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Long-term survey of heavy-metal pollution, biofilm contamination and diatom community structure in the Riou Mort watershed, South-West France

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Periphytic biofilm diatom communities are suitable indicators for the bioassay of elevated levels of metals in contaminated river water.

Abstract

In a metal-polluted stream in the Riou Mort watershed in SW France, periphytic biofilm was analyzed for diatom cell densities and taxonomic composition, dry weight and metal bio-accumulation (cadmium and zinc). Periphytic diatom communities were affected by the metal but displayed induced tolerance, seen through structural impact (dominance of small, adnate species) as well as morphological abnormalities particularly in the genera *Ulnaria* and *Fragilaria*. Species assemblages were characterized by taxa known to occur in metal-polluted environments, and shifts in the community structure expressed seasonal patterns: high numbers of *Eolimna minima*, *Nitzschia palea* and *Pinnularia parvulissima* were recorded in Summer and Autumn, whereas the species *Surirella brebissonii*, *Achnanthidium minutissimum*, *Navicula lanceolata* and *Surirella angusta* were dominant in Winter and Spring. Commonly used indices such as the Shannon diversity index and Specific Pollution Sensitivity Index reflected the level of pollution and suggest seasonal periodicity, the lowest diversities being observed in Summer.

Keywords: Geochemical survey; Cadmium; Zinc; Metal bioaccumulation; Biofilms; Periphytic diatoms; Valve abnormalities

1. Introduction

The Gironde estuary exhibits polymetallic pollution which has notable consequences on the biota in the coastal zone; the estuary has been classified as "zone D" by the National Observation Network (RNO), i.e. it is forbidden to harvest oysters for consumption, production or purification. The largest source of cadmium is located in the former zinc ore treatment area of Decazeville, in the Riou Mort watershed, a small tributary of the Lot River (Boutier et al., 1989; Latouche, 1992). Although metal emissions in the source zones have clearly decreased during the last two decades (Audry et al., 2004), Zn and Cd concentrations in water and suspended particulate matter (SPM) still are high (Coynel, 2005). Moreover, decreasing acceptable threshold concentrations for seafood

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(e.g. Cd: <5 μg g^{-1}, dry weight; European Community, 2002) and the historically high levels of metal in the Lot River sediments (Audry et al., 2004), strengthen the potential socio-economic impact of the Cd pollution, (e.g. for oyster production at Marennes-Oléron, ∼30 km north of the mouth of the Gironde estuary).

Biomonitoring is a powerful tool for assessing aquatic ecosystem health further to physical and chemical analyses. It involves organisms that are likely to reveal the environmental changes brought about by natural and anthropogenic phenomena. In freshwater environments, periphytic algal assemblages are the main primary producers. As they are composed of a large number of species with various ecological preferences (Lange-Bertalot, 1979; Steinberg and Schiefele, 1988; van Dam et al., 1994), and due to their position in the foodweb and their short life cycle, they are powerful ecological indicators. However, few field studies have been carried out to characterize the alterations occurring in periphytic communities owing to long-term metal contamination. Most of the surveys performed one to four samplings per year (Foster, 1982; Lindstrøm and Rørslett, 1991; McFarland et al., 1997; Hill et al., 2000a; Sabater, 2000), but studies on periphyton based on monthly sampling frequencies are rare (Takamura et al., 1990; Nakanishi et al., 2004). In the present study, a long-term geochemical and diatom survey was conducted from April 2004 to March 2005 at a highly metal-polluted site on the Riou Mort River. Our approach aimed to relate benthic community structure to metal exposure. Cadmium and zinc concentrations were measured in water, suspended particulate matter (SPM) and in the biofilm to characterize the geochemical behaviour of Cd and Zn and their impact on bioaccumulation kinetics. Benthic communities are described through diatom assemblages, applying the Specific Pollution Sensitivity Index (SPI; Coste in Cemagref, 1982) commonly used for routine biomonitoring in France. The taxonomic composition of the assemblages, commonly used indices (SPI, Shannon index) and the frequency of morphological aberrations occurring in some diatom species were determined and are discussed as a response to metal contamination.

2. Material and methods

2.1. Study site

The Riou Mort River (watershed area: 155 km^2) drains the Decazeville area known for polymetallic pollution due to former open-cast coal mining and Zn ore treatment. The mean annual discharge of the Riou Mort River at the studied site, Joanis, was ∼1.9 $m^3\ s^{-1}$ during 2000–2003 and ∼0.98 $m^3\ s^{-1}$ during the study period (March 2004 to March 2005, data from the Diren: French regional environment department).

The experimental site on the Riou Mort River, which is located close to the outlet of the watershed and downstream from the former ore treatment site (Fig. 1), receives cadmium- and zinc-enriched water from this pollutant source (Boutier et al., 1989; Latouche, 1992).

A site, located a few kilometres upstream of the Riou Mort confluence but just downstream of the Decazeville agglomeration, was used as a "metal-free" reference when needed for data interpretation.

2.2. Sampling and sample analyses

2.2.1. Stream water physicochemical parameters

Temperature, pH, conductivity and dissolved oxygen were measured in the river every 24 days (WTW, Weilheim, Germany) during the 13-month experimental period (March 2004 to March 2005).

Two-litre stream water samples were simultaneously collected and brought back to the laboratory for nutrient measurements. Phosphate, silica, ammonium, nitrite and nitrate concentrations were determined according to French and international standards (NF T90-023, NF T90-007, NF EN ISO 11732 and NF EN ISO 13395, respectively).

Water and SPM for trace metal analysis were sampled every 24 days using clean techniques. All materials in contact with the water samples were made of polypropylene (PP), carefully decontaminated as previously detailed in Audry et al. (2004). The dissolved phase was sampled using an acid pre-cleaned PP

