Consistency in Diatom Response to Metal-Contaminated Environments

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Abstract Diatoms play a key role in the functioning of streams, and their sensitivity to many environmental factors has led to the development of numerous diatom-based indices used in water quality assessment. Although diatom-based monitoring of metal contamination is not currently included in water quality monitoring programs, the effects of metals on diatom communities have been studied in many polluted watersheds as well as in laboratory experiments, underlying their high potential for metal contamination assessment. Here, we review the response of

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diatoms to metal pollution from individual level (e.g. size, growth form, and morphological abnormalities) to community structure (replacement of sensitive species by tolerant ones). These potential effects are then tested using a large, multi-country database combining diatom and metal information. Metal contamination proved to be a strong driver of the community structure, and enabled for the identification of tolerant species like *Cocconeis placentula* var. *euglypta, Eolimna minima*, *Fragilaria gracilis*, *Nitzschia sociabilis*, *Pinnularia parvulissima*, and *Surirella angusta*. Among the traits tested, diatom cell size and the occurrence of diatom deformities were found to be good indicators of high metal contamination. This work provides a basis for further use of diatoms as indicators of metal pollution.

Keywords Deformities • Metals • Periphytic diatoms • Rivers • Species distribution • Species traits

Contents

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1 Introduction

Biomonitoring has been increasingly used to assess water quality due to the more time-integrative characteristic of the approach compared with punctual chemical measurements. Among the tools used in biomonitoring, diatoms are cosmopolitan aquatic organisms, and are a major component of benthic biofilms. Because diatoms are at the basis of the trophic chain, these microscopic algae respond quickly to environmental changes and are considered good indicators of environmental conditions [1, 2]. They are included in numerous water quality monitoring programs worldwide, and river diatom-based indices have been developed in numerous countries [3–8]. Diatom species distribution is driven by environmental factors acting at different scales, from local (general water quality) to larger scale determinants (biogeography). Therefore, biological monitoring is best achieved considering both local and larger scales. Diatom-based indices developed to assess ecosystems' health often include these different scales of variability. For example, geology has a strong influence on water chemistry, which in turn affects diatom community structure [9–11]. To overcome the natural variability associated with the geological characteristics of the region, diatom-based indices must include larger scale determinants to effectively provide information on the local environment. This explains the fact that diatom-based water quality monitoring is usually country- or even ecoregion-dependent (e.g. [8, 11, 12]). Diatom indices assess the biological status of streams, with reference to trophy, acidity, conductivity, etc., but generally do not take into account toxic pollution. Field studies dealing with metal contaminations in various regions and countries showed quite consistent responses of diatom communities such as higher abundances of small-sized species [13, 14], increasing proportions of metal-tolerant species or significantly higher occurrences of valve deformities.

The main research results dealing with diatoms exposed to metals are reviewed in the first part of this chapter. The impacts most frequently observed on periphytic algae are addressed at different organization levels (from the individual cell to the community structure). Diatom communities may respond similarly to metal pollution, regardless of the region investigated. Consequently, we built a database with diatom species composition and corresponding information on water metal content from six different countries (France, Switzerland, Spain, Vietnam, China and Canada) to investigate the relationships between diatom communities and metal contamination, without considering other determinants (nutrient bioavailability, geographical location, seasonality, stream order, etc). The main goals of this case study based on a multi-country dataset were (1) to investigate how metal exposure drives diatom community patterns in a comparable way among countries, (2) to assess the information brought by nontaxonomical indicators (diatom deformations, cell size and diatom growth forms) for the monitoring of metal pollution, and (3) to determine the indicative value of the most representative species occurring with significant abundances in the studied streams.

2 Effects of Metals on Freshwater Diatom Communities

Metal toxicity on diatoms is linked to different steps in the circulation of the toxicant (Fig. 1) across the membrane (especially uptake mechanisms) and inside the cell, inducing perturbations in the normal functioning of structural/functional intracellular components. Diatom communities exposed to metals have, therefore, variable capacities to tolerate the stress caused by the toxicant. Tolerance (or resistance) is developed at the individual scale (with different levels of sensitivity among species) and also at the community scale where the biofilm acts as a coherent and protective matrix.

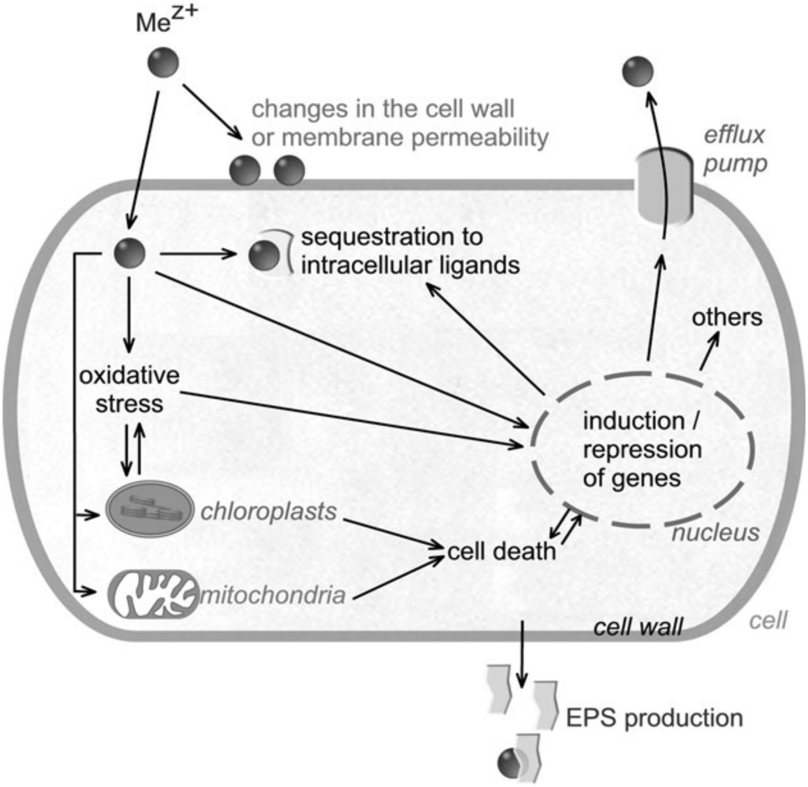


Fig. 1 Metal circulation in the cell and resulting potentially harmful effects at the cellular level. Metal influx can alter the membrane permeability; once in the cell, metals can induce an oxidative stress, affect the photosynthetic apparatus or mitochondria, and modify genetic expression, eventually leading to apoptosis. Several mechanisms are known to protect the cell against these toxic effects, such as metal binding by intracellular ligands, active expulsion, or EPS production for intracellular binding of the metals

