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

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Abstract

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

1. Introduction

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

Pollution and monitoring programmes routinely include the examination of diatoms to investigate the ecological status of fluvial systems (Kelly et al., 1998; Prygiel et al., 2002, Jüttner et al., 2003). It is also recognised diatoms sensitivity to high metal pollution as has been shown in watersheds draining mining areas (Ivorra et al., 1999; Hirst et al., 2002; Nunes et al., 2003; Gold et al., 2003) but also to low metal pollution as described under experimental conditions (Paulsson et al., 2000; Guasch et al., 2002). However, there are few studies relating diatom species composition with metal pollution in fluvial systems draining urban areas or receiving Waste Water Treatment Plant (WWTP) outfalls. The simultaneous occurrence of low metal pollution with eutrophication and organic pollution, may confound the specific effects of metal pollution on diatom communities (Rogers et al., 2002; Hirst et al., 2002; Boisson & Perrodin, 2006). Significant increases in surface water metal concentration can occur in the vicinity of WWTP outfalls and due to urban runoff. In this context, the usual approach used in most of the stream monitoring programs might be confounded by spatial variation, therefore limiting our ability to separate the effect of all sources of human disturbance. Sediments play an important role in reducing water column concentrations following effluent release. The dominance of adsorption and complexation processes in natural systems means that most trace metals are rapidly incorporated within bottom sediments (Bubb & Lester, 1995). Consequently, monitoring programs based on water metal concentration may fail to detect this type of pollution if data concerning metal content in the bottom sediments and biota are not considered.

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

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

2. Methods

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

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

2.1 Area of study

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

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

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

2.2 Field sampling

Regional study. Data were obtained from the monitoring program planned by the Catalan Water Agency (ACA) for the assessment of diatom indices during summer (July-August) 2002 and spring (May-June) 2003. Epilithic diatom samples were taken from the riffle sections at sites covering the major types of geomorphological and physiographical conditions (ACA, 2003). At least five stones were randomly collected from the stream bottom, and brushed to detach the algal communities to a final area of 2-10 cm^2. A pooled sample was obtained from each site. Simultaneously, field physico-chemical variables were measured and site descriptors taken from field observations. Diatom sampling and counting followed the CEN standard protocols (2000, 2001). Algal samples were preserved in formaldehyde 4% (v/v) until analysis.

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

2.3 Water chemistry

Regional study. Water chemistry of the selected sites was obtained from the Catalan Water Agency (ACA) which monitors them monthly. The following variables were included: conductivity, water hardness, dissolved oxygen, TOC, NO_3-N, P_2O_5, SO_4, Cl, HCO_3; NH_4-N, K, Ca, Na (the most common used for monitoring purposes) and water metal concentration (Cd, Cr, Cu, Pb, Ni, Zn, As, Hg). Water samples were analysed following standard procedures (APHA, 1989).

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

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

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

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

Each DGT device consisted of a plastic base (2.5 cm diameter) and a plastic cap with a 2 cm diameter window; this plastic holder was loaded with a filter, a resin gel, a diffusive gel and a second filter. DGT holders (piston type), diffusive gel disks (0.8 mm thick) and Chelex-100 binding resins (0.4 mm thick) were purchased from DGT research (Lancaster, UK); nitrate cellulose filter membranes (0.13 mm thick; 0.45 µm mesh) were purchased from Millipore (Molsheim, France). Three DGTs were deployed at each sampling site (B1, B2, T1, T2, OL, Rd) in two occasions (December and July 04). The DGT holders were placed in a plastic grid role with the window down to avoid sediment settlement on top of them. The grid role was made by a piece of plastic grid (square of 30 * 30 cm with 4 cm holes approx.) which was rolled and the DGT holder was fixed to a small window. The whole device was attached to big stones inside the river during one week. Exact exposure time, temperature, water flow, and pH were recorded for each sampling site. After exposure, the resin gels were eluted in 7 mL 1M HNO_3. Metal concentrations of the DGT resin gel eluates and the acidified (1% HNO_3) water samples were measured by inductively coupled plasma mass spectrometry (ICP-MS; HP 7500c Agilent). The mass of metal sequestered on the DGT resin was evaluated considering an 80% yield of elution (Zhang et al. 1995). Calculations of labile copper were done according to Davison and Zhang (1994). All material used was soaked previously in 10% HNO_3 for 24 h and rinsed with ultrapure water.

