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1 Use of multivariate analyses to investigate the contribution of metal pollution to diatom species
2 composition: search for the most appropriate cases and explanatory variables

3

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10

11 **Abstract**

12 The aim of this study was to elucidate how the spatial scale and the set of variables
13 included influence our ability to detect the effects of different types of pollution on the biota.
14 Using variance partitioning analysis we assessed the individual importance of a set of
15 environmental factors (eutrophication and organic pollution) versus metal level pollution on the
16 community structure of diatom assemblages at different spatial scales. At regional scale
17 environmental factors did not explain more of the variance compared to the watershed study. The
18 results of the watershed scale field survey indicate that diatom community composition was
19 influenced by low metal concentrations but this pattern was only observed by the inclusion of
20 biofilm metal concentration data. We recommend the analysis of metal traces in the water phase
21 and the biota (fluvial biofilms) as well as the use of the Diffusive Gradient in Thin films (DGT)
22 technique to characterize low metal level pollution in freshwater systems.

23 **1. Introduction**

24 The scale-dependence of ecological patterns and processes has been widely recognized by
25 freshwater ecologists, current knowledge of scale effects on monitoring networks is still
26 insufficient. Furthermore, this might be a complicated task in polluted rivers with different types
27 of pollution occurring simultaneously. The recognition of what factors structure benthic
28 communities at different spatial scales is also necessary for water policy makers. In the case of
29 Europe, this knowledge will contribute to the implementation of the European Water Framework
30 Directive (WFD) (Directive 2000/60/EC). The WFD is an important piece of legislation aiming to
31 achieve a “good ecological quality” for all waters in the EU by 2015.

32 Pollution and monitoring programmes routinely include the examination of diatoms to
33 investigate the ecological status of fluvial systems (Kelly et al., 1998; Prygiel et al., 2002, Jüttner
34 et al., 2003). It is also recognised diatoms sensitivity to high metal pollution as has been shown in
35 watersheds draining mining areas (Ivorra et al., 1999; Hirst et al., 2002; Nunes et al., 2003; Gold
36 et al., 2003) but also to low metal pollution as described under experimental conditions (Paulsson
37 et al., 2000; Guasch et al., 2002). However, there are few studies relating diatom species
38 composition with metal pollution in fluvial systems draining urban areas or receiving Waste
39 Water Treatment Plant (WWTP) outfalls. The simultaneous occurrence of low metal pollution
40 with eutrophication and organic pollution, may confound the specific effects of metal pollution on
41 diatom communities (Rogers et al., 2002; Hirst et al., 2002; Boisson & Perrodin, 2006).

42 Significant increases in surface water metal concentration can occur in the vicinity of WWTP
43 outfalls and due to urban runoff. In this context, the usual approach used in most of the stream
44 monitoring programs might be confounded by spatial variation, therefore limiting our ability to
45 separate the effect of all sources of human disturbance. Sediments play an important role in
46 reducing water column concentrations following effluent release. The dominance of adsorption
47 and complexation processes in natural systems means that most trace metals are rapidly
48 incorporated within bottom sediments (Bubb & Lester, 1995). Consequently, monitoring

49 programs based on water metal concentration may fail to detect this type of pollution if data
50 concerning metal content in the bottom sediments and biota are not considered.

51 Particularly, metal concentration in periphyton has been related with metal pollution, but it
52 may also be modulated by differences in metal speciation (Meylan et al., 2003). Metal speciation
53 is a matter of interest as it controls toxicity: assessment of total, dissolved and free (ions) metals is
54 thus needed for understanding the real exposition of organisms. Diffusive Gel in Thin films
55 (DGT) has been proposed as a technique capable of measuring metal speciation “in situ”.

56 The main aim of this study was to elucidate the confounding effects of spatial variation in
57 community structure in order to determine the influence of low metal pollution on the ecological
58 status of fluvial systems. Our specific objectives were to i) describe the pollution gradient and the
59 corresponding diatom community at regional scale, ii) obtain a good characterization of metal
60 loads and metal availability in a watershed chronically exposed to low metal pollution and iii)
61 investigate, at both scales, the relative contribution of different types of pollution: eutrophication
62 and organic pollution vs. metal pollution to diatom species composition using variance
63 partitioning multivariate analyses

64 **2. Methods**

65 Multivariate analysis were applied to two different data-sets, one covering a large area (referred to
66 as regional study) and the second restricted to a small watershed (referred to as watershed study).
67 Data used for the regional study was provided by the Catalan Water Agency. The data set aimed
68 to cover a large environmental gradient. Available data included a characterization of water
69 mineralization, nutrient content, water metal concentration and inventories of diatom species from
70 different watersheds influenced mainly by urban, agricultural and industrial activities (Tornés et
71 al., 2007). Furthermore, and due to the limited data available concerning metal pollution at a
72 regional scale, a more detailed sampling was planned in the Fluvià watershed slightly impacted by
73 agricultural, urban and small industrial activities; a continual in situ sampling (time-integrated)
74 followed by analysis in the laboratory was applied using the Diffusive Gradient in Thin films

75 (DGT) technique. Metal analysis techniques were also improved (reducing detection limits from
76 10 µg/L to 0.1 µg/L); and the analyses of metal concentrations in biofilms was also included.

77 **2.1 Area of study**

78 *Regional study.* Twenty-one sites monitored by the Catalan Water Agency (Catalonia, NE Spain)
79 were included in the regional study. The diversity in morphology, climate and human land uses in
80 the area of study determine an important spatial heterogeneity across the region (Leira & Sabater,
81 2005). The sites were selected to cover a large gradient of metal pollution, ranging from pristine to
82 polluted, with different levels of human disturbance (Table 1 and Fig. 1).

83 *Watershed study.* The watershed study was carried out in the River Fluvià, a Mediterranean
84 calcareous river in Catalonia (NE Spain). The area of study is located within a volcanic zone
85 (declared Natural Park in 1982) in the upper part of the Fluvià watershed (1080 km²). This study
86 included six sampling sites in the headwater of the River Fluvià and its main tributaries (Fig. 1).

87 In spite of being a mainly forested area (68% of the surface), the Fluvià watershed is
88 highly influenced by agricultural activities (25%). The remaining area of the watershed consists of
89 shrub lands (4%), built-up space (2%), and wetland vegetation (<1%). The sampling sites chosen
90 in this study followed a gradient in the dominant land uses from mostly forested area (B1,
91 B2=J070 and T1) to agricultural, urban and industrial uses (Rd=J105, T2=J104, Ol=J013). Two
92 sampling sites receive WWTP outflows: Rd urban wastewaters from a small town (Olot, 32,000
93 inhabitants) and T2 food industry wastewaters (Beguda).

94 **2.2 Field sampling**

95 *Regional study.* Data were obtained from the monitoring program planned by the Catalan Water
96 Agency (ACA) for the assessment of diatom indices during summer (July-August) 2002 and
97 spring (May-June) 2003. Epilithic diatom samples were taken from the riffle sections at sites
98 covering the major types of geomorphological and physiographical conditions (ACA, 2003). At

99 least five stones were randomly collected from the stream bottom, and brushed to detach the algal
100 communities to a final area of 2-10 cm². A pooled sample was obtained from each site.
101 Simultaneously, field physico-chemical variables were measured and site descriptors taken from
102 field observations. Diatom sampling and counting followed the CEN standard protocols (2000,
103 2001). Algal samples were preserved in formaldehyde 4% (v/v) until analysis.

