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Ecotoxicological risk assessment of chemical pollution in four Iberian river basins and its relationship with the aquatic macroinvertebrate community status

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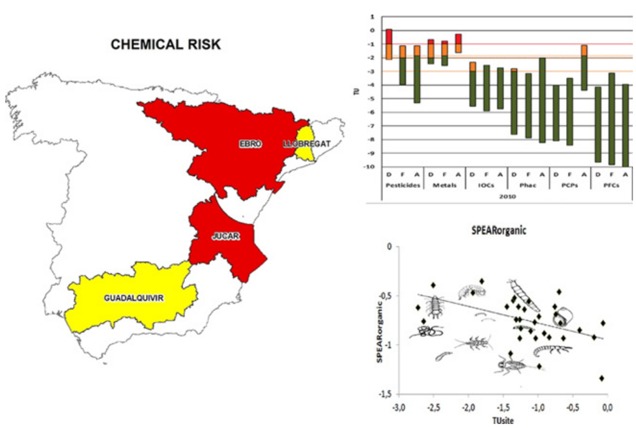
HIGHLIGHTS

- Ecological risk assessment of chemical pollution was performed in four Iberian rivers.
- Relationships between chemical risk and biological responses in situ were examined.
- Acute risk effects at more than 40% and chronic at all sites were present.
- Both regulated and emerging compounds are responsible for chronic risk.
- Decrease of sensitive species was correlated with the increase of the risk.

GRAPHICAL ABSTRACT



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ABSTRACT

Ecotoxicological risk assessment of chemical pollution in four Iberian river basins (Llobregat, Ebro, Júcar and Guadalquivir) was performed. The data set included more than 200 emerging and priority compounds measured at 77 sampling sites along four river basins studied. The toxic units (TU) approach was used to assess the risk of individual compounds and the concentration addition model (CA) to assess the site specific risk. Link between chemical pollution and aquatic macroinvertebrate communities in situ was examined by using four biological indexes; SPEAR ("Species at Risk Index") as the indicator of decline of sensitive species in relation to general organic ($SPEAR_{organic}$) and pesticides ($SPEAR_{pesticides}$) pollution; and Shannon and Margalef biodiversity indexes. The results of the study suggested that organic chemicals posed the risk of acute effects at 42% of the sampling sites and the risk of chronic effects at all the sites. Metals posed the acute risk at 44% of the sites. The main drivers of the risk were mainly pesticides and metals. However, several emerging contaminants (e.g. the antidepressant drug sertraline and the disinfectant triclosan) were contributing to the chronic effects risk. When risk associated

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with metals and organic chemicals was compared, the latter dominated in 2010, mainly due to the presence of highly toxic pesticides, while metals did in 2011. Compounds that are not regulated on the European level were posing the risk of chronic effects at 23% of the sites. The decline of sensitive macroinvertebrate taxa expressed in terms of SPEAR index was correlated with the increase of toxic stress related to organic compounds Biodiversity indexes were negatively correlated with the metals and the urban land use type in the catchment.

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1. Introduction

Aquatic ecosystems are impacted by a variety of stressors, including organic and inorganic stressors, excess input of nutrients, geomorphological alterations, land use changes, hydrological stress, invasive species and pathogens (Vörösmarty et al., 2010). As a consequence, the biodiversity decline is one of the greatest ecological problems threatening aquatic ecosystems (Beketov et al., 2013). However, little is known beyond the described effects of single stressors on specific ecological endpoints (Navarro-Ortega et al., 2015) and our understanding of the main causes for the losses of biodiversity still remains vague (Beketov et al., 2013). Rivers are receiving numerous chemical compounds originated from anthropogenic activities on a daily basis. As a result, complex mixtures of potentially dangerous compounds are present in the aquatic environment. However, site-specific exposures can vary a lot and some sites are likely to be affected more than others due to local conditions and specific vulnerability characteristics (Brack et al., 2015). Thus, the characterization of the constituents of these mixtures and the identification of the compounds of the highest concern in different spatial frameworks is one of the key issues for the protection of natural ecosystems (Vörösmarty et al., 2010).

Besides a number of regulated pollutants which are known to exhibit adverse effects, there is a large number of chemicals currently in use that are not taken into account in the routine water quality monitoring (Barceló and Petrovic, 2007). These compounds are commonly referred to as emerging contaminants. They encompass a variety of substances used both in industry and households; such as pharmaceuticals, personal care products, hormones, industrial chemicals or their byproducts and the transformation products, all together having in common that their environmental allowed levels are not regulated. In the European Union, the Water Framework Directive (WFD) Directive 2013/39/EU (2013) is the legislation concerning the chemical pollution which aims to achieve good chemical status of water bodies by meeting the Environmental Quality Standards (EQS) for the 45 so-called priority substances (PS) and priority hazardous substances (PHS). In addition, under the WFD, the EU member states are obliged to set quality standards for river basin specific pollutants discharged in each water body and to take action to meet these quality standards as a part of ecological status. A question that remains open is to what extent priority pollutants represent chemical status in comparison with unregulated chemicals. Here we address this issue from the perspective of their associated ecotoxicological risk.

Another challenge for the scientist dealing with aquatic risk assessment is revealing the link between water pollution and biological community responses. Due to the presence of multiple stressors, their unknown joint effects and the complexity of the biological responses, it is very difficult to distinguish the influence of particular stressors on affected ecosystems. Moreover, in recent years, studies in ecology are increasingly emphasizing that biodiversity loss implies more than the mere loss of species (i. e. taxonomic diversity) (Feld et al., 2014). Hence, the functional component of biodiversity should rather be addressed by using the concept of biological traits (e.g. generation time, body size) (Beketov and Liess, 2008; Feld et al., 2014). Commonly used taxonomic richness and diversity metrics (e.g. Shannon or Margalef diversity indexes) are dependent on both anthropogenic influences and natural longitudinal gradient of environmental factors in rivers as altitude, temperature, stream width, nutrition status and velocity (Minshall et al., 1985; Beketov and Liess, 2008; Paller et al., 2006) so they might not be able to characterize the toxicant specific influence of ecosystems. To cope with this problem stressor specific, traits based metric SPEAR index was developed for pesticides (Liess and Von Der Ohe, 2005), general organic toxicants (e.g. petrochemicals, synthetic surfactants) (Beketov and Liess, 2008) and salinity (Schäfer et al., 2011a) which is poorly dependent on the natural longitudinal factors (Beketov and Liess, 2008).

In this context our study is addressing the following objectives. First, to assess the area specific levels of the risk posed to aquatic ecosystems on the river basin level for more than 200 emerging and priority pollutants in four Iberian river basins using the toxic unit concept. Second, to evaluate whether the current list of WFD priority pollutants is enough to estimate the ecotoxicological risk in these basins or there are other compounds present that could be more or equally important in terms of risk. And third, to determine the potential relationship between the ecotoxicity associated with local mixtures of pollutants and aquatic macroinvertebrate biological community responses using four different metrics: Shannon and Margalef biodiversity indexes and $SPEAR_{pesticides}$ and $SPEAR_{organic}$.

