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Diatom responses to zinc contamination along a Mediterranean river

Soizic Morin[1,*], Natàlia Corcoll[2,3], Berta Bonet[3], Ahmed Tlili[4] & Helena Guasch[3]

[1]Irstea, UR EABX. 50 avenue de Verdun, FR-33612 Cestas cedex, France
[2]Catalan Institute for Water Research (ICRA), C/ Emili Grahit, 101. ES-17003 Girona, Spain
[3]Institute of Aquatic Ecology, University of Girona, Campus Montilivi. ES-17071 Girona, Spain
[4]Swiss Federal Institute of Aquatic Science and Technology (Eawag), Department of Environmental Toxicology. Überlandstrasse 133, Postfach 611, CH-8600 Dübendorf, Switzerland
*Author for correspondence: soizic.morin@irstea.fr

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Abstract

Background and aims – Diatom-based water quality management is increasing, and specific indicators are required for the assessment of priority substances such as metals. We tested a variety of features of diatom communities, in a river exhibiting a gradient of zinc contamination (the Riera d'Osor, Spain), to determine the most relevant ones.
Key results – Community composition changed over time of exposure, and with the intensity of metal contamination. Species richness was significantly lower at the most contaminated sites. Species composition was more even under background and low exposure levels, but low metal inputs selected for varieties of *Cocconeis placentula* (var. *placentula*, *euglypta* and *lineata*). Small taxa (*Eolimna minina*, or *Achnanthidium minutissimum* and *A. pyrenaicum*) dominated in the most contaminated sites, and deformed diatom cells were found abundant.
Conclusions – Although species composition clearly responded to varied levels of zinc pollution, combining cell size classes or total biovolume of the community and percentages of deformities allowed reliable assessment of the presence, and intensity, of contamination. These descriptors present the major advantage of being independent of regional taxonomic peculiarities, thus providing robust assessment irrespective of the area studied.

Key words – diatoms, community structure, cell size, biovolume, teratologies, zinc.

INTRODUCTION

Periphytic diatoms are widely used to monitor freshwater bodies, as bioindicators of general water quality, and increasingly for toxicant stress. Although many diatom endpoints may respond to metals (as reviewed in Morin et al. 2012), clarifying their relevance and/or complementarity calls for *in situ* validation. The most cited ones are: teratologies (e.g. see Falasco et al. 2009), decrease in individual and global cell sizes (Cattaneo et al. 2004, Morin and Coste, 2006, Morin et al. 2007, Luís et al. 2011), mortality (Torres et al. 1998) and shifts in assemblages towards a dominance of species growing close to the substrate or motile taxa (Medley and Clements, 1998, Cattaneo et al. 2004, Morin et al. 2012). An *in situ* translocation experiment was undertaken in a Mediterranean river (called Riera d'Osor) to assess the responses of periphyton to a gradient of zinc contamination. The Riera d'Osor is a siliceous Mediterranean mountain stream, located in North-East Spain. This second-order river drains a metal-polluted area due to former mining activities. In this river, photosynthetic processes (Corcoll et al. 2012) and biomarkers (antioxidant enzymatic activities, Bonet et al. 2014) were early warnings of metal contamination, whereas algal composition (Corcoll et al. 2012) or tolerance of both heterotrophic and autotrophic compartments (Tlili et al. 2011) were more powerful in detecting chronic exposure or extremely high stress on the short term. In complement to their work, we explored the temporal trajectories of the specific response of diatom features to the metal gradient of Riera d'Osor, using communities with the same exposure history (i.e. previously grown under the same uncontaminated conditions) transferred to sites exhibiting varied zinc contamination. The main objective is to determine which are the most relevant diatom endpoints (among those cited above) for the *in situ* assessment of chronic zinc contamination.

MATERIALS AND METHODS

Experimental design

The experimental set up and physicochemical methods used are described in Bonet et al. (2014) and Corcoll et al. (2012). Briefly, glass slides were immersed as artificial substrates for periphyton colonisation upstream the Riera d'Osor (colonisation site, CS). This river exhibits a gradient of zinc contamination (Tlili et al. 2011, Corcoll et al. 2012, Bonet et al. 2014) downstream CS. After five weeks of colonisation, one series of substrates was kept at CS, and the others were transferred to five downstream sites (called Up, M1, M2, M3, plus one additional site at the mining source, MS) in comparable light and water flow conditions to CS. The sites (fig. 1) were surveyed on the day of translocation (week 0), and one, three and five weeks after translocation (weeks 1, 3 and 5). At each sampling date, water physicochemistry was characterized *in situ* for pH, temperature, conductivity, oxygen, and at the laboratory for dissolved nutrients and metals.

