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

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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 brebissoni* 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.



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47 groups which will be meaningful for biomonitoring pollution in natural aquatic systems.

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# 1 **Introduction**

2           Pollution of aquatic systems by heavy metals is an important environmental problem because  
3 of their potential accumulation and transfer along the food chains, leading to more or less severe  
4 toxic effects on the different biological levels, from the cellular and molecular basis to the  
5 communities and biocenosis. Metal sources for freshwater systems result from natural processes  
6 (weathering of soils and rocks, volcanic eruptions, etc.) and from a variety of human activities  
7 (mining, smelting and agricultural fertilization) (Audry et al., 2004; Ruangsomboona and Wongrat,  
8 2006). Trace metals such as cadmium (Cd) are considered to be non-essential elements for living  
9 organisms; Cd is one of the most toxic metals, with a high solubility in water and a great  
10 bioaccumulation capacity in many aquatic species, notably algae and bivalves (Lee et al., 1996;  
11 Torres et al., 1998; Baudrimont et al., 1997a).

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

22           The use of freshwater algae in general and periphytic diatoms in particular as indicators for  
23 water quality led to the definition of several diatom indices which are currently applied in many  
24 countries (Coste *in* Cemagref, 1982; Watanabe et al., 1986; Kelly, 1998; Prygiel and Coste, 1999).  
25 The use of the structure of diatom communities to assess impacts of metal pollution on freshwater  
26 system has been discussed by several authors (Medley and Clements, 1998; Ivorra et al., 1999; Gold  
27 et al., 2002). Numerous studies, based on biofilm samples collected along pollution gradients or  
28 within indoor or outdoor artificial streams, have investigated metal impacts on periphyton  
29 communities, the majority of the studies being devoted to diatom communities (e.g. Rushforth et al.,  
30 1981; Gustavson and Wängberg, 1995; Soldo and Behra, 2000; Gold et al., 2003a; ; Gold et al.,  
31 2003b; Guasch et al., 2003). The polymetallic pollution of the Lot River in South-West of France  
32 (figure 1), essentially based on Cd and zinc (Zn) discharges from a zinc ore treatment factory via a

1 small tributary (Riou-Mort), represents a remarkable field site for ecotoxicological studies. Gold et  
2 al. (2002; 2003a and 2003b) have investigated metal impacts on periphytic diatom communities  
3 after colonization of artificial substrates introduced along the pollution gradient (Stations 1, 2 and 3:  
4 figure 1). The confrontation between field data and experimental data obtained from indoor artificial  
5 streams enriched with diatom communities collected at the reference station on the Lot River (St. 1)  
6 and contaminated with metals individually added or in combination (Cd, Zn, Cd+Zn), has revealed  
7 the key role played by cadmium towards toxic effects on the diatom communities: significant  
8 decrease of cell density and diversity; presence of abnormal diatom frustules (Gold, 2002; Gold et  
9 al., 2003a; Morin et al., 2008).

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

## 24 **Material and methods**

### 25 *Study area and sampling stations*

26 The study area is located in the industrial basin of Decazeville (SW France), in the middle  
27 section of the Lot River (figure 1). Since the end of the 19<sup>th</sup> century, the Riou-Mort River, a small  
28 tributary of the Lot River, is contaminated by direct discharges of percolation water coming from  
29 the industrial site of Vieille Montagne, specialized in zinc ore treatment. Two sampling stations  
30 were selected (figure 1). The reference station (Firmi), located on the upstream zone of the Riou-  
31 Mort River, with very low metal background levels in the water; the polluted station (Joanis),

1 located in the downstream zone of the Riou-Mort River, at about 3 km downstream its confluence  
2 with the Riou-Viou River, characterized by high concentrations of Cd and Zn in the water column  
3 (Audry et al., 2004; Blanc et al., 1999). All along the Riou-Mort River, organic discharges coming  
4 from urban and touristic activities are observed.

### 5 ***Biofilm collection***

6 Glass slides (600 cm<sup>2</sup> for both sides) were used as artificial substrates for biofilm attachment. At  
7 each sampling station, three plastic racks containing four vertical glass slides were immersed in the  
8 water column, parallel to the current at about 10-15 cm below the water surface (see Gold et al.,  
9 2002 for details). Biofilm samples were collected after 4, 7, 14 and 20 days of colonization, in July  
10 2004 (07/01 to 07/21) and March 2005 (03/03 to 03/23). At each sampling day, one glass slide was  
11 removed randomly from each rack (3 replicates), then scraped using a cutter blade and washed with  
12 mineral water. All biofilm samples were diluted in a standard volume of 100 mL and divided after  
13 homogenisation and addition of 1 mL of formol solution (Formaldehyde 37%, Prolabo, France) for  
14 preservation into three fractions assigned to various analyses: diatom identification; biofilm dry  
15 weight measurement and Cd concentration determination. Remaining sample was set aside for  
16 prospective additional analyses.

### 17 ***Physico-chemical characteristics of the water and Cd concentrations in the dissolved fraction of*** 18 ***the water column***

19 Temperature, conductivity, pH and dissolved oxygen concentration were measured *in situ* at  
20 each sampling date using a multi-probe analyser (WTW, Weilheim, Germany). Water samples were  
21 also collected for nutrients analyses and phosphate, nitrate, nitrite, ammonium and silica  
22 concentrations were determined according to French and International standard methods (NT T 90-  
23 023, NF EN ISO 13395, NF EN ISO 11732 and NF T 90-007, respectively).

24 Dissolved Cd concentration was measured using ICP-MS (X7, THERMO, Elemental, UK)  
25 with external calibration. Indium was used as internal standard and after each batch of five samples,  
26 a calibration blank and one calibration standard were measured to control potential sensitivity  
27 variations or memory effects. The analytical method employed was continuously quality checked by  
28 analysis of certified reference river waters (SLRS-3, SLRS-4). Accuracy was within 5% of the  
29 certified values and the analytical error (relative standard deviation) was generally better than 5%  
30 for concentrations ten times higher than detection limits (see Audry, 2003 for details). Detection  
31 limit were 0.1 µg Cd.L<sup>-1</sup>.



## 1 ***Cd accumulation levels in biofilm***

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

## 13 ***Samples preparation for diatom studies***

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

## 23 ***Data treatment***

24 Statistical analyses were carried out using one-way variance model (ANOVA) to reveal the  
25 effects of colonization duration (days) and sampling stations (Joanis and Firmi) on biofilm dry  
26 weight, and Cd accumulation in biofilm. Tests for normality and homogeneity of variance were  
27 verified by using Cochran's test. If a significant effect was observed, *post-hoc* tests (Least  
28 Significant Difference test, LSD; Newman-Keuls test) were performed to isolate difference at a  
29 significant level of  $p < 0.05$ . All statistical investigations were performed using *STATISTICA* version  
30 7 software, expressed results corresponding to mean values  $\pm$  standard error (SE). A principal  
31 component analysis (PCA) using SPAD Software (version 5.6, Decisia, Paris, France) was

1 performed on the relative abundances of diatom species, in order to reveal taxonomic differences  
2 between communities collected from the two stations during the two seasons (July 2004 and March  
3 2005). Pearson correlation matrix between Cd accumulation levels in biofilms and relative  
4 abundances of the 20 most abundant diatom species were done by PCA, SPAD software (v. 5.6,  
5 Decisia, Paris, France). Indices ( $\lambda_{PS}$  and  $\lambda_{Diversity}$ ) were calculated using OMNIDIA software  
6 (Lecointe et al., 1993).