To tackle these questions we used as case study four rivers of the Iberian Peninsula for which both biological and chemical data were previously gathered (Navarro-Ortega et al., 2012).

2. Materials and methods

2.1. Study area

Four Iberian river basins (Fig. 1) were studied as the representatives of Mediterranean rivers. Detailed description of the study area can be found elsewhere (Kuzmanović et al., 2015).

The Llobregat is the river situated in the North East of Iberian Peninsula. The lower part of the basin is subjected to strong anthropogenic pressures due to high proportion of the urban and industrial land use types in that area. In the middle part of the basin most of the agricultural lands are situated. As a typical Mediterranean river, Llobregat is subjected to decreased flow in the summer periods as a consequence of Mediterranean climate (Gasith and Resh, 1999). The Ebro is the large river situated in North of the Peninsula. The main pressures for water quality are coming from agriculture developed along the river basin. The urban and industrial centers are scattered in the basin, mostly in the North East and central part of the basin. The Júcar basin is situated in the East of Iberian Peninsula characterized by semi-arid climate. The most of the agricultural and urban areas are located in the medium and lower parts. Thus, these areas are receiving the most of the combined pressures together. The Guadalquivir basin, situated in the South of the Peninsula as a consequence of the high population, is subjected to strong anthropogenic pressures that may cause deterioration of water quality. A large portion of the basin is devoted to agricultural use which might result in water quality deterioration due to input of pesticides and fertilizers.

2.2. Sampling

The data used for this study were gathered within the Spanish research SCARCE-CONSOLIDER project (Navarro-Ortega et al., 2012). Extensive monitoring of water, sediment and biota from the four Iberian river basins was carried out in two monitoring campaigns (autumn 2010 and 2011). The autumn of 2010 was characterized by intense

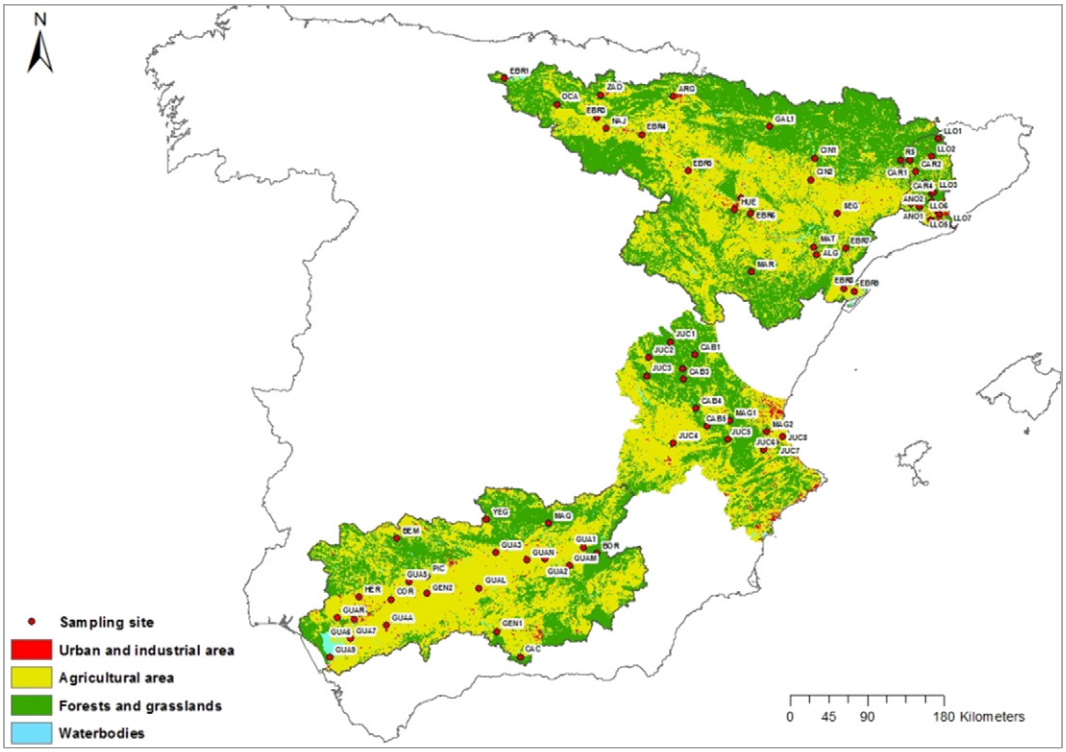

Fig. 1. Studied river basins with the major land use types and the sampling sites indicated.

precipitation, which resulted in a comparatively higher flow of Iberian rivers, while the autumn 2011 was dry and the river flows were low. Grab water samples were collected for chemical characterization at 77 selected locations in the Llobregat (15 sites), Ebro (23 sites), Júcar (15 sites) and Guadalquivir (24 sites) River Basins (Fig. 1). Metals and biological data were measured at 19 sites: Llobregat (5 sites), Ebro (5 sites), Júcar (5 sites) and Guadalquivir (4 sites). Sites were selected in a gradient of pollution from sites presumably less polluted to downstream where pollution was accumulated. The major land use types in the catchments were calculated by simplifying the Corine land cover into three groups: urban, agricultural and natural (including forest and grasslands) by Arc Map 10.1 software.

2.2.1. Macroinvertebrate sampling

Five sediment samples were randomly collected at each site with a polyvinyl sand corer (24 cm^2 area). Samples were sieved through a 500-μm mesh and fixed with 4% formaldehyde. The invertebrates were sorted, counted and identified in the laboratory under a dissecting microscope (Leica Stereomicroscope). The identification was at species level for almost all taxa – including Oligochaeta – with the exception of the Chironomids, which were identified at the genus level, and the Phylum Nematoda. Abundances were referred to the basis of sediment surface area (De Castro-Català et al., 2015).

To examine the biological status and link it with the chemical pollution three indexes were calculated: Shannon diversity index (H′) (Shannon, 1949), Margalef diversity index (d) (Margalef, 1969) and Species at Risk (SPEAR) for general organic pollution SPEAR $_{\text{organic}}$ (Beketov and Liess, 2008) and pesticides SPEAR $_{\text{pesticides}}$ (Liess and Von Der Ohe, 2005) (www.systemecology.eu/spear/spear-calculator/). SPEAR is a species trait based index that links chemical quality and biological community composition. It provides an assessment of the magnitude of the ecological effects of pollution (Liess and Von Der Ohe, 2005). For the calculations the species identified in sediment samples were used. When species was not present in the SPEAR database we selected the higher taxonomical order.

