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19 # BRIDGING LEVELS OF PHARMACEUTICALS IN RIVER WATER WITH BIOLOGICAL

20 # COMMUNITY STRUCTURE IN THE LLOBREGAT RIVER BASIN (NE SPAIN)

21

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Abstract–A wide range of human pharmaceuticals are present at low concentrations, in freshwater systems, particularly in sections of polluted river. These compounds show high biological activity, often associated with a high stability. These characteristics imply a potential impact of these substances on aquatic biota even when present at low environmental concentrations. Low flow conditions in Mediterranean rivers, most of which flow through densely populated areas and are subjected to intensive water use, increase the environmental risk of these emergent compounds. Here we studied whether pharmaceuticals in river water affect the local benthic community structure (diatoms and invertebrates). For this purpose, we analyzed the occurrence of pharmaceuticals along the Llobregat River and examined the benthic community structure (diatoms and invertebrates) of this system. Some pharmaceutical products in the Llobregat River registered concentrations greater than those cited in the literature. 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*). Further interpretation in terms of cause-effect relationships is discussed, however it must be always taken with caution since other pollutants may also have significant contributions. Combined with further community experiments in the laboratory, our approach could be a desirable way to go on in future risk management decisions.

Keywords–Llobregat River, Pharmaceuticals, Invertebrates, Diatoms, Multivariate analysis

64

65 **Introduction**

66

67 Pharmaceuticals comprise an array of products, including a variety of chemical formulations and
68 multiple biological targets. These drugs exert specific biological effects; and are administered for
69 human and veterinary health care. Although a variety of pharmaceutical compounds have been
70 detected in the environment, their potential ecological significance remains unknown and few
71 studies have addressed their impact on non-target species [1].

72

73 As a result of human population density and more intensive animal farming techniques,
74 water catchments are highly susceptible to be at risk for potential contamination by various
75 pharmaceutical products. A wide range of human pharmaceuticals, including analgesics,
76 antibiotics, steroids, cardiovascular drugs, and various drugs used to treat mental illness, are
77 present in effluents from sewage treatment plants that continuously enter freshwater systems [2-
78 4].

79

80 Although concentrations of pharmaceuticals in the aquatic environment are generally in
81 the nanogram-per-liter and low microgram-per-liter range, these compounds show high reactivity
82 with biological systems; and are often highly stable. These characteristics may cause potential
83 toxic effects to aquatic organisms even at low environmental concentrations [5]. Most studies in
84 this field have addressed the effects of pharmaceuticals on aquatic vertebrates, namely fish.
85 Chronic toxicity data for algae and invertebrate have become more available in recent years,
86 although they are usually obtained from aqueous-exposure experiments [6] rather than from field

87 studies. Although pharmaceuticals are often moderately lipophilic [7], few studies have
88 examined their potential impact on benthic communities and sediment organisms.
89
90 The rationale for the present study was to focus on algae and invertebrate fauna as
91 representatives of aquatic benthic communities inhabiting the sediment interphase, with the aim
92 to determine the potential effect of pharmaceuticals on the abundance and community structure
93 of these organisms. These chemicals, transported by water, are adsorbed by particulate matter
94 and accumulated in sediments. After adsorption, chemicals can be remobilized by resuspension
95 or desorption, being a primary source of contamination for benthic organisms.
96 Analyses of chronic toxicity of pharmaceuticals on organisms are essential to obtain a
97 realistic environmental risk assessment, since these substances were designed to exert distinct
98 molecular modes of actions. However, studies in this field usually provide data only for target
99 species and work with concentrations far above those found in nature. Multi-species tests, even
100 in standardized bioassays, allow realism in terms of ascribing biological endpoints to
101 contaminant impact [8]. Data on the responses of natural communities in field conditions to
102 pharmaceuticals may provide the information required to define the ecological relevance of these
103 substances.
104
105 Given that flowing waters are exposed to multiple stressors, the effects of which can be
106 cumulative and interact across spatial and temporal scales, defining causal relationships can be
107 complex. To establish some relationships between environmental stressors and biological
108 response variables, it is necessary to integrate laboratory, field and other experimental
109 approaches such as mesocosms [9]. Field observations are essential for the detection of emerging

consequences of stressors on communities, and for allowing the generation of hypotheses to identify potential cause-and-effect relationships.

The management of Mediterranean river basins implies considering their particular hydrology (low winter and summer discharges and periodical floods in spring and autumn) as well as continuing human pressure on resources and on the ecosystem. Characterized by low flows during normal conditions (~5 m3/s) and extraordinary peak events (maximum recorded of 2500 m3/s in 1971) which periodically resets the system, the Llobregat River (NE Spain) is a good example of a Mediterranean basin. The middle and lower sections of the river drain densely populated and industrialized (tannery, textile, pulp, and paper) areas. The major land use types in this area are: 38% urban and industrial activities and 13% farmlands. Furthermore, the Llobregat provides drinking water to the large conurbation of Barcelona.

Several works have investigated the presence of various synthetic and natural estrogens and progestogens [10, 11] and other pharmaceuticals [12, 13] in scattered locations in the Llobregat river basin. The invertebrate community of this river has been used as indicator of its ecological quality since the early 1980s [14, 15]. Diatom data from this river have also been available since the 1980s [16-18] and a surveillance program including macroinvertebrates and diatoms is currently implemented by the Catalan Water Authority.

Within this framework, here we aimed to analyse the occurrence of pharmaceuticals in the water in the Llobregat River basin; and to find potential relationships between the presence of pharmaceuticals and the structural composition (changes in abundance and biomass) of the

biological community (benthic algae and invertebrates) in this river. A total of 29 pharmaceuticals, including analgesics, anti-inflammatories, lipid regulators, antibiotics, lipid regulators, psychiatric drugs, anti-histamines and Beta blockers, were analyzed in river water. Other physical and chemical variables related to eutrophication and other environmental characteristics were also analyzed to delineate their influence on the natural community structure. Thus, to the best of our knowledge, the present study is the first to provide extensive data on the occurrence of a large number of pharmaceuticals in the aqueous phase of the Llobregat basin, and in a range of locations and time periods. On the basis of our findings, spatial and temporal variations in pharmaceutical load in the river can be deduced, together with the potential effects of these substances on biota.