2.1 Community Size Reduction

Exposure to metals leads to malfunction of cell metabolic processes (primary productivity, respiration, nutrient and oligoelement fluxes) (e.g. [15–17]), and reproductive characteristics (vegetative versus sexual reproduction) [13, 18], as well as increase in cell mortality [19]. Community size may be impaired through three complementary ways: (1) reduction of cell number, (2) selection for small-sized species, and (3) diminution of cell sizes within a given species.

Diatoms can accumulate high amounts of metals [20–24], which affects phosphorus metabolism [16], photosynthesis by production of reactive oxygen species [25] or by alteration of the functioning of the xanthophyll cycle [15, 26, 27], and homeostasis [28]. Thus, diatom growth can be delayed, or inhibited, leading to a reduction of diatom biomass [16, 29, 30]. In addition to lower survival and growth rates [31, 32], changes in emigration/immigration strategies [33] could also be responsible for the reduction in diatom cell densities and biomass [34].

Metal uptake depends on cell surface area exposed to the medium [35, 36], and can be reduced by physical protection offered by the exopolysaccharidic matrix [37] within the biofilm. This mechanical protection can be more effective for small-sized species, and thus this might be a mechanistic explanation for their positive selection under heavy metal pollution [18, 38, 39].

Reduction of cell size within taxa with metal exposure is probably linked to the mitotic division peculiar to diatoms, an important feature distinguishing these organisms from other algae. Hence, each division results in two daughter cells, one of which has the same size as the mother cell, and the other being smaller. As a consequence, average cell size at the population level is reduced with each successive round of mitosis [40]. Because the vegetative reproduction is the dominant mode of multiplication in diatoms [41], the decrease in size of many taxa observed in metal-contaminated environments [14, 42–44] could be a result of higher cell division rate inherent to organisms inhabiting in stressed ecosystems [45, 46].

Altogether, these combined effects of metal stress on diatom community would explain the significantly lower diatom biomass that is often observed in metal-contaminated environments.

2.2 Selection of Diatom Growth Forms

Metal exposure may modify the three-dimensional architecture of the diatom community by favouring some growth forms and constraining the development of others. Species colonization/growth strategies are driven by metal levels [47]: the communities that develop in such heavily impacted environments are dominated, even over long-term periods, by pioneer, substrate-adherent species that are more metal tolerant according to Medley and Clements [38]. It is the case, for example, of the cosmopolitan diatom *Achnanthidium minutissimum* frequently dominating in lotic environments exposed to toxic events, and considered as an indicator of metal pollution [48, 49]. Subsequent colonizers are generally stalked or filamentous, even motile species, and constitute the external layers of the biofilm under undisturbed conditions. Their development is less important in case of metal exposure, which results in the formation of thinner biofilms [50–52].

2.3 Diatom Teratologic Forms

The appearance of abnormal individuals is among the most striking effects of metals on diatom metabolism and has been widely reported in highly contaminated environments (see review in [53]). The deformities can affect the general shape of the frustule, and/or its ornamentations, which led Falasco et al. [54] to the description of seven types of abnormalities (Table 1). The most frequently observed are distortions of the cell outline (in particular in Araphid diatoms) [53–56], and changes in striation patterns. Deformities can be initiated at different stages throughout the diatom life cycle, and the processes leading to abnormal cell formation are yet unsolved. Current knowledge of diatom morphogenesis suggests direct and indirect effects consecutive to metal uptake on many cytoplasmic components involved in valve formation. The most documented negative effects of metal contamination on diatoms are nucleus alterations (e.g. [57]) and/or poisoning of the microtubular system involved in the transport of silica towards silica deposition vesicles [58].

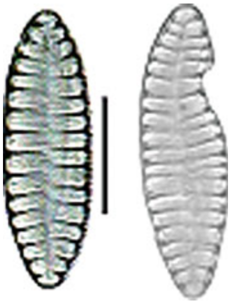
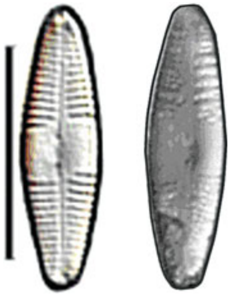
Although no standard based on diatom teratologies has yet been established, many authors suggested using their occurrence as potential indicators of high metal pollution [48, 59–62].

2.4 Selection of Tolerant Species

Diatom species composition is driven by several environmental factors. Among the chemical parameters, the exposure to toxic agents such as metals can be a major determinant. Metal contamination selects for species able to tolerate metal-related stresses, whereas sensitive species tend to decrease in number or ultimately disappear. This is the conceptual basis of the Pollution-Induced Community Tolerance (PICT) concept developed by Blanck et al. [63], where the structure of a stressed community is rearranged in a manner that increases the overall community tolerance to the toxicant. Because the impacts specifically caused by metals are generally difficult to separate from other stressors, there is no agreement on the sensitivity or tolerance for some particular species. The influence on biofilm structure of other environmental parameters such as physical characteristics, nutrient availability or even biological interactions [64–68] may be one of the major reasons of these contradictory results.

