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Encyonema minutum, Surirella angusta, and Gomphonema parvulum and cadmium accumulation were observed, indicating that these species are tolerant to high levels of cadmium. On the other hand, negative correlations of Cyclotella meneghiniana, Navicula gregaria, Navicula lanceolata, Melosira varians and Nitzschia dissipata with cadmium qualify them as sensitive diatom species. Periphytic diatom composition through the presence of specific species highlight metal tolerant indicator diatom groups which will be meaningful for biomonitoring pollution in natural aquatic systems.

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Seasonal effects of cadmium accumulation in periphytic diatom communities of freshwater biofilms

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Key words: Biofilms; Diatom communities; Cadmium; Seasonal effect

Abstract

The relationships between diatom species and cadmium (Cd) accumulated in biofilms of the Riou-Mort River (SW, France) were studied in July 2004 and March 2005. Biofilms were sampled from artificial substrates immersed along a metallic pollution gradient during twenty days. Dynamics of diatom communities and cadmium accumulation were followed by collecting samples after 4, 7, 14 and 20 days of biofilm colonization. Cd accumulation in biofilms during experiment was significantly higher in Cd polluted station (Joanis) than in reference station (Firmi) for both seasons. Periphytic diatom composition varied between sites and seasons. At Firmi station, seasonal dynamics of diatom communities were stable with the dominance of *Cyclotella meneghiniana* and *Melosira varians* in July and *Surirella brebissonni* and *Navicula gregaria* in March. At Joanis station, diatom communities mainly responded to high levels of metal by a high proportion of small, adnate species. Positive correlations between *Eolimna minima*, *Nitzschia palea*, *Encyonema minutum, Surirella angusta*, *and Gomphonema parvulum* and cadmium accumulation were observed, indicating that these species are tolerant to high levels of cadmium. On the other hand, negative correlations of *Cyclotella meneghiniana*, *Navicula gregaria, Navicula lanceolata, Melosira varians* and *Nitzschia dissipata* with cadmium qualify them as sensitive diatom species. Periphytic diatom composition through the presence of specific species highlight metal tolerant indicator diatom groups which will be meaningful for biomonitoring pollution in natural aquatic systems.

Introduction

Pollution of aquatic systems by heavy metals is an important environmental problem because of their potential accumulation and transfer along the food chains, leading to more or less severe toxic effects on the different biological levels, from the cellular and molecular basis to the communities and biocenosis. Metal sources for freshwater systems result from natural processes (weathering of soils and rocks, volcanic eruptions, etc.) and from a variety of human activities (mining, smelting and agricultural fertilization) (Audry et al., 2004; Ruangsomboona and Wongrat, 2006). Trace metals such as cadmium (Cd) are considered to be non-essential elements for living organisms; Cd is one of the most toxic metals, with a high solubility in water and a great bioaccumulation capacity in many aquatic species, notably algae and bivalves (Lee et al., 1996; Torres et al., 1998; Baudrimont et al., 1997a).

In freshwater ecosystems, biofilms are complex matrices attached to submerged substrata, made of periphytic algae, bacteria, fungi and their secretory products such as extracellular polymeric substances (EPS) and organic and inorganic non living materials (Newman and McIntosh, 1989; Sekar et al., 2002; Burkholder, 1996). The capacity of freshwater periphytic algae to accumulate metals has been reported and discussed in several papers (Whitton and Say, 1975; Newman and McIntosh., 1989; Clements, 1991; Ramelow et al., 1992). Metal contents in algae are used to reflect their bioavailability from the aquatic biotopes, especially when their concentrations in water are too low to be detectable by routine analyses (Foster, 1982; Clements, 1991; Berha et al., 2002). Three main processes are involved in metal accumulation by periphytic algae: (i) binding to EPS; (ii) cell surface adsorption; (iii) intracellular uptake (Holding et al., 2003).

The use of freshwater algae in general and periphytic diatoms in particular as indicators for water quality led to the definition of several diatom indices which are currently applied in many countries (Coste *in* Cemagref, 1982; Watanabe et al., 1986; Kelly, 1998; Prygiel and Coste, 1999). The use of the structure of diatom communities to assess impacts of metal pollution on freshwater system has been discussed by several authors (Medley and Clements, 1998; Ivorra et al., 1999; Gold et al., 2002). Numerous studies, based on biofilm samples collected along pollution gradients or within indoor or outdoor artificial streams, have investigated metal impacts on periphyton communities, the majority of the studies being devoted to diatom communities (e.g. Rushforth et al., 1981; Gustavson and Wängberg, 1995; Soldo and Behra, 2000; Gold et al., 2003a; ; Gold et al., 2003b; Guasch et al., 2003). The polymetallic pollution of the Lot River in South-West of France (figure 1), essentially based on Cd and zinc (Zn) discharges from a zinc ore treatment factory via a small tributary (Riou-Mort), represents a remarkable field site for ecotoxicological studies. Gold et al. (2002; 2003a and 2003b) have investigated metal impacts on periphytic diatom communities after colonization of artificial substrates introduced along the pollution gradient (Stations 1, 2 and 3: figure 1). The confrontation between field data and experimental data obtained from indoor artificial streams enriched with diatom communities collected at the reference station on the Lot River (St. 1) and contaminated with metals individually added or in combination (Cd, Zn, Cd+Zn), has revealed the key role played by cadmium towards toxic effects on the diatom communities: significant decrease of cell density and diversity; presence of abnormal diatom frustules (Gold, 2002; Gold et al., 2003a; Morin et al., 2008).

In this paper, we present a new field study focused on the upstream zone where metal effluents are originated in order to investigate the relationships between Cd accumulation levels in biofilms and the structural characteristics of periphytic diatom communities after 4, 7, 14 and 20 days of colonization on artificial substrates during two different seasons. Besides metal pollution impact, this zone is subjected to anthropic pressure by a constant, even if not massive, touristic and urban development along the Riou-Mort River which generates an organic overload. All the previous studies have solely taken into account the contamination pressure via the determination of metal concentrations in the dissolved fraction of the water column during the different colonization phases. Cd concentrations within biofilms ensues from the metal bioavailability and the structural and functional properties of biofilms; both sets of factors being highly linked to environmental conditions and therefore to seasonal variations. In order to investigate these seasonal effects, this comparative study was set up between July 2004 and March 2005, using identical artificial substrates introduced in the sites located up and downstream the metallic factory discharges from the Riou-Viou tributary.

Material and methods

Study area and sampling stations

The study area is located in the industrial basin of Decazeville (SW France), in the middle section of the Lot River (figure 1). Since the end of the 19th century, the Riou-Mort River, a small tributary of the Lot River, is contaminated by direct discharges of percolation water coming from the industrial site of Vieille Montagne, specialized in zinc ore treatment. Two sampling stations were selected (figure 1). The reference station (Firmi), located on the upstream zone of the Riou-Mort River, with very low metal background levels in the water; the polluted station (Joanis), located in the downstream zone of the Riou-Mort River, at about 3 km downstream its confluence with the Riou-Viou River, characterized by high concentrations of Cd and Zn in the water column (Audry et al., 2004; Blanc et al., 1999). All along the Riou-Mort River, organic discharges coming from urban and touristic activities are observed.

Biofilm collection

Glass slides (600 cm^2 for both sides) were used as artificial substrates for biofilm attachment. At each sampling station, three plastic racks containing four vertical glass slides were immersed in the water column, parallel to the current at about 10-15 cm below the water surface (see Gold et al., 2002 for details). Biofilm samples were collected after 4, 7, 14 and 20 days of colonization, in July 2004 (07/01 to 07/21) and March 2005 (03/03 to 03/23). At each sampling day, one glass slide was removed randomly from each rack (3 replicates), then scraped using a cutter blade and washed with mineral water. All biofilm samples were diluted in a standard volume of 100 mL and divided after homogenisation and addition of 1 mL of formol solution (Formaldehyde 37%, Prolabo, France) for preservation into three fractions assigned to various analyses: diatom identification; biofilm dry weight measurement and Cd concentration determination. Remaining sample was set aside for prospective additional analyses.

Physico-chemical characteristics of the water and Cd concentrations in the dissolved fraction of the water column

Temperature, conductivity, pH and dissolved oxygen concentration were measured *in situ* at each sampling date using a multi-probe analyser (WTW, Weilheim, Germany). Water samples were also collected for nutrients analyses and phosphate, nitrate, nitrite, ammonium and silica concentrations were determined according to French and International standard methods (NT T 90-023, NF EN ISO 13395, NF EN ISO 11732 and NF T 90-007, respectively).

Dissolved Cd concentration was measured using ICP-MS (X7, THERMO, Elemental, UK) 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 control potential sensitivity variations or memory effects. The analytical method employed was continuously quality checked by analysis of certified reference 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 (see Audry, 2003 for details). Detection limit were 0.1 µg $Cd.L^{-1}$.

Cd accumulation levels in biofilm

Biofilm samples (50 mL) were filtered through metal-free filters (47 mm, 0.45 µm pore size, Millipore). Filters were dried at 60°C for 48 h and weighed, to determine the total dry weights (dw), expressed in $\mu g.cm^{-2}$. The filters were digested for Cd analysis by nitric acid attack (3 mL HNO_3, Merck, Darmstadt, Germany) in a pressurized medium at 100°C for 3 h (hot block CAL 3300, Environmental Express, USA). Digestates were then diluted up to 23 mL with ultra-pure water (Milli Q, Bedford, MA, USA). Cd concentrations were determined by flame atomic absorption spectrometry (Varian AA20, Australia), with detection limit of 15 $\mu g.L^{-1}$. The validity of the method was checked periodically with certified biological reference materials (Tort 2, lobster hepatopancreas; Dolt 2, dogfish liver from NRCC-CNRC, Ottawa, Canada). Values were consistently within the certified ranges (data not shown). Cd concentrations in biofilms were expressed in $ng.cm^{-2}$.

Samples preparation for diatom studies

In the laboratory, after homogenization, each sample collected for diatom studies were heated at 100 °C with hydrogen peroxide (30 %) and hydrochloric acid (35 %) to remove organic matter and dissolve calcium carbonates. The cleaned frustules were then mounted on a microscope glass slide in a high refractive index medium (Brunel Microscopes Ltd, UK; RI = 1.74). Up to 400 diatom frustules were counted and identified on each slide at 1000x magnification, following the Süßwasserflora classification (Krammer and Lange-Bertalot, 1986-1991). Relative abundances (%) of each diatom species and species richness were estimated and diversity index was calculated using the Shannon-Weaver index (Shannon and Weaver, 1963). Anomalous forms of diatom species with abnormal general shape and/or species with deformed valve wall ornamentation were estimated.