Material and Methods

Study site

The Llobregat River (NE Spain) is 156.5 km long and covers a catchment area of approximately 4948 km2. The Llobregat River watershed is heavily populated (3,089,465 inhabitants in 1999). Together with its two main tributaries, the River Cardener and the River Anoia, the Llobregat is a paradigm of overexploited Mediterranean rivers. The river has a mean annual discharge of 693,000,000 m3 and near 30% is used for drinking water. The Llobregat receives extensive urban and industrial wastewater discharges (137,000,000 m3/year; 92% comes from the wastewater treatment plants) that cannot be diluted by its natural flow (0.68-6.5 m3/s basal flow). Forty-eight percent of these point sources are located in the studied area. In addition, the

156 middle part of the basin receives natural salt slurries from salt formations, which have caused an
157 increase in water salinity downstream.
158
159 Four sampling sites were selected from the middle and lower part of the Llobregat main
160 channel and another three were selected in the Anoia tributary (**Fig. 1**). These sites were part of a
161 pollution gradient: sites LL1 and A1 were the least polluted but received some industrial
162 effluents and surface runoff from agricultural areas. Sites A3 and LL4 were located in the last
163 section of the two rivers, and were the most polluted sites. Site A2 was located in a highly
164 polluted area receiving wastewaters from tannery, textile and paper industries. Sites LL2 and
165 LL3 were located in a densely inhabited area and received urban and industrial wastewaters
166 inputs.
167
168 Water samples for chemical (nutrients, cations and anions) and pharmaceutical analysis
169 were collected simultaneously with the biological samples from the sediments. Sampling was
170 performed in early June 2005, November 2005, and late May 2006. These three periods covered
171 two of the most relevant periods (spring and autumn) in the system in terms of its hydrology.
172 Samples for grain size characterization were taken in the two first samplings. Ninety-eight
173 percent of the sediment was gravel and sand in all sites. Sites A1, A2, LL1, and LL2 had a
174 lightly more proportion of gravel and A3 a higher proportion of fine sand respect to the others
175 sites.
176
177 *Water quality parameters*
178

179 The pH, water temperature, conductivity and oxygen concentration were measured in situ with
180 appropriate probes. Water samples for nutrient analysis were collected in triplicate in each
181 sampling period. The samples were filtered immediately (Nylon Membrane Filters 0.2 mm,
182 Whatman) and frozen until analysis. Nitrate, sulphate, chloride, sodium, potassium, and calcium
183 were determined by ion chromatography (761 Compact IC, Metrohm, Herisau Switzerland).
184 Soluble reactive phosphate and ammonium were measured following standard procedures.
185
186 *Analysis of pharmaceuticals*
187 A total of 29 pharmaceuticals, belonging to the classes of analgesics and non-steroidal anti-
188 inflammatories, lipid regulators, psychiatric drugs, anti-histamines, anti-ulcer agents, antibiotics
189 and Beta-blockers were measured in water by means of off-line solid-phase extraction (SPE)
190 followed by liquid chromatography-tandem mass spectrometry. Briefly, water samples (400 ml),
191 previously passed through 0.45 µm nylon membrane filters, were preconcentrated on Oasis ®
192 HLB (hydrophilic-lipophilic balance; 60 mg, from Waters (Milford, USA) cartridges, which
193 were further rinsed with 5 ml of high-performance liquid chromatography-grade water, dried
194 under vacuum for 15 to 20 min, and eluted with 2x4 ml of methanol. The extracts were then
195 evaporated under a gentle nitrogen stream, reconstituted with 1ml of methanol-water (25:75,
196 v/v), and added to 10µl of an internal standard mixture. Analysis of the extracts was performed
197 by liquid chromatography-triple quadrupole-tandem mass spectrometry, equipped with an
198 electrospray interface, operating in the SRM mode. Two SRM transitions were monitored per
199 compound. Nine of the 29 compounds were measured in negative ion mode and the remaining 20
200 in positive ion mode. This method and its validation (linearity, detection and quantification
201 limits, repeatability, etc.) are described in detail in Gros et al. [19].

202

203 *Diatom analysis*

204 The sampling of biofilms for diatom analysis included those growing on large sediment particles
205 (cobbles and gravel; epilithic biofilms) and sand (in the site LL4). Sand samples (2-5cm depth)
206 were collected with a polyvinyl corer, and top sub-samples were taken by an untapped syringe.
207 Epilithic biofilms samples were collected by scrapping a known surface (1cm2) of gravel or
208 cobbles with a knife. Samples were fixed with 4% formaldehyde for further identification and
209 counting. One replicate from each sampling site was cleaned by means of acid oxidation. The
210 cleaned frustules were mounted on resin (Naphrax, refraction index 1.74; Brunel Microscope) on
211 permanent slides. The diatom community was observed and identified under a light microscope
212 (Nikon Eclipse 600W) using phase-contrast and Nomarski differential interference contrast
213 optics at a magnification of
214 x1,000. At least 400 valves were counted and identified to establish the relative abundance of the
215 diatom species present in each sample.

216

217 *Invertebrate analysis*

218

219 Five sediment samples (10-15 cm depth) were randomly collected with a polyvinyl sand corer in
220 each sampling site. The animals retained when sieving the sediment through a 500 mm mesh (the
221 benthic macrofauna) were fixed with 4% formaldehyde. The invertebrates were sorted, counted
222 and identified in the laboratory under a dissecting stereoscopic microscope. Identification was at
223 family level for Oligochaeta and at the genus or species level for the rest of the groups present
224 (mainly Chironomidae and Ephemeroptera). Biomass was calculated as a dry weight from length

dimension using exponential equations [20]. Abundance and biomass were referred to the sediment surface area.

Data analysis

Diatom and invertebrate taxa accounting for more than 1% of total abundance in at least two of the samples were included in the analyses. Taxa abundances were square root transformed prior to analysis. A preliminary analysis based on the Bray-Curtis similarity index for abundance data was conducted to examine the extent to which macroinvertebrate and diatom assemblages varied among samples. This index calculates the similarity between two sites on the basis of species composition. A non-metric multi-dimensional scaling (MDS) procedure was used to ordinate communities (and hence sites) on the basis of the similarity between each pair of samples estimated with the Bray-Curtis similarity index for abundances and Euclidean distances for invertebrate biomass data [8]. The MDS draws the sample distribution in a space delimited by a maximum of three axes. Short distances indicate high similarity in community composition between sites. The stress index measures the goodness of fit between the rank order of similarities and the rank order of distances [8]. Stress values higher than 0.3 indicates that the points are closed to being arbitrarily placed in the two dimensional ordination space.