Simultaneously, periphyton was scraped from artificial substrates to quantify total zinc bioaccumulation (Bonet et al. 2014) and characterize diatom communities (five replicate slides on week 0, then three replicates per site and per sampling date). Diatom cell density and mortality were determined from fresh material using a Nageotte counting chamber (Morin et al. 2010). Then diatom samples prepared according to the European standard EN ISO 13946 were counted using a Leica photomicroscope (x1000 magnification); a minimum of 400 individuals per sample (EN ISO 14407) was identified using appropriate literature (Krammer & Lange-Bertalot, 1986, 1988, 1991a, 1991b and recent nomenclatural updates).

The distribution of growth forms (i.e. prostrate, tightly attached cells; cells close to the substrate either erected or with a short stalk, cells forming rosettes, clumps or long stalk; and solitary or colonial non-attached cells) was determined for each genera or occasionally species, according to the observations made by Hoagland et al. (1982), Hudon & Bourget (1983), Hudon et al. (1987), Katoh (1992), Kelly et al. (2005) and Tuji (2000). Specific biovolumes were calculated from theoretical dimensions found in the literature using the formulae of Hillebrand et al. (1999). Species and traits distributions were analyzed in terms of their relative abundances and contribution to the total biovolume of the community. Deformed diatoms, also named teratologies (cells with abnormal general shape and / or diatoms with deformed valve wall ornamentation), were inventoried, and grouped together under the code TRTG for subsequent analyses.

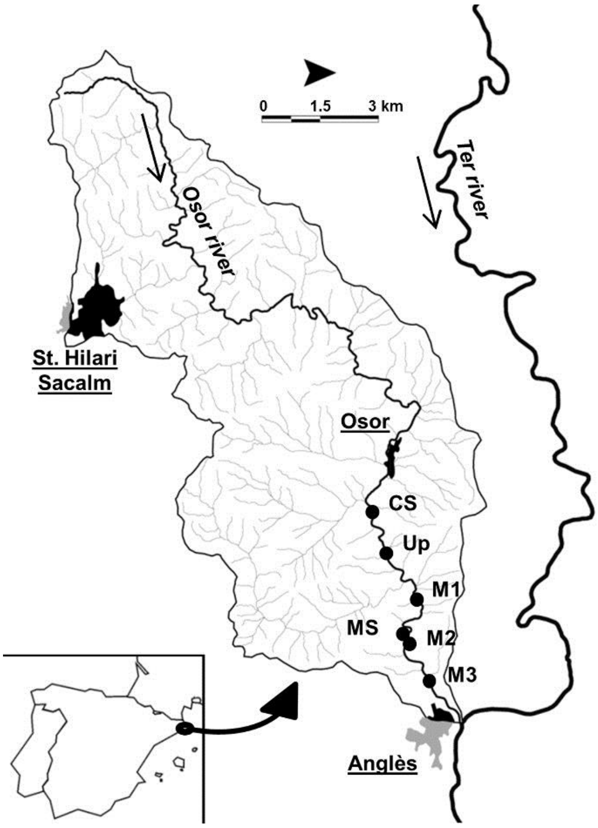

Figure 1 – Location of the sampling sites in the Riera d'Osor.

Data analysis

Significant differences in water chemical parameters between sites were identified by one-way ANOVA followed by a Tukey's HSD test, using XL-STAT (AddinSoft, Paris, France, 2010). Dissolved metal concentrations were computed into Cumulative Criterion Units (CCU) to estimate potential toxicity to aquatic organisms, following Clements et al. (2000). They consist in a score based on US Environmental Protection Agency's National Water Quality Criteria (http://www.epa.gov/waterscience/criteria/wqctable/) for each metal and their concentration measured *in situ*. Three categories are defined: "background" are characterized by CCUs below 1, "low" by CCUs between 1 and 2, and "moderate" by CCUs higher than 2. Based on taxa relative abundances, we analyzed the temporal trajectories of exposed communities, compared to the non-linear changes of the reference (CS) community, by