A list of the presumptive sensitivity or tolerance of species based on those reported in the literature to occur, or disappear, in metal-contaminated

Table 1 Types of diatom deformities (from [54]). Scale bar: 10 µm

Teratology	Description	Example of normal vs. deformed individuals
Type 1	Deformed valve outline (loss of symmetry, pentagonal or trilobate shapes, abnormal outline)	 *Surirella angusta*, Charest river, Canada
Type 2	Changes in striation pattern, costae and septae	 *Caloneis bacillum*, Nant d'Avril river, Switzerland
Type 3	Changes in shape, size and position of the longitudinal and central area (e.g. displaced, doubled, abnormally enlarged, absent)	 *Planothidium frequentissimum*, Riou Mort river, France
Type 4	Raphe modifications (split, sinuate or fragmented, changes in orientation, occasionally absent)	

(continued)

Table 1 (continued)

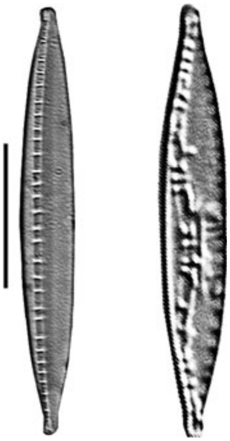
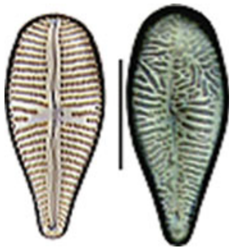
Teratology	Description	Example of normal vs. deformed individuals
		 *Gomphonema micropus* (SEM), Riou-Mort river, France
Type 5	Raphe canal system modifications (distorted, displaced, stretched out fibulae)	 *Nitzschia dissipata*, Osor river, Spain
Type 6	Unusual arrangement of the cells forming colonies	
Type 7	Mixed type in which one valve shows more than one kind of teratology	 *Gomphonema truncatum*, Deûle river, France

Table 2 List of species that are described in the literature to disappear or be favoured in metal-contaminated environments

Species	Decrease/disappear with metals	Increase/still present with metals
Achnanthidium minutissimum (Kützing) Czarnecki	++	++++
Asterionella formosa Hassall	++	
Cocconeis placentula Ehrenberg var. *placentula*	++	+
Cyclotella meneghiniana Kützing	+	++
Diatoma vulgaris Bory	+	+
Encyonema minutum (Hilse *in* Rabhenhorst) D.G. Mann	+	++
Eolimna minima (Grunow) Lange-Bertalot		++++
Eunotia exigua (Brébisson ex Kützing) Rabenhorst		++
Fragilaria capucina Desmazières var. *capucina*	+	+++
Fragilaria capucina Desmazières var. *vaucheriae* (Kützing) Lange-Bertalot		++
Fragilaria crotonensis Kitton	+	++
Fragilaria rumpens (Kützing) G.W.F.Carlson	+	+
Gomphonema parvulum (Kützing) Kützing var. *parvulum*	+	++++
Mayamaea permitis (Hustedt) Bruder & Medlin	+	++
Melosira varians Agardh	+++	+
Navicula lanceolata (Agardh) Ehrenberg	+	+
Navicula tripunctata (O.F.Müller) Bory	+	++
Naviculadicta seminulum (Grunow) Lange Bertalot	+	+++
Nitzschia dissipata (Kützing) Grunow var. *dissipata*	++	++
Nitzschia linearis (Agardh) W.M.Smith var. *linearis*	+	++
Nitzschia palea (Kützing) W.Smith		++++
Pinnularia parvulissima Krammer		++
Planothidium frequentissimum (Lange-Bertalot) Lange-Bertalot	+	++
Planothidium lanceolatum (Brébisson ex Kützing) Lange-Bertalot		++
Staurosira construens Ehrenberg	++	+
Surirella *angusta* Kützing		+++
Surirella brebissonii Krammer & Lange-Bertalot var. *brebissonii*	+	++
Tabellaria flocculosa (Roth) Kützing	++	
Ulnaria ulna (Nitzsch) Compère	++	+++

Cited: +: more than once, ++: in more than five references, +++: in more than 10 references, ++++: in more than 20 references

environments [13, 14, 21, 29, 38, 39, 52, 56, 60, 61, 64–67, 69–125] is presented in Table 2. This table includes only the species that were cited in at least five papers; species that were found in both categories (either to tolerate or to be sensitive, depending on the references) are also listed. The diatoms *Eolimna minima,* *Gomphonema parvulum* or *Nitzschia palea* are described as metal tolerant in a number of studies, whereas the sensitivity of species seems to be more difficult to determine, with only *Melosira varians* cited more than ten times to disappear in metal-contaminated environments. For some controversial species, such as *Ulnaria ulna*, Duong et al. [78] found different sensitivities that they ascribed to seasonal variability. The species *Achnanthidium minutissimum*, frequently dominant in lotic environments subjected to toxic events, is generally considered as indicator of metal pollution [14, 48, 49], but also indicates good general water quality (e.g. [3, 8]). Indeed, this species has been found to remain in highly metal-polluted conditions but to disappear with increasing trophy [61, 126, 127].

2.5 Tolerance Mechanisms

Diatoms present constitutive (phenotypic) and adaptative mechanisms to cope with elevated metal concentrations [128]. Defence and detoxification mechanisms mitigate perturbations in cell homeostasis caused by metal exposure. Regulation of metal fluxes through the cell may be driven by limitation of the influx, storage of the metal in the cytosol in insoluble form, neutralization of oxidative stress and active expulsion out of the cell (Fig. 1).

