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Environmental risk assessment of pharmaceuticals in rivers: Relationships between hazard indexes and aquatic macroinvertebrate diversity indexes in the Llobregat River (NE Spain)

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ABSTRACT

Continuous input of pharmaceuticals into rivers, through wastewater treatment systems, may cause adverse effects on the aquatic ecosystems of the receiving waterbodies, due to the intrinsic biological activity of these compounds. To investigate this issue, we have carried out an Environmental Risk Assessment in the lower part of the Llobregat River basin (NE Spain). The survey was carried out along three campaigns in 7 sampling points, located in the main river and in one of its tributaries (Anoia River). In each sample, 29 commonly used pharmaceuticals, belonging to different therapeutical classes (analgesics and non-steroidal anti-inflammatories (NSAIDs), lipid regulators, psychiatric drugs, anti-histamines, anti-ulcer agents, antibiotics and β-blockers) have been determined. Simultaneously, the macroinvertebrate community status of the same points has been also studied.

Hazard quotient indexes have been estimated for the most representative compounds as the ratio between concentrations and EC_{50} reported values, for three bioassays commonly used in environmental toxicology, namely, fish, *Daphnia* and algae.

Hazard indexes are obtained for each sample by summing up the hazard quotients of all the compounds present, and taking its average along the three sampling campaigns. In general, hazard quotients tend to increase when going downstream. Only those points located most upstream of the two rivers can be qualified under low risk for the three bioassays. The most sensitive bioassay seems to be algae, followed by *Daphnia* and fish.

Log-transformed hazard indexes show fairly good inverse correlations ($r = -0.58$ to -0.93, $p < 0.05$) with Shannon diversity indexes of macroinvertebrates, determined from both densities and biomasses. Best correlations are obtained for *Daphnia* based hazard indexes, as expected from its taxonomical proximity to macroinvertebrates. The abnormal correlation behaviour found in one point located in the Anoia River is explained by the presence of other previously reported pollutants of industrial origin, generated by the nearby existing industry.

1. Introduction

Occurrence of pharmaceuticals in the aquatic environment is nowadays a well established issue (Daughton and Ternes, 1999) and has become a matter of both scientific and public concern. Tons of different classes of pharmaceuticals find their way to the environment at variable degrees, after their use and excretion through wastewater and sewage treatment systems (Gros et al., 2007, 2009; Joss et al., 2005; Carballa et al., 2005; Heberer et al., 2002; Kolpin et al., 2004; Santos et al., 2007; Vieno et al., 2007; Suárez et al., 2008). The progress on the development of new and more powerful analytical multi-residue chromatographic methods has made available their detection and quantification in natural water bodies and wastewater, at concentrations in the range of ng/L (Petrovic et al., 2005; Martínez Bueno et al., 2007; Radjenovic et al., 2007; Gros et al., 2006a,b, 2009).

Whereas their occurrence is fairly well established, their long-term effects and environmental consequences are still a matter of active research (Sanderson et al., 2004; Hernando et al., 2007; Fent et al., 2006;

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Farré et al., 2001; Isidori et al., 2005; Nunes et al., 2005). However, there is a general consensus on the main features of the problem which can be summarized on the following facts:

(a) Pharmaceuticals are intrinsically bioactive compounds; therefore, they are able to cause potential effects on living systems
(b) There is a continuous and worldwide increase on their use and, thus, on their subsequent input into the environment.
(c) Hundreds of different pharmaceuticals are currently and regularly used simultaneously, which are susceptible to interaction and synergistic effects, that are basically unknown (Pomati et al., 2008; Cleuvers, 2003, 2004; Richards et al., 2004; Escher et al., 2005) and
(d) Data regarding effects on the aquatic ecosystems resulting from long-term low-dose exposure to pharmaceuticals are almost lacking.

Direct estimation of effects caused by environmental pollutants on ecosystems is not a straightforward task. In the case of pharmaceuticals, development of environmental risk assessment procedures (ERA) has been sometimes attempted, based on ecotoxicological data, mostly through the estimation of the so called hazard quotients (*HQ*), which are defined as the ratio between predicted or measured environmental concentrations (PEC or MEC, respectively) and their chronic toxicity, usually expressed as NOEC (non-observed effect concentrations) or PNEC (predicted non-effect concentrations) values (Bound and Voulvoulis, 2006; Castiglioni et al., 2004; Carballa et al., 2005; Cooper et al., 2008). When NOEC values are not available, EC_{50} or LC_{50} (50% effect concentration or 50% lethal concentration, respectively) values from standard ecotoxicological tests can be used, eventually after correction by an assessment factor. These *HQ* values are determined for every compound present (or predicted) on the environment, and can be aggregated, usually by simple addition. Under this framework, if *HQ* equals to unity there is a potential environmental risky situation (i.e., $HQ \geq 1$ is interpreted as environmental risk, while $HQ < 1$ should indicate a no risk).

Current European and US regulatory registration procedures for new pharmaceuticals intend to face this issue, requiring to provide ecotoxicological information , i.e., to carry out standard acute toxicity tests based on aquatic organisms like algae, *Daphnia magna* and fish, if the predicted or measured environmental concentration (PEC or MEC) of the active ingredient surpasses certain levels. For instance, the European Medicines Agency (EMEA), has set a threshold safety value at 10 ng/L, and compounds whose PEC exceeds this quantity have to be subjected to toxicity tests and can be considered as potential candidates to be included in monitoring programmes (Straub, 2002). Moreover, in the second tier, an ERA as described above is prescribed (Sanderson et al., 2003). Then, *HQ* is determined, and if $HQ < 1$, no further assessment is necessary (EMEA, 2001; Sanderson et al., 2004).

This approach, which is actually based on short term ecotoxicological lab determinations, is valuable since it provides an a priori identification (and quantification) of risk. However, it does not necessarily reflect the real ecosystem situation, since many additional concurrent factors (stressors) can be equally present. Furthermore, up to date there are still very few studies available on their effects on real ecosystems (Hernando et al., 2006; Crane et al., 2006; Sanderson et al., 2004; Fent et al., 2006; Nunes et al., 2005; Pascoe et al., 2003).