Physicochemical variables, except pH were transformed to reduce skewed distributions. Values of environmental and pharmaceutical variables were normalized by subtracting them from the mean and dividing this number by the standard deviation before their inclusion in the analyses. To avoid co-linearity, the variables Ca+ (correlated with Mg+, r=0.95) and ranitidine concentration (correlated with sotalol, r=0.96) were unselected. When the pharmaceutical

248 concentrations were below method detection limits, a value equal to one-half the method
249 detection limit was assigned to these data in the statistical analyses.
250
251 The optimal match between the community patterns and the environmental variables
252 associated with those samples was explored with the BEST (Bio-Env-Stepwise) procedure. This
253 procedure searches for the highest rank correlation (Spearman correlation) between the species
254 similarity matrix (calculated with abundances and biomass for invertebrates) and the
255 environmental matrix (based on Euclidean distances). Spearman rank correlations were
256 calculated by matching element to element [21]. The significance of the rank was assessed using
257 the Monte Carlo permutation test (999 unrestricted permutations). These analyses were
258 performed with the PRIMER® 6 statistical package (Plymouth Marine Laboratory, UK, 2001).
259
260 In addition, invertebrate data were analysed by detrended correspondence analysis to
261 explore the species responses along an ordination axis. The maximum length of the gradient
262 obtained with the detrended correspondence analysis was 2.44, indicating that linear methods
263 were appropriate [22]. Consequently, we carried out a redundancy analysis, in which species data
264 were constrained by environmental variables. This ordination assumes a linear combination of
265 the species along the environmental gradients preserving the Euclidean distances. The
266 redundancy analysis was performed with CANOCO® software, version 4.5 [22].
267
268
269

Results and Discussion

Water quality parameters

Average values of the water quality parameters in the studied sites are shown in **Table 1.** The Llobregat and Anoia waters were characterized with high conductivity values and elevated ion concentrations. The upstream Anoia site had waters with high sulphate concentrations because of its gypsum bedrock. Salt (mainly KCl and NaCl) outcrops in the Llobregat basin caused the high concentration of these ions and the corresponding high water conductivity.

The sites A2, LL3 and LL4 showed the highest nutrient concentrations and lowest dissolved oxygen. Water flow was low in the two spring seasons (2005, mean discharge in June of 4.79 m3/s, and in 2006, 5.67 m3/s, at LL2 site) and nutrient loads and conductivity increased substantially from upper to downstream reaches. In autumn 2005 the river discharge was higher (mean discharge in October: 8.71 m3/s) and most of the concentrations reached their lowest values.

Pharmaceuticals in the Llobregat River

Twenty-one of the 29 compounds studied were present in at least one of the samples analyzed (**Table 2**). Twelve of these compounds presented maximum concentrations above 1 µg/L and three (the analgesic diclofenac, the lipid regulator bezafribate, and the antibiotic sulfamethoxazole) exceeded 10 µg/L. Mean concentrations higher than 1µg/L were found for eight analytes. The highest mean concentrations were observed for ibuprofen (in sites A3, LL4),

293 diclofenac (A2, LL4), clofibric acid (LL4), and ofloxacin (LL4), mainly as a result of punctual
294 maximum concentrations in specific sites. Analgesics, anti-inflammatories, lipid regulators and
295 antibiotics were the families with the highest concentrations.
296
297 No significant seasonal variations in pharmaceutical occurrence were detected (analysis of
298 variance, time as a factor, $p>0.05$) but a clear spatial pattern was observed in sites A2, A3, and
299 especially LL4, which registered the highest concentrations (analysis of variance, site as a factor
300 and post-hoc comparisons with Tukey test, $p<0.05$) (**Fig. 2**).
301
302 The pharmaceutical products observed in the Llobregat closely matched those identified by the
303 Spanish National Health System as those most consumed. These are mostly analgesics, drugs to
304 treat ulcer, anti-histamines, antibiotics, and antidepressants. The concentrations of
305 pharmaceuticals that we recorded in the Llobregat River were higher than those reported by Gros
306 et al [3] in the Ebro River, where concentrations ranged from 0.1 to 0.6 µg/L. In low flow
307 periods in a Mediterranean river in France, Comoretto and Chiron [23] reported similar
308 concentrations for carbamezapine and bezafribrate to those monitored in our present study. The
309 concentration of pharmaceuticals in the Llobregat River were in general in the range described
310 by Fent et al. [2], except for ketoprofen, diclofenac, gemfibrazil, bezafibrate, and ranitidine
311 which were detected at higher mean concentrations in our present study.
312
313 *Biological community structure in the Llobregat River*
314

315 The ordination of the diatom assemblages (**Fig. 3A**) was derived from the two-dimensional MDS
316 based on Bray-Curtis similarities calculated from root-transformed diatom abundances. Diatom
317 ordination was highly similar between the least polluted sites (A1 and LL1), which showed the
318 highest species richness. The community was dominated by the taxa *Navicula cryptocephala* in
319 A1 and *Nitzschia inconspicua* in LL1. The community composition of site A2, one of the most
320 polluted, clearly differed from the other sites.
321
322 The plot carried out with the data on invertebrate abundance (**Fig. 3B**) separated one
323 group with the most polluted sites A2 and LL4. Most of the sites (except A1 and A3) in the
324 autumn sampling formed a separate group related to the general low abundance found in these
325 sites as a result of the higher discharge in that period. The rest of the sites presented a more
326 diverse community characterized by the presence of mayflies and several families of worms. The
327 ordination carried out with the data on invertebrate biomass (**Fig. 3C**) showed sites A2 and LL4
328 to be separated from the others. These sites were characterized by the dominance of the non-
329 biting midge *Chironomus* spp (mainly *bernensis* and *plumosus*) both in terms of abundance and
330 biomass (**Fig. 4A**). Site A3 was characterized in the first spring sampling by the dominance of
331 Oligochaeta (mainly *Tubifex tubifex*, **Fig**. **4B**). This taxon was also present in the other sampling
332 periods but with moderate values.
333
334 Communities of diatoms and invertebrates found in this study are characteristics of a
335 perturbed fluvial system. There was a general decrease of species richness downstream and
336 prevalent abundance of the most tolerant species to organic and chemical pollution. Salinity,

high nutrient concentration and low flow are considered to be responsible for biologically poor communities, made up of tolerant taxa [15, 17].