The limitation of the amount of metal entering the cell is linked to the decrease in free, i.e. bioavailable, ion concentration. Exposure enhances the production of polysaccharidic exudates (e.g. extracellular polymeric substances, EPS) able to bind metals outside of the cell [37, 106], in general proportionally to the concentration of metal exposed [129], thus leading to immobilization of the complexes outside the cell in a less bioavailable form. Recent studies indicate that frustulines, membrane-bound peptides linked to the diatom frustule resistance, may also play a role in metal binding [130]. Other regulation mechanisms have been described to occur at the cell surface. Some of the metals may be entrapped by iron or manganese hydroxides covering the cell wall [32]. Pokrovsky et al. [108] described the saturation of ligands (phosphoryl, sulfydryl) on diatom surfaces in highly polluted environments, leading to reduced adsorption capacities. Alterations of the membrane during metal internalization can lead to a decrease in membrane permeability [131, 132].

Internal mechanisms of storage contribute to efficient tolerance to metals. Metal induces the production of thiol-rich polypeptides known as phytochelatins [133, 134] or polyphosphate bodies [135], which are polymers that sequester intracellularly the excess of metal in a stable, detoxified form [27, 136, 137]. Resulting tolerance is variable among diatom species [138] and metals: sequestration capacities of the metal/protein complex depend, for example, on their valence characteristics [22, 137].

Cell defence against harmful effects of oxidative stress caused by metals relies on two main mechanisms. Increase in the production of proline [139, 140] and low-molecular-weight thiols (especially glutathione) [141–143] plays an antioxidant and detoxifying role. Metal exposure also induces activation of enzymes like superoxide-dismutase [142, 144] that convert superoxide anions into a less toxic form.

Excretion mechanisms of complexing compounds contribute to tolerance to toxicants [145]. Exposure to metals leads to increasing production of polysaccharides, which can bind metals externally after being exported in the extracellular environment [106]. Moreover, Lee et al. [146] described efflux of phytochelatin/cadmium complexes in *Thalassiosira weissflogii* exposed to high cadmium concentrations. Active expulsion by ATPase pumps as described in bacteria could also play a role in detoxification and survival of phytoplankton species [147].

The protective role of the matrix towards metals has been attributed to many features of the biofilm: metal-binding capacities of the polysaccharidic secretions [106, 148], local pH and hypoxia conditions in the internal layers of thick biofilms [149, 150], species interactions [151] and reduction of the exchanges between the inner cells and the environment [93, 152] partially linked to the presence of a superficial layer of dead cells [145].

As presented in this literature review, many studies generally performed at the watershed scale described the effects of metal contamination on cell size, growth forms, cell morphology, as well as diatom community structure. The second part of this chapter consists in assessing the relevance of these endpoints on a larger scale, using a multi-country dataset of diatom samples.

3 Case Study: A Multi-Country Database

3.1 Sites Studied

The diatom database consists of 202 samples of mature biofilms collected from hard substrates in rivers of circumneutral pH between 1999 and 2009. At each sampling unit, benthic diatoms were scraped from randomly collected substrates to form one composite sample, and preservatives (formaldehyde 4% or concentrated Lugol's iodine) were added to stop cell division and prevent organic matter decomposition. Most of them come from different parts of Europe: France (61 samples), Switzerland (15 samples) and Spain (71 samples); data from Eastern Canada (23 samples), Vietnam (18 samples) and China (14 samples) were also included. Some of the data have already been published for other purposes [12, 39, 61, 77, 90, 126, 153–155].

3.2 Diatom Analyses

Periphytic samples were cleaned of organic material before mounting permanent slides with Naphrax® (Brunel Microscopes Ltd, UK) for diatom identification based on observation of the frustule. Transects were scanned randomly under light microscopy at a magnification of ×1,000 until at least 400 valves were identified. Taxa were identified to the lowest possible taxonomical level, according to standard floras [156–162] and recent nomenclature updates that are listed in https://hydrobio-dce.cemagref.fr/en-cours-deau/cours-deau/Telecharger/indice_biologique_diatomee-ibd/. The diatom database was harmonized for taxonomy, leading to a final list of 640 taxa.

3.3 Determination of Metal Exposure

The metal data used come from surveys conducted simultaneously with diatom sampling. The water samples were collected in streams from various watersheds, where diatom communities were exposed to mixtures of dissolved metals (mainly Al, As, Cd, Cr, Cu, Fe, Hg, Ni, Pb, Se and Zn) in variable concentrations. To facilitate data interpretation, we used an estimate of metal concentration and toxicity developed by [163] and already used to investigate the responses of aquatic organisms to metals [163, 90, 91]. The CCU (cumulative criterion unit) is a score based on the sum of the ratios between metal concentrations measured in filtered waters and the corresponding criterion value (US EPA's National Recommended Water Quality Criteria, http://www.epa.gov/waterscience/criteria/wqctable/). Four categories of CCU were used following the thresholds determined by Guasch et al. [90]. CCU below 1.0 corresponded to background levels (B), low metal category (L) was characterized by CCUs between 1.0 and 2.0, and intermediate metal category (M) by CCUs between 2.0 and 10.0. For scores above 10.0, we added a high metal category (H) to Guasch´s classification.

Metals in the water samples ranged from undetectable concentrations (leading to CCU values of 0.0) to CCU scores higher than 1,000 (Spain, Rio Cea, June 2007). The distribution of the samples according to CCU categories in the different countries is given in Table 3, showing a balanced repartition of the samples in the different categories. The four CCU classes were all found in samples from France, Spain and Switzerland, but were unequally distributed among the other countries. Globally, CCU scores were due to various metals that were different between countries. Indeed, in some cases, we found two main metals contributing to CCU scores, such as in the samples from China (Cr $>$ Pb), France (Cd $>$ Zn), Switzerland (Cu $>$ Zn) and Vietnam (Cd $>$ Pb). On the contrary, much more metals were found in Canada (Zn $>$ Cd $>$ Al $>$ Pb) and Spain (Se $>$ Ni $>$ Zn $>$ Pb $>$ Al, with one different dominant contributor in most of the samples). Metals were also unequally distributed between CCU categories. The highest values (H) were generally due to Cd, Zn, and to a lower extent, Pb, whereas in the L and M categories contributions were quite balanced and mostly involved Se, Pb, Cu and Cd.