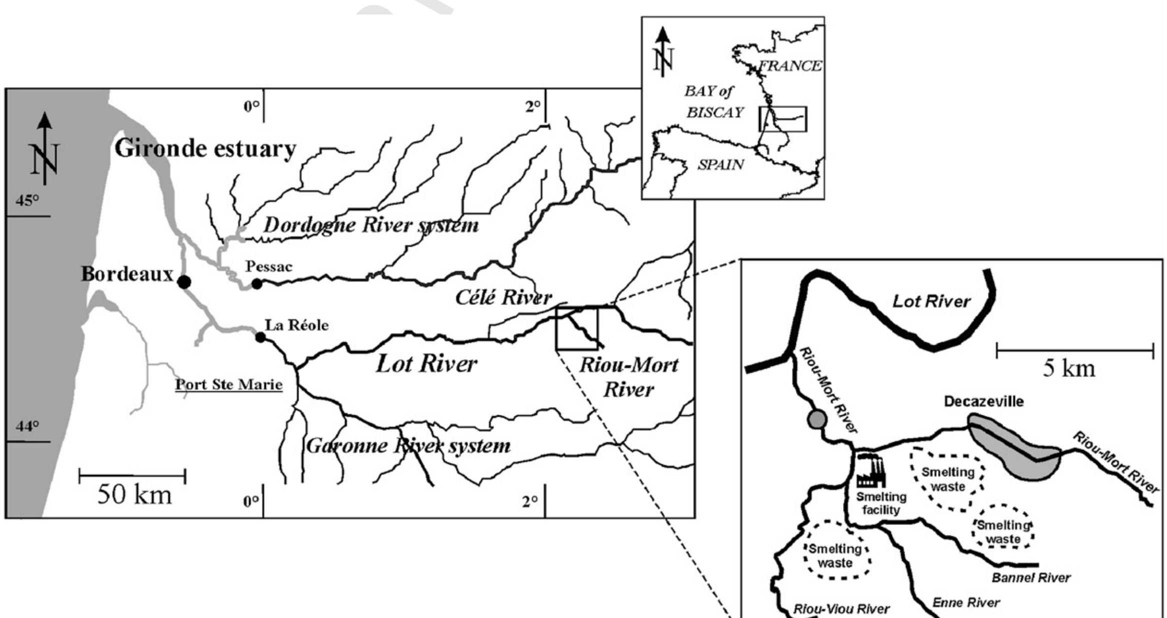

Fig. 1. Map of the Lot–Garonne fluvial system and location of the study site (shaded circle).

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bottle (1 L), previously rinsed with the river water from the site. Water samples were filtered immediately through 0.2 µm Nucleopore® polycarbonate filters in a glove box (laboratory van). Filtrates were collected in an acid-washed PP bottle after thoroughly rinsing it with an aliquot of the filtrate, acidified (0.1% ultrapure HNO_3, Merck, Darmstadt, Germany) and stored in the dark at 4 °C until analysis (Blanc et al., 1999).

Particulate matter for trace element analyses was retrieved by pumping up to 200 L of river water (1 m from the bank at 0.3 m depth) using an all PP-pump with PP-tubing followed by centrifugation (Westfalia, Oelde, Germany; 12,000 × *g*). This technique is considered a practicable and reliable method for SPM sampling in all hydrological situations (Lapaquellerie et al., 1996; Schäfer and Blanc, 2002).

Daily cumulative 1-litre samples of river water consisting of 8 subsamples taken at regular time intervals (every 3 h) were obtained using an automated sampling system (Sigma 900P, American Sigma, Hach, Loveland, CO). The samples were recovered every 24 days and filtered through preweighed 0.7 µm glass fibre filters (Whatman GF/F) to obtain daily SPM concentrations (Schäfer et al., 2002; Coynel et al., 2004). During low and/or stable hydrological conditions, water aliquots and SPM of up to six daily samples were then cumulated and were considered representative of up to a six-day period whereas samples representing particular hydrological events (e.g. floods, high SPM concentrations) were analyzed separately. Cumulative or isolated samples were filtered in the laboratory using 0.2 µm Sartorius® polycarbonate filters. Filtrates were collected in 60 cc polypropylene bottles, previously decontaminated and thoroughly rinsed with the filtrate, acidified (HNO_3 ultrapure grade; 1:1000) and stored at 4 °C until analysis (Schäfer et al., 2002).

Representative sub-samples (30 mg of dry, powdered and homogenized material) were digested in closed Teflon reactors (Savillex®) using 750 µl HCl (12 M, suprapur), 250 µl HNO_3 (14 M, suprapur) and 2 ml HF (22 M, suprapur). The reactors were then heated to 110 °C for 2 h. After complete cooling, the solutions were evaporated to dryness and then brought to 10 ml using 150 µl HNO_3 (suprapur) and ultrapure (Milli-Q®) water (Blanc et al., 1999; Audry et al., 2005).

Dissolved and particulate metals were measured using ICP-MS (X7, Thermo) with external calibration. Indium was used as internal standard and after each batch of five samples, a calibration blank and one calibration standard were measured to check potential sensitivity variations or memory effects. The analytical methods employed were continuously quality checked by analysis of certified reference sediments (PACS-1, BCR 320, SL-1) and river waters (SLRS-3, SLRS-4). Accuracy was within 5% of the certified values and the analytical error (relative standard deviation) was generally better than 5% for concentrations ten times higher than detection limits.

2.2.2. Biofilm characteristics

Biofilms were grown on glass slides (total surface area reaching 300 cm^2) used as artificial substrates (Fig. 2). After a 24-day immersion, the slides were removed from the water and scraped into a standardized volume of 200 ml mineral water. Three replicate samples per sampling date were collected and separated into aliquots.

Another aliquot of 20 ml was used for particulate matter analysis: biofilm dry weights (DW) were determined following the European standard NF EN 872. After filtration through pre-weighed glass fibre filters (Sartorius, Göttingen, Germany), samples were dried at 105 °C for 1 h. The amount of material retained (i.e. biofilm DW, as expressed in mg cm^{-2} glass substrate) was determined by re-weighing.

Aliquots of 20 ml were used for metal measurements (cadmium and zinc). Periphyton samples were filtered with an aspiration pump, through a pre-weighed metal-free filter paper (0.45 µm membrane, Millipore) to obtain the dry weight (DW) of each sample, after drying at 60 °C for 48 h in incubation tubes. Then, the filters were digested using nitric acid (3 ml of 65%HNO_3) in a pressurized container at 100 °C for 3 h (hot block CAL 3300, Environmental Express, USA). Digestates were then diluted with ultra-pure water (Milli Q, Bedford, MA, USA), and Cd and Zn concentrations measured by flame atomic absorption spectrometry (Varian AA20), with a detection limit of 15 µg Cd L^{-1} and 10 µg Zn L^{-1}. The validity of the method was checked periodically by parallel analysis of certified biological reference materials (Tort 2: lobster hepatopancreas and Dolt 2: dogfish liver from NRCC-CNRC, Ottawa, Canada). Reference samples were measured in triplicate. Values for Cd and Zn were consistently within the certified ranges for each metal and each standard reference (data not shown).