On a previous work (Muñoz et al., in press) evidence of biological benthic community (macroinvertebrates and diatoms) impairment associated to the presence of pharmaceutical drugs in the Llobregat (NE Spain) has been reported. Some pharmaceutical products in this river registered concentrations on the high range of those cited in the literature. For instance, multivariate analyses revealed a potential causal association between the concentration of some anti-inflammatories and beta-blockers and the abundance and biomass of several benthic invertebrates (*Chironomus* spp. and *Tubifex tubifex*). Though pharmaceutical products are, until now, not included in the list of priority or dangerous priority substances, according to the Water Framework Directive (Directive 2000/60/EC), and thus no environmental quality standards are stipulated (Directive 2008/105/EC), it is clearly established in the same directive that substances discharged into the basin should be controlled. This is the case of pharmaceuticals, which are able to reach surface waters through domestic wastewaters, even after its treatment in wastewater treatment plants (WWTPs), especially if they can cause adverse effects on the ecosystems.

Therefore, our aim on the present study is to progress further on that issue, carrying out an environmental risk assessment of pharmaceuticals present in the Llobregat River basin, showing more general relationships between hazard quotients and ecological status, quantified by means of some commonly used diversity indexes. The present study is thus full in line with the requirements of both the WFD and the EMEA.

2. Experimental

2.1. Study site

The Llobregat River (NE Spain) is 156 km long and covers a catchment's area of about 4957 km^2. From the hydrologic point of view, the Llobregat is a typical Mediterranean river, its flow being characterized by a high variability, which is closely controlled by seasonal rainfall. The mean annual precipitation is 3330 Hm^3 and it has an annual average discharge of 693 Hm^3. Its watershed is heavily populated with more than 3 million inhabitants living therein. Together with its two main tributaries, River Cardener and River Anoia, the Llobregat is subjected to a heavy anthropogenic pressure, receiving extensive urban and industrial waste water discharges (137 Hm^3/year; 92% coming from the waste water treatment plants), which constitutes a significant part of its natural flow. 48% of these point sources are located in the studied area. Furthermore, in the middle part of the basin it receives brine leachates from natural salt formations and mining operations, which have caused an increase in water salinity downstream. The Llobregat is thus an illustrative example of overexploited river.

Four sampling sites were selected from the middle and lower part of the Llobregat main channel and another three were selected in the Anoia tributary (Fig. 1). These sites were part of a pollution gradient: sites LL1 and A1 were the least polluted but received some industrial effluents and surface runoff from agricultural areas. Sites A3 and LL4 were located in the last section of the two rivers, and were the most polluted sites according to the monitoring data of the Water Authority (Catalonian Water Agency). Site A2 was located in a highly polluted area receiving waste waters from tannery, textile and paper industries. Sites LL2 and LL3 were located in a densely inhabited area and receive urban and industrial wastewaters inputs.

Sampling was performed in early June 2005, November 2005 and late May 2006. These three periods covered two of the most relevant periods (spring and autumn) in the system in terms of its hydrology. Samples for grain size characterization were taken in the two first samplings. 98% of the collected sediment was gravel and sand in all sites. Sites A1, A2, LL1 and LL2 had slightly more proportion of gravel and A3 higher proportion of fine sand with respect to the other sites.

2.2. Analysis of pharmaceuticals

A total of 29 pharmaceuticals, belonging to the classes of analgesics and non-steroidal anti-inflammatories (NSAIDs), lipid regulators, psychiatric drugs, anti-histamines, anti-ulcer agents, antibiotics and Beta-blockers were measured in water by means of off-line solid-phase extraction (SPE) followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Briefly, water samples (400 ml), previously passed through 0.45 μm nylon membrane filters, were preconcentrated on Oasis HLB cartridges, which were further rinsed

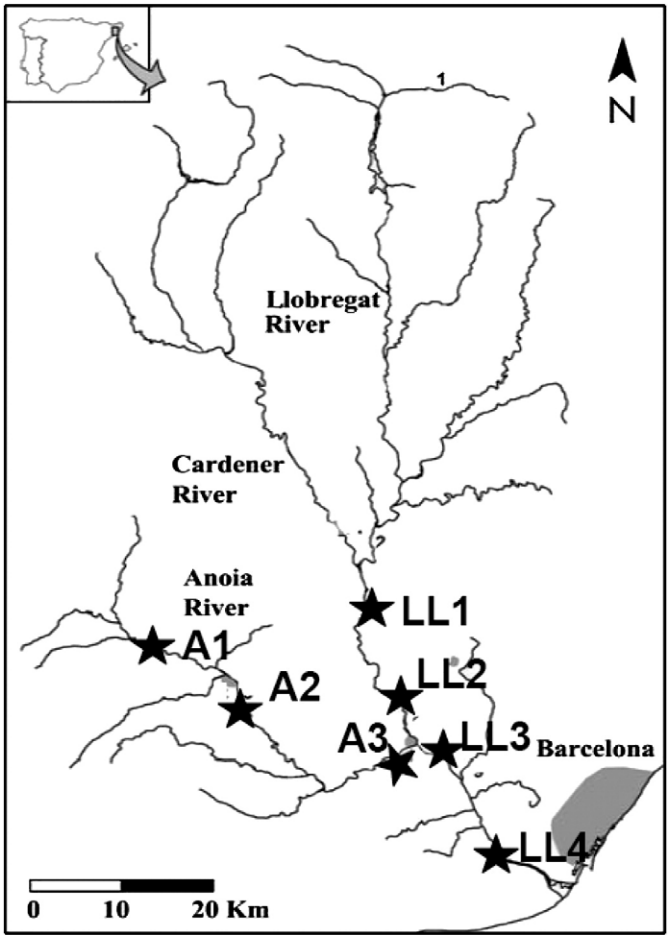

Fig. 1. Llobregat River: map of the basin indicating the sampling sites.

with 5 ml of HPLC-grade water, dried under vacuum for 15–20 min, and eluted with 2×4 ml of methanol. The extracts were then evaporated under a gentle nitrogen stream, reconstituted with 1 ml of methanol–water (25:75, v/v), and added to 10 µl of an internal standard mixture. Analysis of the extracts was performed by liquid chromatography–electrospray–triple quadrupole-tandem mass spectrometry (LC–ESI–MS/MS), operating in the selected reaction monitoring (SRM) mode. Two SRM transitions were monitored per compound. Nine of the 29 compounds were measured in negative ion mode and the remaining 20 in positive ion mode. This method and its validation (linearity, detection and quantification limits, repeatability, etc.) are described in detail in (Gros et al., 2006a,b).