Principal Response Curves (Van den Brink & ter Braak, 1999) conducted using the vegan package in R (Oksanen et al. 2010) on the centred data matrix of species with relative abundances exceeding 2% in at least one sample. The significance of the PRC was tested through Monte Carlo test (199 permutations).
Linear regressions were calculated between non-taxonomic diatom endpoints (cell densities, diatom mortality, species richness, percentage of abnormalities, biovolume) and zinc bioaccumulation, being a more integrative measurement of exposure than water concentrations, especially in the case of long-term exposure experiments as bioaccumulation reflects the real exposure of organisms to the toxicant. The data were plotted in ternary plots to describe the distribution of cell sizes in the samples with time of exposure, using PAST software v2.16 (Hammer et al. 2001).

RESULTS

Table 1 summarizes the main physicochemical characteristics of the six sites studied along the Riera d'Osor. These sites exhibited different levels of metals (mainly zinc, iron, aluminium and nickel) and CCUs discriminated M3 (background level), from CS, Up and M1 (low level) and MS and M2 (moderate exposure). Bonet et al. (2014) demonstrated that zinc concentrations were the driving factor along the Riera d'Osor gradient, and that bioaccumulation in the periphyton reached a plateau after one week of exposure. Zinc accumulation at week 5 classified the sites as follows: Up ~ CS << M3 < M1 << M2 << MS. In M3, dissolved metals were very low but were observable from bioaccumulation data. Nutrient concentrations were somewhat lower in MS (table 1).

Table 1 – Average (± standard error) values of environmental parameters during the translocation period (n=4) and zinc bioaccumulation in the biofilms on week 5 (n = 3).
For each parameter, different letters indicate significant differences (p<0.05) between sites after ANOVA and Tukey's HSD test. n.s.: not significant.

Parameter	CS	Up	M1	MS	M2	M3	p-value ANOVA
pH	8.2±0.0	8.1±0.1	7.9±0.1	8.0±0.1	8.1±0.1	7.8±0.2	n.s.
Conductivity (µS/cm)	227±12	233±15	448±246	649±276	394±93	220±12	n.s.
Dissolved oxygen (%)	98.3±2.3	98.2±1.9	93.3±4.1	95.1±3.6	99.3±2.8	98.2±2.3	n.s.
Temperature (°C)	16.3±1.7	16.8±1.6	17.1±1.7	16.5±1.4	17.8±1.8	16.9±1.5	n.s.
PO_4^{3-} (mg/L)	$0.37\pm0.05^{a,b}$	0.40 ± 0.06^{a}	$0.34\pm0.03^{a,b}$	$0.05\pm0.02^{a,b}$	0.02 ± 0.01^{b}	$0.33\pm0.04^{a,b}$	0.030
NO_3^- (mg/L)	3.5 ± 0.3^{a}	3.2 ± 0.3^{a}	$2.3\pm0.2^{a,b}$	0.7 ± 0.3^{b}	$1.9\pm0.3^{a,b}$	3.1 ± 0.2^{a}	0.009
$[Zn]_{water}$ (µg/L)	35 ± 19^{a}	28 ± 15^{a}	44 ± 17^{a}	2481 ± 463^{b}	341 ± 69^{a}	31 ± 16^{a}	0.0004
$[Zn]_{biofilm}$ (µg/$g_{dry\ weight}$)	148±11	84±6	640±168	8249±1619	1975±230	437±7	

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A total number of 105 diatom taxa were identified in the Riera d'Osor (see electronic appendix 1 for species list and their relative abundances on week 5). From the initial community structure (CS, week 0), the trajectories based on diatom composition (using the 33 dominant taxa) diverged accordingly to exposure categories. The Principle Response Curve (eigenvalue 0.3343) was highly significant, with a *p*-value of 0.005. As shown in fig. 2, diatom communities were similar at CS and Up, diverged slightly from M1 and M3 and clearly at MS and M2. Species richness at CS, Up, M1 and M3 were high (c. forty species per sample), whereas the number of taxa decreased significantly at MS (-20%) and M2 (-35%). Moreover, communities were very diversified, except at M2 and MS were species diversity decreased: communities were dominated by *Eolimna minima* (in MS: 26.0 ± 3.1%) or *Achnanthidium minutissimum* (in M2: 20.3 ± 0.7%) in conditions exhibiting the most pronounced zinc contamination. At these sites, elevated percentages of abnormal diatoms belonging to thirty species were also found (3.4 ± 0.4%, figs 2–3). The architecture of the communities (electronic appendix 2) also changed with zinc contamination, exhibiting lower percentages of adherent species, higher abundances of species not attached to the substrate and potentially motile.

