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Physiological responses of three mono-species phototrophic biofilms exposed to copper and zinc

Emilie Loustau^{1,2} · Jessica Ferriol¹ · Shams Koteiche¹ · Léo Gerlin¹ · Joséphine Leflaive¹ · Frédéric Moulin³ · Elisabeth Girbal-Neuhauser² · Jean-Luc Rols¹

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Abstract

In freshwater ecosystem, phototrophic biofilms play a crucial role through adsorption and sequestration of organic and inorganic pollutants. However, extracellular polymeric substance (EPS) secretion by phototrophic biofilms exposed to metals is poorly documented. This work evaluated the physiological responses of phototrophic biofilms by exposing three microorganisms (cyanobacterium *Phormidium autumnale*, diatom *Nitzschia palea* and green alga *Uronema confervicolum*) to 20 and 200 $\mu\text{g L}^{-1}$ of Cu or 60 and 600 $\mu\text{g L}^{-1}$ of Zn, both individually and in combination. Analysis of metal effects on algal biomass and photosynthetic efficiency showed that metals were toxic at higher concentrations for these two parameters together and that all the strains were more sensitive to Cu than to Zn. *U. confervicolum* was the most impacted in terms of growth, while *P. autumnale* was the most impacted in terms of photosynthetic efficiency. In consequence to metal exposure at higher concentrations (Cu200, Zn600 and Cu200Zn600), a higher EPS production was measured in diatom and cyanobacterium biofilms, essentially caused by an overproduction of protein-like polymers. On the other hand, the amount of secreted polysaccharides decreased during metal exposure of the diatom and green alga biofilms. Size exclusion chromatography revealed specific EPS molecular fingerprints in *P. autumnale* and *N. palea* biofilms that have secreted different protein-like polymers during their development in the presence of Zn600. These proteins were not detected in the presence of Cu200 despite an increase of proteins in the EPS extracts compared to the control. These results highlight interesting divergent responses between the three mono-species biofilms and suggest that increasing protein production in EPS biofilms may be a fingerprint of natural biofilm against metal pollutants in freshwater rivers.

Keywords Metal toxicity · Photosynthetic efficiency · EPS production · Protein stimulation

Introduction

Soils and rivers are largely contaminated through anthropogenic activities by receiving regularly metals from industrialization,

urbanization, mining, fertilizers and pesticide applications (Pacyna et al. 2007). Over a billion tons of toxic metals have been released into the environment during the last 30 years (Haferburg and Kothe 2012). One consequence is the degradation of surface water quality in many parts of the world with the associated risks for human health (Lone et al. 2008). Copper and zinc are released into the environment mainly by mining and agriculture (Hogsden and Harding 2012; Johnson and Hallberg 2005). The contamination by these metals has been increasing in aquatic environment through leaching of soils and run-off from agricultural land (Weingerl and Kerin 2000; Rabiet et al. 2015). Copper is essential for photosynthesis and mitochondrial respiration, for carbon and nitrogen metabolisms and for oxidative stress protection and is required for cell wall synthesis (Hänsch and Mendel 2009). Zinc plays critical roles in a wide variety of biochemical processes (Hänsch and Mendel 2009; Li et al. 2012); it is also important for energy production and maintains

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the structural integrity of bio-membranes. If copper and zinc are essential micronutrients for algae, when their concentration is largely above those required for optimal growth, they are deleterious to the cells, causing serious threat to aquatic ecosystems and becoming a global issue.

Many prokaryotic and eukaryotic microorganisms such as bacteria, fungi and microalgae colonize the substratum in a shallow aquatic environment to form phototrophic biofilms (Kroll et al. 2016). Phototrophic biofilms are known for their essential ecological functions such as primary production, oxygen production and nutrient cycling (Lamberti 1996). They serve as habitat for different protists and invertebrates and have the capacity to respond quickly to environmental stressors (Lyautey et al. 2005). In aggregated form, microorganisms are known to produce a complex matrix of extracellular polymeric substances (EPS) largely composed of polysaccharides and proteins. EPS are the major contributor to the mass of phototrophic biofilm.

The toxic effects of metals on the microbial community of phototrophic biofilms are essential to know because these biofilms represent the first link in the food web of many aquatic environments (Fabrega et al. 2011). Since the early investigations by Reese (1937) and Williams and Mount (1965), the effects of metals on phototrophic biofilm communities have been largely studied. It was reported that exposures to metals such as Cu or Zn induced changes in the abundance of species and the composition of the microbial community. Differences in sensitivity to metals among species composing biofilm communities induce community tolerance, a concept introduced by Blanck et al. (1988). This tolerance can be explained by the development of acquiescence mechanisms or by a natural selection of species: biofilms treated with various concentrations of Cu (0.63 or 6.35 mg L⁻¹) or Zn (7.85 mg L⁻¹) showed a clear shift in species composition (García-Meza et al. 2005; Xu et al. 2016). Compared to other metals such as mercury, an exposure to Cu resulted in a greater growth inhibition of freshwater diatom even at low concentration (100 µg L⁻¹) and after short duration (24 h) of exposure (Mu et al. 2017). Concerning chronic exposure during 28 days, cyanobacterium cells were more affected by Cu (100 to 300 µg L⁻¹) compared with other divalent metals (Pb, Cd or Li) with higher exposure concentrations (Mota et al. 2015). The growth rate inhibition of two freshwater green algae species increased as Cu concentration (1 and 5 mg L⁻¹) or exposure time (1 to 72 h) increased (Angel et al. 2017). On the contrary, exposure to low Zn concentrations (under 250 µg L⁻¹) accelerated the growth of green alga species (Cao et al. 2015).

EPS participate to the maintenance of the biofilm integrity (Bellinger et al. 2010; Sheng et al. 2010; Stewart et al. 2013). EPS production can serve as a protection mechanism against various stresses (Levy et al. 2007; Staats et al. 2000) and can mediate the exchanges of substances between benthic organisms and water column. EPS production is considered as the first protective barrier against toxic substances and, in particular, against metal income into the cells (Pistocchi et al. 2000;

Sheng and Liu 2011). Extracellular polysaccharides produced by phototrophic biofilms contain acidic sugars that show a high affinity for bivalent metal cations (Rossi et al. 2012), suggesting their ability to adsorb and accumulate metal cations and nanoparticles present in the water column (Coutaud et al. 2014, 2018, 2019). However, the secretion of EPS, in response to a stress situation like nutrient limitation, is extremely dependent on the existing environmental conditions (Stewart and Franklin 2008). Using confocal laser-scanning microscopy, García-Meza et al. (2005) visualised an increase of EPS production by a phototrophic biofilm mostly composed of green algae and cyanobacteria under Cu exposure (0.635 and 6.35 mg L⁻¹). In contrast, Cao et al. (2015) showed only a small increase of extracellular protein production by the green alga *Cladophora* after treatment with Cu and Zn, especially at low concentrations (100 to 500 µg L⁻¹), and the amount of polysaccharides produced by the cyanobacterium *Cyanothece* sp. was not enhanced by the presence of these metals (Mota et al. 2015). These few results show that the effects of metals on the qualitative and quantitative composition of EPS in the extracellular matrix of phototrophic biofilm are still poorly understood and remain largely under-investigated.

