

Chl-*a* fluorescence parameters as biomarkers of metal toxicity in fluvial biofilms: an experimental study

Natàlia Corcoll · Berta Bonet · Manel Leira ·
Helena Guasch

Received: 18 October 2010 / Revised: 3 May 2011 / Accepted: 15 May 2011 / Published online: 27 May 2011
© Springer Science+Business Media B.V. 2011

Abstract A study was carried out to evaluate the sensitivity of different chlorophyll-*a* (chl-*a*) fluorescence parameters measured in freshwater biofilms as metal pollution biomarkers of short- and long-term metal exposures at environmentally realistic concentrations. A microcosm experiment was performed using indoor channels. Mature biofilms were exposed from hours to weeks to three different treatments: No-Metal, Zn (400 $\mu\text{g l}^{-1}$); and Zn plus Cd (400 $\mu\text{g l}^{-1}$ and 20 $\mu\text{g l}^{-1}$, respectively). Metal concentration was based on a real case study: the Riou-Mort River (France). Biofilms exposed to Zn bioaccumulated similar Zn contents per dry weight to those exposed to the mixture (Zn plus Cd) causing a similar inhibition of the effective quantum yield (Φ'_M) during the first hours of exposure. A reduction of the algal biomass, a shift in the community composition (a high reduction of diatoms), a reduction of the maximal quantum yield (Φ_M) and a strong reduction of non-photochemical quenching (NPQ) were observed from day 14

until the end of the experiment (35 days). The results indicate that the effects of the metal mixture present in the Riou-Mort on biofilms could be attributed to Zn toxicity. The use of a set of chl-*a* fluorescence measurements, including photochemical and NPQ parameters, are recommended as a reliable biomarker tool box to evaluate both short- and long-term effects of metals on biofilms containing oxygenic photoautotrophs, suggesting its use in field applications.

Keywords Fluvial biofilm · Metals · Pulse-amplitude-modulated (PAM) fluorometry · Bioaccumulation · Indoor channels

Introduction

Among the great varieties of pollutants interfering with the metabolism of photosynthetic organisms at different stages, trace metals are one of the most common non-biodegradable pollutants reported at elevated concentrations in many parts of the world (Mallick & Mohn, 2003). Metal pollution is of great concern in freshwater systems affected by agricultural, mining and industrial activities. Recent studies performed in the Riou-Mort (Bonnineau et al. 2011), a river draining an active mining area in France, have demonstrated that the mixture of metals found is toxic to biofilms causing structural alterations, such as growth inhibition, and changes in the architecture

Handling editor: Judit Padisak

N. Corcoll (✉) · B. Bonet · H. Guasch
Institute of Aquatic Ecology, University of Girona,
Campus de Montilivi, 17071 Girona, Spain
e-mail: natalia.corcoll@udg.edu

M. Leira
Faculty of Sciences, University of A Coruña, Campus da
Zapateira, 15071 A Coruña, Spain

and composition of the diatom community (Gold et al., 2003a; Duong et al., 2010). It is not clear, however, whether the effects reported were caused by Zn, Cd or the mixture of both.

Zn ions at low concentrations are indispensable for algal growth, but at high concentration they become toxic (Silver, 1998). Zinc toxicity on algae may affect the oxidizing side of PSII by noncompetitively inhibiting Ca^{2+} and Mn^{2+} binding at their native sites on the water oxidizing complex (Rashid et al., 1994). Based on algal monocultures, Zn toxicity is reported at very different concentrations depending on the species tested and the biomarker assessed: Chaloub et al. (2005) found Zn toxicity on cyanobacteria cultures between 30 and 332 mg l^{-1} , and Posthuma et al. (2001) describe an EC_{50} of 15 $\mu\text{g l}^{-1}$ in *Selenastrum capricornutum*. Cd is considered as an important contaminant of natural waters because of its high toxicity at low concentration (Deckert, 2005). Cd displaces metal cofactors (Zn^{2+} and Ca^{2+}) from undefined protein targets or directly binds amino acid residues, including cysteine, glutamate, aspartate and histidine (Faller et al., 2005). However, Cd toxicity to autotrophic organisms at environmentally realistic concentrations is less documented. Studies performed with *Selenastrum acutus* (Turpin) Kützing (Ilangovan et al., 1998) have shown Cd toxicity at high Cd concentration ($\text{EC}_{50} = 500 \mu\text{g Cd l}^{-1}$). Zhou et al. (2006) observed Cd toxicity on cyanobacteria cultures of *Microcystis aeruginosa* 448 $\mu\text{g Cd l}^{-1}$.

In our investigation, metal toxicity was assessed on biofilms (growing on artificial substrata), because of its capacity to detect early effects produced by toxic substances (Sabater et al., 2007) and to provide a community ecotoxicology approach with higher ecological relevance than using single species (Clements & Newman, 2002; Guasch et al., 2010a). Biofilms, also known as microphytobenthos or periphyton, are complex biological structures composed mainly by algae, cyanobacteria, bacteria, fungi and microfauna, located in close physical contact and embedded in a mucopolysaccharide matrix (Lock et al., 1984). In rivers and streams, the biofilm has an important role in the primary production (Guasch & Sabater, 1998) and in the organic matter processing (Romaní & Sabater, 2001).

Metal toxicity on biofilms differs from the results obtained with free-living organisms. Paulsson et al. (2000) reported an EC_{50} of 3,640 $\mu\text{g Zn l}^{-1}$ based on

the photosynthetic activity. The same authors, in another study (Paulsson et al., 2002) observed that Zn produced indirect effects on biofilms between 6 and 25 $\mu\text{g l}^{-1}$. Guasch et al. (2003), reported that Zn toxicity on biofilms was highly related to algal biomass and, for this reason, the range of EC_{10} values based on photosynthesis tests was very broad (455–65,000 $\mu\text{g Zn l}^{-1}$). In the case of Cd, previous studies (Gold et al., 2003b; Morin et al., 2008) showed slight effects at 10 $\mu\text{g l}^{-1}$, and a marked reduction of the biofilm biomass and diatom density, after chronic exposure to 100 $\mu\text{g l}^{-1}$ (Gold et al., 2003b; Morin et al., 2008).

Based on the results reported above concerning fluvial biofilms, it is expected that the type of pollution present in the Riou-Mort may cause marked effects because of chronic Zn pollution (around 400 $\mu\text{g Zn l}^{-1}$) and slight effects because of Cd (around 17 $\mu\text{g Cd l}^{-1}$). However, it is not clear whether the effects of the exposure to both metals will be similar, higher or lower than the exposure to Zn alone. While Morin et al. (2008) earlier investigated the response of biofilm to Cd exposure, showing slight effects. In the present investigation, we focus on the difference between the effects of Zn alone and Zn with Cd. This approach will allow us to investigate the effects of the type of metal pollution found in the field and to elucidate the specific influence of Zn on freshwater biofilms. Zn and Cd pollutions have been described in other metal-polluted streams at higher concentrations: in the Dommel stream in Belgium (Ivorra et al., 1999) reaching up to 282 $\mu\text{g Cd l}^{-1}$ and 3,147 $\mu\text{g Zn l}^{-1}$ or the Guadiamar stream, in Spain, (Santos et al., 2010) presenting up to 286 $\mu\text{g Cd l}^{-1}$ and 1,001 $\mu\text{g Zn l}^{-1}$. On the other hand, lower levels of Zn have been reported in other metal-polluted areas; i.e. around 600 $\mu\text{g Zn l}^{-1}$ in Osor River, Spain (ACA) or around 25 $\mu\text{g Zn l}^{-1}$ in Furtbach River, Switzerland and East Fork Polar Creek, USA (Le Faucheur et al., 2005; Hill et al., 2010, respectively).

Toxic concentrations of trace metals have been reported to act on the photosynthesis in higher plants (Sheoran & Sing, 1992), green algae (Samson et al., 1988), cyanobacteria and diatoms (Juneau et al., 2001). The pulse-amplitude-modulated (PAM) fluorimetry technique can be used to assess the direct and indirect effects of toxicants on photosynthetic processes (Ralph et al., 2007). In particular, the electron

transport rate (Ralph & Burchett, 1998) or the water-splitting apparatus (Moustakas et al., 1994) can be inhibited. Within biofilms, PAM fluorimetry has been used in several studies to evaluate metal toxicity based on the effective quantum yield (Φ'_M) and the maximal photosynthetic capacity (Φ_M) parameters (e.g. Guasch et al., 2002; Barranguet et al., 2003; Serra et al., 2009). However, the effects of metals in the use of the energy designated to radiative (qP) or non-radiative processes (qN and NPQ) is less documented in biofilm studies. These quenching parameters were incorporated in our study to widen the applications of the fluorescence parameters for the assessment of metal pollution effects on biofilm communities. Recent studies based on biofilms showed the potential use of the non-photochemical parameter (NPQ) as a pertinent fluorescence parameter to assess the effects of organic toxic substances (Laviale et al., 2010; Ricart et al., 2010a).

The duration of metal exposure is of great relevance for biofilms since metal bio-accumulation and toxicity increase with the time of exposure. Short-term metal toxicity on biofilms is usually found at concentrations higher than those described in free-living organisms (Guasch et al., 2010a, b). This marked difference is explained by the structure of biofilms, reducing metal penetration into the cells. On the other hand, longer metal exposures may allow the metals to reach the different types of organisms embedded in the biofilm (Guasch et al., 2003) increasing metal toxicity, after the toxic compounds have entered into the cell (Escher & Hermens, 2002). In field situations, point-sources of metal pollution might cause chronic and variable metal exposure depending on flow conditions (of lower concentration under high-flow conditions due to dilution) whereas diffuse sources such as the urban runoff would cause short-term metal exposures directly linked with rainfall episodes (Guasch et al., 2010a). Aquatic organisms will accumulate less metal during a short exposure causing a transient metabolic and physiological response. Internal concentration will increase after longer metal exposure causing increases in toxicity and/or the development of mechanisms to regulate the accumulation of metals (e.g. Hassler & Wilkinson, 2003 or Hassler et al., 2005).

Since the different species of algae and cyanobacteria found in a biofilm may differ in their metal sensitivity, it is also expected that long-term exposures to low metal concentrations may lead to changes in

their competitive interactions producing community changes as it was observed by Serra et al. (2009) in an experiment of chronic exposure of biofilms to Cu. In this study, cyanobacteria were the autotrophic group better adapted to Cu exposure. Thus, it is of great interest to investigate both short- and long-term effects of metal exposure providing the most appropriate endpoints for each temporal scale of exposure.