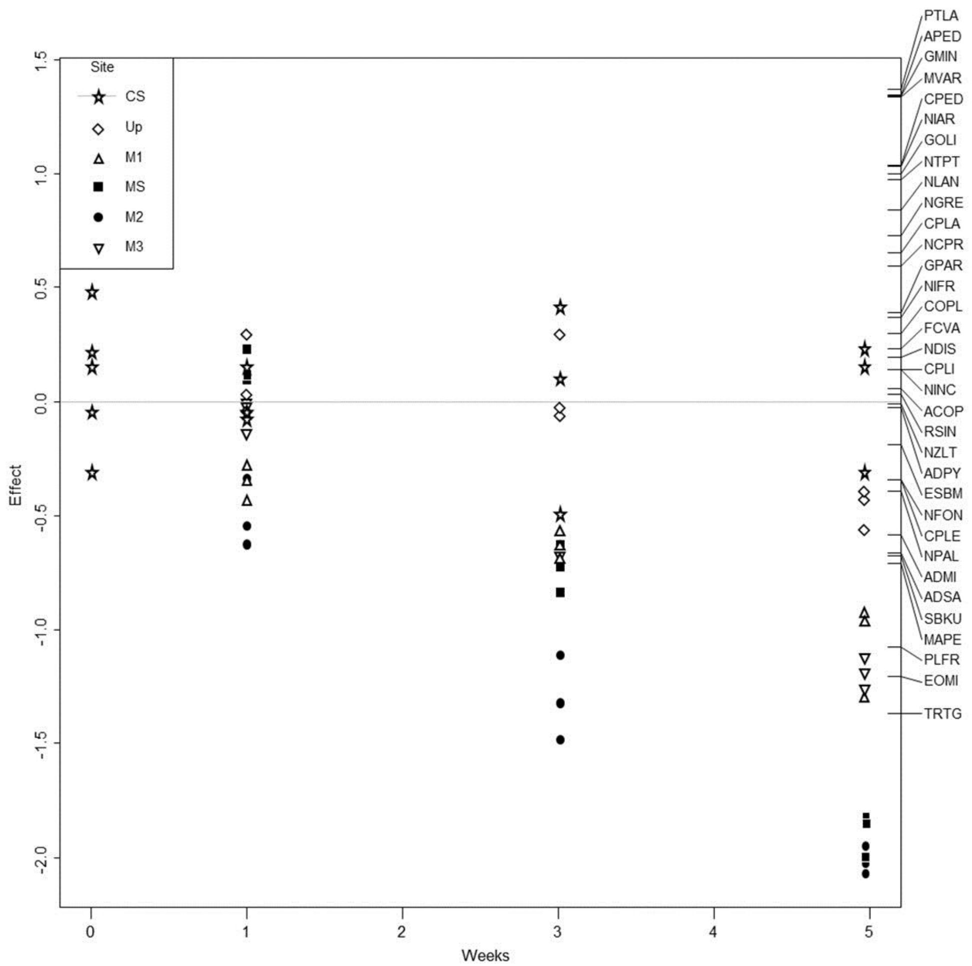

Figure 2 – Time-dependant multivariate changes in diatom species composition in the six sites studied. The grey horizontal line represents the mean trajectory of CS communities.