104 *Watershed study.* Sampling of the six sites within the Fluvià watershed took place at three
105 different periods: December 2003, February and July 2004. During each sampling period,
106 physico-chemical parameters were measured in the field. Samples of benthic diatoms were taken
107 as for the regional study.

108 **2.3 Water chemistry**

109 *Regional study.* Water chemistry of the selected sites was obtained from the Catalan Water
110 Agency (ACA) which monitors them monthly. The following variables were included:
111 conductivity, water hardness, dissolved oxygen, TOC, NO₃-N, P₂O₅, SO₄, Cl, HCO₃; NH₄-N, K,
112 Ca, Na (the most common used for monitoring purposes) and water metal concentration (Cd, Cr,
113 Cu, Pb, Ni, Zn, As, Hg). Water samples were analysed following standard procedures (APHA,
114 1989).

115 *Watershed study.* Physicochemical parameters of each point were taken at 3 sampling dates,
116 December 2003, February and July 2004. At each sampling date, temperature, pH, dissolved
117 oxygen and conductivity were measured in the field using WTW probes, and water samples (1
118 litre per site) were collected and transported refrigerated for their analysis in the laboratory.
119 Samples were filtered by GF/C Whatman glass microfibre filters, refrigerated (4°C). They were
120 analysed within 24h for ammonium measurements and frozen for SRP, nitrate and nitrite analyses
121 following standard procedures (APHA, 1989). Naturally derived DOC is relatively low in the area
122 of study ranging between 1-3 mg/L (Blanck et al 2003). DOC values above 5 mg/L are indicative,
123 in this area, of anthropogenic organic pollution.

124 Metals in water. Total dissolved metal concentration measurements were carried out in
125 triplicates of water samples collected in the field at each sampling point. Samples were
126 immediately filtered (Whatman nylon filters 0.2 μm) and acidified with nitric acid (Supra pure,
127 Merck; 1%).

128 Metals in biofilms. Biofilms were scratched from three different stones randomly collected
129 from the stream bottom at each sampling site by using a clean microscope slide. After the
130 collection, biofilms were lyophilized and weighed. Dry samples were digested with 4ml of
131 concentrated nitric acid (Supra pure) and 1mL of 30 % of hydrogen peroxide in a high
132 performance microwave oven (Milestone, Ethos sel) and were thereafter diluted to 25 mL with
133 milli-Q water. Metal concentrations of water and biofilm samples were determined by inductively
134 coupled plasma mass spectroscopy (ICP-MS; HP 7500c, Agilent).

135 DGT. Diffusive Gradient in Thin films has been proposed as a technique capable of
136 measuring metal speciation “in situ” (Davison et al. 2000). DGT was applied to measure free and
137 labile copper, which is supposed to be a good estimation of the bioavailable part in the water
138 (Davison and Zhang, 1994). This technique has recently been validated in fluvial systems
139 (Tusseau-Vuillemin et al., 2007).

140 Each DGT device consisted of a plastic base (2.5 cm diameter) and a plastic cap with a
141 2 cm diameter window; this plastic holder was loaded with a filter, a resin gel, a diffusive gel and
142 a second filter. DGT holders (piston type), diffusive gel disks (0.8 mm thick) and Chelex-100
143 binding resins (0.4 mm thick) were purchased from DGT research (Lancaster, UK); nitrate
144 cellulose filter membranes (0.13 mm thick; 0.45 μm mesh) were purchased from Millipore
145 (Molsheim, France). Three DGTs were deployed at each sampling site (B1, B2, T1, T2, OL, Rd)
146 in two occasions (December and July 04). The DGT holders were placed in a plastic grid role with
147 the window down to avoid sediment settlement on top of them. The grid role was made by a piece
148 of plastic grid (square of 30 * 30 cm with 4 cm holes approx.) which was rolled and the DGT

149 holder was fixed to a small window. The whole device was attached to big stones inside the river
150 during one week. Exact exposure time, temperature, water flow, and pH were recorded for each
151 sampling site. After exposure, the resin gels were eluted in 7 mL 1M HNO₃. Metal concentrations
152 of the DGT resin gel eluates and the acidified (1% HNO₃) water samples were measured by
153 inductively coupled plasma mass spectrometry (ICP-MS; HP 7500c Agilent). The mass of metal
154 sequestered on the DGT resin was evaluated considering an 80% yield of elution (Zhang et al.
155 1995). Calculations of labile copper were done according to Davison and Zhang (1994). All
156 material used was soaked previously in 10% HNO₃ for 24 h and rinsed with ultrapure water.

157 **2.4. Diatom analyses**

158 Diatom frustules were cleaned of organic material by acid oxidation using sulphuric acid,
159 dichromate potassium and hydrogen peroxide. Permanent slides were mounted using Naphrax (r.i.
160 1.74). At least 400 valves were counted on each slide by performing random transects under light
161 microscopy using Nomarski differential interference contrast optics at magnification of 1000 x.
162 Taxa were identified mainly according to standard floras (Krammer & Lange-Bertalot 1991-1997;
163 and Lange-Bertalot 2001).

164 **2.5. Data analysis**

165 Polluted sites are likely to contain a mixture of metals which in turn might have additive effects
166 even at chronic concentration. Consequently, we used a measure of total metal concentration and
167 toxicity; the cumulative criterion unit (CCU; Clements et al., 2000) which has been already
168 applied to analyse the response of different organisms to metals in streams (Clements et al., 2000;
169 Hickey and Golding, 2002; Hirst et al., 2002). However, so far, the CCU is untested in the field in
170 continental Europe. Cumulative criterion unit (CCU) scores were calculated from metal
171 concentrations after EPA (2006) as:

$$172 \quad CCU = \sum m_i / c_i$$

173 where m_i is the total recoverable metal concentration and c_i is the criterion value for the i th metal.
174 The criterion value is based on U.S. EPA guidelines on critical concentrations, which, when
175 exceeded, may harm aquatic organisms. Metals that were below detection were not included in the
176 CCU. Thus, a CCU value of 1.0 represents a conservative estimate of the criterion value for all
177 metals based at any given sampling site. We calculated the relative percentage contribution of
178 each metal to the CCU values to evaluate which metals contribute most to toxicity. We placed the
179 sampling sites into one of three categories based on the measure CCU. Background sites were
180 defined as sites with CCU values below 1.0. Assuming that metal effects are additive, a value of
181 1.0 represents the point at which we would expect to notice adverse effects on the aquatic
182 organisms. The low metal category consisted of those sites with a CCU value between 1.0 and 2.0.
183 The medium metal category consisted of sites with CCU values between 2.0 and 10.0. We
184 selected these cutoff values because we were interested in quantifying the effect of metals at low
185 and moderate concentrations, so the medium level category would be ideal to test if the diatom
186 communities would become tolerant when metal concentrations exceed the limit criterion value by
187 a factor of only 2 or less. As water hardness affects the toxicity and bioavailability of some
188 metals, criterion values for Cd, Cu, Pb and Zn may vary, and were modified to account for
189 variation in water hardness between sites.