The main aim of this study was to evaluate the sensitivity of different chlorophyll-*a* (chl-*a*) fluorescence parameters as metal pollution biomarkers of short- and long-term metal exposures under realistic metal-exposure conditions. The Riou-Mort, characterized by elevated Cd and Zn concentrations (Morin et al., 2008) was chosen as model case study. With this aim, biofilm communities were exposed, under controlled conditions, to 400 $\mu\text{g Zn l}^{-1}$ and 20 $\mu\text{g Cd l}^{-1}$. Moreover, the study also aimed to investigate the contribution of Zn, the metal found at higher concentration, on the effects produced by Zn plus Cd. With this aim, the effects of Zn alone were also investigated.

Our hypotheses are that (1) The set of parameters studied will allow us to show the effects of metal pollution (Zn + Cd) at realistic concentrations; and (2) Short-term exposure will mainly cause functional alterations, whereas structural changes will appear after long-term exposure.

Functional alterations will cause changes in qP and Φ'_M , closely related to the electron transport chain, and NPQ and qN activated to avoid stress. Structural damages of the photosynthetic apparatus such as the ultra-structure of the thylakoid membrane and the pigments composition, will lead, respectively, to a reduction of the Φ_M and the NPQ. Shifts in algal composition will also occur because of selection pressure according to differences in the species metal tolerances or sensitivities.

Materials and methods

Experimental design

The experiment was carried out indoors in recirculating channels. The microcosm system was made of nine independent Perspex channels (each being 170 cm long, 9 cm wide and 9 cm high) as described in Serra et al. (2009), except for the light intensity

that was between 100 and 120 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. In each channel, two sizes of glass substrates (8.5×12 and 8.5×2 cm) were placed horizontally to facilitate biofilm growth. Water was recirculated by means of water pumps and renewed every 2–3 days to minimize water chemistry changes.

The experiment lasted for 10 weeks: the first 5 weeks were dedicated for colonization, and the next 5 weeks for metal exposure. Sampling strategy was planned to monitor the colonization (referred to as colonization samples) and to compare mature biofilms before metal exposure (referred to as Pre-exposure) and during metal exposure (referred to as short-term and long-term exposures). Samples corresponding to the short-term exposure phase were taken after 6, 24 and 72 h of exposure whereas those corresponding to the long-term exposure were taken at weeks 1, 2, 3, 4 and 5.

Pre-exposure samples were analysed at three different moments at the end of the colonization period: 5 and 3 days before exposure and the same day that metal additions started. These three samples are labelled as -5 , -3 and 0 days, respectively; and the moment when metal addition started is considered time zero (T0). From this moment on, the three channels with no metal addition will be referred to as 'No-Metal treatment'; the three with Zn addition as 'Zn treatment' and the three channels with the addition of Zn and Cd as 'Zn + Cd treatment'.

Colonization

In the channels system described above, inocula obtained from natural biofilms were used to colonize the surface of etched glass substrates during 5 weeks, as described in Serra et al. (2009). Natural biofilms were sampled from the Llémena River, a small calcareous tributary of the Ter River (North-east Spain). This river is relatively unpolluted by metals (ACA) making it possible to avoid communities already pre-selected for zinc or cadmium tolerance. No metals were added during the whole colonization period (5 weeks).

Metal exposure

After the 5 weeks of biofilm colonization, the additions of the Zn and Zn + Cd started (T0). From

T0 until week 5, three channels were exposed to a nominal concentration $400 \mu\text{g Zn l}^{-1}$ (Zn treatment), another three to nominal concentrations of $400 \mu\text{g Zn l}^{-1}$ plus $20 \mu\text{g Cd l}^{-1}$ (Zn + Cd treatment) and the remaining three were not exposed to metals (No-Metal treatment). Zn and Cd metal solutions were prepared from ZnCl_2 and CdCl_2 from Tritisol (Merck, Darmstadt, Germany) with deionised water (18Ω Q-H₂O grade Barnstead Nanopure). Equilibration of the metal with the water was done in carboys during a period of 24 h at room temperature, before water renewals.

Water sampling

During the colonization period, water samples were taken to characterize the content in dissolved organic carbon (DOC; $n = 3$), alkalinity ($n = 15$), anions ($n = 39$) and cations ($n = 20$).

Physical and chemical parameters (temperature, pH, dissolved oxygen and conductivity) were measured before and after each water renewal (three times per week in three different channels each time) during the colonization period ($n = 114$) and during metal exposure ($n = 57$). Water samples (10 ml) were taken in triplicate to determine phosphate concentration during the colonization ($n = 54$) and during the metal exposure ($n = 27$). Triplicate water samples (5 ml) were also taken before and after each water renewal for Zn and Cd analysis during the colonization ($n = 18$) and during the metal exposure ($n = 27$). All water samples were immediately filtered using $0.2 \mu\text{M}$ nylon membrane filters (Whatman), and samples for metal analysis were also acidified with 1% HNO_3 (65% HNO_3 , Suprapur, Merck, Germany). Samples were stored at 4°C .

Biofilm sampling

In order to monitor the changes in algal biomass during the colonization period and to know the growth phase of biofilm communities at the moment when metal additions began, biofilms were sampled twice a week (total $n = 49$). During every sampling time, three colonized glass substrata (17 cm^2) were randomly removed from each channel using a random number generator from excel application. These samples were used for Fo (fluorescence yield) measurements using a Phyto-PAM fluorometer

(version EDF, Walz, Effeltrich, Germany); see PAM measurements paragraph. Since Phyto-PAM measurement is a non-destructive technique, glass substrata were returned back to the channels after these fluorescence measurements.

During Pre-exposure (−5, −3 and 0 days; $n = 27$ for each treatment), Short-term exposure (6, 24 and 72 h; $n = 9$ for each treatment) and Chronic exposure (wk1, wk2, wk3, wk4 and wk5; $n = 15$ for each treatment), three glass substrates (17 cm²) were also removed at random from each treatment and fluorescence parameters measured. Specifically, following measurements were done (1) the fluorescence of the different algal classes composing the biofilm communities, (2) the F_o as indirect measure of algal biomass and (3) Φ_M , Φ_M' , qP, qN and NPQ (see PAM measurements).

A total number of 81 biofilm samples were taken for chl-*a* concentration and used to further calculate the carotenoids ratio (430:665): during the Pre-exposure (at −5, −3 and 0 days—three samples per treatment), during Short-term exposure (at hours 6, 24 and 72 h—the same number of samples per treatment) and during Long-term exposure (at weeks W1, W3, and W5—the same number of samples). Samples for chl-*a* analysis were obtained after scraping biofilms from a small substratum (17 cm²) and were kept in polypropylene vials at −4°C until further analysis (see biofilm analysis).

A total of 72 samples were taken for bioaccumulation analyses. Each sample corresponded to 51 cm² of colonized substrata (half of a big substratum). The biofilm was scraped and stored at −80°C until their laboratory analysis (see biofilm analysis). Biofilms were taken in triplicate two times (at −5 and 0 days) per treatment during the Pre-exposure; three times (at 6, 24 and 72 h) per treatment during the Short-term exposure; and three more times (at wk1, wk3 and wk5) per treatment during Long-term exposure. Substrata were always randomly chosen.

Water analysis

Physical and chemical parameters (water temperature, dissolved oxygen and pH) were measured using a multi-parametric probe (WWT). Total dissolved Zn and Cd concentrations in water were measured by ICP-MS (7500c Agilent Technologies, Inc. Wilmington, DE). The detection limit for Zn was 2.61 $\mu\text{g l}^{-1}$

and for Cd was 2.05 $\mu\text{g l}^{-1}$. Ga and Rh were used as internal standards. The accuracy of the analytical method was checked periodically using certified water reference (SPS-SW2 Batch 113, Oslo, Norway). The average of the Zn certified water reference was 100.2 $\mu\text{g l}^{-1}$, and for Cd it was 3.79 $\mu\text{g l}^{-1}$.

The phosphate concentration was analysed using the Murphy and Riley (1962) molybdenum blue colorimetric method. Anions (Cl^- , NO_3^- and SO_4^{2-}) and cations (K^+ , Na^+ , NH_4^+ , Ca^{2+} and Mg^+) were analysed by ion chromatography (761 Compact IC, METROHM, Herisau, Switzerland). The DOC was measured on a Shimadzu TOC 5000. The total alkalinity was analysed following the APHA method (APHA, 1989).

Biofilm analysis

Chl-a concentration

Chl-*a* and the carotenoids ratio (430:665) were obtained from biofilm samples after extraction with 90% acetone, and measured spectrophotometrically (Jeffrey & Humphrey, 1975). Chl-*a* concentration was used as a measure of algal biomass, and the carotenoids ratio was calculated as the quotient between optical densities at 430 and 665 nm (Margalef, 1983), and it was used as an indicator of the proportion of protection pigments such as carotenoids per unit of active chlorophyll.

Metal bioaccumulation

In order to measure the total amount of Zn and Cd accumulated in the biofilm, dried biofilm samples were lyophilized and weighed (g) to determine the dry weight (DW) (Meylan et al., 2003). Then, c. 200 mg of DW were digested with 4 ml of concentrated HNO_3 (65% HNO_3 , Suprapur, Merck, Germany) and 1 ml of H_2O_2 (31% H_2O_2 , Suprapur, Merck, Germany). After dilution with MiliQ water (75 times), water samples were acidified (1%) with HNO_3 (65% HNO_3 , Suprapur, Merck, Germany) and stored at −4°C. Digested samples were analysed following the procedure described for total Zn and Cd dissolved in water (see paragraph water analysis). Metal bio-accumulation was expressed as dissolved metal contents (μg) per dry weight (g DW^{-1}). The detection limit was 2.6 $\mu\text{g Zn g DW}^{-1}$ for Zn and

1.8 $\mu\text{g Cd g DW}^{-1}$ for Cd. The accuracy of the analytical methods was checked periodically using certified reference materials (Trace Elements in Plankton (CRM 414), Community Bureau of Reference (BCR), Brussels, Belgium).

PAM measurements

Measurements of *in vivo* chl-*a* fluorescence of biofilms were performed using the Phyto-PAM fluorometer version EDF (Walz, Effeltrich, Germany), based on the method described by Schreiber (1998).