Data treatment

Statistical analyses were carried out using one-way variance model (ANOVA) to reveal the effects of colonization duration (days) and sampling stations (Joanis and Firmi) on biofilm dry weight, and Cd accumulation in biofilm. Tests for normality and homogeneity of variance were verified by using Cochran's test. If a significant effect was observed, *post-hoc* tests (Least Significant Difference test, LSD; Newman-Keuls test) were performed to isolate difference at a significant level of $p < 0.05$. All statistical investigations were performed using *STATISTICA* version 7 software, expressed results corresponding to mean values ± standard error (SE). A principal component analysis (PCA) using SPAD Software (version 5.6, Decisia, Paris, France) was performed on the relative abundances of diatom species, in order to reveal taxonomic differences between communities collected from the two stations during the two seasons (July 2004 and March 2005). Pearson correlation matrix between Cd accumulation levels in biofilms and relative abundances of the 20 most abundant diatom species were done by PCA, SPAD software (v. 5.6, Decisia, Paris, France). Indices (IPS and Diversity) were calculated using OMNIDIA software (Lecointe et al., 1993).

Results

Environmental characteristics of the two sampling stations

Values of physico-chemical parameters measured at Firmi and Joanis stations during the two 20-day sampling periods in July 2004 and March 2005 are summarized in table 1. The average values for pH were 7.6 for Firmi and 7.8 for Joanis, without significant difference ($p<0.05$) between the two seasons. Extreme water temperatures ranged from 2.8 °C (Joanis, March, + 4 days) to 24.8 °C (Joanis, July, + 20 days). The average temperature values for Firmi in July and March were 19.4 and 7.8 °C, respectively; and for Joanis, 19.7 and 8.2 °C, respectively, without significant differences between the two stations. No significant difference was observed neither between the two stations for the oxygen concentrations during each sampling periods: 10.0 $mg.L^{-1}$ in March and 7.5 $mg.L^{-1}$ in July. No significant difference in conductivity was observed between the two stations in July (Firmi: 1580 ± 259 $\mu S.cm^{-1}$ and Joanis: 1359 ± 46 $\mu S.cm^{-1}$); but in March, values from Joanis were significantly higher (1451 ± 240 and 608 ± 31 $\mu S.cm^{-1}$, respectively). Marked differences were observed between ammonium, nitrate, nitrite and phosphate concentrations, in favour of Joanis: overall for $N\text{-}NO_3$. Silica determination in water samples showed weak differences between the two stations but a significant difference was observed between the two seasons (7.4 ± 0.7 in March and 12.4 ± 0.7 in July). Cd concentrations measured in the dissolved fraction (< 0.45 µm) of the water column were systematically below the detection limit (0.1 $\mu g.L^{-1}$) at the reference station (Firmi). Higher Cd concentrations were measured at the polluted site (Joanis), with mean values of 25.6 and 17.4 $\mu g.L^{-1}$ in July 2004 and March 2005, respectively.

Biofilm total dry weight

Evolutions of biofilm dry weights during the 20-day colonization periods for the two stations in July 2004 and March 2005 are shown in figure 2. After 14 and 20 days of colonization, significant differences were observed between the two stations in favour of Joanis: at the end of the experiment, Joanis mean values were 824 ± 80 versus Firmi at 405 ± 128 $\mu g.cm^{-2}$ in July and 446 ±

29 (Joanis) versus 287 ± 28 µg.cm^{-2} (Firmi) in March. No significant difference was observed after 4 and 7 days. The dry weight increase was progressive, with tendencies close to linearity, excepted at Joanis in March where a decrease was observed between 14 and 20 days of colonization.

Cd accumulation in biofilms

Kinetics of Cd accumulation in biofilm samples are shown in figure 3. At the reference station (Firmi), Cd levels were very low, close to background levels: after 20 days' colonization, mean values were 0.75 ± 0.52 ng.cm^{-2} in July and 1.8 ± 0.4 ng.cm^{-2} in March. These accumulation levels, expressed on the biofilm dry weight basis, were 1.8 and 6.3 ng.mg^{-1}, respectively. At the opposite, very high values were observed at Joanis at the end of the experiment: 1100 ± 237 ng.cm^{-2} (corresponding to 1327 ng.mg^{-1}) in July and 254 ± 88 ng.cm^{-2} (corresponding to 784 ng.mg^{-1}) in March. The evolution tendencies are different between the two seasons at the polluted station: in July, Cd accumulation levels increase progressively between + 4 and + 20 days; in March, the mean values measured after 14 and 20 days are not significantly different.

Diatom community characteristics

Over 200 diatom taxa were identified from the different biofilm samples collected. Species richness S and diversity index (H') were quite similar between the two stations and significantly higher in March (table 2). Mean values of IPS index ranged from 7.2 (Joanis, July) to 10.7 (Firmi, March), and indicated poor to moderate water quality. The calculated IPS values were higher in Firmi station than in Joanis station and comparatively lower in July than in March.

Taxonomic composition and relative abundance of diatom species were markedly different between the two stations and the two seasons (figure 4). In July, at Firmi station, the relative abundances of the seven most abundant species were quite similar during the 20-day colonization: *Cyclotella meneghiniana* (CMEN) was the dominant species, with more than 50 % of the total diatom communities. In addition, *Navicula gregaria* (NGRE) and *Melosira varians* (MVAR) represented 8.4 and 9.2 % respectively. In Joanis, still in July, *Eolimna minima* (EOMI) was dominant, with a mean relative abundance close to 50 %. For *Cyclotella meneghiniana* (CMEN), *Nitzschia palea* (NPAL), *Ulnaria ulna* (UULN) and *Gomphonema parvulum* (GPAR), the mean values were 12.1%, 5.4%, 4.6% and 2.7%, respectively. In March, relative abundances of the main diatom species at Firmi station were comparable after 4, 7, 14 and 20 days' colonization. The dominant planctonic species CMEN in July was replaced by two species: *Surirella brebissonii* (SBRE, 18 % on average) and *Ulnaria ulna* (UULN, 10 %), with a marked increase of *Navicula* *gregaria* (NGRE, 19 %). At Joanis station (March), a marked decrease of UULN relative abundances was recorded (44 % after 4 days and 6 % after 20 days) with in parallel an increase of the relative abundance of *Surirella angusta* (SANG) from 2 to 19 %, respectively. The two species *Nitzschia palea* (NPAL) and *Gomphonema parvulum* (GPAR) were still represented in March: 8 and 5 % respectively. A global approach, via a Principal Component Analysis (PCA), was based on the relative abundances of the 50 diatom species with the highest cumulative abundances from the two stations and the four sampling dates during the two seasons (figure 5a and b). On the first plan defined by the two axis 1 and 2, which represents more than 50 % of the total variance, the four conditions are clearly individualized: the two stations Firmi and Joanis in July are localized in the right superior quarter; Joanis station in March in the middle of the inferior part; Firmi in March in the left superior quarter (figure 5a). The correlation circle (figure 5b) indicates the links between diatom species and the four studied conditions. Besides the ten species which were dominant according to their relative abundances, Joanis' diatom communities in July were characterized by small species such as *Achnanthidium minutissimum* (ADMI), *Achnanthidium saprophila* (ADSA), *Mayamaea atomus* var. *permitis* (MAPE) and *Navicula seminulum* (NVDS). In March, numerous species were associated to this polluted site: the dominant ones *Surirella angusta* (SANG), *Nitzschia palea* (NPAL), *Ulnaria ulna* (UULN), *Encyonema minutum* (ENMI), *Cocconeis placentula* (CPLA), *Gomphonema olivaceum* (GOLI) or *Navicula veneta* (NVEN). For the reference site (Firmi), only three species were associated with the July period: *Cyclotella meneghiniana* (CMEN) (dominant species), *Melosira varians* (MVAR) and *Parlibellus protracta* (PPRO). At the opposite, more than fifteen species were representative of the March period, with the dominant taxa such as *Surirella brebissonii* (SBRE), *Navicula gregaria* (NGRE), *Navicula lanceolata* (NLAN), *Nitzschia dissipata* (NDIS), *Gomphonema micropus* (GMIC), *Nitzschia recta* (NREC). The relationships between relative abundances of diatom species and Cd accumulation levels in biofilms were investigated via Pearson correlations, one for each season: among them, twenty diatom species have relative abundances higher than 10 % in July and 5 % in March (table 3). The species *Eolimna minima* (EOMI), *Nitzschia palea* (NPAL), *Encyonema minutum* ENMI), *Gomphonema parvulum* (GPAR) and *Surirella angusta* (SANG)) are strongly positively correlated to Cd content in biofilms, and *Cyclotella meneghiniana* (CMEN), *Navicula lanceolata* (NLAN), *Navicula gregaria* (NGRE) , *Surirella brebissonii* (SBRE) and *Melosira varians* (MVAR) are negatively correlated to Cd accumulation in biofilms.

Teratological frustules of diatom species which consisted in twisted valves in their apical axis or irregularity in striae arrangement were rather frequently observed at Joanis station in both seasons (table 4, and figure 6). The percentage of deformations in the total assemblage at Joanis station was higher in July than in March and ranged around 27.3‰ and 23.0‰ (mean value) respectively. Among them, araphids diatoms such as *Ulnaria ulna* occupied larger proportion with 14.5‰ (mean value) in July and 16.7 ‰ (mean value) in March. In addition, other abnormal frustules belonging to the raphids were also quite abundant in biofilm samples at Joanis station at both seasons, with *Gomphonema parvulum* which presented a percentage of 2.3 ‰ in July and 1.6 ‰ in March; *Eolimna minima* which showed 2.6 ‰ in July and 0.5 ‰ in March and *Nitzschia palea* in July and March assemblages appearing with 1 and 0.3 ‰ respectively. Besides the abnormal forms of species cited above, araphids, monoraphids and centric diatom deformed appeared in both seasons with low proportions of *Diatoma vulgaris*) *Achnanthidium minutissimum*, *Achnanthidium saprophila* and *Cyclotella meneghiniana*.

Discussion

In the present study, the development of periphytic biofilms along metallic pollution gradient was investigated at two stations during two seasons. The dynamics of periphytic diatom communities and cadmium accumulation in natural biofilm varied considerably between sites and seasons. Biofilm dry weights gradually increased and reached a maximum value after 20 days of colonization in both stations and seasons. Despite a high level of metal in Joanis station (table 1), biofilm biomass in this station was higher than in Firmi station, and corresponded to a higher availability in nitrates in Joanis for both seasons which seemed to contribute to an increasing growth of biofilm at this station. However, increase in periphytic biomass was more acute in Joanis in March after 7 days of colonization than in July. This might be related to the difference of environmental conditions between seasons with well oxygenated waters induced by cold temperature, higher flow ~~usually~~ measured in winter (Poff et al., 1990; Ghosh and Gaur., 1998), associated with good nutrients conditions (Lozano and Pratt., 1994; Lawrence et al., 2004) promoting a swift growth of the biofilm and overall of its diatomic fraction, which is predominant in winter. On the contrary, a gradual increase occurred in summer, when a more diversified biofilm was developed with an important fraction of green algae and a more important inter specific competition between algal groups for spatial and trophic resources.

