn.

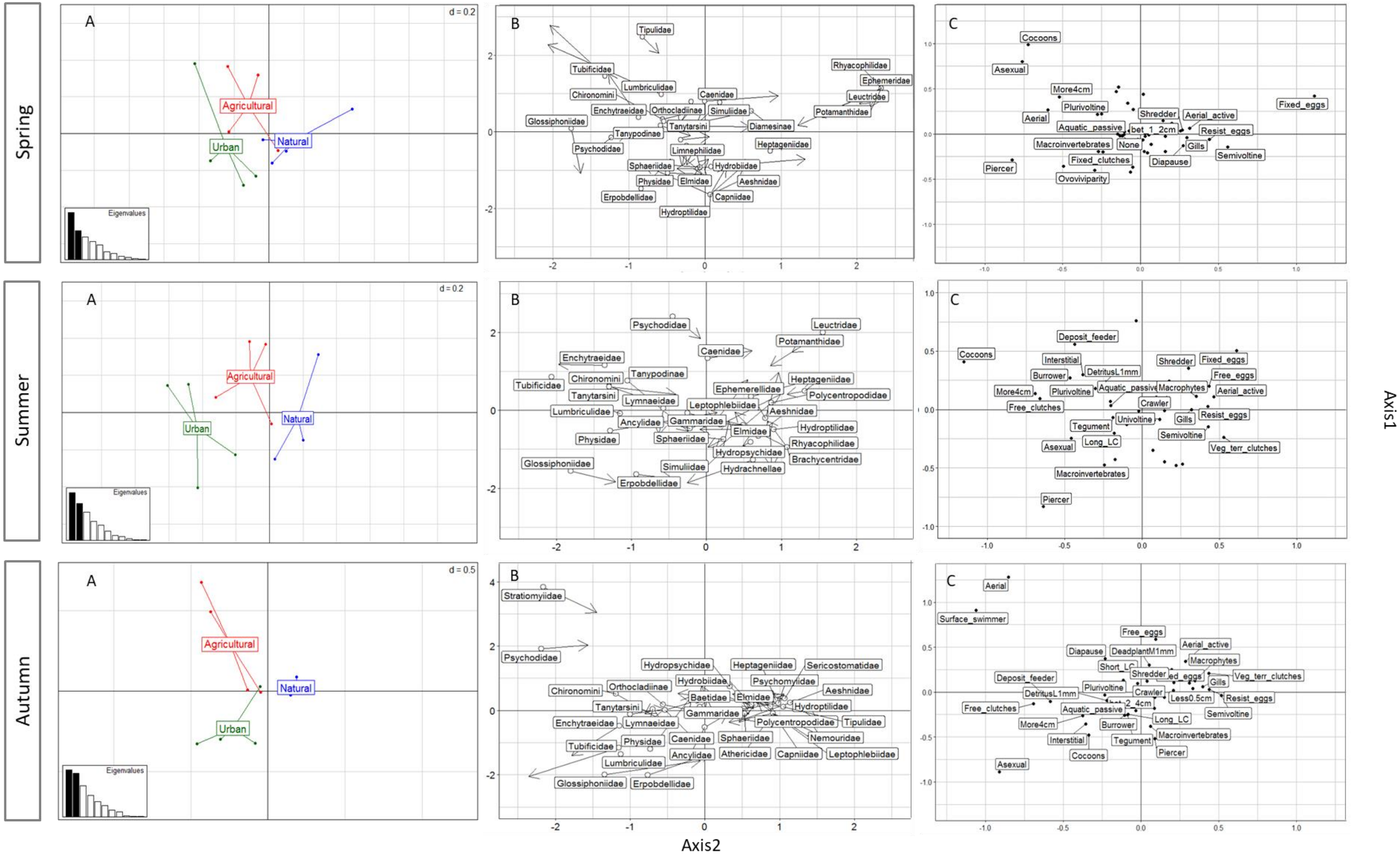


Figure 4. Scores of sites (A), taxa (B) and trait categories (C) along the first and second axes of the co-inertia analysis performed separately on each season. Insert gives the diagram of eigenvalues. In (A) sites are grouped by level of pollution and hydrological variation (see acronyms in the text). In (B) arrows represent the difference between the position of each taxon from its abundance in sites and from its trait profile. Longer arrows mean less coherence for a given family.