In order to obtain a good representation of the algal and cyanobacteria heterogeneity within each colonized substratum, four different spots (1 cm of diameter) were measured in each slide. Accessory equipment was designed to adapt Phyto-PAM fluorescence measurements to colonized glass substrata. This equipment consisted of (1) a set of transparent Perspex boxes ($9 \times 9 \times 6 \text{ cm}^3$) used to incubate biofilm samples and measure fluorescence parameters, avoiding the manipulation of the samples; and (2) a micrometric device used for fixing the sensor of the Phyto-PAM under the transparent boxes which contained the biofilm samples. During PAM measurements biofilm samples were submerged to avoid desiccation. The Phyto-PAM sensor was placed below the box at 6 mm of total distance from the biofilm samples. The measuring distance was kept constant for all PAM measurements. Measurements were performed in dark conditions at room temperature (20°C).

Fo measurements

Biofilm samples were incubated for 15 min in dark conditions to ensure that all reaction centres were open, and then a weak measuring light was applied to record the minimum Fo. Phyto-PAM employs an

array of light-emitting diodes (LEDs) to excite chlorophyll fluorescence at different measuring lights (470, 520, 645 and 665 nm), and to illuminate samples with actinic light (AL) and saturation pulses (SPs). The Fo of dark-adapted cells at 665 nm, is proportional to chl-*a* concentration (Rysgaard et al., 2001); thus, Fo was used for following the growth of the biofilm during the colonization. In our case, chl-*a* concentration was low enough ($<7.5 \mu\text{g cm}^{-2}$) to follow a linear relationship with Fo (Schmitt-Jansen & Altenburger, 2008). In this study, the de-convolution of the overall fluorescence signal (Fo) into the contributions made by algal groups (cyanobacteria-Fo(BI), diatoms-Fo(Br) and green algae-Fo(Gr)) from biofilm samples was based on internal ‘reference excitation spectra’ of pure cultures which have previously been validated on fluvial biofilms by Schmitt-Jansen & Altenburger (2008) and used also in several biofilm studies (e.g. Serra et al., 2009 or Rotter et al., 2011). These data were used for evaluating the relative contribution (in percentage) of each class to the whole community and follow their temporal changes.

Quantum yields and quenching parameters

Dark-adapted samples used for the measurement of Fo, were thereafter exposed to a short SP of light of $8,000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ to induce the Fm. Biofilm samples were then illuminated ($120 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$) by AL during 10 min to induce the typical Kautsky effect (Kautsky & Hirsch, 1931), and the change of the Fm’ in light conditions was induced by five SPs at the end of this AL period. Afterwards, the AL was turned off, and then the reoxidation of the photosynthetic pigments the Fo’ was recorded. Using both dark and light fluorescence measurements, several fluorescence parameters could be calculated (Table 1).

Table 1 Chl-*a* fluorescence parameters obtained by Phyto-PAM measurements

Parameter	Equation	Reference
Φ_M	$\Phi_M = (F_m - F_o)/F_m$	Genty et al. (1989)
Φ'_M	$\Phi'_M = (F'_m - F)/F'_m$	Genty et al. (1989)
qP	$qP = (F'_m - F)/(F'_m - F_o')$	Schreiber et al. (1986)
qN	$qN = 1 - [(F'_m - F_o')/(F_m - F_o)]$	Schreiber et al. (1986)
NPQ	$NPQ = (F_m - F'_m)/F'_m$	Bilger & Björkman (1990)

The effective quantum yield (Φ'_M) represents the photosynthetic efficiency at the steady state of electron transport. The photochemical quenching (qP) is used to monitor the energy used by the electron transport chain which leads to carbon fixation. The maximal quantum yield (Φ_M) is a potential estimate that provides information about the maximum electron flow. Both non-photochemical quenching parameters, qN and NPQ, represent the energy dissipated in non-radiative energy processes (non photochemical ways) as heat to protect the photosynthetic apparatus. Ralph et al. (2007) recommended a method for calculating the non-photochemical quenching process using the NPQ equation instead of qN, because NPQ does not require the determination of the F_o' , a parameter difficult to obtain. In our study both parameters were measured for comparison.

Data analysis

Changes in algal biomass (F_o) throughout the 10 weeks of the experiment (from the beginning of colonization until the end of the metal-exposure period) were adjusted to a logistic model to compare the growth curves between channels throughout the different stages of growth (including the periods of colonization and metal exposure). Data from the three channels assigned to each different treatment were used to fit the corresponding growth curve. Data were fitted to a logistic model using the SIGMAPLOT software.

$$y = \frac{k}{1 + \exp\left[-\frac{x-x_0}{b}\right]} \quad (1)$$

The $1/b$ parameter represents the growth rate (r), the x_0 parameter represents the time when maximal community growth rate is achieved, and the k parameter represents the carrying capacity. In our

experiment, the r parameter was used to compare the growth rates during the colonization period, the x_0 parameter to evaluate when biofilms reached the maximal growth rate and the k parameter to evaluate the carrying capacity (Romaní, 2010).

An ANOVA of repeated measures was applied to examine the temporal tendency of each biological parameter (chl-*a*, 430:665, $\mu\text{g Zn g DW}^{-1}$, $\mu\text{g Cd g DW}^{-1}$, $F_o(\text{BI})$, $F_o(\text{Gr})$, $F_o(\text{Br})$, Φ_M , Φ'_M , qP, qN and NPQ) during the Pre-exposure, the Short- and Long-term exposure periods. The post-hoc Tukey's HSD test was applied to check if tendencies were different between treatments. ANOVA analyses were realized by SPSS software.

Principal Component Analysis (PCA) was employed to explore the ordination of samples according to the different biofilm variables measured. The data set included photosynthetic parameters, structural parameters (pigment analysis, F_o and the fluorescence of each algal class) and bio-accumulation values. A preliminary PCA was done using all the sampling times to check the supposed difference in responses during short- and long-term exposures.

The PCA analyses were done after elimination of auto-correlated variables ($R^2 > 0.8$), standardization and normalization of remaining variables (chl-*a*, 430:665, $\mu\text{g Zn g DW}^{-1}$, $\mu\text{g Cd g DW}^{-1}$, $F_o(\text{BI})$, $F_o(\text{Gr})$, $F_o(\text{Br})$, Φ_M , Φ'_M , qP, qN and NPQ) (PRIMER, Clarke & Warwick, 2001).

Results

Physical and chemical characteristics of the water from the artificial channels system

The major anions and cations, as well as alkalinity and DOC concentrations, are summarized in Table 2. Physical and chemical conditions remained stable

Table 2 Chemical characteristics of water channels

	SO_4^{2-} (mg l^{-1})	Cl^- (mg l^{-1})	K^+ (mg l^{-1})	Na^+ (mg l^{-1})	Ca^{2+} (mg l^{-1})	Mg^{2+} (mg l^{-1})	Alkalinity (mEq l^{-1})	NO_3^- (mg l^{-1})	NH_4^+ (mg l^{-1})	DOC (mg l^{-1})
AVG	73.33	86.13	3.34	24.88	41.4	8.06	1.47	4.353	0.05	4.92
SE	± 8.75	± 3.98	± 0.11	± 0.94	± 1.75	± 0.30	± 0.03	± 0.204	± 0.03	± 0.95
<i>n</i>	39	39	20	20	20	20	14	20	20	3

Average (AVG) and standard error (SE) of water samples (*n*) taken throughout the duration of the experiment

Table 3 Summary of dissolved oxygen (Oxy), water temperature (Temp), conductivity (Cond), phosphate (PO_4^{3-}), zinc (Zn) and cadmium (Cd) concentrations during the colonization and metal-exposure periods for each treatment (No-Metal, Zn and Zn + Cd)

	Colonization			Metal-exposure								
	AVG	SE	<i>n</i>	No-Metal treatment			Zn treatment			Zn + Cd treatment		
				AVG	SE	<i>n</i>	AVG	SE	<i>n</i>	AVG	SE	<i>n</i>
Oxy. (mg l^{-1})	8.82	0.04	114	9.44	0.05	57	9.08	0.03	57	9.15	0.04	57
Temp. ($^{\circ}\text{C}$)	19.29	0.14	114	18.55	0.14	57	18.40	0.15	57	18.41	0.02	57
pH	8.36	0.02	114	8.61	0.03	57	8.52	0.02	57	8.53	0.02	57
Cond. ($\mu\text{S cm}^{-1}$)	326.72	1.40	114	310.72	3.62	57	341.72	2.58	57	341.51	2.66	57
PO_3^{-4} ($\mu\text{g l}^{-1}$)	29.18	2.59	54	23.12	2.08	27	28.81	3.66	27	29.20	3.26	27
Zn ($\mu\text{g l}^{-1}$)	8.64	0.76	18	4.88	0.75	27	317.53	24.94	27	321.44	22.08	27
Cd ($\mu\text{g l}^{-1}$)	b.d.l.		18	b.d.l.		27	b.d.l.		27	14.30	1.09	27

Each value corresponds to the average (AVG) of before and after water renewals and \pm standard error (SE)

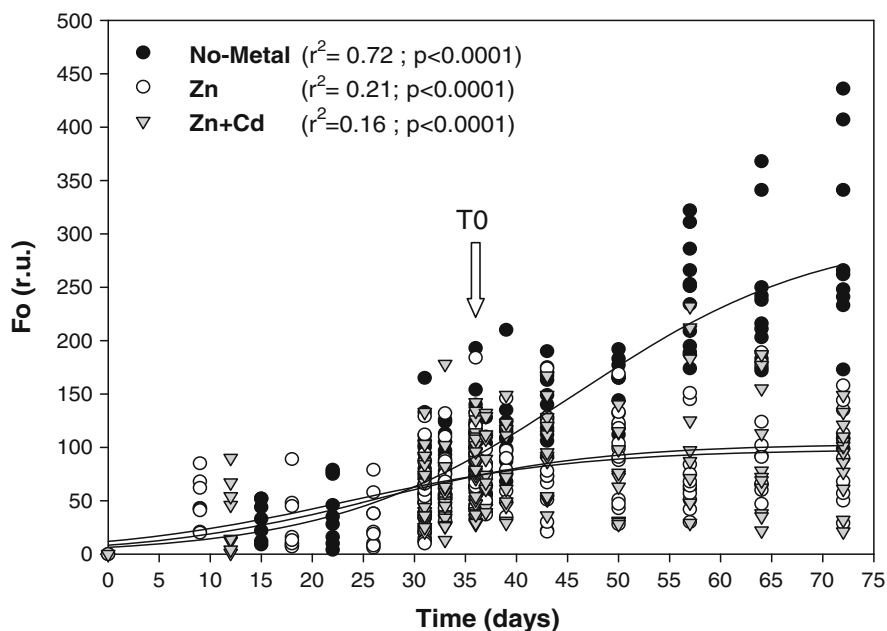
b.d.l. below detection limit

during the colonization and metal-exposure periods and were similar between treatments (Table 3). Metal concentration in the No-Metal treatment was always $<10 \mu\text{g l}^{-1}$ (Table 3). Zn concentration was similar in the Zn and Zn + Cd treatments. Zn concentrations were 21 and 20% below nominal concentration ($400 \mu\text{g Zn l}^{-1}$), respectively, in the Zn treatment and in the Zn + Cd treatment; Cd concentration in the Zn + Cd treatment was 28% below nominal concentration ($20 \mu\text{g Cd l}^{-1}$).