## 7 **Results**

### 8 *Environmental characteristics of the two sampling stations*

9 Values of physico-chemical parameters measured at Firmi and Joanis stations during the two  
10 20-day sampling periods in July 2004 and March 2005 are summarized in table 1. The average  
11 values for pH were 7.6 for Firmi and 7.8 for Joanis, without significant difference ( $p < 0.05$ ) between  
12 the two seasons. Extreme water temperatures ranged from 2.8 °C (Joanis, March, + 4 days) to  
13 24.8 °C (Joanis, July, + 20 days). The average temperature values for Firmi in July and March were  
14 19.4 and 7.8 °C, respectively; and for Joanis, 19.7 and 8.2 °C, respectively, without significant  
15 differences between the two stations. No significant difference was observed neither between the  
16 two stations for the oxygen concentrations during each sampling periods: 10.0 mg.L<sup>-1</sup> in March and  
17 7.5 mg.L<sup>-1</sup> in July. No significant difference in conductivity was observed between the two stations  
18 in July (Firmi: 1580 ± 259 μS.cm<sup>-1</sup> and Joanis: 1359 ± 46 μS.cm<sup>-1</sup>); but in March, values from  
19 Joanis were significantly higher (1451 ± 240 and 608 ± 31 μS.cm<sup>-1</sup>, respectively). Marked  
20 differences were observed between ammonium, nitrate, nitrite and phosphate concentrations, in  
21 favour of Joanis: overall for N-NO<sub>3</sub>. Silica determination in water samples showed weak differences  
22 between the two stations but a significant difference was observed between the two seasons (7.4 ±  
23 0.7 in March and 12.4 ± 0.7 in July). Cd concentrations measured in the dissolved fraction (< 0.45  
24 μm) of the water column were systematically below the detection limit (0.1 μg.L<sup>-1</sup>) at the reference  
25 station (Firmi). Higher Cd concentrations were measured at the polluted site (Joanis), with mean  
26 values of 25.6 and 17.4 μg.L<sup>-1</sup> in July 2004 and March 2005, respectively.

### 27 *Biofilm total dry weight*

28 Evolutions of biofilm dry weights during the 20-day colonization periods for the two stations  
29 in July 2004 and March 2005 are shown in figure 2. After 14 and 20 days of colonization,  
30 significant differences were observed between the two stations in favour of Joanis: at the end of the  
31 experiment, Joanis mean values were 824 ± 80 versus Firmi at 405 ± 128 μg.cm<sup>-2</sup> in July and 446 ±

1 29 (Joanis) versus  $287 \pm 28 \mu\text{g}\cdot\text{cm}^{-2}$  (Firmi) in March. No significant difference was observed after  
2 4 and 7 days. The dry weight increase was progressive, with tendencies close to linearity, excepted  
3 at Joanis in March where a decrease was observed between 14 and 20 days of colonization.

#### 4 ***Cd accumulation in biofilms***

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

#### 14 ***Diatom community characteristics***

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

20 Taxonomic composition and relative abundance of diatom species were markedly different  
21 between the two stations and the two seasons (figure 4). In July, at Firmi station, the relative  
22 abundances of the seven most abundant species were quite similar during the 20-day colonization:  
23 *Cyclotella meneghiniana* (CMEN) was the dominant species, with more than 50 % of the total  
24 diatom communities. In addition, *Navicula gregaria* (NGRE) and *Melosira varians* (MVAR)  
25 represented 8.4 and 9.2 % respectively. In Joanis, still in July, *Eolimna minima* (EOMI) was  
26 dominant, with a mean relative abundance close to 50 %. For *Cyclotella meneghiniana* (CMEN),  
27 *Nitzschia palea* (NPAL), *Ulnaria ulna* (UULN) and *Gomphonema parvulum* (GPAR), the mean  
28 values were 12.1%, 5.4%, 4.6% and 2.7%, respectively. In March, relative abundances of the main  
29 diatom species at Firmi station were comparable after 4, 7, 14 and 20 days' colonization. The  
30 dominant planctonic species CMEN in July was replaced by two species: *Surirella brebissonii*  
31 (SBRE, 18 % on average) and *Ulnaria ulna* (UULN, 10 %), with a marked increase of *Navicula*

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

32 Teratological frustules of diatom species which consisted in twisted valves in their apical  
33 axis or irregularity in striae arrangement were rather frequently observed at Joanis station in both

1 seasons (table 4, and figure 6). The percentage of deformations in the total assemblage at Joanis  
2 station was higher in July than in March and ranged around 27.3‰ and 23.0‰ (mean value)  
3 respectively. Among them, araphids diatoms such as *Ulnaria ulna* occupied larger proportion with  
4 14.5‰ (mean value) in July and 16.7 ‰ (mean value) in March. In addition, other abnormal  
5 frustules belonging to the raphids were also quite abundant in biofilm samples at Joanis station at  
6 both seasons, with *Gomphonema parvulum* which presented a percentage of 2.3 ‰ in July and 1.6  
7 ‰ in March; *Eolimna minima* which showed 2.6 ‰ in July and 0.5 ‰ in March and *Nitzschia palea*  
8 in July and March assemblages appearing with 1 and 0.3 ‰ respectively. Besides the abnormal  
9 forms of species cited above, araphids, monoraphids and centric diatom deformed appeared in both  
10 seasons with low proportions of *Diatoma vulgare*) *Achnantheidium minutissimum*, *Achnantheidium*  
11 *saprophila* and *Cyclotella meneghiniana*.

## 12 **Discussion**

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

29 Although dissolved Cd concentrations in water were not detectable in the reference site  
30 (Firmi), metal levels were measured in natural biofilms at Firmi station, however not as much as in  
31 Joanis station which allows biofilm to accumulate metal overall in July, and underlines its utilization  
32 as a useful tool to indicate metallic pollution (Ramelow et al., 1992). Cd accumulation in natural

1 biofilm reflected their exposure history (figure 3). In our study, Cd contents in biofilm from polluted  
2 station (Joanis) increased gradually and reached their maximum value at the end of the experiment  
3 in July whereas in March Cd content in biofilm increased till the day 14 and then stayed constant  
4 This could be related to the saturation of binding sites in biofilm leading to a limitation of Cd  
5 accumulation in biofilm on the day 20. A large number of metal binding sites in the biofilm (cell  
6 surface, organic particles embedded in the matrix) have been found to play an important role in  
7 metal sorption from water column (Pistocchi et al., 1997; Decho, 2000; Barranguet et al., 2000).  
8 Increase of biofilm dry weights containing, bacteria, fungi, algae and their secretory products such  
9 as extracellular polymeric substances which have been reported to act as a trap for nutrients or  
10 metals (Sekar et al., 2002), could explain the continuous sorption of Cd by biofilms in growth. By  
11 using X-ray analysis to observe metals contained in algae collected from heavy metal pollution sites,  
12 Lai et al. (2003), Chien (2004) and Nakanishi et al. (2004) showed that heavy metal elements could  
13 be found in diatom species such as *Nitzschia palea*, *Achnantheidium minutissimum* and *Fragilaria*  
14 *tenera*. Moreover, according to Khoshmanesh et al. (1997), smaller cells of algae have  
15 proportionally larger surface area and more sites for metal binding than those of larger cells. So, the  
16 abundance of small diatom species in biofilm of Joanis station in July (figure 5b) could explain  
17 higher metal concentrations accumulated from surrounding water (figure 3).

18         Beside the higher species richness and diversity in March, IPS values obtained from this  
19 investigation indicated rather poor to moderate quality status of water for the two stations and  
20 reflected the organic overload of the two sampling stations located in an urban area (table 1).  
21 Though, nutrients availability was found all along the Riou Mort River, metal pollution solely  
22 detected in Joanis station, downstream the confluence with the Riou-Viou River (figure 1) seems to  
23 determine the structure of diatom assemblages as expected and shown by the distribution and the  
24 presence of particular diatom species in the Riou-Mort River reflecting metal levels along the river  
25 between Firmi and Joanis stations. Moreover, our results showed marked differences in periphytic  
26 diatoms composition between stations and seasons. Throughout the summer experiment, relative  
27 abundances of dominant diatom species at Joanis and Firmi stations were developed stably  
28 (figure 4). Planktonic species such as *Cyclotella meneghiniana* (CMEN), *Melosira varians* (MVAR)  
29 and raphid species *Navicula gregaria* (NGRE) presented large proportions in Firmi biofilms.  
30 Several authors have found these species to be sensitive to metal pollution (Genter et al., 1987;  
31 Medley and Clements, 1998; Morin et al., 2007). This is confirmed in table 3 by negative  
32 correlations of these species to Cd accumulation in biofilm in both seasons and by the low  
33 proportion of these species observed in the polluted site (Joanis). More adapted to high levels of Cd

1 in both water column and biofilm, diatom communities in Joanis station in July tend to be  
2 dominated by *Eolimna minima* (EOMI) (around 50% of the total community) (figure 4) and other  
3 small forms of diatom species such as *Mayamaea atomus* (MAPE), *Achnantheidium saprophila*  
4 (ADSA), *Aulacoseira subarctica* (AUSU) (figure 4 and 5). *E. minima* (EOMI) with its high  
5 proportion is known to be metal resistant species (Gold et al., 2002; Szabó et al., 2005). Additive  
6 presence of many small forms in communities compared to diatom communities from reference  
7 station (Firmi) (figure 5a and b) supports the hypothesis that effects of metallic pollution on diatoms  
8 composition are detectable even in overload zone like Joanis station which receives organic  
9 discharges according to wastewater treatment plant activity. In accordance with Medley and  
10 Clements' observations (1998), high proportions of adnate and small species were underscored in  
11 our results and were strongly related to Cd presented in table 3.. Similarly, considerable increases of  
12 small diatom species under high Cd concentration in laboratory experiment were also observed by  
13 Pérès (1995).