190 Water metal concentration and metal concentrations in the biofilm were also used to
191 calculate cumulative criterion units for the watershed study following the procedures described
192 above and referred to as CCUw and CCUb, respectively. In the case of CCUb, criterion values
193 were not corrected for water hardness due to the lack of information concerning water chemistry
194 in the biofilm. However, differences in metal availability due to hardness are expected to be small
195 since all sites belong to the same geologic area and have similar water hardness.

196 ***2.6. Statistical methods***

197 Physico-chemical variability over time was first explored. In the regional study, temporal

198 variability was evaluated by comparing spring and summer average values. In the watershed
199 study, differences between the three sampling times were evaluated using ANOVA using the time
200 as a factor.

201 Multivariate techniques (ter Braak & Verdonschot, 1995) allow the elucidation of
202 ecological factors, which may explain the variation in diatom communities. Constrained
203 ordination does not allow to estimate the unique effect of a set of explanatory variables. However,
204 variation partitioning or partial canonical analysis (Borcard et al., 1992) may help to estimate the
205 fraction of the variance in diatom community distribution explained by different sets of
206 environmental factors influencing. Multivariate data analyses were performed on the diatom
207 dataset to explore the main gradients of floristic variation and to detect and visualize similarities
208 in the diatom samples. Diatom data were analysed by means of detrended correspondence analysis
209 (DCA) (Hill & Gauch, 1980) to determine the length of the gradient. DCA revealed that the
210 gradient was greater than 3 standard deviation units (4.3) in the regional study, therefore unimodal
211 ordination techniques would be more appropriate. However, the gradient length was smaller than
212 3 standard deviation units (2.7) in the watershed study and linear ordination techniques should be
213 applied (ter Braak and Šmilauer, 2002). Constrained ordination, canonical correspondence
214 analysis (CCA) and Redundancy Analyses (RDA) were used to relate diatom assemblage structure
215 to all predictor environmental variables and to explore the relationships among and between
216 species and the environment (Ter Braak & Verdonschot, 1995). According to this preliminary
217 CCA and RDA, we identified collinear variables and selected a subset on inspection of variance
218 inflation factors ($VIF < 20$) (ter Braak & Šmilauer, 2002. Forward). Explanatory variables were
219 submitted to the step-wise forward selection and procedure in which the statistical significance of
220 each variable was tested by the Monte Carlo permutation test (ter Braak & Šmilauer, 2002), (999
221 permutations). Probabilities for multiple comparison were corrected using the Bonferroni
222 correction. Then, partial CCA and RDA were used to separate and examine the relative
223 importance for the species data of two sets of explanatory variables on the diatom assemblage

224 (Borcard et al., 1992). We were interested in separating metal pollution from all the other
225 variables and then testing whether these two different groups were redundant to each other, or
226 whether they each explained unique aspects of species composition. The variance partitioning was
227 conducted in different steps: (1) CCA/RDA of the species matrix constrained by the
228 environmental matrix; (2) CCA/RDA of the species matrix constrained by the metal pollution
229 matrix; (3) partial CCA/RDA of the species matrix constrained by the environmental matrix and
230 using the metal pollution matrix as covariables; (4) partial CCA/RDA of the species matrix
231 constrained by the metal pollution matrix and using the environmental matrix as covariables. The
232 data set included two sampling times (summer 2002 and spring 2003) in the regional study, and
233 three sampling times (December 2003, February and July 2004) in the watershed study. The set of
234 variables included are detailed in Tables 2, 4 and 5.

235 Only diatom taxa occurring in more than 2 samples with a relative proportion $\geq 1\%$ were
236 included in the analyses. Taxa abundance was square root transformed to reduce the effect of
237 highly variable population densities on ordination scores. Environmental data (except pH) were
238 logarithmically transformed before analysis to reduce skewed distributions. All ordinations were
239 performed using CANOCO version 4.5 (ter Braak & Šmilauer, 2002).

240 **3. Results**

241 ***3.1. Regional study***

242 *Physical, chemical and biological characteristics.* Streams were characterized by conductivities
243 from 57 to 2750 $\mu\text{S cm}^{-1}$ and a variety of nutrient levels (Table 2). Metal concentration ranged
244 between $<0.1\text{-}0.8 \mu\text{g/L}$ for Cd and Hg, $<1\text{-}10 \mu\text{g/L}$ for As, Cr and Pb and $<10\text{-}80 \mu\text{g/L}$ for Cu, Ni
245 and Zn. Comparing spring and summer data, average values of dissolved salts (conductivity and
246 chloride), nutrients (mainly phosphate) and metal CCU were slightly higher in summer than in
247 spring, but the variability between sites (see the standard error of the mean) was always much
248 higher than temporal variability (Table 2). Over 288 taxa of diatoms were identified from the

249 study sites. Of those, twenty-three diatom taxa were included (typically represented > 1% of the
250 diatom community at all sites).

251 *Metal Cumulative Concentration Units categories.* Heavy metal concentrations, expressed as
252 CCU do not differ greatly among stations and ranged from 0.065 to 6.383 (mean 1.906) (Table 2)
253 and were less than 2.0 at the majority of sites (68%). The relative importance of As, Cd, Cr and Ni
254 was < 5% to the overall CCU. Pb was the most important metal and accounted for 53% of all the
255 CCU followed by Cu (24 %), Hg (12%) and Zn (8 %). The relative contribution of each metal to
256 the CCU differed among the three metal categories (Fig. 2). The contribution of Cu and Zn was c.
257 57% and 37% respectively at background sites. Pb was the most important metal at low and
258 medium level sites (CCU > 1.0). The relative importance of Cu and Zn decreased at low and
259 medium level sites, while Hg and Pb increased. Zn had the highest absolute concentration in the
260 streams, but its contribution to the CCU was considerably smaller than that of Cu and Pb once the
261 criterion values and the effects of water hardness on bioavailability were taken into account.

262 *Relative importance of metal pollution vs. other environmental factors.* The summary of the
263 ordination gives an overall measurement of how much variation can be related to both explanatory
264 variables. However, not all diatom species are equally well explained by the same set of
265 environmental variables. The fit for species can be used as a measurement to find out which
266 species are better represented and the percentage of variance fit by each set of explanatory
267 variables.

268 The first run of the data analysis (i.e. environmental variables as explanatory variables)
269 showed that organic pollution and conductivity were statistically significant. In the second run
270 (i.e. metal pollution as explanatory variables) chromium and CCU turned out to be statistically
271 significant. Environmental variables alone accounted for 22.6% of explained variation. The metal
272 pollution represented 9.8% of explained variation. The results showed that 5.1% of the diatom
273 data variation was shared by the environmental and metal pollution variables. Finally, the

274 unexplained variation corresponded to 62.5%.

275 The percentage of variance explained by metal pollution and other environmental factors
276 with respect to the unconstrained variance differs among the different species (Table 3). *Navicula*
277 *saprohila* has its distribution mostly explained by metal pollution (Fig 2). *Gomphonema pumilum*,
278 *Gomphonema parvulum*, *Nitzschia palea*, *Cymbella sinuata*, *Cymbella minuta*, *Nitzschia*
279 *frustulum*, *Cyclotella meneghiniana*, *Achnanthes minutissima*, *Gomphonema minutm*, *Nitzschia*
280 *amphibia*, *Navicula minima*, and *Cocconeis placentula* were mostly related to the other
281 environmental factors. *Navicula seminulum* and *Navicula veneta* had both large amounts of their
282 distribution explained by the two sets of explanatory variables (Table 3).

