The effect of metals on photosynthesis processes and diatoms metrics of biofilm from a metal contaminated river: a translocation experiment

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Abstract

A field study in a metal polluted river (the Osor River, NE Spain) was carried out by performing a biofilm translocation experimental design in order to evaluate cause-effect relationships between metal pollution and toxicity on biofilm structure and function, based on photosynthesis alterations and diatoms community. For this propose a range of biofilm metrics, including chl-a fluorescence parameters, pigments composition and diatom metrics were monitored over time (acute and chronic exposures) on biofilm growth in a non polluted site and translocated to 4 downstream sties presenting a gradient of metals. By using multivariate statistical techniques we elucidated metal toxicity (Zn and Pb bioaccumulation) from other stressors (phosphate concentration in water) and found the most pertinent endpoints to assess acute and chronic metal toxicity on biofilms. Our findings showed that the metal pollution of the Osor River was mainly attributed to Zn bioaccumulation during an acute exposure of time and, to Zn and Pb

bioaccumulation during a chronic exposure. During acute exposures, toxicity was observed by a decrease of the photosynthesis efficiency of biofilm and enhancing the xanthophylls cycle of green algae. Meanwhile, during chronic exposures, toxicity was observed at level of a reduction of the maximal photosynthetic capacity of biofilm in concomitance with an alteration of the photosynthetic pigments and a high decrease of the diatoms richness, the index pollution sensitivity and the mean biovolume of diatoms community. Our study supports one of the ambitions of the European Water Framework Directive (2000/60/EC) which propose the relevance of evaluating the effects of pollutants on biota and to look for appropriates biota indicators.

Key words: fluvial ecotoxicology, field study, metal pollution, biofilm, PAM techniques, multivariate analysis

1. Introduction

Metal pollution in freshwater ecosystems is in great concern with urban and mining activities (Beasley and Kneale, 2002; Burton and Pitt, 2002). Commonly they present the occurrence of poly-metal contamination (Robson et al. 2006; Guasch et al. 2009; Ferreira da Silva et al. 2009; Sierra, M.V. and Gómez, N. 2010). For example, the common abundance of Zn, Cu and Pb in urban runoff is well reported (Kennedy, 1999; ARC, 2007; Kayhanian et al., 2007). The response of aquatic organisms to trace metals depends on metal speciation that determines bioavailability and toxicity (Tessier et al. 1995; Morel, F. M.M. and Hering, J.G. 1993) and metal accumulation (Meylan et al. 2004). Several studies have shown that freshwater biofilm accumulated metals to great concentrations (Behra et al. 2002, Serra et al. 2009). Accumulated metals can then be transferred to fish and invertebrates feeding on biofilm (Rhea et al. 2006; Farag et al. 2007).

The WFD (Directive 2000/60/EC) aims to reduce chemical pollution in water bodies by 2015. This directive highlights the importance of evaluating the effects of Priority Pollutant on the biota. Based on this directive, chemical status of Spanish water bodies during 2009 was in many cases attributed to metal contamination (informe IMPRESS, 2009), highlighting that metal contamination is still a matter of concern. In comparison to the Directive 1005/2008, where 33 priority substances were proposed (including Pb and its compounds), 43 priority substances were proposed for 2010, underling the occurrence of Zn and its compounds as a new priority substance. Zn pollution has been described in several rivers in Europe; in Oiartzun River, Spain, dissolved Zn concentrations ranged from 26 to 160 µg/L (UHO, 2003), in the Furtbach River, Switzerland, ranged from 150 to 400 µg/L (Le Faucheur et al. 2005), in Dommel River,

Belgium, ranged from 76 to 2216 µg/L (Ivorra et al. 200), in Riou-Mort River, France, ranged from 300 to 2000 µg/L (Morin et al. 2007) and in Osor River, Spain, our case of study, between 25 and 450 µg/L. The Zn toxicity threshold, according to the Evaluation Pollution Agency (EPA) of United State, is between 36 and 71 µg Zn/L in soft waters and between 30 and 81 µg Zn/L in hard waters. So, according to this directive, Zn concentrations reported in these polluted cases are above the toxicity thresholds. The occurrence of Pb in fluvial ecosystems is often more associated to sediment (Robson et al. 2006; Sierra, M.V. and Gómez, N. 2010) and biofilm accumulation (Guasch et al. 2010; Ancion, P.Y. et al. 2010) than its presence in water surface. Probably, this can be explained on the one hand by the handicap to determine its low concentrations in water bodies by the used instruments with high detection limits, and on the other hand by the high affinity that display the lead ions to be accumulate by organisms, in comparison to other metals (Guasch et al. 2010, Ancion, P.Y. et al. 2010).

Fluvial biofilms, is a complex phytobenthic communities which live attached on submerged substrata composed mainly by algae and bacteria, as well as protozoa and fungi. Due the situation of biofilms, at the interference between benthos and flowing water, they play an important role in the primary production (Guasch and Sabater 1998) and nutrient recycling and trophic cascade (Romaní et al. 2004, Romaní, 2010). Biofilms, have been widely used as biondicators of pollution due to its capacity to detect early effects produced by toxic substances, as are heavy metals (Sabater et al. 2007). The use of biofilms provide an ecotoxciology approach at community level with high ecological relevance, more than using bioassay with mono-specific algal cultures (Clements and Newman 2002, Guasch et al. 2010a). Biofilms, especially in natural ecosystems, are exposed to any disturbance which might be buffered or enhanced by complex biological interactions (Geiszinger et al. 2009). Besides, in natural ecosystems

the occurrence of confounding factors is common (Guasch et al. 2010) and found causality, understood as chemical toxicity and biofilm response, is not always easy. The use of multivariate analysis seems to be a good technique to evaluate the combined effects of environmental factors and toxicants substances on biofilm (Guasch et al. 2009, Ricart et al. 2010,).

Zn is a micronutrient for algae but at high concentrations become toxic. Zn, as other metals, has an unspecific mode of action on algae. Zn toxicity on photosynthesis processes has been described at level of the light harvesting complex (LHC), the oxygen evolution complex (OEC), the cytocrom complex, the pool of plastoquinones or the ferrodoxin protein (Prysard et al. 1999). Pb has no known biological function (Stewart, 1977; Pawlik-kowronska, 2000; Lamai et al., 2005). Compared to other heavy metals, Pb is not considered particularly toxic although it can be accumulated to a very high degree (Clark, 2001). At high concentrations, Pb exerts adverse effects on microalgal morphology, growth and photosynthesis (Pawlik- Skowronska, 2002). From this perspective, toxicity on photosynthetic processes and pigments composition on biofilm exposed to these metals can be expected. The use of the relative abundance of photosynthetic pigments as potential metal biomarker is based on the knowledge that the occurrences of accessory pigments of the photosynthetic apparatus are indicative of its physiological status (Lohr and Wilhelm, 1999). Besides, the correlation between pigments involved in the electron transport via photosystem II (PSII) and xanthophylls cycle with chl-a fluorescence parameters (Gilmere et al. 1993, Goss et al. 1998) offer the possibility to compare results from two different approaches and evaluated deeply metal toxicity on the function and structure of the global photosynthesis from biofilm. The use of chl-a fluorescence techniques has widely been used to assess chemicals

effects on autotrophic organisms, especially in bioassays with algal monocultures (Barranguet et al. 2000, Ivorra et al. 2002, Juneau et al. 2002). In laboratory biofilm studies, its use has been increased in the last years (Serra et al. 2009, Bonnineau et al. 2010, Ricart et al. 2010, Laviale et al. 2010) but in field studies focused on natural biofilm communities its use is still low and few fluorescence parameters are commonly applied in comparison to laboratory studies. Probably this lack of application in field studies could be related with limitations in the developed protocols and instruments. In a previous laboratory study (Corcoll et al. submitted), we found that the use of several fluorescence parameters was pertinent to assess structural and functional effects of Zn (400µg/L) on biofilms. We found that the non-photochemical quenching (NPQ), the effective quantum yield (Φ'_M) and the maximal quantum yield (Φ_M) parameters were sensitive biomarkers to evaluate Zn and Zn plus Cd toxicity on biofilms exposed during acute and chronic periods of time to metals. Many studies support the use of diatoms metrics to assess metal pollution. They respond to environmental degradation not only at the community level through sifts in dominant taxes and diversity patterns but also at the individual level with changes in frustules deformities or size decrease (Gold et al. 2003, Morin et al. 2007, Falasco et al. 2009, Da Silva et al. 2009).

The main aim of this study was to evaluate the convenience of using a set of biofilm metrics, including chl-a fluorescence parameters, pigments composition and diatom metrics to assess the effects of metals in a polluted river, the Osor River (NE, Spain), due the wastes from abandoned mining activities (F, Zn and Pb explotation) in its watershed. To reach this goal, a translocation experiment was performed in Spring 2009. Biofilms grown in a non-polluted site were translocated to 4 down-stream sites with different water metal concentrations. After few hours and several weeks, biofilm

metrics were measured to assess acute and chronic metal toxicity, based on photosynthetic and diatoms metrics responses. Deriving from this experiment, other issues have been evaluated for other authors by using different metrics: Tlili et al. (in prep) evaluated the induction of tolerance to Zn of heterotrophic and autotrophic communities from biofilm and Bonet et al. (in prep), the role of the antioxidant enzyme activities as mechanism to avoid metal stress in bioiflms .