14 Composition of benthic diatom communities in March were diversified in both stations and  
15 differed from those in July (figure 4 and 5b). The differences observed in diatom communities might  
16 be attributed to changes in the environment (table 1) with lower metal concentrations in water  
17 column in March leading to a lower accumulation in biofilms. Seasonal variations of metal  
18 concentrations have been suggested to influence periphytic diatom composition (Medley and  
19 Clements, 1998). Firmi's diatom communities were characterized by quite stable abundances of  
20 *Navicula gregaria* (NGRE) and *Surirella brebissonii* (SBRE) throughout the study, and  
21 corresponding to a total ratio of 40 %. Unlike diatom composition in July, under lower cadmium  
22 concentration, in March, Joanis's communities showed presence of a few big diatom species such as  
23 *Nitzschia linearis* (NLIN), *Fragilaria ulna* var. *acus* (FUAC), *Gomphonema olivaceum* (GOLI)  
24 (figure 4 and 5b); although some small forms of diatoms were still well represented. The  
25 modifications in diatom structure at Joanis station compared to communities in Firmi station were  
26 noticeable with the decrease of *Ulnaria ulna* (UULN) species and increase of *Surirella angusta*  
27 (SANG) and *Nitzschia palea* (NPAL) (figure 4). The gradual increase of these species, accompanied  
28 and relatively correlated with a considerable increase of Cd content in biofilm, from its initial to  
29 mature stage, could therefore be indicators of high level of Cd. Indeed, relative abundances of  
30 *Surirella angusta* (SANG) occupying around 50% of total diatom community with high  
31 concentration of metals in biofilm were found in Kakehashi River (Nakanishi et al., 2004). Complex  
32 relationships between *Ulnaria ulna* (UULN) and several metals were recorded on attached diatoms  
33 in the Uintah basin of Utah (Rushforth et al., 1981) with a positive correlation of *Ulnaria ulna* with

1 cadmium, copper and mercury in spring season, and on the contrary a negative correlation with  
2 copper in winter season. In the present study, this phenomenon was revealed by Pearson correlations  
3 for *Ulnaria ulna* (UULN) and cadmium between summer (July) and spring (March) seasons (table  
4 3), and we could suggest that such varying relations of diatom species to heavy metals may also be  
5 attributed to seasonal differences.

6 Heavy metals do not only affect diatom community structure but also the formation of their  
7 valves. Morphological aberrations of diatom species under environment stress have been reported by  
8 several authors (Ruggiu et al., 1998; Yang and Duthie, 1993; Gold et al., 2003; Cattaneo et al.,  
9 2004). They suggested that increasing metals concentration (in water or sediment) could trigger the  
10 formation of deformed valves within some diatom genera. In this study, Cd contamination in water  
11 column and significant Cd accumulation in natural biofilm at the polluted station (Joanis) have led  
12 to the frequent emergence of abnormal forms at this station. The occurrence of deformed valves  
13 within *Fragilaria* genus (synonym *Ulnaria* or *Synedra*) have been widely reported, for example by  
14 McFarland et al. (1997) for *F. capucina*, by Ruggiu et al. (1998) for *Synedra tenera*, by Gold et al.  
15 (2003 a) for *F. capucina* var. *gracilis* and by Nunes et al. (2003) for *F. crotonensis* and *F. capucina*  
16 var. *rumpens*. In addition, our study has also presented other morphological abnormal forms  
17 belonging to centric and raphids species.

18 In conclusion, our study shows that, cadmium accumulation in natural biofilm was detected  
19 despite non acute Cd concentration levels in water column and in an environment characterized by  
20 an organic overload. Hence, using biofilm method is suitable for heavy metal monitoring. Due to the  
21 different seasons and levels of metal load in an organic overload medium, Cd accumulation in  
22 natural biofilms and periphytic diatom communities significantly varied. Season appears as an  
23 important factor not to overlook. Metal pollution caused changes in periphytic diatom composition  
24 along the river. Diatom assemblages, dominated by small, adnate diatom species and the presence of  
25 teratological forms at polluted station, could be valid indicators of contamination by metals. Positive  
26 or negative correlations of dominant diatom taxa to cadmium accumulation in natural biofilm  
27 indicates groups of species tolerant or sensitive to metal, which will be meaningful for  
28 biomonitoring in aquatic systems. Such interpretations could be confirmed for validation by further  
29 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
<b>July 2004</b>								
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 <sup>-1</sup> )	1377	1223	1410	1427	2350	1426	1290	1257
O <sub>2</sub> (mg. L <sup>-1</sup> )	7.0	8.0	9.3	nm	7.2	7.3	6.4	nm
NH <sub>4</sub> (mg. L <sup>-1</sup> )	0.54	0.71	1.33	2.34	0.77	1.95	2.5	3.43
NO <sub>3</sub> (mg. L <sup>-1</sup> )	3	3.6	2.5	2.9	23.7	54.4	38.4	36
NO <sub>2</sub> (mg. L <sup>-1</sup> )	0.18	0.18	0.23	0.24	0.9	1	0.9	1.3
PO <sub>4</sub> (mg. L <sup>-1</sup> )	0.11	0.1	0.1	0.04	0.58	0.87	0.82	2.86
Si (mg. L <sup>-1</sup> )	10.5	13.5	12	15	12	11.5	12	12.5
Cd (µg. L <sup>-1</sup> )	< 0.1	< 0.1	< 0.1	< 0.1	26	26	27	24
<b>March 2005</b>								
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 <sup>-1</sup> )	678	538	639	578	735	1602	1723	1744
O <sub>2</sub> (mg. L <sup>-1</sup> )	11,1	9,4	9,1	10.5	10,3	9,3	9,9	10.1
NH <sub>4</sub> (mg. L <sup>-1</sup> )	0.88	0.39	0.61	0.62	0.92	3.44	3.54	2.98
NO <sub>3</sub> (mg. L <sup>-1</sup> )	5	3.9	2.8	2.7	33.8	11.6	26.6	37.3
NO <sub>2</sub> (mg. L <sup>-1</sup> )	0.07	0.04	0.08	0.1	0.7	0.5	1.1	1.1
PO <sub>4</sub> (mg. L <sup>-1</sup> )	0.24	0.08	0.2	0.1	0.2	1.5	1.4	1.3
Si (mg. L <sup>-1</sup> )	7	7.5	5.5	6.5	6.5	9	9.5	8
Cd (µg. L <sup>-1</sup> )	< 0.1	< 0.1	< 0.1	< 0.1	17.1	19.8	21.6	11.2

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

3  
 4

	July 2004		March 2005	
	Firmi	Joaanis	Firmi	Joaanis
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<sup>-2</sup>).

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  $\pm$  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  $\pm$  standard deviation; n=3.