2.3. Macroinvertebrate analysis

Five sediment samples (5.5 cm diameter, 10–15 cm depth) were randomly collected with a polyvinyl sand corer in each sampling site and period. 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 stereoscopic microscope. Identification was at family level for Oligochaeta and at the genus or species level for the rest of the groups present (mainly Chironomidae and Ephemeroptera). Biomass was calculated as a dry weight from length dimension using exponential equations (Benke et al., 1999). Abundance and biomass were referred to the sediment surface area. Taxa considered in the present study are reported in Table 1.

Table 1
Macroinvertebrate taxa used in the determination of diversity indexes.

Taxon
Tubificidae
Enchytraeidae
Naididae
Ephemerella sp.
Baetis sp.
Caenis sp.
Alcalcarella sp.
Chironomus spp.
Cricotopus sp.
Cryptochironomus sp.
Nanocladius bicolor
Orthocladiinae
Paracladius conversus
Paratanytarsus sp.
Polypedilum spp.
Pottastia sp.
Prodiamesa olivacea
Rheocricotopus sp.
Stictochironomus sp.
Tanytarsus sp.
Corynoneura
Tanypodinae

3. Results and discussion

3.1. Presence of pharmaceuticals

29 different commonly prescribed drugs, distributed among different therapeutic classes (Table 2), namely, analgesics and NSAIDs (8), lipid regulators and cholesterol lowering (5), psychiatric drugs (3), anti-ulcer agents (1), histamine H1 and H2 antagonists (3), antibiotics (5) and β-blockers (4), were monitored. Target compounds were selected according to the information found in the literature on their occurrence and ubiquity in the aquatic environment, as well as their high human use and consumption worldwide (Petrovic et al., 2006). Typical concentrations found were in the range of 10 to 10^{-2} µg/L (75th percentiles). These values are in the upper side of

Table 2
Average, minimum and maximum concentrations (µg/L) of pharmaceuticals in the water samples analyzed, and concentration at the 75th percentile ($n=21$).

		Mean	Min	Max	75th	MDL (ng/L)
Analgesics and anti-inflammatories	Ketoprofen	0.79	0.16	2.71	0.71	30
	Naproxen	0.53	0.02	2.06	0.65	7
	Ibuprofen	1.37	0.16	9.89	1.51	8
	Indomethacine	0.16	0.05	0.38	0.26	6
	Diclofenac	2.20	0.08	18.74	1.49	2
	Mefenamic acid	0.02	0.01	0.04	0.03	0.5
	Acetaminophen	0.42	0.06	2.42	0.45	17
	Propyphenazone	0.09	0.03	0.18	0.15	3
Lipid regulators and cholesterol lowering statin drugs	Clofibric acid	2.28	0.01	7.91	3.29	1
	Gemfibrozil	1.42	0.04	7.78	1.42	1
	Bezafibrate	1.02	0.03	15.06	0.35	1
	Pravastatin	<MDL				47
	Mevastatin	<MDL				7
Psychiatric drugs	Carbamazepine	1.07	0.08	3.09	1.93	2
	Fluoxetine	<MDL				20
	Paroxetine	<MDL				8
Anti-ulcer agent	Lansoprazole	<MDL				5
Histamine H1 and H2 receptor antagonists	Loratadine	<MDL				2
	Famotidine	<MDL				5
	Ranitidine	0.11	0.01	0.57	0.09	2
Antibiotics	Erythromycin	0.03	0.01	0.07	0.06	4
	Azythromycin	<MDL				1
	Sulfamethoxazole	1.11	0.03	11.92	0.44	5
	Trimethoprim	0.14	0.02	0.47	0.21	1
	Ofloxacin	2.11	0.19	8.77	1.70	16
Beta-blockers	Atenolol	0.22	0.05	0.67	0.32	9
	Sotalol	0.57	0.11	1.82	0.63	18
	Metoprolol	0.05	0.01	0.18	0.06	3
	Propranolol	0.03	0.01	0.06	0.06	2

MDL (method detection level). (Muñoz et al., in press).

those reported for other Mediterranean rivers like the Ebro (Kuster et al., 2008; Gros et al., 2007; Comoretto and Chiron, 2005), but this fact can be explained if one takes into account the high demographic pressure exerted in the low part of the Llobregat basin, together with the limited dilution capacity of this river. Peak concentrations exceeding 5 µg/L corresponded to some NSAIDs, such as ibuprofen or diclofenac, lipid lowering agents related to clofibric acid (clofibric acid itself, gemfibrozil and bezafibrate) and two antibiotics (sulfamethoxazole and ofloxacin). This profile is consistent with their market penetration (prescription pattern), as well as, their reluctance to biodegradation in standard WWTPs (Gros et al., 2009). Detection frequencies, expressed as percentages, are shown in Fig. 2, and they also support this statement. Three compounds are present in all the samples analyzed (diclofenac, gemfibrozil and ibuprofen), and 16 in 50% of them. Only 6 compounds (azythromycin, famotidine, fluoxetine, paroxetine, mevastatin and pravastatin) were below their detection limits in all the samples analyzed. This behaviour is in partial coincidence with studies carried out in other representative Iberian river, as it is the Ebro (Gros et al., 2009), where famotidine, mevastatin and paroxetin were also not observed.

3.2. Diversity indexes

Diversity indexes *DI* were calculated for each sample, using both taxa densities and biomasses, expressed as frequencies after normalization to unity, using the well known Shannon information-theoretical equation (Margalef, 1991):

$$DI = \sum_i f_i \log_2 f_i$$

where the sum extends over all the i taxa considered, and $\sum_i f_i = 1$.

Diversity Indexes obtained (expressed in *bit* units), are summarized in Fig. 3 for the various sampling points. As expected, no relevant differences are observed between density based or biomass based indexes. Both of them show a general tendency to decrease when going downstream, thus reflecting the corresponding increase of anthropogenic pressure existing in the two rivers studied, Anoia and Llobregat. Values obtained are comprised between ca. 3 and 0.4 bits, but the ranges are highly variable, depending on the sampling point. For instance, point A2 shows a fairly constant value of about 0.5 bits for the density based diversity index, while point Ll2 shows a wide variation of *ca.* one order of magnitude (0.36 to 3.36 bits).