2.3. Chemical analysis

Compounds were measured using previously published analytical techniques based on gas chromatography-tandem mass spectrometry and liquid chromatography-tandem and hybrid mass spectrometry (Table S1). Water phase concentration data of 200 compounds belonging to different groups of priority and emerging contaminants: a) pesticides (48), b) pharmaceuticals and hormones (90) c) perflourinated compounds (21) d) akylphenols and other industrial organic compounds (14) e) drugs of abuse (8) and f) personal care products (17) and g) metals (8) were used for this study. Compounds below their limit of detection (LOD) were excluded from study. List of measured compounds and analytical methods used are available in Supporting Information (Tables S1 and S2, Supplementary information). Of 45 WFD priority pollutants, our dataset included seven pesticides, two industrial organic compounds and two metals (Table S2). Metals concentrations were transformed to bioavailable fraction using biotic ligand model (BLM) (Di Toro et al., 2001). The final number of number of chemicals that were used for risk assessment in this study (i. e. they were measured above their LOD is and their toxicity data was available) was 142.

2.4. Toxicity assessment

The toxic unit (TU) approach (Sprague, 1970) was used for the ecotoxicological risk assessment of measured concentrations of compounds (C_i). The TU of each compound was based on acute toxicity values i.e. EC_{50} (50% effective concentration) for reproduction and immobilization for algae and invertebrates respectively and LC_{50} (50% lethal concentration) for fish (Eq. (1)).

$$\boldsymbol{TU_i}_{\,(\textit{algae},\ \textit{invertebrates},\ \textit{fish})} = \frac{\boldsymbol{C_i}}{\mathrm{EC50_i}} \tag{1}$$

where TU_i is the toxic unit of a compound i; C_i measured concentration (μg/L) of the compound in the water phase; $\mathrm{EC50_i}$ or $\mathrm{LC50_i}$ (μg/L) effective or lethal concentration of 50% of individuals when exposed to the substance concerned. The toxicity data of each chemical was collected for three standard test species (green algae *Pseudokirchneriella subcapitata*, invertebrate *Daphnia magna* and fish *Pimephales promelas* or *Oncorhynchus mykiss*) from the literature and the databases when available, mainly ECOTOX (USEPA, 2008) and Pesticides Properties Database (PAN, 2015). Missing toxicity data were estimated by ECOSAR v.1.11. To determine site specific toxic stress and compare it with biological quality, we used the classical concept of concentration addition (CA). It allows the prediction of the mixture toxicity from concentration and toxicity of constituents of the mixture (Backhaus and Faust, 2012) but without regarding possible synergistic and antagonistic effects between chemicals. Site specific toxic stress (TU_{site}) was calculated by summing all the individual TU_i of each detected compound at all of the 77 studied sites. Since different effects in ecosystem are expected from metals and organic compounds (López-Doval et al., 2012), toxic units for metals (TU_{metals}) and organic compounds ($TU_{organic}$) were calculated separately. Additionally, in order to find out how risk is allocated between regulated and unregulated compounds in our dataset we grouped the compounds in the following manner. Firstly, we excluded the WFD priority pollutants from our dataset and examined which part of total risk is allocated to "non-priority contaminants" ($TU_{non\text{-}priority}$) (Table S2) by summing the toxic units of all the compounds detected in each sample except the WFD priority pollutants. Secondly, besides WFD priority pollutants, we excluded the other compounds regulated in European Union (i.e. banned pesticides) (Table S3). In that way, we examined the risk posed by the unregulated contaminants ($TU_{unregulated}$) only. Finally, the site specific risk was expressed as the logarithm of the mixture toxicity for: metals, all the detected organic compounds, "non-priority compounds" and unregulated compounds (Eq. (2)):

$$\boldsymbol{TU}_{\boldsymbol{SITE}(\boldsymbol{metals},\ \boldsymbol{organic},\ \boldsymbol{non-priority\ and\ unregulated})} = \log\sum_{i=1}^{n}\boldsymbol{TU_i} \qquad (2)$$

where, TU_i is the toxic unit of each of individual compound at the site. For convenience, along the present article TU associated with each site is expressed in log units. Having in mind the possible different modes of action of the studied compounds, there is a possible overestimation of risk. However, since the modes of action of many studied compounds are still unknown, we used the CA approach which is generally accepted as a first tier approach (Backhaus and Faust, 2012). Additionally, it was showed that the toxicity of the mixture predicted by CA correlated with the SPEAR index (Schäfer et al., 2013) suggesting this is a valid approach for predicting the toxic stress for biological communities in situ (McKnight et al., 2015).

2.5. Effects thresholds selection

To determine the potential effects of chemical pollution on the biological communities in situ we used the effect thresholds as proposed by Malaj et al. (2014). The acute risk threshold was set at the TU ≥ −1 (1/10 of EC_{50} or LC_{50}) for all three test species, since the acute effects in the ecosystem are generally expected at that level (Schäfer et al., 2011b; Schäfer et al., 2012; Van Wijngaarden et al., 2005). For the invertebrates, chronic risk threshold value of TU ≥ −3 (1/1000 of EC_{50}) was used. Changes in communities have been observed above that threshold i.e., decrease of sensitive species and shift towards more resistant species assemblages (Beketov et al., 2013; Liess and Von Der Ohe, 2005; Schäfer et al., 2012). However, this threshold is based on the field studies of effects of pesticides on biological communities. Therefore, extrapolating this threshold to other groups of compounds could lead to over or underestimation of the risk for some of the compounds. Also, those studies used maximum toxic unit (TU_{max}) in the sample, indicating the minimum estimated toxicity of the mixture as the toxicity of the most potent compound (Schäfer et al., 2013). In the case when the sum of toxic units is used to represent the mixture toxicity it should be noted that this is a bit more conservative approach but in line with the principle of screening-level risk assessments (McKnight et al., 2015). Due to the absence of studies relating pollution and long term effects in communities, chronic risk thresholds for algae and fish were based on acute to chronic ratio (Malaj et al., 2014). For algae the acute to chronic factor 5 was used and for fish factor 10 (Ahlers et al., 2006; Heger et al., 1995; Länge et al., 1998).

2.6. Statistical analysis

Analyses of variability and relations of toxic stress and biological indexes were performed by Principal Component Analysis (PCA) using Microsoft Excel XLSTAT statistical software. Toxic stress was characterized as the sum of TU (for invertebrates) per compound families, namely organic micropollutants and metals. Organic micropollutants were when necessary, grouped in several sub-classes, namely, pesticides, industrial organic chemicals (IOCs), pharmaceuticals, personal care products (PCPs) and perflourinated compounds (PFCs) (Table S2).

Linear regression and non-parametric correlations (Spearman correlation coefficient) were used to capture the relationships between toxic stress and changes in aquatic macroinvertebrate communities in situ.