The hypothesis of this study are that: 1) Increasing metal exposure will cause increasing effects on biofilms structure and function, 2) Changes in chl-a fluorescence parameters will be supported by changes in pigment composition and 3) the different parameters will respond differently depending on the time of metal exposure, specifically functional responses will be detected after short-term metal exposure and structural changes will be found in the long-term. In this last scenario, changes in the diatom community will also be found.

2. Material and methods

2.1. Study site

The translocation experiment was conducted in the Osor River, a second order river situated in Girona (NE, Spain) tributary of Ter River (Fig.1). The Osor river is 23.5 km long and drains a catchment area (from Guilleries Mountains) of 8890 ha. The geological substratum of this river is mainly siliceous; with moderate mineralization (173 $CaCO_3$ mg /L, ACA 2009). In terms of hydro morphology it presents riffles and pools. The substratum is mainly of cobbles with some graves and organic debris in zones with low velocity. The riparian vegetation is well developed reaching a high cover on spring. It is mainly composed for deciduous autochthonous trees (*Alnus*

glutinosa, *Salix alba*, *Populos nigra* or *Fraxinus excelsior*) and few allochthonous species (*Robinia pseudoacacia* or *Platanus x hispanica*) that allow refuge for a high diversity of invertebrates, birds, micromammals and few mammals (i.e.*Lutra lutra*) that is in the top of this trophic food chain. The river, is a relatively well preserved with low anthropic pressures, being mainly affected by continuous (mine effluent) and diffuse metal inputs (mine run-over) remaining from former mining activities (finished in 1980). The mine ore exploited were fluorite (CaF_2). sphalerite (ZnS) and galena (PbS) (Bruguera, F. 2004). Urban activities are small, receiving small amounts of residual urban waters from the Osor village (354 inhabitants) and from a wastewater treatment plant located upstream (St. Hilari Sacalm with 5064 inhabitants). The climate of this region is Mediterranean. Annual average precipitations are 950 L/m^2 and median temperature 12ºC.

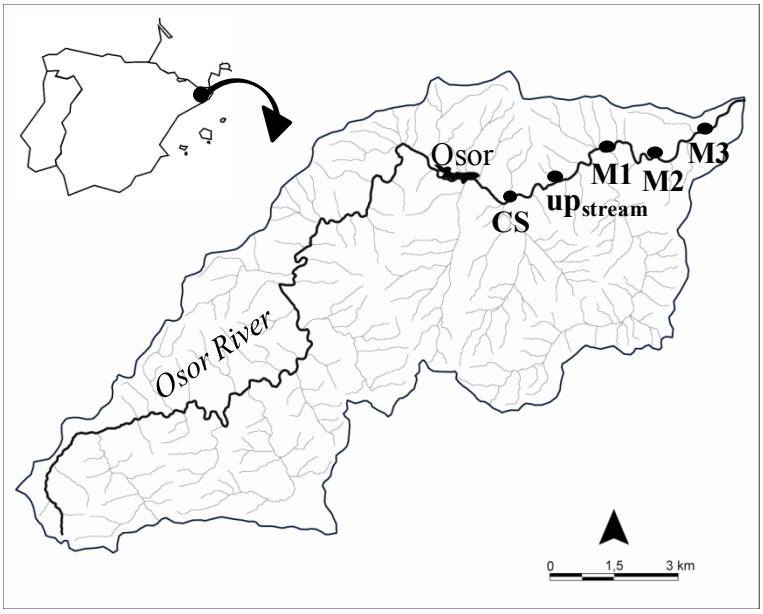


Fig. 1. Situation of the Osor watershed and the 5 sites involved in the biofilm translocation experiment (CS: Colonization site, up_{stream}: upstream site, M1: mining 1 site, M2: mining 2 site and M3: mining 3 site).

2.2 Experimental design

For this translocation study, 5 sites located on the main course of the Osor river (Fig.1) were chosen. The experiment was conducted from May to June 2009.

Biofilms were grown on submerged artificial substrata. Each artificial substratum was made up of heavy stoneware (40x40cm) with attached sand-blasted glasses (70 units of 8.5x2cm and 80 units of 1.2x1.2cm) in horizontal position. All substrata were first placed in the same site, called Colonization site (CS) (Fig.1), and let for 5 weeks to obtain mature biofilms. The Colonization site, is located upstream the mining area. Metal inputs are expected to be low, mainly coming from urban and agricultural activities.

Mature biofilms from CS were translocated to four sites (Fig. 1): the upstream site (up_{stream}), and three sites located within the mining are: metal 1 (M1), metal 2 (M2) and metal 3 (M3). By this way, all translocated biofilms had the same origin and maturity and suffered comparable stress caused by their transport. This moment was considered as T0 of the translocation. The up_{stream} site is located after the Colonization site and before the mine effluent (Fig.1). It is considered a reference site, similar to the Colonization site, with good ecological status and background metal pollution. The M1 site is situated before the main mine effluent and expected to receive diffuse metal sources (Fig.1). The M2 site, is located after the mine effluent (Fig.1), receiving continuously metals from the mine effluent. The M3 site is placed downstream. This site is expected to have lower metal concentrations than M2 due metal retention in zones of sediment deposition (Fig.1).

2.3 Sampling

During the whole experiment, water was sampled 9 times and biofilms 4 times. In the colonization site, water samples were taken at days 29 and 31 (6 and 4 days before T0, respectively). In the translocation sites (up_{stream}, M1, M2 and M3), water samples were taken after 6, 24, and 72 hours (acute exposure) and after 1, 3 and 5 weeks (chronic exposure). Translocated biofilm were sampled after 6h and 24h (acute exposure) and after 3 and 5 weeks (chronic exposure). During the course of the experiment, high precipitations were recorded between week 3 and week5 which could influence the metals dilution factor of the continuous source as well as diffuse input via run-off.

Each sampling time, temperature, conductivity, pH and dissolved oxygen were measured *in situ* by a multi-parametric probe (WTW, Meters, Weilheim, Germany). Water samples were taken for the analysis of phosphate, nitrate, dissolved organic carbon (DOC) and dissolved metals concentrations. Samples were taken in triplicate: 10 mL for phosphate and nitrate analyses; 20 mL for DOC and 5 mL for dissolved metals. All water samples were immediately filtered by using 0.2 µM nylon membrane filters (Whatman), and samples for metal analysis were also acidified with 1% HNO_3 (65 % suprapure, Merck) and transported to the laboratory and stored until their analyses.

All biofilm samples were taken in triplicate. For metal bioaccumulation, biofilms from two big sand-blasted glasses (8.5 x 2 cm) were scrapped and used for each replicate. For chl-a fluorescence analysis a small sand-blasted glass (1.5 x 1.5 cm^2) was used for each replicate. Each glass was individually placed on the bottom of a transparent glass vial filled with 10ml of water from the corresponding sampling site. As chl-a fluorescence measurement is a non destructive technique; the same samples were fixed after measurements with formaldehyde 4% for further diatoms analysis. For pigment analysis, a small glass was used for each replicate. All biofilm samples were transported

into the laboratory in a dark cool box. Samples for pigments and bioaccumulation analysis were kept frozen until analysis.

2.4 Water analysis

Phosphate concentration was analysed using the Murphy and Riley (1992) molybdenum blue colorimetric method and nitrate concentration was analysed by ion chromatography (761 Compact IC, METROHM, Herisau, Switzerland). DOC was measured on a Shimadzu TOC 5000. Dissolved metals (Al, Fe, Zn, Cd, Cu, Pb, Ni, Sb) were analysed by ICP-MS (7500c Agilent Technologies, Inc. Wilmington, DE). The detection limit for Cu was 0.12 $\mu g \cdot L^{-1}$, for Zn was 2.82 $\mu g \cdot L^{-1}$, for Al was 0.72 $\mu g \cdot L^{-1}$, for Pb was 0.40 $\mu g \cdot L^{-1}$, for Ni was 2.44 $\mu g \cdot L^{-1}$, for Fe was 17.18 $\mu g \cdot L^{-1}$ and for Cd was 0.0 $\mu g \cdot L^{-1}$. 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)

2.5. Biofilm analysis

2.5.1 Metal bioaccumulation

In order to measure the total amount of metals accumulated in the biofilm, dried biofilm samples were lyophylized 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%, suprapure) and 1 mL of H_2O_2 (31%, suprapure). After dilution with MiliQ water, water samples were acidified (1% nitric acid suprapure) and stored at -4ºC. Digested samples were analyzed following the procedure described for total metals dissolved in water (see water analysis paragraph). Metal bio-accumulation was expressed as dissolved metal contents (µg) per dry weight (gDW^{-1}). If concentrations

were below method detection limits, a value equal to half of the method detection limit was assigned to these data in the statistical analyses (Helsel, 1990).

2.5.2. Chla-fluorescence measurements

In order to characterize the photosynthetic and non-photochemical quenching parameters of the biofilm; the optimal quantum yield (Φ_M), the effective quantum yield (Φ'_M) and the non-photochemical quenching (NPQ) parameters were determined by measuring the biofilm chl-a fluorescence emission by a Mini-PAM (Pulse Amplitude Modulated) fluorometer (Heinz,Walz, GmbH). The Φ_M was used as a biofilm indicator of the maximal photosynthetic capacity, the Φ'_M as an indicator of the photosynthetic efficiency of the photosythem II (PSII) and the NPQ as an indicator of the dissipation of light energy in non-photochemical quenching processes (Screiber et al. 1998). Before performing PAM measurements, biofilm samples were dark adapted (15 minutes) to remove any energy-dependent quenching and to open all photosynthetic reaction centres to gain the minimal fluorescence (Fo) and the maximal (Fm) fluorescence under dark conditions. Afterwards, samples were illuminated by PAR light (150 µmol photons$\cdot m^{-2} \cdot sec^{-1}$) during 15 minutes and then the minimal (F) and the maximal (Fm') fluorescence's values under light conditions were recorded by the Mini-PAM. Fluorescence measurements obtained were used to calculate the Φ_M (Φ_M = (Fm – Fo) / Fm) and Φ'_M (Φ'_M = (Fm' – F) / Fm') parameters according to Gently et al. (1989) and the NPQ parameter (NPQ = (Fm - F'm)/ F'm) according to Bilger and Björkman 1990.