Biofilm growth curves

The growth curves of the biofilm based on Fo showed significant fits to the logistic model (Fig. 1). All growth curves had a similar growth rate ($r = 0.083 \text{ day}^{-1}$, $P < 0.0001$ for No-metal; $r = 0.085 \text{ day}^{-1}$, $P < 0.001$ for Zn and $r = 0.092 \text{ day}^{-1}$, $P < 0.05$ for Zn + Cd) indicating that biofilms presented comparable growth rates during the colonization (until day 36).

Fig. 1 Growth curves of biofilms from the nine channels where the No-Metal, Zn and Zn + Cd treatments were applied. Fo measurements were recorded throughout the duration of 10 weeks of the experiment. Lines represent curves fitted to the sigmoidal model. T0 corresponds to the time when metal exposure began (day 36)



However, the time to archive the maximal community growth rate (x_0) differed between treatments. In the No-Metal treatment, it was 46 days ($P < 0.0001$), slower than in the Zn treatment ($x_0 = 23$ days; $P < 0.0001$) or in the Zn + Cd treatment ($x_0 = 26$ days, $P < 0.0001$). The carrying capacity (k) of biofilm from No-Metal treatment was 300 units of Fo, 98 for the Zn treatment, and 103 for the Zn + Cd treatment (67 and 65% lower than k in the No-Metal treatment, respectively).

Biofilm characterization

Chl-a concentration

During Pre-exposure and the first days of exposure (Short-term exposure period), chl-*a* concentration was low and remained stable in all treatments (Table 4). The carotenoids ratio (430:665) was also similar between treatments (Table 4). However, after longer exposure (Long-term exposure period), chl-*a* concentration and the carotenoids ratio showed significant differences between treatments (ANOVA of repeated measures; $F = 52.53$, $P < 0.001$ and $F = 11.96$, $P < 0.001$, respectively). Chl-*a* increased progressively over time in the No-Metal treatment, reaching values three times higher than those measured in the Zn and Zn + Cd treatments (Table 4). The carotenoids ratio showed a trend to decrease in the No-Metal treatment, while it remained stable in the Zn + Cd treatment and increased in the Zn treatment as well, showing significant differences between the No-Metal and the Zn treatments (Table 4).

Bioaccumulation

During Pre-exposure, biofilm metal contents remained low and stable (Table 4).

Metal bioaccumulation increased over time in the biofilms exposed to metals. A positive trend was observed for Zn during both Short- and Long-term exposure periods in both metal treatments (ANOVA of repeated measures; $F = 101.14$, $P < 0.05$ and $F = 45$, $P < 0.05$, respectively) reaching in both cases values between 1 and 2 orders of magnitude higher than controls (Table 4). Cd bioaccumulation was also observed in the Zn + Cd treatment, both during Short- and Long-term exposures (ANOVA of

repeated measures; $F = 57.55$, $P < 0.0001$ and $F = 36.88$, $P < 0.0001$, respectively), reaching up to $74 \mu\text{g Cd g DW}^{-1}$ (Table 4).

The algal classes

Cyanobacteria, diatoms and green algae were detected with the Phyto-PAM. The percentage of each group was stable during Pre-exposure (data not shown) and Short-term exposure in all treatments (Fig. 2). However, during Long-term exposure, a marked reduction in the percentage of diatoms was observed in both metal treatments, differing from the No-Metal treatment (ANOVA of repeated measures; $F = 9.715$, $P < 0.05$) (Fig. 2). At week 5, the reduction in Fo(Br) reached 88% of the No-Metal values in the Zn treatment, and a 99% in the Zn + Cd treatment (Fig. 2).

Photosynthetic parameters

All photosynthetic parameters remained stable and were similar in the different channels before metal exposure (Pre-exposure). The qP and qN were stable over time showing similar values in all treatments. Φ'_M ranged between 0.1 ± 0.01 and 0.2 ± 0.02 (AVG \pm SE; $n = 3$), Φ_M between 0.41 ± 0.02 and 0.54 ± 0.05 and NPQ between 0.53 ± 0.03 and 1.02 ± 0.13 in the No-Metal treatment, and were differently affected by metal exposure.

Short-term exposure The Φ'_M parameter was inhibited in both metal treatments showing significant differences with the No-Metal treatment and also between them (ANOVA of repeated measures: $F = 37.157$, $P < 0.001$). It was 23% below controls in the Zn treatment and 8% below controls in the Zn + Cd treatment (Fig. 3).

Metal-exposure effects on NPQ were less consistent, showing a significant difference between the No-Metal and the Zn + Cd treatments after 6 h of exposure (ANOVA one way: $F = 13.220$, $P < 0.05$) as it is shown in (Fig. 3) in percentage of controls.

Long-term exposure Φ_M decreased in both metal treatments (ANOVA of repeated measures; $F = 22.35$, $P < 0.01$) reaching values up to 38% below controls (Fig. 3). Φ'_M slightly decreased over time in biofilms from No-Metal treatment and was not

Table 4 Biofilm values throughout the three periods: Pre exposure, Short- and Long-term metal exposures in the three treatments: No-Metal, Zn and Zn + Cd

Treatments	Pre-exposure			Short-term exposure			Long-term exposure			Tukey test		
	-5 days	-3 days	0 day	Tukey test	6 h	24 h	72 h	Tukey test	W1		W3	W5
Chl-α ($\mu\text{g cm}^{-2}$)												
No-Metal	1.16 \pm 0.37	1.02 \pm 0.05	0.88 \pm 0.13	n.s.	0.95 \pm 0.14	1.00 \pm 0.19	1.22 \pm 0.09	n.s.	1.50 \pm 0.20	2.51 \pm 0.49	3.59 \pm 1.19	a
Zn	0.98 \pm 0.23	0.91 \pm 0.14	1.35 \pm 0.42		1.41 \pm 0.38	1.11 \pm 0.22	0.82 \pm 0.14		0.77 \pm 0.06	0.70 \pm 0.06	0.87 \pm 0.37	b
Zn + Cd	1.49 \pm 0.33	0.82 \pm 0.12	0.68 \pm 0.01		0.81 \pm 0.10	0.89 \pm 0.20	1.05 \pm 0.22		0.73 \pm 0.15	1.09 \pm 0.38	0.99 \pm 0.36	b
430:665												
No-Metal	3.04 \pm 0.19	3.4 \pm 0.09	2.90 \pm 0.10	n.s.	2.95 \pm 0.14	2.85 \pm 0.03	2.79 \pm 0.03	n.s.	2.84 \pm 0.09	2.57 \pm 0.13	2.48 \pm 0.02	a
Zn	2.96 \pm 0.25	3.13 \pm 0.05	3.19 \pm 0.13		2.99 \pm 0.21	2.94 \pm 0.07	2.98 \pm 0.10		3.06 \pm 0.19	3.39 \pm 0.39	3.48 \pm 0.15	b
Zn + Cd	2.82 \pm 0.00	2.99 \pm 0.15	3.08 \pm 0.33		2.99 \pm 0.11	2.92 \pm 0.32	3.00 \pm 0.15		3.08 \pm 0.21	2.90 \pm 0.22	3.02 \pm 0.20	ab
$\mu\text{g Zn g DW}^{-1}$												
No-Metal	25.74 \pm 4.91	n.d.	17.87 \pm 4.19	n.s.	33.87 \pm 7.56	26.55 \pm 2.83	14.86 \pm 3.44	a	18.16 \pm 2.10	17.09 \pm 0.52	8.78 \pm 2.97	a
Zn	23.3 \pm 3.06	n.d.	17.34 \pm 1.26		146.6 \pm 21.22	239.87 \pm 9.14	220.97 \pm 9.53	b	574.38 \pm 49.34	735.01 \pm 32.60	1571.24 \pm 186.53	b
Zn + Cd	31.63 \pm 13.16	n.d.	20.91 \pm 10.94		205.84 \pm 42.10	183.44 \pm 7.19	224.31 \pm 8.64	b	371.38 \pm 16.59	1051.16 \pm 237.77	1570.91 \pm 136.04	b
$\mu\text{g Cd g DW}^{-1}$												
No-Metal	b.d.l.	n.d.	b.d.l.	n.s.	b.d.l.	b.d.l.	b.d.l.	a	b.d.l.	b.d.l.	b.d.l.	a
Zn	1.96 \pm 0.62	n.d.	b.d.l.		b.d.l.	b.d.l.	b.d.l.	a	2.23 \pm 0.23	b.d.l.	b.d.l.	a
Zn + Cd	2.34 \pm 1.01	n.d.	b.d.l.		6.3 \pm 3.26	9.84 \pm 1.22	10.62 \pm 0.41	b	25.47 \pm 1.44	57.73 \pm 9.31	74.35 \pm 16.87	b

Chl- α concentration ($\mu\text{g chl-}\alpha\text{ cm}^{-2}$), the carotenoids ratio (430:665), the Zn content per dry weight ($\mu\text{g Zn g DW}^{-1}$), the Cd content per dry weight ($\mu\text{g Cd g DW}^{-1}$). Each value corresponds to the AVG \pm SE; $n = 3$. For each parameter, different letters indicate significant differences ($P < 0.01$) between treatments during the exposure period analysed (Tukey's HSD test from ANOVA of repeated measures)

b.d.l. below detection limit, n.d. no data, n.s. non significant differences between treatments