2.4. Diatom analyses

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

2.5. Data analysis

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

$$CCU = \sum m_i / c_i$$

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

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

2.6. Statistical methods

Physico-chemical variability over time was first explored. In the regional study, temporal variability was evaluated by comparing spring and summer average values. In the watershed study, differences between the three sampling times were evaluated using ANOVA using the time as a factor.

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

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

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

3. Results

3.1. Regional study

Physical, chemical and biological characteristics. Streams were characterized by conductivities from 57 to 2750 µS cm^{-1} and a variety of nutrient levels (Table 2). Metal concentration ranged between <0.1-0.8 µg/L for Cd and Hg, <1-10 µg/L for As, Cr and Pb and <10-80 µg/L for Cu, Ni and Zn. Comparing spring and summer data, average values of dissolved salts (conductivity and chloride), nutrients (mainly phosphate) and metal CCU were slightly higher in summer than in spring, but the variability between sites (see the standard error of the mean) was always much higher than temporal variability (Table 2). Over 288 taxa of diatoms were identified from the study sites. Of those, twenty-three diatom taxa were included (typically represented > 1% of the diatom community at all sites).

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

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

The first run of the data analysis (i.e. environmental variables as explanatory variables) showed that organic pollution and conductivity were statistically significant. In the second run (i.e. metal pollution as explanatory variables) chromium and CCU turned out to be statistically significant. Environmental variables alone accounted for 22.6% of explained variation. The metal pollution represented 9.8% of explained variation. The results showed that 5.1% of the diatom data variation was shared by the environmental and metal pollution variables. Finally, the unexplained variation corresponded to 62.5%.

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

3.2. Watershed study

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

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

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

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

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

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

4. Discussion

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

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

In contrast with the regional case-study, study sites in the Fluvià watershed were selected in order to provide the minimum physiographical and geochemical variability. All sampling sites had similar hydromorphology (2-3 order streams) draining the same geological area. In this case diatoms followed a clear eutrophication gradient. Repetitive sampling over time (2-3 times) provided temporal variability which was also influencing diatom species due to seasonal water temperature changes.

Focusing on the second type of pollution analyzed, i.e. metal pollution, the percentage of variance explained in the regional study was lower (<10%) than in the Fluvià watershed (21.1 %). This difference was mainly due to the variables included in the analysis: water metal concentration in the first case and both, water and biofilm metal concentrations in the second one. As it was expected, water metal content was a better predictor of the diatom community structure in the regional than in the watershed study. Taking the CCU as a summary of water metal pollution, all sampling points included in the Fluvià case-study correspond to the lower metal category of the regional data set. In addition, while metal loads exceeded expected toxicity thresholds in some of the cases included in the regional study we did not expect metal toxicity in the watershed study. The Fluvià case-study is a clear example of low-metal pollution from diffuse (B2 and Ol) and point sources (the outflow of relatively small WWTP in T2 and Rd). In spite of the fact that metal loads were relatively high, the percentage of variance explained by metal pollution in the water phase in the regional case was low, and the partition of variance included only the most polluted cases. It was related with only one diatom species: *Navicula saprophila*, considered to be very tolerant to pollution (Soininen 2002, Rakowska 2004, Tornés et al. 2007). *Navicula saprophila* was a dominant taxon (>60% relative abundance) during spring in the lower part of the Besós (J034 and J048) and Francolí rivers (J079). One of the sites (J048) is located in an industrial area and has the highest metal loads which exceed quality thresholds (0.80 µg/L Cd, 4 µg/L Hg and 6.26 CCU). Water metal concentration was also above the quality threshold at J048 (3.58 CCU), but relatively low at J034 (0.29 CCU). Differences in TOC were very broad and may have modulated periphyton metal bioavailability. Furthermore, it has been reported that Cu and Zn toxicity on periphyton are influenced by nutrient loads (Ivorra et al., 2002; Guasch et al., 2004, Paulsson et al., 2000). Differences in phosphate and nitrate concentration may also influence the sensitivity of periphyton modulating the response of diatoms to metal under different nutrient conditions.