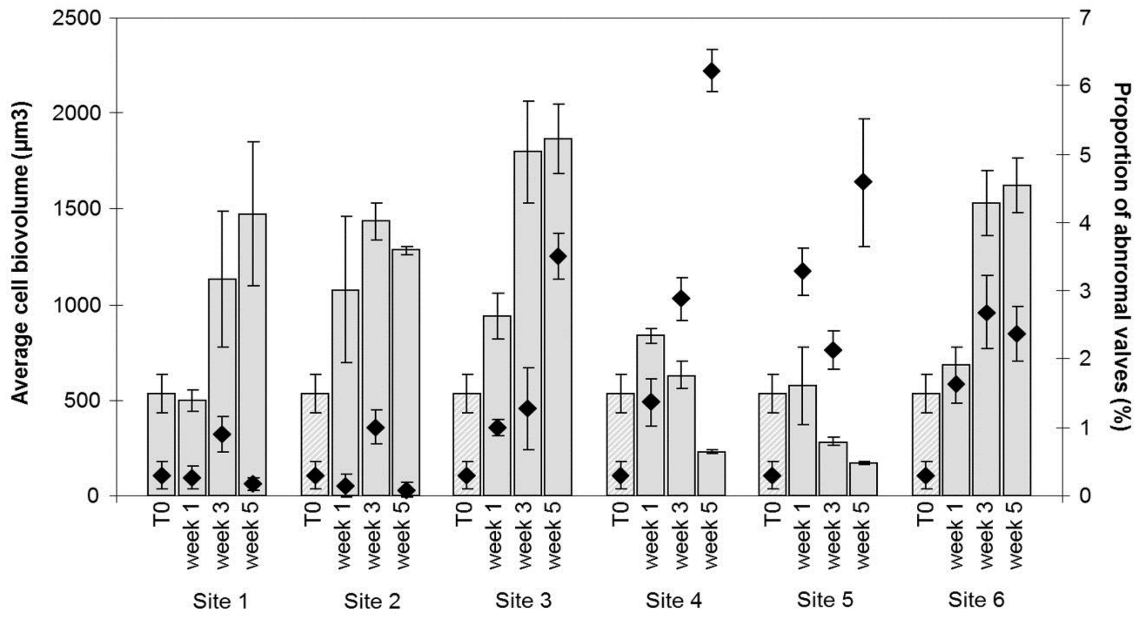

Figure 3 – Temporal changes cell biovolume of the communities sampled (grey histograms) and percentage of diatom deformities (black diamonds). Values are average ± standard error (n = 3).

Metal accumulation was significantly correlated with a decrease in diatom densities and an increase in mortality (respectively $R^2 = 0.21$, $p = 0.04$ and $R^2 = 0.27$, $p < 0.01$, data not shown).
Average cell biovolume tended to decrease with metal exposure ($R^2 = 0.02$, $p = 0.08$, and fig. 3); the distribution of size classes was also modified at MS and M2 (fig. 4). From an initial diatom community (week 0) composed of medium-sized species (59 ± 3%) associated with smaller taxa (25 ± 5%), community composition in CS evolved towards higher abundances of larger cells (41 ± 13%) after five weeks. This pattern was also observed in Up, M1 and M3 sites, although it was delayed in M3 and the trajectory slightly diverged in M1 on week 1 with higher proportions of small diatoms. At the end of the experiment large species such as the varieties *euglypta*, *lineata* and *placentula* of *Cocconeis placentula* (individual biovolumes higher than 2500 μm^3) contributed most to the total biomass in Up, CS, M1 and M3, consequently increasing average biovolume in these sites (fig. 3). Conversely, communities in MS and M2 shifted from week 3 towards a dominance of small species. For instance, on week 5, *Eolimna minima* (63 ± 8%) and *Achnanthidium minutissimum* (51 ± 1%) were the most dominant species at M2 and MS respectively.