3. Results and discussion

3.1. Ecotoxicological risk assessment: Acute and chronic risk

3.1.1. Acute effects risk in Iberian rivers

The toxic units ($TU_{organic}$) indicated that there was a risk of the acute effects in biological communities posed by organic compounds at 42% of the sampling sites (Fig. S1) and risk of chronic effects at all the studied sites (Fig. 5A). Of the three test species used for risk assessment, invertebrates were the most sensitive group (Supplementary Figs. S2–4) due to the presence of highly toxic insecticides at many sampling sites. Considering the four studied rivers the total number of sites with exceedance of the acute risk threshold was higher in 2010 (42% for invertebrates, 3% for fish and none for algae), than in 2011 (20% for invertebrates and no exceedance for algae and fish). The highest number of sites exceeding the acute threshold was in Ebro in 2010 (74% of sites) and in Júcar (67% and 60% in 2010 and 2011, respectively) (Fig. 2) mostly due to the presence of insecticides chlorpyriphos, chlorfenvinphos and ethion. On the contrary, in 2011 there was no exceedance of acute risk threshold in the Ebro due to relatively lower concentrations of those pesticides (Fig. 4). In Llobregat and Guadalquivir there was exceedance of acute risk threshold at less than 25% of the sites (Fig. 2). In 2011, the only area where acute risk was increased compared to previous year was in the lower part of the Llobregat basin (Fig. 3).

Of all the organic compounds measured in water the major contributors to the chemical risk were pesticides (Fig. 4). The compounds responsible for acute risk in Llobregat were chlorpyriphos and azinphos ethyl and ethion. In Guadalquivir there was acute risk at only 4 sites in 2010 and 3 sites in 2011 (Fig. 4) where high concentrations of chlorpyriphos, ethion and chlorfenvinphos were measured. In general, several pesticides were related with risk of acute effects (Fig. 4) of which the most important were the insecticides chlorfenvinphos (29% of sites with acute risk exceedance in 2010) and chlorpyriphos (15% sites in 2010). They are both classified by WFD as priority compounds and were identified as the compounds of highest ecotoxicological concern in studied river basins (Kuzmanović et al., 2015). Conversely, in 2011 they were not present in water at such high concentrations and thus the resulting acute risk exceedance was evidently lower, especially in the case of Ebro where chlorfenvinphos was detected only at one site in that year's sampling campaign (Fig. S5). The lower acute risk in 2011 might be an underestimation due to sampling in the dry period with the absence of precipitation which can trigger for the runoff effect of pesticides which were the most toxic compounds measured. Other pesticides not covered by WFD, but banned in the European Union

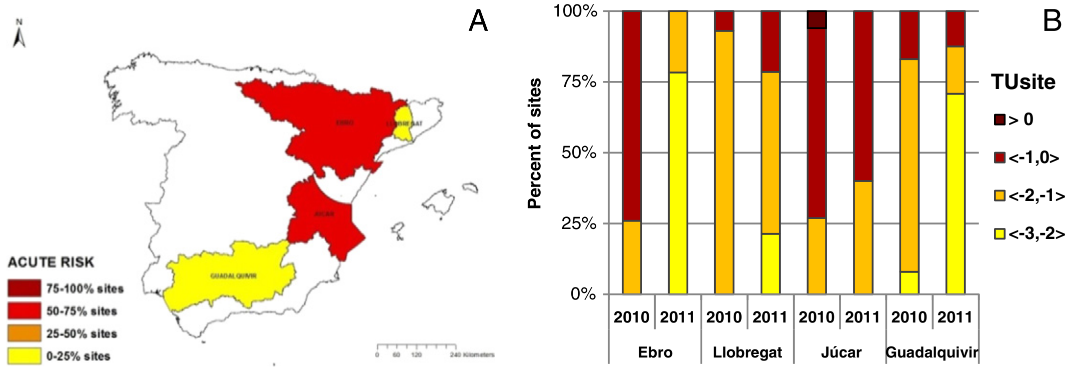

Fig. 2. Percentage of sampling sites A) with acute risk exceedance and B) with TU_{site} (most sensitive test species) belonging to one of four toxic unit ranges for each of four river basins in 2010 and 2011.

(Table S3) were also detected in water at high toxic units (e. g. ethion up to TU = −0.36 in the Júcar).

3.1.2. Chronic effects risk in Iberian rivers

The chronic risk threshold was exceeded at all of the sampling sites (Fig. 5A) for at least one of the test species. In 2011, the exceedance was the highest in the Júcar (all sites), the Llobregat (80% of the sites), the Ebro (61% of the sites) and the Guadalquivir (55% of the sites) (Figs. S2–4). While only pesticides and metals were responsible for acute risk, all measured compound groups except perflourinated compounds exceeded the chronic risk threshold for at least one test species (Fig. 4). Perflourinated compounds were in low TU at all the sampling sites (Fig. 4). Industrial organic compounds exceeded the chronic risk threshold at several sampling sites, mostly in the Guadalquivir (54%) and in the lower part of the Llobregat basin (50%). Of that group, the WFD priority compounds alkyphenols and their ethoxylate derivatives were the main contributors to toxic load among compounds detected. Personal care products exceeded algae chronic threshold (Fig. 4) mostly due to triclosan, that was detected around industrial and urban areas (lower part of the Llobregat and the Júcar basins, northern part of the Ebro basin (Fig. 1)). Pharmaceuticals exceeded chronic risk threshold in the Llobregat basin in 2010 with the antidepressant sertraline as the compound mostly responsible for threshold exceedance.

However, in this study we used acute toxicity data to assess the risk of both acute and chronic effects. Despite the fact that long term chronic exposure to pollutants is more realistic scenario (Eggen et al., 2004) there is a paucity of chronic toxicity data, especially for emerging contaminants. As stated by Calow and Forbes (Calow and Forbes, 2003), there is uncertainty in extrapolating results from effects caused after short, high dose exposure to effects caused after long time exposures to low doses of chemicals. There are indications that chronic responses to some chemicals may be greater than expected from risk assessment procedures similar to the one we followed. The chemicals causing endocrine disrupting effects at low environmental concentrations are the example for that, and it is reasonable to expect other types of specific chronic effects in the future caused by different compounds (Calow and Forbes, 2003).