Total metal concentration in the water has been considered a poor indicator of metal availability (Campbell, 1995; Tusseau et al., 2007). Metal loads are derived from single water samples which only record a limited time period. Point water sampling cannot account for the temporal variability and detecting the occurrence of higher concentration episodes that may have been recorded either with passive samplers (integrating up to the 7 previous days) or benthic biota (fluvial biofilms may integrate several weeks of exposure). Furthermore, it is well known that biologically active surfaces (periphyton and sediments) play an important role in reducing water column concentrations following effluent release via adsorption and complexation processes (Bubb & Lester, 1995) providing an integrated representation of the accumulation of toxicants in the benthic environment (Meylan et al., 2004). In our study, metal content in biofilms accounted for a significant portion of diatom species variance. The partial RDA identifies seven diatom species related with biofilm metal concentration. Three different patterns were observed. Two species, (*Navicula gregaria* and *Rhoicosphenia abbreviata)* which were not found in the reference sites, increased linearly with biofilm metal concentration. In the case of *Navicula gregaria*, the relative abundance increased from 0 to 12%. In the context of our area of study, this species may be considered indicative of slight metal concentration increase. In agreement with our results, *N. gregaria* was considered to be tolerant to metal pollution by Chanson et al. (2005) but also to organic pollution (Gomà et al., 2005). Another diatom species (*Nizschia tripunctata*), already present in the reference sites, showed a slight increase at higher metal concentration. Finally, *Nitzschia fonticola* and *Navicula cryptotenella* showed a non-linear increase with metal concentration. Overall, this group of species increased from <5% to more than 30% of the total abundance with increasing metal concentration (Fig. 3). Rogers et al. (2002) also found a good correlation between metal concentration in vegetative habitats and the corresponding biological condition using macroinvertebrate communities. The percentage of variance explained by metal concentration in vegetative habitats was 22%, very close to the 23% variance explained by metal concentration in the biofilms of our investigation.

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

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

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

Looking for generalizations, large scale studies are a tempting approach for research and also management purposes. However, they are influenced by regional scale variability that may conceal the effects of human impacts. Conversely, human impacts are better detected in small scale studies, including reference and impacted situations, within a restricted range of environmental variability. However, any conclusion drawn from these studies is restricted to the area of study and cannot be easily generalized. Surprisingly, few studies have addressed the importance of scale. The comparison between the two case studies here illustrates the relevance of the scale but also the importance of using a good set of explanatory variables. In addition, the results obtained in our local case study, the Fluvià watershed, reveal that diatom assemblages may respond to low levels of metal pollution. Given the regional differences in climate and land use we were not surprised to find differences in community composition at the regional scale that were correlated with these strong ecological gradients, although the finding that at watershed level streams were responding to low metal pollution was of interest. This finding was possible by means of a good sampling strategy, but also thanks to the use of highly sensitive analytical techniques for the study of metal concentration and bioavailability. Analyses of metal traces in the water phase and the biota (fluvial biofilms), together with the use of passive samplers are recommended for assessing low metal pollution.

5. Conclusions

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

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

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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

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

	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 (µS /cm)	1142	203.8	853.6	196.3	57.0	2750.0	
Temperature (ºC)	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 (µg/L)	<0.1		0.07	0.07	<0.1	0.8	0.25
Cr (µg/L)	<0.1		0.93	0.66	<0.1	6.60	74
Cu (µg/L)	9.40	3.34	5.03	1.42	<5	50.0	9
Pb (µg/L)	1.07	0.77	<1		<1	10.0	2.5
Ni (µg/L)	14.64	6.51	8.20	4.44	<1	80.0	52
Zn (µg/L)	37.45	4.75	35.11	4.37	6	63.0	120
As (µg/L)	<1		2.31	1.21	<1	10.0	150
Hg (µg/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

Table 2. Physico-chemical variables used in the CCA of the regional study (Catalonia, NE Spain). Average and standard error of summer (n=14) and spring (n=11) data, minimum (Min.); Maximum (Max.) values recorded (n=25) and corresponding toxicity threshold.

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

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

site	Cond (µS/cm)	phosphate (µM)	Nitrate (µM)	Ammonium (µM)	DOC (mg/L)	Oxygen (mg/L)	pH	Temp (ºC)
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

Table 4. Physico-chemical variables used in the RDA of the watershed study (Fluvià river). Average and standard deviation for the three different sampling periods (December, February and July) of conductivity (Cond), nutrient concentration, dissolved organic carbon (DOC), oxygen concentration, pH and temperature at the different sampling sites (n=17).