Although dissolved Cd concentrations in water were not detectable in the reference site (Firmi), metal levels were measured in natural biofilms at Firmi station, however not as much as in Joanis station which allows biofilm to accumulate metal overall in July, and underlines its utilization as a useful tool to indicate metallic pollution (Ramelow et al., 1992). Cd accumulation in natural biofilm reflected their exposure history (figure 3). In our study, Cd contents in biofilm from polluted station (Joanis) increased gradually and reached their maximum value at the end of the experiment in July whereas in March Cd content in biofilm increased till the day 14 and then stayed constant This could be related to the saturation of binding sites in biofilm leading to a limitation of Cd accumulation in biofilm on the day 20. A large number of metal binding sites in the biofilm (cell surface, organic particles embedded in the matrix) have been found to play an important role in metal sorption from water column (Pistocchi et al., 1997; Decho, 2000; Barranguet et al., 2000). Increase of biofilm dry weights containing, bacteria, fungi, algae and their secretory products such as extracellular polymeric substances which have been reported to act as a trap for nutrients or metals (Sekar et al., 2002), could explain the continuous sorption of Cd by biofilms in growth. By using X-ray analysis to observe metals contained in algae collected from heavy metal pollution sites, Lai et al. (2003), Chien (2004) and Nakanishi et al. (2004) showed that heavy metal elements could be found in diatom species such as *Nitzschia palea, Achnanthidium minutissimum* and *Fragilaria tenera.* Moreover, according to Khoshmanesh et al. (1997), smaller cells of algae have proportionally larger surface area and more sites for metal binding than those of larger cells. So, the abundance of small diatom species in biofilm of Joanis station in July (figure 5b) could explain higher metal concentrations accumulated from surrounding water (figure 3).

Beside the higher species richness and diversity in March, IPS values obtained from this investigation indicated rather poor to moderate quality status of water for the two stations and reflected the organic overload of the two sampling stations located in an urban area (table 1). Though, nutrients availability was found all along the Riou Mort River, metal pollution solely detected in Joanis station, downstream the confluence with the Riou-Viou River (figure 1) seems to determine the structure of diatom assemblages as expected and shown by the distribution and the presence of particular diatom species in the Riou-Mort River reflecting metal levels along the river between Firmi and Joanis stations. Morover, our results showed marked differences in periphytic diatoms composition between stations and seasons. Throughout the summer experiment, relative abundances of dominant diatom species at Joanis and Firmi stations were developed stably (figure 4). Planktonic species such as *Cyclotella meneghiniana* (CMEN), *Melosira varians* (MVAR) and raphid species *Navicula gregaria* (NGRE) presented large proportions in Firmi biofilms. Several authors have found these species to be sensitive to metal pollution (Genter et al., 1987; Medley and Clements, 1998; Morin et al., 2007). This is confirmed in table 3 by negative correlations of these species to Cd accumulation in biofilm in both seasons and by the low proportion of these species observed in the polluted site (Joanis). More adapted to high levels of Cd in both water column and biofilm, diatom communities in Joanis station in July tend to be dominated by *Eolimna minima* (EOMI) (around 50% of the total community) (figure 4) and other small forms of diatom species such as *Mayamaea atomus* (MAPE), *Achnanthidium saprophila* (ADSA), *Aulacoseira subarctica* (AUSU) (figure 4 and 5). *E. minima* (EOMI) with its high proportion is known to be metal resistant species (Gold et al., 2002; Szabó et al., 2005). Additive presence of many small forms in communities compared to diatom communities from reference station (Firmi) (figure 5a and b) supports the hypothesis that effects of metallic pollution on diatoms composition are detectable even in overload zone like Joanis station which receives organic discharges according to wastewater treatment plant activity. In accordance with Medley and Clements' observations (1998), high proportions of adnate and small species were underscored in our results and were strongly related to Cd presented in table 3.. Similarly, considerable increases of small diatom species under high Cd concentration in laboratory experiment were also observed by Pérès (1995).

Composition of benthic diatom communities in March were diversified in both stations and differed from those in July (figure 4 and 5b). The differences observed in diatom communities might be attributed to changes in the environment (table 1) with lower metal concentrations in water column in March leading to a lower accumulation in biofilms. Seasonal variations of metal concentrations have been suggested to influence periphytic diatom composition (Medley and Clements, 1998). Firmi's diatom communities were characterized by quite stable abundances of *Navicula gregaria* (NGRE) and *Surirella brebissonii* (SBRE) throughout the study, and corresponding to a total ratio of 40 %. Unlike diatom composition in July, under lower cadmium concentration, in March, Joanis's communities showed presence of a few big diatom species such as *Nitzschia linearis* (NLIN), *Fragilaria ulna* var. *acus* (FUAC), *Gomphonema olivaceum (*GOLI) (figure 4 and 5b); although some small forms of diatoms were still well represented. The modifications in diatom structure at Joanis station compared to communities in Firmi station were noticeable with the decrease of *Ulnaria ulna* (UULN) species and increase of *Surirella angusta* (SANG) and *Nitzschia palea* (NPAL) (figure 4). The gradual increase of these species, accompanied and relatively correlated with a considerable increase of Cd content in biofilm, from its initial to mature stage, could therefore be indicators of high level of Cd. Indeed, relative abundances of *Surirella angusta* (SANG) occupying around 50% of total diatom community with high concentration of metals in biofilm were found in Kakehashi River (Nakanishi et al., 2004). Complex relationships between *Ulnaria ulna* (UULN) and several metals were recorded on attached diatoms in the Uintah basin of Utah (Rushforth et al., 1981) with a positive correlation of *Ulnaria ulna* with cadmium, copper and mercury in spring season, and on the contrary a negative correlation with copper in winter season. In the present study, this phenomenon was revealed by Pearson correlations for *Ulnaria ulna* (UULN) and cadmium between summer (July) and spring (March) seasons (table 3), and we could suggest that such varying relations of diatom species to heavy metals may also be attributed to seasonal differences.

Heavy metals do not only affect diatom community structure but also the formation of their valves. Morphological aberrations of diatom species under environment stress have been reported by several authors (Ruggiu et al., 1998; Yang and Duthie, 1993; Gold et al., 2003; Cattaneo et al., 2004). They suggested that increasing metals concentration (in water or sediment) could trigger the formation of deformed valves within some diatom genera. In this study, Cd contamination in water column and significant Cd accumulation in natural biofilm at the polluted station (Joanis) have led to the frequent emergence of abnormal forms at this station. The occurrence of deformed valves within *Fragilaria* genus (synonym *Ulnaria* or *Synedra*) have been widely reported, for example by McFarland et al. (1997) for *F. capucina*, by Ruggiu et al. (1998) for *Synedra tenera*, by Gold et al. (2003 a) for *F. capucina* var. *gracilis* and by Nunes et al. (2003) for *F. crotonensis* and *F.capucina* var. *rumpens*. In addition, our study has also presented other morphological abnormal forms belonging to centric and raphids species.

In conclusion, our study shows that, cadmium accumulation in natural biofilm was detected despite non acute Cd concentration levels in water column and in an environment characterized by an organic overload. Hence, using biofilm method is suitable for heavy metal monitoring. Due to the different seasons and levels of metal load in an organic overload medium, Cd accumulation in natural biofilms and periphytic diatom communities significantly varied. Season appears as an important factor not to overlook. Metal pollution caused changes in periphytic diatom composition along the river. Diatom assemblages, dominated by small, adnate diatom species and the presence of teratological forms at polluted station, could be valid indicators of contamination by metals. Positive or negative correlations of dominant diatom taxa to cadmium accumulation in natural biofilm indicates groups of species tolerant or sensitive to metal, which will be meaningful for biomomitoring in aquatic systems. Such interpretations could be confirmed for validation by further indoor studies concerning metal bioaccumulation in biofilms and their relation with diatoms.

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TABLES

Table 1: Physical and chemical characteristics of waters at Firmi and Joanis) stations during experimental period in July 2004 and March 2005. (nm: not measured).

Parameters	Firmi				Joanis			
	4 days	7 days	14 days	20 days	4 days	7 days	14 days	20 days
July 2004								
pH	7.4	7.4	7.4	7.3	8.0	7.7	7.7	7.7
T (°C)	17.6	16.4	19.4	24	19.7	17.6	16.7	24.8
Cond ($\mu S.cm^{-1}$)	1377	1223	1410	1427	2350	1426	1290	1257
O_2 (mg. L^{-1})	7.0	8.0	9.3	nm	7.2	7.3	6.4	nm
NH_4 (mg. L^{-1})	0.54	0.71	1.33	2.34	0.77	1.95	2.5	3.43
NO_3 (mg. L^{-1})	3	3.6	2.5	2.9	23.7	54.4	38.4	36
NO_2 (mg. L^{-1})	0.18	0.18	0.23	0.24	0.9	1	0.9	1.3
PO_4 (mg. L^{-1})	0.11	0.1	0.1	0.04	0.58	0.87	0.82	2.86
Si (mg. L^{-1})	10.5	13.5	12	15	12	11.5	12	12.5
Cd (μg. L^{-1})	< 0.1	< 0.1	< 0.1	< 0.1	26	26	27	24
March 2005								
pH	7.6	7.6	7.9	8	7.5	7.8	7.9	8.2
T (°C)	3,4	4,5	10,3	13	2,8	6,1	10	13,9
Cond ($\mu S.cm^{-1}$)	678	538	639	578	735	1602	1723	1744
O_2 (mg. L^{-1})	11,1	9,4	9,1	10.5	10,3	9,3	9,9	10.1
NH_4 (mg. L^{-1})	0.88	0.39	0.61	0.62	0.92	3.44	3.54	2.98
NO_3 (mg. L^{-1})	5	3.9	2.8	2.7	33.8	11.6	26.6	37.3
NO_2 (mg. L^{-1})	0.07	0.04	0.08	0.1	0.7	0.5	1.1	1.1
PO_4 (mg. L^{-1})	0.24	0.08	0.2	0.1	0.2	1.5	1.4	1.3
Si (mg. L^{-1})	7	7.5	5.5	6.5	6.5	9	9.5	8
Cd (μg. L^{-1})	< 0.1	< 0.1	< 0.1	< 0.1	17.1	19.8	21.6	11.2

Table 2: Species richness, diversity index and IPS (mean value and standard error; n = 3) of the reference Firmi and polluted Joanis sites in July 2004 and March 2005.