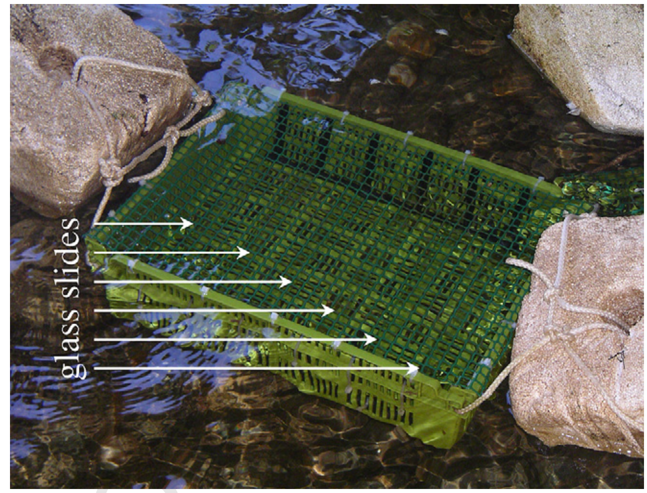

Fig. 2. Artificial substrates used for algal attachment.

The primary algal colonizers are frequently diatoms, for this reason only this particular phylum was studied: 5 ml were finally preserved in a formalin solution for quantitative counting and diatom identifications to the species level. Enumeration was done in each ''fixed'' sample using a Nageotte counting chamber: the total number of cells counted in 10 fields (1.25 µl each, 0.5 mm depth) using light microscopy at 400× magnification (photomicroscope Leica DMRB, Wetzlar, Germany) was then recorded as cells per unit area of sampled substrate (number of diatom cells cm^{-2}). Subsamples were also assigned to taxonomic analysis of diatom assemblages, they were prepared according to ANSP (Academy of Natural Sciences of Philadelphia) protocols (Charles et al., 2002), i.e. digestion in boiling hydrogen peroxide (30% H_2O_2) and hydrochloric acid (35%) followed by three cycles of centrifugation of the sample and pellet rinsing with distilled water. After the last treatment, the pellet was once again resuspended in distilled water, and this solution was pipetted onto coverslips which were mounted onto slides after air drying, using the high refractive index (1.74) medium Naphrax (Brunel Microscopes Ltd, UK). Diatom counts were conducted at a magnification of 1000×; individual fields were scanned until at least 400 valves had been identified using taxonomic literature from central Europe (Krammer and Lange-Bertalot, 1986–1991) from which theoretical biovolumes of each species were also extracted. Diatom species diversity was calculated using the Shannon index $H' = -\Sigma p_i \log_2 p_i$ (where p_i: relative abundances of species *i*; Shannon and Weaver, 1949). Individual deformities (cells with abnormal general shape and/or diatoms with deformed valve wall ornamentation) were observed and their frequency determined.

2.2.3. Statistical analyses

Major differences in physicochemical parameters between sampling dates were investigated using Principal Components Analysis (PCA) performed with PC-ORD software (McCune and Mefford, 1999). Accumulation rates of metals in the biofilm as well as correlations between DW and diatom densities were determined by linear regression on the raw data set and tested for statistical significance. Regression least-squares assumptions (homoscedasticity and normality of the error term) were checked using the residual term (Levene test and Chi^2 and Kolmogorov–Smirnov goodness-of-fit tests). Commonly used indices (SPI, Shannon index) were calculated using Omnidia software (Lecointe et al., 1993). Statistical significance of metal contents in biofilm and particulate matter was examined using analysis of variance. For all statistical results, a probability of $p < 0.05$ was considered significant. Values are means ± standard error (SE).

3. Results

3.1. Field colonization conditions

3.1.1. Physical and chemical characteristics

The sampling site is next to a permanent station of the regional environment department (Diren), measuring water levels for discharge observation. The hydrological evaluation of the study period is based on the data available at http://hydro.rnde.tm.fr/. The mean water discharge for this period was $1.5 \pm 2.0\ m^3\ s^{-1}$, and the study period is quite representative of the situation observed for several years. Typical, short flood events occurred in winter, when discharge reached $54\ m^3\ s^{-1}$ in mid-March.

The physical and chemical parameters measured in the sampled waters are shown in Table 1.

During this study, water had a uniform pH (around 7.7 ± 0.3) and silica levels were found at concentrations sufficient for diatom development (Paasche, 1980). Temperature showed strong seasonal variations, ranging from about 5 °C in March to almost 25 °C in July. Accordingly, the dissolved oxygen level was minimal during the summer. Nutrient values differed greatly among sampling dates. Located downstream of the urban area of Decazeville, the Joanis site is strongly impacted by quite high levels of organic and domestic contamination (Lemaire et al., 2006): orthophosphates as well as high levels of ionic forms of nitrogen (nitrates, nitrites and ammonia; Table 1). Average conductivity values were quite high ($1130 \pm 320\ \mu S\ cm^{-1}$) during almost the whole observation period and drastically decreased to $414\ \mu S\ cm^{-1}$ in May, corresponding to a short flood event ($2.3\ m^3\ s^{-1}$). The relatively high conductivity on 3 March 2005, during moderately high discharge cannot be explained by the measured parameters, but may be attributed to wastewater treatment (e.g. pH adjustment) on the mining site (Audry et al., 2006).

3.1.2. Dissolved and particulate metal concentrations

The average dissolved Cd and Zn concentrations in the samples obtained every 24 days and filtered immediately after sampling ranged from 5.2 to $49.2\ \mu g\ L^{-1}$ (average $26.2\ \mu g\ L^{-1}$) and from 259 to $2965\ \mu g\ L^{-1}$ (average $1458\ \mu g\ L^{-1}$), respectively (Table 1). The dissolved Cd and Zn concentrations measured in samples retrieved by the automatic sampling system and filtered after several days of storage in the sampling containers ranged from 0.223 to $54.3\ \mu g\ L^{-1}$ (average $22.2\ \mu g\ L^{-1}$) and from 28.0 to $3490\ \mu g\ L^{-1}$ (average $1280\ \mu g\ L^{-1}$; data not shown), respectively. Average particulate metal concentrations in SPM sampled manually and automatically ranged from 170 to $1160\ mg\ kg^{-1}$ (average $560\ mg\ kg^{-1}$) for Cd and from 4750 to $26{,}540\ mg\ kg^{-1}$ (average $13{,}120\ mg\ kg^{-1}$) for Zn. No clear differences in particulate metal concentrations obtained from the two sample types were observed.