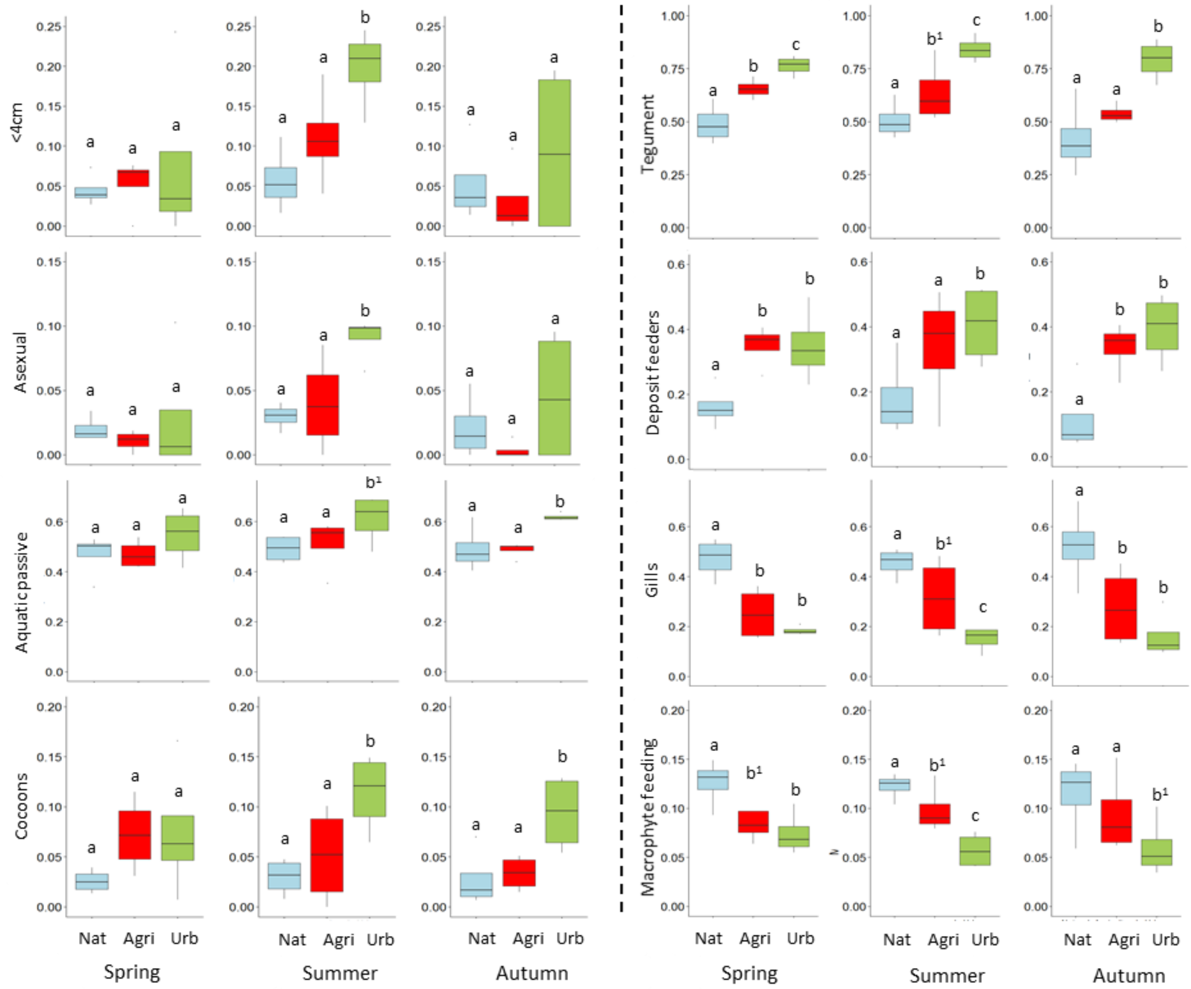


Figure 5. Trait categories showing significant differences (ANOVA p-value<0.05) in at least one season between natural, agricultural and urban sites, with notable change in trait frequencies between spring, summer and autumn. Lowercase letters indicate significant differences (pairwise t-test, p-value<0.05) between groups of sites within each season. [1]0.1>p-value>0.05.