Relationships between chemical and biological parameters

The results of the BEST procedure showed no significant correlation between the diatom community and the physicochemical and pharmaceutical variables. However, a significant rank correlation (0.492, p=0.001) was observed between invertebrate abundance and the concentrations of indomethacine and propranolol, as well as with water temperature. Invertebrate biomass also showed a significant rank correlation (0.810, p=0.002) with the concentration of ibuprofen, atenolol and propranolol. On the basis of the results from the Best analysis, if the true driving abiotic variables are selected, and two sites have very similar suites of values for these, then the assemblages would also be expected to be similar (and vice-versa). This was the case in sites A3 and LL4, where the reduced invertebrate community, mostly made up of midge larvae (*Chironomus* spp.) and Oligocaheta showed higher abundances and biomass when the river carried higher concentrations of anti-inflammatories and Beta-blockers (**Fig. 4D**). Remarkably, temperature was the only selected environmental parameter related to invertebrate community abundance. This observation probably reflects the spatial arrangement of the polluted sites. Neither nutrients nor conductivity were selected as significant variables correlated with invertebrate community in these sites, regardless of their relevance as by-products of human activities in the Llobregat River and their established effect on the biological communities [15]. The finding that diatom distribution was not affected by the pharmaceutical products is also relevant, however, this observation may be attributed to the mode of action of these products, which does not directly affect the primary producers at the observed concentrations [2].

The RDA determined that the concentration of some anti-inflammatories and propranolol, as well as temperature, explained 71% of the taxonomic variance in invertebrate density (**Fig. 5**). The first RDA axis reflected the distribution of sites along the presence of indomethacine and propranolol but also on the increase of temperature. Ibuprofen was the variable most correlated with axis 2; temperature was also correlated with this axis (**Table 3**). Sites A2 and LL4 were associated with high concentrations of these pharmaceuticals, in contrast to the remaining sites. The position of the samples in the second axis was associated with the high concentration of ibuprofen in site A3, especially in the first sampling period. *Chironomus* spp. abundance was closely associated with the highest concentrations of propranolol and indomethacine and colder waters that were characteristic of site LL4 (**Fig. 5B**). Instead, the abundance of families of Oligochaeta (Naididae, Enchytraeidae, and Tubificidae) was found to be related to higher ibuprofen concentrations (site 3). The remaining taxa were related to low concentrations of pharmaceuticals (Fig. 5B).

The RDA analysis for diatoms showed a slight correlation of the environmental variables with the axis, and no significance of the Monte Carlo test was found.

The results of both multivariate analyses for invertebrate assemblage were similar, sites with higher concentrations of anti-inflammatories and Beta blockers and higher temperatures were characterized by a greater abundance and biomass of *Chironomus* spp. and *Tubifex tubifex*. Several studies on the effects of chronic exposure indicate that ibuprofen has very little impact on aquatic environments [24-26]. However, an increase in the somatic growth of *Daphnia magna*

383 population when exposed to ibuprofen (20 mg/L) and a reduction in reproduction has been
384 described [27]. These authors suggested that the negative impact on reproduction would release
385 energy to be invested in growth. Preliminary results (López-Doval, unpublished results) of a
386 chronic laboratory test with *Chironomus riparius* exposed to sediments spiked with
387 indomethacine (120 µg/g sediment), show an increase of nearly 60% of individual growth
388 respect to the control treatment.
389
390 Other authors listed numerous effects of anti-inflammatories in freshwater organisms, for
391 example, Schwaiger et al. [28] and Triebskorn et al. [29] detected bioaccumulation and
392 hystopathological alterations in kidney and gills in rainbow trout and common carp exposed to
393 diclofenac (lowest-observed-effect concentrations: 1-5 mg/L) at a concentration range found in
394 our present study. Pharmaceuticals into the environment may affect the same pathways in
395 animals with similar target organs, tissues, cells or biomolecules. Knowledge about these targets
396 exists primarily for fish but less is known in invertebrates or other phyla [2].
397
398 The toxicity of the Beta-blockers is difficult to ascertain in invertebrates since most
399 studies have analyzed the effects only in *D. magna* [30]. Propranolol shows the highest acute
400 toxicity and highest log K_{OW} among beta-blockers. Stanley et al. [31] found 48-h propranolol
401 median effective concentration value for *D. magna*.of 1.67 mg/L. These experimental
402 concentrations were extremely higher than those we registered in the Llobregat water. Beta
403 blockers may have several effects on fish, such as cardiovascular dysfunction and impairment of
404 fitness [32] or reproduction [33]. Propranolol swells fish erythrocytes, thereby affecting oxygen
405 uptake.

406 Some psychiatric drugs, like carbamazepine, show low acute toxicity in aquatic
407 organisms, particularly invertebrates [34]. However, in response to chronic sediment-exposure,
408 the midge *Chironomus riparius* shows a blockage of pupation and emergence (10% effective
409 concentration values 70 to 210 mg/kg dry wt). Although information on the concentrations of
410 pharmaceuticals in sediment is limited, it is feasible that these compounds accumulate in this
411 compartment [35], thereby posing a risk for the survival of populations of benthic organisms.
412
413 Some of the mechanisms of action of pharmaceuticals described in the literature could
414 explain the association between these substances and the invertebrates found in the Llobregat
415 River. However, the correlational findings could also be the result of cumulative or synergistic
416 effects caused by several stressors that co-occur in the system. Pharmaceuticals do not appear as
417 isolated compounds in river water, but as a mixture, and data on the responses of aquatic
418 organisms to a mixture of pharmaceuticals is very limited. Escher et al. [36] reported higher
419 toxicity of a pharmaceutical mixture than separated products. Flaherty et al. [37] indicated
420 unpredictable and complex effects of a pharmaceutical mixture on *Daphnia* survival, growth and
421 reproduction. Moreover, other indirect effects can be influential factors, such as habitat and food
422 availability, species competition or predator-prey interactions. Although habitat characterization
423 (sediment grain size) showed slight differences in the studied sites, little is known about the other
424 parameters. Therefore, our results should be taken as indicative and require further experimental
425 testing in controlled conditions such as mesocosms [38-40].
426
427
428

Conclusion

Several pharmaceutical products in the Llobregat River were found at concentrations higher than those cited in the literature. The low water flow of Mediterranean rivers increases the potential environmental risk of these emerging water contaminants. Thus, much of the aquatic biota will be exposed, throughout their lives, to complex mixtures of these compounds. Like other contaminants, the best way to reduce the ecological impact of pharmaceuticals in rivers may be to prevent input by improving sewage treatment procedures.