	July 2004		March 2005	
	Firmi	Joanis	Firmi	Joanis
Species richness (S)	41 ± 0.5	46 ± 4.0	54 ± 4.0	55 ± 3.0
Diversity index (H')	3.0 ± 0.02	3.2 ± 0.1	4.2 ± 0.1	4.1 ± 0.2
IPS	8.5 ± 0.34	7.2 ± 0.34	10.7 ± 0.21	10.0 ± 1.03

Table 3: Pearson correlations between relative abundances of the 20 main diatom species from the two stations: Firmi and Joanis during two sampling periods (July 2004 and March 2005) and cadmium accumulation levels in biofilms ([Cd], $ng.cm^{-2}$).

	July 2004																				
	CMEN	**EOMI**	**NPAL**	**NGRE**	**MVAR**	**UULN**	**GPAR**	**SBRE**	**NLAN**	**MAPE**	**ADMI**	**PLFR**	**ADSA**	**PTLA**	**NVDS**	**NDIS**	**ESBM**	**AUSU**	**ADMM**	**ENMI**	**[Cd]**
[Cd]	-0.57	0.55	0.83	-0.72	-0.60	0.79	0.73	-0.64	-0.73	0.31	-0.52	0.15	0.53	0.68	0.17	-0.21	-0.01	0.33	0.35	0.67	1.0
	March 2005																				
	UULN	**NGRE**	**NPAL**	**SBRE**	**NLIN**	**GPAR**	**SANG**	**NACI**	**NLAN**	**NZLT**	**ENMI**	**CMEN**	**GMIC**	**FUAC**	**EOMI**	**NDIS**	**PLFR**	**PTLA**	**NCPL**	**NIPU**	**[Cd]**
[Cd]	-0.38	-0.49	0.81	-0.18	-0.05	0.20	0.98	-0.42	-0.59	-0.32	0.76	-0.44	-0.52	-0.50	0.98	-0.72	0.68	0.22	0.49	-0.10	1.0

Table 4: Abnormal diatom frustules (‰) at two sampling stations: Firmi and Joanis) along metallic pollution gradient during two sampling periods (July 2004 and March 2005) after 4, 7, 14, and 20 days of colonization.

Colonization duration (days)	July 2004		March 2005	
	Firmi	Joanis	Firmi	Joanis
4	0	8.0 ± 0.7	0.7 ± 0.6	13.7 ± 0.3
7	1.0 ± 0.7	19.5 ± 3.2	4.3 ± 1.3	43.0 ± 9.3
14	0	33.5 ± 0.4	2.3 ± 2.0	17.7 ± 3.1
20	0	48.5 ± 1.1	6.0 ± 0.9	17.3 ± 1.3

FIGURES CAPTIONS

Figure 1: Sampling stations in the Riou-Mort River

Figure 2: Dry weights of biofilms collected from Firmi and Joanis stations in July 2004 and March 2005 after 4, 7, 14 and 20 days of colonization. Mean ± standard deviation; n=3.

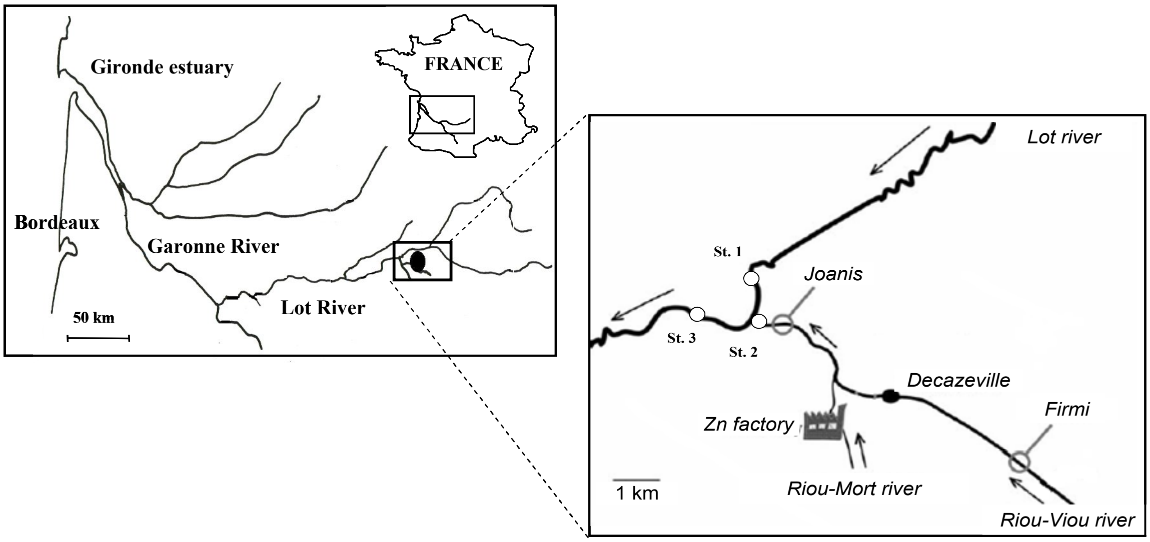
Figure 3: Cd accumulated in biofilms at Firmi and Joanis stations in July 2004 and March 2005 after 4, 7, 14 and 20 days of colonization. Mean ± standard deviation; n=3.

Figure 4: Relative abundances of 10 diatom species (mean values; n=3) with relative abundances ≥ 10% in July and ≥ 5% in March 2005 after after 4, 7, 14 and 20 days of colonization at Firmi and Joanis stations. (CMEN: *Cyclotella meneghiniana;* EOMI: *Eolimna minima;* GPAR: *Gomphonema parvulum;* MVAR: *Melosira varians;* NGRE: *Navicula gregaria;* NLIN: *Nitzschia linearis;* NPAL: *Nitzschia palea;* SANG: *Surirella angusta;* SBRE: *Surirella brebissonii;* UULN: *Ulnaria ulna*).

Figure 5 a and b: Principal Component Analysis (PCA) based on the taxonomic composition of the diatom communities collected at Firmi and Joanis along metallic pollution during two periods (July 2004 and March 2005) after 4, 7, 14 and 20 days colonization. (a): Projection of the communities on the two first principal components axes (3 replicates per site). Colonization duration (4, 7, 14 and 20 days) of each site and sampling period is pointed out in brackets. b: Projection of the diatom species on the correlation circle and 50 diatom species with highest cumulative abundances are plotted on the graph.

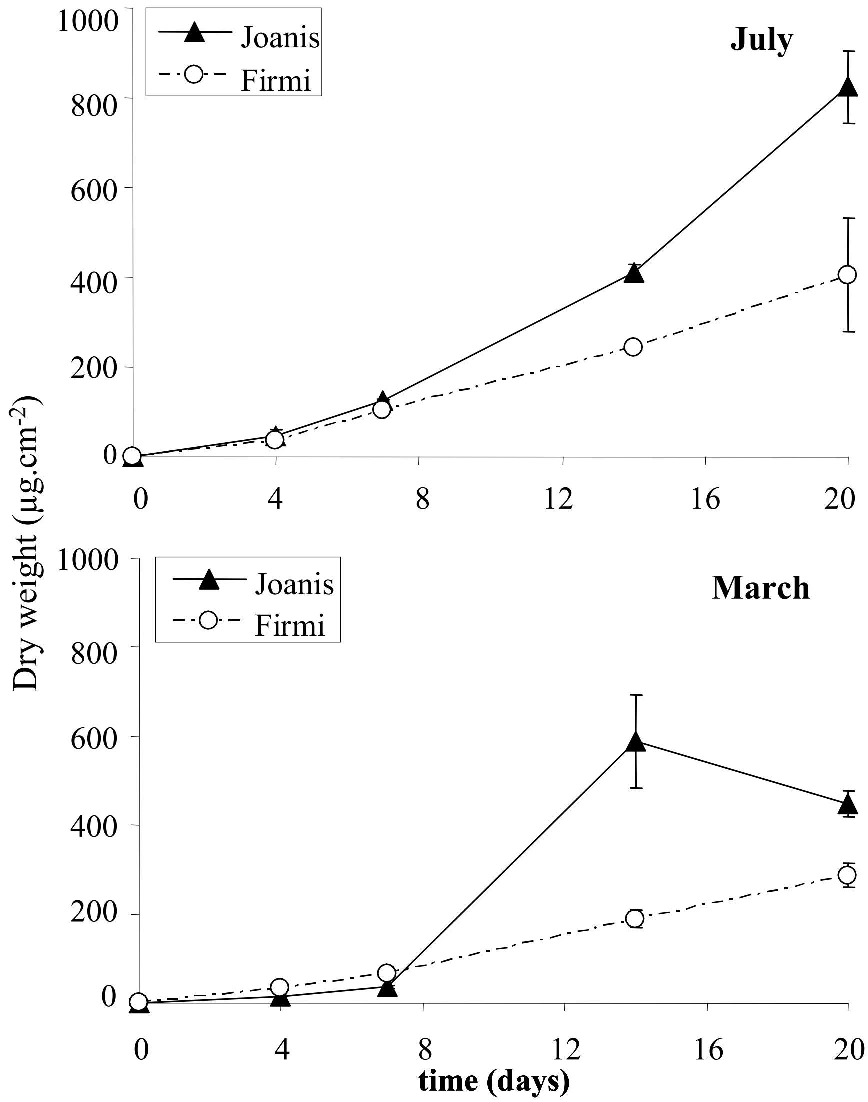
Figure 6: Abnormal forms of *Ulnaria ulna* (UULN) (1), *Fragilaria capucina* (FCAP) (2) and *Gomphonema parvulum* (GPAR) (3) found in Joanis station (nf: normal form and ab: abnormal form)