283 **3.2. Watershed study**

284 *Physical, chemical and biological characteristics.* Based on the physical and chemical variables
285 analyzed, two sampling sites had low nutrient concentration (B1 and T1), another two sites were
286 slightly eutrophic due to diffuse pollution (B2 and Ol), and the remaining two (T2 and Rd) had the
287 highest nutrient concentration 3-10 times higher ammonia, phosphate and DOC (Table 4). There
288 were no significant differences between sampling times (ANOVA, $p < 0.001$) since the variability
289 between sampling sites was always higher than temporal variability (Table 4).

290 *Metal contents.* The water metal concentration (Table 5) followed a similar pattern to nutrients
291 and conductivity but differences between sites were less evident. Metal concentration was lower at
292 B1, T1 and Ol; and slightly higher at B2 and Rd; while metal levels in biofilms were lower at B1,
293 B2 and T1, higher at Ol and T2 and reaching maximum at Rd (Table 5). The bioavailable metal
294 portion in water – estimated with DGT analysis – followed the same pattern but amounted to 16 -
295 23% of total dissolved metal content (average percentage Ni: 23,1%; Cu: 23,2%; Zn: 16,7%; Cd:
296 15,6%; and Pb: 19,6% respectively). A good correlation between water concentration and metal
297 levels in biofilms was obtained for Zn ($r = 0,86$); Cu, As, Cd, and Pb concentrations in water were
298 very low (< 5 ug/L), close to the detection level of the method showing a high scatter and were not

299 significantly correlated with biofilm metal concentration.

300 CCU calculated using water concentrations (CCUw) ranged between 0.06 and 0.75
301 (average 0.34). The relative importance of As, Hg and Ni to the overall CCUw was < 10%. Cd
302 was the most important metal and accounted for 25% of all the CCUw followed by Zn and Cu (24
303 %) and Pb (17%). In order to increase sensitivity in the detection of changes due to metal
304 pollution, biofilm metal concentration was used to calculate CCUb. CCUb values ranged between
305 0.90 and 15.4. The relative contribution of each metal to the CCUb was similar among the three
306 metal categories (Fig. 3a). In this case the relative importance of As, Ni, Zn and Cu was < 10% to
307 the overall CCUb. Pb was the most important metal and accounted for 68% of all the CCUb
308 followed by Cd (18 %).

309 *Diatom community responses to metal levels.* Phosphate, nitrate and water temperature were the
310 environmental variables with a significant effect on the diatom distribution. These environmental
311 variables alone accounted for 23.2% of explained variation. Metal concentration and CCU units in
312 biofilm (Cu-biofilm, Pb-biofilm and CCU biofilm) turned out to be statistically significant
313 amongst the metal pollution variables. Metal pollution represented 21.1% of the explained
314 variation. Up to 15.9% of the diatom data variation was shared by the environmental and metal
315 pollution variables. Finally, the unexplained variation corresponded to 39.8%.

316 The percentage of variance explained by both sets of variables independently with respect
317 to the unconstrained variance differs among the different species. *Navicula gregaria*, *Amphora*
318 *pediculus*, *Rhoiscosphenia abbreviate*, *Navicula tripunctata*, *Nitzschia fonticola*, *Navicula*
319 *subhamulata* and *Navicula cryptotenella* had their distribution mostly explained by metal
320 pollution (Table 6). In fact, the RDA first axis scores differ between CCU metal levels (ANOVA;
321 $p=0.059$; $F= 3.469$). Overall, this group of species is absent or present with low abundance at the
322 lowest metal category, increase with metal content, and rise up to more 30% of total abundance in
323 the highest metal category (Fig. 3 b, c, d, e and f).. The most significant case is *Navicula gregaria*.

324 In this case, metal pollution explains 42% of variance. This taxon is absent at sites with low metal
325 content in biofilms, and increases with increasing metal content (Fig 3b) presenting significant
326 differences in the abundance between CCU metal categories (ANOVA; $p=0.03$; $F=8.93$).
327 *Gomphonema minutum*, *Gomphonema parvulum*, *Cocconeis pediculus* were amongst the most
328 related to other environmental factors. Finally, *Nitzschia frustulum*, *Cymbella minuta*, *Achnanthes*
329 *minutissima*, *Melosira varians*, *Navicula menisculus* var. *grunowii*, *Gomphonema micropus*,
330 *Cocconeis placentula*, *Navicula capitatoradiata* and *Nitzschia dissipata* were both explained by
331 metal pollution and other environmental factors (Table 6).

332 **4. Discussion**

333 Based on the opportunity to evaluate the response of numerous species simultaneously,
334 community ecotoxicology can provide a much broader context for the assessment of
335 environmental contamination than the study of individual species (Clements and Newman, 2002).
336 Different species in a community respond differentially to contaminants and other stressors
337 because of differences in life history characteristics and tolerance. Thus, the composition of
338 communities at different locations, or at different points in time, provide useful information about
339 the environmental conditions.

340 Diatom communities are used in this study as indicators of different types of pollution:
341 eutrophication and organic pollution vs. metal pollution. The relative contribution of each type of
342 pollution and the respective key environmental variables differ between the two case-studies.
343 Focusing on the first type of pollution (eutrophication and organic pollution), the percentage of
344 variance explained was similar between both cases (22.6% and 23.2%, respectively), but the key
345 environmental variables were different: organic pollution and conductivity in the regional study
346 and nutrients (nitrate and phosphate) and water temperature in the Fluvià watershed. Many studies
347 show that key environmental variables which are important determinants of freshwater
348 communities function and structure are influenced by factors operating at different temporal and
349 spatial scales. The regional study included sites of different order from different watersheds
350 covering a broad range of conditions at the highest spatial level. Diatoms followed a gradient of
351 pollution but also differed in the growing conditions for total dissolved salts (57-2750 $\mu\text{S}/\text{cm}$).
352 Environmental factors explaining diatom assemblage distribution at a large spatial scale have been
353 clearly shown to be predominantly related to physiographic characteristics (Leira & Sabater,
354 2005) and water mineralization (Potapova & Charles, 2003).

355 In contrast with the regional case-study, study sites in the Fluvià watershed were selected
356 in order to provide the minimum physiographical and geochemical variability. All sampling sites

357 had similar hydromorphology (2-3 order streams) draining the same geological area. In this case
358 diatoms followed a clear eutrophication gradient. Repetitive sampling over time (2-3 times)
359 provided temporal variability which was also influencing diatom species due to seasonal water
360 temperature changes.