		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

* n=2

Table 5. Metal concentration variables included in the RDA: metal concentration in biofilms (µg/g) and water (µg/L). For each site, values are the average and standard error of three sampling. Average metal concentrations estimated with DGT (µg/L) were not included in the RDA and are the average of two sampling.

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

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

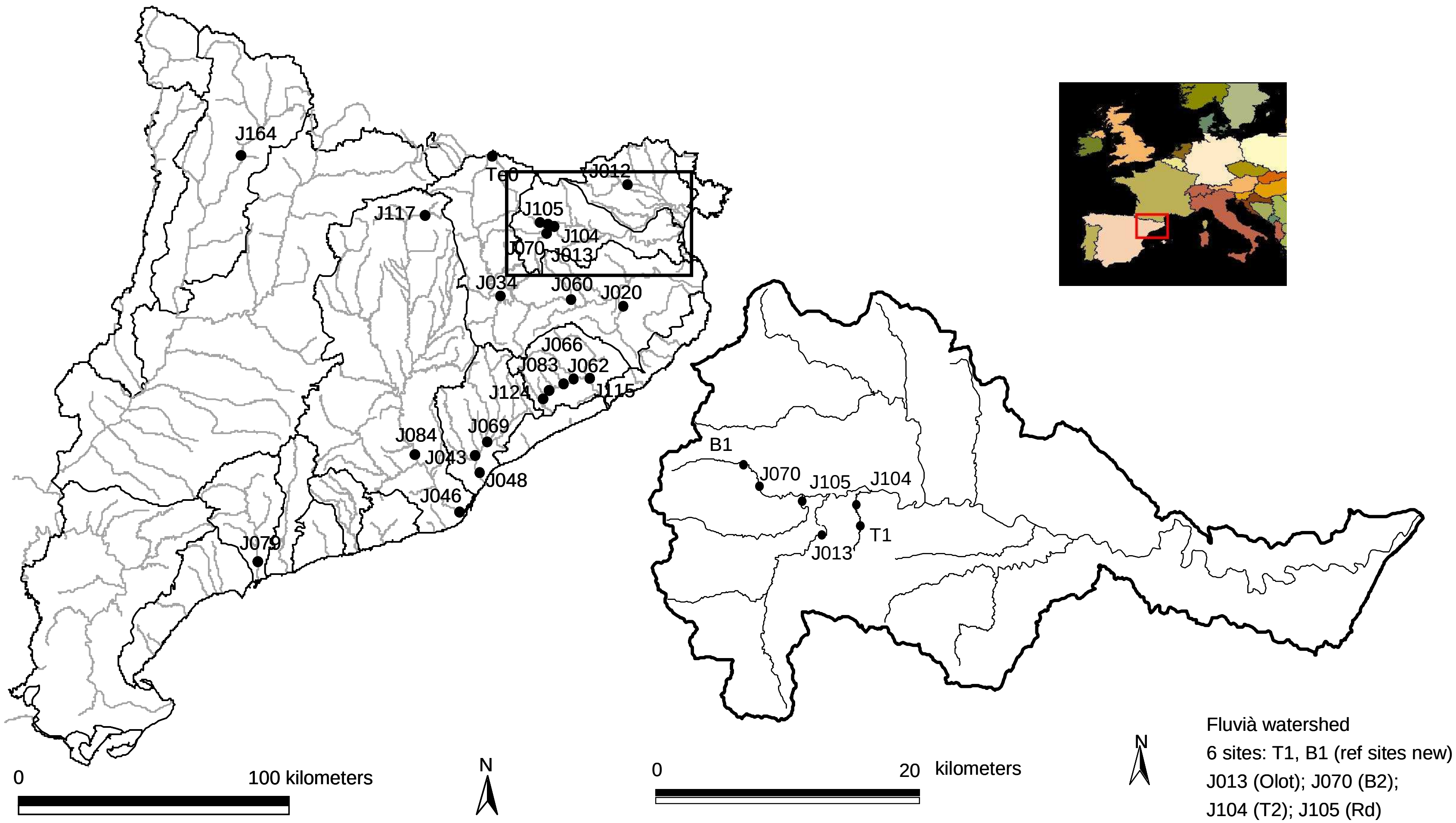

Figure 1. Location of the area of study (Catalonia, NE Spain). Study sites included in the regional and watershed studies (left and right maps, respectively). The code assigned by Catalan Water Agency. The location of the Fluvià watershed in the regional map is also indicated.