Table 3 Data are average ± standard error

CCU category	B	L	M	H
Distribution of samples				
Number of samples	58	37	50	57
Minimum CCU value;		1.02	2.04	7.04
metal mixture (μg/L)		Cu (3.2), Se (3.0), Zn (8.0)	Se(10.0), Zn (4.8)	Al (15.3), Cd (0.6), Cu (0.9), Fe (92.4), Ni (1.6), Zn (514.3)
Maximum CCU value;		1.96	6.26	1271.0
metal mixture (μg/L)		Cu (5.7), Se (6.0), Zn (15.7)	Pb (15.4), Zn (13.6)	As (50.0), Cd (315.0), Hg (8.2)
Countries	CA, CH, CN, ES, FR	CH, CN, ES, FR	CA, CH, CN, ES, FR, VN	CA, CH, ES, FR
Distribution of cell sizes				
Mean biovolume per cell (μm^3)	774 ± 67^a	676 ± 79^a	1,293 ± 134^b	524 ± 51^a
% of small-sized taxa (<100 μm^3)	25.7 ± 2.5^a	31.4 ± 3.3^{a,b}	24.7 ± 3.5^a	43.1 ± 3.9^b
% of medium-sized taxa (100–1,000 μm^3)	59.1 ± 2.0^a	50.9 ± 3.5^{a,b}	42.3 ± 2.6^b	42.2 ± 3.5^b
% of larger taxa (>1,000 μm^3)	15.2 ± 1.8^a	17.6 ± 2.7^a	33.0 ± 3.6^b	14.7 ± 2.2^a
Occurrences of valve deformities				
Abnormal diatom valves (‰)	3.6 ± 0.7^a	2.7 ± 0.7^a	4.3 ± 1.0^a	9.4 ± 1.7^b
Distribution of growth forms and postures				
Solitary not attached (%)	47.6 ± 3.4	36.0 ± 4.7	50.0 ± 4.3	45.1 ± 4.0
Solitary prostrate (%)	17.6 ± 2.6^{a,b}	22.6 ± 3.6^a	9.5 ± 1.7^b	21.2 ± 3.2^a
Solitary erected (%)	19.5 ± 2.1	29.4 ± 3.7	16.8 ± 2.6	22.1 ± 3.0
Clump-forming (%)	0.8 ± 0.2	0.7 ± 0.2	0.7 ± 0.3	1.5 ± 0.5
Filamentous (%)	10.1 ± 1.7^a	10.9 ± 2.7^{a,b}	21.8 ± 3.4^b	9.2 ± 1.7^a
Diversity indices				
Species richness	42.0 ± 2.8^a	44.9 ± 3.9^a	47.8 ± 3.5^a	29.1 ± 2.5^b
Shannon diversity index	2.46 ± 0.07^a	2.39 ± 0.13^{a,b}	2.29 ± 0.11^{a,b}	2.01 ± 0.11^b

Countries' abbreviations: CA Canada, CH Switzerland, CN China, ES Spain, FR France, VN Vietnam. Homogenous groups are noted with the same letters (a, b)

3.4 Non Taxonomical Indicators

Traits like diatom cell size, distribution of growth forms and postures (diatoms forming filaments, clumps or solitary forms including erected, prostrate and not attached cells) and proportion of valve abnormalities were investigated. Those descriptors present the major interest of being independent of the biogeographical variability of the natural communities. They strongly suggested in many cases to be indicative of perturbations such as toxicant exposure [13, 14, 38, 39, 60, 61], and were thus tested using the complete dataset based on the exhaustive list of 640 taxa. Specific biovolumes were calculated from the average dimensions provided in the floras for each taxon and using the formulae of Hillebrand et al. [164] established for the different geometrical shapes. The proportion of valve abnormalities was directly inferred from the taxonomical counts, by adding up the relative abundances of the individuals that had unusual shape and/or ornamentation of the frustule, and expressed in ‰. The distribution of growth forms and postures (in the case of solitary cells) was determined for each genera or occasionally species, according to the observations of Hoagland et al. [165], Hudon and Bourget [166], Hudon et al. [167], Katoh [168], Kelly et al. [169] and Tuji [170].

The patterns of those traits in the four CCU categories were compared using 1-way ANOVA (Statistica v5.1, StatSoft Inc., Tulsa, USA) after checking for normality of the data. Statistically significant probability level was set at $p < 0.01$. Spjotvoll/Stoline HSD tests for unequal sample sizes were performed for post hoc comparisons.

Valve abnormalities. Abnormal diatoms are generally observed in very low relative abundances, and authors agree that an average value of 10‰ is a significant threshold for metal-induced teratologies [14, 60, 61, 78, 171]. Indeed, occurrences of 3.5 ± 0.5‰ (i.e. values recorded in the B, L and M metal categories that were not discriminated by post hoc tests) can be considered as naturally occurring, or "background" levels. Previous laboratory experiments with Cd demonstrated that the percentage of valve abnormalities was not linearly correlated with metal concentration, but could be attributable, when above 10‰, to toxicity caused by concentrations above a given threshold [21, 100]. Thus we can suppose that teratologies occur in nature when a certain level of metal contamination is reached. When examining the distribution of abnormalities of this dataset along CCU scores (Fig. 2), statistical tests separated two sets of data: CCU values higher than 7.0 with average abnormalities frequency reaching values of about 10‰, and CCU values below 7.0 with average abnormalities frequency of ca. 3.5‰. This field-based evidence allowed us to refine the arbitrary threshold of the H category (CCU = 10.0) to a new threshold value of 7.0 that was used further on in the study.