3.2. Regulated vs. unregulated contaminants

The WFD priority contaminants list includes a limited number of priority and hazardous substances for chemical status regulation. However, the reality in the aquatic ecosystems is far more complex and those compounds that might be the most toxic are in fact just "the top of the iceberg". There are numerous unregulated compounds present in the environment and their potential adverse effects should not be overlooked. Besides, some banned pesticides can still be found in the aquatic environment and pose the threat to biological communities. In this study, the "non-priority" contaminants ($TU_{non\text{-}priority}$) (i.e., those left when WFD priority compounds were excluded from the dataset) exceeded the chronic threshold at 98% of the studied sites (Fig. 5B). However, the acute risk threshold was exceeded at six sites only. In any case, it is clear that we cannot exclude the risk for biological communities of studied rivers by regulating just WFD priority pollutants. Furthermore, when we excluded both the banned pesticides and the WFD priority pollutants from the dataset, the unregulated contaminants ($TU_{unregulated}$) exceeded the chronic risk threshold at 23% of sites. More precisely in Llobregat and Júcar (25–50% of sites) while in Ebro and Guadalquivir the exceedance of threshold happened at several sites only (Fig. 5C). In that group, the compounds responsible for chronic risk threshold exceedance were mainly unregulated pesticides, biocide

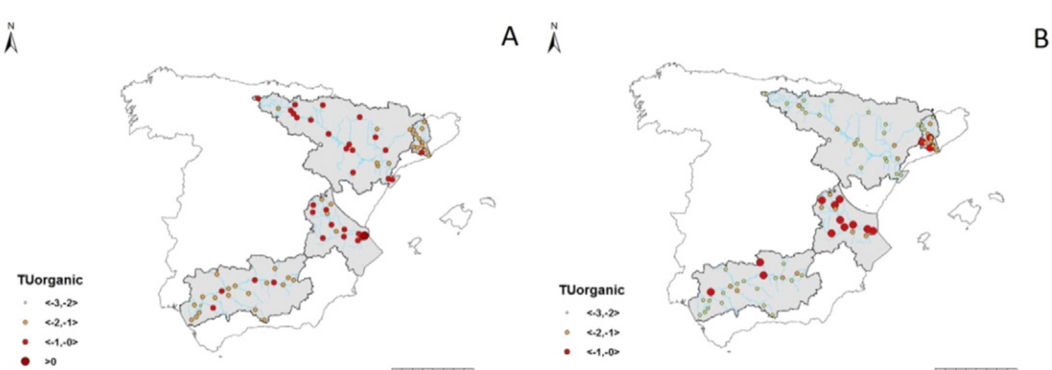

Fig. 3. Toxic units (TU_{site}) (for the most sensitive test species) for organic compounds at 77 sampling sites in A) 2010 and B) 2011.

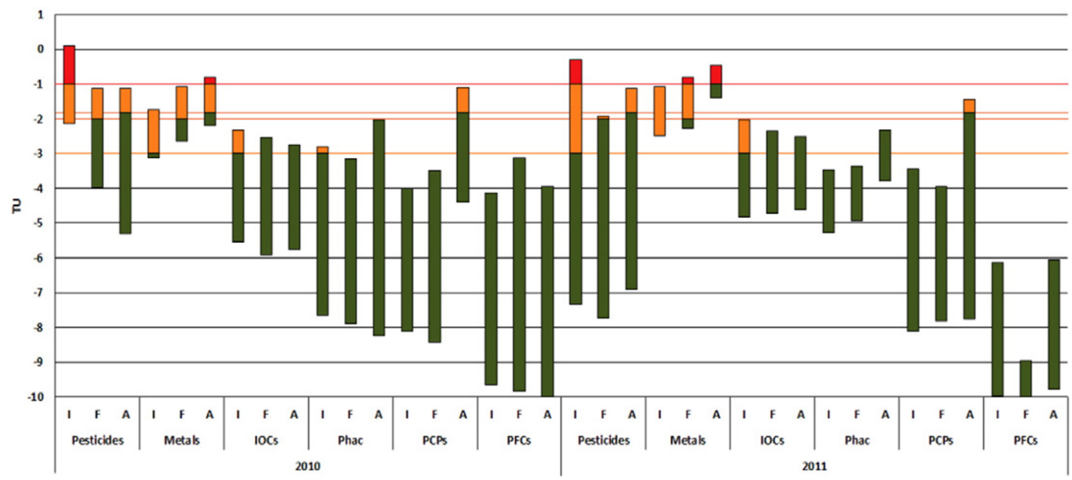

Fig. 4. Minimum and maximum plot for TU summed for families of measured compounds at sampling sites (organic compounds, n = 77; metals n = 18) for algae (A), invertebrates (I) and fish (F) in 2010 and 2011. Red color indicates the exceedance of acute risk threshold for the species concerned. Orange indicates the exceedance of a chronic risk threshold (TU −3 for invertebrates., −2 for fish and −1.69 for algae). IOCs—industrial organics, Phac—pharmaceuticals, PCPs—personal care products, PFCs—perfluoralkyl compounds (list of all the compounds is available in Supplementary material (Table S1)).

triclosan and the antidepressant sertraline. Remarkably, some banned pesticides such as e.g. chlorfenvinphos or ethion have been found in the water at the levels high enough even to pose acute risk and even more of those that were posing a chronic risk (e.g. diclofenthion, parathion-ethyl etc.) The question remains, why banned pesticides are still found in water at such levels that pose threat to aquatic life. In some cases, European legislation bans the pesticides for agricultural purposes, but the product still can be used in urban settlements as biocide, thus could reach the rivers. In other cases the ban of the pesticides can be implied just for some types of the crops while it can be used for other crops. On the other hand, McKnight et al. (2015) found several pesticides in Danish streams that were not authorized for use in that country for long time periods. They related the presence of banned pesticides (mostly herbicides) in stream water with the groundwater input as one of the important pathways. Another possible source could be the remobilization of legacy pesticides from sediment. Obviously, both currently used and banned pesticides are still posing the risk for aquatic life in studied rivers and both should be considered for risk assessment purposes. Especially important would be to determine the sources of the banned pesticides. In general, the overall risk for