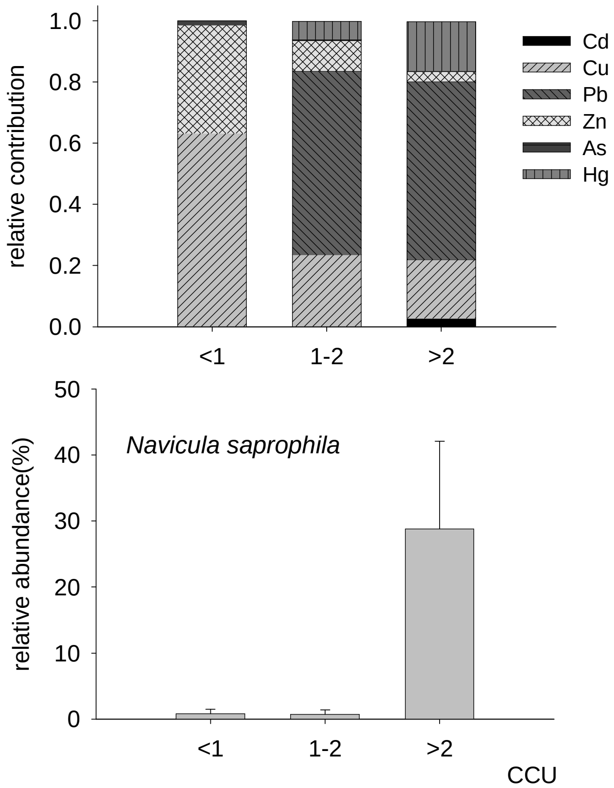

Fig 2. Regional case-study. Relative contribution of various metals to the cumulative criterion unit (CCU) at background (CCU<1, n=10); low metal (1<CCU<2, n=7); and medium metal (2<CCU<10, n=8) categories. Average and standard error, for each metal category, of the abundance of diatom taxon related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

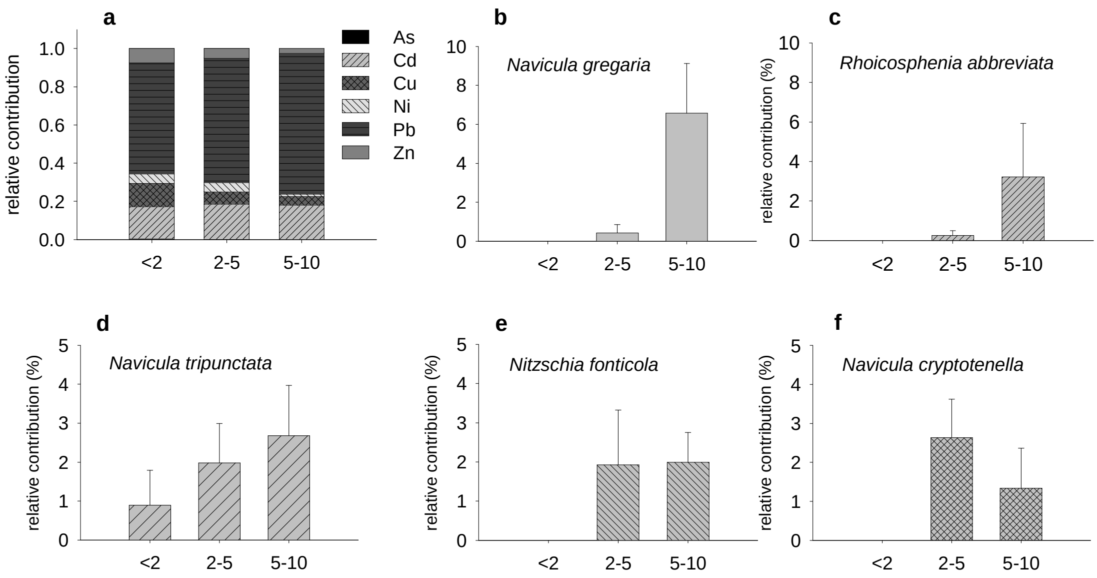

Fig 3. Relative contribution of various metals to the cumulative criterion unit calculated using biofilm concentrations (CCUb) at increasing metal categories in the Fluvia case-study. CCUb<2 (n=3); 2<CCUb<5 (n=10); and CCu>5 (n=4). Average and standard error, for each metal category, of the abundance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