The aim of the present study was to test the hypotheses that in realistic hydrodynamic conditions (i) exposure to Cu and Zn will modify qualitatively and quantitatively the EPS produced by benthic algae and (ii) these modifications will be species-dependent and (iii) will occur at non-inhibiting concentrations. In this purpose, three species largely represented in phototrophic biofilms, i.e. the diatom *Nitzschia palea*, the cyanobacterium *Phormidium autumnale* and the green alga *Uronema confervicolum*, were used to form mono-species biofilms. In order to simulate natural water flow conditions, these strains were cultivated in artificial streams. The tolerance of these microorganisms was tested at values of 60 and 600 µg L⁻¹ for Zn and 20 and 200 µg L⁻¹ for Cu, corresponding to concentrations less than the half maximal inhibitory concentration (IC₅₀) obtained by Coutaud et al. (2019) for Zn (around 13 mg L⁻¹) and Cu (around 4 mg L⁻¹) with a phototrophic biofilm rich in cyanobacteria. The physiological response of the biofilms towards metal exposure was investigated through their effect on biomass growth, on photosynthetic efficiency and on EPS production. Polysaccharides and proteins were extracted from the biofilms and quantified as an estimation of EPS composition. Molecular weight (MW) distribution of extracted EPS was suggested as a useful tool for EPS fingerprint characterisation.

Material and methods

Maintenance of algal strains

The three benthic phototrophic microorganisms used in this study, the cyanobacterium *Phormidium autumnale*, the

diatom *Nitzschia palea* and the green alga *Uronema confervicolum*, were isolated from phototrophic biofilms in the rivers Tarn and Garonne (southwestern France). These three species are maintained in non-axenic conditions in the algae collection of EcoLab (Toulouse, France) at 18 °C under white light of $30 \pm 5 \mu\text{mol s}^{-1} \text{m}^{-2}$ with light/dark periods of 16 h/8 h. Individual pre-cultures of the three species were prepared for 21 days in 500 mL Erlenmeyer flasks with adapted nutrient media: BG11 medium (Stanier et al. 1971) for *P. autumnale* and Combo medium (Kilham et al. 1998) for *N. palea* and *U. confervicolum*. Before inoculation in the hydraulic mini-channels, the algal biomass was homogenised by using an Ultra-Turrax disperser (T25, Janke-Kunkel, 13,500 rpm, 1 min) and subdivided in equal inocula.

Experimental setup

The experiment was carried out in a dedicated device composed of several hydraulic mini-channels (see Supplementary data section A, Fig. A.1). The study was conducted with a series of three experiments, each experiment corresponding to the use of one of the three species of phototrophic microorganisms (Fig. 1). For one monospecific biofilm, three modules were used, each one composed of a 12 mini-channel plate devised in 3 blocks. The operating conditions were the following. Physico-chemical parameters fixed for the circulating water in the mini-channels were a temperature of 20 ± 0.2 °C, a pH of 8 ± 0.1 (pH meter Multi 3430 SET C) during the light period and a flow rate of 300 L h^{-1} obtained with the combination of the manual valve and a slope of 1.2%. The choice of this flow rate resulted from the analysis of flow regime in mini-channels (homogeneous turbulent flow on the coupon along the mini-channels, see Supplementary data section B) and the elemental mixing in each block (mixing time < 1 min, see Supplementary data section C). The light intensity was

$71.5 \pm 13.5 \mu\text{mol s}^{-1} \text{m}^{-2}$ with a circadian cycle of light/dark periods of 16 h/8 h. For each condition, the lower tanks were filled with 50 L of nutrient media adapted to each species and a suspension of biofilm biomass was added. After 5 days, the time required for the colonisation of coupons by species, copper (CuSO_4) or zinc ($\text{ZnSO}_4 \cdot \text{H}_2\text{O}$) was added in the circulating water to reach the final concentrations, specific to each block of four mini-channels. In each module, one block was a control (without metal addition) and the two others contained one or two metals at two different concentrations: 20 and $200 \mu\text{g L}^{-1}$ for Cu (Cu20 and Cu200, respectively), 60 and $600 \mu\text{g L}^{-1}$ for Zn (Zn60 and Zn600, respectively) or a mix of the metals Cu20Zn60 and Cu200Zn600. The lowest concentrations are closed to concentrations recorded in polluted rivers (Montuelle et al. 2010; Gaillardet et al. 2014). Incubations with metals were conducted during 16 days. To avoid nutrient depletion, 100 mL of a concentrated solution were added every week, consisting of NO_3^- at 2.44 g L^{-1} and PO_4^{3-} at 0.228 g L^{-1} for the three species and additional SiO_2 at 5 g L^{-1} for the diatom. Finally, at day 21, biofilms were scrapped from the coupons with a sterile toothbrush. One replicate was sampled in each mini-channel, thus giving four replicates per block. Replicates were constituted by 10 coupons suspended in 15 mL of culture media for the characterisation of biofilm biomass, an over identical suspension for bacterial community structure, and by 15 coupons suspended in 100 mL for EPS extraction.

Thermodynamic modelling

The concentration and speciation of dissolved Cu and Zn in BG11 and Combo nutrient media as well as solution saturation degree with respect to Cu and Zn minerals were calculated using Visual MINTEQ (Gustafsson 2011, version 3.1 for Windows, October 2014) in conjunction with a database and

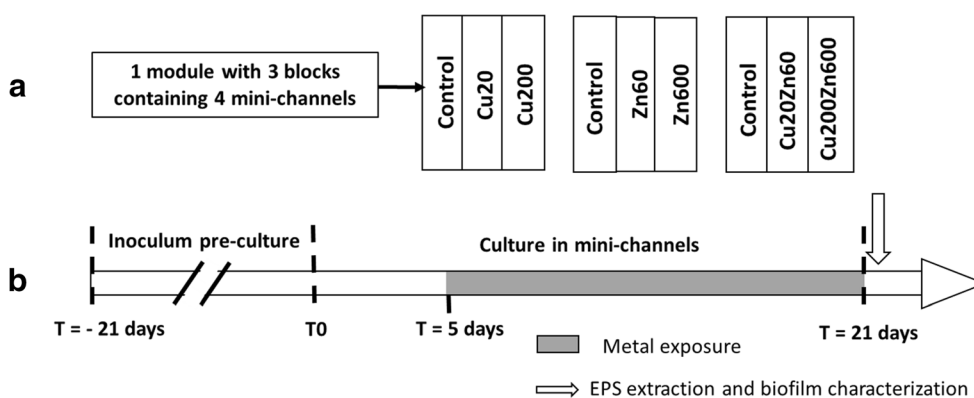


Fig. 1 a Experimental design realised for the three species of phototrophic microorganisms. In each module, one experimental condition is the same in the four mini-channels of one block. Seven different conditions were tested: control (biofilms without metal exposure), Cu20 or Cu200 (biofilms exposed to 20 or $200 \mu\text{g L}^{-1}$ of Cu,