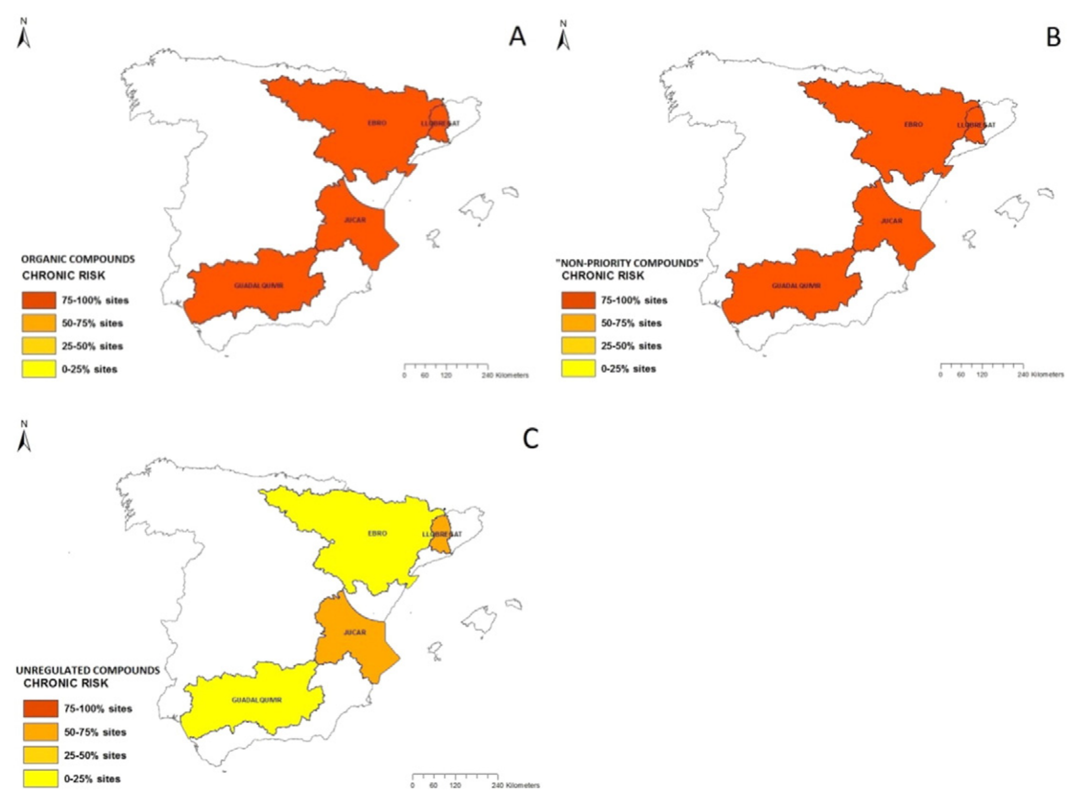

Fig. 5. Chronic risk 2010 and 2011—percentage of sites with exceedance of chronic risk threshold for at least one of three standard test species for A) organic compounds—the whole dataset, B) "non-priority" compounds and C) unregulated compounds.

aquatic ecosystems may often be dominated by a few components of the mixtures (Kortenkamp and Faust, 2010), which in this case were pesticides both classified as WFD priority pollutants and others (Kuzmanović et al., 2015). However, the risk of chronic effects of less toxic compounds is still present. Therefore, the risk of adverse effects in biological communities of studied rivers cannot be excluded by setting environmental quality standards just for the WFD priority compounds. Rather, a variety of chemicals present in the environment should be taken into account for the proper risk characterization. Moreover, in this study, there is a possible underestimation of risk because other toxic compounds could be present in the river water but they have not been covered here. Also, the influence of the synergistic and antagonistic effects between chemicals on the overall risk was not considered by this study.

3.3. Ecotoxicological risk — metals vs. organic micropollutants

Metals were measured in high toxic units at majority of the sampling sites ($n = 18$) (Fig. S6). While invertebrates were the most sensitive test species for organic chemicals, algae were the most sensitive species for metals (Fig. 4). When compared the risk at sampling sites where both organic compounds and metals were measured ($n = 18$), organic compounds risk was higher at majority of the sites in 2010 (Fig. S6) due to the presence of highly toxic pesticides in water. This could be related to the hydrometeorological situation of that year characterized by intense precipitation that could have triggered runoff of pesticides from the surrounding agricultural fields. On the contrary, in 2011 metals risk was higher at majority of sites in Ebro, Llobregat and Guadalqivir due to higher concentrations of metals in water and the lower concentrations of some pesticides.

Among metals measured, copper and zinc contributed mostly to the overall toxicity. The acute risk threshold ($TU_{metals} \geq -1$) was exceeded at 11% of the sites in 2010 and at 44% of the sites in 2011. It was found in previous studies based on routine monitoring that metals (especially zinc and copper) were the most important compounds in terms of toxic units in the studied area, while organic chemicals monitored only slightly contributed to the risk (López-Doval et al., 2012). These findings should be taken with some caution since the number of organic micropollutants analyzed was limited. A study of Catalan river basins based on the species sensitivity distribution (Carafa et al., 2011) and routine monitoring data carried out by the local authorities found an increase of toxic risk associated with urban and industrial areas of the Llobregat river basin was likely attributable to metals, surfactants (e.g. nonylphenol) and the pesticide chlorpyrifos (Carafa et al., 2011). Again, this study only included a limited number of organic pollutants (mostly priority compounds).

3.4. Biological status

Both diversity indexes (Shannon and Margalef) showed similar trends, decreasing downstream (Table 1) as a result of the reduction of the number and the abundance of species. The same trend was also observed in previous studies in the case of Llobregat river basin (López-Doval et al., 2010; Ginebreda et al., 2013).

In addition to the general tendency to decrease downstream, low values of diversity were also found in some sites located relatively upstream (e.g. EBR2, JUC2 and GUA2). According to SPEAR index, biological status of most of the sampling sites was moderate to bad (Fig. S7). However, this general status should be taken with caution. SPEAR metric has been developed to evaluate the risk of the whole invertebrate community inhabiting all the habitats present in the river. In this study, we sampled only the sediment and only few species living in this habitat are actually classified "at risk" in the SPEAR metric. Most of the species found in our sediments are part of the family Chironomidae and the order Oligochaeta. The SPEAR determines both taxonomical groups as "not at risk" without distinction between species. Even though the described limitations, SPEAR index has been used previously to assess biological status of sediment community with satisfactory results (Wolfram et al., 2012).

The invertebrates TU for the different compounds are suggesting several degrees of risk for biological communities and this could explain the community impairment observed with the biological indexes in all the sampling sites. Changes in the community structure due to priority and emerging pollutants have been described previously in Mediterranean rivers (Brix et al., 2012; Muñoz et al., 2009; Ricart et al., 2010), indicating the general biological impairment in relation to pollution.

3.4.1. Relationship between toxic stress and biological status

The only statistically significant correlation (Spearman, $p < 0.05$) between toxic stress of organic compounds and biological community descriptors was between $SPEAR_{organic}$ and $TU_{organic}$ ($r = -0.490$) and $TU_{pesticides}$ ($r = -0.431$) (Table 2). Neither Shannon nor Margalef indexes were showing significant correlation with $TU_{organic}$ (Table 2). Moreover, diversity indexes were not correlated with $SPEAR_{pesticides}$ and $SPEAR_{organic}$. It has been reported in several studies, that Shannon and similar biodiversity indexes were not suitable to identify the effects of pesticides at community level (Ippolito et al., 2012) and are influenced by different natural and anthropogenic factors (Beketov and

Table 1
Biological descriptors for macroinvertebrates in sediment at the different sampling sites (EBR: Ebro; LLO: Llobregat; JUC: Jucar; GUA: Guadalquivir).