FIGURE 1



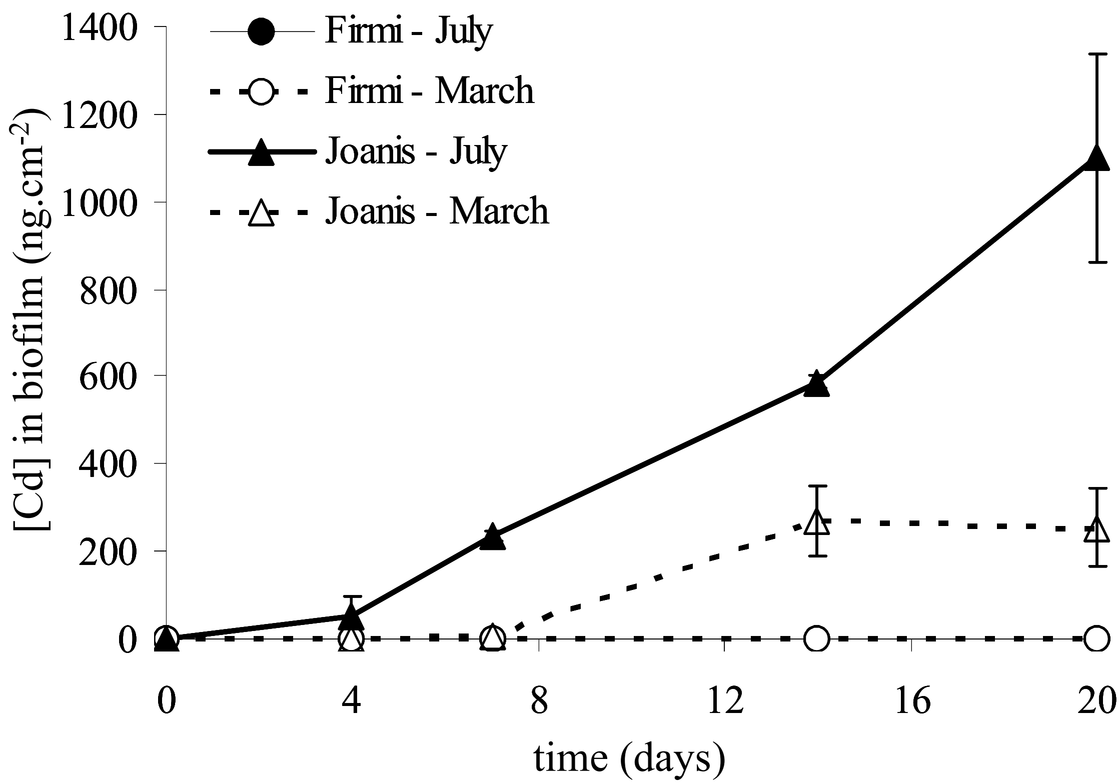
Gironde estuary
FRANCE
Bordeaux
Garonne River
Lot River
50 km
Lot river
St. 1
Joanis
St. 3
St. 2
Decazeville
Firmi
Zn factory
1 km
Riou-Mort river
Riou-Viou river

FIGURE 2



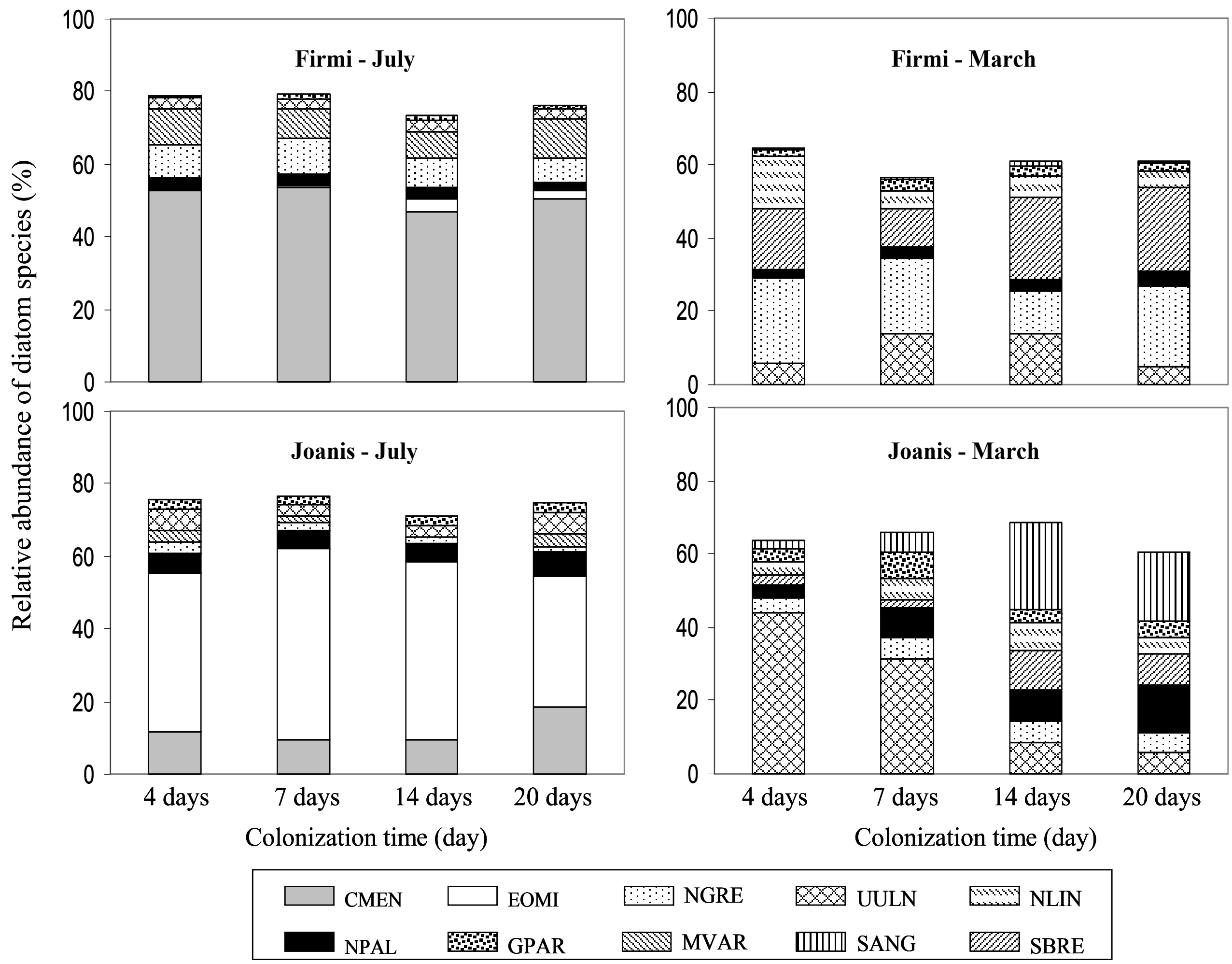
Joanis
Firmi
July
Joanis
Firmi
March
Dry weight (µg.cm-2)
1000
800
600
400
200
0
0
4
8
12
16
20
time (days)

FIGURE 3



[Cd] in biofilm (ng.cm^{-2})

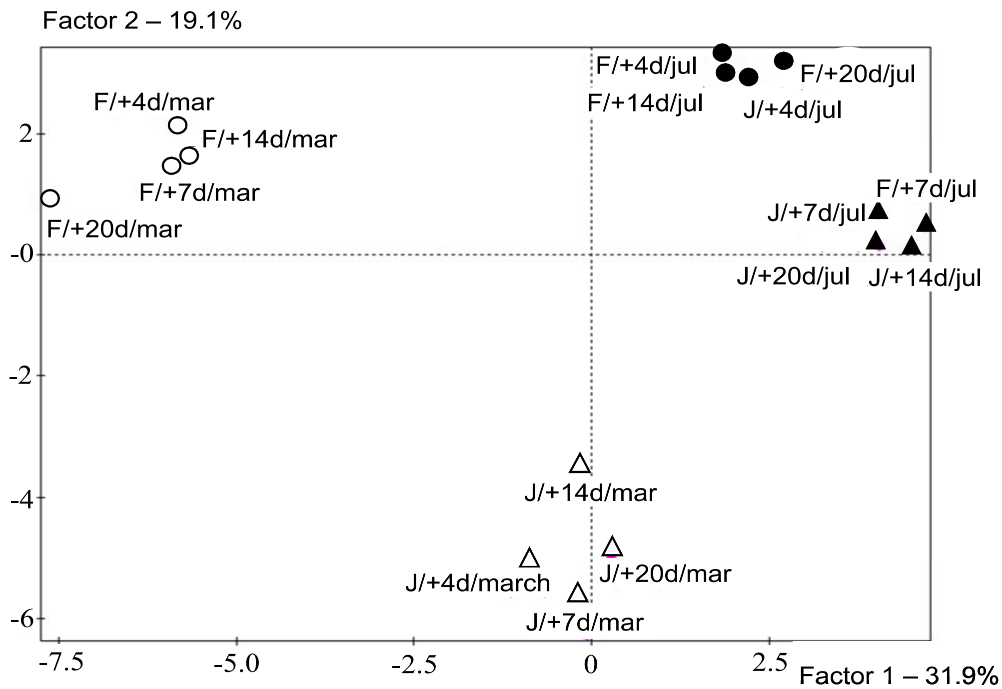
FIGURE 4



Firmi - July
Firmi - March
Joanis - July
Joanis - March
Relative abundance of diatom species (%)
0
20
40
60
80
100
4 days
7 days
14 days
20 days
Colonization time (day)
CMEN
EOMI
NGRE
UULN
NLIN
NPAL
GPAR
MVAR
SANG
SBRE

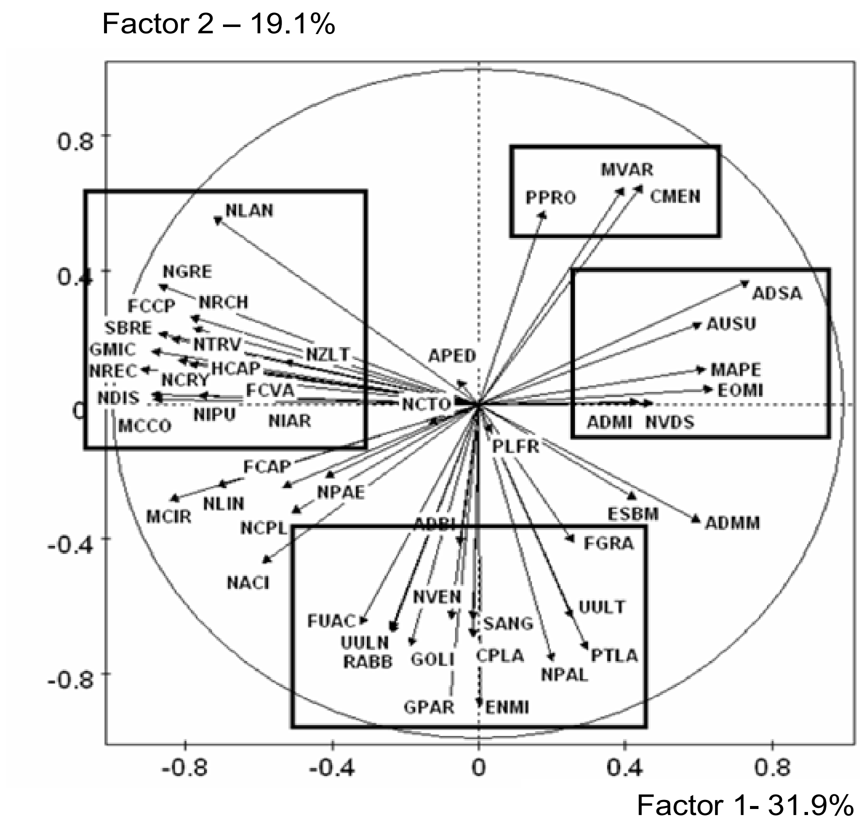
FIGURE 5

a)