When considering the type of diatom deformities, most of the cases concerned both global shape and ornamentation. In the Canadian samples, however, only the outline of the frustules was affected (Type 1 deformities as defined by [54]). The contribution of metals to the final CCU score was also different between the four countries where values higher than 7.0 were found. In most cases, deformities were

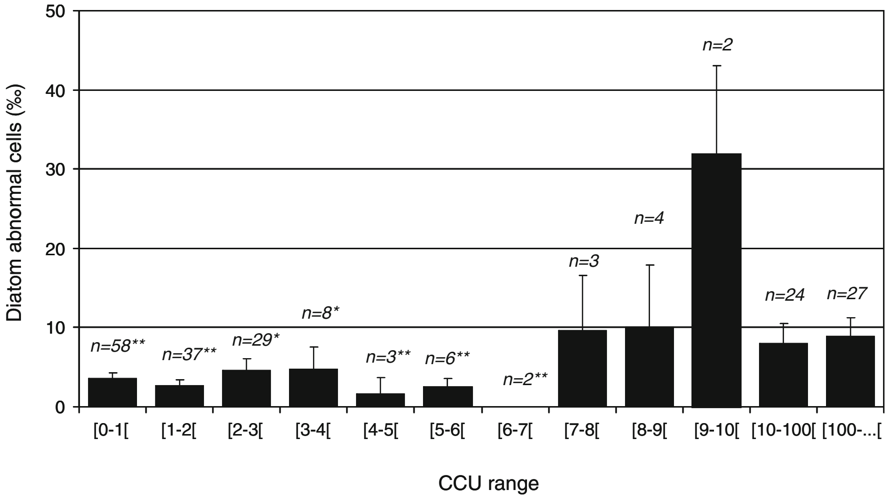


Fig. 2 Distribution of valves abnormalities within the CCU ranges. n = number of samples per CCU range. Statistically different from CCU range [9–10]: *: $p < 0.05$; **: $p < 0.01$

estimated to be caused by a "dominant" metal, as reflected by diversity indices based on metal contributions (metal diversity = 0.44 ± 0.06), whereas in Canada CCU scores were explained by a more balanced contribution of different metals (metal diversity = 1.31 ± 0.12; [172]). The calculation of CCUs is based upon the assumption that the adverse effects of metals are additive. However, the nature of the deformities observed indicates that there are differences in effects that could be linked to the balance between the metals contributing to the CCU scores, suggesting that alternative methods are needed to explain differences between the types of deformities. Guanzon et al. [22] evidenced competition between the metals that coexist in the medium, for the fixation on membrane binding sites. In their experimental exposures to binary and ternary mixtures of Cu, Zn and Cd, the diatom *Aulacoseira granulata* adsorbed and accumulated reduced quantities when compared to single-metal exposures. Since deformity formation is likely to be provoked by the metals absorbed, we can thus suppose that metal toxicities are not purely additive (in the particular case of abnormalities induction), with lower "teratogenic" power in the case of mixtures, or that some metals are more "teratogenic" than others and can also have different pathways. On the other hand, deformities have been widely described in long-term cultures [173, 174], and have been ascribed to somatic alterations linked to artificial conditions. The balance between metals was not taken into consideration, as metal concentrations in the culture medium were generally low. However, the consumption by the cultured cells of some of the oligoelements may modify, in the long term, the balance between essential and non-essential metals in the environment, which could be an alternative explanation of the occurrence of teratology in laboratory cultures.

Cell biovolumes. It has been demonstrated that small-sized species dominate in metal-contaminated environments (Sect. 2.1). Using this large database, we tried to link mean community biovolume with the gradient in CCUs, but there was no significant trend of cell size reduction with increasing metal pollution (Table 3). Diatom mean biovolume was, moreover, significantly higher in the M metal category than in the other ones, linked to higher abundances of larger taxa. Medium-sized taxa were found in higher abundances in the B and L metal categories, and a quite significant increase in small-sized taxa abundances was observed in the H categories. Indeed, there is not necessarily a decrease in average community cell size with increasing metal pollution, but higher amounts of small-sized taxa, which could in many cases not be sufficient to result in a significant decrease in mean biovolume.

Growth forms. Diatom growth forms' distribution was highly variable within CCU groups, somehow more than between categories. Samples were dominated by motile, non-attached species. In the high metal categories (M and H), these species tended to be more abundant (47.4 ± 2.9 % vs. 43.1 ± 2.8 % in B and L categories), although this trend was not statistically significant. Some studies evidenced that motile species would be less disfavoured than attached ones in metal-stressed environments [175]; however, we were unable to demonstrate this clearly using our database. Mitigation of the effects of metals by the environmental conditions [68, 70] should also be considered and we can suppose that, in a given watershed, this estimate is a good indicator of increasing metal pollution. However, changes in the community structure and thus in growth forms depend on the pool of species present (i.e. constrained by environmental drivers). The results from this study suggest that general environmental differences are likely to have stronger effects than species selection by metal contaminations. For this reason, the use of growth forms for biomonitoring metal pollution would not represent a reliable approach applicable in a large-scale context.

3.5 Global Patterns of Diatom Communities in Response to Metal Contamination

To investigate the common patterns in diatom communities between countries, the taxa that were only present in one country were removed from the analysis to exclude species that are either endemic or identified differently by operators/countries, and to reduce errors associated with the morphological approach to diatom identification, especially in the case of ambiguous species (e.g. [176]). Moreover, rare species (i.e., those that were observed in less than 5% of the samples and/or that had maximal relative abundances $< 1\%$) were not included in the analysis. This selection of data led to a final set of 152 taxa out of the initial list of 640. Prior to analysis, diatom counts were log-transformed, centred and scaled. A linear discriminant analysis (LDA) was then performed using the ade4 package

[177] implemented in the R statistical software [178]. The LDA was used to classify the dataset into exclusive groups corresponding to the four CCU categories as described above, using a M/H boundary of 7.0. The IndVal method of Dufrêne and Legendre [179] was used to identify the indicator value of each species to determine the most structuring ones for each CCU category.