One of the major objectives for environmental scientists is to establish causal links between stressors and the quality of ecological systems. Procedures that identify causality allow corrective action measures for habitat recovery and control. Identifying the cause of biological impairment in freshwater systems is also an essential objective of the European Water Framework Directive. Our results reveal a potential causal relationship between the concentration of a number of pharmaceutical products and the abundance and biomass of several key benthic invertebrates. Although our assessment has been based on field data, it provides evidence on how pharmaceuticals disrupt biological communities. Although strict guidelines for developing cause and effect relationships are not well established, some criteria support causality in environmental impact studies [40]. Experiments that include evidence from multiple field and laboratory studies are stronger than those based alone on only one kind of data. Knowledge of field patterns will help us to focus subsequent monitoring efforts and to identify key taxa for use as ecological indicators. This approach might be useful to find spatial and temporal correlation of stressor and effects along gradients, although it should be combined with community experiments in the laboratory to examine hypotheses generated from field studies. This could be a desirable way to go on in future risk management decisions for these emerging water

452 contaminants taking into account both the scarcity of experimental studies with durations longer
453 than a few weeks and the few works that used multi-species in standardized bioassays.
454
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460
461
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575

576 **Figure legends**

577

578 Figure 1. Map of the sampling sites (Spain).

579 Figure 2. Concentrations (µg/L) of the families of pharmaceuticals in the sites studied for the
580 three sampling campaigns.

581 Figure 3. (**A**) Non metric multidimensional scaling (MDS) ordination of the sites using a Bray-
582 Curtis similarities on root-transformed diatom species relative abundances. Lines delimit the
583 groups formed by a cluster analysis (group average linked) at 40 % of similarity, (**B**) MDS site
584 ordination for invertebrate abundance (individual/cm2) using the same similarity index, (**C**)
585 MDS site ordination using Euclidean distances for invertebrate biomass (mg/cm2), lines delimit
586 the groups formed by cluster analysis at 1.1 distance index. Site codes: (A) spring 2005; (B)
587 autumn 2005; (C) spring 2006.

588 Figure 4. (**A**-**B**) Invertebrate abundance multidimensional scaling (MDS) plot from Figure 3,
589 with values of *Chironomus* spp. and Tubificidae biomass (mg/m2) at distinct sites, superimposed
590 as circles of different sizes on the basis and proportional to biomass values, (**C**-**D**) the same
591 MDS plot on the basis and proportional to values of ibuprofen and propranolol concentrations
592 (É g/L) in water samples.

593 Figure 5. (**A**) Redundancy analysis (RDA) ordination plot for sites and environmental
594 relationships based on the abundance of invertebrate taxa. The environmental variables more
595 significantly correlated with the RDA axes are represented in the byplot by arrows, which point
596 was in the direction of maximum change in the value of associated variable. (**B**) Ordination of
597 invertebrate species on the first two environmental axes of the RDA.

598

Table 1. Physicochemical parameters and nutrients measured in the water samples collected at the selected sites. Average values and standard deviation (SD) are showed (*n*=3). SRP: Soluble reactive phosphate

	A1		A2		A3		LL1		LL2		LL3		LL4	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
pH	7.89	0.08	7.61	0.17	8.20	0.23	8.32	0.11	7.83	0.03	8.08	0.07	7.73	0.12
Temperature (ºC)	14.67	1.12	23.07	3.49	21.87	4.88	20.33	4.65	20.77	3.36	21.30	3.41	24.47	2.76
Conductivity (µS/cm)	3163.33	271.54	3863.33	443.77	2177.67	294.31	1457.33	65.74	1624.33	145.11	1833.00	40.71	2766.67	457.86
Oxygen (mg/L)	8.31	1.72	6.74	2.01	10.25	5.44	11.00	1.08	7.90	0.13	7.63	1.64	6.96	2.04
NO3 (mg/L)	6.92	5.99	24.05	20.09	4.84	4.10	6.19	6.40	6.64	4.85	7.98	5.34	4.52	3.31
SO4 (mg/L)	837.90	237.97	528.89	162.85	372.90	111.20	140.38	8.64	125.92	34.99	239.01	85.04	282.42	71.42
Cl (mg/L)	300.03	78.50	521.43	254.10	278.05	95.93	308.69	121.42	259.47	104.78	315.74	47.12	479.31	131.38
NO2 (mg/L)	0.10	0.07	0.59	0.73	0.43	0.35	0.08	0.08	0.14	0.06	-	-	0.99	0.64
SRP (µgPO4/L)	32.09	18.05	598.98	162.33	313.35	65.26	200.62	43.80	117.11	57.78	350.33	236.19	635.14	164.71
Na (mg/L)	195.38	30.02	349.87	202.38	209.85	76.08	131.26	13.45	146.53	43.10	168.80	42.68	284.65	40.95
K (mg/L)	12.12	9.23	23.65	13.33	28.42	24.97	45.27	25.11	46.42	29.36	23.06	19.39	72.47	50.23
Ca (mg/L)	207.64	21.70	119.34	37.85	113.72	24.87	68.24	4.09	59.98	7.07	71.84	10.41	77.37	6.67
Mg (mg/L)	79.16	19.46	45.68	13.83	43.45	13.13	24.09	6.17	23.15	7.52	29.25	9.66	27.24	13.30
NH4 (mg/L)	0.33	0.32	0.95	0.06	0.47	0.41	0.24	0.34	0.67	0.54	2.31	1.32	0.65	0.18