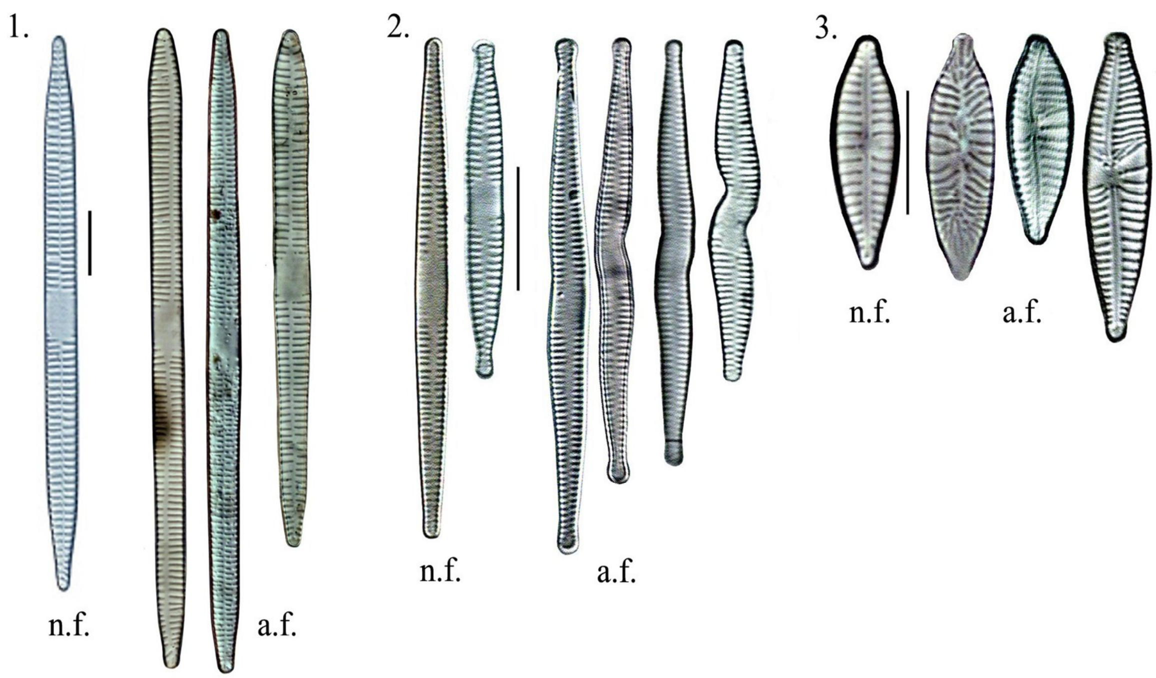
Factor 2 – 19.1%
F/+4d/mar
F/+14d/mar
F/+7d/mar
F/+20d/mar
F/+4d/jul
F/+14d/jul
F/+20d/jul
J/+4d/jul
F/+7d/jul
J/+7d/jul
J/+20d/jul
J/+14d/jul
J/+14d/mar
J/+4d/march
J/+20d/mar
J/+7d/mar
2
-0
-2
-4
-6
-7.5
-5.0
-2.5
0
2.5
Factor 1 – 31.9%

b)



Factor 2 – 19.1%
0.8
0.4
0
-0.4
-0.8
-0.8
-0.4
0
0.4
0.8
Factor 1- 31.9%
NLAN
NGRE
FCCP
NRCH
SBRE
NTRV
NZLT
GMIC
NREC
HCAP
NCRY
NDIS
FCVA
NIPU
NIAR
MCCO
NCTO
APED
PPRO
MVAR
CMEN
ADSA
AUSU
MAPE
EOMI
ADMI
NVDS
PLFR
FCAP
NPAE
MCIR
NLIN
NCPL
NACI
ADBI
ESBM
ADMM
FGRA
NVEN
FUAC
UULN
RABB
GOLI
SANG
CPLA
GPAR
ENMI
UULT
PTLA
NPAL

FIGURE 6



Figure(s)

FIGURES CAPTIONS

Figure 1: Sampling stations in the Riou-Mort River

Figure 2: Dry weights of biofilms collected from Firmi and Joanis stations in July 2004 and March 2005 after 4, 7, 14 and 20 days of colonization. Mean ± standard deviation; n=3.

Figure 3: Cd accumulated in biofilms at Firmi and Joanis stations in July 2004 and March 2005 after 4, 7, 14 and 20 days of colonization. Mean ± standard deviation; n=3.

Figure 4: Relative abundances of 10 diatom species (mean values; n=3) with relative abundances ≥ 10% in July and ≥ 5% in March 2005 after after 4, 7, 14 and 20 days of colonization at Firmi and Joanis stations. (CMEN: *Cyclotella meneghiniana;* EOMI: *Eolimna minima;* GPAR: *Gomphonema parvulum;* MVAR: *Melosira varians;* NGRE: *Navicula gregaria;* NLIN: *Nitzschia linearis;* NPAL: *Nitzschia palea;* SANG: *Surirella angusta;* SBRE: *Surirella brebissonii;* UULN: *Ulnaria ulna*).

Figure 5 a and b: Principal Component Analysis (PCA) based on the taxonomic composition of the diatom communities collected at Firmi and Joanis along metallic pollution during two periods (July 2004 and March 2005) after 4, 7, 14 and 20 days colonization. (a): Projection of the communities on the two first principal components axes (3 replicates per site). Colonization duration (4, 7, 14 and 20 days) of each site and sampling period is pointed out in brackets. b: Projection of the diatom species on the correlation circle and 50 diatom species with highest cumulative abundances are plotted on the graph.

Figure 6: Abnormal forms of *Ulnaria ulna* (UULN) (1), *Fragilaria capucina* (FCAP) (2) and *Gomphonema parvulum* (GPAR) (3) found in Joanis station (nf: normal form and ab: abnormal form)

FIGURE 1

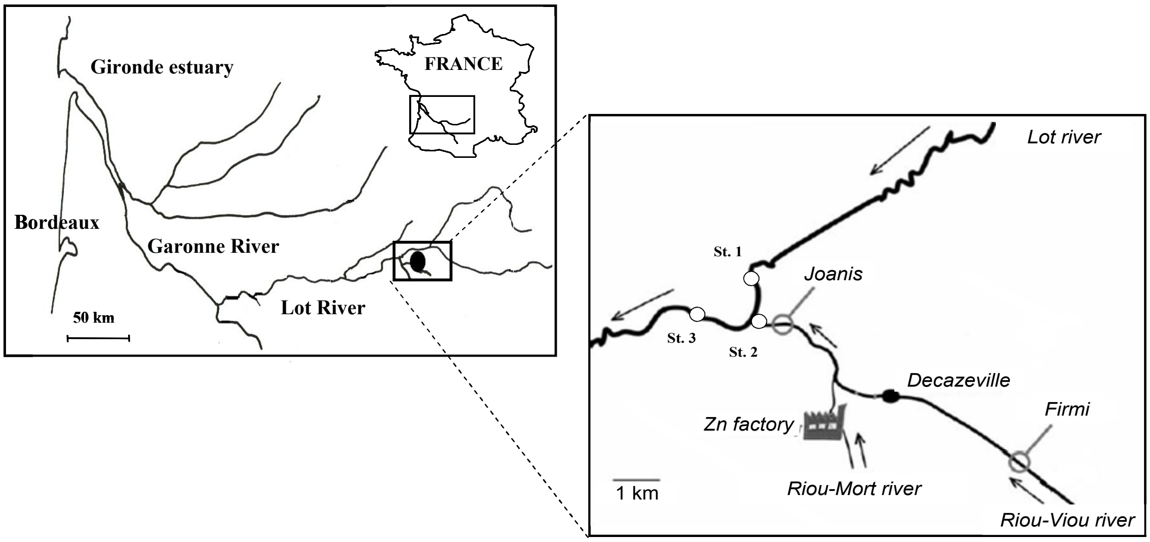
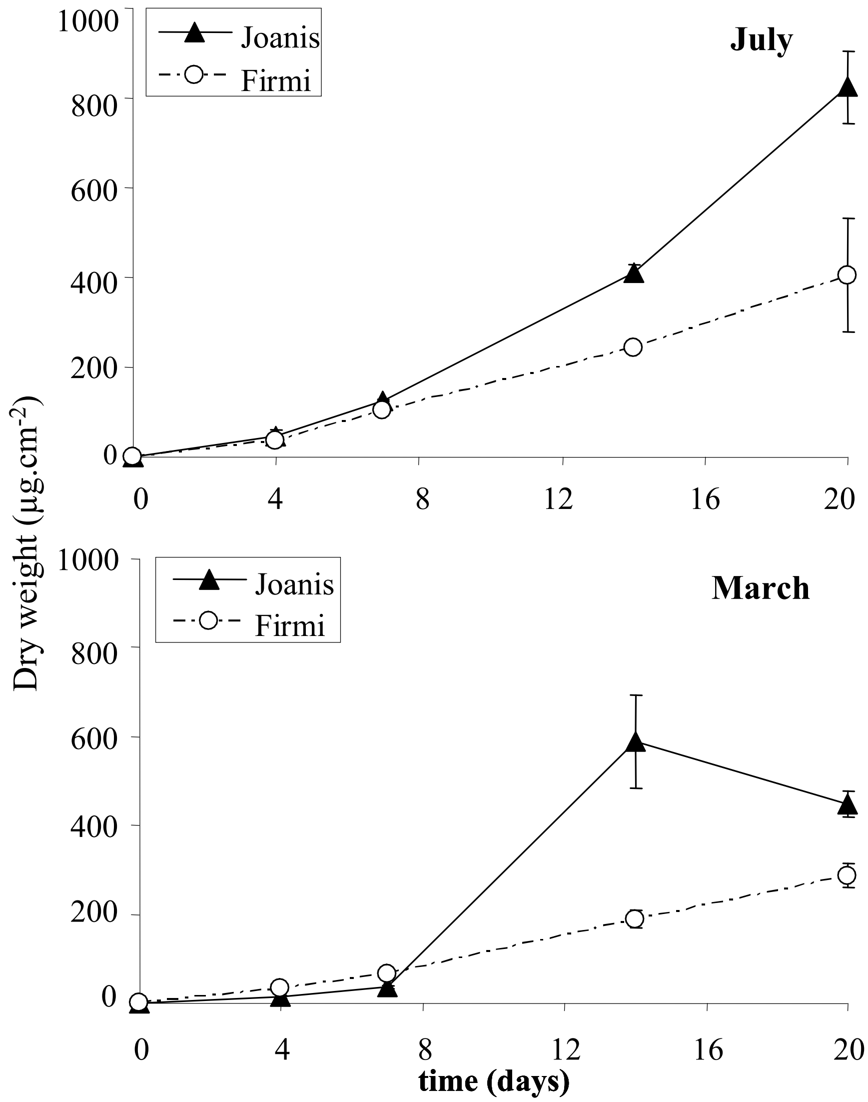
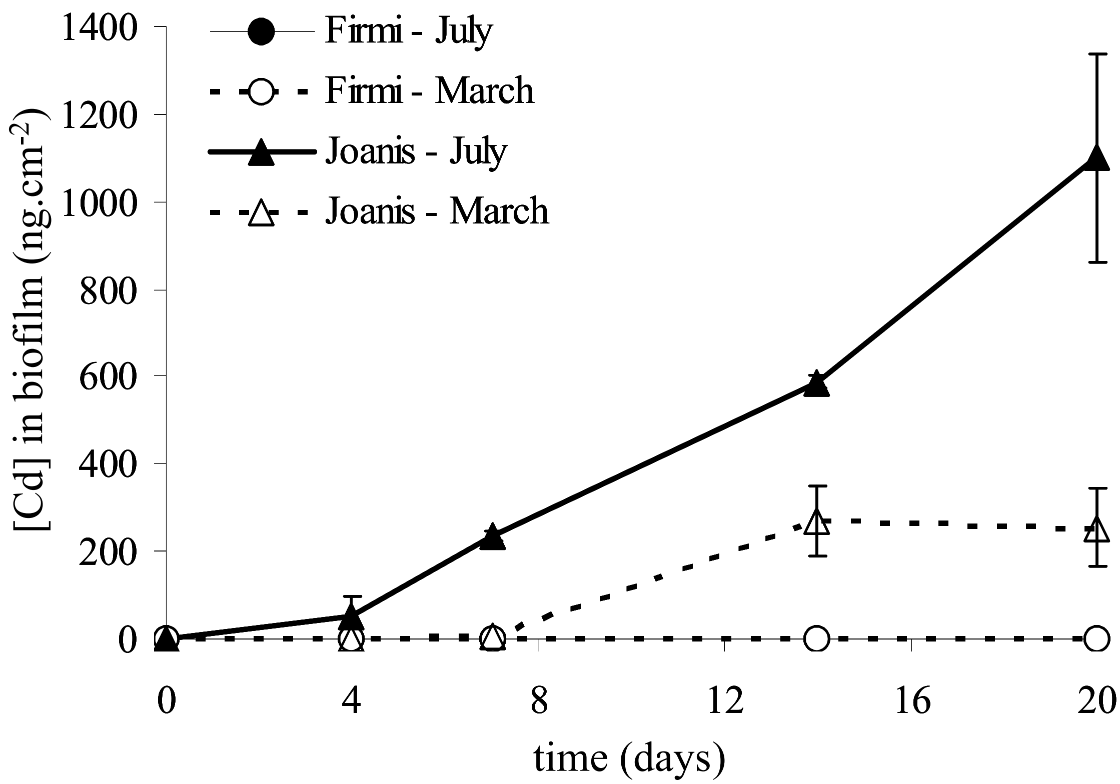