The analysis of trait responses showed that during the low-flow period, differences in trait category profiles tended to be more pronounced between groups of sites, indicating a change in the ecology and functional characteristics of the macroinvertebrate communities. It is important to note that the differences in the relative frequency of those trait categories between agricultural and natural sites were lower than those between urban and natural sites, oppositely to what was shown by most

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biological indexes. This could be related to the fact that urban sites present a more pollution tolerant and stable macroinvertebrate functional community composition over time.

In our study, the frequency of trait categories such as tegument respiration and deposit feeders increased in polluted rivers, principally in urban sites. On the other hand, gill breathers and macrophyte feeders were less prominent in such environments. In principle, respiration via gills or tegument have been associated with a higher chemical uptake and sensitivity to toxicants (Rico et al., 2015), and with a high susceptibility to organic matter loads and limited dissolved oxygen concentrations (Statzner and Bêche, 2010; Table 1). Nevertheless, other authors have found a high abundance of tegument respiration organisms downstream of wastewater treatment plants (Charvet et al., 1998), and argued that cuticular respiration may be sufficient to supply the oxygen needs of relatively inactive organisms (Williams and Feltmate, 1992). In our study, the most prominent tegument respiration organisms in the monitored polluted sites included gastropods (Ancylidae, Physidae) and annelids (Erpobdellidae; Figure 4B), which are usually within the most tolerant taxa to pesticides and other organic compounds with unspecific modes of action (Rico et al., 2015; Van den Berg et al., 2019). The higher prevalence of deposited feeders in the polluted sites in comparison to the natural ones is in line with our water quality measurements, which indicated higher DOC levels (Table A3) in polluted sites, particularly in the urban ones. Similarly, the low abundance of macrophyte feeders in those environments was expected as the high turbidity and substrate characteristics impeded the growth of sediment-rooted macrophytes (Figure A2).

The effects of pollution (agricultural and urban) during low flow periods (principally during the summer season) showed a more prominent increase in the proportion of individuals with large size, asexual reproduction, aquatic passive dispersal habits and using cocoons as resistance form. These results are in line with our expectations (Table 1). On the other hand, opposite to our expectations, the frequency of long life cycles had a positive response to chemical pollution in summer and autumn (Figure 4C, Table A7) (Table 1). This trait category was not assessed as a potential indicator of hydrological stress or pollution since it is most likely correlated with other trait categories with high affinity for highly polluted sites in summer and autumn (Figure 4C, Table A7). For example, the proportion of individuals feeding on living macroinvertebrates (i.e. predators) was high in urban sites (Figure 4C; Table A7), and these organisms normally present a larger size, which may reduce their level of internal chemical exposure (Table 1), and which is often correlated with longer life cycles (for a description of trait correlations and syndromes, see Poff et al., 2006; Statzner and Bêche, 2010). Yet, the increased affinity of large size organisms for polluted sites in summer is in line with our expectations and does have a mechanistic explanation (Table 1), which makes this category a good indicator of pollution and hydrological stress in our study.

4.4 Study limitations

One of the main limitations of this study was the low number of sampling sites included, which was partly a consequence of time and economic constraints. Despite this, we believe the site selection followed a thorough land use and hydrological variation study, which renders sufficient credibility on the obtained outcomes. Another potential limitation was the lack of unpolluted sampling sites that are not subjected to large annual hydrological variation. An effort was made during the site selection process to include them, but we were unsuccessful in identifying them. This is mainly because rivers with relatively constant flow (and limited pollution impact) are often affected by other sources of anthropogenic impact such as damming for hydroelectric power generation. Further evaluations of biological effects produced by flow variation in combination with pollution should therefore include such pollution controls and potentially be done under semi-controlled conditions (e.g. making use of experimental streams or mesocosms). In this way the separate and combined effects of these two stressors could be better evaluated.