361 Focusing on the second type of pollution analyzed, i.e. metal pollution, the percentage of
362 variance explained in the regional study was lower (<10%) than in the Fluvià watershed (21.1 %).
363 This difference was mainly due to the variables included in the analysis: water metal
364 concentration in the first case and both, water and biofilm metal concentrations in the second one.
365 As it was expected, water metal content was a better predictor of the diatom community structure
366 in the regional than in the watershed study. Taking the CCU as a summary of water metal
367 pollution, all sampling points included in the Fluvià case-study correspond to the lower metal
368 category of the regional data set. In addition, while metal loads exceeded expected toxicity
369 thresholds in some of the cases included in the regional study we did not expect metal toxicity in
370 the watershed study. The Fluvià case-study is a clear example of low-metal pollution from diffuse
371 (B2 and Ol) and point sources (the outflow of relatively small WWTP in T2 and Rd). In spite of
372 the fact that metal loads were relatively high, the percentage of variance explained by metal
373 pollution in the water phase in the regional case was low, and the partition of variance included
374 only the most polluted cases. It was related with only one diatom species: *Navicula saprophila*,
375 considered to be very tolerant to pollution (Soininen 2002, Rakowska 2004, Tornés et al. 2007).
376 *Navicula saprophila* was a dominant taxon (>60% relative abundance) during spring in the lower
377 part of the Besós (J034 and J048) and Francolí rivers (J079). One of the sites (J048) is located in
378 an industrial area and has the highest metal loads which exceed quality thresholds (0.80 µg/L Cd,
379 4 µg/L Hg and 6.26 CCU). Water metal concentration was also above the quality threshold at
380 J048 (3.58 CCU), but relatively low at J034 (0.29 CCU). Differences in TOC were very broad and
381 may have modulated periphyton metal bioavailability. Furthermore, it has been reported that Cu
382 and Zn toxicity on periphyton are influenced by nutrient loads (Ivorra et al., 2002; Guasch et al.,

383 2004, Paulsson et al., 2000). Differences in phosphate and nitrate concentration may also
384 influence the sensitivity of periphyton modulating the response of diatoms to metal under different
385 nutrient conditions.

386 Total metal concentration in the water has been considered a poor indicator of metal
387 availability (Campbell, 1995; Tusseau et al., 2007). Metal loads are derived from single water
388 samples which only record a limited time period. Point water sampling cannot account for the
389 temporal variability and detecting the occurrence of higher concentration episodes that may have
390 been recorded either with passive samplers (integrating up to the 7 previous days) or benthic biota
391 (fluvial biofilms may integrate several weeks of exposure). Furthermore, it is well known that
392 biologically active surfaces (periphyton and sediments) play an important role in reducing water
393 column concentrations following effluent release via adsorption and complexation processes
394 (Bubb & Lester, 1995) providing an integrated representation of the accumulation of toxicants in
395 the benthic environment (Meylan et al., 2004). In our study, metal content in biofilms accounted
396 for a significant portion of diatom species variance. The partial RDA identifies seven diatom
397 species related with biofilm metal concentration. Three different patterns were observed. Two
398 species, (*Navicula gregaria* and *Rhoicosphenia abbreviata*) which were not found in the reference
399 sites, increased linearly with biofilm metal concentration. In the case of *Navicula gregaria*, the
400 relative abundance increased from 0 to 12%. In the context of our area of study, this species may
401 be considered indicative of slight metal concentration increase. In agreement with our results, *N.*
402 *gregaria* was considered to be tolerant to metal pollution by Chanson et al. (2005) but also to
403 organic pollution (Gomà et al., 2005). Another diatom species (*Nitzschia tripunctata*), already
404 present in the reference sites, showed a slight increase at higher metal concentration. Finally,
405 *Nitzschia fonticola* and *Navicula cryptotenella* showed a non-linear increase with metal
406 concentration. Overall, this group of species increased from <5% to more than 30% of the total
407 abundance with increasing metal concentration (Fig. 3). Rogers et al. (2002) also found a good
408 correlation between metal concentration in vegetative habitats and the corresponding biological

409 condition using macroinvertebrate communities. The percentage of variance explained by metal
410 concentration in vegetative habitats was 22%, very close to the 23% variance explained by metal
411 concentration in the biofilms of our investigation.

412 Behra et al. (2002) indicate that metal concentration in biofilms is influenced by metal
413 speciation and may be subject to temporal dynamics in the environment. The used DGT technique
414 is believed to give an estimation of the labile metal fraction in the water. The average percentage
415 of DGT labile metals of the total dissolved metal concentrations in this study were in good
416 agreement with results obtained by Odzak et al. (2002), revealing that only a minor fraction of the
417 total dissolved metal content is bioavailable.

418 The consistency between temporal replicates, and the results obtained by passive samplers
419 indicate that biofilm metal concentration can be used to evaluate aquatic metal contamination and
420 bioavailability. Furthermore, since water metal concentration was near detection limits in some
421 reference sites, the higher metal contents measured in biofilms allowed a better characterization of
422 metal pollution. This measurement provided a higher sensitivity for the elements such as As or Cd
423 that occurred at very low concentration in the aqueous phase.

424 The results obtained in the Fluvia watershed indicate that the biofilm metal analysis may
425 have improved the assessment of metal pollution effects at a broader scale.

426 Looking for generalizations, large scale studies are a tempting approach for research and
427 also management purposes. However, they are influenced by regional scale variability that may
428 conceal the effects of human impacts. Conversely, human impacts are better detected in small
429 scale studies, including reference and impacted situations, within a restricted range of
430 environmental variability. However, any conclusion drawn from these studies is restricted to the
431 area of study and cannot be easily generalized. Surprisingly, few studies have addressed the
432 importance of scale. The comparison between the two case studies here illustrates the relevance of
433 the scale but also the importance of using a good set of explanatory variables. In addition, the

434 results obtained in our local case study, the Fluvià watershed, reveal that diatom assemblages may
435 respond to low levels of metal pollution. Given the regional differences in climate and land use we
436 were not surprised to find differences in community composition at the regional scale that were
437 correlated with these strong ecological gradients, although the finding that at watershed level
438 streams were responding to low metal pollution was of interest. This finding was possible by
439 means of a good sampling strategy, but also thanks to the use of highly sensitive analytical
440 techniques for the study of metal concentration and bioavailability. Analyses of metal traces in the
441 water phase and the biota (fluvial biofilms), together with the use of passive samplers are
442 recommended for assessing low metal pollution.

443 **5. Conclusions**

444 The results obtained illustrate two key points in field sampling: the scale of study and the
445 importance of choosing a good set of explanatory variables. Large-scale studies of human-derived
446 impacts are complicated by the natural variability between sites, requiring the inclusion of many
447 variables accounting for all sources of variation occurring at different scales (physiographical,
448 geochemical and biological). This difficulty can partially be solved if human-impact is analyzed at
449 a smaller scale, including contrasted reference and impacted sites. Focusing on metal pollution,
450 metal water concentration is a poor indicator of metal availability in chronic low metal pollution
451 scenarios. Analyses of metal traces in the water phase and the biota (fluvial biofilms) together
452 with the use of passive samplers are, therefore, recommended.

453 In addition, the cases presented in this paper show the usefulness of variance partitioning
454 analysis to evaluate the relative contribution of different sources of pollution to the ecological
455 status of fluvial systems when they occur simultaneously, i.e. low metal pollution, eutrophication
456 and organic pollution. Application of these techniques allow to assess changes in the composition
457 of diatom communities at different locations, or at different points in time, in response to
458 overlapping environmental condition gradients, including situations of low metal pollution.