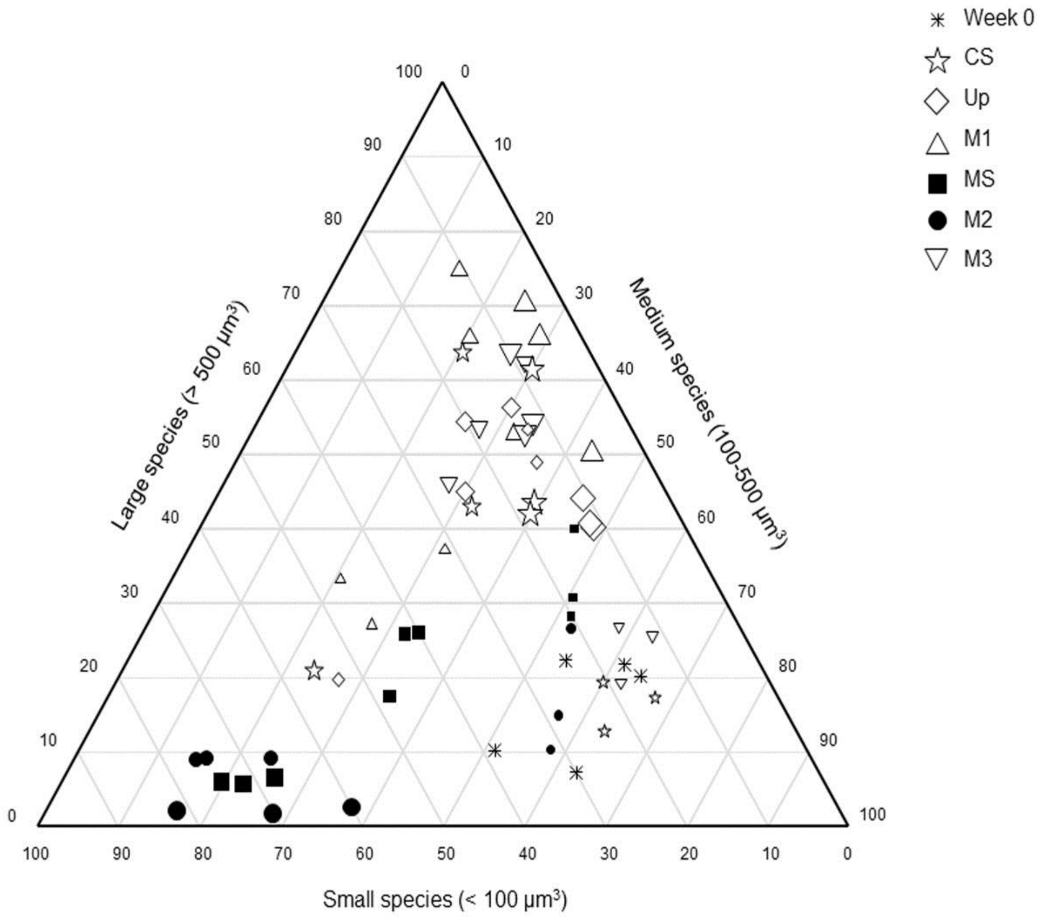

Figure 4 – Relative abundances (%) of small, medium and large species in the assemblages, using the same size classes of biovolume as Morin et al. (2007). Sampling dates are week 0 (*), week 1, 3 and 5. The larger the symbol, the longer the duration of translocation.

DISCUSSION

Metal contamination along the Riera d'Osor

Translocation in the Riera d'Osor allowed an ecologically relevant assessment of contrasted zinc contaminations along the river, thus reducing the influence of environmental factors other than metal pollution (in particular, local geology or species pool), contrarily to previous translocation studies in a different river (Ivorra et al. 1999, Gold et al. 2002). Such experimental watersheds are rare, and provide the opportunity for effect-based assessment under "uncontrolled" and natural conditions along a contamination gradient, displaying a potential metal toxicity ranging from background (almost unimpacted) in M3, and low in CS, Up and M1 to moderate levels in M2 and MS (Bonet et al. 2014). The presence of a dam between M2 and M3 caused very low metal concentrations in the water downstream (retention and/or dilution) but sporadic transport of contaminated matter may explain some accumulation in M3 periphyton.
Tlili et al. (2011), Corcoll et al. (2012) and Bonet et al. (2014) demonstrated that metals, zinc in particular, were the main driving factor of periphytic responses in the Riera d'Osor. Zinc

accumulation globally reflected the gradient of contamination, and rapidly reached a stable concentration, providing a reliable estimate of exposure. Changes in diatom features are developed below with respect to their findings, i.e. in relation to zinc pollution.

Metal contamination impacts diatom growth

A significant decrease in cell densities was observed with increasing zinc contamination, likely reflecting lower growth rates under metal exposure (Morin et al. 2008). However, cell densities may also be affected by many other factors such as nutrients or light availability, but also grazing pressure (e.g. Guasch et al. submitted). Cell mortality is in turn a direct expression of toxicity; the significant increase in diatom dead cell number was, here, directly related to zinc pollution. The highest mortality values were observed on week 3 when diatom densities were also particularly low downstream, probably due to self-detachment (Boulêtreau et al. 2006) under the combination of hydraulic and toxic factors.