respectively), Zn60 or Zn600 (biofilms exposed to 60 or $600 \mu\text{g L}^{-1}$ of Zn, respectively), Cu20Zn60 (biofilm exposed to $20 \mu\text{g L}^{-1}$ of Cu and $60 \mu\text{g L}^{-1}$ of Zn) and Cu200Zn600 (biofilm exposed to $200 \mu\text{g L}^{-1}$ of Cu and $600 \mu\text{g L}^{-1}$ of Zn). (b) Experimental setup where T0 = time of inoculation of the mini-channels

the NICA-Donnan humic ion binding model (Benedetti et al. 1995; Milne et al. 2003). The calculations were performed for two nutrient media without metal addition and with Cu and Zn at two different concentrations: 20 and 200 $\mu\text{g L}^{-1}$ for Cu and 60 and 600 $\mu\text{g L}^{-1}$ for Zn. The input parameters of the model were pH, temperature, concentrations of dissolved organic carbon, Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- , SO_4^{2-} , PO_4^{3-} , NO_3^- , CO_3^{2-} , H_4SiO_4 , H_3BO_3 , MoO_4^{2-} , Se, EDTA^{4-} , citrate^{3-} , NH_4^+ , Fe^{3+} , Zn^{2+} and Cu^{2+} . The model allowed solids with positive saturation index (S.I.) values to precipitate.

Biofilm biomass

Biofilm suspension obtained as described above was used to assess biofilm biomass by measuring the dry weight (DW) after 72 h of drying at 70 °C.

PhytoPAM measurements

PhytoPAM measurements were performed directly in the mini-channels by measuring fluorescence response to a saturating light flash after 20 min of dark adaptation (dark-adapted state (DAS) conditions) (Baker 2008). For each condition in one block, 32 total values were performed (eight measures per mini-channel).

The physiological state of algae was estimated from the maximal efficiency of the photosystem II (yield), corresponding to the transfer rate of electrons, thanks to the formula defined by Roháček and Barták (Roháček and Barták 1999):

$$\Phi_{PSII} (\text{DAS conditions}) = 1 - (F_0 / F_M); 0 < \Phi_{PSII} < 1 \quad (1)$$

F_M represents the maximal fluorescence and F_0 corresponds to the minimal fluorescence of the photosystem II, in DAS conditions. The photosynthetic efficiency is specific to each algal group, so no comparison is permitted between the values of the different species used here.

Prokaryotic community structure

In this experiment, the algal strains were not axenic. The metals potentially impacted the heterotrophic bacteria associated with each alga, heterotrophic bacteria that are also EPS producers. We assumed that the variations of EPS production we observed were linked to algal physiology and not to modifications of the associated heterotrophic bacteria, notably because alga represented by far most of the biomass. Genetic prokaryote diversities were monitored with ARISA (Automated Ribosomal Intergenic Spacer Analysis) (Fisher and Triplett 1999). The DNA of biofilm communities was extracted with the DNeasy Plant Mini Kit (Qiagen), the lysis buffer of the first step being directly applied on the pellet. ARISA PCR was performed using the universal bacterial

primers FAM-labelled ITSReub (GCCAAGGCATCCACC) and ITSr (GTCGTAACAAGGTAGCCGTA) (Cardinale et al. 2004). An initial denaturation step at 94 °C for 5 min was followed by 35 cycles at 91 °C for 1 min, 56 °C for 1 min and 72 °C for 2 min. Finally, an extension was carried out at 72 °C for 10 min.

After denaturation at 96 °C for 3 min in the presence of formamide (37%), the PCR products were analysed on 3130 xl Capillary Genetic Analyser (Applied Biosystems) together with a DNA size standard ladder LIZ1200 (Applied Biosystems), and the results were analysed with the Peak Scanner 1.0 software (Applied Biosystems). The size of DNA fragments was rounded to the nearest whole number. Fragments shorter than 200 bp were excluded from the analysis. The area under the peaks was normalised to the total area of the sample, and peaks corresponding to less than 0.1% of total area were excluded from the analysis. For each couple of primers, peaks on the chromatogram, corresponding to OTUs, were scored as present or absent from the chromatogram analysis and gathered in taxa (OTU)-presence matrix.

Extracellular polymeric substance assays

EPS extraction

EPS were extracted from biofilms following the sequential method developed previously by Loustau et al. (2018). The biomass suspension was firstly homogenised by pre-treatment with an Ultra-Turrax disperser (T25, Janke-Kunkel) at 13,500 rpm for 1 min at room temperature. Then, a volume of the homogenised suspension corresponding to 10 mg DW was centrifuged at 4000 g for 15 min. The pellet was washed with PBS solution (phosphate buffer saline, 10 mM $\text{HPO}_4^{2-}/\text{H}_2\text{PO}_4^-$ at pH 7.4, with 2.7 mmol L^{-1} of KCl and 137 mmol L^{-1} of NaCl) and centrifuged again. Two EPS extraction steps were applied in sequence with intermediate centrifugations (4000 g for 15 min) to collect the supernatants containing the solubilised EPS. In the first step, the biomass pellet was suspended in 2 mL of PBS solution containing 12 μL of 37% formamide, and the tube was shaken gently on a rotary disc shaker at 150 rpm and 4 °C for 60 min. Centrifugation at 4000 g for 15 min allowed to recover the EPS released in the supernatant (called "S-F"). In the second step, the biomass pellet was re-suspended with 2 mL of PBS solution with addition of cation exchange resin (CER, Dowex Marathon) with a final dosage of 50 g g^{-1} DW. The suspension was stirred for 90 min at 150 rpm and 4 °C. Then, the sample was centrifuged at 4000 g for 15 min to recover the supernatant (called "S-CER1"). PBS was added again on the pellet to clean up the beads allowing a second supernatant to be collected after centrifugation (called "S-CER2"). The amount of EPS extracted during the CER extraction step (S-

CER) was the sum of the EPS amounts contained in both supernatants ($S\text{-CER} = S\text{-CER1} + S\text{-CER2}$).

The cellular integrity of phototrophic cells during the extraction steps was controlled by the measurement of chlorophyll *a* release in the EPS extracts (Loustau et al. 2018). It allows to estimate for each biofilm sample the percentage of phototrophic cell lysis due to the extraction treatments. It is assumed that the lysis of heterotrophic bacteria growing in the non-axenic phototrophic biofilms is negligible, the conditions of EPS extraction being gentle compared to those used for example for activated sludge with sonication as first step and higher centrifugation rate (Ras et al. 2008).