Among the 640 taxa, 16 were found in the six countries covered in this study (see Fig. 3b), and 56 were observed in at least five of the countries, pointing out high "cosmopolitanism". An overall decrease in community complexity, i.e. declining species richness (ANOVA, $p < 0.001$) and diversity ($p = 0.0077$), was observed in the H metal category. Indeed, in the most contaminated cases, communities were dominated by one single species, representing on average 43.3% relative abundances and reaching in many cases values higher than 90%. The concomitant loss of sensitive species with development of more resistant species allowed for a clear discrimination by the LDA of three subsets of data, grouping B and L communities together, and separating them from M and H categories (Fig. 3). The species that were found in at least three countries and that had highly significant IndVals ($p < 0.05$) are given in Table 4. The tolerant indicator species identified were generally not those that showed most deformities, and many taxa that were not structuring the dataset also exhibited teratologies. Globally, the taxa characterizing M and H categories were in accordance with literature data (see Table 2), whereas contradictory results were found for some of the B and L categories. For example *Encyonema minutum, Mayamea permitis* and *Planothidium lanceolatum* are generally described as tolerant taxa, but were mostly found in the B and L categories. The extinction of sensitive species would be a strong signal to use for biomonitoring purposes; however, it seems that sensitivity to metals is more difficult to unequivocally determine than tolerance, maybe because of the importance of other environmental factors. Indeed, under non-contaminated conditions, competition for resource utilization selects for the species best adapted to their specific environment, whereas in metal-polluted conditions sensitive taxa tend to disappear leading to reduced competitive exclusion among species and the selective development of tolerant species, whatever their resource-competitive abilities. Diatom-based monitoring of metal pollutions would then be more relevant using the occurrences of metal-tolerant species than using a metric combining both sensitivities and tolerances of all species in the community.

The high cosmopolitanism observed indicates that metal-tolerant species derived from this study could be used to develop metal pollution diatom indices with a broad geographical application. Moreover, the specific information obtained in each non-taxonomical endpoint (e.g. relative abundances of small-sized species, of morphological abnormalities) could be used to improve sensitivity of such indicator for regional applications.

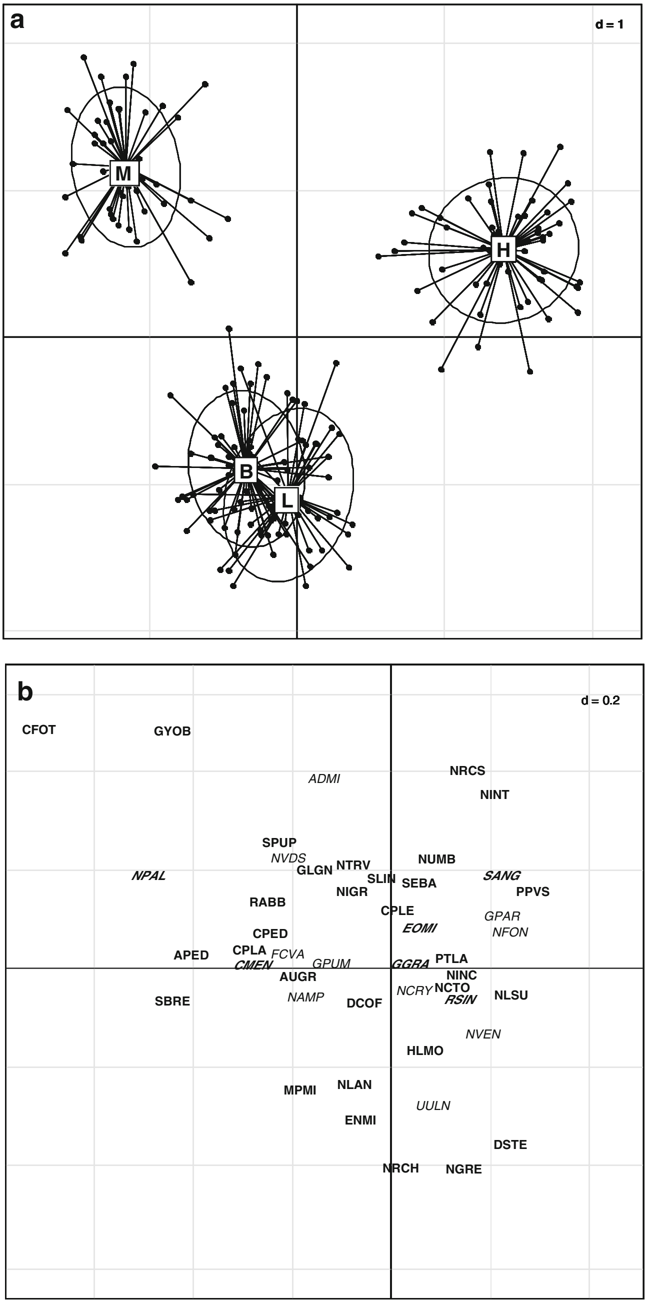


Fig. 3 Linear Discriminant Analysis of diatom community structure, constrained by CCU categories. (**a**) Projection of the samples, grouped by CCU category; (**b**) diatom species with highest indicative values (*in bold*) and the taxa common to the six countries (*in italics*). Species abbreviations: ADMI *Achnanthidium minutissimum*, FCVA *Fragilaria capucina* var. *vaucheriae*, NCRY *Navicula cryptocephala*, NVEN *N. veneta*, NVDS *Naviculadicta seminulum*, NAMP *Nitzschia amphibia*, NFON *N. fonticola*, UULN *Ulnaria ulna* and see Table 4