3.1.3. Seasonal variability

We applied the PCA method to a data matrix of 13 months observed on 14 variables. These presentations based on stream physicochemical parameters (Fig. 3) revealed great differences between four groups of sampling dates and underlined the

Table 1
Physicochemical parameters measured in stream waters at each sampling date

Sampling date	pH	Temperature (°C)	Conductivity ($\mu S\ cm^{-1}$)	Dissolved oxygen ($mg\ L^{-1}$)	NO_3 ($mg\ L^{-1}$)	NO_2 ($mg\ L^{-1}$)	NH_4 ($mg\ L^{-1}$)	PO_4 ($mg\ L^{-1}$)	Si ($mg\ L^{-1}$)	Discharge ($m^3\ s^{-1}$)	Dissolved Cd ($\mu g\ L^{-1}$)	Particulate Cd ($mg\ kg^{-1}$)	Dissolved Zn ($\mu g\ L^{-1}$)	Particulate Zn ($mg\ kg^{-1}$)
04/15/04	8.2	12.4	867	9.3	15.6	0.4	1.0	0.4	8.0	1.4	8.4 ± 0.5	447 ± 39	371 ± 19	8910 ± 455
05/13/04	7.8	14.2	414	8.9	9.5	0.2	0.6	0.3	9.0	2.3	5.2 ± 0.5	170 ± 29	259 ± 47	4750 ± 756
06/02/04	7.9	17.7	780	8.8	8.6	0.5	1.0	0.8	10.0	0.9	13.5 ± 1.0	460 ± 16	510 ± 39	13850 ± 355
07/21/04	7.5	24.8	1220	n.m.	36.0	1.3	3.4	2.9	12.5	0.1	24.4 ± 0.7	1020 ± 65	1963 ± 118	15790 ± 806
08/17/04	7.5	20.9	1210	n.m.	7.3	0.5	1.9	1.2	13.0	0.3	22.7 ± 0.9	1160 ± 55	1138 ± 62	26540 ± 1450
09/07/04	7.3	21.9	1280	5.7	71.3	1.6	3.3	2.3	12.0	0.2	28.5 ± 1.8	558 ± 55	1292 ± 80	15790 ± 1290
10/06/04	7.5	19.3	1220	4.8	n.m.	n.m.	n.m.	n.m.	n.m.	0.1	47.7 ± 0.6	515 ± 10	2965 ± 44	15020 ± 137
10/27/04	7.0	13.2	1110	8.3	6.8	1.6	2.2	1.5	11.0	0.1	32.6 ± 2.8	579 ± 41	2628 ± 170	14080 ± 1550
11/24/04	7.8	9.3	1200	7.2	41.4	1.2	6.3	2.9	8.5	0.2	49.2 ± 1.4	560 ± 21	2525 ± 63	14330 ± 453
12/15/04	7.6	7.0	1240	9.0	38.6	1.2	7.6	2.7	12.0	0.2	43.5 ± 0.8	788 ± 52	2510 ± 66	18920 ± 974
01/11/05	7.8	6.7	862	9.6	22.1	0.5	3.7	1.6	9.5	0.4	35.2 ± 1.3	456 ± 19	1985 ± 119	7860 ± 608
02/02/05	7.8	6.1	1230	8.9	18.5	0.4	2.2	1.3	8.0	0.5	18.1 ± 1.7	359 ± 43	805 ± 76	9290 ± 965
03/03/05	8.0	4.5	1600	9.6	25.5	0.75	2.9	0.9	10.5	0.5	13.7 ± 0.8	464 ± 17	695 ± 66	9670 ± 974

Metal concentrations are mean values (±SE) for the previous 24 days. n.m., not measured.

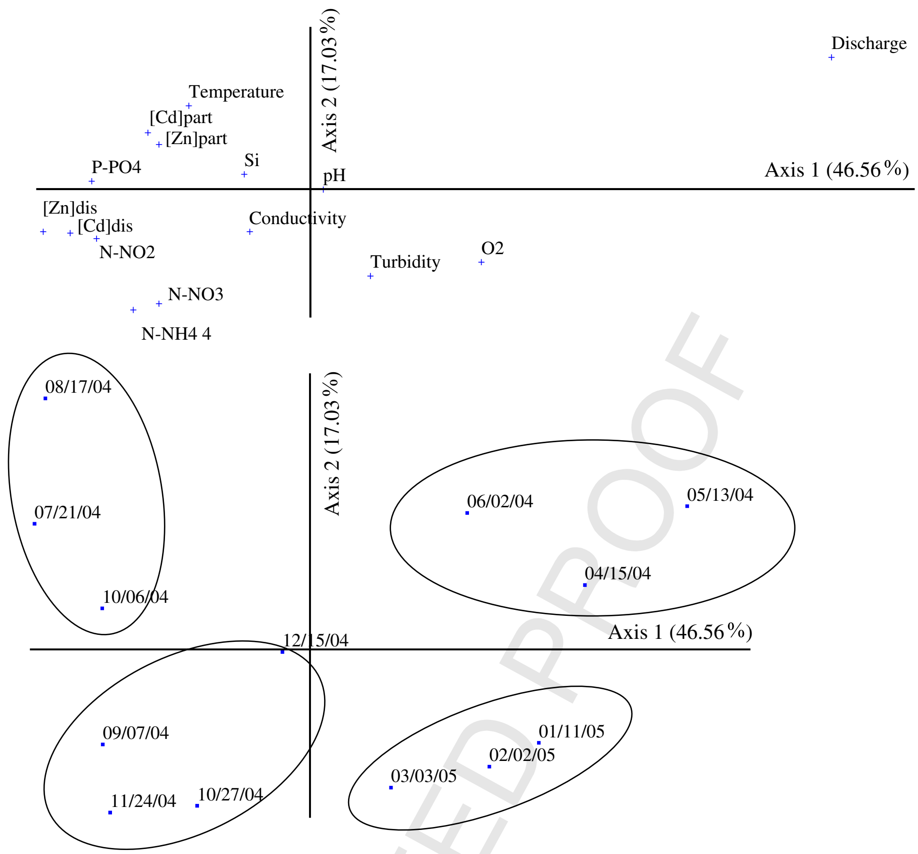

Fig. 3. Principal Components Analysis (PCA) of physicochemical stream characteristics. The explained variance for each axis is presented in brackets.

seasonal gradient. The results of this analysis showed that the first two principal components mainly account for more than 60% of the total variability. For this reason, due to the number of variables, the projection in a plane spanned by the first two principal components explains the structure of the data with a good clarification power. It is worth noting that the same tendencies were observed in planes 1–3.

Axis 1 separated Winter and Spring samples on the right-half plane (positive values) and Summer and Autumn water on the left-half plane (negative values). Separation along Axis 2 expressed the differences between water collected in Spring and Summer on the one hand, and those sampled in Autumn and Winter on the other hand. PCA discriminated sampling dates according to the most important seasonal characteristics. Summer samples were correlated with high temperatures and strong particulate metal contamination levels while Winter samples were characterized by low temperatures and the highest dissolved oxygen concentrations as well as elevated turbidity. Autumn samples exhibited the highest levels of dissolved metals and nutrients, with nitrate concentrations reaching 71 mg L^{-1} in September 2004 (Table 1).