Table 2. Average, minimum and maximum concentrations (µg/L) of pharmaceuticals in the water samples analysed, and concentration at the 75th percentile (*n*=21).
MDL=method detection level

		Mean	Min	Max	75th	MDL (ng/L)
Analgesics and anti-inflamatories	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
Antiulcer 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

Table 3. Correlation between axes and environmental variables following redundancy analysis of invertebrate species abundance data from Llobregat River

	Axis 1	Axis 2
Propranolol	0.90	-0.13
Ibuprofen	0.11	0.63
Indomethacine	0.72	-0.12
Temperature	0.52	0.47
Species-environment	0.92	0.81
Eigenvalues	0.44	0.22

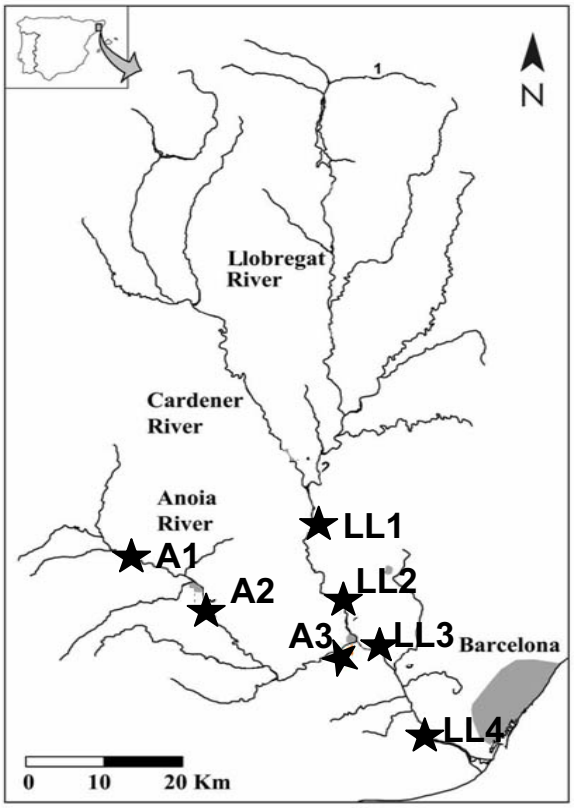

N
Llobregat River
Cardener River
Anoia River
LL1
A1
LL2
A2
A3
LL3
Barcelona
LL4
0
10
20 Km

Figure 1

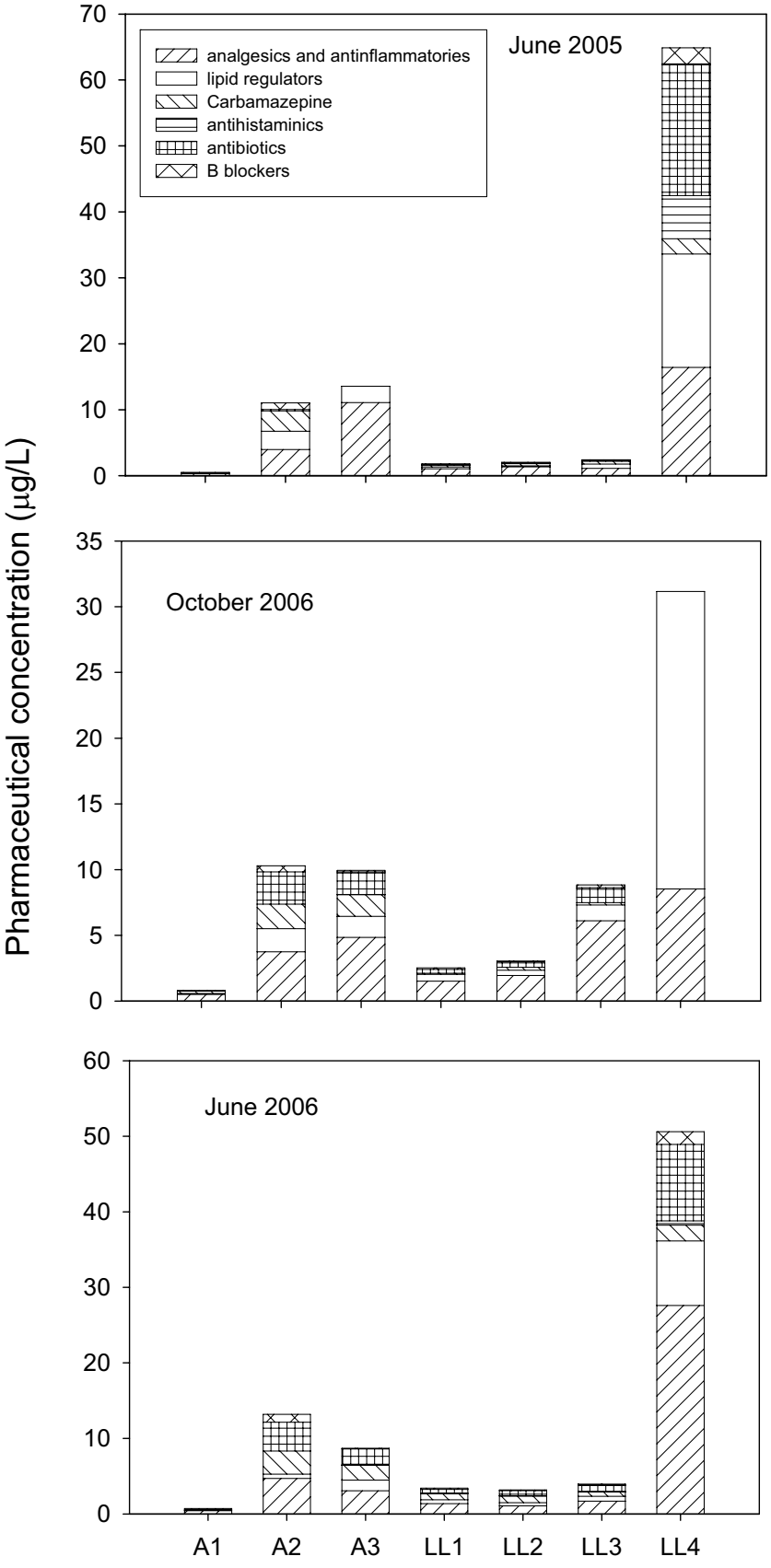
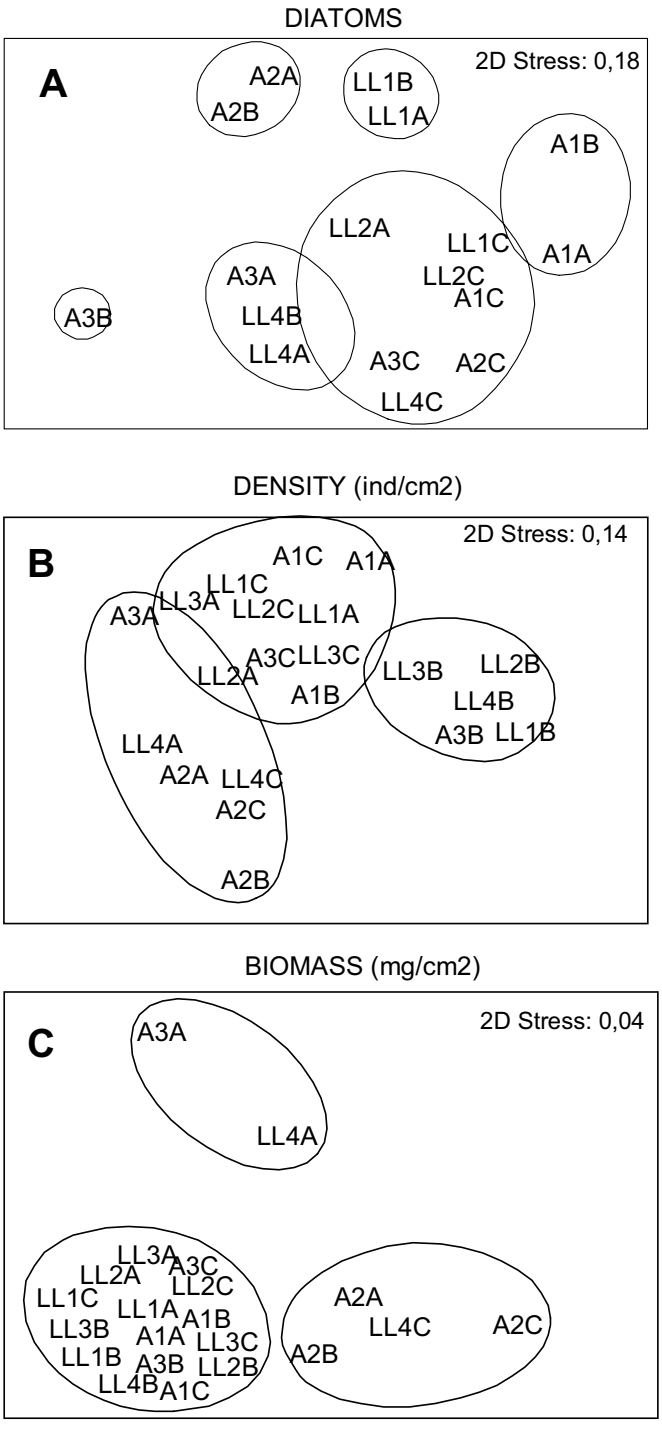