2.5.3. Pigment analysis

In order to assess photosynthetic parameters relation with pigments composition, several biofilm pigments (Chla, Lutein, Diatoxanthin, Violaxanthin, Diadinoxanthin,

Dinoxanthin, Pheophytin-a, b-Carotene, Anheroxanthin, Zeaxanthin) related to the photosynthetic apparatus were analysed by high pressure liquid chromatography (HPLC) according to Tlili et al. (2008). Five milliliters of each biofilm suspension were centrifuged for 30 min (9750×*g*, 0◦C), and kept at −25 ◦C until analysis. Pellets were placed in individual centrifuge tubes (Corning) and pigments were extracted with 4mL of methanol/0.5M ammonium acetate (98/2, v/v) solution as described in Dorigo et al. (2007). An injection volume of 100 μL of purified biofilm extract was used to determine the lipophilic pigment composition of the biofilm by HPLC. Pigments were separated on a 4.6mm×250mm column (Waters Spherisorb ODS5 25_m). Each pigment was identified from its retention time and absorption spectrum using DAD according to SCOR (Jeffrey et al., 1997). Each pigment was expressed as the percentage of the sum of the areas for all the pigments in the sample.

2.5.4. Diatom metrics

Several metrics from the diatom community were assessed: species richness (S), cell biovolume (biovol), % teratological forms (% teratos) and the Index Pollution Sensitivity (IPS). For identification to the species level, diatoms were cleaned with hydrogen peroxide (30%) and hydrochloric acid (35%) to remove organic material and dissolve calcium carbonates. Cleaned frustules were mounted in a microscope glass slide in a high refractive index medium (Brunel Microscopes Ltd, UK; RI=1.74). A minimum of 400 diatom frustules were identified and counted in each slide using a light microscope at 1000X magnification. The floras of Krammer and Lange-Betarlot (1986–1991) were used as references for identification and from which theoretical biovolumes of each species were also extracted. The richness species index-s and the specific Index

Pollution Sensitivity-IPS (Coste in CEMAGREF, 1982) were calculated using Omnidia 4.1 software (Lecointe et al. 1993). Individual deformities (cells with abnormal general shape and/or diatoms with deformed valve wall ornamentation) were observed and their frequency determined. The cell biovolumen was expressed as the mean bioavolume per sample.

2.6. Data analysis

Only diatom taxa and phototrophic pigments accounting for at least more than 2% in two samples were included in the PCA (Guasch et al. 2009).

One way ANOVA were performed to detect significant differences in the physical and chemical characteristics of water from each site and differences between the biofilm metrics (chl-a fluorescence parameters, metal accumulation, pigments analysis and diatoms metrics) in translocated biofilms during the acute and the chronic exposures. Data were transformed to achieve normality. Tukey's HSD test was applied to identify which sites were statistically different to the up_{stream} site. ANOVA analyses were realized by SPSS software. A 5% significance level ($p<0.05$) was used for rejection of null hypothesis in all cases.

Multivariate analyses were performed using the CANOCO software version 4.5 (ter Braak and Smilauer, 1998). Except pH, the rest of variables were previously transformed by $\log_{10}(x+1)$ to reduce skewed distributions. The ordination of sampling sites on the water physic and chemical characteristics was carried out with principal component analysis (PCA). If metal concentrations were below method detection limits,

a value equal to half of the method detection limit was assigned to these data in the statistical analyses (Helsel, 1990).

Relationship between biofilm metrics and metals concentrations and water physical-chemical variables during the acute and the chronic exposures were assessed with redundancy detrended analysis (RDA). For the RDA analysis deriving from acute exposure, data from Chl-a fluorescence parameters and pigments composition were used to determine the respective influence of metals concentrations (dissolved in water and bioaccumulated) and other water physical-chemical variables on their distribution. For the RDA analysis deriving from chronic exposure, to part to use the same metric variables that were used for the RDA of the acute exposure, variables related to the diatom metrics were added.

The maximum gradient length for biofilm metrics was determined using detrended correspondence analysis (DCA). For the RDA of acute exposure: the maximum amount of variation in the Chl-a fluorescence parameters was 0.553 and 1.212 for pigments data, indicating that linear methods would be appropriate (ter Brasak and Smilauer, 2002). For the RDA of chronic exposure: the maximum amount of variation in the Chl-a fluorescence parameters was 0.556, 0.846 for pigments and 0.504 for diatom metrics data, indicating also that linear methods would be appropriate (ter Brasak and Smilauer, 2002). Consequently, we carried out various redundancy analyses (RDA) on both acute exposure and chronic exposure analysis, whereby data were constrained by metals concentrations and water physical-chemical variables. To avoid co-linearity, the variables were selected based on the inspection of variance inflation factors (VIF<20) (ter Braak and Smilauer, 1998). Forward selection was used to reduce

the environmental variables that significantly explained the distribution pattern of the diatoms and invertebrates at a cut-off point of p=0.1.

The significance of the RDA axes was assessed using the Monte Carlo permutation test (999 unrestricted permutations). Probabilities for multiple comparisons were corrected by applying the Bonferroni correction.

To separate the effects of metals from those of other chemical and physical variables on biofilm metrics distribution, the variance partitioning technique was applied. This technique enabled us to assess the fractions of the explained variance that are shared by two predictor variables, and to determine which of them could be uniquely attributed to each of them (Borcard et al., 1992). The explanatory variables were grouped into two subsets: (a) physical and chemical variables and (b) metals. The following sequences of RDAs and partial RDAs were performed for both biofilm metrics datasets (acute exposure and chronic exposure): (a) RDA of biofilm metrics constrained with physical and chemical variables, (b) RDA of biofilm metrics constrained with metals, (c) partial RDA of biofilm metrics constrained with physical and chemical variables and using the metals as covariables and (d) partial RDA of the biofilm metrics constrained with metals using the physical and chemical variables as covariables. These analyses were useful in evaluating whether these groups of variables were redundant or explained unique aspects of the metrics. The explanatory variables were grouped into two subsets: (a) physical and chemical variables and (b) metals.

3. Results

3.1 Physical and chemical water characterization

The pH was slightly basic in all sites except at M3 site where was more neutral (Table 1) and oxygen around saturation. Conductivity ranged between 170 and 440 µS/cm and it was comparable between sites (Table 1). Lower temperature was observed at "Colonization site" in comparison to the other sites, probably related to the earlier sampling period at this site in comparison to the other ones. Nutrient (phosphate and nitrate) concentrations were lower and Zn concentrations higher in the M2 site, due to the dilution (in the first case) or input (in the second case) exerted by the mining source with high metal concentration but low nutrient levels (data not shown). Fe concentrations ranged from 90 to 500 µg/L. Higher concentrations were recorded in M2 and M3 sites in comparison to up_{stream} site. Ni and Al concentrations were low in all sampling sites (<15 µg/L). Cu, Pb and Cd concentration were in most cases (94%) below detection limit. Maximum concentrations were 0.29 µg Cu/L, 0.48 µg Pb/L and 0.39 µg Cd/L. According to these results, Cu, Pb and Cd concentrations were considered of low significance and not included in the multivariate analysis. Based on the calculated Concentration Cumulative Unites (CCU), the highest value was obtained in M2 with a moderate potential toxicity level. Up_{stream} and M1 sites had a low potential toxicity level while M3 had a background potential toxicity value (Table 1).

The PCA analysis of water chemistry shows the ordination of sampling points with respect to two principal axes (Fig. 2). The first axis explained the 58.8% variance and separates the most polluted site (M2) with high Zn and Fe concentrations (290 µg/L and 456 µg/L, respectively), from the less metal polluted ones (Colonization site, $up_{stream,}$, M1 and M3 sites) where Zn concentration did not exceed 31 µg/L and Fe was below 313 µg/L (Fig.2., Table 1). Also this axis separates the sites with high nitrate (Colonization site, up_{stream} , M1 and M3) from the M2 site, with low nitrate

concentration. The second axis explained a 25.2 % variance and separates the sites with highest Al, pH Fe and Ni (Colonization site, $up_{stream,}$ and M1) from those with lower Al and Fe concentrations and lower pH (M3) (Fig.2). Colonization and the up_{stream} sites were plotted close together in the PCA, indicating that the physicochemical characteristics of these two sites are very similar.

3.2 Metal bioaccumulation

Biofilms translocated to up_{stream}, M1, M2 and M3 sites accumulated different amounts of metals depending on the translocation site and the time of exposure (acute vs. chronic) (Fig.3). Cd bioaccumulation was always below detection limit. During acute exposure (after 6 and 24h), Zn bioaccumulation was 2.5 times higher in M1, M2 and M3 sites than in up_{stream} site (Fig.3). Al bioaccumulation was high in all sites (Table 1). Fe bioaccumulation was high and comparable in all sites except at M3 site, where Fe bioaccumulation was lower than in up_{stream} site (Fig. 3). Cu and Pb bioaccumulation were low and comparable between sites (Fig.3). Ni accumulation was also low in all sites but in M3 site it was slightly higher than to up_{stream} site (Fig.3).