3.2. Biofilm characteristics and structure of diatom assemblages

3.2.1. Trace metal concentrations in the biofilm.

The concentration of the metals Cd and Zn that had accumulated in biofilm are presented in Table 2. They varied considerably throughout the study. Zn was very high in August ($23,750 \pm 2470$ µg g^{-1}) whereas Cd was elevated during summer from July to August, then in October and in December. The highest levels of Cd in biofilm were observed in August with an average of around 1809 ± 200 µg g^{-1}. There were strong relationships between metal concentrations in biofilm and those in SPM (Fig. 4). In fact, biofilm Cd and Zn concentrations showed significant linear regressions (Cd: $R^2 = 0.71$, $p < 0.05$ and Zn: $R^2 = 0.46$, $p < 0.05$) with the respective concentrations in SPM. In contrast, no significant correlation was observed between dissolved metal concentrations in the streams and its concentration in the biofilm. (Cd: $R^2 = 0.01$, $p = 0.77$ and Zn: $R^2 = 0.01$, $p = 0.74$).

3.2.2. Species composition and standing crop

The results of the quantitative measurements of periphytic biomass, as expressed by dry weight and cell density, are shown in Table 2. The solid inorganic material in the biofilms represented from ~50% of the total dry weight to about 80% in spring and autumn (data not shown). These data reveal a consistent trend of co-variation between mean dry weights and mean densities ($R^2 = 0.31$, $p < 0.05$). Average cell densities during this 13-month survey were $\sim 12,200 \pm 2000$ cells cm^{-2}. The significant peaks that were observed for cell densities (see Section 3.2.3) were concomitant with peaks in biofilm dry weights and better correlated with dry weights than the organic fraction of the biofilms and diatom densities ($R^2 = 0.14$, $p = 0.17$).

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Table 2
Biofilm and diatom assemblage characteristics (mean value ± SE)

Sampling date	Dry weight ($mg\ cm^{-2}$)	Density ($cells\ cm^{-2}$)	Cd ($\mu g\ g^{-1}$ DW)	Zn ($\mu g\ g^{-1}$ DW)	Shannon index	SPI value	Biovolume ($\mu m^3\ cell^{-1}$)	Deformed valves (‰)
04/15/04	0.97 ± 0.11	13800 ± 520	278 ± 34	9007 ± 274	4.63 ± 0.06	12.4 ± 0.2	894 ± 65	14.6 ± 7.4
05/13/04	2.68 ± 0.20	11860 ± 590	170 ± 2	3958 ± 41	4.57 ± 0.32	13.2 ± 0.2	804 ± 6	12.5 ± 8.1
06/02/04	0.25 ± 0.03	11920 ± 500	743 ± 31	19149 ± 1014	4.63 ± 0.11	10.5 ± 0.2	795 ± 47	32.4 ± 8.0
07/21/04	2.72 ± 0.33	19870 ± 390	1711 ± 65	4171 ± 181	3.51 ± 0.47	9.2 ± 2.4	612 ± 52	11.8 ± 6.8
08/17/04	0.12 ± 0.01	3650 ± 1550	1809 ± 201	23750 ± 2469	3.81 ± 0.34	9.0 ± 2.2	836 ± 9	10.6 ± 4.6
09/07/04	2.24 ± 0.15	15600 ± 500	143 ± 8	6070 ± 225	4.79 ± 0.10	10.9 ± 0.8	1018 ± 17	6.1 ± 2.6
10/06/04	0.36 ± 0.00	14380 ± 750	1062 ± 542	19166 ± 70	4.15 ± 0.41	7.8 ± 2.0	766 ± 70	3.7 ± 0.8
10/27/04	3.63 ± 0.62	26250 ± 2520	228 ± 4	6714 ± 290	4.66 ± 0.04	10.2 ± 1.4	1289 ± 194	5.8 ± 3.1
11/24/04	0.91 ± 0.30	5760 ± 100	180 ± 39	10062 ± 379	4.27 ± 0.20	8.4 ± 2.2	1204 ± 246	5.1 ± 2.3
12/15/04	3.73 ± 0.78	27450 ± 1430	729 ± 20	8446 ± 108	4.57 ± 0.36	9.1 ± 2.4	872 ± 111	7.7 ± 3.3
01/11/05	0.37 ± 0.03	2240 ± 410	399 ± 89	6885 ± 1235	5.17 ± 0.04	12.0 ± 1.9	846 ± 110	4.2 ± 2.2
02/02/05	1.09 ± 0.08	6174 ± 340	287 ± 5	4239 ± 67	4.96 ± 0.14	12.5 ± 1.7	822 ± 115	1.8 ± 1.1
03/03/05	3.75 ± 0.28	4171 ± 80	399 ± 2	4348 ± 4	5.17 ± 0.03	11.5 ± 0.2	890 ± 72	9.7 ± 1.6

Diatom assemblages were characterized by an association of Naviculaceae, Nitzschiaceae and Araphideae, the most abundant species being *Eolimna minima* (Grunow) Lange-Bertalot (16.4% of the total annual standing crop), *Nitzschia palea* (Kützing) W. Smith (7.0%) and *Pinnularia parvulissima* Krammer (6.8%). During the study, 255 species were identified; the most abundant and frequent are presented with their individual biovolumes in Table 3. Biovolumes inferred from individual sizes and relative abundances ranged from 800 to more than 1000 μm^3 in autumn, and averaged 900 ± 55 μm^3 per diatom cell (Table 2). Although the statistical correlation is not strong ($R^2 = 0.34$, $p < 0.05$), there is a general trend of negative relationships between average diatom biovolume and Cd accumulation in the biofilms.

Various valve abnormalities affecting general cell shape and/or valve ornamentation were observed in a total of 28 species representing 17 genera, with frequencies reaching 3.2% in June 2004. Malformations (Fig. 5) were more often observed in genera such as *Ulnaria* (53% of the teratologies enumerated), *Fragilaria* (23%) and some Raphids (22%) than in others (e.g. abnormal valves of *Cocconeis pediculus* Ehrenberg, *C. placentula* Ehrenberg and *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot represented together less than 2%). The closest correlations between abnormality occurrences and metals were observed with dissolved metals (Cd: $R^2 = 0.28$, $p = 0.062$ and Zn: $R^2 = 0.28$, $p = 0.068$).