Figure 4: Relative abundances of 10 diatom species (mean values; n=3) with relative abundances  $\geq 10\%$  in July and  $\geq 5\%$  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

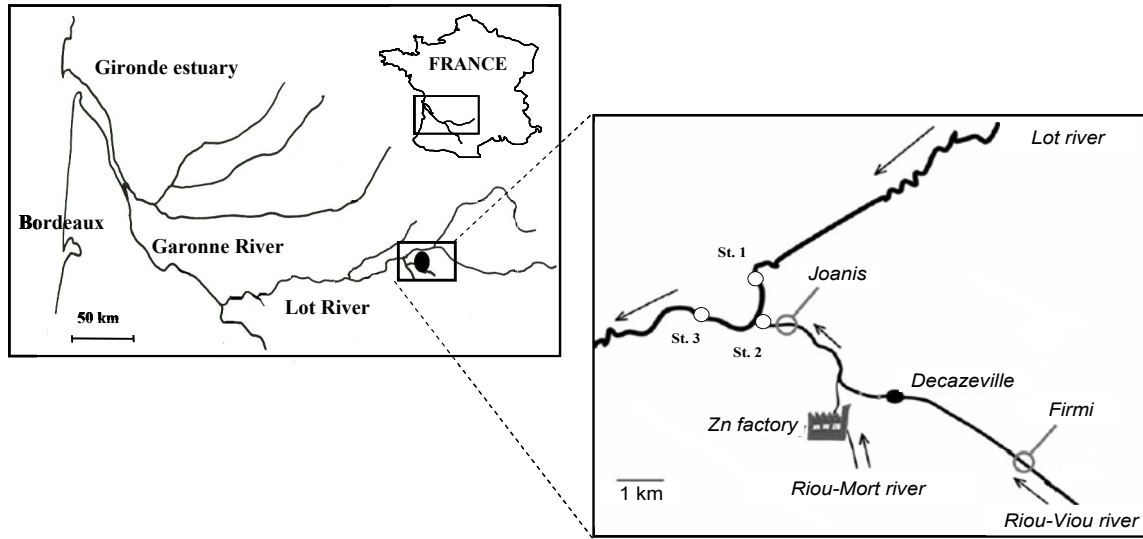


FIGURE 2

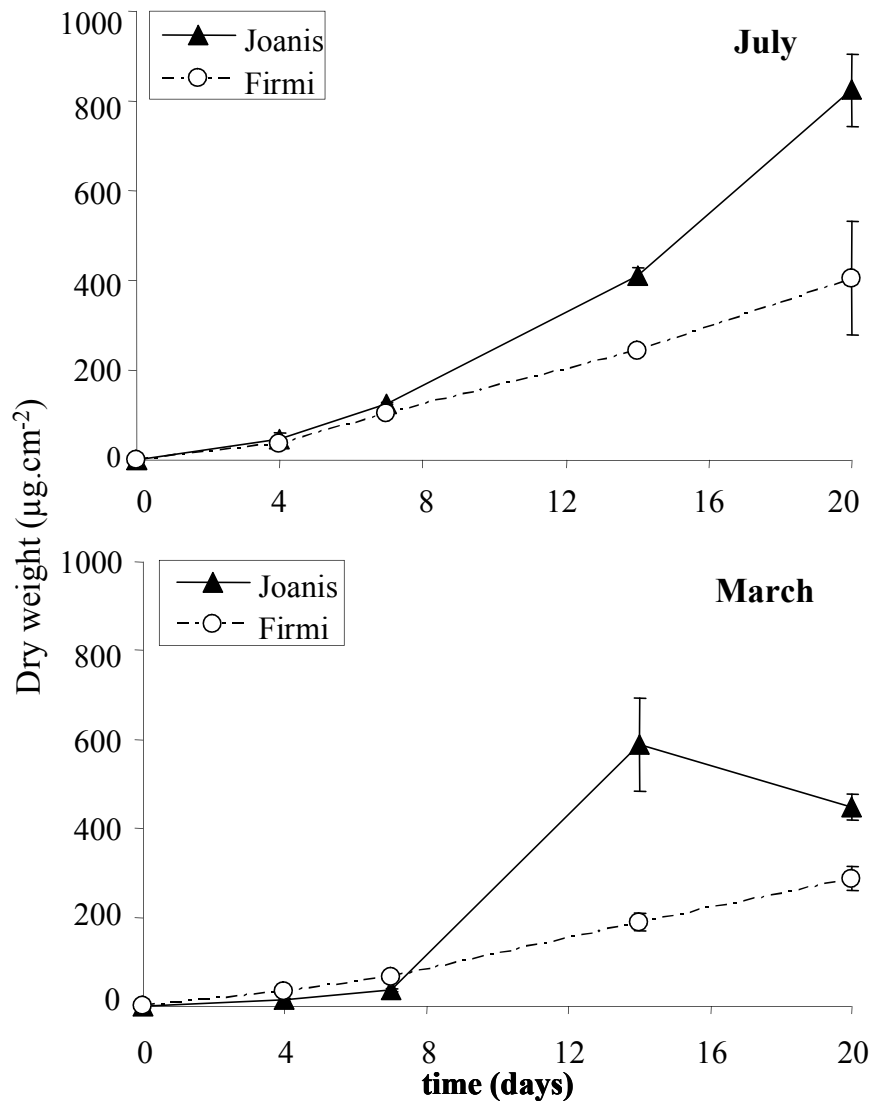
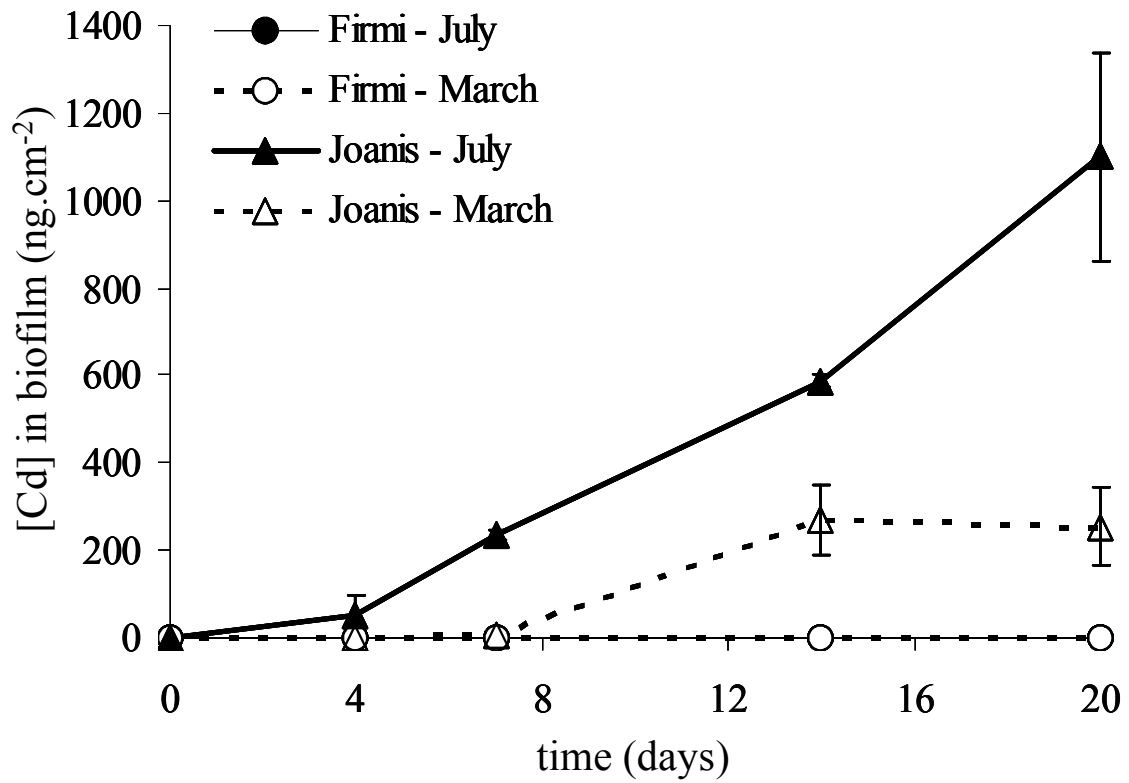


FIGURE 3



[Cd] in biofilm ( $\text{ng}\cdot\text{cm}^{-2}$ )