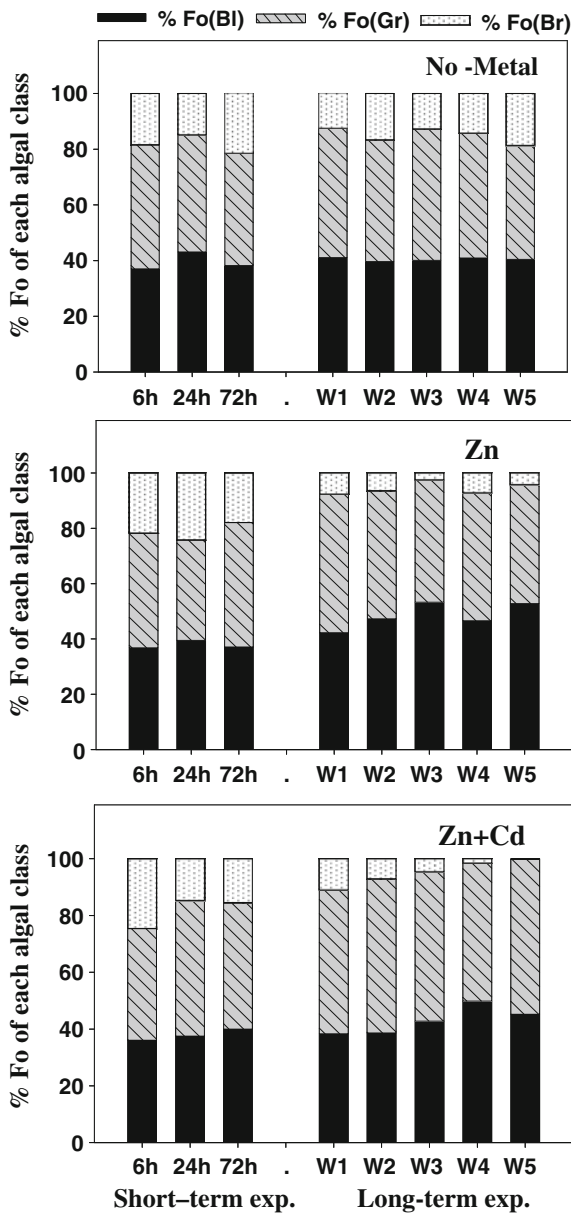


Fig. 2 Percentage of fluorescence of each class: cyanobacteria (Fo(BI)), green algae (Fo(Gr)) and diatoms (Fo(Br)) from biofilms of No-Metal, Zn and Zn + Cd treatments in each sampling time during Short- and Long-term metal exposures (AVG; $n = 3$)

affected by metal exposure (Φ'_M from metal treatments was not significantly different from the No-Metal treatment). Effects of metal exposure on NPQ were significant (ANOVA of repeated measures; $F = 26.57$, $P < 0.01$), reaching, in both metal treatments, values up to 74% below controls (Fig. 3).

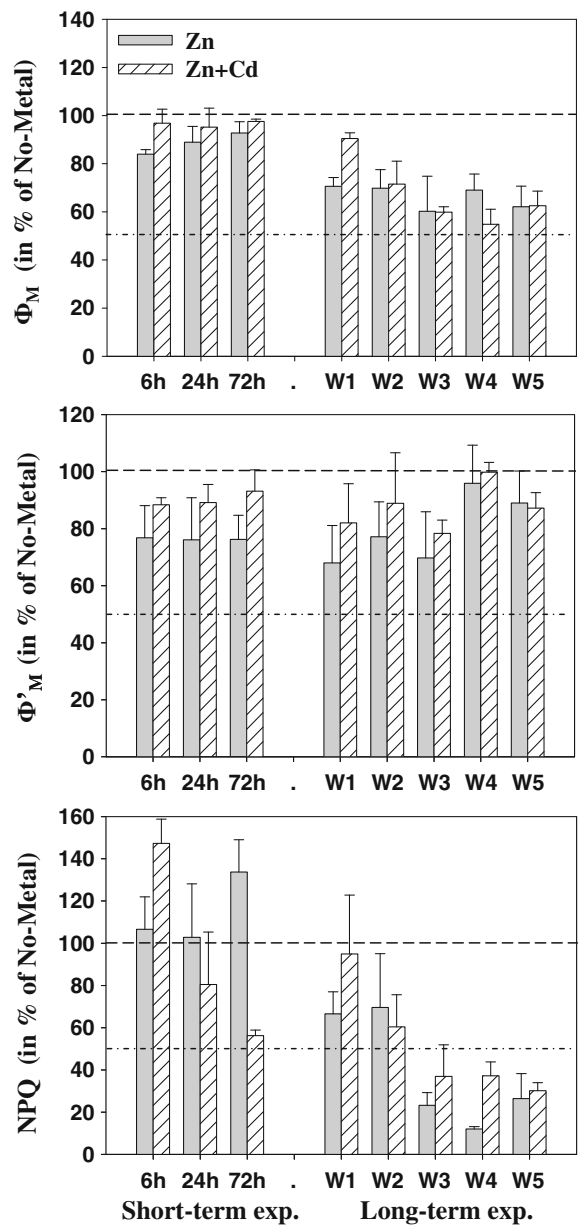


Fig. 3 Maximal quantum yield (Φ_M), effective quantum yield (Φ'_M) and non-photochemical quenching (NPQ) of biofilms from the Zn treatment (grey bars) and the Zn + Cd treatment (dashed bars), in percentage of the No-Metal treatment, during the Short- and the Long-term metal-exposure periods (AVG + SE; $n = 3$)

Multivariate analysis

The PCA carried out with all sampling times and variables separated data from T0 up to 72 h (Short-term exposure) from data obtained after W1

(Long-term exposure), and therefore, these two periods were analysed separately supporting the initial separation between Short- and Long-term exposure.

The PCA first axis of the Pre-exposure and Short-term exposure periods explained 22.9% of the variance while the second axis explained 16.5% (Fig. 4A). Biofilm samples were separated along the first axis based on their metal bioaccumulation: Zn and Cd bioaccumulation negatively and NPQ, Φ_M and Φ'_M positively (Table 5; Fig. 4A). The second axis represents a gradient based positively with the Φ'_M and negatively with non-photochemical quenching parameters (qN and NPQ) and the cyanobacteria and green-algae groups (Table 5; Fig. 4A).

The first axis of the PCA carried out with data from the Pre-exposure and Long-term exposure periods explained a 41.7% of the variance (Fig. 4B). This first axis was related with metal toxicity. Samples with high Zn and Cd bioaccumulation values on the left (see Table 5; Fig. 4B) and those with low NPQ, Φ_M and Fo(Br) values on the right (see Table 5; Fig. 4B). The second axis explained a 23.2% of the variance and was related with the maturity of the community, thus positively with the chl-*a* content, and the relative abundance of green algae (Fo(Gr)) and cyanobacteria (Fo(BI)), and

negatively with the degradation state of the pigments (430:665) and the effective quantum yield (Φ'_M).

Discussion

In agreement with our hypothesis, metal pollution (Zn and Zn + Cd) at realistic concentrations and exposure time (hours to weeks) lead to a variety of biofilm community alterations including changes in the kinetics of algal growth, metal bioaccumulation, chl-*a* concentration, community composition, and different PSII-related fluorescence parameters (Φ_M , Φ'_M and NPQ) that are discussed below.

The kinetics of growth summarizes the integrated response of biofilms throughout the entire metal-exposure period. The effect of metals on the growth curves was very marked, and no differences were observed between metal treatments (Zn or Zn + Cd). In contrast to Non-exposed biofilms that reached the highest biomass at the end of the experiment, algal growth in metal-exposed biofilms was clearly reaching plateau values up to three times lower than non-exposed biofilms (considered as controls). In comparison with controls, plateau values were around 66–67% lower in both metal treatments indicating a lower carrying capacity,

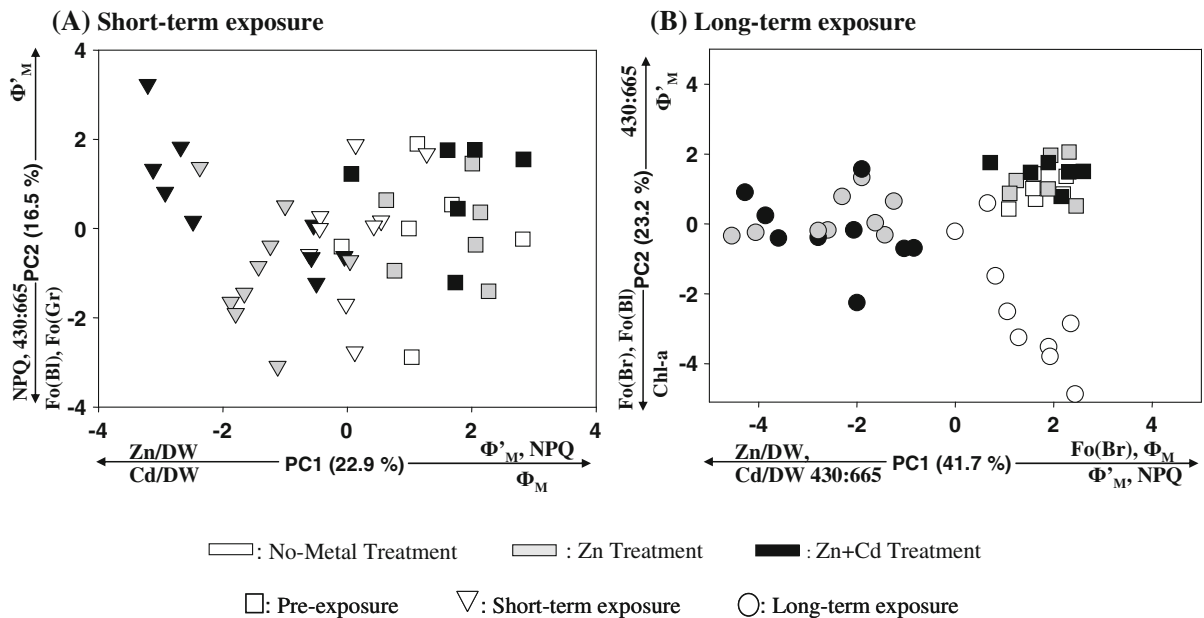


Fig. 4 PCA of biological variables from biofilms in the No-Metal, Zn and Zn + Cd treatments during the Pre-exposure and Short-term exposure (A) and during the Pre-exposure and Long-term exposure (B)

Table 5 Axis loadings of biological variables for PCA of the biological correlation matrix (Short- and Long-term exposures)