3.3. Hazard quotient indexes

In order to estimate the environmental risk posed by certain contaminants on aquatic ecosystems, the WFD states the convenience of assessment using taxa of three different representative trophic levels of the ecosystem, such as algae, daphnids and fish. Therefore, following these guidelines, in the present study we have determined the hazard quotients (*HQ*) associated to the most representative pharmaceuticals found in the low Llobregat River. PNEC values were estimated for (a) fish, (b) daphnids and (c) algae from data literature on acute toxicity. Specifically, dividing EC_{50} values by an arbitrary safety factor, in this case, typically 1000, PNEC were derived (Sanderson et al., 2004). EC_{50} values used in this study were collected from the literature and are summarized in Table 3. It is worth noting that when more than one EC_{50} value was found, the lowest values were taken into consideration. When no experimental values were available, we used EC_{50} estimated with ECOSAR, as reported (Sanderson et al., 2003). For erythromycin a PNEC (µg/L) of 0.02 was taken for daphnids (Lee et al., 2008) and for sulfamethoxazole a PNEC (µg/L) of 0.12 was taken for algae (Grung et al., 2008).

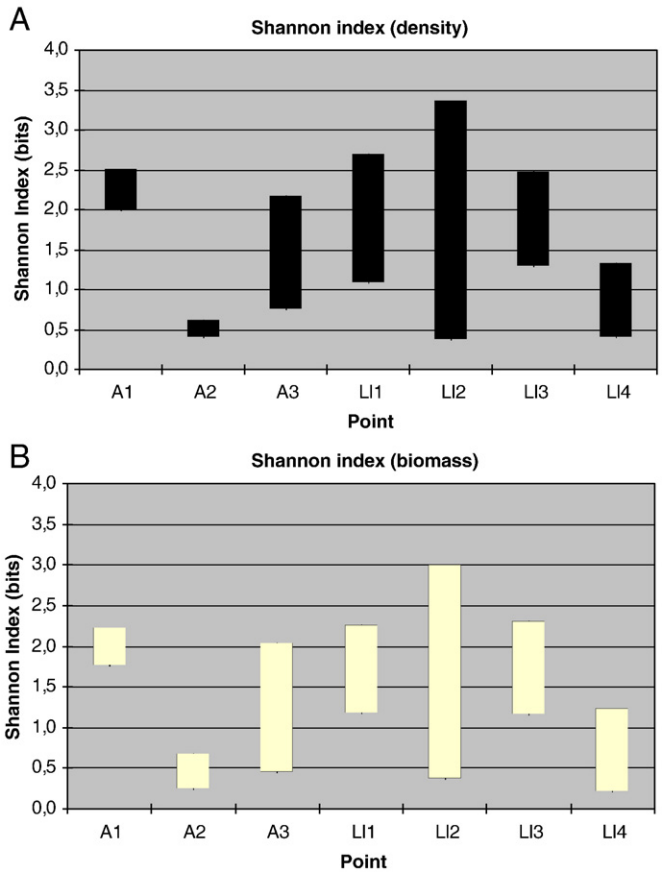

Fig. 3. Shannon diversity indexes of benthic macroinvertebrates for every sampling point, (A) calculated from taxa densities, (B) calculated from taxa biomasses.

Hazard quotient hq_{ij} associated to compound j in the i measurement is given by the following expression:

$$hq_{ij} = \frac{c_{ij}}{PNEC_j}$$

where c_{ij} is the concentration of compound j in sample i and $PNEC_j$ is the 'Predicted Non-Effect Concentration' for compound j, as described above:

$$PNEC_j = \frac{EC50_j}{1000}$$

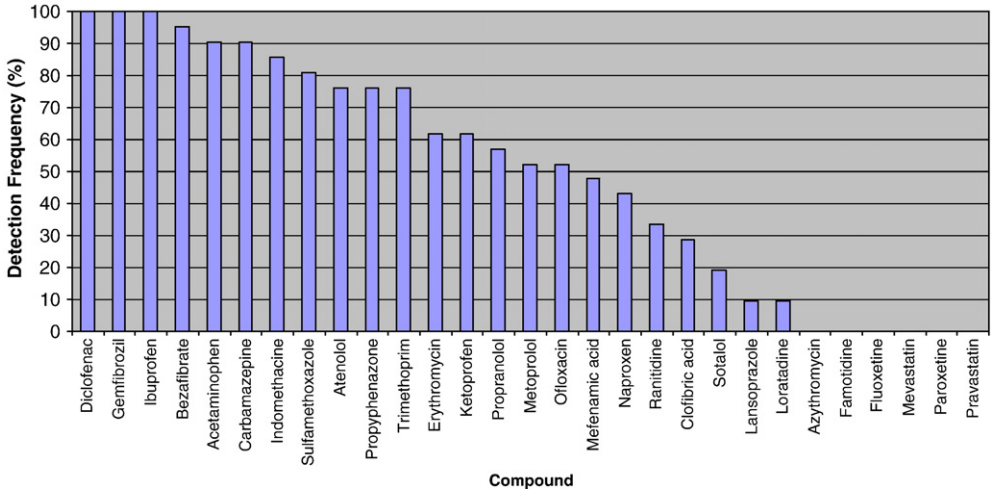

Fig. 2. Detection frequencies, expressed as percentage, of the compounds analyzed.

Table 3
EC_{50} (in mg/L) used to calculate PNEC (by dividing EC_{50} by an assessment factor of 1000, except for those cases indicated below), for fish, *Daphnia* (*Daphnia magna*) and algae for the most ubiquitous pharmaceuticals detected in the Llobregat River basin.