459

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River	Tributary	Code	Utm_x	Utm_y	type of pollution
Ebre	Noguera Pallaresa	J164	346200	4697150	Reference site
Francolí	main course	J079	351880	4557749	Industrial and agricultural
Llobregat	main course	J117	414345	4676946	Reference site
	main course	J084	409900	4595207	Urban, industrial and agricultural
	main course	J046	426317	4575155	Urban, industrial and agricultural
Besòs	main course	J043	432745	4593970	Industrial, urban and agricultural
	main course	J048	433470	4589092	Industrial, urban and agricultural
	main course	J069	436397	4599652	Industrial, urban and agricultural
Tordera	main course	J083	458991	4615836	Agricultural
	main course	J062	474623	4621201	Urban
	Vallfogona	J124	457436	4614528	Agricultural
	Arbúcies	J066	468683	4620831	Urban and industrial
Ter	main course	Te0	438961	4697736	Reference site
	main course	J034	440624	4648776	Agricultural
	main course	J060	467193	4648385	Agricultural
	Onyar	J020	486931	4646082	Agricultural
Fluvià	main course	J013	459669	4671043	Agricultural
	Bianya	J070	459601	4673988	Agricultural
	Turonell	J104	462182	4673130	Urban, agricultural and industrial
	Ridaura	J105	457655	4674088	Urban, agricultural and industrial
Muga	Muga	J012	488502	4687167	Agricultural

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640 Table 1. Summary of the location (river basin, tributary, ACA code and UTM coordinates) and
641 main type of pollution (in order of importance) of the cases selected in the regional study.

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	Summer		Spring		Min.	Max.	Tox. threshold
	Mean	s.e.	Mean	s.e.			
pH	7.57	0.12	7.95	0.10	6.7	8.5	
Conductivity (μ S /cm)	1142	203.8	853.6	196.3	57.0	2750.0	
Temperature ($^{\circ}$ C)	20.49	0.77	17.28	1.53	8.0	25.0	
Dissolved oxygen (mg/L)	7.42	0.75	14.96	5.98	4.0	74.6	
Nitrate (mg/L)	11.11	1.83	10.99	2.01	0.4	22.3	
Phosphate (mg/L)	2.40	0.87	0.62	0.18	0.1	12.3	
Sulphate (mg/L)	113.6	27.27	118.9	29.38	4.0	384.8	
Chloride (mg/L)	299.7	140.5	126.2	37.38	2.4	2041.0	
Bicarbonate (mg/L)	265.6	30.6	253.0	36.26	35.0	417.4	
Ammonium (mg/L)	3.73	1.72	4.02	2.01	0.1	20.9	
Potassium (mg/L)	18.24	9.72	9.23	2.69	0.1	141.7	
Calcium (mg/L)	93.23	13.14	98.81	14.46	11.6	220.3	
Magnesium (mg/L)	19.83	6.24	22.14	5.04	1.0	98.0	
Sodium (mg/L)	179.6	72.97	93.11	29.46	1.4	1045.3	
TOC (mg/L)	5.27	1.37	5.05	1.05	0.5	16.5	
Cd (μ g/L)	<0.1		0.07	0.07	<0.1	0.8	0.25
Cr (μ g/L)	<0.1		0.93	0.66	<0.1	6.60	74
Cu (μ g/L)	9.40	3.34	5.03	1.42	<5	50.0	9
Pb (μ g/L)	1.07	0.77	<1		<1	10.0	2.5
Ni (μ g/L)	14.64	6.51	8.20	4.44	<1	80.0	52
Zn (μ g/L)	37.45	4.75	35.11	4.37	6	63.0	120
As (μ g/L)	<1		2.31	1.21	<1	10.0	150
Hg (μ g/L)	0.04	0.04	0.36	0.36	<0.1	4.00	0.77
CCU	3.56	0.67	3.30	0.92	0.065	6.383	2

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649 Table 2. Physico-chemical variables used in the CCA of the regional study (Catalonia, NE Spain).

650 Average and standard error of summer (n=14) and spring (n=11) data, minimum (Min.);

651 Maximum (Max.) values recorded (n=25) and corresponding toxicity threshold.

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Code	Taxon	Fraction of total variance		Fraction of explained variance	
		Environ.	metals	Environ.	Metals
NVEN	<i>Navicula veneta</i> Kützing	61.21	19.76	75.6	24.4
GPUM	<i>Gomphonema pumilum</i> (Grunow) Reichardt & Lange-Bertalot	52.90	4.07	92.9	7.1
GPAR	<i>Gomphonema parvulum</i> Kützing	52.72	7.8	87.1	12.9
NPAL	<i>Nitzschia palea</i> (Kütz) Smith	47.86	10.89	81.5	18.5
CSIN	<i>Cymbella sinuata</i> Gregory	45.63	2.73	94.4	5.6
CMIN	<i>Cymbella minuta</i> Hilse ex Rabenhorst	43.24	0.93	97.9	2.1
NIFR	<i>Nitzschia frustulum</i> (Kütingow) Grun.	41.26	4.97	89.2	10.8
CMEN	<i>Cyclotella meneghiniana</i> Kützing	33.44	3.62	90.2	9.8
AMIN	<i>Achnanthes minutissima</i> Kützing	31.82	3.46	90.2	9.8
GMIN	<i>Gomphonema minutum</i> (Ag.) Agardh	29.63	2.66	91.8	8.2
NAMP	<i>Nitzschia amphibia</i> Grunow	28.10	1.19	95.9	4.1
NMIN	<i>Navicula minima</i> Grunow	27.52	5.82	82.5	17.5
CPLA	<i>Cocconeis placentula</i> Ehrenberg	26.41	0.58	97.9	2.1
NSEM	<i>Navicula seminulum</i> Grunow	25.29	14.72	63.2	36.8
NSAP	<i>Navicula saprophila</i> Lange-Bertalot & Bonik	1.22	47.12	2.5	97.5

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658 Table 3. Regional study. Results of the partial canonical correspondence analysis (CCA) with
659 forward selection of environmental variables.

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site	Cond ($\mu\text{S/cm}$)	phosphate (μM)	Nitrate (μM)	Ammonium (μM)	DOC (mg/L)	Oxygen (mg/L)	pH	Temp ($^{\circ}\text{C}$)
B1	533 ± 25	0.860 ± 0.912	49.4 ± 8.6	0.781 ± 0.487	1.129	10.77 ± 1.01	7.80 ± 0.39	9.97 ± 7.11
T1	587 ± 18	0.272 ± 0.156	78.9 ± 63.8	0.495 ± 0.008	0.610	10.68 ± 1.30	7.93 ± 0.33	10.03 ± 6.41
B2	658 ± 90	0.283 ± 0.250	97.6 ± 32.5	0.497 ± 0.006	0.957	11.27 ± 0.96	7.73 ± 0.20	11.57 ± 5.95
Ol	597 ± 20	0.564 ± 0.534	104.0 ± 71.5	1.862 ± 1.825	1.301	12.43 ± 0.79	8.24 ± 0.23	11.97 ± 6.97
T2*	1457	115.9	205.0	15.22	0.990	10.42	8.25	12.95
Rd	1687 ± 219	21.29 ± 3.309	38.7 ± 53.8	4.709 ± 1.318	8.557	11.17 ± 4.12	8.30 ± 0.19	16.77 ± 5.83

* n=2

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677 Table 4. Physico-chemical variables used in the RDA of the watershed study (Fluvià river).