2010	d	H′	$SPEAR_{organic}$	$SPEAR_{pesticides}$	2011	d	H′	$SPEAR_{organic}$	$SPEAR_{pesticides}$
EBR1	2.08	3.29	−0.92	0	2EBR1	2.38	2.78	/	/
EBR2	0.32	1.25	−0.93	0	2EBR2	1.10	2.72	−0.74	0
EBR3	1.04	2.97	−0.61	0	2EBR3	0.69	1.92	−0.76	0
EBR4	/	/	/	/	2EBR4	0.45	1.69	−0.39	0
EBR5	0.50	1.92	−0.78	0	2EBR5	0.58	1.63	−0.47	22.47
LLO3	1.45	2.57	−0.61	8.13	2LLO3	2.02	3.76	−0.77	11.82
LLO4	0.57	2.16	−0.55	19.23	2LLO4	0.78	2.47	−0.56	24.99
LLO5	0.61	1.80	−0.83	0	2LLO5	0.92	2.40	−0.35	44.54
LLO6	0.17	0.81	−0.93	0	2LLO6	0.44	1.49	−0.61	0
LLO7	0.46	1.81	−0.64	22.27	2LLO7	0.34	1.50	−0.93	0
JUC1	3.06	3.73	−0.88	6.35	2JUC1	2.44	2.61	−1.09	10.52
JUC2	0.79	1.40	−0.92	0	2JUC2	0.70	1.35	−1.22	0
JUC4	1.57	3.24	−0.78	14.37	2JUC4	1.12	2.76	−0.69	0
JUC5	1.06	2.55	−0.85	19.02	2JUC5	1.44	2.58	−0.86	13.12
JUC6	0.34	1.50	−1.34	0	2GUA2	0.57	1.98	−0.74	0
GUA1	2.82	3.74	−0.71	0	2GUA3	1.34	3.30	−0.46	20.82
GUA4	0.72	2.28	−0.52	0	2GUA4	0.91	2.48	−0.62	0

d—Margalef richness index, H′—Shannon diversity index—SPEAR: Species at Risk Index.

Table 2
Correlation matrix based on Spearman rank correlation test (in bold, $p < 0.05$).

Variables	Urban	Agricultural	Natural	d	H′	$SPEAR_{pesticides}$	$SPEAR_{organic}$	TU_{metals}	TU_{IOC}	TU_{PCP}	$TU_{pharmaceuticals}$	$TU_{pesticides}$	$TU_{organic}$
Urban	**1**	–	–	–	–	–	–	–	–	–	–	–	–
Agricultural	0.134	**1**	–	–	–	–	–	–	–	–	–	–	–
Natural	**−0.497**	**−0.817**	**1**	–	–	–	–	–	–	–	–	–	–
d	**−0.672**	−0.068	**0.375**	**1**	–	—	–	–	–	–	–	–	–
H′	**−0.436**	0.134	0.140	**0.883**	**1**	–	–	–	–	–	–	–	–
$SPEAR_{pesticides}$	0.120	0.014	0.028	0.232	0.269	**1**	–	–	–	–	–	–	–
$SPEAR_{organic}$	0.339	0.337	−0.306	0.088	0.286	**0.481**	**1**	–	–	–	–	–	–
TU_{metals}	**0.600**	0.010	−0.295	**−0.515**	−0.268	0.043	0.330	**1**	–	–	–	–	–
TU_{IOC}	0.045	0.018	−0.063	0.004	−0.004	−0.117	−0.127	0.007	**1**	–	–	–	–
TU_{PCP}	0.248	0.036	−0.151	−0.129	−0.092	0.061	0.105	0.210	**−0.585**	**1**	–	–	–
$TU_{pharmaceuticals}$	**0.490**	−0.010	−0.243	−0.232	−0.151	0.344	0.303	**0.492**	**−0.389**	**0.674**	**1**	–	–
$TU_{pesticides}$	**−0.412**	0.160	0.020	0.140	0.156	−0.229	**−0.431**	**−0.405**	0.323	**−0.404**	**−0.606**	**1**	–
$TU_{organic}$	**−0.394**	−0.012	0.128	0.175	0.155	−0.073	**−0.490**	**−0.459**	/	/	/	/	**1**

Liess, 2008). In this study they were negatively correlated with metals (TU_{metals}) (Table 2). However, only Margalef index was significantly correlated with the metals toxic units TU_{metals} ($r = -0.515$) (Table 2). Metals toxic units were significantly and positively correlated with urban land use type, while Shannon and Margalef indexes were correlated negatively (Table 2). That is, we can relate the decrease of macroinvertebrate biodiversity to urban areas. Nevertheless, urban rivers are highly impacted by a variety of stressors and it is known that in some cases, more environmental stressors can interact with the toxicants (Liess et al., 2013). Besides chemical pollution, in urban rivers there are often present habitat changes, temperature alterations and other stressors (Vörösmarty et al., 2010). Also, the natural gradient of environmental factors along the rivers is one of the most important sources of differences between biological communities (Beketov and Liess, 2008) and each site has its unique combination of natural factors (Schäfer et al., 2007) it should be taken into account when interpreting the macroinvertebrate biodiversity change along the river. The relation between biodiversity indexes and urban land use could be reflecting the response of the community to a variety of stressors present at the urban areas that are acting together along with the pollution.

Linear regression line between $SPEAR_{organic}$ and total organic stress at site ($TU_{organic}$) was significant with $r^2 = 0.235$ ($p > 0.05$) and a relationship between $SPEAR_{pesticides}$ and $TU_{pesticides}$ with $r^2 = 0.104$ ($p > 0.1$). Scatter plots show the relationship between losses of sensitive species with the increase of toxic stress of organic compound (Fig. 6A) and pesticides (Fig. 6B). All the sites were characterized by medium to high toxic stress (TU from-2.7 to 0) therefore the gradient of toxicity was relatively low and we could not observe the communities composition in pollution free conditions (i.e., reference conditions).

Even though SPEARindex is designed to be a stressor specific indicator it cannot be excluded that other stressors might have influenced the loss of sensitive species. This could be the case, especially since studied rivers are impacted by a multitude of anthropogenic stressors and some stressors are expected to cause similar changes in trait categories (Rasmussen et al., 2013; Statzner and Bêche, 2010). Besides, different co-occurring stressors (Liess and Beketov, 2011) and their complex relationships with biological communities (Liess et al., 2008) can mask the effects of single toxicant. Naturally, the use of $SPEAR_{pesticides}$ was showing the best results in agricultural streams where pesticides are the predominant stressors (Beketov et al., 2013; Schäfer et al., 2007). However, since only macroinvertebrates in the sediment were sampled in this study, the low values of SPEAR $_{pesticides}$ could be attributed to a relatively large proportion of tolerant species in that habitat (von der Ohe and Goedkoop, 2012; Wolfram et al., 2012) and the starting bias in the data makes any conclusion difficult. However, $SPEAR_{organic}$ as a less specific indicator seems to be more suitable for the multi chemical polluted rivers. In conclusion, when all four biological indexes used in this study are compared, the most suitable to relate changes in biological communities (i.e. decrease of sensitive species) to organic stress was the $SPEAR_{organic}$ indicator.