Although cell densities, or even mortality, are very rough descriptors, they integrate a wide range of environmental constraints and, when affected, highlight marked and unequivocal impacts, especially in long term studies. In our study, extreme toxicity occurred on global community dynamics at MS and M2, but mortality slightly recovered on week 5 (electronic appendix 1) due to likely adaptation of the communities settled. At this date, mortality was around 20% in all sites, and probably more related to general community ageing.

Metal contamination drives changes in community structure

In long term surveys, the characterisation of trajectories in community structure is particularly relevant (Van den Brink & ter Braak, 1999), as it allows observing discrete shifts in species composition or progressive transitions to alternative stable states upon exposure to a particular environmental factor (here, zinc exposure). Community responses to changes in exposure also encompass the influence of species dispersal and colonisation by new immigrants from upstream drift.

The Principal Response Curve (PRC, fig. 2) based on non-rare taxa indicated shifts in community composition over time and upon exposure to zinc. Based on community temporal trajectories, three groups of sites were identified. These groups were slightly distinct when sites are classified into CCU categories (M3 grouped with M1) and rather followed zinc content in the periphyton. Indeed, CS and Up had similar trajectories, then from week 3 M1 and M3 slightly diverged from them, and were clearly separated from M2 and MS. The relative abundances of *Eolimna minima* and *Planothidium frequentissimum* significantly increased with the most pronounced zinc contamination (MS and M2), compared to CS. Both species are generally considered as indicative of metal contamination (see review by Morin et al. 2012). Opposite behaviors between *P. lanceolatum* and *P. frequentissimum* confirmed the higher tolerance of the latter to a wide range of pollutions, as also shown by their ecological profiles in Coste et al. (2009). Diatom community composition, and consequently the architecture of the communities, were congruent with patterns identified in various metal contaminated areas characteristic of moderate contaminations (Morin et al. 2012), such as increased abundances of small species, potentially motile. A rise in the proportion of erected diatoms occurred in M2, mainly related to the presence of *Achnanthidium minutissimum* clumps. This is in accordance with Stevenson and Bahls (1999) who use this species as an indicator of metal pollution. Moreover, higher abundances of deformed diatoms (TRTG) were observed in zinc polluted sites, as expected from the literature (Falasco et al. 2009, Morin et al. 2012). No difference in trajectories was observed between the two most contaminated sites, M2 and MS, although metal accumulation was four times higher in the latter. However, diatom communities were slightly different on week 3, and then clearly converged on week 5, probably due to these differences in exposure level. This suggests a shift of composition, above a certain level, towards a “metal adapted” community able to cope with various levels of metal exposure. However, more species belonging to the “non-attached” group (electronic appendix 2) were found in M2, suggesting higher contribution of species drift to community colonization at this site. Besides confirming the metal tolerance of these species, the PRC indicated similar trajectories of CS and Up sites (between-replicates differences within the same range of variation), and community changes intermediate between upstream sites and the most contaminated ones at M1 and M3, which was congruent with the contamination levels. The intermediate position of these sites in the PRC between the unpolluted and the highly polluted sites highlighted the progressive selection of species driven by the gradient of metal exposure.

Metal contamination modifies cell size distributions
Communities from CS, Up, M1 and M3 shifted towards an increase of larger cells (fig. 4, electronic appendix 2). The ternary plot permitted an easy visualisation of the patterns of translocated communities deviating from the reference (CS) communities over time. The trends observed in fig.4 were consistent with the results of the PRC (fig. 2). Community composition shifted from an association of medium-sized species and smaller taxa on week 0 towards higher abundances of larger cells after 5 weeks in CS, Up, M1 and M3, the two latter slightly diverging from the upstream sites. These differential responses were probably linked to slightly higher zinc accumulation in the periphyton, driving intermediate trajectories between the less and the most exposed communities. On week 5, the communities in Up, CS, M1 and M3 had relatively high average biovolume (fig. 3), whereas communities in MS and M2 shifted from week 3 towards a dominance of small species, confirming that metal contamination selects for smaller taxa (Morin et al. 2012). A decrease in cell biovolumes accounts for a long-term process of species selection under metal contamination (Medley and Clements, 1998, Morin et al. 2007, Morin et al. 2012), i.e. transition towards dominance by smaller size classes denoting species selection: death, or limited growth, of big taxa, concomitant to the development of small species (abundances higher than 50%). Therefore a decrease in large cell abundances (< 10%) displays high metal impact (concentration and chronic exposure).