678 Average and standard deviation for the three different sampling periods (December, February and
 679 July) of conductivity (Cond), nutrient concentration, dissolved organic carbon (DOC), oxygen
 680 concentration, pH and temperature at the different sampling sites (n=17).

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		B1	T1	B2	OL	T2*	RD	Avg	±s.e.
Ni	Biofilm	4.11 ±0.01	15.30 ±17.71	12.51 ±14.63	2.08 ±1.20	4.20	9.77 ±3.62	8.00	±2.17
	water	19.54 ±26.17	3.12 ±2.44	1.18 ±0.32	3.03 ±2.50	1.71	6.02 ±0.84	5.77	±2.84
	DGT	0.21	0.13	0.22	0.35	0.40	3.40	0.78	±1.28
Cu	biofilm	1.93 ±0.47	0.85 ±0.27	1.03 ±0.60	1.26 ±0.97	3.35	8.06 ±3.33	2.74	±1.13
	water	0.65 ±0.19	1.44 ±1.00	0.99 ±0.35	1.11 ±0.15	4.93	4.23 ±0.11	2.23	±0.76
	DGT	0.20	0.12	0.18	0.40	0.46	0.32	0.28	±0.13
Zn	biofilm	23.84 ±6.35	5.27 ±5.56	5.71 ±4.74	8.15 ±8.20	44.46	80.98 ±4.29	28.07	±12.25
	water	16.58	5.54	12.56	4.90	30.3	79.9	25.0	±11.6
	DGT	5.26	5.44	3.82	4.24	12.49	13.59	7.47	±4.37
As	biofilm	1.696 ±0.185	0.508 ±0.249	0.888 ±0.676	0.420 ±0.255	0.690	1.507 ±0.707	0.95	±0.22
	water	0.635 ±0.184	0.514 ±0.023	0.702 ±0.033	0.849 ±0.334	1.326	1.153 ±0.222	0.86	±0.13
Cd	biofilm	0.218 ±0.214	0.098 ±0.101	0.122 ±0.068	0.133 ±0.024	0.187	0.440 ±0.448	0.20	±0.05
	water	0.055 ±0.029	0.057 ±0.033	0.057 ±0.025	0.047 ±0.061	0.058	0.077 0.083	0.06	±0.00
	DGT	0.01	0.01	0.01	0.01	0.01	0.01	0.01	±0.001
Pb	biofilm	3.452 ±1.129	2.662 ±1.836	5.225 ±0.238	8.637 ±1.626	7.207	15.7 ±14.4	7.15	±1.94
	water	0.370 ±0.332	0.589 ±0.303	0.413 ±0.117	0.381 ±0.087	0.675	0.751 0.047	0.53	±0.07
	DGT	0,09	0,07	0,05	0,08	0,08	0,08	0,08	±0.019
CCUw		0.277 ±0.169	0.180 ±0.099	0.241 ±0.154	0.255 ±0.194	0.540	0.590 ±0.146	0.339	±0.049
CCUb		2.895 ±0.551	1.817 ±0.578	3.233 ±0.286	4.696 ±0.552	4.457	9.482 ±3.255	4.428	±0.818

685 * n=2

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687 Table 5. Metal concentration variables included in the RDA: metal concentration in biofilms

688 (µg/g) and water (µg/L). For each site, values are the average and standard error of three

689 sampling. Average metal concentrations estimated with DGT (µg/L) were not included in the

690 RDA and are the average of two sampling.

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Code	Taxon	Fraction of total variance		Fraction of explained variance	
		Environ.	Metals	Environ.	Metals
GMIN	<i>Gomphonema minutum</i> (Ag.) Agardh	37.02	7.38	83.4	16.6
GPAR	<i>Gomphonema parvulum</i> Kützing	31.27	4.18	88.2	11.8
CPED	<i>Cocconeis pediculus</i> Ehrenberg	33.56	5.83	85.4	14.6
NIFR	<i>Nitzschia frustulum</i> (Kützing) Grunow	46.98	21.83	68.3	31.7
CMIN	<i>Cymbella minuta</i> Hilse ex orst&Rabenh.	39.66	33.9	53.9	46.1
AMIN	<i>Achnanthes minutissima</i> Kützing	35.67	16.83	67.9	32.1
MVAR	<i>Melosira varians</i> Agardh	33.27	32.77	50.4	49.6
NMEG	<i>Navicula menisculus</i> Schuman var. grunowii Lange-Bertalot	33.14	44.87	42.5	57.5
GMIC	<i>Gomphonema micropus</i> Kützing	29.77	26.66	52.7	47.2
CPLA	<i>Cocconeis placentula</i> Ehrenberg	29.4	31.33	48.4	51.6
NCPR	<i>Navicula capitatoradiata</i> Germain	27.42	35.83	43.3	56.6
NDIS	<i>Nitzschia dissipata</i> (Kützing) Grunow	21.13	43.47	32.7	67.3
NGRE	<i>Navicula gregaria</i> Donkin	11.52	42.08	21.5	78.5
APED	<i>Amphora pediculus</i> (Kützing) Grunow	7.5	38.13	16.4	83.6
RABB	<i>Rhoicosphenia abbreviata</i> (C.Agardh) Lange-Bertalot	6.57	28.19	18.9	81.1
NTPT	<i>Navicula tripunctata</i> (O.F.Müller) Bory	3.32	28.05	10.6	89.4
NFON	<i>Nitzschia fonticola</i> Grunow	2.98	23.48	11.3	88.7
NSBH	<i>Navicula subhamulata</i> Grunow	2.56	37.07	6.5	93.5
NCTE	<i>Navicula cryptotenella</i> Lange-Bertalot	2.19	38.73	5.3	94.6

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Table 6. Watershed study. Results of the partial canonical correspondence analysis (RDA) with forward selection of environmental variables.