FIGURE 4

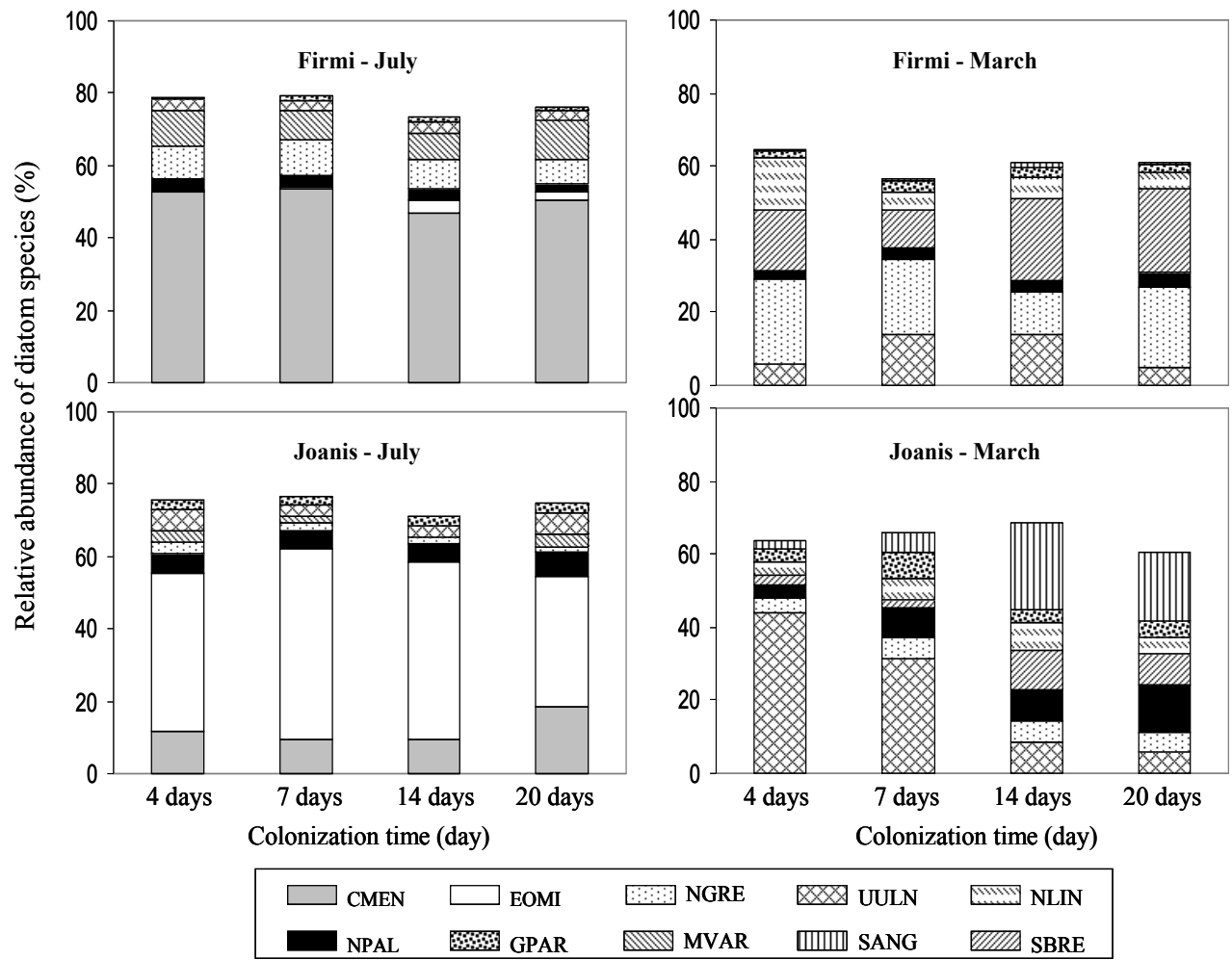




FIGURE 5

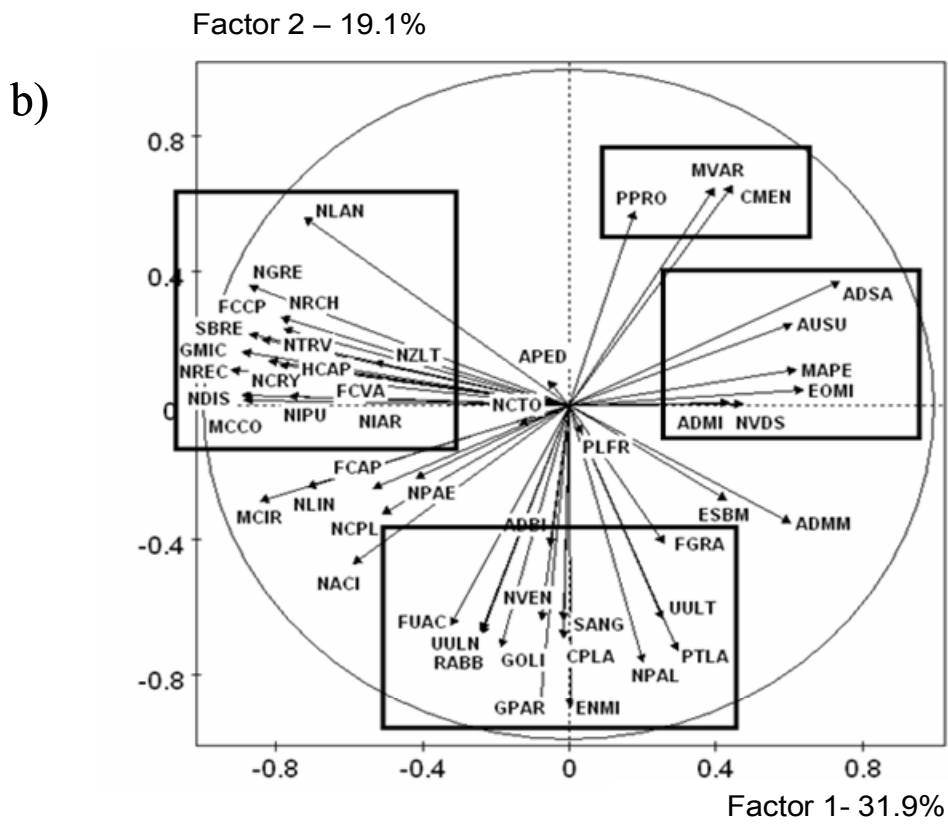
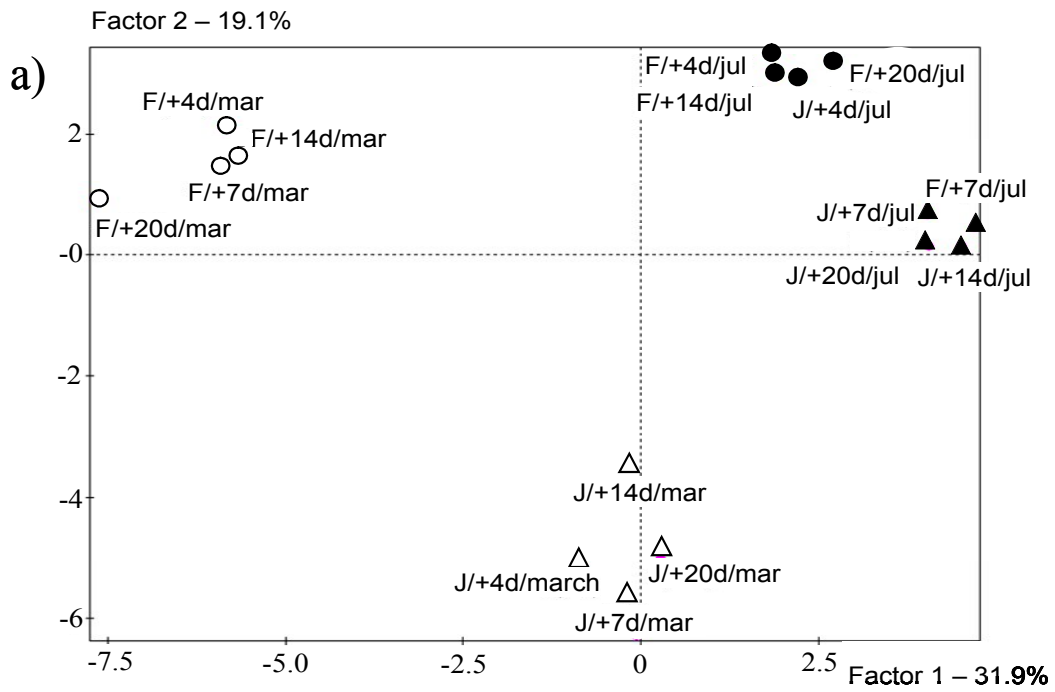
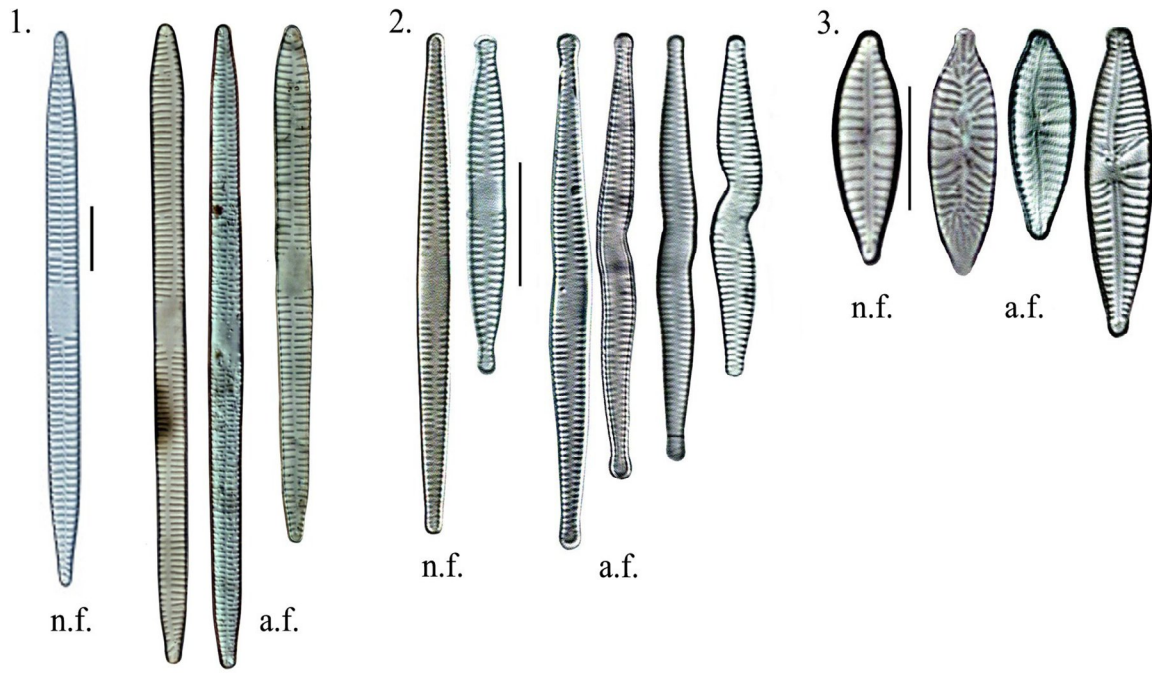