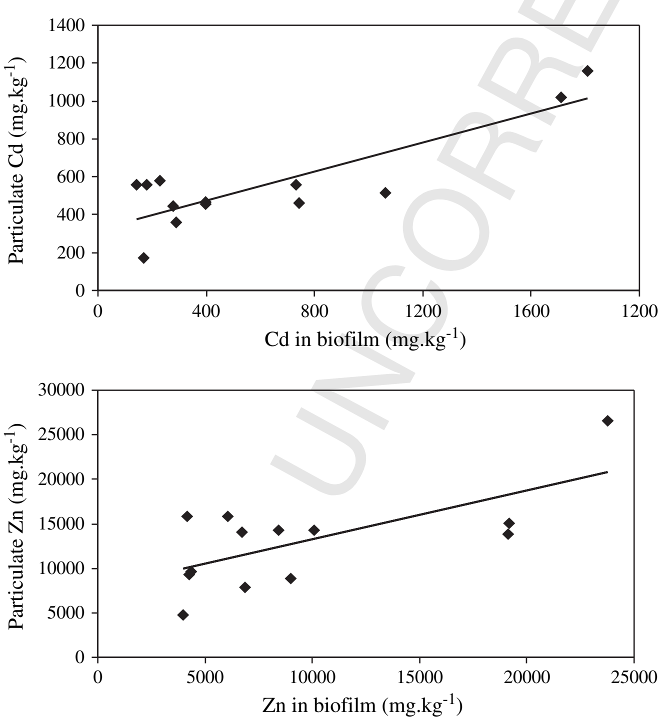

Fig. 4. Relationships between metal concentrations in the water particulate phase and in the biofilm.

3.2.3. Seasonal variations

Seasonal variations of the periphytic biomass and diatom cell densities are reported in Table 2. Dry weights and cell densities displayed a similar seasonal pattern with significant peaks in July, September, October and December 2004.

Calculations of indices also exhibited seasonal variations. For example, SPI values were generally from 8 to 10, except for spring values which reached up to 13, and were correlated with PO_4 and NO_2 measurements (PO_4: $R^2 = 0.42$, $p < 0.05$ and NO_2: $R^2 = 0.38$, $p < 0.05$). SPI values were also closely related to dissolved (Cd: $R^2 = 0.57$, $p < 0.05$ and Zn: $R^2 = 0.56$, $p < 0.05$) and particulate (Zn: $R^2 = 0.58$, $p < 0.05$) metal concentrations. Species diversity was generally lowest in summer, when only 40–50 taxa were enumerated. The highest Shannon index values were observed in winter and peaked to 5.17 in January and March 2005.

We also observed seasonal cycles of the principal species: *E. minima*, *N. palea*, *P. parvulissima* and *Cyclotella meneghiniana* Kützing were dominant in Summer and Autumn. The species *Surirella brebissonii* Krammer & Lange-Bertalot, *Achnanthidium minutissimum* (Kützing) Czarnecki, *Navicula lanceolata* (Agardh) Ehrenberg and *Surirella angusta* Kützing reached peak abundances in Winter and Spring.

4. Discussion

4.1. Geochemical scenario

The average dissolved metal concentrations in the cumulated samples were ∼30% lower than average values in the

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Table 3
Average relative abundances (R.A., %) of the most common diatom species at each sampling date

	Sampling date	*Achnanthidium minutissimum*	*Cyclotella meneghiniana*	*Eolimna minima*	*Navicula gregaria*	*Navicula lanceolata*	*Nitzschia palea*	*Pinnularia parvulissima*	*Surirella angusta*	*Surirella brebissonii*
Biovolume (μm^3)		76	1240	88	485	1230	391	2650	1820	2650
R.A. (%)	04/15/04	4.1	0.2	3.5	12.7	18.0	2.3	0.0	1.0	12.4
	05/13/04	6.8	0.0	3.7	20.3	15.3	1.9	0.0	0.3	3.8
	06/02/04	8.7	0.2	14.7	7.9	10.8	6.9	0.1	1.6	3.5
	07/21/04	0.5	18.7	36.3	2.0	1.0	9.0	4.0	0.5	0.3
	08/17/04	2.0	21.3	16.4	2.4	2.4	5.3	8.0	0.3	0.8
	09/07/04	1.0	4.0	20.8	1.0	0.7	11.9	34.7	0.1	0.7
	10/06/04	1.7	10.9	20.4	2.2	1.2	6.3	9.3	1.0	0.6
	10/27/04	1.6	6.2	15.4	0.8	1.0	13.1	18.6	1.7	0.7
	11/24/04	1.2	3.0	10.0	0.6	1.4	7.1	16.0	20.9	0.3
	12/15/04	6.4	3.7	14.1	2.3	1.0	4.8	2.7	3.2	0.3
	01/11/05	8.4	4.1	12.4	4.0	0.9	5.6	0.5	1.6	0.7
	02/02/05	5.0	3.8	7.5	7.7	1.6	3.8	0.2	6.7	1.6

manually retrieved and immediately filtered samples. This may be partly due to sorption onto the container walls and/or onto SPM in the samples during the storage period (up to 24 days) in the automatic sampling system. However, these samples integrate 8 sub-samples per day over 5–6 days including possible diurnal variations due to urban and/or industrial activities in the watershed. Therefore, these samples may be more representative of real conditions integrated by the biofilms, than the samples taken by hand during the daytime every 24 days. However, given the generally very high dissolved Cd and Zn concentrations compared to uncontaminated sites (data not shown), the differences between the freshly assayed and 24-day sample types are irrelevant in the interpretation of the effects.

The observed relatively high dissolved and particulate Cd and Zn concentrations are typical for the Riou Mort River, i.e. similar to the concentration ranges observed during our permanent observation (since 2000) at the same site. Indeed, these levels are 2–3 orders of magnitude higher than those measured in the Riou Mort River tributaries (e.g. the Riou Viou, Enne and Banel Rivers) upstream from the former mining and ore treatment area (Coynel, 2005). A large part of the