Compounds	Fish	*Daphnia*	Algae	References
	EC_{50} (mg/L)	EC_{50} (mg/L)	EC_{50} (mg/L)	
Naproxen	34*	15*	22*	Sanderson et al. (2003)
Ibuprofen	5*	9.02	4	(Quinn et al., 2008; Sanderson et al., 2003; Stauer-Lauridsen et al., 2000)
Diclofenac	532*	22	14.5	Grung et al. (2008)
Acetaminophen	378	9.2	134	Grung et al. (2008)
Salicylic acid	1.29*	59*	48*	(Grung et al., 2008; Sanderson et al., 2003; Stauer-Lauridsen et al., 2000)
Ketoprofen	32*	248*	164	Sanderson et al. (2003)
Indomethacine	3.90*	26*	18*	Sanderson et al. (2003)
Phenazone	3*	6.7*	1.1*	Sanderson et al. (2003)
Propyphenazone	0.8*	3.5*	1.0*	Sanderson et al. (2003)
Bezafibrate	6	30	18	Hernando et al. (2007)
Gemfibrozil	0.9	10.4	4	Hernando et al. (2007)
Pravastatin	1.8	–	–	Hernando et al. (2007)
Clofibric acid	53	0.11	192	Hernando et al. (2007)
Fluoxetine	1.70*	0.51	0.80*	Henry et al. (2004)
Carbamazepine	35.4	76.3	85	(Kim et al., 2007; Huschek et al., 2004)
Ranitidine	1076*	63*	66*	Sanderson et al. (2003)
Ofloxacin	–	31.75	–	Isidori et al. (2005)
Sulfamethoxazole	562.5	25.20	(b)	(Kim et al., 2007; Isidori et al., 2005)
Sulfamethazine	517*	4*	38*	Sanderson et al. (2003)
Erythromycin	61.5	(a)	4.3*	(Lee et al., 2008; Sanderson et al., 2003)
Tetracycline	220	6*	4*	Grung et al. (2008)
Trimethoprim	795*	120.7	16	(Kim et al., 2007; Grung et al., 2008; Sanderson et al., 2003)
Atenolol	–	200	–	Hernando et al. (2007)
Metoprolol	944	63.9	7.9	(Huschek et al., 2004; Grung et al., 2008)

Values indicated with * mean that the EC_{50} is estimated with ECOSAR. Data were taken from Sanderson et al., 2003.
(a) For Erythromycin a PNEC (μg/L) of 0.02 was taken for daphnids from Lee et al., 2008
(b) For Sulfamethoxazole a PNEC (μg/L) of 0.12 was taken for algae from Grung et al., 2008.

Since more than one compound is usually present, their joint effects must be somehow considered. According to the aforesaid, *hq* indexes have been supposed to follow an additive model for the different compounds involved (Von der Ohe et al., 2009). Therefore the overall hazard quotient for determination (sample) *i* is:

$$HQ_i = \sum_j hq_{ij}$$

HQ indexes obtained according to this way are reported in Table 4, for the three bioassays considered. It is worth to assess the relative contribution weight of the different compounds to overall *HQ* indexes. For that purpose, it seemed more intuitive to estimate the contribution of each compound within every single HQ_i in terms of its percentage. This is carried out by normalization of hq_{ij} to 100, using the following expression:

$$hq'_{ij}(\%) = \frac{hq_{ij}}{\sum_j hq_{ij}} \times 100 = \frac{hq_{ij}}{HQ_i} \times 100$$

(where *i* and *j* stand as above).

In this way

$$\sum_j hq'_{ij}(\%) = 100$$

In Fig. 4 are depicted the percentage ranges hq'_{ij} (%) for every compound *j* contributing to the set of 21 *HQ* determinations (7 points and 3 campaigns make a total of $i = 21$ *HQ* determinations or samples). This has been done for each one of the three bioassays, namely, fish (a), *Daphnia* (b) and algae (c).

Table 4
Hazard indexes (*HQ*) for fish, *Daphnia* and algae in the different sampling points and campaigns.

Point	Sampling campaign	Fish (*HQ*)	Daphnia (*HQ*)	Algae (*HQ*)
A1	a	0.113	0.038	0.065
A1	b	0.117	0.047	0.323
A1	c	0.106	0.047	1.154
Mean		0.112	0.044	0.514
A2	a	3.328	3.014	1.103
A2	b	2.241	3.955	2.142
A2	c	1.336	1.576	22.815
Mean		**2.302**	**2.848**	**8.687**
A3	a	4.554	1.365	3.102
A3	b	2.300	2.123	1.521
A3	c	1.876	3.565	11.360
Mean		**2.910**	**2.351**	**5.328**
Ll1	a	0.451	0.607	0.712
Ll1	b	0.693	0.149	0.851
Ll1	c	0.725	1.187	3.876
Mean		0.623	0.648	**1.813**
Ll2	a	0.305	0.124	0.821
Ll2	b	0.712	0.739	0.964
Ll2	c	0.587	0.756	3.895
Mean		0.535	0.540	**1.893**
Ll3	a	0.884	0.666	0.947
Ll3	b	1.858	1.669	2.327
Ll3	c	0.887	0.328	3.879
Mean		**1.210**	0.888	**2.385**
Ll4	a	11.103	77.375	103.022
Ll4	b	7.846	35.004	2.556
Ll4	c	9.217	24.485	11.512
Mean		**9.389**	**45.622**	**39.030**

Sampling campaigns: a: June 2005; b: November 2005; c: May 2006. Mean *HQ* values greater than 1 are indicated in bold.

As expected, since the respective toxicities are different for the three bioassays, each one shows its own sensitivity respect to certain compounds. Thus, for the fish based bioassay, major contribution is due to gemfibrozil, followed by ibuprofen and diclofenac. Other compounds with significative effect are propyphenazone and bezafibrate. For *Daphnia*, major contributions are attributable to erythromycin, ibuprofen and clofibric acid in point Ll4 and, to a less extent, to diclofenac, acetaminophen and sufamethoxazole. Finally, because of its high toxicity reported towards algae (Grung et al., 2008), this bioassay appears to be mostly dependent of sulfamethoxazole, followed by ibuprofen and gemfibrozil.

3.4. Relationship (correlations) between HQ and diversity indexes

The key point of the present study is addressed to ascertain the existence of possible relationships between diversity and ecotoxicity, respectively expressed, as explained above, in terms of Shannon diversity indexes and hazard quotients.

After long time exposure to stressor factors, ecosystems are supposed to get integrative responses adapted (until certain extent) to them. Therefore, since we are assessing long-term situations, for a given point we considered more realistic to reflect its diversity status as the average of the results obtained along the three sampling campaigns. The same rationale holds for hazard quotients.