Protein and polysaccharide quantification

For each EPS extract, protein concentration was measured by the bicinchoninic acid (BCA) method (Smith et al. 1985). For 25 μL of each sample, 200 μL of BCA reagent (Sigma-Aldrich) were incubated in a 96-well microplate (15 min at 60 °C) before measuring the optical density at 540 nm with a microplate reader (Synergy Mx Biotek). Bovine serum albumin (BSA) was used as a standard with concentrations ranging from 0 to 800 mg L^{-1} .

Polysaccharides concentrations were estimated using the anthrone method (Dreywood 1946) allowing total carbohydrates to be quantified: 100 μL of each sample and 200 μL of anthrone reagent (2% anthrone in 96% sulfuric acid) were incubated in a 96-well microplate (30 min at 60 °C), cooled at room temperature for 10 min before measuring the optical density at 620 nm with the microplate reader. Glucose was used as a standard with concentrations ranging from 0 to 100 mg L^{-1} .

For each replicate from one mini-channel, the quantification of the amounts of polysaccharides and proteins was realised three times.

Size exclusion chromatography

Size exclusion chromatography (SEC) can be used to characterise the fingerprint and/or the molecular weight distribution of EPS (Sheng et al. 2010). The size distribution of extracted EPS (S-CER) was investigated by SEC using an AKTA Purifier System (GE Healthcare), equipped with a 250- μL injection loop and two columns: a Superdex peptide 10/300 GL (GE Healthcare) with a resolving range 0.3–10 kDa, followed by a Superdex 200 10/300 GL (GE Healthcare) with a resolving range 10–600 kDa. The exclusion volume of this chromatographic system was estimated to be 15 mL and the total permeation volume to be 46 mL. All measurements were performed using PBS as eluent with a flow rate of 0.4 mL min^{-1} . Detection of total organic and inorganic compounds was carried out at 210 nm, and detection of protein-like polymers was performed with fluorescence by excitation

and emission wavelengths of 221 nm/350 nm allowing the detection of tryptophan fluorescence (Bhatia et al. 2013).

The apparent molecular weight of the eluted molecules was calculated from a calibration curve obtained after injection of a mix of different standard proteins purchased from GE Healthcare (thyroglobulin, 669 kDa; aldolase, 158 kDa; conalbumin, 75 kDa; carbonic anhydrase, 29 kDa; ribonuclease A, 13.7 kDa; aprotinin, 6.511 kDa) or from Sigma-Aldrich (angiotensin II, 1.046 kDa; leucine enkephaline, 0.556 kDa; thyrotropin, 0.362 kDa).

The following equation was obtained for this calibration curve:

$$\text{Log (MW)} = -0.1421 \text{ Ve} + 8.316 \quad (R^2 = 0.9923) \quad (2)$$

with MW (molecular weight) expressed in dalton and Ve (peak elution volume) in millilitres.

For the three alga species, the extracted EPS by CER from each replicate were pooled by block. The size distribution was defined for the experiments with Cu200 and Zn600 and their controls.

Statistical analyses

Statistical analyses were performed using the PAST software (Paleontological Statistics, version 2.17 and 3.06) (Hammer et al. 2001). For all analyses, the normality was checked on each dataset (EPS quantity, biomass, PhytoPam) with the Shapiro–Wilk test and data were transformed if needed. When data were normally distributed, two-way ANOVAs were used to test the effects of copper and zinc, followed by a Tukey post hoc test. The non-parametric Kruskal–Wallis test was used with Mann–Whitney test for pairwise comparisons of non-parametric data. Data given in the text are means \pm standard error (SE). For all statistical analyses, significance was inferred at $p < 0.05$.

Results

Concentration and speciation of dissolved Cu and Zn in nutrient media

The speciation calculation using vMinteq has shown that Cu and Zn are fully dissolved in both nutrient media Combo and BG11, except for BG11 medium with Cu200, where the percentage of dissolved Cu is of 61% (see Supplementary data section D, Table D.1). Both nutrient media were undersaturated with respect to Cu and Zn minerals (S.I. < 0), but BG11 medium with Cu200 was found to be oversaturated with respect to cupric ferrite (S.I. = 19.8), which leads to precipitation of 39% of Cu in apparent equilibrium (S.I. = 0).

For both media, simulation with vMinteq has showed that major parts of Cu and Zn are in the form of complexes mostly with EDTA anions: for the Combo medium 74.24–97.96% of Cu and 94.55–99.98% of Zn (see Supplementary data section D, Table D.2) and for the BG11 medium 66.31–99.87% of Cu and 99.9% of Zn for control and with Zn60 (see Supplementary data section D, Table D.3). Dissolved Zn in the BG11 medium with Zn600 is in the form of complexes with citrate anions (53.61%) and with EDTA anions (14.22%) and 25.9% of Zn is in the form of free ions.

Effect of metals on biomass growth

Compared to their controls, no significant effects were observed on biomass growth for *P. autumnale* and *U. confervicolum* biofilms exposed to copper and/or zinc at the lower concentrations (Cu20 or Zn60 or Cu20Zn60). Even at these low concentrations, *N. palea* biofilm appeared more sensitive to Cu than to Zn exposure showing a $27 \pm 2\%$ decrease in biofilm biomass with Cu20 ($p = 0.043$) (Fig. 2). At the higher metal concentrations (Cu200 or Zn600, respectively), significant biomass reduction was observed for the *N. palea* biofilm— $38.1 \pm 4.6\%$ ($p = 0.019$) and $29.3 \pm 0.8\%$ ($p = 0.022$)—and for the *U. confervicolum* biofilm— $80.8 \pm 5.7\%$ ($p = 0.021$) and $42.2 \pm 9.4\%$ ($p = 0.042$). Noticeably, at similar concentrations, no effect was detected on the growth of

the cyanobacteria *P. autumnale*. When the two metals were added simultaneously (Cu200Zn600), a synergetic effect was nevertheless observed for this cyanobacteria with a decrease in biomass growth above 50% ($p = 0.016$). This was not the case for the two other species since no significant differences in biofilm growth between Cu200 and Cu200Zn600 exposure were observed for *N. palea* and *U. confervicolum* biofilms.

Effect of metals on photosynthetic efficiency

For the three phototrophic species and compared to their controls, no significant deleterious effect on Φ_{PSII} was exerted with Cu20 or Zn60 (Fig. 3). Important decrease by $39.2 \pm 5.87\%$ ($p < 0.0001$) was nevertheless observed after exposure of *P. autumnale* species to a mixture of the two metals at similar low concentrations (Cu20Zn60).

At the higher metal concentrations, the photosynthesis efficiency was particularly reduced for *P. autumnale* species with a decrease of 32.8 ± 3.4 and $20.5 \pm 2.5\%$ with Cu200 and Zn600, respectively. In these conditions, photosynthesis efficiency was only reduced by 10.2 ± 4.2 and $8.5 \pm 2.1\%$ for *N. palea* species and by 17.8 ± 2.6 and $7.5 \pm 1.3\%$ for *U. confervicolum* species.