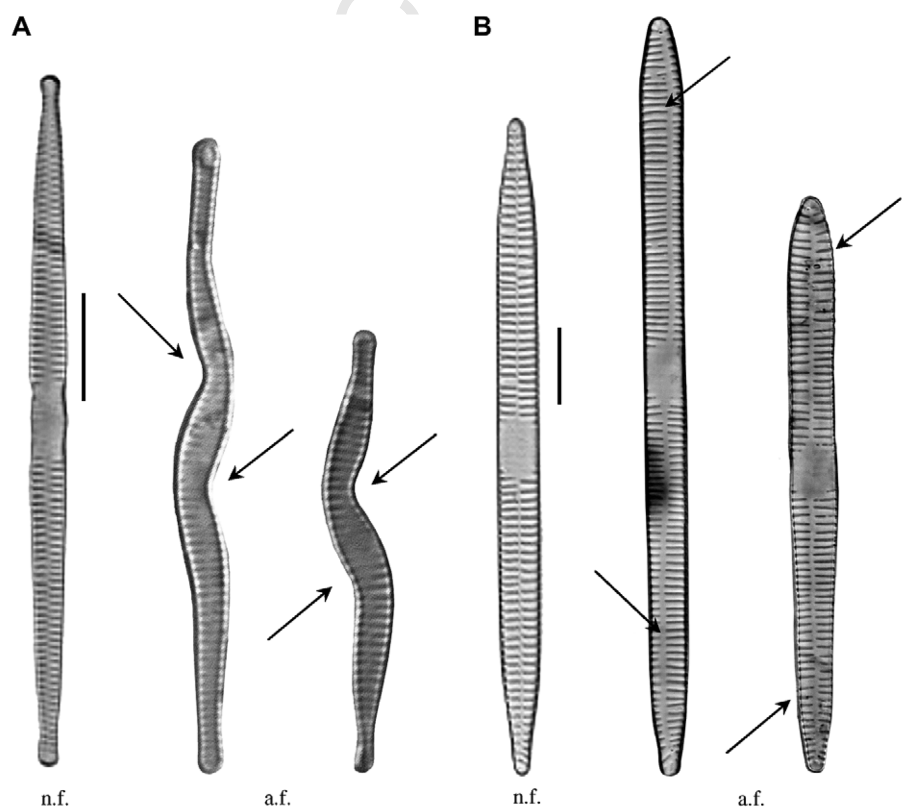

Fig. 5. Morphological variants of *Fragilaria capucina* var. *gracilis* (A) and *Ulnaria ulna* (B) originated from Joanis (n.f., normal forms; a.f., abnormal forms; scale bar = 10 μm). The arrows indicate the location of valve distortion, abnormal central area location and irregular striation.

metal load in the Riou Mort River has been attributed to sulphide oxidation of Zn ore treatment residues (Audry et al., 2005). However, hydrological variations may induce complex responses in dissolved metal concentrations due to dilution effects, leaching of diverse industrial residues and changing interactions between groundwater and surface water (Coynel, 2005). During the period studied, dissolved metal concentrations tended to increase with decreasing discharge. In contrast, SPM were mainly derived from erosion processes and transported during short floods. Therefore, particulate metal concentrations depend on the particle type, the source and, to a lesser extent, on sorption processes, i.e. exchanges between the dissolved and the particulate phases (Coynel, 2005). During the period studied, particulate metal fluxes were relatively low due to the atypically dry autumn and winter season 2004/2005 (Diren; data not shown).

In the system studied, seasonal variations in dissolved and particulate metal concentrations are generally related to hydrology, with higher concentrations during low discharge. However, in polluted watersheds, short intense floods (e.g. after rainstorms) may temporarily increase concentrations due to leaching and erosion of waste heaps and sediments (Coynel et al., in press a). Moreover, metal inputs from polluted groundwater, more or less independent of discharge, may strongly modify dissolved and, to a lesser extent, particulate metal concentrations in the Riou Mort River upstream from the Joanis site (Coynel et al., in press b).

4.2. Metal bioaccumulation in biofilms

The present study shows that high levels of Cd and Zn accumulated in biofim at the Joanis station on the Riou Mort River. High levels of metals in this area have been documented in several studies (Andres et al., 1999; Audry et al., 2005). This suggested that metals can be detected reliably by measuring the metal content of biofilm. High levels of trace elements are reported to accumulate in natural biofilm in acute metal pollution conditions (Ivorra et al., 1999) and it has been suggested that biofilm could serve as a biological monitor for anthropogenic waste (Newman et al., 1985; Hill et al., 2000b). The metals contained in biofilm were used to provide an indication of both the biological availability of metals and their ambient concentrations over relatively long periods (Foster, 1982; Behra et al., 2002). Our results revealed correlations between metal concentration in biofilm and SPM, especially Cd, in contrast to several reports concluding that metal concentrations in water samples reflected metal concentration in biofilm (Ivorra et al., 1999; Behra et al., 2002). The reason may be that the metals accumulated in biofilm are contained into two fractions, i.e. the biotic (algae, bacteria) and abiotic (silt, particulate matter) components of the biofilm. Total metal contents as measured in this study did not distinguish intracellular metal and SPM entrapped in the organic matrix; however, high positive correlations were found between intracellular metal content in biofilm and the exchangeable inorganic fraction for Cd, Cu and Zn metals (Behra et al., 2002; Holding et al., 2003). Metal levels in periphyton were used here as an estimate of effective metal exposure, assuming that inorganic entrapped SPM may also become biologically available when subjected to local environmental conditions. Accumulation of metals depended on natural variation of their concentration, according to observations made by Meylan et al. (2003). Correlation values between metals in biofilms and in waters were lower for dissolved than for particulate metals, but this does not demonstrate the absence of such correlations. It is more plausible that metal accumulation by the biofilms relies on both mechanisms of dissolved and particular metal uptake. Further studies should be performed to better understand metal accumulation processes within the periphyton both in short-term and long-term metal exposure.

4.3. Diatom community responses to heavy metal contamination

4.3.1. Characterization of the community structure

We established that biofilm dry weights and diatom densities at Joanis were somehow correlated. This means that, instead of counting the number of cells per unit area, which is too elaborate and time consuming for routine biomonitoring programs, further shifts in the standing crop at Joanis may be assessed through DW measurements.

Interpretation of DW and density data in the present study is however quite difficult, because they are highly dependent on nutrient and toxicant concentrations as well as on natural disturbances. For example, high discharge may scour the substrate and possibly lead to erroneous estimates of the number of organisms present (Ghosh and Gaur, 1998), although our data did not reveal any correlations between dry weights and monthly discharge nor extreme short-term events. It cannot be excluded that the size of the standing crop is influenced by grazing from invertebrates and fish, which can complicate interpretation.

SPI values indicated a moderate to poor quality status of the waters sampled. Water quality assessed by the SPI index, correlated to phosphorus, nitrite and metal concentrations, reflects the nutrient contamination level rather than Cd or Zn concentrations, although it is also known to be sensitive to micropollutants in some cases. As the calculation of this index was originally based on datasets taking into account classical water physicochemical parameters (e.g. nutrient concentrations, pH, conductivity, etc.), the low values of the SPI index cannot be considered as conclusive of metal pollution level so these data should be given additional support. Indeed, species sensitivity to metals is variable and is not necessarily correlated to their sensitivity to organic load. In this study however, nutrient and metal concentrations in waters varied together, which does not allow discrimination of independent effects between organic and metallic contaminations.