Roughly speaking, both types of indicators are mutually consistent and evidence a decrease of quality when going downstream. This can be observed in both the Llobregat and in its tributary Anoia.

As said before, it is assumed that *HQ*s below unity are indicative of no risk. This situation is only observed in points A1, Ll1 and Ll2 (in the later two, only for fish and *Daphnia* bioassays). Conversely, the worst hazard quotient is obtained for Ll4, exceeding in one to two orders of magnitude the threshold value. Points A2, A3 and Ll3 are on an intermediate situation with an *HQ* index above 1. When the three bioassays are compared, algae appear to be the most sensitive, followed by fish and *Daphnia*. The highest sensitivity of algae was also reported by Gros et al. (2009) in the Ebro River.

Careful comparison between both diversity and hazard quotient indexes reveals some abnormal behaviour of point A2, whose poor diversity is only partially explained by its pharmaceutical based *HQ*. Therefore, occurrence of additional factors must be taken into consideration. Point A2 is strongly affected by the discharge of a WWTP, which receives industrial effluents from the tannery industries located in its neighbourhood. Previous results obtained in our lab showed the presence of several contaminants, such as trivalent chromium, ethoxylated alkylphenols and their degradation products (nonyl and octylphenol) (Céspedes et al., 2005), alkylarylsulfonated derivatives, etc. (Alonso et al., 2005).

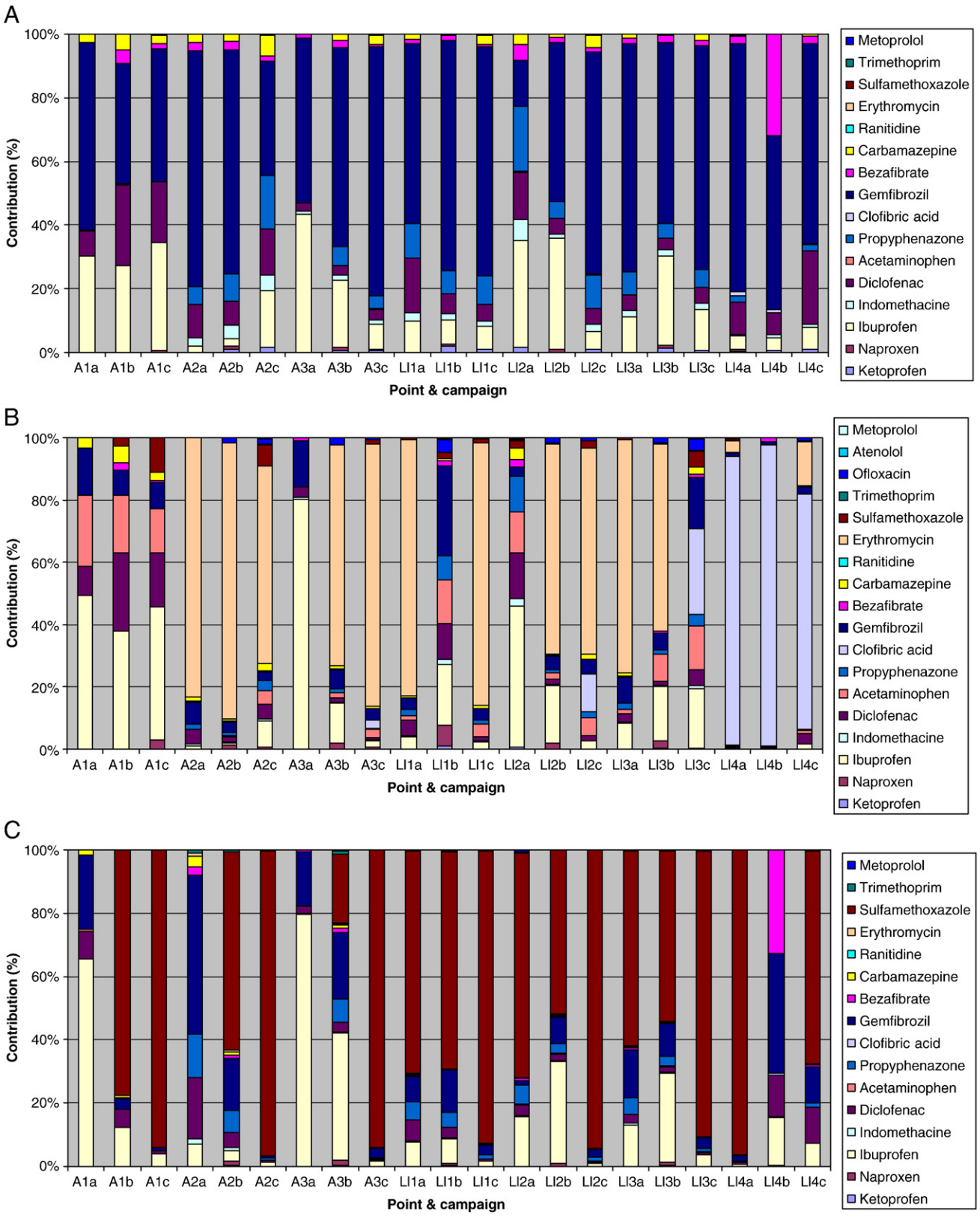

Fig. 4. Percent contribution of different compounds to hazard indexes for (A) fish, (B) *Daphnia* and (C) algae.

Fig. 5A shows the plots obtained between log-transformed *HQs* and diversity indexes (density based or biomass based, respectively) for the three bioassays considered, i.e., fish, *Daphnia* and algae. The corresponding regression lines are also included, thus showing a fairly good inverse correlation ($r = -0.58$ to -0.78, $p < 0.05$). Concerning the three bioassays considered, the best correlations correspond to *Daphnia* and algae ($r = -0.76$ to -0.78, $p < 0.05$), while fish bioassay shows a slightly poorer one ($r = -0.58$, $p < 0.05$). In the case of *Daphnia* this can be explained due to its closer taxonomic proximity to macroinvertebrates. When point A2 is excluded (due to the

aforementioned abnormal behaviour), there is a substantial improvement on the correlations, as it is evident from the correlation coefficients $r = -0.7$ to -0.93, $p < 0.05$ (Fig. 5B). Again, the best correlations are observed for *Daphnia* and algae. These results are summarized in Table 5.