Gironde estuary
FRANCE
Bordeaux
Garonne River
Lot River
50 km
Lot river
St. 1
Joanis
St. 3
St. 2
Decazeville
Firmi
Zn factory
1 km
Riou-Mort river
Riou-Viou river

FIGURE 2



July
March
Joanis
Firmi
Joanis
Firmi
Dry weight (µg.cm-2)
1000
800
600
400
200
0
0
4
8
12
16
20
time (days)

FIGURE 3



[Cd] in biofilm (ng.cm^{-2})

FIGURE 4

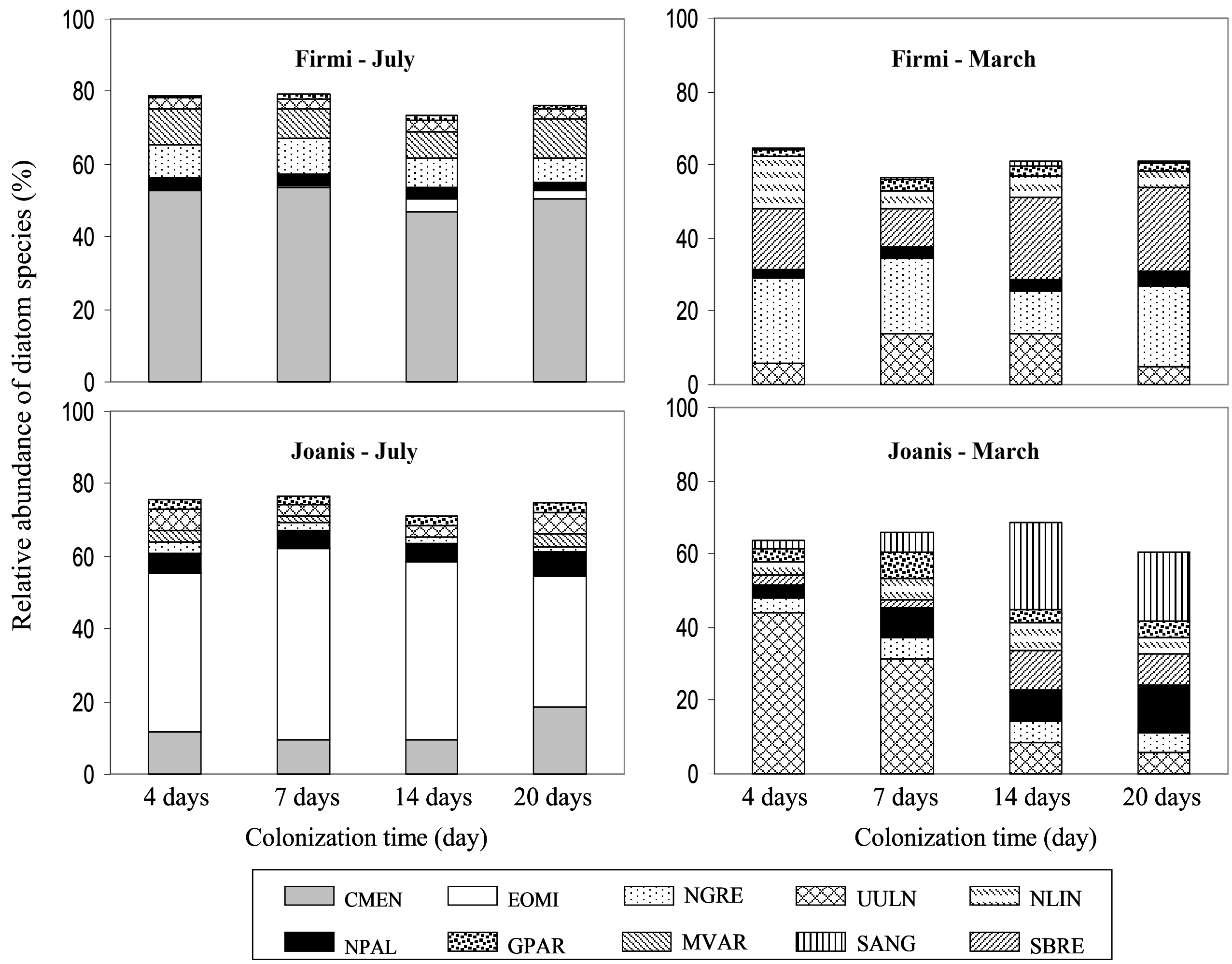
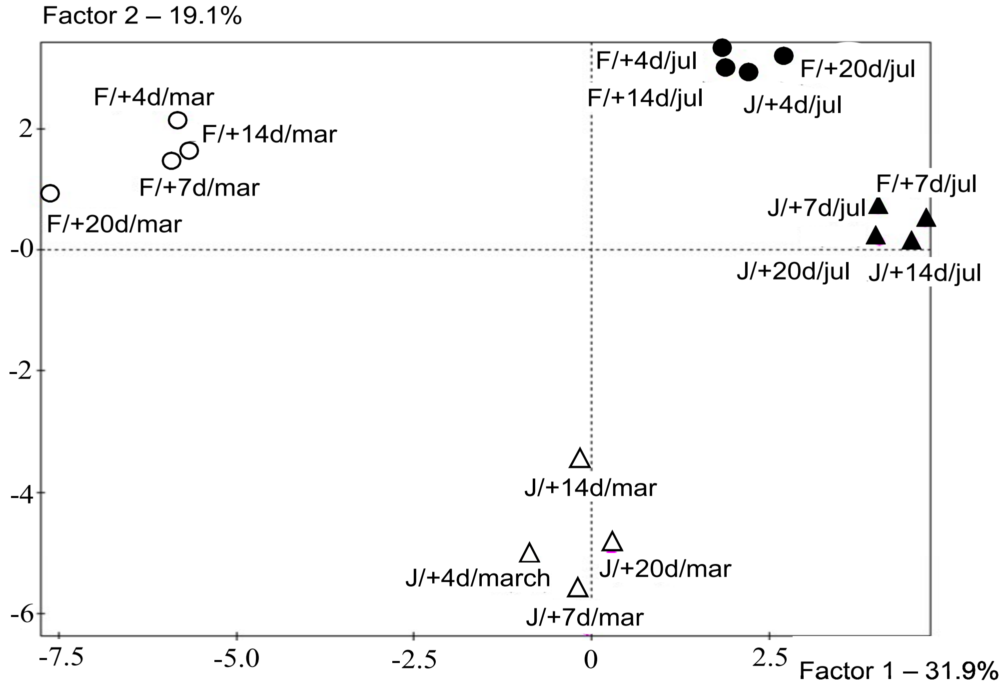

Firmi - July
Firmi - March
Joanis - July
Joanis - March
Relative abundance of diatom species (%)
0
20
40
60
80
100
4 days
7 days
14 days
20 days
Colonization time (day)
CMEN
EOMI
NGRE
UULN
NLIN
NPAL
GPAR
MVAR
SANG
SBRE