The highest decreases of the Φ_{PSII} compared to the controls were observed with Cu200Zn600 giving values of $29.3 \pm 1.7\%$ ($p = 0.008$) for *N. palea* species, $52.1 \pm 4.2\%$

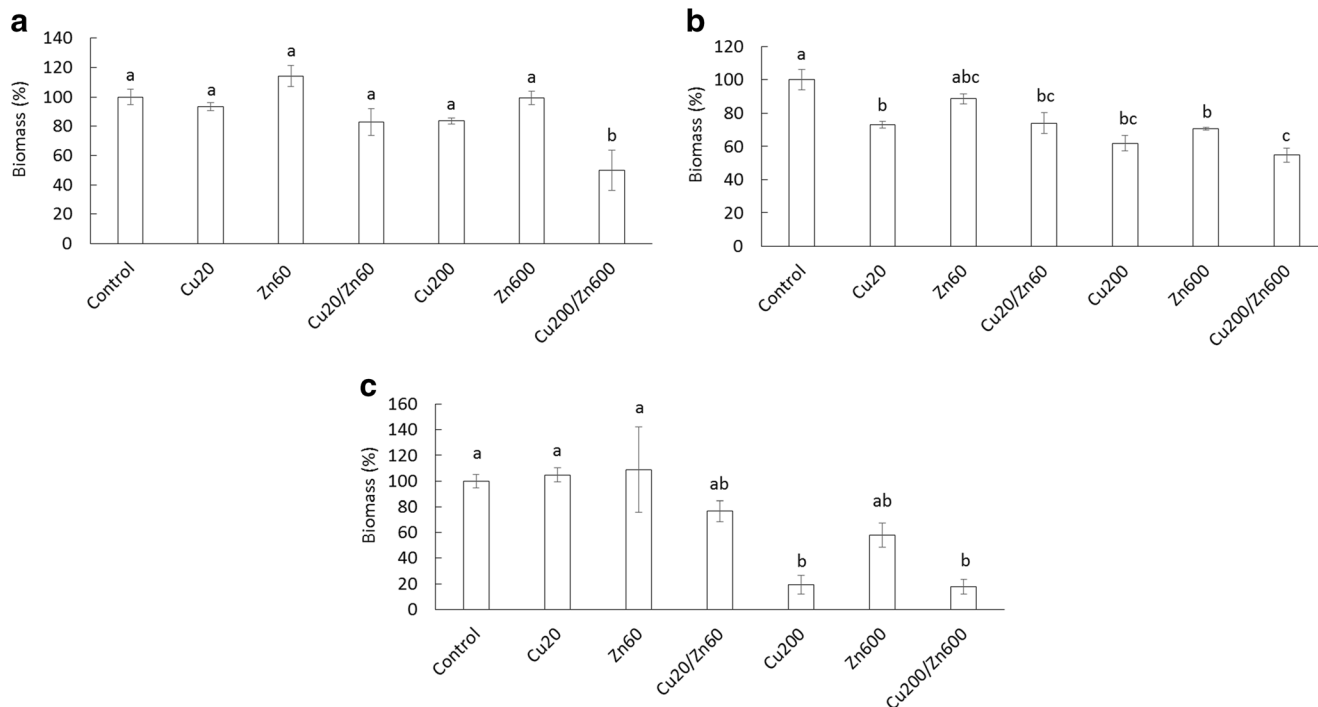


Fig. 2 Effects of copper and zinc exposure on the cyanobacterium (*P. autumnale*; a), the diatom (*N. palea*; b) and the green alga (*U. confervicolum*; c) biomasses ($n = 4$, mean \pm SD) compared to the control biomass ($n = 12$, mean \pm SD). The mean control value was fixed at 100% (5.4 ± 0.3 , 3.0 ± 0.2 and 2.1 ± 0.1 mg DW cm^{-2} for *P. autumnale*,

N. palea and *U. confervicolum* biofilms, respectively). The biofilms were exposed under 16 days to copper at $20 \mu\text{g L}^{-1}$ (Cu20) or $200 \mu\text{g L}^{-1}$ (Cu200), to zinc at $60 \mu\text{g L}^{-1}$ (Zn60) or $600 \mu\text{g L}^{-1}$ (Zn600) and to a mixture of the metals (Cu20Zn60 or Cu200Zn600)

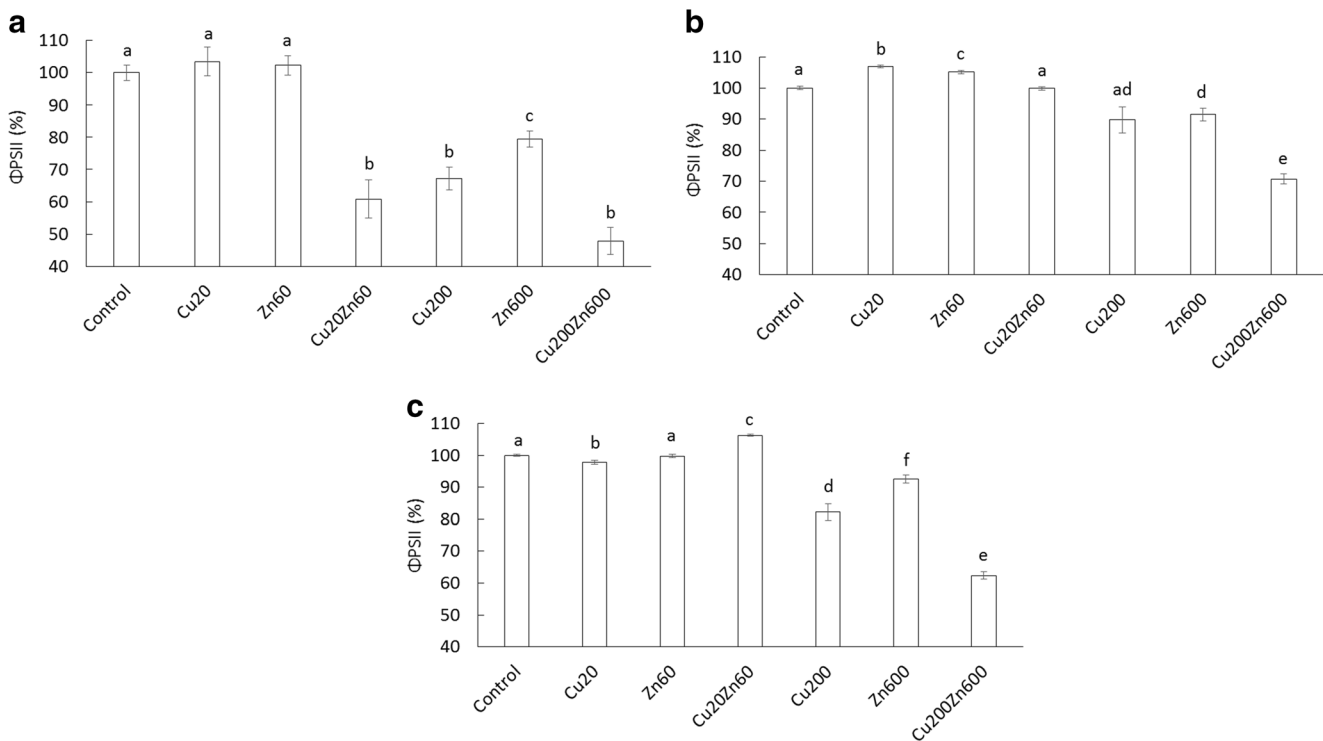


Fig. 3 Effects of copper and zinc exposure on the cyanobacterium (*P. autumnale*; a), the diatom (*N. palea*; b) and the green alga (*U. confervicolum*; c) photosynthetic efficiencies (Φ_{PSII}) (n = 32, mean \pm SD) compared to the control (n = 96, mean \pm SD). The mean control value was fixed at 100% (0.26 ± 0.01 , 0.62 ± 0.01 and 0.72 ± 0.01 for