During chronic exposure (after 3 and 5 weeks of started translocation), Zn bioaccumulation in M1, M2 and M3 sites were higher than during the acute exposure. Different amounts of Zn were bioaccumulated between sites. M2 bioaccumlated the highest amounts of Zn in comparison to comparison to up_{stream} (43 times more) in agreement with the high Zn concentration in water observed (Fig.3). Zn bioaccumulations in M1 and M3 sites were comparable and were 6 and 7 times higher than in up_{stream}. The pattern of Pb bioaccumulation between sites was similar to that one observed for Zn bioaccumulation, but with lower concentrations. Pb bioaccumulation was linked to Pb inputs from the mining area and the mine source. In M2, Pb

bioaccumulation was 9 times higher than in up_{stream} site, and in M1 and M3 Zn bioaccumulation was similar and 6 times higher than in up_{stream} site (Fig.3). Biofilms bioaccumulated different amounts of Al, Fe and Cu deepening on the site by similar pattern Al, Fe and Cu bioaccumulation were higher in M1 and M3sites than in up_{stream} site (Fig.3). Ni bioaccumulation during chronic exposure was comparable to that one observed during acute exposure, indicating that the amounts observed could be considered the background contents for the biofilm of this river. In M1, Ni bioaccumulation was lower in comparison to up_{stream} site probably to the intermittence of its inputs from the mining area.

3.3 Chl-a fluorescence parameters

Biofilms translocated presented differences in all the photosynthetic parameters in function of the translocation site and the time of exposure (acute vs. chronic).

Acute exposure (after 6 and 24h of translocation started):

At 6h, the Φ'_M was inhibited in M2, the most Zn polluted site, as well as in the M1 and M3 sites, which presented intermediate metal pollution, in comparison to up_{stream} site (Fig.4). The NPQ increased in M1, M2 and M3 in comparison to up_{stream} site. The Φ_M increased in M2 and M3 in comparison to up_{stream} site (Fig.4). After 24h of started the translocation, the Φ'_M was comparable between sites and the Φ_M was inhibited at M1 and M2 sites. The NPQ was inhibited in M2 and M3 sites in comparison to up_{stream} site.

Chronic exposure (after 3 and 5weeks of translocation started):

During chronic exposure, photosynthetic parameters showed different responses to that were observed during the acute exposure (Fig.4). The Φ'_M of biofilm from M1 site was inhibited after 3 weeks in comparison to up_{stream} site (Fig.4). The Φ'_M of biofilom

translocated to M3 was higher at week 5 than that one observed in up_{stream} site. During chronic exposure, Φ_M of all sites b reached values lowers than during acute exposure. After 3 weeks of started the translocation the Φ_M of biofilom from M3 sites was lower than in up_{stream} site (Fig.4). The Φ_M values reached during the week 5 were comparable between sites and remained lower in comparison to week 3 or during the acute exposure (Fig.4). During the week 5, the NPQ was higher in M1, M2 and M3 sites in comparison to up_{stream} site (Fig.4).

3.4 Pigments composition

Ten pigments related to the photosynthetic processes were identified: chlorophyll a (Chla), Lutein (Lut), Diatoxanthin (Diat), Violaxanthin (Viol), Diadionoxanthin (Diadino), Dinoxanthin (Dino), Pheophytin-a (Pheo), β-caroten (β-Car), Antheroxanthin (Anth) and Zeaxanthin (Zea). During acute exposure, the pigment with higher relative abundance in all sites was Chla (Taable 4). Biofilms from mining sites (M1, M2 and M3) showed a decrease of the violaxanthin abundance in comparison to up_{stream} site (Table 4). In M1, violaxanthin abundance was 5 times lower than in up_{stream} site, in M2 10 times and in M3 11 times respectively (Table 2) . Also during acute exposure, biofilm translocated to mining sites (M1, M2 and M3) showed an increase of the abundance relative of dinoxanthin in comparison to up_{stream} site; concretely in M1 and M3 this increase was of 6 time and in M2 was of 8 times. During chronic exposure, also the pigment with a high relative abundance was the Chla, presenting a higher amount in M3 sites than up_{stream} site (Table 2). The abundance of lutein, diatoxathin and zeaxanthin was higher in M1 site than in up_{stream} site, and the abundance of lutein and zeaxanthin also was high in M2 site than in up_{stream} site (Table 2).

3.5 Diatoms metrics

During chronic exposure, diatoms metrics of biofilm translocated presented high differences depending on the translocation site (Table 3). The species richness (S) of biofilm translocated to M2 site decrease in comparison to up_{stream} site, indicating a decrease of the diatoms diversity in this site. The percentage of teratoforms was higher in mining sites (M1, M2 and M3) than in up_{stream} site, reaching up 6 times higher in M2 site (Table 3). The medium biovolume of the diatoms in M2 site was highly reduced (6 times) in comparison to up_{stream} site (Table 3). The index pollution sensitivity at: up_{stream} site, M1and M3 sites had values that qualify its biological status of "Good quality" according to the WFD (Directive 2000/60/EC). Meanwhile in M2 site, the IPS value was low, situating it in the biological status of "Deficient" according to the same directive.

3.6 Redundancy Analysis (RDA)

3.6.1 Acute exposure

The RDA of acute exposure (Fig. 5) showed that Zn bioaccumulation in biofilm and phosphate concentration in water significantly influenced the biofilm metrics (Table 4). This analysis explains a 35.50 % of the variance, where the first axis which explains an 18.6 % of the variance shows the distribution of sites along a gradient of Zn bioaccumulation. Zn correlation to the second axis was high (Table 4). The relative abundance of lutein, diatoxanthin, dinoxanthin, antheroxantin and the Φ'_M were closely related with sites presented different amounts of Zn bioaccumulated in biofilm during an acute exposure period of time. Biofilm from translocated sites with higher amounts of Zn bioaccumulated, sites M2 and M3 at 24h, presented a major relative abundance of antheroxanthin and dinoxanthin in comparison to other sites with less amounts of Zn bioaccumulated (Fig.5). The second axis explains a 16.9% of the total variance and

ordinate biofilm samples in function a phosphate gradient (Fig.5). Phosphate concentration showed a lower correlation to axis 2 in comparison to the correlation observed for Zn bioaccumulation with axis 1 (Table 4). Biofilm presenting higher relative abundance of violaxanthin and higher Φ'_M were related to sites with higher phosphate concentrations in water (up_{stream} site) (Fig.5).

The potential contribution of each of the two sets of variables (physicochemicals and metals variables) to the variance of the biofilm metrics revelled that in terms of metals variables, Zn bioaccumulation was the statistically significant metal variable and accounted for an 18.4% of the total variation (35.5%), being the Zn bioaccumulation the only significant variable. In terms of physicochemical variables, phosphate had a significant effect on the biofilm metrics, explaining a 17.20%. . No shared variance between physicochemical and metal variables was represented (Fig. 5).

During acute exposure, biofilm metrics presenting a fraction of explained variance related only to metal toxicity were the Φ'_M and the relative abundance of lutein, diatoxantin, dinoxantin and antheroxhantin pigments (Table 5). Meanwhile, the biofilm metrics presenting a fraction of explained variance mainly related to physical and chemical variables were the Φ_M and the pheophytin-a and β-carotene relative abundances attributed (Table 5).

3.6.2 Chronic exposure

The results of RDA analysis for chronic exposure (Fig. 5) showed that: Zn and Pb bioaccumulations on biofilm and phosphate concentration in water significantly influenced the biofilm metrics. This analysis explains a 57.2 % of the variance, where the first axis which explains a 43.20 % of the variance ordinates the distribution of sites along a joint gradient of metal accumulation (Zn and Pb)

and phosphate in water. Zn bioaccumulation presented a high correlation with the first axis (Table 4); higher to that one was observed for Pb bioaccumulation or phosphate concentration (Table 4). According to axis 1 ordination, biofilms from M2 site were separated to those from M1, M3 and up_{stream} sites. Biofilm community from M2 site presented high amounts of Zn and Pb bioaccomulated and it was characterized for presenting a low IPS index, a low diatom's biovolume and a low diatom' species richness. Besides to these characteristics related to diatoms metrics, biofilm from M2 site presented a high relative abundance of zeaxanthin and diadinonxanthin pigments (Fig. 5b). The second axis explained only a 11.10 % of the total variance and showed mainly a gradient of Zn and Pb bioaccumulation (Fig.5b). Zn and Pb bioaccumulation were the variables that presented more correlation with the second axis (Table 4). Accordingly to this axis, biofilm from up_{stream} was separated of biofilm from mining sites (M1, M2 and M3) (Fig.5b). Biofilm from up_{stream} was characterized for presenting higher Φ'_{M}, a higher number of diatom' species richness and a higher relative abundance of pheophytin-a and dinoxanthin pigment than biofilms from mining sites (Fig. 5b). Biofilm from M1 and M3 sites presented high NPQ values and high percentage of diatoms with teratorforms (Fig.4b).

Related to the results from the partitioning of the variance analysis for evaluate the potential contribution of the two sets of variables (physicochemicals and metals variables) to the variance of the biofilm metrics, it was observed that metals accounted for a 48.6 % of the total variation and physical-chemical variables, accounted for a 31.9 % of the total variance (57.2 %). Shared variance represented a 23.31% of the total variance. The percentage of variance explained by metals and physical and chemical variables differed between the biofilm metrics (Table 6). During chronic exposure, the biofilm metrics that were mainly explained by the presence of Zn and Pb bioaccumulated in biofilm were: the maximal photosynthetic capacity which explained, the diatoms richness species, the percentage of diatoms presenting teratoforms, the IPS index and the diatom'

biovolum, and the relative abundance of chla, lutein, diatoxantin, dianoxanthin, pheophytin-a and zeaxanthin. The only biofim metric that was explained for physiochemical variables during chronic exposure was the relative abundance of β-carotene pigment (Table 6).