FIGURE 6



## 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  $\pm$  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  $\pm$  standard deviation; n=3.

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FIGURE 1

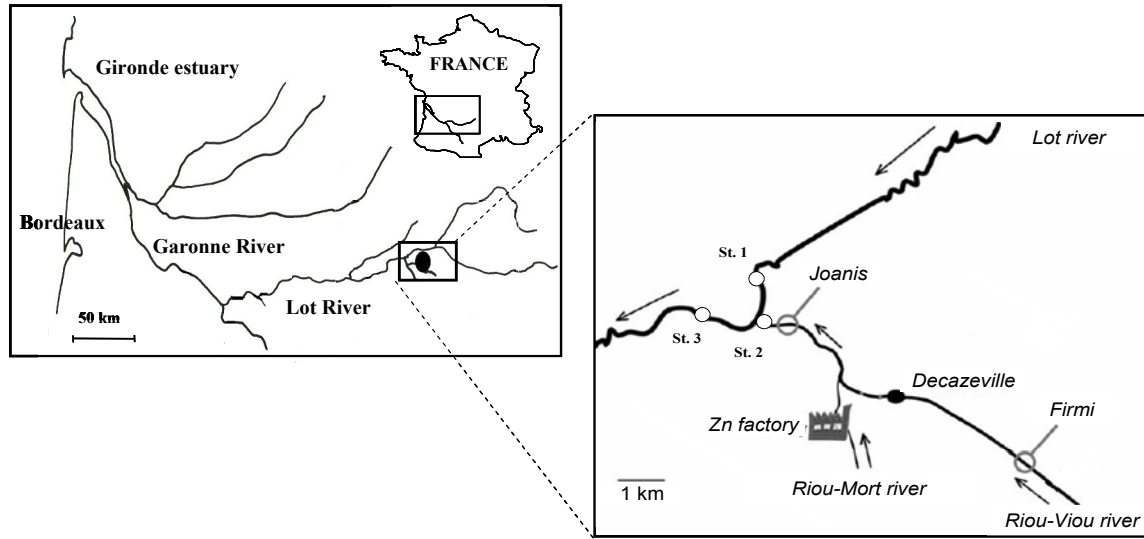


FIGURE 2

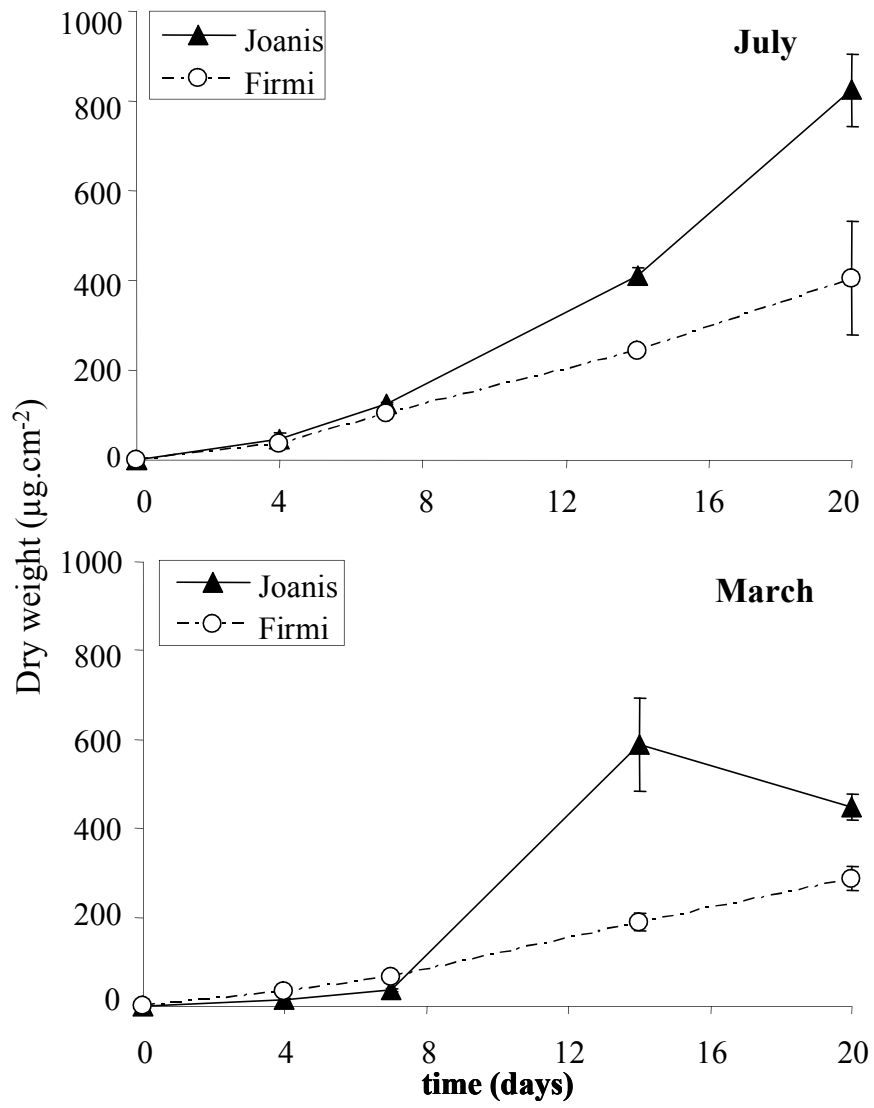
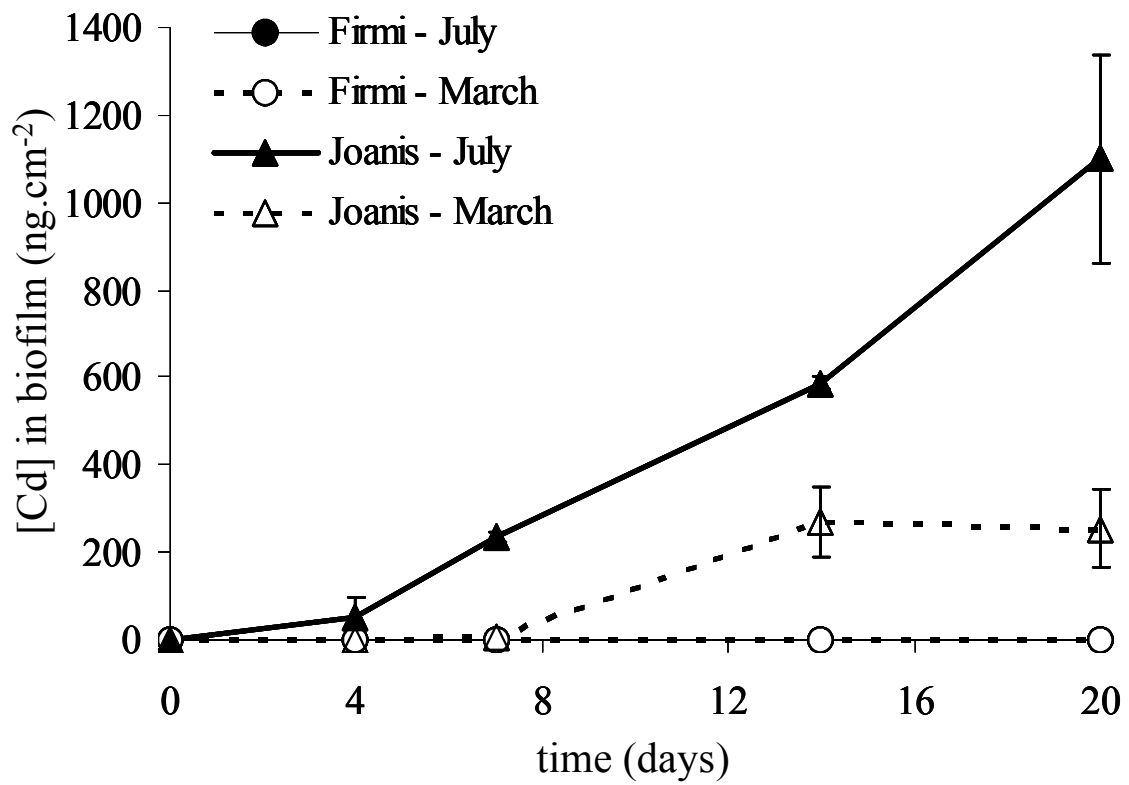