	PCA loadings			
	PCA: Short-term exposure		PCA: Long-term exposure	
	First axis	Second axis	First axis	Second axis
Eigenvalues	2.74	1.99	5	2.78
% Variation	22.9	16.5	41.7	23.2
Biological variable	Eigenvectors PC1	Eigenvectors PC2	Eigenvectors PC1	Eigenvectors PC2
Chl- <i>a</i>	0.109	−0.034	0.201	−0.451
430:665	0.043	−0.338	−0.235	0.322
qP	0.128	−0.067	0.107	−0.132
qN	0.177	−0.534	0.236	−0.104
NPQ	0.306	−0.359	0.358	−0.176
Φ_M	0.461	−0.036	0.409	0.153
Φ'_M	0.376	0.424	0.331	0.266
Fo(BI)	−0.262	−0.339	−0.165	−0.515
Fo(Gr)	−0.286	−0.301	−0.162	−0.513
Fo(Br)	0.183	−0.222	0.378	−0.09
Zn/DW	−0.464	−0.014	−0.415	0.049
Cd/DW	−0.302	0.172	−0.252	−0.016

Zn/DW the Zn content per dry weight ($\mu\text{g Zn g DW}^{-1}$), Cd/DW the Cd content per dry weight ($\mu\text{g Cd g DW}^{-1}$)

which is in agreement with the chl-*a* difference observed at the end of the experiment (63–65% below controls). Serra et al. (2009) also found that biofilms exposed to Cu reached a plateau state (in terms of Fo) before than controls. The observed growth reduction may have marked effects on the fluvial ecosystem due to their role in the uptake or retention of inorganic and organic nutrients (Fishcer et al., 2002; Romaní et al., 2004) affecting self-depuration processes, and transfer of energy to higher trophic levels (Schmitt et al., 1995; Sheldon & Walker, 1997; Hall & Meyer, 1998). On the other hand, biofilms exposed to Zn and Zn + Cd bioaccumulated these metals reaching values 1–2 orders of magnitude higher than the non-metal-exposed communities. Metal bioaccumulation can have adverse effects for fish and invertebrates that prey on it due to biomagnification (Rhea et al., 2006; Farag et al., 2007; Hill et al., 2010).

Zn and Cd effects depending on the time of exposure

After short-term metal exposure, the effects observed were of low magnitude, always below 30% in

comparison to controls, and this was attributed to the relatively low metal-exposure conditions simulated. NPQ, Φ_M and Φ'_M , decreased as metal bioaccumulation increased (Fig. 4A; Table 5), but these changes explained a small portion of total variance based on the PCA analysis (22.9% of the variance). Zn bioaccumulation was eight times higher in metal-exposed biofilms than in the non-exposed ones, causing, in agreement with our hypothesis, mainly functional community responses. The Φ'_M decreased (23 and 8% in the Zn and Zn + Cd treatments, respectively) indicating an inhibition in the electron transport flow during the light-dependent photosynthesis activity (Juneau et al., 2007). This effect could also be attributed to the ‘in vivo’ substitution of magnesium, the central atom of chlorophyll, by heavy metals leading to a breakdown in photosynthesis (Küpper et al., 1998; Zvezdanovic & Markovic, 2009). This damage-causing mechanism, reported in heavy metal-stressed plants, was not directly addressed in this investigation. NPQ response was less consistent. Although a tendency to decrease with exposure is indicated by the PCA analyses, a significant increase was observed after 6 h of exposure in the Zn + Cd treatment (Fig. 3). Niyogi

(2000) described an increase in the NPQ as a mechanism to avoid damage on the photosynthetic apparatus produced by an excess of light reaching the PSII which cannot be used in the photosynthetic process. Similar NPQ responses have been observed in bioassays with algal monocultures (Perales-Vega et al., 2007) and cyanobacteria (Juneau et al., 2001) exposed during few hours to metals. The maximal photosynthetic activity (Φ_M) showed a similar pattern to Φ'_M (Fig. 4), indicating that structural damage was already occurring during the first hours of metal exposures.

Other studies indicate that higher Zn concentrations ($450 \mu\text{g Zn l}^{-1}$ to 40 mg Zn l^{-1}) are required to affect biofilm photosynthesis (50% inhibitions) after short-term exposure (Admiraal et al., 1999; Blanck et al., 2003; Guasch et al., 2003). The structure of biofilm could play an important protective role against Zn toxicity (Guasch et al., 2010a) since metal toxicity is linked to metal penetration into cells (Escher & Hermens, 2002) and biofilm thickness reduces Zn and Cd diffusion (Ivorra et al., 2000; Duong et al., 2010). Since our biofilm was exposed after 4 weeks of colonization, a slow rate of diffusion into the biofilm matrix was expected during the first hours.

During long-term metal exposures, clear effects were observed on metal-exposed biofilms: algal growth was deeply inhibited (above 50% of controls), community composition was altered and most chl-*a* fluorescence parameters remained affected. In agreement with what was expected, these marked effects were linked to a higher metal bioaccumulation, result of a longer time of metal exposure. According to PCA analysis, metal-exposure effects account for a high portion (around 42%) of the total variance (Fig. 4B; Table 5). Zn bioaccumulation increased progressively over time, reaching values 1–2 orders of magnitude higher than the non metal-exposed communities, within the range found in Zn polluted rivers (Behra et al., 2002; Morin et al., 2008; Hill et al., 2010). Biofilm endpoints linked to Zn and Cd bioaccumulation were NPQ, Fo(Br) and Φ_M . In NPQ inhibition was very marked (above 50% controls in both metal treatments), indicating that this non-photochemical quenching mechanism did not take place. It supports the hypothesis that metal toxicity caused structural effects, and these effects included

the damage of pigments where NPQ processes occur. Ricart et al. (2010a) also found a drastic reduction of this parameter when biofilms were exposed during a long period of time to triclosan and they also attributed these findings to an alteration of the photosynthetic pigments. In our study, the NPQ was even more sensitive than qN to metal stress, so it can, therefore be considered the most sensitive photosynthesis-related parameter evaluated. This high sensitivity of NPQ versus qN was reported recently in a review study about the use of PAM fluorometry to assess herbicides toxicity on monocultures (Juneau et al., 2007). In contrast, slight effects were observed in the Φ_M . The reduction of the Φ_M indicates that the structure of the PSII complex was indeed damaged by metals. The maximal photosynthetic capacity is a parameter extremely sensitive to alterations of the PSII/LHCII complex in the thylakoids (Rosenqvist & Van Kooten, 2003). Lazar (1999) also suggested that a destruction of the antennae pigments may be partially responsible for the reduction of Φ_M (Lazar, 1999). An increase in the carotenoids ratio (430:665) was also observed (mainly in the Zn treatment), supporting the occurrence of structural damage (Martínez-Abaigar & Núñez-Olivera, 1998). Photosynthesis efficiency was slightly affected after long-term metal exposures following the same pattern observed during the first hours of exposure. Φ'_M remained inhibited, indicating a persistent alteration on the electron transport chain when a metal exposure takes place over a long period. However, photosynthesis inhibition decreased over time reaching values similar to controls. This apparent lack of inhibition can be explained by the patterns observed in controls, decreasing their Φ'_M because of maturity.

The community composition of biofilm was highly altered due metal toxicity. Metal-exposed biofilms suffered a marked decrease of the relative abundance of diatoms (between an 80 and 90% in comparison to controls) while the relative abundance of cyanobacteria and green algae remained stable. These results indicate that cyanobacteria and green algae are more tolerant to Zn and Zn plus Cd exposures than diatoms. Other studies, based on biofilms, have already reported a metal resistance of green algae to Zn (Ivorra et al., 2000) as well as resistance of cyanobacteria to Zn (Genter et al., 1987) and Zn mixtures (Takamura et al., 1989). Serra et al. (2009)

also observed a higher tolerance of cyanobacteria than green algae or diatoms, when biofilms were chronically exposed to Cu.

On the other hand, changes in the Φ'_M and the carotenoid ratio were mainly attributed to biofilm's maturity (23.3% of the total variance, Table 5). It is well reported that photosynthetic activity declines with biofilm's age (Hill & Boston, 1991; Hawes, 1993) because of an increase of self-shading and a decrease of nutrient availability with biofilm thickness (Paul & Duthie, 1989; Dodds et al., 1999).

Our experimental results allowed to link short-term metal exposure (at the concentrations used) with functional alterations in biofilm: mainly the Φ'_M that may therefore be used as biomarkers of pollution. As such, they provide an early indicator of toxic exposure that can be later confirmed by even structural alterations.

Zn versus Zn + Cd effects on biofilm

In agreement with our expectation, the effects caused by Zn + Cd could be attributed to Zn toxicity. The influence of Cd bioaccumulation during the short-term exposure was evident in the short-term exposure according to the multivariate analysis (Fig. 4A; Table 5), showing that biofilms exposed to Zn + Cd presented higher photosynthetic efficiency than those exposed to Zn alone. However, Cd had a low weight in the long-term exposure, indicating a lack of difference between the two metal treatments applied. Wang et al. (1995) reported, in a study on marine algae, that when the Cd concentration was low and Zn concentration was high, the toxicity depends upon Zn concentration.

As was pointed out before, the Zn and Cd concentrations were very different: Zn concentration was 22 times higher than Cd concentration, and so no equal toxicity can be expected. The similarities observed between Zn and Zn + Cd treatments agree with the results obtained by Gold et al. (2003b), as shown in laboratory biofilms' low effects of $10 \mu\text{g Cd l}^{-1}$, but clear effects of Zn + Cd in the field (Gold et al. 2003a). Ivorra et al. (2000), based on different endpoints to those we use (chl-*a* concentration and diatoms taxonomy), also found that biofilm suffered similar effects when it was exposed to Zn + Cd or Zn alone.

The use of chl-*a* fluorescence parameters in biofilms as early warning systems for metal toxicity detection

The set of chl-*a* fluorescence parameters included in this experimental study was very sensitive to metal exposure. However, its sensitivity to detect metal effects in field studies may be lower due to a lack of specificity and for this reason supplementary chemical analysis are needed. In our experiment, the effects observed after short-term exposure may be indicative of changes that will be detected after longer exposure. The selection towards a metal-adapted community had an energetic cost detected after short-term exposure as a physiological alteration leading to marked growth changes. Based on the results of this study, the reduction of Φ'_M and the alterations on Φ_M and NPQ in biofilm communities could be used as indicative biomarkers of short-term metal impact if they are compared to controls and complemented with metal exposure and accumulation data.