Table 4 Main structuring species of the different metal categories

Species	Abbreviation	CCU category	IndVal (%)	*p*-value
*Amphora pediculus**	APED	B-L	28.5	0.029
*Cocconeis placentula**	CPLA	B-L	29.5	0.003
Cocconeis pseudolineata	COPL	B-L	10.7	0.039
Encyonema minutum	ENMI	B-L	31.2	0.010
Fragilaria virescens	FVIR	B-L	9.8	0.018
Gomphonema gracile	GGRA	B-L	16.3	0.039
Gomphonema pumilum	GPUM	B-L	14.0	0.027
*Mayamaea permitis**	MPMI	B-L	28.7	0.005
Navicula gregaria	NGRE	B-L	40.5	0.001
Navicula lanceolata	NLAN	B-L	27.3	0.005
Navicula notha	NNOT	B-L	12.0	0.033
Navicula reichardtiana	NRCH	B-L	20.3	0.038
Nitzschia hantzschiana	NHAN	B-L	9.4	0.044
*Nitzschia inconspicua**	NINC	B-L	25.9	0.010
Parlibellus protracta	PPRO	B-L	13.6	0.019
*Planothidium lanceolatum**	PTLA	B-L	32.2	0.004
*Reimeria sinuata**	RSIN	B-L	23.4	0.037
*Rhoicosphenia abbreviata**	RABB	B-L	25.3	0.015
*Achnanthidium subatomus**	ADSU	M	15.0	0.016
Aulacoseira ambigua	AAMB	M	13.4	0.007
Aulacoseira granulata	AUGR	M	44.9	0.001
Bacillaria paxillifera	BPAX	M	11.7	0.017
Cocconeis pediculus	CPED	M	21.3	0.022
Cyclotella fottii	CFOT	M	32.4	0.001
*Cyclotella meneghiniana**	CMEN	M	26.5	0.026
Cyclostephanos invisitatus	CINV	M	12.1	0.034
Cymbella tumida	CTUM	M	12.9	0.024
Cymbella turgidula	CTGL	M	12.0	0.003
Diadesmis confervacea	DCOF	M	19.8	0.001
Discostella pseudostelligera	DPST	M	11.3	0.031
Discostella stelligera	DSTE	M	19.2	0.001
Gomphonema lagenula	GLGN	M	15.8	0.007
Gyrosigma obtusatum	GYOB	M	33.2	0.001
Halamphora montana	HLMO	M	31.1	0.001
*Lemnicola hungarica**	LHUN	M	12.3	0.002
Luticola mutica	LMUT	M	11.9	0.016
Navicula catalanogermanica	NCAT	M	13.5	0.018
Navicula cryptotenelloides	NCTO	M	19.0	0.008
*Navicula recens**	NRCS	M	31.8	0.001
Navicula trivialis	NTRV	M	27.2	0.001
Nitzschia filiformis	NFIL	M	9.5	0.049
Nitzschia gracilis	NIGR	M	15.2	0.004
Nitzschia intermedia	NINT	M	30.9	0.001
Nitzschia linearis var. *subtilis*	NLSU	M	16.5	0.001
*Nitzschia palea**	NPAL	M	36.3	0.007
*Nitzschia umbonata**	NUMB	M	28.5	0.001

(continued)

Table 4 (continued)

Species	Abbreviation	CCU category	IndVal (%)	*p*-value
Sellaphora bacillum	SEBA	M	17.6	0.001
Sellaphora pupula	SPUP	M	24.1	0.006
Surirella brebissonii	SBRE	M	28.7	0.010
Surirella linearis	SLIN	M	20.1	0.001
Surirella minuta	SUMI	M	12.5	0.003
Tabularia fasciculata	TFAS	M	13.2	0.003
Cocconeis placentula var. *euglypta*	CPLE	H	20.2	0.002
*Eolimna minima**	EOMI	H	35.3	0.006
*Fragilaria gracilis**	FGRA	H	12.3	0.032
Nitzschia sociabilis	NSOC	H	12.3	0.015
*Pinnularia parvulissima**	PPVS	H	18.2	0.003
*Surirella angusta**	SANG	H	26.6	0.039

*Species for which deformities were observed

4 Conclusions

Diatoms are ubiquitous and often predominant constituents of the primary producers in streams, and are sensitive to many environmental changes including metal concentrations. Even if many other environmental factors have a predominant influence on diatom community structure, the broad-scale patterns observed proved that diatom-based approaches are adequate for the monitoring of metal pollution, bringing ecological relevance based on specific sensitivities/tolerances at the community level.

The effects of metals can be observed at different levels, from the individual (deformations of the frustule) to the structure of the community. From a literature review and the analysis of indicator values determined from our large database, we provide lists of species that are likely to disappear, or to develop, with increasing metal contamination, thus providing a basis for the development of monitoring methods.

The CCU approach used in this study offers a satisfactory alternative for assessing the relationships between diatom communities and complex mixtures of metals in the field. A further step would be the development of indices taking into account metal diversity and potential toxicity to improve metal assessment in the field.

Finally, we can observe that, on the contrary to what was expected, the responses of diatoms were markedly different between rivers with intermediate and high metal pollution. In particular the increasing percentage of valve deformities proposed by many authors to be indicative of metal pollutions is, in fact, observed above all in cases of high metal contamination (H category). Our database allowed for the determination of a naturally occurring, or "background", abundance of deformed cells (3.5 ± 0.5‰) in environmental samples. A significant increase of abnormal cells was used to re-define a new threshold value for the H category from CCU = 10 to 7.

In the natural environment, conditions corresponding to the H category are expected to happen less and less frequently, especially with the development of sustainable practices of industries and of site remediation. However, worldwide, many rehabilitation programs are being implemented in historical mining sites, and there is public demand for the evaluation of restoration success. Multiple abiotic and biotic criteria can be used to qualify/quantify the changes in "stream health" during and after rehabilitation programs. Diatom-based indicators would thus be an appropriate tool for assessing the success of rehabilitation actions and justify, through a recovery of the aquatic biota, the rehabilitation programs undergone and corresponding investments.

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