FIGURE 3



[Cd] in biofilm ( $\text{ng}\cdot\text{cm}^{-2}$ )

FIGURE 4

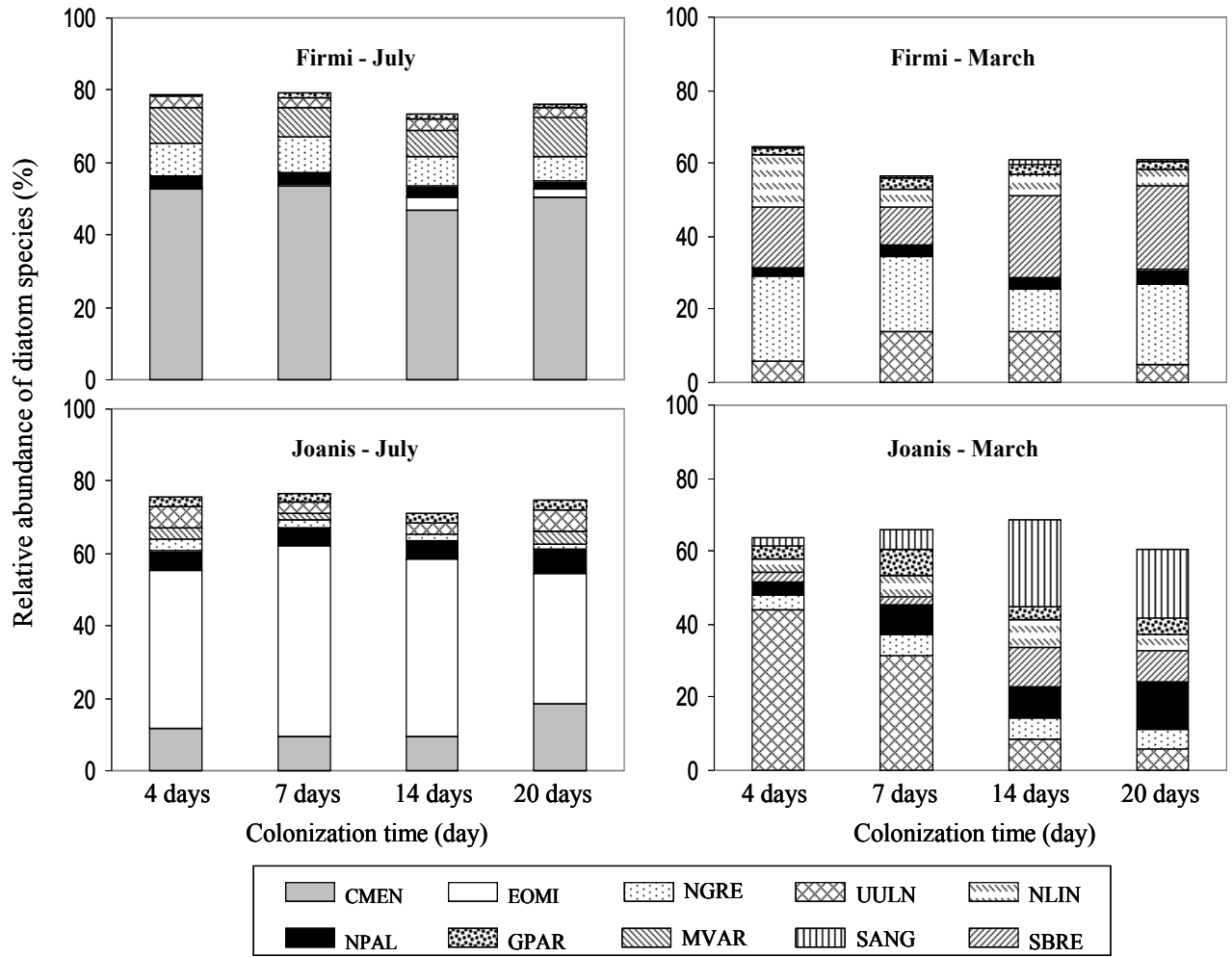


FIGURE 5

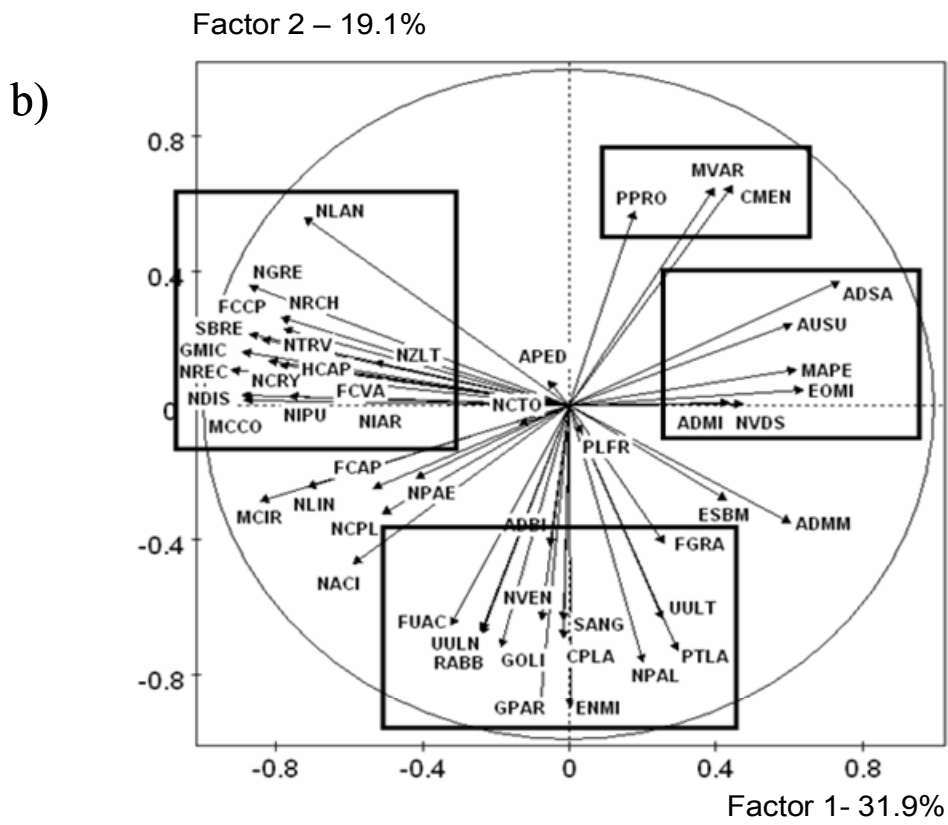
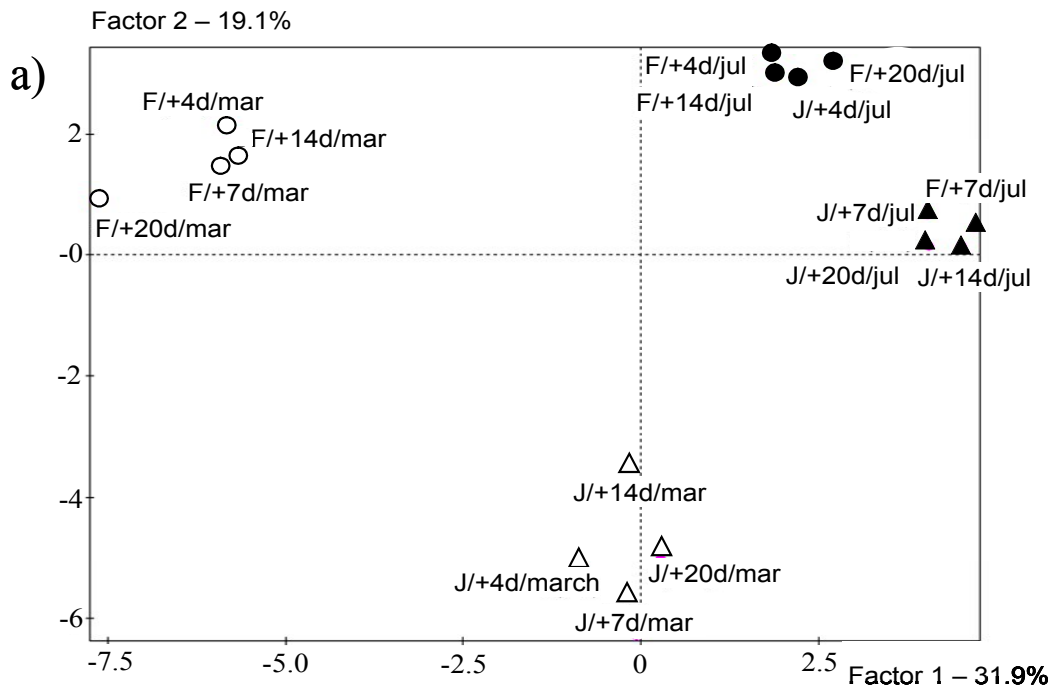
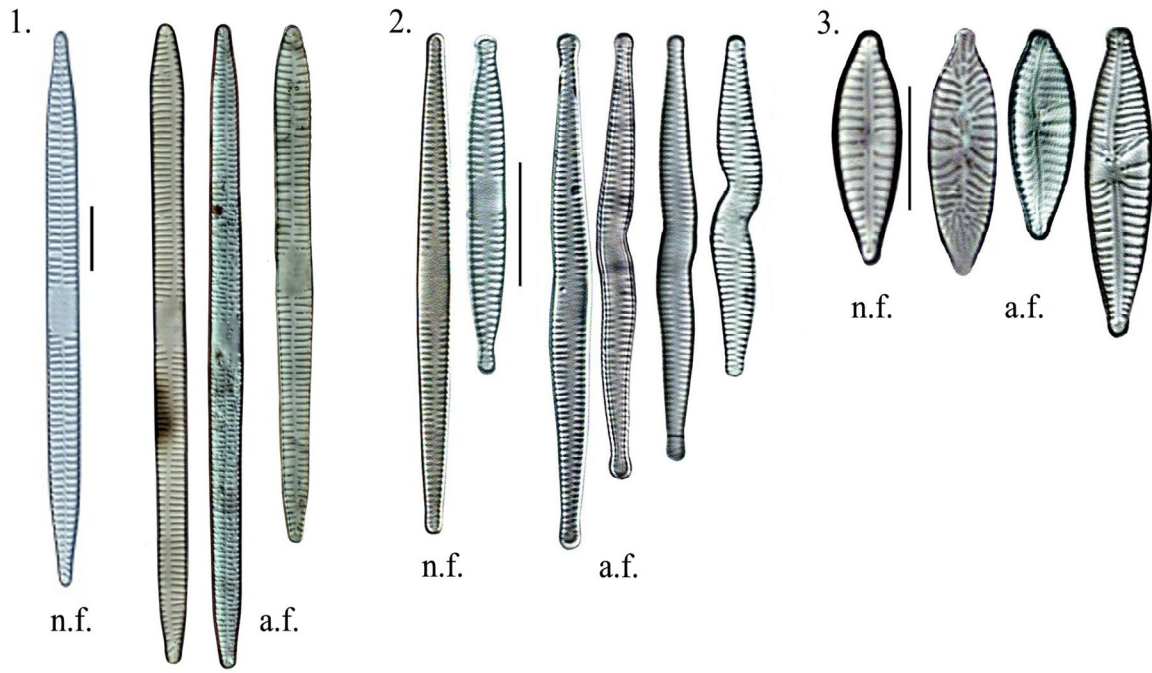




FIGURE 6



## 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
<b>July 2004</b>								
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 ( $\mu\text{S.cm}^{-1}$ )	1377	1223	1410	1427	2350	1426	1290	1257
O <sub>2</sub> (mg. L <sup>-1</sup> )	7.0	8.0	9.3	nm	7.2	7.3	6.4	nm
NH <sub>4</sub> (mg. L <sup>-1</sup> )	0.54	0.71	1.33	2.34	0.77	1.95	2.5	3.43
NO <sub>3</sub> (mg. L <sup>-1</sup> )	3	3.6	2.5	2.9	23.7	54.4	38.4	36
NO <sub>2</sub> (mg. L <sup>-1</sup> )	0.18	0.18	0.23	0.24	0.9	1	0.9	1.3
PO <sub>4</sub> (mg. L <sup>-1</sup> )	0.11	0.1	0.1	0.04	0.58	0.87	0.82	2.86
Si (mg. L <sup>-1</sup> )	10.5	13.5	12	15	12	11.5	12	12.5
Cd ( $\mu\text{g. L}^{-1}$ )	< 0.1	< 0.1	< 0.1	< 0.1	26	26	27	24