3.5. Multivariate analysis: Biological descriptors and toxic units of chemical groups

A principle components analysis was performed, including variables representing the toxic stress of chemical families studied (i.e. sum of toxic units of each group of chemicals), biological indexes and land uses expressed in percentage of agricultural, urban and natural respectively. The first two components were interpretable which explained 48% of the total variance (30.67% and 17.42% respectively).

The first component can be mainly related to pharmaceuticals, metals and personal care products that were grouped together and were related to urban land use type (Fig. 7). On the other hand, pesticides, industrial organic compounds (IOC) and biodiversity contribute

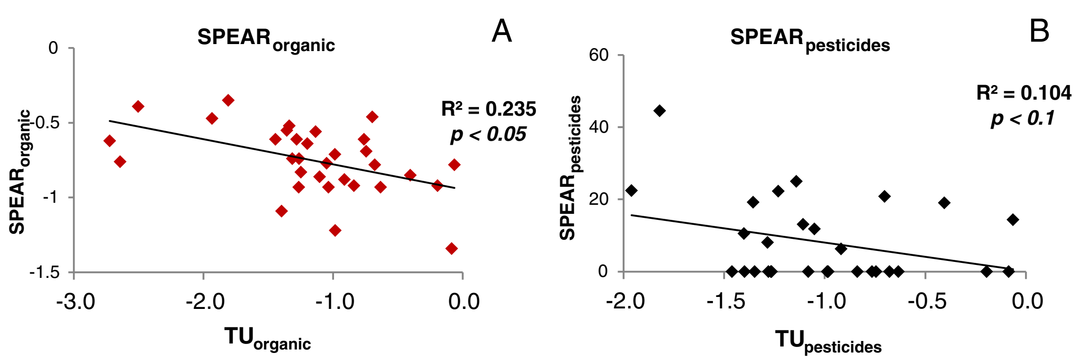

Fig. 6. Relationship between invertebrate communities in situ and the toxic stress. A) Expressed as $SPEAR_{organic}$ and toxic units of organic compounds ($TU_{organic}$ invertebrates). Linear regression is significant with $r^2 = 0.235$, $p > 0.05$. B) Expressed as $SPEAR_{pesticides}$ and toxic units of pesticides ($TU_{pesticides,}$ invertebrates). Linear regression is significant with $r^2 = 0.104$ at $p > 0.1$.

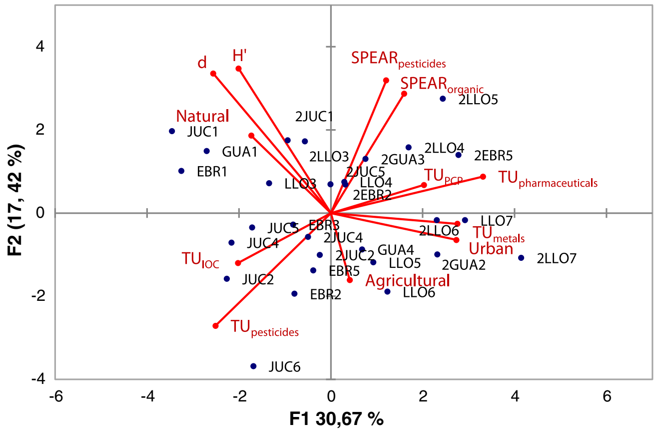

Fig. 7. Biplot of the first two principal components. The first two components of the PCA explain 48% of the total variance (30.67% and 17.42%, respectively).

negatively. This component could be tentatively interpreted as representative of the stressors related to urban areas. Sites with higher diversity indexes coincide with the upstream areas and natural land use type (forests and grasslands). Second component roughly informs about biological quality with positive contributions of biological indexes (H′, d, and SPEAR) and natural land use type, and negative contributions of industrial organic chemicals, pesticides and agricultural land use type. The distribution of the sites is consistent with the above interpretation. Polluted sites subjected to high urban pressure such as those in the lower part of Llobregat (LLO6 and LLO7) are distributed along the first component. $SPEAR_{pesticides}$ and $SPEAR_{organic}$ were negatively correlated with toxic stress of pesticides and industrial organic chemicals but were not reflecting the effect of urban origin (pharmaceuticals and PCPs). Therefore, we could assume pesticides and IOCs were those the compounds mostly influencing the decline of sensitive species.

4. Conclusions

In the present article we have assessed the environmental risk associated to chemical pollution on the basis of their ecotoxicological properties. To that end the toxic units approach based on three trophic levels (algae, invertebrates and fish) was used and applied to four Iberian river basins. Spatial ecotoxicological risk was characterized using available occurrence concentration data of more than 200 organic chemicals and metals transformed into toxic units and subsequently aggregated using widely accepted mixture toxicity criteria (i.e., concentration addition). This methodology enabled to quantify and depict in risk maps both acute and chronic potential effects that can be of great value for water management purposes. Both organic micropollutants (particularly pesticides) and metals significantly contribute to acute ecotoxicological risk.

The used methodology also enabled to differentiate the respective contributions to environmental risk between regulated and unregulated compounds, thus showing that both categories of compounds need to be taken into account for proper risk assessment. Banned pesticides are still present in river water in high toxic units and could be causing acute and chronic effects in biological communities. The unregulated contaminants alone posed the chronic risk at 23% of the studied sites. These findings have obvious management implications, for instance in the design of adequate monitoring campaigns.

Chemical and ecological status of water ecosystems are key aspects of the WFD and both are explicitly considered. However, their interrelation is not always clear. Here we used ecotoxciological assessment as an explanatory “bridge” between both. The combined use of toxicity indexes, conventional diversity indexes and traits-based indexes helped disentangle the relationships between macroinvertebrate communities and the different co-occurring stressors. Specifically, we found that the decline of aquatic macroinvertebrate sensitive species based on trait indexes (SPEAR) was correlated with the increase of organic load quantified in toxic units. Diversity indexes reflected in a general way the multiple stress conditions that the studied rivers were subjected to. These results were supported by multivariate statistical analysis in which both biological, land use and pollutants' ecotoxicological risk variables were used to satisfactorily to explain the observed variability among sites.

However, more work needs to be done in order to better understand the effects of co-occurring stressors in aquatic ecosystems. The appropriate combination of different community indicators and endpoints (e.g. behavior or functioning) will help to improve toxicological risk assessment of aquatic ecosystems.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.06.112.

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