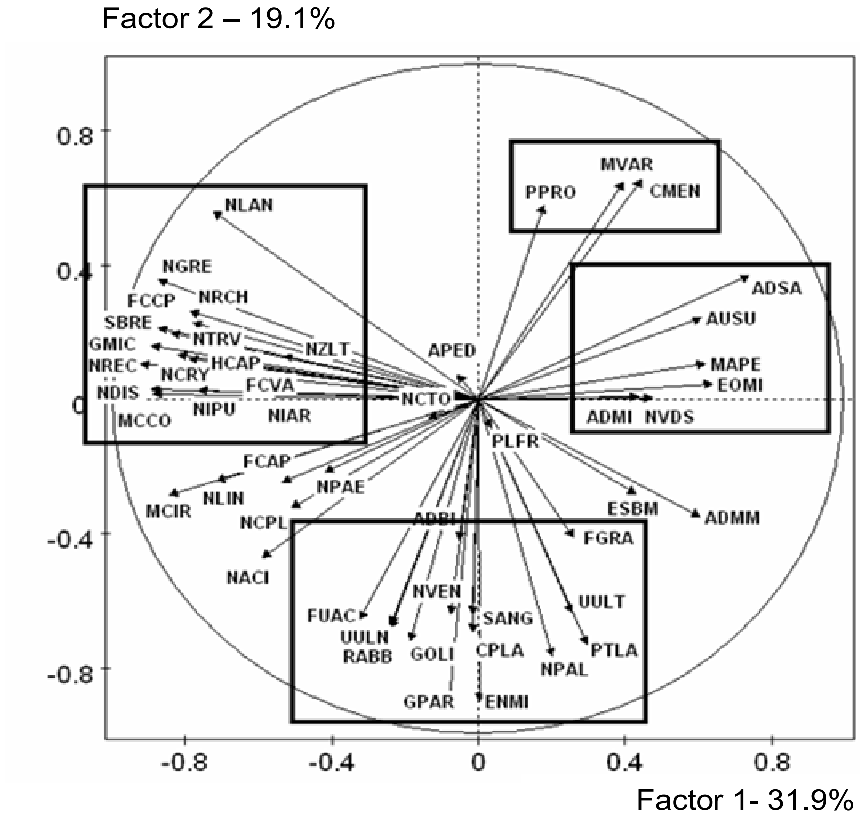
FIGURE 5

a)



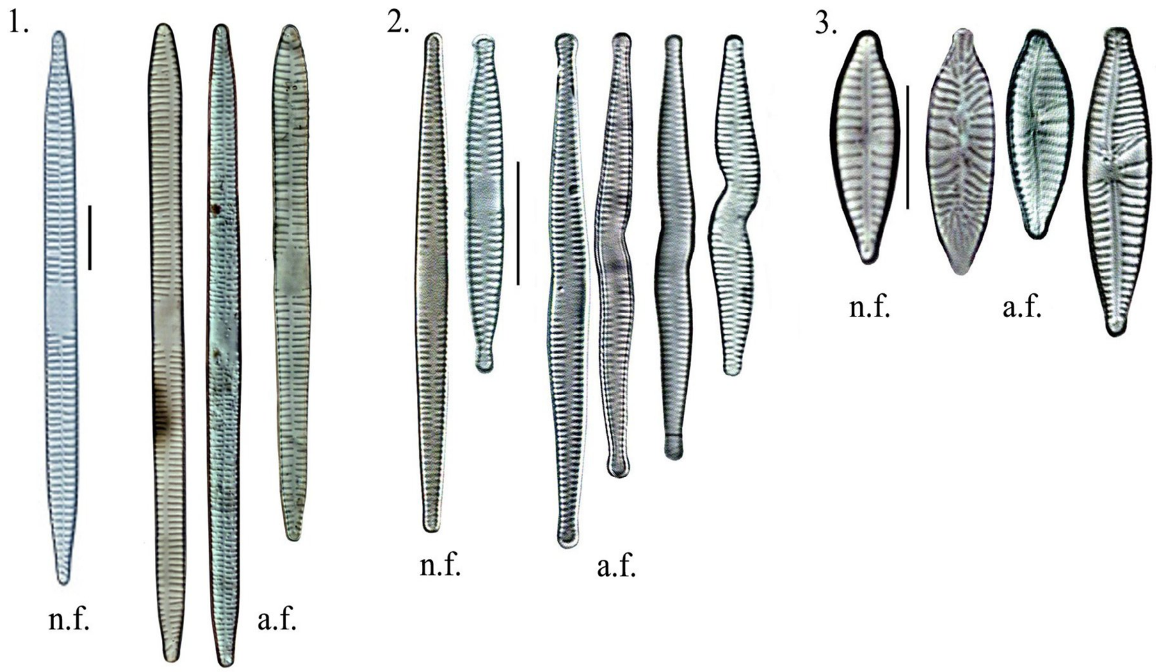
Factor 2 – 19.1%
F/+4d/jul
F/+20d/jul
F/+14d/jul
J/+4d/jul
F/+4d/mar
F/+14d/mar
F/+7d/mar
F/+20d/mar
F/+7d/jul
J/+7d/jul
J/+20d/jul
J/+14d/jul
J/+14d/mar
J/+20d/mar
J/+4d/march
J/+7d/mar
2
-0
-2
-4
-6
-7.5
-5.0
-2.5
0
2.5
Factor 1 – 31.9%

b)



Factor 2 – 19.1%
0.8
0.4
0
-0.4
-0.8
-0.8
-0.4
0
0.4
0.8
Factor 1- 31.9%
MVAR
PPRO
CMEN
NLAN
NGRE
NRCH
FCCP
SBRE
NTRV
GMIC
NZLT
APED
NREC
HCAP
NCRY
FCVA
NDIS
NCTO
NIPU
NIAR
MCCO
ADSA
AUSU
MAPE
EOMI
ADMI
NVDS
PLFR
FCAP
NPAE
NLIN
MCIR
NCPL
NACI
ESBM
ADMM
FGRA
ADBI
NVEN
UULT
FUAC
SANG
UULN
RABB
GOLI
CPLA
PTLA
NPAL
GPAR
ENMI

FIGURE 6



TABLES

Table 1: Physical and chemical characteristics of waters at Firmi and Joanis) stations during experimental period in July 2004 and March 2005. (nm: not measured).

Parameters	Firmi				Joanis			
	4 days	7 days	14 days	20 days	4 days	7 days	14 days	20 days
July 2004								
pH	7.4	7.4	7.4	7.3	8.0	7.7	7.7	7.7
T (°C)	17.6	16.4	19.4	24	19.7	17.6	16.7	24.8
Cond (µS.cm^{-1})	1377	1223	1410	1427	2350	1426	1290	1257
O_2 (mg. L^{-1})	7.0	8.0	9.3	nm	7.2	7.3	6.4	nm
NH_4 (mg. L^{-1})	0.54	0.71	1.33	2.34	0.77	1.95	2.5	3.43
NO_3 (mg. L^{-1})	3	3.6	2.5	2.9	23.7	54.4	38.4	36
NO_2 (mg. L^{-1})	0.18	0.18	0.23	0.24	0.9	1	0.9	1.3
PO_4 (mg. L^{-1})	0.11	0.1	0.1	0.04	0.58	0.87	0.82	2.86
Si (mg. L^{-1})	10.5	13.5	12	15	12	11.5	12	12.5
Cd (µg. L^{-1})	< 0.1	< 0.1	< 0.1	< 0.1	26	26	27	24
March 2005								
pH	7.6	7.6	7.9	8	7.5	7.8	7.9	8.2
T (°C)	3,4	4,5	10,3	13	2,8	6,1	10	13,9
Cond (µS.cm^{-1})	678	538	639	578	735	1602	1723	1744
O_2 (mg. L^{-1})	11,1	9,4	9,1	10.5	10,3	9,3	9,9	10.1
NH_4 (mg. L^{-1})	0.88	0.39	0.61	0.62	0.92	3.44	3.54	2.98
NO_3 (mg. L^{-1})	5	3.9	2.8	2.7	33.8	11.6	26.6	37.3
NO_2 (mg. L^{-1})	0.07	0.04	0.08	0.1	0.7	0.5	1.1	1.1
PO_4 (mg. L^{-1})	0.24	0.08	0.2	0.1	0.2	1.5	1.4	1.3
Si (mg. L^{-1})	7	7.5	5.5	6.5	6.5	9	9.5	8
Cd (µg. L^{-1})	< 0.1	< 0.1	< 0.1	< 0.1	17.1	19.8	21.6	11.2

Table 2: Species richness, diversity index and IPS (mean value and standard error; n = 3) of the reference Firmi and polluted Joanis sites in July 2004 and March 2005.

	July 2004		March 2005	
	Firmi	Joanis	Firmi	Joanis
Species richness (S)	41 ± 0.5	46 ± 4.0	54 ± 4.0	55 ± 3.0
Diversity index (H')	3.0 ± 0.02	3.2 ± 0.1	4.2 ± 0.1	4.1 ± 0.2
IPS	8.5 ± 0.34	7.2 ± 0.34	10.7 ± 0.21	10.0 ± 1.03

Table 3: Pearson correlations between relative abundances of the 20 main diatom species from the two stations: Firmi and Joanis during two sampling periods (July 2004 and March 2005) and cadmium accumulation levels in biofilms ([Cd], $ng.cm^{-2}$).

	July 2004																				
	CMEN	**EOMI**	**NPAL**	**NGRE**	**MVAR**	**UULN**	**GPAR**	**SBRE**	**NLAN**	**MAPE**	**ADMI**	**PLFR**	**ADSA**	**PTLA**	**NVDS**	**NDIS**	**ESBM**	**AUSU**	**ADMM**	**ENMI**	**[Cd]**
[Cd]	-0.57	0.55	0.83	-0.72	-0.60	0.79	0.73	-0.64	-0.73	0.31	-0.52	0.15	0.53	0.68	0.17	-0.21	-0.01	0.33	0.35	0.67	1.0
	March 2005																				
	UULN	**NGRE**	**NPAL**	**SBRE**	**NLIN**	**GPAR**	**SANG**	**NACI**	**NLAN**	**NZLT**	**ENMI**	**CMEN**	**GMIC**	**FUAC**	**EOMI**	**NDIS**	**PLFR**	**PTLA**	**NCPL**	**NIPU**	**[Cd]**
[Cd]	-0.38	-0.49	0.81	-0.18	-0.05	0.20	0.98	-0.42	-0.59	-0.32	0.76	-0.44	-0.52	-0.50	0.98	-0.72	0.68	0.22	0.49	-0.10	1.0

Table 4: Abnormal diatom frustules (‰) at two sampling stations: Firmi and Joanis) along metallic pollution gradient during two sampling periods (July 2004 and March 2005) after 4, 7, 14, and 20 days of colonization.

Colonization duration (days)	July 2004		March 2005	
	Firmi	Joanis	Firmi	Joanis
4	0	8.0 ± 0.7	0.7 ± 0.6	13.7 ± 0.3
7	1.0 ± 0.7	19.5 ± 3.2	4.3 ± 1.3	43.0 ± 9.3
14	0	33.5 ± 0.4	2.3 ± 2.0	17.7 ± 3.1
20	0	48.5 ± 1.1	6.0 ± 0.9	17.3 ± 1.3