Conclusions

Our results indicate that chronic exposure of biofilms to the Zn concentrations found in several fluvial systems (Blanck et al., 2003; Morin et al., 2007), may have negative effects on biofilms. Our experimental study demonstrates that in natural streams such as the Riou-Mort, with a chronic pollution of Zn plus Cd, most of the metal toxicity reported on the autotrophic component of biofilms could be attributed to the metal found at higher concentration (in this case Zn). The use of chl-*a* fluorescence parameters seems to be a potential tool to assess metal pollution in natural ecosystems. Metals' effects can be assessed 'in vivo' at different temporal scales, following a community ecotoxicology approach. Its use can inform about (a) functional and quick alterations on photosynthesis processes (which could occur after a punctual spill during a short period of time); and (b) structural changes or functional alterations linked to changes in the community composition (which could occur in situations of chronic metal pollution). However, chemical analysis and comparisons between metal-impacted and non-metal-impacted (reference) sites

should also be performed because of its lack of specificity.

Acknowledgments This study was supported by the Spanish Ministry project FLUVIALFITOMARC (CGL2006-12785), FLUVIALMULTISTRESS (CTM2009-14111-C02-01), and the EC project KEYBIOEFFECTS (MRTN-CT-2006-035695). The authors express their thanks to the ‘Serveis Tècnics de Recerca’ of the University of Girona for offering their facilities and technical assistance during the metal analysis.

References

- ACA-Catalan water agency [available on internet at <http://aca-web.gencat.cat>].
- Admiraal, W., H. Blanck, M. Buckert-de Jong, H. Guasch, N. Ivorra, V. Lehmann, B. A. H. Nystroo, M. Paulsson & S. Sabater, 1999. Short-term toxicity of zinc to microbenthic algae and bacteria in a metal polluted stream. *Water Research* 33: 1989–1996.
- American public health association (APHA), 1989. Standard methods for the examination of water and wastewater, 17 edition. -APHA, Washington, DC.
- Barranguet, C., F. P. Van den Ende, M. Rugers, A. M. Breure, M. Greudanus, J. J. Sinke & W. Admiraal, 2003. Copper-induced modifications of the trophic relations in riverine algal-bacterial biofilms. *Environmental Toxicology and Chemistry* 22: 1340–1349.
- Behra, R., R. Landwehrjohann, K. Vogel, B. Wagner & L. Sigg, 2002. Copper and zinc content of periphyton from two rivers as a function of dissolved metal concentration. *Aquatic Science* 64: 300–306.
- Bilger, W. & O. Björkman, 1990. Role of xanthophyll cycle in photoprotection elucidated by measurements of light-induced absorbance changes, fluorescence and photosynthesis in leaves of *Hedera canariensis*. *Photosynthesis Research* 25: 174–185.
- Blanck, H., W. Admiraal, R. F. M. J. Cleven, H. Guasch, M. A. G. T. van den Hoop, N. Ivorra, B. Nyström, M. Paulsson, R. P. Petterson, S. Sabater & G. M. J. Tubbing, 2003. Variability in zinc tolerance, measured as incorporation of radio-labeled carbon dioxide and thymidine, in periphyton communities sampled from 15 European river stretches. *Archives of Environmental Contamination and Toxicology* 44: 17–29.
- Bonnineau, C., B. Bonet, N. Corcoll & H. Guasch, 2011. Catalase in fluvial biofilms: a comparison between different extraction methods and example of application in a metal-polluted river. *Ecotoxicology* 20: 293–303.
- Chaloub, R. M., C. C. P. de Magalhaes & C. P. dos Santos, 2005. Early toxic effects of Zinc on PSII of *Synechocystis aquatilis* F. *aquatilis* (Cyanophyceae). *Journal of Phycology* 41: 1162–1168.
- Clarke, K. R. & R. M. Warwick, 2001. Changes in Marine Communities: An Approach to Statistical Analysis and Interpretation, 2nd ed. PRIMER-E, Plymouth, UK.
- Clements, W. H. & M. C. Newman, 2002. Community Ecotoxicology. John Wiley & Sons, LTD, West Sussex, UK: 336.
- Deckert, J., 2005. Cadmium toxicity in plants: Is there any analogy to its carcinogenic effect in mammalian cells? *BioMetals* 18: 475–481.
- Dodds, W. K., B. J. F. Biggs & R. L. Lowe, 1999. Photosynthesis irradiance patterns in benthic microalgae: variations as a function of assemblage thickness and community structure. *Journal of Phycology* 35: 42–53.
- Duong, T. T., S. Morin, M. Coste, O. Herlory, A. Feurtet-Mazel & A. Boudou, 2010. Experimental toxicity and bio-accumulation of cadmium in freshwater periphytic diatoms in relation with biofilm maturity. *Science of the Total Environment* 408: 552–562.
- Escher, B. I. & J. L. M. Hermens, 2002. Modes of action in ecotoxicology: their role in body burdens, species sensitivity, QSARs, and mixture effects. *Environmental Science and Technology* 20: 4201–4217.
- Faller, P., K. Kienzler & A. Krieger-Liszkay, 2005. Mechanism of Cd²⁺ toxicity: Cd²⁺ inhibits photoactivation of Photosystem II by competitive binding to the essential Ca²⁺ site. *Biochimica et Biophysica Acta* 1706: 158–164.
- Farag, A. M., D. A. Nimick, B. A. Kimball, S. E. Church, D. D. Harper & W. G. Brumbaugh, 2007. Concentrations of metals in water, sediment, biofilm, benthic macroinvertebrates, and fish in the Boulder River watershed, Montana, and the role of colloids in metal uptake. *Archives of Environmental Contamination and Toxicology* 52: 397–409.
- Fishcer, H., A. Sachse, C. E. W. Steinberg & M. Pusch, 2002. Differential retention and utilization of dissolved organic carbon by bacteria in river sediments. *Limnology and Oceanography* 47: 1702–1711.
- Genter, R. B., D. S. Cherry, E. P. Smith & J. J. Cairns, 1987. Algal-periphyton population and community changes from zinc stress in stream mesocosms. *Hydrobiologia* 153: 261–275.
- Genty, B., J.-M. Briantais & N. R. Baker, 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochimica et Biophysica Acta* 990: 87–92.
- Gold, C., A. Feurtet-Mazel, M. Coste & A. Boudou, 2003a. Impacts of Cd and Zn on the development of periphytic diatom communities in artificial streams located along a river pollution gradient. *Archives of Environmental Contamination and Toxicology* 44: 189–197.
- Gold, C., A. Feurtet-Mazel, M. Coste & A. Boudou, 2003b. Effects of cadmium stress on periphytic diatom communities in indoor artificial streams. *Freshwater Biology* 48: 316–328.
- Guasch, H. & S. Sabater, 1998. Estimation of the annual primary production of stream epilithic biofilms based on photosynthesis-irradiance relations. *Archiv für Hydrobiologie* 141: 469–481.
- Guasch, H., M. Paulsson & S. Sabater, 2002. Effect of copper on algal communities from oligotrophic calcareous streams. *Journal of Phycology* 38: 241–248.
- Guasch, H., W. Admiraal & S. Sabater, 2003. Contrasting effects of organic and inorganic toxicants on freshwater periphyton. *Aquatic Toxicology* 64: 165–175.
- Guasch, H., A. Serra, N. Corcoll, B. Bonet & M. Leira, 2010a. Metal ecotoxicology in fluvial biofilms: potential influence of water scarcity. In Sabater, S. & D. Barceló (eds),

- Water Scarcity in the Mediterranean: Perspectives Under Global Change. Springer, Berlin, Germany: 41–54.
- Guasch, H., G. Atli, B. Bonet, N. Corcoll, M. Leira & A. Serra, 2010b. Discharge and the response of biofilms to metal exposure in Mediterranean rivers. *Hydrobiologia* 657: 143–157.
- Hall, R. O. J. & J. L. Meyer, 1998. The trophic significance of bacteria in a detritus-based stream food web. *Ecology* 79: 1995–2012.
- Hassler, C. S. & K. J. Wilkinson, 2003. Failure of the biotic ligand and free-ion activity models to explain zinc bioaccumulation by *Chlorella kesslerii*. *Environmental Toxicology and Chemistry* 22: 620–626.
- Hassler, C. S., R. Behra & K. J. Wilkinson, 2005. Impact of zinc acclimation on bioaccumulation and homeostasis in *Chlorella kesslerii*. *Aquatic Toxicology* 74: 139–149.
- Hawes, I., 1993. Photosynthesis in thick cyanobacterial films: a comparison of annual and perennial antarctic mat communities. *Hydrobiologia* 252: 203–209.
- Hill, W. R. & H. L. Boston, 1991. Community development alters photosynthesis-irradiance relations in stream periphyton. *Limnology and Oceanography* 36: 1375–1389.
- Hill, W. R., M. G. Ryon, J. G. Smith, S. M. Adams, H. L. Boston & A. J. Stewart, 2010. The role of periphyton in mediating the effects of pollution in a stream ecosystem. *Environmental Management* 45: 563–576.
- Ilangovan, K., R. O. Cañizares-Villanueva, S. González-Moreno & D. Voltolina, 1998. Effect of cadmium and zinc on respiration and photosynthesis in suspended and immobilized cultures of *Chlorella vulgaris* and *Scenedesmus acutus*. *Bulletin of Environmental Contamination and Toxicology* 60: 936–943.
- Ivorra, N., J. Hettelaar, G. M. J. Tubbing, M. H. S. Kraak & W. Admiraal, 1999. Translocation of microbenthic algal assemblages used for in situ analysis of metal pollution in rivers. *Archives of Environmental Contamination and Toxicology* 37: 19–28.
- Ivorra, N., S. Bremer, H. Guasch, M. H. S. Kraak & W. Admiraal, 2000. Differences in the sensitivity of benthic microalgae to Zn and Cd regarding biofilm development and exposure history. *Environmental Toxicology and Chemistry* 19: 1332–1339.
- Jeffrey, S. W. & G. F. Humphrey, 1975. New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. *Biochemical Physiology Pflanz* 167: 191–194.
- Juneau, P., D. Dewez, S. Matsui, S. G. Kim & R. Popovic, 2001. Evaluation of different algal species sensitivity to mercury and metolachlor by PAM-fluorometry. *Chemosphere* 45: 589–598.
- Juneau, P., B. Qiu & C. Deblois, 2007. Use of chlorophyll fluorescence as a tool for determination of herbicide toxic effect: review. *Toxicological and Environmental Chemistry* 89: 309–325.
- Kautsky, H. & A. Hirsch, 1931. Neue Versuche zur Kohlensäureassimilation. *Naturwissenschaften* 19: 964.
- Küpper, H., F. Küpper & M. Spiller, 1998. In situ detection of heavy metal substituted chlorophylls in water plants. *Photosynthesis Research* 58: 123–133.
- Laviale, M., J. Prygiel & A. Créach, 2010. Light modulated toxicity of isoproturon toward natural stream periphyton photosynthesis: a comparison between constant and dynamic light conditions. *Aquatic Toxicology* 97: 334–342.
- Lazar, D., 1999. Chlorophyll *a* fluorescence induction. *Biochimica et Biophysica Acta* 1412: 1–28.
- Le Faucheur, S., R. Behra & L. Sigg, 2005. Thiol and metal contents in periphyton exposed to elevated copper and zinc concentrations: a field and microcosm study. *Environmental Science and Technology* 39: 8099–8107.
- Lock, M. A., R. R. Wallace, J. W. Costerton, R. M. Ventullo & S. E. Charlton, 1984. River epilithon: toward a structural-functional model. *Oikos* 42: 10–22.
- Mallick, N. & F. H. Mohn, 2003. Use of chlorophyll fluorescence in metal-stress research: a case study with the green microalgae *Scenedesmus*. *Ecotoxicology and Environmental Safety* 55: 64–69.
- Margalef, R., 1983. *Limnología*. Ediciones Omega, Barcelona: 1010 pp.
- Martínez-Abaigar, J. & E. Núñez-Olivera, 1998. Ecophysiology of photosynthetic pigments in aquatic bryophytes. In Bates, J. W., N. W. Ashton & J. G. Duckett (eds), *Bryology for the Twenty-First Century*. Maney Publishing and the British Bryological Society, Leeds, UK: 277–292.
- Meylan, S., R. Behra & L. Sigg, 2003. Accumulation of copper and zinc in periphyton in response to dynamic variations of metal speciation in freshwater. *Environmental Science and Technology* 37: 5204–5212.
- Morin, S., M. Vivas-Nogues, T. T. Duong, A. Boudou, M. Coste & F. Delmas, 2007. Dynamics of benthic diatom colonization in a cadmium/zinc-polluted river (Riou-Mort, France). *Fundamental and Applied Limnology* 168: 179–187.
- Morin, S., T. T. Duong, A. Dabrin, A. Coynel, O. Herlory, M. Baudrimont, F. Delmas, G. Durrieu, J. Schäfer, P. Winterton, G. Blanc & M. Coste, 2008. Long-term survey of heavy-metal pollution, biofilm contamination and diatom community structure in the Riou Mort watershed, South-West France. *Environmental Pollution* 151: 532–542.
- Moustakas, M., T. Lanaras, L. Symeonidis & S. Karataglis, 1994. Growth and some photosynthetic characteristics of field grown *Avena sativa* under copper and lead stress. *Photosynthetica* 30: 389–396.
- Murphy, J. & J. P. Riley, 1962. A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta* 27: 31–36.
- Niyogi, K. K., 2000. Safety valves for photosynthesis. *Current Opinion in Plant Biology* 3: 455–460.
- Paul, B. J. & H. C. Duthie, 1989. Nutrient cycling in the epilithon of running waters. *Canadian Journal of Botany* 67: 2302–2309.
- Paulsson, M., B. Nyström & H. Blanck, 2000. Long-term toxicity of zinc to bacteria and algae in periphyton communities from the river Göta Älv, based on a microcosm study. *Aquatic Toxicology* 47: 243–257.
- Paulsson, M., V. Mansson & H. Blanck, 2002. Effects of zinc on the phosphorus availability to periphyton communities from the river Göta Älv. *Aquatic Toxicology* 56: 103–113.