Even though the correlations found are quite significant, one has to be cautious when pointing out to pharmaceuticals as the unique compounds responsible for the loss of diversity. Since pharmaceuticals are clearly associated with domestic wastewater discharges, it must be kept in mind that they are just one part of a more general chemical pollution load. In fact, the presence of pesticides (Ricart et al., in press), alkylphenolic compounds and estrogens (Brix et al., submitted for publication), and other common physical–chemical parameters and nutrients (Muñoz et al., in press) has also been reported.

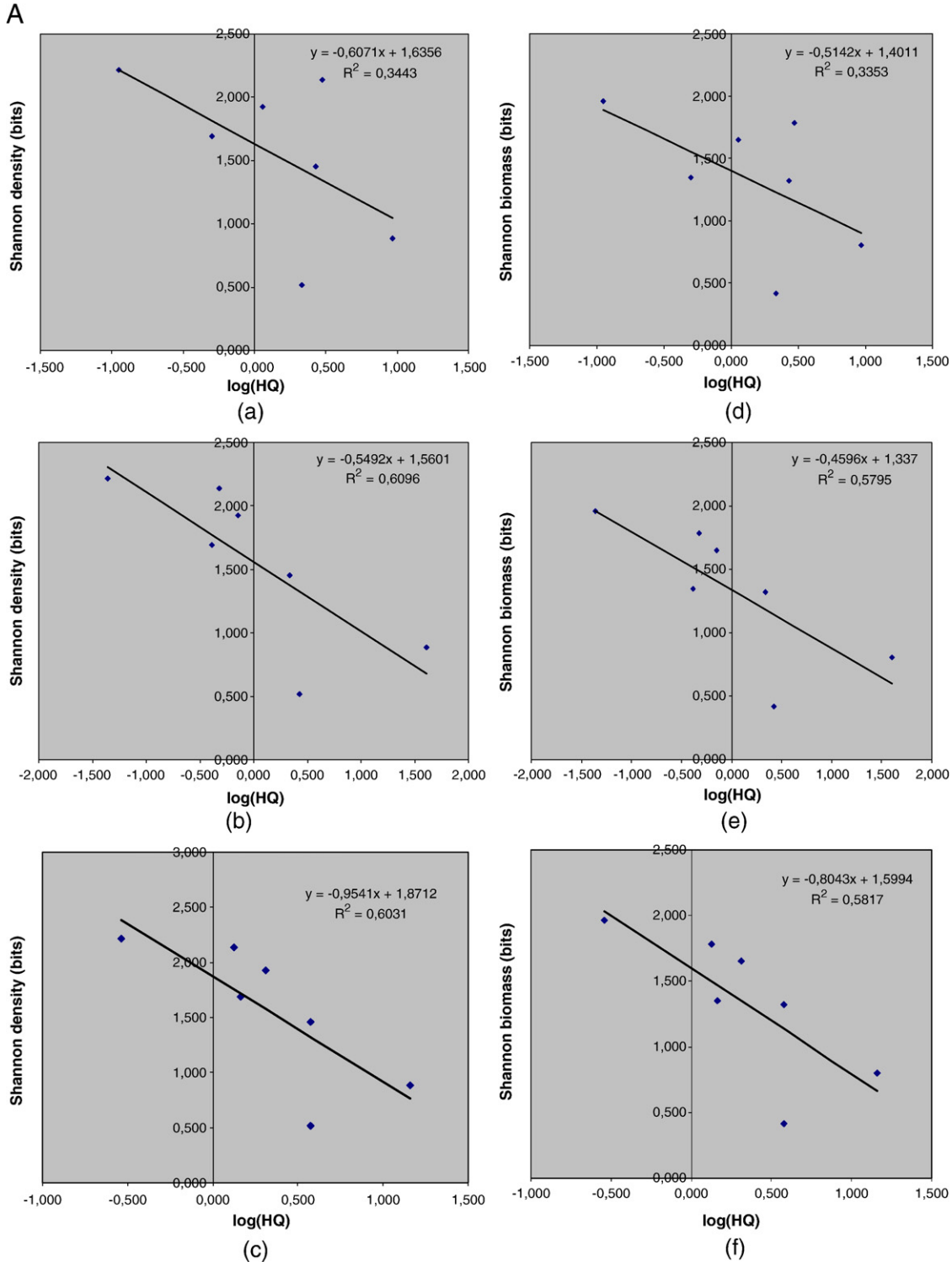

Fig. 5. A. Correlations between log-transformed total hazard indexes log(*HQ*) and Shannon diversity indexes. (a) Fish toxicity vs. Shannon diversity (density), (b) *Daphnia magna* toxicity *vs.* Shannon diversity (density), (c) Algae toxicity *vs.* Shannon diversity (density) (d) Fish toxicity *vs.* Shannon diversity (biomass), (e) *Daphnia magna* toxicity *vs.* Shannon diversity (biomass), (f) Algae toxicity *vs.* Shannon diversity (biomass). B. Correlations between log-transformed total hazard indexes log(*HQ*) and Shannon diversity indexes, excluding point A2. (a) Fish toxicity *vs.* Shannon diversity (density), (b) *Daphnia magna* toxicity *vs.* Shannon diversity (density), (c) Algae toxicity *vs.* Shannon diversity (density) (d) Fish toxicity *vs.* Shannon diversity (biomass) (e) *Daphnia magna* toxicity *vs.* Shannon diversity (biomass) (f) Algae toxicity *vs.* Shannon diversity (biomass).