<b>March 2005</b>								
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 ( $\mu\text{S.cm}^{-1}$ )	678	538	639	578	735	1602	1723	1744
O <sub>2</sub> (mg. L <sup>-1</sup> )	11,1	9,4	9,1	10.5	10,3	9,3	9,9	10.1
NH <sub>4</sub> (mg. L <sup>-1</sup> )	0.88	0.39	0.61	0.62	0.92	3.44	3.54	2.98
NO <sub>3</sub> (mg. L <sup>-1</sup> )	5	3.9	2.8	2.7	33.8	11.6	26.6	37.3
NO <sub>2</sub> (mg. L <sup>-1</sup> )	0.07	0.04	0.08	0.1	0.7	0.5	1.1	1.1
PO <sub>4</sub> (mg. L <sup>-1</sup> )	0.24	0.08	0.2	0.1	0.2	1.5	1.4	1.3
Si (mg. L <sup>-1</sup> )	7	7.5	5.5	6.5	6.5	9	9.5	8
Cd ( $\mu\text{g. L}^{-1}$ )	< 0.1	< 0.1	< 0.1	< 0.1	17.1	19.8	21.6	11.2

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

3  
 4

	July 2004		March 2005	
	Firmi	Joaanis	Firmi	Joaanis
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<sup>-2</sup>).

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	Joaanis	Firmi	Joaanis
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