Discussion

Elucidate metal effects from other stressors is in great concern in ecotoxicological field studies (Culp et al. 2000). The intermittence of the water flow (typical from Mediterranean streams), nutrients occurrence, water physicochemical characteristics or the natural variability of the samples are factors that could contribute to mask metal toxicity based on biofilm responses. In this study, two different approaches were used to abort this problem. On the one hand, an experimental design based on a biofilm translocation experiment was applied, and on the other hand, multivariate statistical techniques were used for data analysis. The biofilm translocation experimental design applied allowed us: 1) to reduce samples variability because all biofilm translocated was growth in the same site and had initially comparable community composition and maturity and 2) to control the time of exposure in the translocated sites, which presented different metal concentrations. Similar experimental designs have been applied in field studies to evaluate metal toxicity on biofilm (Ivorra et al. 1999). It is reported that the use of multivariate statistical techniques is useful in determining spatial and temporal relationships between stressors and their effects along a gradient on the biota (Muñoz et al. 2009). Consequently, in the last years an increase of ecotoxicological field studies has applied these multivariate analyses (Guasch et al. 2009, Ferreira da Silva et al. 2009, Ricart et al. 2010). Our findings based on redundancy data analysis (RDA) and the partitioning of variance showed that during the acute period of exposure, biofilm metrics were mainly affected for metals and physicochemical variables. Metal toxicity was attributed to Zn bioaccumulation and accounted for 18.6 % of the variance. Physicochemical effects were attributed to phosphate concentration in water and accounted for a 16.9 % of the variance. Stronger effects

were observed during chronic exposure. They also were attributed to metal toxicity and physicochemical variables. But during chronic exposure, metal toxicity was attributed to two metals: Zn and Pb bioaacumulation, which accounted for a 48.6% of the variance. The physico-chemical variance was attributed to phosphate in water, as in the acute exposure, and accounted for a 31.9 % of the variance. So in both periods of time exposure (acute and chronic), metal toxicity in terms of metals bioaccumulation was the main factor attribute to the observed changes on the structure and function of the evaluated biofilm metrics. Our results are in agreement with other studies in which also observed that metal toxicity is more related to metal bioaccumulation than metal concentration in water (Bradac et al. 2009). Metal bioaccumulation is the result of different biotic and abiotic involved processes that included metal speciation and species uptake. Metal pollution in Osor River was mainly attributed to Zn and secondly to Pb. Still aluminum and iron concentrations in water and in biofilm were much higher than that's ones observed for Zn or Pb, no toxicity was attributed to them. During chronic exposure, it was observed that biofilm translocated to Mining 2 site, bioaccumulate high amounts of Zn and Pb and lower amounts of Al and Fe in comparison to that one observed in M1 and M3 sites. Since Al and Fe concentrations in water were comparable between M1, M2 and M3 sites, probably a competition for the binding sites between Zn and Pb versus Al and Fe could explain our results. Differences in metal affinity for autotrhophic species have been reported; Ancion et al. (2010) observed in experimental conditions that the affinity of the biofilm for lead ions was much higher than for Zn or Cu. Basile et al. (2007), compared the metal bioaccumulation capacity of an epiphytic moss and an epiphytic lichen and they found that Pb was the element most highly accumulated in both organisms and Fe the least. In experimental studies with tobacco (Yamamoto et al. 1997, Chang et al. 1999), it was observed that Al accumulation in cells was closely related to the Fe(II) presence in the medium, which enhancing its bioaccumulation.

Biosorption of trace metals by algal cells generally increase with time of exposure (Collard and Matagne, 1994). According to Escher et al. 2002, it is expected that an organism bioaccumulate less metal during a short exposure than during a longer metal exposure, causing firstly metabolic and functional alterations and secondly structural and community composition alterations.

Metal toxicity on biofilm after acute time of exposure: looking for the best biofilm biomarker

During acute exposure, metal toxicity was attributed to Zn bioaccumulation representing an 18.6 % according to the RDA performed. The photosynthesis efficiency (Φ'_M) was inhibited in miming sites and its variation was attributed to Zn toxicity (72.03%), indicating that Zn toxicity altered the electron transport of the PSII. In consequence, exes of excited energy could be expected to be quenched in non radiate ways to avoid PSII damage and the generation of reactive oxygen species (Demming et al. 1987). During acute exposure, the maximal photosynthetic capacity (Φ_M) remained practically undisturbed in mining sites, and its variation (72.43 %) was attributed to physicochemical parameters. This suggested that not damage in PSII structure took place. The relative abundance of Chl-a, the most important pigment of the PSII, remained stable suggesting that no structural damage took place in the reaction centers of PSII. The non-photochemical quenching (NPQ) was firstly enhanced, after 6h of exposure and after 24h was inhibited. According to the RDA analysis, the variation of this parameter was responding neither to physico-chemical variables nor to metal pollution. The NPQ parameter, which is assessed by Chla-fluorescence techniques, is a deexictation measure that depends on a large trans-thylakoid proton gradient that is established in excessive light and its development correlates with the synthesis of zeaxanthin and antheroxanthin from violaxanthin via the xanthophylls cycle (Gilmere et al. 1993, Goss et al. 1998). It is described that the light harvesting complex (LHC) composed mainly for accessories PSII pigments as carotenoids (lutein, β-carotene and xanthophylls) could change they pigments ratio to avoid photoinhibition under stress conditions produced for high light irradiances or chemical

substances that could potentially block light-dependent photosynthesis reactions, i.g. metals. The xanthophylls cycle is an important way to use the excess excited energy to avoid photoinhibition by performing de-epoxidation reactions. In green algae, the violaxanthin (Viol) is transformed to zeaxanthin (Zea) and/or antheroxantin (Anth) pigments (Lohr and Wilhelm, 1999). In diatoms and dinoflagelats, the diatoxanthin is transformed to diadinoxanthin or dianoxanthin, respectively (Lohr and Wilhelm, 1999). In cyanobacteria, no xanthophylls cycle has been described. The de-epoxidation reaction is a mechanism to provide protection to photoxidative stress (Jin et al. 2003). A higher relative abundance of violaxanthih was characterized of biofilm communities from up_{stream} and M1 sites in comparison to M2 and M3 sites, but it not was related neither with physicochemical or metal variables. The relative abundance of antheroxahtin was fairly increased in M2 and M3 sites and related to metal toxicity. This result appointed that the xanthophylls cycle of green algae from biofilm translocated to M2 and M3 sites took place during acute exposure but at low levels. In contrast, diatoms from biofilm translocated to mining sites, especially M2 site, seems to lost the xanthophylls cycle based on the no observed synthesis of diatoxanthin from diadinoxanthin by deepoxidation reaction. In contrast, biofilm from upstream site and M1 presented a higher relative abundance of diatoxanthin pigment. According to RDA analysis, diatoxanthin was related to metal toxicity. By using two different approaches to evaluate the deconvolution of excess light arriving on PSII of biofilms, NPQ values and xantohpyll cycle quantification, comparable results were found; biofilm exposed during an acute period of time to Zn no develop reliable mechanisms for dissipate excess light energy on PSII. Consequently potential photoinhibition could be expected if the stress persist on the time (Demming et al. 1987). In this last scenario, severe effects could take place; from firstly the occurrence of oxidative stress going to produce structural damage in the photosynthesis apparatus to changes in the morphology of the species living in the biofilm community. The relative abundance of lutein was related to metal toxicity. Its relative abundance was higher in mining sites than in up_{stream} site. Its biological significance is related to act, as other carotenoids, as

an antioxidant (Armstron and Hearst, 1996). The results suggest that is occurrence could be linked as a protective mechanism of autotrophic groups from biofilm to avoid oxidative stress caused by Zn bioaccumulation.

Based on the set of biofilm metrics used to evaluate metal toxicity on photosynthesis from bioifilm during an acute exposure, we found that the sensitive order was: lutein > diatoxanthin > dinoxanthin > antheroxanthin > Φ'_M.