Pharmaceutical concentration (µg/L)
June 2005
analgesics and antinflammatories
lipid regulators
Carbamazepine
antihistaminics
antibiotics
B blockers
October 2006
June 2006
A1
A2
A3
LL1
LL2
LL3
LL4

Figure 2


DIATOMS
A
2D Stress: 0,18
A2A
A2B
LL1B
LL1A
A1B
LL2A
LL1C
A1A
A3A
LL2C
A1C
A3B
LL4B
LL4A
A3C
A2C
LL4C
DENSITY (ind/cm2)
B
2D Stress: 0,14
A1C
A1A
LL1C
LL3A
LL2C
LL1A
A3A
A3C
LL3C
LL3B
LL2B
LL2A
A1B
LL4B
A3B
LL1B
LL4A
A2A
LL4C
A2C
A2B
BIOMASS (mg/cm2)
C
2D Stress: 0,04
A3A
LL4A
LL3A
A3C
LL2A
LL2C
LL1C
LL1A
A1B
LL3B
A1A
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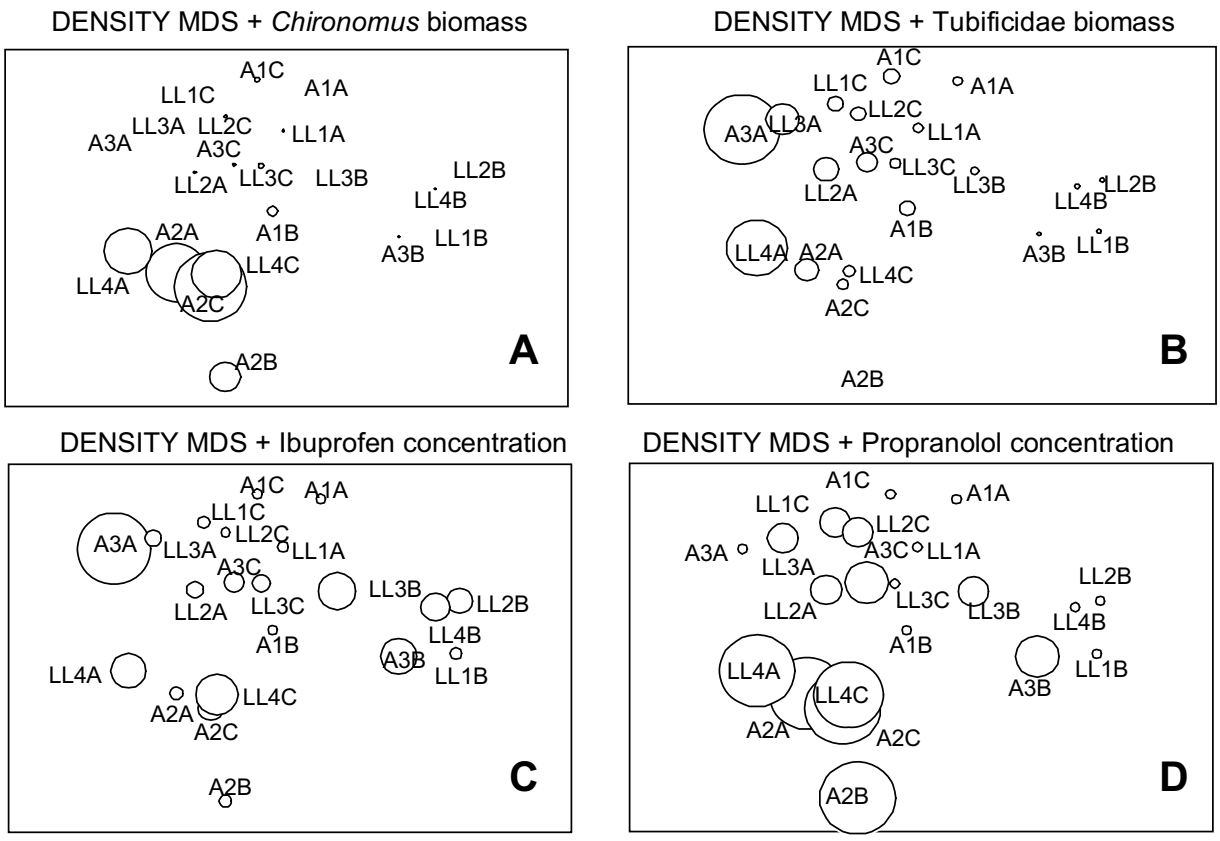
LL4C
A2C
A2B