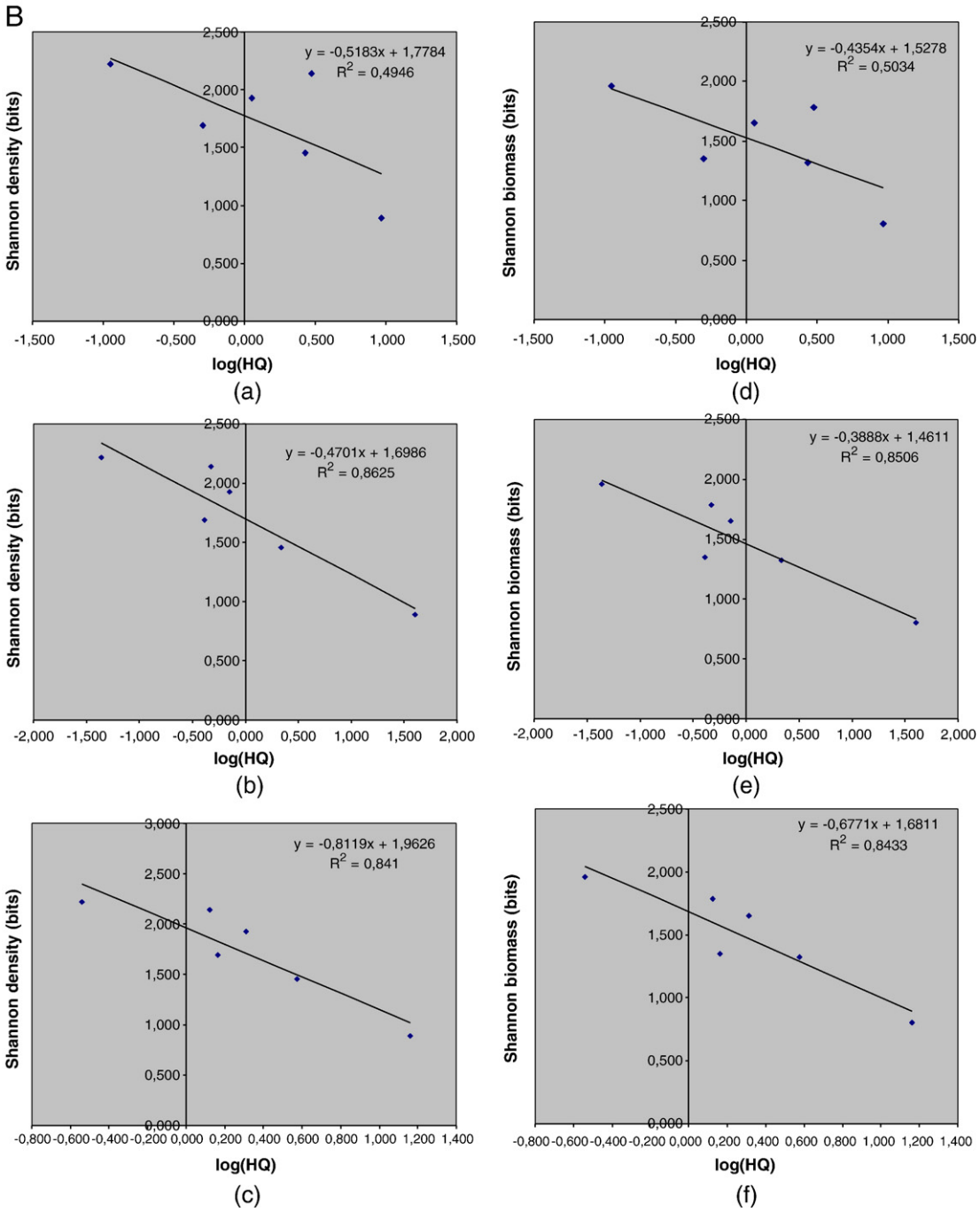

Fig. 5 (*continued*).

4. Conclusions

The relationship between ecological status and chemical status can be considered as one of the key issues of the Water Framework Directive. In the present study we have tried to put together both aspects of aquatic quality. Data concerning both ecological status, expressed in terms of diversity, and chemical contamination, for a specific family of micropollutants, i.e., pharmaceutical products, have been presented. A risk assessment study has been carried out through the determination of hazard quotient indexes, calculated from measured environmental concentrations and ecotoxicity values using three different bioassays, namely, fishes, *Daphnia* and algae. As expected, each bioassay shows its own sensitivity with respect to certain compounds. Whereas some compounds, such as ibuprofen, diclofenac and fibrates (clofibric, gemfibrozil) have influence in the three bioassays, others, like erythromycin for *Daphnia*, or sulfamethoxazole for algae, show much more specificity.

In general, hazard quotients tend to increase when going downstream. Assuming that *HQ*s lower than unity are indicative of no risk, it must be noticed that this situation is only observed in 3 sites (out of the 7 studied) for fish, 4 for *Daphnia* and 1 for algae, which are located upstream of the two rivers studied. Among them, only one site, located in the upper Anoia tributary, shows the three indexes below unity. When the three bioassays are compared, algae appear to be the most sensitive, followed by fish and *Daphnia*. The results found show a clear inverse linear correlation between macroinvertebrate

Table 5
Linear correlation equations between Shannon diversity indexes (*DI*) and log-transformed hazard indexes log(*HQ*).

y	*x*	$y = ax + b$	*n*	*r*	Remarks
DI(density)	log(*HQ*) fish	$y = -0.6071x + 1.6356$	7	−0.587	–
DI(density)	log(*HQ*) *Daphnia*	$y = -0.5492x + 1.5601$	7	−0.781	–
DI(density)	log(*HQ*) algae	$y = -0.9541x + 1.8712$	7	−0.777	–
DI(biomass)	log(*HQ*) fish	$y = -0.5142x + 1.4011$	7	−0.579	–
DI(biomass)	log(*HQ*) *Daphnia*	$y = -0.4596x + 1.3370$	7	−0.761	–
DI(biomass)	log(*HQ*) algae	$y = -0.8043x + 1.5994$	7	−0.763	–
DI(density)	log(*HQ*) fish	$y = -0.5183x + 1.7784$	6	−0.703	A2 excluded
DI(density)	log(*HQ*) *Daphnia*	$y = -0.4701x + 1.6986$	6	−0.929	A2 excluded
DI(density)	log(*HQ*) algae	$y = -0.8119x + 1.9626$	6	−0.917	A2 excluded
DI(biomass)	log(*HQ*) fish	$y = -0.4354x + 1.5278$	6	−0.710	A2 excluded
DI(biomass)	log(*HQ*) *Daphnia*	$y = -0.3888x + 1.4611$	6	−0.922	A2 excluded
DI(biomass)	log(*HQ*) algae	$y = -0.6771x + 1.6811$	6	−0.918	A2 excluded

(*a* slope; *b* intercept; *n* number of points; *r* correlation coefficient, $p < 0.05$).

diversity indexes and hazard quotients that can be tentatively (but not conclusively) interpreted as cause–effect. Further work on that subject appears necessary.

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