Metal toxicity on biofilm after chronic time of exposure: looking for the best biofilm biomarker

During chronic exposure, metal toxicity was attributed to Zn and Pb bioaccumulation representing a 48.6% according to the RDA performed. The maximal photosynthesis capacity (Φ_M) was inhibited in miming sites and its variation was attributed to metal toxicity (97.34%), indicating that metal toxicity at level of the photosynthesis activity was higher than during acute exposure, where photosynthesis was altered only at functional level of its efficiency (Φ'_M). The decrease of the Φ_M could indicate damage on the structure of the photosynthetic apparatus of algae from biofilm. The observed increase of the relative abundance of the chla, could be linked with an increase of the number of PSII, chla is the main pigment involved in the PSII (Falkowski and Raven, 2007), in order to supply the decrease of Φ_M. It is described that algae may adjust their intracellular concentration of photosynthetic pigments in response to environmental conditions (Kana et al., 1997). Similar results have been observed in other ecotoxicological studies on biofilm (Ricart et al. 2009). Lutein, during chronic exposure, as during the acute exposure, was related to metal toxicity. The increase of its relative abundance in biofilms from mining sites, especially in M2, could be interpreted as a response to avoid metal toxicity under persistent metal exposures in order to avoid oxidative stress (Armstrong and Hearst, 1996). The pheophytin-a pigment was also related to metal toxicity, this is a pigment involved in the early steps of photosynthetic solar energy conversion as the primary electron acceptor of PSII (Klimov 2003). Its abundance was higher in the non polluted

site, up_{stream} site, than in mining sites. The elevate abundance of pheophytin-a seemed to be related to a higher Φ'_M, indicating that biofilm not exposed to mining sites presented a better physiological status of their photosynthetic apparatus than that one presented in M2 site. Zeaxanthin abundance was related to metal toxicity, being higher in mining sites, especially in M2. Its occurrence has been related to the activation of the violaxanthin cycle by green algae in order to provide protection to photoxidative stress deriving by any stressor causing an excess of light energy arriving on reaction centers (Lohr and Wilhelm, 1999; Jin et al. 2003). Diatoxanthin abundance, related to the xanthophylls cycle of diatoms, was related to metal toxicity. Biofilm from mining sites 1 and 2, presenting moderate Zn and Pb bioaccumulation was characterized for having a higher abundance of diatoxanthin than that one observed in non polluted site (up_{stream} site) and the highest polluted site (M2 sites). Suggesting that biofilm exposed to moderate but longer time of exposure to metal pollution, enhanced the activation of this mechanism to avoid photoxidative stress. The fact, that biofilm from these intermediates polluted mining sites presented higher abundances of diatoms with malformations (% teratoforms), also linked to metal toxicity, support the linked between toxicity on diatoms community at level of the photosynthetic apparatus and morphology. In contrast, biofilms from the highest polluted site (M2) exposed during a long period of time to metal pollution no presented an increase of the abundance of the diatoxanthin pigment, suggesting that this diatoms' community no responded to metal pollution by an activation of the xanthophylls cycle. However, in concomitance to this observation a decrease of diatoms richness and IPS index, also linked to metal toxicity, was observed. Suggesting, that higher metal toxicity happened on diatoms from M2 by producing lethal effects on no tolerant diatom's species. Also, biofilm from the highest polluted site, M2, presented a mean biovolume of diatoms lower than that one observed in up_{stream} site. The decrease in the diatom biovolume is associated with the predominance of smaller growth forms in the biofilms (Sabater and Admiraal, 2005). These results appointed that diatoms community of

biofilms from chronic metal polluted sites presented smaller growth forms than that's ones are found in non polluted sites.

Based on the set of biofilm metrics used to evaluate metal toxicity on photosynthesis and diatoms structure from bioifilm during a chronic exposure, we found that the sensitive order was: chla> Φ_M. > S (diatoms richness) > pheophytin-a abundance > diatoxanthin abundance > the % of diatoms presenting teratoforms > lutein abundance > zeaxanthin abundance > IPS index > biovolume of diatoms cells

5. Conclusion

6. References

Table 1. Physical and chemical parameters of water from Osor River; in the Colonization site (during the colonization period; n=9) and in the 4 translocation sites (during the translocation period; n= 18). Each value corresponds to the average (± standard error). ANOVA results are presented. Tt: Toxicity threshold.

Parameter	Colonization Site	up_{stream}	M1	M2	M3	Tt	ANOVA results	
	AVG (± SE)	AVG (± SE)	AVG (± SE)	AVG (± SE)	AVG (± SE)		$F_{(4,76)}$	p value
pH	8.14 (0.02)	8.14 (0.04)	8.13 (0.07)	8.15 (0.04)	7.68 (0.07)*		13.91	<0.00
Cond.(µS/cm)	183.33 (6.60)	226.72 (5.17)	370.15 (77.84)	356.83 (29.80)*	222.95 (4.03)		7.43	<0.00
Oxy. (mg/L)	9.40 (0.06)	9.12 (0.09)	8.84 (0.18)	9.11 (0.07)	9.23 (0.09)		3.47	0.012
TºC	14.27 (0.43)*	18.02 (0.27)	18.22 (0.23)	18.6 (0.2)	17.63 (0.19)		35.31	<0.00
DOC (mg/L)	4.69 (0.92)	3.45 (0.23)	3.59 (0.25)	3.29 (0.28)	4.21 (0.39)		1.84	0.130
PO_4^{3-} (µg/L)	220.8(18.89)*	380.2 (33.60)	316.5 (20.75)	218.4 (10.02)*	298.6 (29.44)		4.71	0.002
NO^{3-} (µg/L)	3.04 (0.12)	3.05 (0.21)	2.24 (0.14)*	1.87 (0.18)*	2.89 (0.16)		9.94	<0.00
Al (µg/L)	11.89 (3.32)*	5.52 (1.43)	9.74 (1.95)	0.88 (0.29)*	bdl*	nd	23.99	<0.00
Fe (µg/L)	280.44(13.45)	295.84 (4.8)	303.96 (8.74)	455.97 (40.80)	88.17 (27.42)*	nd	36.28	<0.00
Ni (µg/L)	3.65 (0.42)	3.26 (0.27)	9.53 (3.73)	bdl*	bdl*	52	7.22	<0.00
Zn (µg/L)	bdl	19.19 (9.65)	31.11 (11.24)	289.99 (44.19)*	21.04 (10.71)	120	34.29	<0.00
CCU	1.74 (0.17)	1.17 (0.06)	1.36 (0.09)	3.04 (0.33)*	0.93 (0.09)	2	31.07	<0.00

Notes: F and P values are from one-way ANOVA testing for differences among the sites. bdl: below detection limit; nd: non determined.

*: sites significantly different ($p<0.05$) from up_{stream} site based on the Tukey-HSD Post Hoc test.

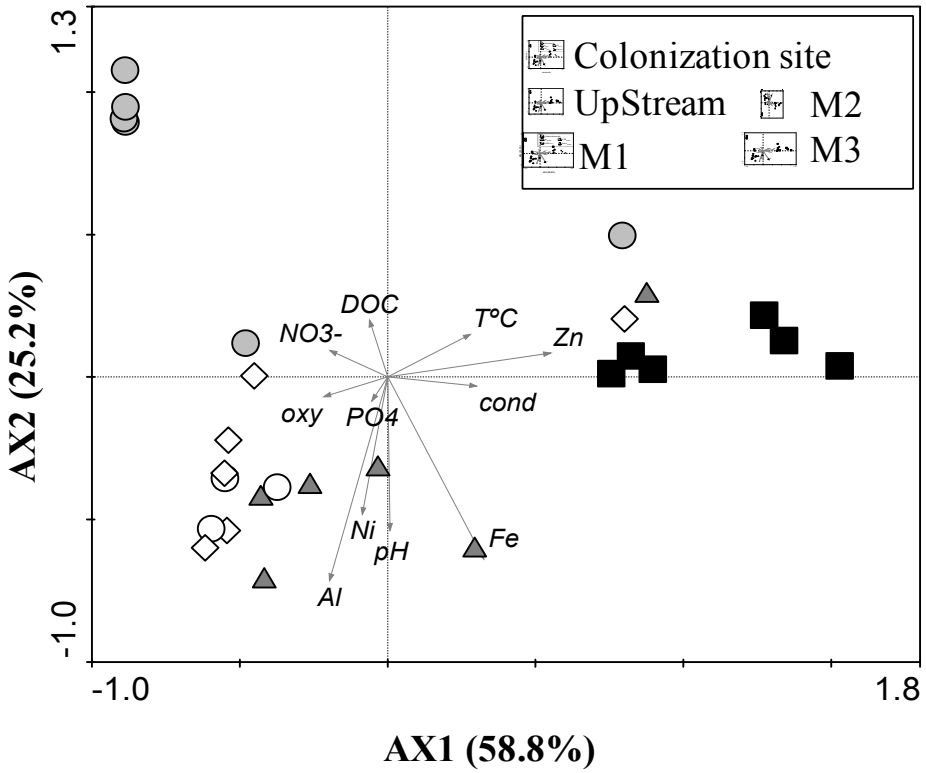


Fig. 2. Principal Components Analysis based on the physicochemical parameters of stream water measured in each site during the experiment summarized in Table 1.