P. autumnale, *N. palea* and *U. confervicolum* biofilms, respectively). The biofilm was exposed under 16 days to copper at $20 \mu\text{g L}^{-1}$ (Cu20) or $200 \mu\text{g L}^{-1}$ (Cu200), to zinc at $60 \mu\text{g L}^{-1}$ (Zn60) or $600 \mu\text{g L}^{-1}$ (Zn600) and to a mix of the metals (Cu20Zn60 or Cu200Zn600)

($p < 0.0001$) for *P. autumnale* species and $37.6 \pm 1.2\%$ ($p < 0.0001$) for *U. confervicolum* species. For *N. palea* and *U. confervicolum* species, a synergetic effect was obtained on the Φ_{PSII} compared to those obtained with Cu200 or Zn600.

Effect of metals on EPS production

Polysaccharide and protein contents in extracted EPS

After EPS extraction from the various biofilms, polysaccharides and proteins were quantified using colorimetric assays and referred to glucose equivalent or BSA equivalent, respectively (Table 1). It can be noticed that using such relative quantification, the amounts of extracted polysaccharides were higher than the amounts of proteins.

Compared to their controls, no significant effects were observed on the amounts of polysaccharides and proteins extracted for the three biofilms with Zn60, except for the green alga with a decrease of 17% for proteins. Besides, the protein amount also decreased with Cu20 by 34% for *U. confervicolum* biofilm. Always with Cu20, the polysaccharide amount increased by 82% for the *P. autumnale* biofilm and decreased by 15% for the *U. confervicolum* biofilm. The effect of the metal mixture Cu20Zn60 compared to metals alone gave an increase of the

protein amount by 28% for the *P. autumnale* biofilm and by 100% for the *N. palea* biofilm.

With Cu200 or Zn600, the polysaccharide amount increased in *P. autumnale* biofilm and decreased in *U. confervicolum* and *N. palea* biofilms compared to their controls. Besides, the protein amount increased for the three biofilms with Zn600, while this increase was observed just for the *P. autumnale* biofilm with Cu200. The effect with Cu200Zn60 compared to the exposure with metals alone was limited to a decrease of the polysaccharide amount by 38% for the *N. palea* biofilm.

Comparison of exposure between lower (Cu20 or Zn60) and higher (Cu200 or Zn600) metal concentrations, respectively, for an increase of Cu or Zn alone indicated that the amount of extracted polysaccharides was not significantly modified for the *P. autumnale* biofilm, decreased by 39 and 29% for the *N. palea* biofilm and decreased by 22 and 10% for the *U. confervicolum* biofilm. For the same conditions of comparison, respectively for Cu or Zn exposure, the amount of extracted proteins increased by 36 and 60% for the *P. autumnale* biofilm, by 22 and 88% for the *N. palea* biofilm and by 74 and 79% for the *U. confervicolum* biofilm. For the three phototrophic species, the increase of protein amount was more important with the increase of Zn concentration (Zn60 vs Zn600) than with the increase of Cu concentration (Cu20 vs Cu200).

Table 1 Effects of copper and/or zinc exposure ($n = 12$ for metal exposure and $n = 36$ for the control, mean \pm SD) during the biofilm growth for the cyanobacterium *P. autumnale*, the diatom *N. palea* and the green alga *U. confervicolum* on the total amounts extracted (S-F + S-CER) as

proteins (PN, in mg eqBSA g^{-1} DW) and polysaccharides (PS, in mg eqGlc g^{-1} DW). Two groups without any letter in common in a same line are significantly different ($p < 0.05$). The values in bold were significantly different from the control, and underlined when higher than the control

		Control	Cu20	Zn60	Cu20Zn60	Cu200	Zn600	Cu200Zn600
Cyanobacterium <i>P. autumnale</i>	PS	16.1 \pm 1.1 _a	29.3 \pm 2.6_b	22.0 \pm 2.7 _{acd}	16.1 \pm 1.8 _a	26.6 \pm 1.5_{bd}	22.8 \pm 2.0_{bd}	14.9 \pm 1.1 _{ac}
	PN	10.4 \pm 0.5 _a	11.4 \pm 0.5 _a	11.2 \pm 0.2 _a	14.5 \pm 0.9_b	15.5 \pm 0.5_{bc}	17.9 \pm 0.9_{bc}	18.6 \pm 1.2_c
Diatom <i>N. palea</i>	PS	25.9 \pm 1.4 _{ab}	30.8 \pm 2.6 _a	24.6 \pm 2.4 _{abc}	22.1 \pm 1.7 _{bcd}	18.7 \pm 1.6_{cd}	17.5 \pm 0.9_d	11.2 \pm 0.9_e
	PN	15.1 \pm 0.6 _{ac}	13.3 \pm 0.9 _a	14.3 \pm 0.8 _{ac}	27.6 \pm 1.7_b	16.2 \pm 0.2 _c	26.9 \pm 0.9_b	23.6 \pm 0.4_d
Green alga <i>U. confervicolum</i>	PS	42.0 \pm 1.8 _a	35.6 \pm 1.4_b	43.9 \pm 2.9 _a	40.8 \pm 1.3_{bc}	27.9 \pm 1.4_{cd}	39.3 \pm 1.3_{bc}	23.8 \pm 1.0_d
	PN	4.1 \pm 0.1 _a	2.7 \pm 0.3_b	3.4 \pm 0.2_c	3.6 \pm 0.4 _{ac}	4.7 \pm 0.4 _{ae}	6.1 \pm 0.2_{de}	5.8 \pm 0.1_e