Metal contamination induces teratologies
Teratologies responded quickly to metal exposure, confirming the findings of, e.g. Duong et al. (2010). Deformed *A. minutissimum* were abundant in MS (5% on week 5), in accordance with the findings of Cantonati et al. (2014) on this species. Total diatom deformities above 1% specifically point out metal contamination, indicating levels above the tolerance range of diatoms (Morin et al. 2012). This threshold was exceeded from the first week following translocation in M1, M2, M3 and MS, and increased with time of exposure, highlighting metal contamination. Abnormalities are increasingly used to evidence the deleterious impacts of metals in the field (Duong et al. 2008, Ferreira da Silva et al. 2009, Luís et al. 2011, Corcoll et al. 2012, Lavoie et al. 2012). Here, the correlation with zinc bioaccumulation (R^2 = 0.25, p = 0.03) confirmed the reliability of this endpoint.

Complementary descriptors to detect the presence and intensity of metal contamination
Metal concentrations in waters and in the periphyton were globally correlated; however the biological responses slightly diverged. Global community biovolume significantly decreased

at M2 and MS (199 ± 10 μm^3, *vs.* 1560 ± 180 μm^3 in the other sites). Among the integrative descriptors studied, the percentage of teratologies was the most sensitive along the gradient of zinc contamination: from site M1, abnormal diatoms emphasized a certain level of metal stress. Metal teratologies were higher than the 0.35% threshold (established by Morin et al. 2012) indicative of toxic stress. Although M3 had background CCU and slightly low zinc bioaccumulation, significant numbers of deformed diatoms were recorded.
Combining both descriptors (biovolumes and teratologies) allowed establishing the presence of metal pollution through abnormalities (at M1, M2, M3 and MS) and its intensity through biovolumes or cell sizes distribution (significantly lower at M2 and MS). The fact that cell abnormalities were still observable at M3 expressed that toxic pressure remained downstream. Although the dissolved metals measured in the water samples were not high enough to classify the site as contaminated by the CCU approach, metal accumulation allowed detecting exposure at this site, as teratologies also did.

GENERAL CONCLUSIONS

Diatom community responses along the gradient of zinc contamination in the Riera d'Osor were in accordance with many previous reports (Morin et al. 2012). The time scale of response to changes in metal exposure after translocation was quite short (within three weeks), but longer-term assessment confirmed the trends observed, with even exacerbated effects.
Indicators based on diatom specific composition have been developed worldwide, but due to varied species distribution they are generally relevant only in the area they were created for. Comparing trajectories of species distributions is particularly interesting, with the use of appropriate statistics such as Principle Response Curves in studies in a specific area with a homogenous pool of species. Larger scale assessment calls for reliable indicators of water quality totally independent of the area surveyed. This can be achieved through a generic classification of local species *preferenda* or the implementation of intercomparisons between water quality indices (as done in the WFD intercalibration exercices) to prescind from ecoregional differences. Traits approaches also provide a valuable alternative, especially with a scope of simplifying diatom bioassessment (Berthon et al. 2011). The morphological features analyzed here have great potential for larger scale applications. In particular, we used the complementary information of biovolumes (or size distributions) and teratologies. Deformities above 1% confirmed metal presence in M1, M2, M3 and MS, and higher concentrations were demonstrated in M2 and MS where low biovolume was concomitant with high percentages of deformities, thus refining the assessment of metal contamination. As raised recently by Chapman (2011), one index alone results of an information loss; here the combination of varied descriptors indicated metal contamination, but also informed on its dramaticity.

SUPPLEMENTARY DATA

Supplementary data are available in pdf at *Plant Ecology and Evolution*, Supplementary Data Site (http://www.ingentaconnect.com/content/botbel/plecevo.supp-data), and consist of (1) diatom species list and relative abundances (in %) in biofilms translocated before transfer, and five weeks after translocation to Upstream, M1, MS, M2 and M3 in the Riera d'Osor; and (2) temporal variation of cell sizes and biofilm architecture at each site.

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