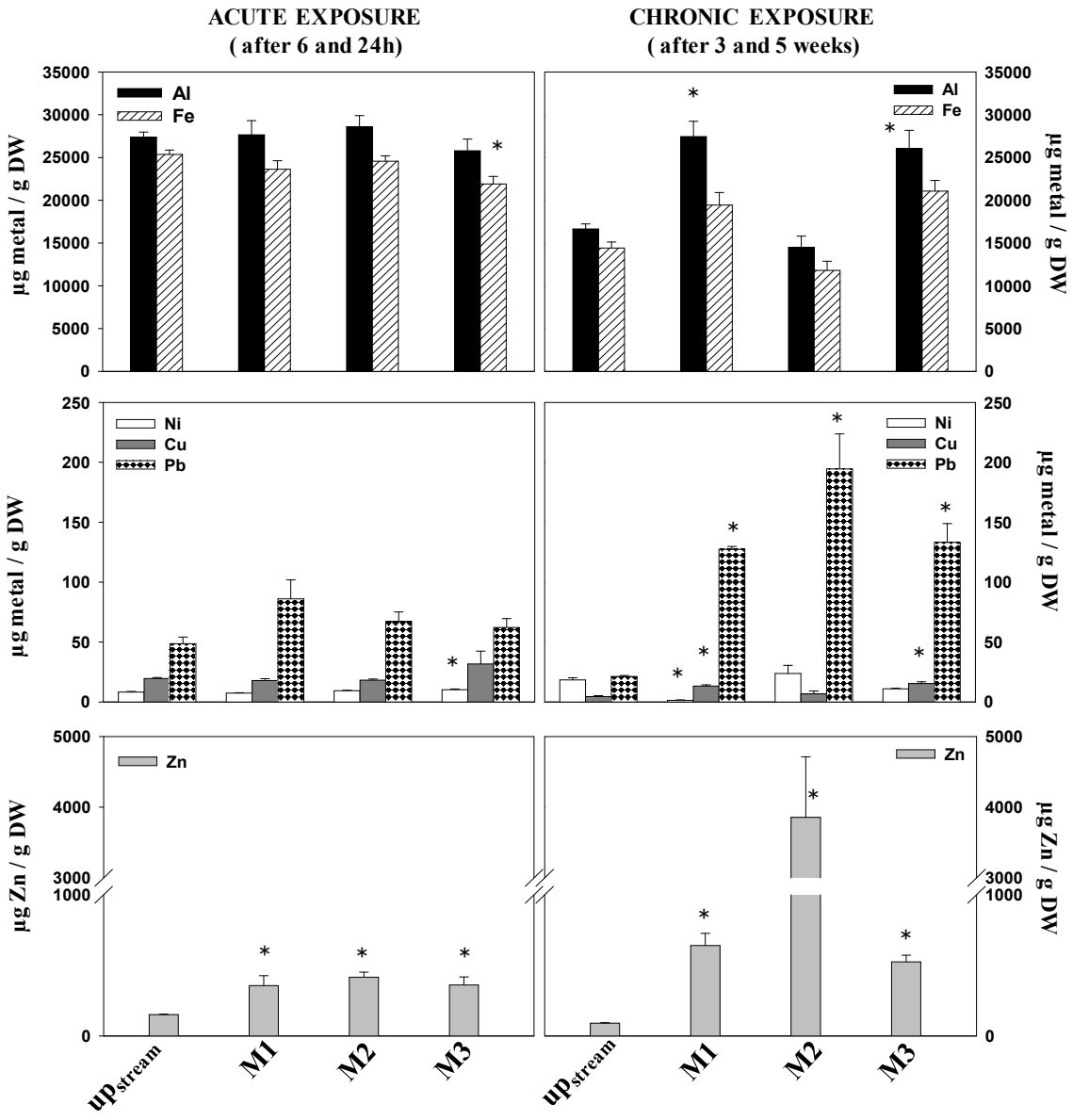


Fig.3. Metal bioaccumulation in biofilms translocated to up_{stream}, M1, M2 and M3 sites, after acute (6 and 24h) and chronic (3 and 5 weeks) times of exposure. Each column corresponds to the AVG±S.E; n=6. (*) indicate significant differences ($p<0.05$) from up_{stream} during the acute and chronic time of exposure analysed (Tukey's HSD test from ANOVA one way).

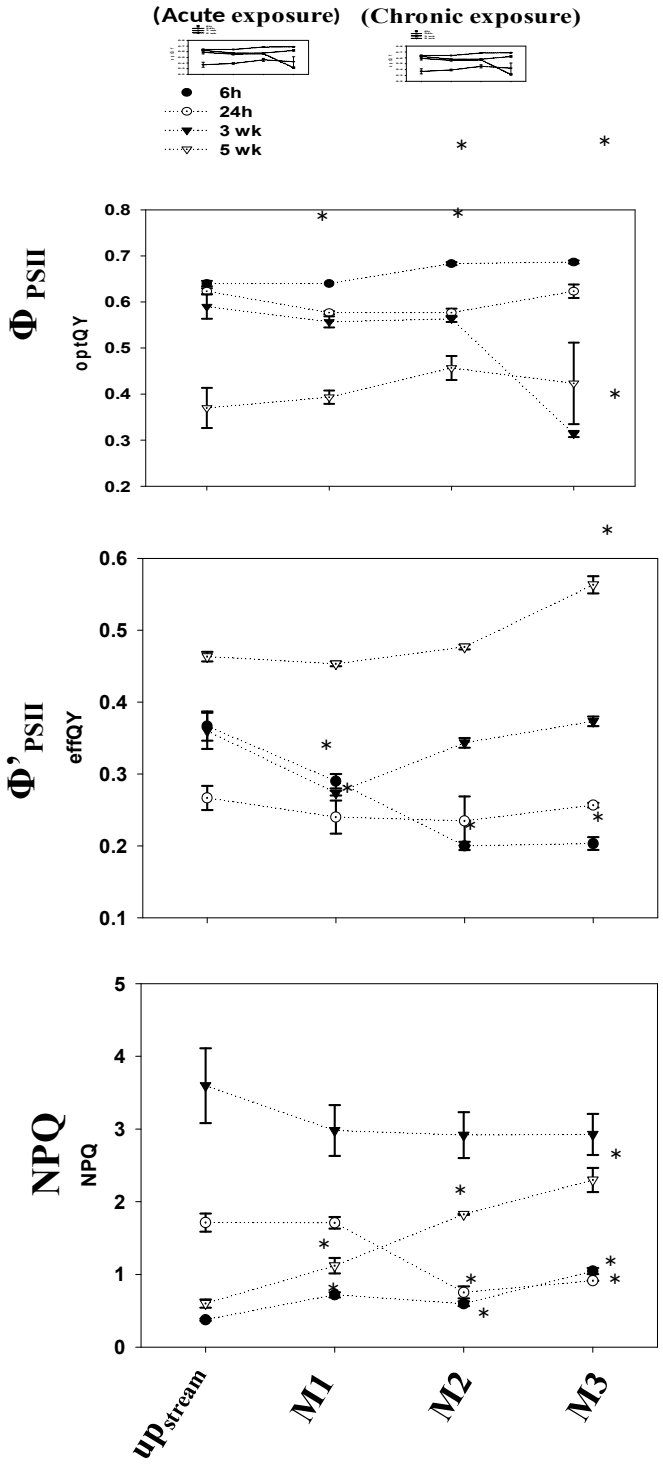


Fig.4. Chl-a fluorescence parameters of biofilm translocated to up_{stream}, M1, M2 and M3 sites after 6 and 24 hours of exposition (acute exposure) and after 3 and 5 weeks(chronic exposure). Each value corresponds to the AVG±S.E; n=3. (*) indicate significant differences ($p<0.05$) from up_{stream} during the exposure periods analysed (Tukey's HSD test from ANOVA one way).

Table 2. Relative abundance of pigments (%) from biofilm translocated after acute exposure (6 and 24h) and chronic exposure (3 and 5 weeks). Each value corresponds to the AVG±SE; n=3. Results of ANOVA one way tests are presented.

Acute exposure	up_{stream}	M1	M2	M3	ANOVA	
					$F_{(3,20)}$	p value
Chla	48.9 (1.45)	43.4 (2.35)	41.88(1.32)	49.85 (3.42)	2.91	0.06
Lutein	2.32 (0.14)	1.92 (0.28)	1.55 (0.18)	1.68 (0.36)	1.93	0.16
Diatoxanthin	0.44 (0.2)	0.12 (0.12)	nd	nd	3.04	0.53
Violaxanthin	2.81 (0.47)	0.59 (0.12)*	0.29(0.11)*	0.25 (0.11)*	27.94	0.00
Diadinoxanthin	3.22 (1.0)	4.05 (0.72)	4.61 (0.19)	3.81 (0.29)	1.357	0.284
Dinoxanthin	0.32 (0.16)	1.91 (0.46)*	2.47(0.14)*	1.99 (0.43)*	7.312	0.002
Pheophytin-a	0.33 (0.16)	0.58 (0.25)	0.34 (0.13)	0.41 (0.02)	0.404	0.752
β-Carotene	0.37 (0.24)	18 (0.18)	0.03 (0.01)	1.6 (0.89)	2.481	0.09
Antheroxanthin	nd	nd	0.08 (0.04)	0.04 (0.02)	3.524	0.034
Zeaxanthin	nd	nd	nd	nd		

Chronic exposure	up_{stream}	M1	M2	M3	ANOVA	
					$F_{(3,20)}$	p value
Chla	68.26 (0.71)	72.1 (0.81)	72.79(1.76)	74.17 (1.76)*	3.581	0.032
Lutein	0.8 (0.45)	0.26 (0.12)	3.07(0.35)*	1.62 (0.15)	15.332	<0.00
Diatoxanthin	0.05 (0.03)	0.42 (0.15)*	0.13 (0.09)	0.26 (0.04)	3.659	0.03
Violaxanthin	0.19 (0.03)	0.2 (0.03)	0.17 (0.04)	0.13 (0.01)	1.078	0.381
Diadinoxanthin	0.91 (0.08)	1.02 (0.04)	1.21 (0.37)	0.83 (0.07)	0.516	0.676
Dinoxanthin	1.1 (0.12)	1.05 (0.15)	0.82 (0.18)	0.67 (0.02)	2.482	0.09
Pheophytin-a	0.59 (0.13)	0.47 (0.03)	0.35 (0.07)	0.44 (0.06)	1.539	0.235
β-Carotene	0.27 (0.11)	0.11 (0.02)	0.14 (0.03)	0.34 (0.08)	2.454	0.093
Antheroxanthin	nd	0.19 (0.09)*	nd	0.01 (0.01)	4.461	0.015
Zeaxanthin	nd	0.72 (0.05)*	1.45(0.26)*	0.27 (0.13)	24.711	<0.00