Characterization of extracted EPS by size exclusion chromatography

A global SEC fingerprint monitored at 210 nm was undertaken for each EPS extract obtained after CER step treatment (S-CER) for the three species exposed to the higher metal concentrations (Cu200 or Zn600) and their controls (Fig. 4a, c and e). The EPS profiles of the green alga *U. confervicolum* were considered as similar under metal exposure (Cu200 or Zn600) or control condition (Fig. 4e). On the contrary, the exposure of the diatom *N. palea* with Zn600 has affected the EPS profile

leading to an increase in the peak amplitude of almost all the eluted molecules, while it was not the case when the diatom was exposed with Cu200 (Fig. 4c). Concerning the cyanobacterium *P. autumnale*, some modifications could also be noticed in the EPS profile with Zn600: the absorbance intensities of the peaks located between elution volumes from 30 to 37 mL and around 45 mL were slightly increased (Fig. 4a). However, it was difficult to correlate this increase of UV absorbance in the Zn600 EPS profiles to an overexpression of polysaccharides or protein-like polymers because 210 nm monitoring can detect very diversified organic and inorganic

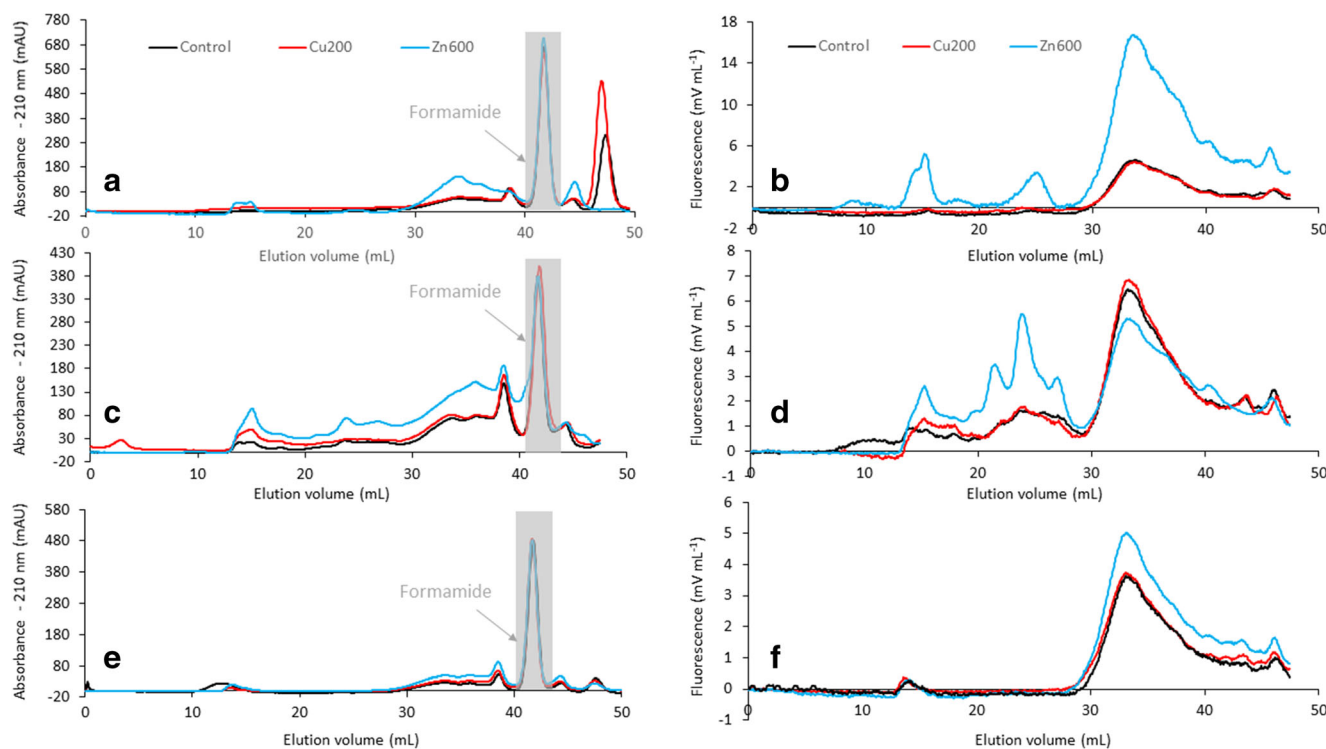


Fig. 4 Size exclusion chromatography of the EPS extracts (S-CER) measured at 210 nm (a, c and e) and the proteins obtained with fluorescence (Excitation / Emission: 221 nm / 350 nm) (b, d and f) recovered from *P. autumnale* (a and b), *N. palea* (c and d) and *U. confervicolum* (e and f) species for the control or Cu200 and Zn600 exposure. The vertical arrows

indicate the position of the peak obtained after injection of PBS buffer containing 0.22% formamide. The molecular weight of eluted molecules was evaluated as 360 kDa for the elution volume of 20 mL, 6 kDa for 30 mL and 0.1 kDa for 40 mL

compounds. As an example, formamide used during the first step of the extraction procedure was detected at 210 nm as a single peak eluted at 41.7 mL (Fig. 4a, c and e).

A more specific detection of protein-like polymers was performed based on the detection of tryptophan fluorescence that can be more specific and sensitive to analyse the eluted protein-like polymers (Fig. 4b, d, f). Fluorescence monitoring evidenced an increase in protein-like molecules in the EPS extracted after Zn exposure of both cyanobacterium and diatom biofilms (Fig. 4b, d), while it was not the case for the green alga biofilm (Fig. 4f). Comparison of the size profiles of the protein-like molecules extracted from the *P. autumnale* biofilm exposed or not to Zn clearly showed three increased populations: one corresponding to high molecular weight proteins (eluted from 13 to 17 mL, size up to 360 kDa), a second to intermediate size proteins (eluted from 22 to 27 mL, size from 6 to 360 kDa) and a last population of low molecular weight molecules (from 30 to 40 mL, 0.1 to 6 kDa). The effect of Zn exposure on the *N. palea* biofilm was somewhat different since in comparison with the unexposed biofilm, the last population of low molecular weight protein-like molecules (30 to 40 mL) decreased. As for the *P. autumnale* biofilm, an increase in the secretion of high molecular weight proteins (13 to 17 mL) was observed after Zn exposure of the *N. palea* biofilm. Noticeably, the intermediate size molecules exhibited a specific pattern since a group of three peaks could be clearly visualised on the *N. palea* profile while only one was detected in the *P. autumnale* profile. These data confirm that high Zn concentration exposure significantly stimulates protein secretion by cyanobacterium and diatom biofilms. In addition, they point out the fact that the secreted molecules might be divergent since specific fluorescent profiles were obtained for the intermediate size proteins extracted from these two biofilms.

Characterisation of the prokaryotic community in the controls

In order to evaluate the possible contribution of prokaryotes associated with each alga to the EPS variations, attention has been paid to the composition of the prokaryote community in each channel. In the experiments with the cyanobacterial strain, this species was certainly detected by ARISA, but since it was present in each mini-channel, it did not contribute to the differences between the communities. The composition of these communities was compared with Jaccard similarity indices, calculated from the matrixes obtained with ARISA fingerprinting. For control experiments, our results showed that the main factor controlling the structure of biofilm prokaryote community was the experimental module. Indeed, for the controls, the similarity within modules was higher than the similarity between modules (Table 2). Conversely, for the three alga species, the module did not significantly influence the amount of EPS extracted (pool of PS + PN): for control

replicates realised in different modules, no significant difference was observed. Therefore, a modification in prokaryote community composition did not influence the quantity of EPS extracted.