The mild influence of hydrological variation on macroinvertebrate communities observed in this study may be related with the fact that 2016 was a humid year, with an average precipitation of 682 mm that was 5% over the reference value for the period 1981-2010, as described by the Meteorological State Authority (MAPAMA, 2016). Furthermore, there are a number of factors that were not measured and that could have also contributed to the observed variability, such as the abundance and type of riparian vegetation and sediment inputs. Follow-up studies including a larger number of sites and longer time periods, and more frequent sampling, are recommended to increase spatial and temporal representativeness in the evaluated responses.

Our study was based on taxonomic identifications done to the lowest practical and possible resolution given the number of samples, and the matching between taxonomy and traits was done at the family level. Some authors have argued that identification at a family level could be sufficient when assessing the responses on functional descriptors along a stress gradient (Gayraud et al., 2003; Sajan et al., 2010). However, it is evident that we have possibly lost some sensitivity in biological responses; the more since it is known that pollution and other stressor's sensitivity may differ markedly between species belonging to the same genera, as Berger et al. (2018) demonstrated for Dipterans in their study of taxonomic and traits responses to stressors.

Finally, it is important to note that the implementation of the TU approach as a mixture toxicity measure to predict chemical impacts on macroinvertebrate communities based on grab sampling is just an approximation to reality. First, because the relative sensitivity of macroinvertebrates to that of *D. magna* varies significantly across taxa and chemical classes (Rico et al., 2015). And, second, because the load of certain substances that are known to significantly affect invertebrates over the short term (i.e., metals, insecticides) and the temporal variation of their aquatic exposure was not evaluated in detail. Biological responses may be a consequence of this unmeasured variability, and therefore follow-up studies should consider the implementation of mixture toxicity approaches that integrate chemical sensitivity differences among species and integrative chemical sampling methods over suitable time windows.

5. Conclusions

This study shows that the impact of anthropogenic pollution on the taxonomic and functional composition of aquatic macroinvertebrate communities is larger during low flow periods (i.e., typically in summer, but also in early autumn). This was potentially due to lowered dilution capacity and physically altered habitats, but further studies should be performed to confirm the individual or combined factors related with pollution and low flows affecting this response. In general, pollution resulted in more tolerant and less diverse communities, with polluted sites suffering from severe hydrological variation (agricultural sites) showing a trend towards lower richness and functional richness, and significantly lower functional diversity. In addition, our study shows that the IBMWP index can identify differences between polluted and non-polluted sites, but lacks sensitivity to assess macroinvertebrate responses to seasonal hydrological and chemical pollution alterations. Therefore, this study supports the evaluation of other taxonomic indexes and the integration of trait-based indices and approaches for the ecological status assessment of Mediterranean rivers.

The functional analysis across the different sampling seasons allowed identification of trait categories related to pollution, and to the co-occurrence of pollution and hydrological stress. The main trait categories showing an overall increase in polluted sites (principally urban) were tegument respiration and deposit feeders; while the ones showing a relative decrease were respiration by gills and feeding on macrophytes. The trait categories that showed a relative increase in polluted sites during low flow periods (agricultural) were large size, asexual reproduction, aquatic passive dispersal and the capacity to produce cocoons as resistance forms.

The development of future monitoring campaigns and biological quality indices should consider the combined effects of multiple stressors and focus on at least one period of maximal interaction among them. The outcomes of our study indicate that late summer or early autumn seems to be the period with the largest functional biodiversity impacts by pollution and low flow conditions in Mediterranean rivers. Therefore, further studies and monitoring campaigns assessing the effects of chemical pollution within these periods are recommended.

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