- Perales-Vega, H. V., S. González-Moreno, C. Montes-Horcasitas & R. O. Cañizares-Villanueva, 2007. Growth, photosynthetic and respiratory responses to sub-lethal copper concentrations in *Scenedesmus incrassatulus* (Chlorophyceae). *Chemosphere* 67: 2274–2281.
- Posthuma, L., G. W. Suter & T. P. Traas, 2001. Species Sensitivity Distributions in Ecotoxicology. CRC Press, Boca Raton, FL.
- Ralph, P. J. & M. D. Burchett, 1998. Photosynthetic response of *Halophila ovalis* to heavy metal stress. *Environmental Pollution* 103: 91–101.
- Ralph, P. J., R. A. Smith, C. M. O. Macinnis-Ng & C. R. Seery, 2007. Use of fluorescence-based ecotoxicological bioassays in monitoring toxicants and pollution in aquatic systems: review. *Toxicological and Environmental Chemistry* 89: 589–607.
- Rashid, A., E. L. Camm & A. K. M. Ekramodoullah, 1994. Molecular mechanism of action of Pb^{2+} and Zn^{2+} on water oxidizing complex of photosystem II. *FEBS Letters* 350: 296–298.
- Rhea, D. T., D. D. Harper, A. M. Farag & W. G. Brumbaugh, 2006. Biomonitoring in the Boulder river watershed, Montana, USA: metal concentrations in biofilm and macroinvertebrates, and relations with macroinvertebrate assemblage. *Environmental Monitoring and Assessment* 115: 381–393.
- Ricart, M., H. Guasch, M. Alberch, D. Barceló, C. Bonineau, A. Geiszinger, M. Farré, J. Ferrer, F. Ricciardi, A. M. Romaní, S. Morin, L. Proia, L. Sala, D. Sureda & S. Sabater, 2010a. Triclosan persistence through wastewater treatment plants and its potential toxic effects on river biofilms. *Aquatic Toxicology* 100: 346–353.
- Ricart, M., H. Guasch, D. Barceló, R. Brix, M. H. Conceição, A. Geiszinger, M. J. de López Alda, J. C. López-Doval, I. Muñoz, C. Postigo, A. M. Romaní, M. Villagrana & S. Sabater, 2010b. Primary and complex stressors in polluted mediterranean rivers: pesticide effects on biological communities. *Journal of Hydrology* 383: 52–61.
- Romaní, A. M., 2010. Freshwater Biofilms. In Dürr, S. & J. C. Thomason (eds), *Biofouling*. Wiley-Blackwell, Oxford, UK: 137–163.
- Romaní, A. M. & S. Sabater, 2001. Structure and activity of rock and sand biofilms in a Mediterranean stream. *Ecology* 82: 3232–3245.
- Romaní, A. M., A. Giorgi, V. Acuña & S. Sabater, 2004. The influence of substratum type and nutrient supply on biofilm organic matter utilization in streams. *Limnology and Oceanography* 49: 1713–1721.
- Rosenqvist, E. & O. Van Kooten, 2003. Chlorophyll fluorescence: a general description and nomenclature. In DeEll, J. R. & P. M. A. Toivonen (eds), *Practical Applications of Chlorophyll Fluorescence in Plant Biology*. Kluwer Academic Publishers, Dordrecht, Netherlands.
- Rotter, S., F. Sans-Piché, G. Streck, R. Altenburger & M. Schmitt-Jansen, 2011. Active bio-monitoring of contamination in aquatic systems—an in situ translocation experiment applying the PICT concept. *Aquatic Toxicology* 101: 228–236.
- Rysgaard, S., M. Kühl, R. N. Glud & J. W. Hansen, 2001. Biomass, production and horizontal patchiness of sea ice algae in a high-Arctic fjord (Young Sound, NE Greenland). *Marine Ecology Progress Series* 223: 15–26.
- Sabater, S., H. Guasch, M. Ricart, A. Romaní, G. Vidal, C. Klünder & M. Schmitt-Jansen, 2007. Monitoring the effect of chemicals on biological communities: the biofilm as an interface. *Analytical and Bioanalytical Chemistry* 387: 1425–1434.
- Samson, G., J. C. Morissette & R. Popovic, 1988. Copper quenching of the variable fluorescence in *Dunaliella tertiolecta*. New evidence for a copper inhibition effect on PSII photoinhibitory. *Photochemistry and Photobiology* 48: 329–332.
- Santos, A., J. L. Santos, I. Aparicio & E. Alonso, 2010. Fractionation and distribution of metals in Guadiamar river sediments (SW Spain). *Water, Air, and Soil Pollution* 207: 103–113.
- Schmitt, J., D. Nivens, D. C. White & H. C. Flemming, 1995. Changes of biofilm properties in response to sorbed substances—an FTIR-ATR study. *Water Science and Technology* 32: 149–155.
- Schmitt-Jansen, M. & R. Altenburger, 2008. Community-level microalgal toxicity assessment by multiwavelength-excitation PAM fluorometry. *Aquatic Toxicology* 86: 49–58.
- Schreiber, U., 1998. Chlorophyll fluorescence: new instruments for special applications. In Garab, G. (ed.), *Photosynthesis: Mechanisms and Effects*, Vol. V. Kluwer Academic Publishers, Dordrecht: 4253–4258.
- Schreiber, U., U. Schliwa & W. Bilger, 1986. Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. *Photosynthesis Research* 10: 51–62.
- Serra, A., N. Corcoll & H. Guasch, 2009. Copper accumulation and toxicity in fluvial periphyton: the influence of exposure history. *Chemosphere* 74: 633–664.
- Sheldon, F. & K. F. Walker, 1997. Changes in biofilms induced by flow regulation could explain extinctions of aquatic snails in the lower River Murray, Australia. *Hydrobiologia* 347: 97–108.
- Sheoran, I. S. & R. Sing, 1992. Effect of heavy metals on photosynthesis in higher plants. In Arbol, Y. P., P. Mohanty & Govindjee (eds), *Photosynthesis: Photoreaction to Plant Productivity*. Kluwer Academic Publishers, The Netherlands: 225–255.
- Silver, S., 1998. Genes for all metals—a bacterial view of the periodic table. The 1996 Thom Award Lecture. *Journal of Industrial Microbiology & Biotechnology* 20: 1–12.
- Takamura, N., F. Kasai & M. M. Watanabe, 1989. Effect of Cu, Cd and Zn on photosynthesis of freshwater benthic algae. *Journal of Applied Phycology* 1: 39–52.
- Wang, J., M. Zhang, J. Xu & Y. Wang, 1995. Reciprocal effect of Cu, Cd, Zn on a kind of marine alga. *Water Research* 29: 209–214.
- Zhou, W., P. Juneau & B. Qiu, 2006. Growth and photosynthetic responses of the bloom-forming cyanobacterium *Microcystis aeruginosa* to elevated levels of cadmium. *Chemosphere* 65: 1738–1746.
- Zvezdanovic, J. & D. Markovic, 2009. Copper, iron, and zinc interactions with chlorophyll in extracts of photosynthetic pigments studied by VIS spectroscopy. *Russian Journal of Physical Chemistry A* 83: 1542–1546.