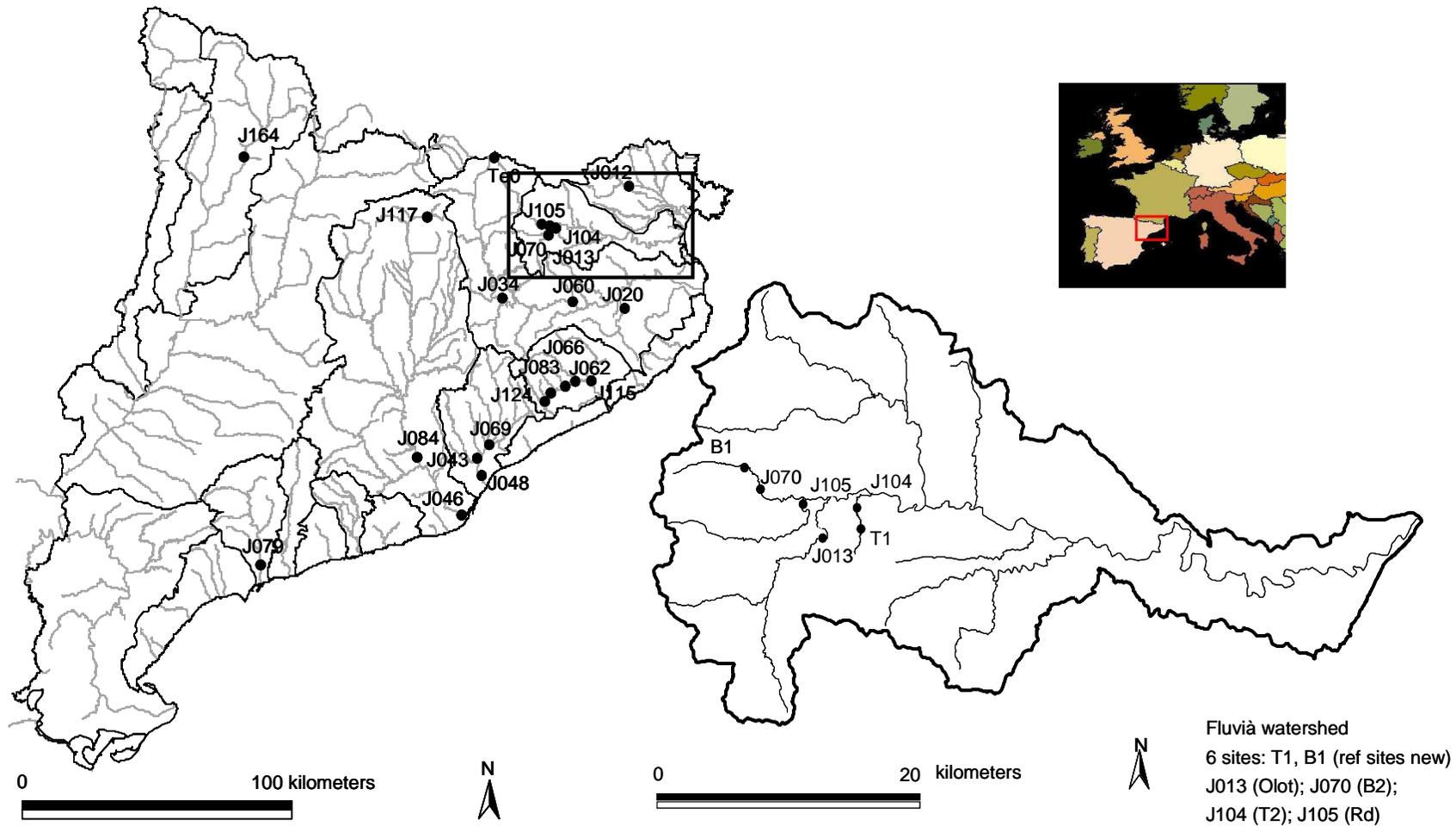
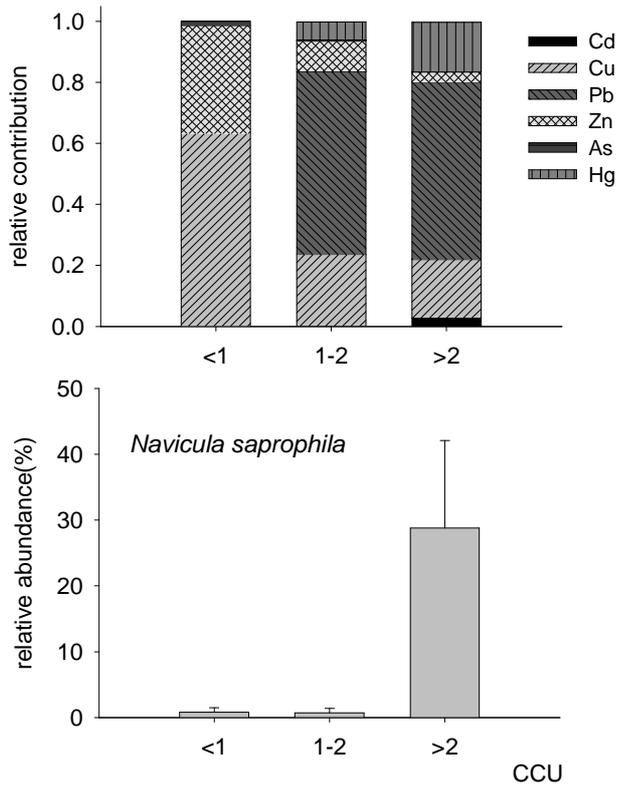


Figure 1. Location of the area of study (Catalonia, NE Spain). Study sites included in the regional and watershed studies (left and right maps, respectively). The code assigned by Catalan Water Agency. The location of the Fluvia watershed in the regional map is also indicated.

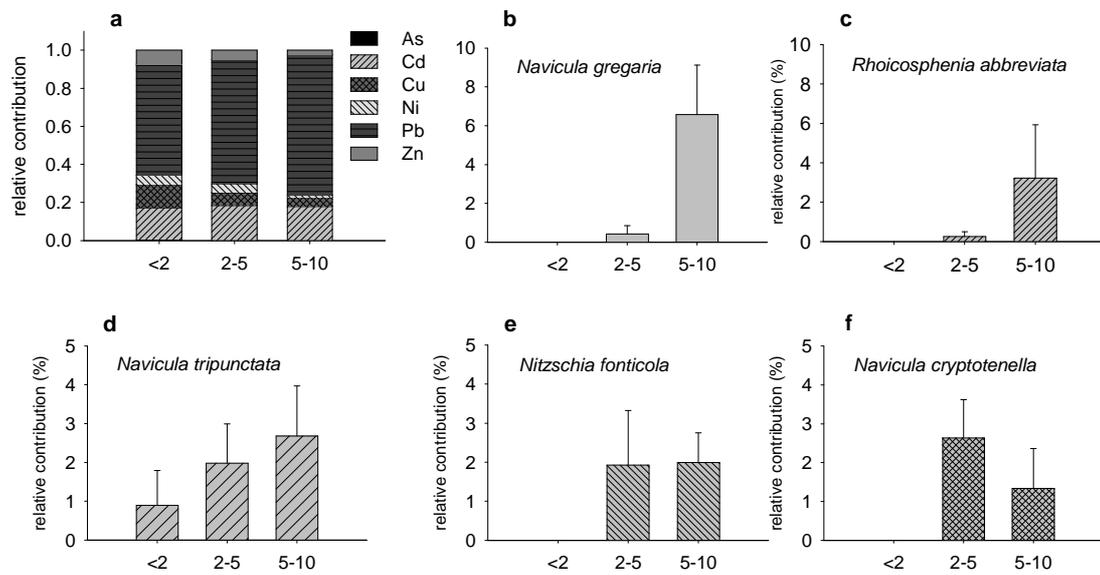
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Fig 2. Regional case-study. Relative contribution of various metals to the cumulative criterion unit (CCU) at background (CCU<1, n=10); low metal (1<CCU<2, n=7); and medium metal (2<CCU<10, n=8) categories. Average and standard error, for each metal category, of the abundance of diatom taxon related to metal content.



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Fig 3. Relative contribution of various metals to the cumulative criterion unit calculated using biofilm concentrations (CCU_b) at increasing metal categories in the Fluvia case-study. CCU_b<2 (n=3); 2<CCU_b<5 (n=10); and CCU_b>5 (n=4). Average and standard error, for each metal category, of the abundance of diatom taxa related to metal content.