Figure 3


DENSITY MDS + *Chironomus* biomass
A1C
A1A
LL1C
LL3A
LL2C
LL1A
A3A
A3C
LL2A
LL3C
LL3B
LL2B
LL4B
A2A
A1B
A3B
LL1B
LL4C
LL4A
A2C
A2B
A
DENSITY MDS + Tubificidae biomass
A1C
LL1C
A1A
LL2C
A3A
LL3A
LL1A
A3C
LL3C
LL2A
LL3B
LL2B
LL4B
A1B
LL4A
A2A
A3B
LL1B
LL4C
A2C
A2B
B
DENSITY MDS + Ibuprofen concentration
A1C
A1A
LL1C
LL2C
A3A
LL3A
LL1A
A3C
LL3B
LL2A
LL3C
LL2B
LL4B
A1B
A3B
LL4A
LL1B
LL4C
A2A
A2C
A2B
C
DENSITY MDS + Propranolol concentration
A1C
A1A
LL1C
LL2C
A3A
A3C
LL1A
LL3A
LL2B
LL2A
LL3C
LL3B
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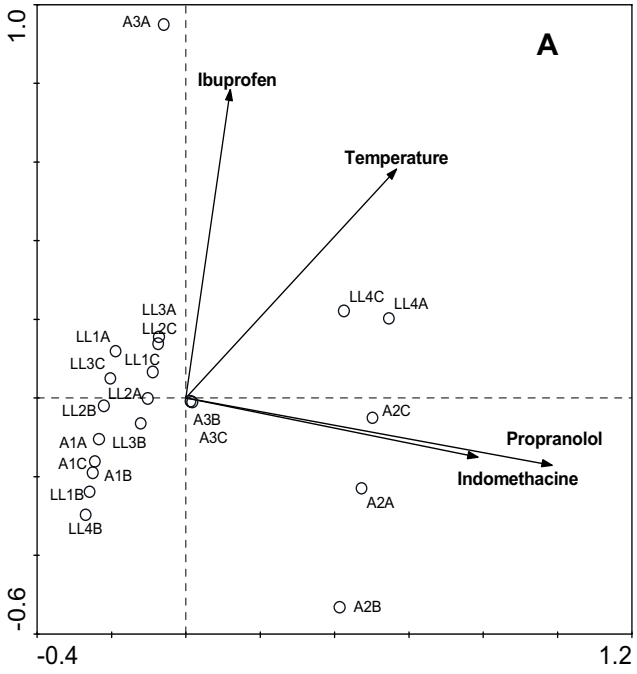
A3B
LL4C
A2A
A2C
A2B
D

Figure 4


A
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-0.4
1.2
A3A
Ibuprofen
Temperature
LL4C
LL4A
LL3A
LL2C
LL1A
LL3C
LL1C
LL2A
LL2B
A3B
A3C
A2C
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LL3B
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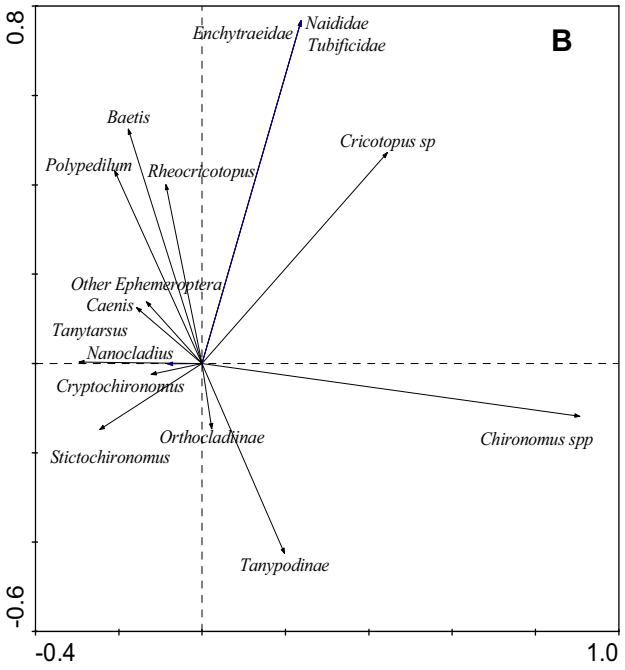
A1B
LL1B
LL4B
Propranolol
Indomethacine
A2A
A2B


B
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-0.6
-0.4
1.0
Enchytraeidae
Naididae
Tubificidae
Baetis
Cricotopus sp
Polypedilum
Rheocricotopus
Other Ephemeroptera
Caenis
Tanytarsus
Nanocladius
Cryptochironomus
Orthocladinae
Chironomus spp
Stictochironomus
Tanypodinae

Figure 5