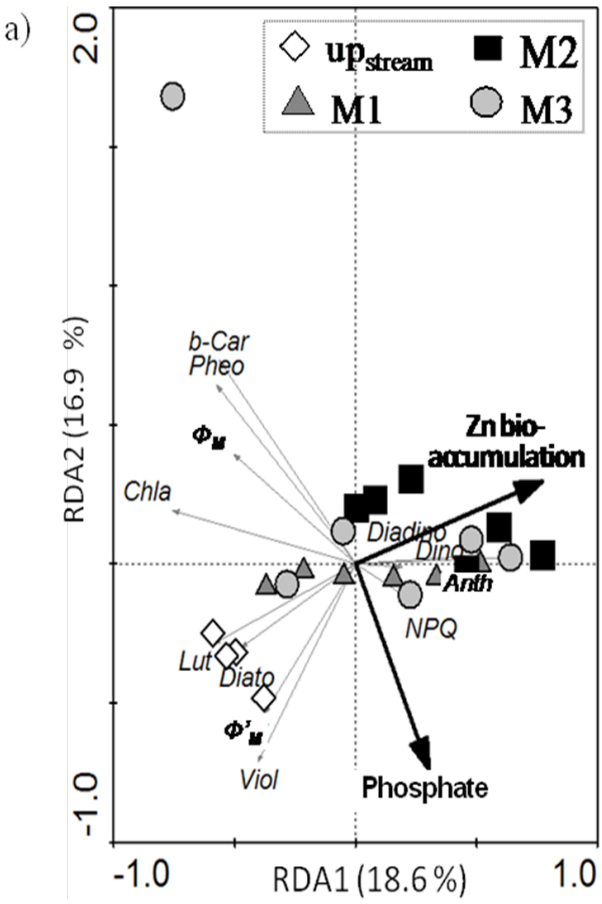
Notes: F and P values are from one-way ANOVA testing for differences among the sites. *p<0.05: sites significantly different from up_{stream} site based on the Tukey-HSD Post Hoc test. nd: non determined.

Table 3. Diatom metrics from biofilm translocated to up_{stream}, M1, M2 and M3 sites after 3 and 5 weeks of exposure. Each value corresponds to the AVG± SE; n=3. Results from ANOVA one way are presented.

Chronic exposure	up_{stream}	M1	M2	M3	ANOVA	
					$F_{(3,8)}$	p value
S	37.83 (1.27)	35.0 (1.93)	29.17 (1.30)*	39.17 (2.06)	7.173	0.002
teratoforms (%)	0.54 (00.23)	2.39 (0.56)*	3.35 (0.65)*	2.52 (0.25)*	10.33	<0.00
biovolume	1358.15 (49.44)	1830.43(118.7)	225.34 (26.45)*	1573.78 (83.65)	178.788	<0.00
IPS	15.22 (0.16)	14.25 (0.31)	8.62 (0.23)*	13.55 (0.21)*	159.408	<0.00

Notes: F and P values are from one-way ANOVA testing for differences among the sites. *p<0.05: sites significantly different from up_{stream} site based on the Tukey-HSD Post Hoc test.

a)


upstream
M2
M1
M3
2.0
-1.0
RDA2 (16.9 %)
-1.0
RDA1 (18.6 %)
1.0
b-Car
Pheo
Φ'M
Chla
Zn bio-accumulation
Diadino
Dino
Anth
NPQ
Lut
Diato
Φ'M
Viol
Phosphate

b)

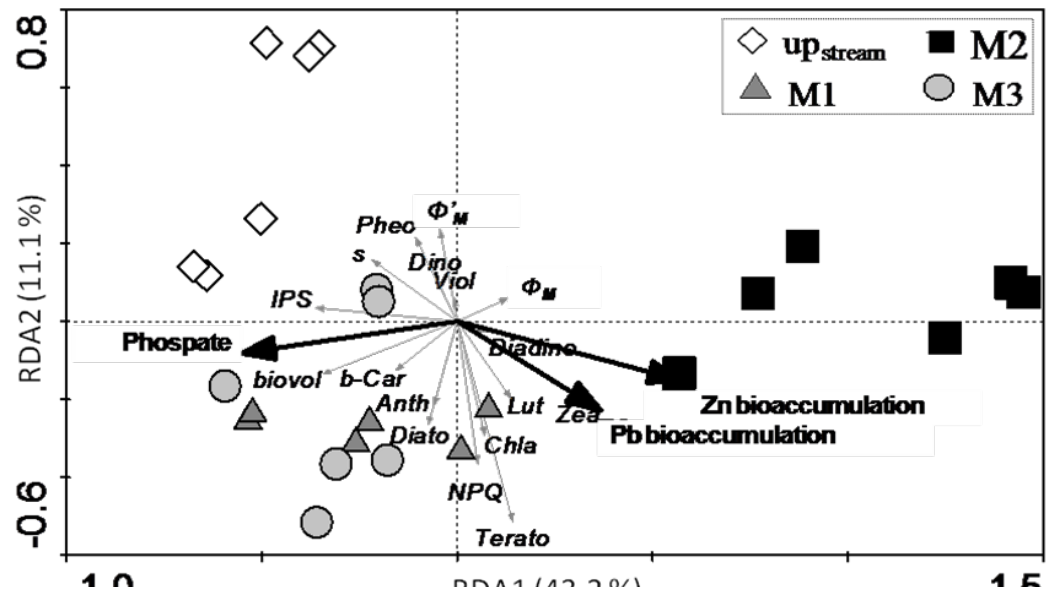

upstream
M2
M1
M3
0.8
-0.6
RDA2 (11.1 %)
Pheo
Φ'M
s
Dino
Viol
ΦM
IPS
Phospate
Diadino
biovol
b-Car
Anth
Lut
Zea
Zn bioaccumulation
Pb bioaccumulation
Diato
Chla
NPQ
Terato

Fig.5. Triplot based on redundancy analysis of biofilm metrics during the acute exposure (a): Chla fluorescence parameters and pigments composition and during the chronic exposure (b): Chla fluorescence parameters, pigments composition and diatom metrics.

Table 4. Correlation between physicochemical variables and metals with the axes (RDA1 and RDA2) of the Redundancy Analysis (RDA) performed with acute and chronic data (Fig. 4).

Acute exposure	**AX1**	**AX2**
Phosphate	0.270	-0.768
Zn bioaccumulation	0.686	0.308

Chronic exposure	**AX1**	**AX2**
Phosphate	-0.727	-0.203
Zn bioaccumulation	0.758	-0.379
Pb bioaccumulation	0.483	-0.569

Table 5. Results of the partial Redundancy Analysis (RDA) during acute exposure (Fig.4a). On the left the fraction of total variance is shown; bold is used to indicate the total variance higher than 10%. On the right, the fraction of explained variance (%) is shown: bold and underlined values indicate biofilm metrics mainly related with physicochemical or metal variables (% of explained variance >60 % and <30%).

Biofilm metrics	Fraction of total variance		Fraction of explained variance (%)	
	Physical and chemical variables	Metals	Physical and chemical variables	Metals
Φ_M	**24.59**	9.36	**<u>72.43</u>**	27.57
Φ'_M	9.5	**24.46**	27.97	**<u>72.03</u>**
NPQ	3.29	2.28	59.07	40.93
Chla	**17.38**	**34.46**	33.53	66.47
Lutein	0.1	**35.51**	0.28	**<u>99.72</u>**
Diatoxanthin	0.66	**25.21**	2.55	**<u>97.45</u>**
Violaxanthin	**19.59**	**33.11**	37.17	62.83
Diadinoxanthin	0.57	2.6	17.98	82.02
Dinoxanthin	2.41	**21.34**	10.15	**<u>89.85</u>**
Pheophytin-a	**52.13**	8.22	**<u>86.38</u>**	13.62
β-Carotene	**62.48**	6.2	**<u>90.97</u>**	9.03
Antheroxanthin	3.76	**28.38**	11.70	**<u>88.30</u>**

Table 6. Results of the partial Redundancy Analysis (RDA) during chronic exposure (Fig. 4b). On the left the fraction of total variance is shown; bold is used to indicate the total variance higher than 10%. On the right, the fraction of explained variance (%) is shown: bold and underlined values indicate biofilm metrics mainly related with physicochemical or metal variables (% of explained variance >60 % and <30%).

Biofilm metrics	Fraction of total variance		Fraction of explained variance (%)	
	Physical and chemical variables	Metals	Physical and chemical variables	Metals
Φ_M	0.44	**16.11**	2.66	**<u>97.34</u>**
Φ'_M	**27.77**	**29.9**	48.15	51.85
NPQ	**29.69**	**33.98**	46.63	53.37
Chla	0.57	**27.19**	2.05	**<u>97.95</u>**
Lutein	4.02	**18.6**	17.77	**<u>82.23</u>**
Diatoxanthin	1.54	**17.56**	8.06	**<u>91.94</u>**
Violaxanthin	0.64	3.22	16.58	83.42
Diadinoxanthin	5.79	**17.39**	24.98	**<u>75.02</u>**
Dinoxanthin	2.71	8.66	23.83	76.17
Pheophytin-a	1.03	**11.96**	7.93	**<u>92.07</u>**
β-Carotene	**18.61**	0.85	**<u>95.63</u>**	4.37
Antheroxanthin	2.1	9.03	18.87	81.13
Zeaxanthin	4.14	**15.38**	21.21	**<u>78.79</u>**
S	2.09	**27.45**	7.08	**<u>92.92</u>**
teratoforms (%)	8.14	**54.23**	13.05	**<u>86.95</u>**
IPS	5.75	**20.54**	21.87	**<u>78.13</u>**
biovolumne	8.65	**20.92**	29.25	**<u>70.75</u>**