According to species richness or Shannon index values, the best time for sampling diatoms is Summer, as Winter samples as well as those collected in Spring and Autumn may contain too many species with less defined dominance (John, 2000). Micropollutant alterations are probably best estimated in Summer i.e. under extreme conditions, typically exhibiting the lowest discharges and highest metal concentrations.

Low average biovolume expressed the dominance of species like *E. minima* or *A. minutissimum*, suggesting competitive exclusion mechanisms (see Section 4.3.2). Although several authors have reported such small, adnate species to present the highest abundances in metal-polluted environments (Medley and Clements, 1998; Cattaneo et al., 2004), very few studies have attempted to link community biovolume to metal contamination. Size reduction of the global community (compared to the non-exposed communities found upstream; Morin and Coste, 2006) cannot here be unequivocally ascribed to Cd or Zn, but depended on metal enrichment and appeared to be an excellent specific indicator of metal contamination, more compatible with routine biomonitoring than measurements of individuals, which necessitates an additional counting effort.

4.3.2. Sensitivities of algal species

The majority of the commonly occurring species in the Riou Mort River are quite cosmopolitan in distribution and their ecological preferences in this study were consistent with those described in the literature. Indeed, most of them had already been recorded in metal-contaminated sites. Jaccard similarity indices between the impacted site and the upstream site (ranging from 0.44 to 0.65) underlined the fact that many species found at Joanis were not recorded upstream. Joanis individuals came from the same available pool of species as upstream; however only some of them were able to grow. For instance, high relative abundances of *E. minima* (in July 2004 this species represented around 50% of the total community) were in accordance with several indications of its tolerance to heavy metals found in the literature (Peres et al., 1997; Gold, 2002; Feurtet-Mazel et al., 2003; Szabó et al., 2005). This study also confirmed the resistance of *N. lanceolata* (Szabó et al., 2005), *N. palea* (Peres et al., 1997; Medley and Clements, 1998; Lai et al., 2003; Whitton, 2003) as well as *S. angusta* (Takamura et al., 1989; Gold, 2002; Feurtet-Mazel et al., 2003). The species *P. parvulissima,* which represented less than 0.2% relative abundances upstream, was found to be tolerant to heavy metals. Many studies have already revealed the presence of the genus *Pinnularia* (Admiraal et al., 1999; Gold, 2002; Hirst et al., 2002) exposed to metal contamination. The status of *A. minutissimum* is still a matter of debate, Sabater (2000) and Blanck et al. (2003) report that it is quite sensitive to metal exposure, while many authors have found this species tolerant to heavy metals (Ivorra et al., 2000; Gold et al., 2002; Feurtet-Mazel et al., 2003; Nunes et al., 2003; Cattaneo et al., 2004; Guasch et al., 2004; Nakanishi et al., 2004; Szabó et al., 2005). It has also been shown that small species tightly attached to the substrate like *A. minutissimum* are more likely to survive under metal stress, as they are embedded in an organic matrix acting like a boundary towards metal toxicity (Burkolder et al., 1990). On the contrary, the ability of filamentous or motile diatoms to extend beyond the boundary layer would probably make them more vulnerable to toxicants.

The presence of *C. meneghiniana* in Summer and Autumn was quite surprising, since species from the genus *Cyclotella* have been described as metal-sensitive by several authors (Ruggiu et al., 1998; Shehata et al., 1999; van Dam and Mertens, 1990); it was probably linked to high nutrient availability at this period, which favoured this species' development. The presence of metals probably created an environmental competitive exclusion scenario where competition between metal-tolerant species and cells from various positions in the biofilm matrix, combined with other environmental conditions (organic enrichment), determined the ultimate community structure. Although most of the taxa recorded here are found in uncontaminated sites as well, their joint presence and co-dominance may be considered as an indicator of metal pollution.

4.3.3. Utility of valve abnormalities in biomonitoring studies

Many deformed valves were evidenced during this study. The most difficult types of abnormalities to assess were those involving changes in the sculpting of the valve surface, particularly in small diatom species. This may be attributed to the limited resolution of the photomicroscopes used here, but may also signify that small species are less susceptible to morphological deformations.

Work on the valve abnormalities has established that both the incidence of abnormal light or the concentration of salts in the growth medium can produce teratological results, which appear identical to metal-induced deformations to the observer. However, several observations have reported extreme changes in valve morphology associated with heavy metal pollution in freshwater environments (e.g. Yang and Duthie, 1993; McFarland et al., 1997; Peres, 2000; Nunes et al., 2003; Szabó et al., 2005; Morin et al., 2007), in seawater (Thomas et al., 1980; Dickman, 1998) and in sediment cores (Ruggiu et al., 1998; Cattaneo et al., 2004). In our case, the deformities were thought to be related to the acute toxicity of Cd and Zn, the amounts of abnormal diatoms being significantly higher than the "background levels" of deformities found in the upstream Riou Mort site (less than 0.5%). Dickman (1998) and Stevenson and Bahls (1999) have suggested that morphological aberrations are good indicators of heavy metal contamination. Here, we quantified many abnormal diatoms, but there are two major limitations for their use in routine biomonitoring. Firstly, the observation and enumeration of abnormal valves assumes a good knowledge of diatom taxonomy and hence requires experienced operators. Secondly, deformed cells are found in very low abundances, suggesting that a better estimate would be obtained by increasing the counting effort and by performing abnormality enumerations separately from the taxonomic identifications. This study demonstrates that the rarely reported effects of heavy metals on the morphology of diatoms need to be studied more thoroughly and that diatom morphology may provide an efficient indicator.

5. Conclusions

This multidisciplinary study has provided an accurate characterization of the environmental conditions of Joanis waters. It has also shown the general relationship between metal contamination in water and in biofilms at this site and confirmed the tolerance to heavy metals of several periphytic species. The distribution patterns of the diatoms are influenced by metal contamination at the Joanis site: under metal stress, diatom communities are dominated by small adnate species and exhibit anomalous proportions of deformed individuals, providing a foundation for periphyton-based assessment of metal pollution. Even though the techniques proposed are less straightforward than robust chemical methods, substantial evidence has been gathered to justify the use of biofilms, and particularly diatoms, as rapid and integrative (over time) indicators of heavy metal contamination.

Uncited references

Cunningham et al., 2003; Navarro et al., 2002; Ramelow et al., 1992.

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