Discussion

Copper and zinc are present in nutrient media at different concentrations. The total amounts of metal exposure at the initial concentration in the recirculating loop of each experiment were the following:

- For Cu in Combo medium: control = $0.3 \mu\text{g L}^{-1}$, Cu20 = $20.3 \mu\text{g L}^{-1}$ and Cu200 = $200.3 \mu\text{g L}^{-1}$.
- For Cu in BG11 medium: control = $25 \mu\text{g L}^{-1}$, Cu20 = $45 \mu\text{g L}^{-1}$ and Cu200 = $225 \mu\text{g L}^{-1}$.
- For Zn in Combo medium: control = $5 \mu\text{g L}^{-1}$, Zn60 = $65 \mu\text{g L}^{-1}$ and Zn600 = $605 \mu\text{g L}^{-1}$.
- For Zn in BG11 medium: control = $46 \mu\text{g L}^{-1}$, Zn60 = $106 \mu\text{g L}^{-1}$ and Zn600 = $646 \mu\text{g L}^{-1}$.

These metals were in the dissolved fraction of the medium, excepted for Cu200 in BG11 for which 39% of Cupric Ferrite were precipitated. The potential of metals bioavailability was moderate when associated with EDTA and high with citrate and Fulvic Acids.

Effects of Cu and Zn on biomass growth and photosystem II efficiency

In this study, three mono-species phototrophic biofilms cultivated in hydraulic mini-channels in non-axenic conditions were exposed to contrasted concentrations of Cu or Zn. The physiological response of each species was first evaluated at the cellular level, measuring the growth inhibition and photosynthesis activity decrease. The three species, chosen to cover the major groups of benthic algae present in river biofilms, showed contrasted responses.

In terms of biomass growth, the cyanobacterium *P. autumnale* was the most resistant species. The diatom *N. palea* was the only species whose biomass production decreased because of metal exposure at environmental concentrations (Cu20 or Zn60). Besides, for the green alga *U. confervicolum*, the synergic effect of metals (Cu20Zn60) seems to provide some toxicity. Cu and Zn have been shown to inhibit cell division of green microalgae (Perales-Vela et al. 2007; Sabatini et al. 2009), diatom species (Anu et al. 2016) and cyanobacterium species (Miao et al. 2009); they also affect cell size and morphology of diatom microalgae (Manimaran et al. 2012; Morin et al. 2007). Our results are partly in agreement with González et al. (2016) who observed a decrease of *N. palea* biomass under low Ag concentration

Table 2 Comparison of similarity index (Jaccard) of bacterial community ($n = 3$ for one module, mean \pm SD) and the EPS extracted ($n = 4$ for one module, mean \pm SD) for biofilm (represented by *P. autumnale*, *N. palea* or *U. confervicolum*) grown without metal exposure (control) within the same or different module (intra or inter-module respectively). For each species and each parameter (distance index or EPS (here EPS = PS + PN)), two groups without any letter in common are significantly different ($p > 0.05$)

	Similarity index (Jaccard)		EPS extracted, for each module (mg g ⁻¹ DW)
	intra-module	inter-module	
Cyanobacterium			28.4 \pm 2.2 _a
<i>P. autumnale</i>	0.546 \pm 0.052 _a	0.149 \pm 0.009 _b	27.1 \pm 1.1 _a 24.0 \pm 2.7 _a
Diatom			40.1 \pm 2.8 _a
<i>N. palea</i>	0.694 \pm 0.042 _a	0.133 \pm 0.006 _b	41.7 \pm 1.6 _a 40.0 \pm 1.8 _a
Green alga			43.8 \pm 2.6 _a
<i>U. confervicolum</i>	0.639 \pm 0.115 _a	0.059 \pm 0.014 _b	48.5 \pm 1.6 _a

exposure for 14 days, whereas *U. confervicolum* biomass was affected at higher Ag concentration. A more drastic reduction in algal biomass has been reported in other studies at moderate Cu concentration compared to our findings. As an example, a reduction of 42% in algal biomass was observed under 32.5 $\mu\text{g L}^{-1}$ of Cu in fluvial phototrophic biofilm for an exposition of six weeks, resulting in a change in the community with a higher proportion of green algae and a lower proportion of diatom (Serra and Guasch 2009).

At high metals concentrations, the growth of *U. confervicolum* was more affected than the one of the other species. Serra et al. (2009) also observed, associated to a modification of the structure of the community, a higher biomass tolerance of cyanobacteria and diatom than green algae when biofilm were chronically exposed to Cu at 100 $\mu\text{g L}^{-1}$. Our data indicate that among the three strains tested, the green alga *U. confervicolum* was the most sensitive to metals for its biomass growth, while the cyanobacteria *P. autumnale* was the most resistant when metals were added alone.

At environmental concentrations, Cu and Zn showed no deleterious effects on the photosynthetic activity of the three species. This is consistent with the results of Lambert et al. (2016) who found that Φ_{PSII} value of phototrophic biofilm was higher in microcosm containing 15 $\mu\text{g L}^{-1}$ of Cu compared to the control at a temperature of 23 °C and after a biofilm growth of 6 weeks. In contrast, higher concentration of metals (Cu200 or Zn600) have decreased the photosynthetic activity by 8 to 33%, *P. autumnale* being the more sensitive species for this parameter. These observations are in agreement with published data indicating that for alga species isolated from phototrophic biofilms sampled from polluted rivers running through Cu or Zn mining regions, the photosynthetic

activity of cyanobacteria is less resistant than that of green algae to Cu exposure at 645 $\mu\text{g L}^{-1}$ (Takamura et al. 1989; Takamura et al. 1990). Our data showed that with the tested metal concentrations, *P. autumnale* and *U. confervicolum* species were more sensitive to Cu than to Zn, while no significant differences were observed for *N. palea* species. The relative tolerance of the diatom may be explained by the presence of the frustule, the siliceous extracellular skeleton of diatom cells, which acts as protection barrier against molecules and plays an important protective role against Zn and Cu toxicity (Guasch et al. 2002).

Cu and Zn stresses are known to functionally impair phototrophic communities by reducing photosynthetic activity (Cao et al. 2015; Lambert et al. 2012; Perales-Vela et al. 2007; Xu et al. 2016). A significant decrease of the chlorophyll *a* content with an increase of Cu concentration was reported for marine diatom (50 to 850 $\mu\text{g L}^{-1}$) (Anu et al. 2016; Manimaran et al. 2012) and green alga species (10 to 1000 $\mu\text{g L}^{-1}$) (Levy et al. 2008). Concerning Zn exposure, Xu et al. (2016) observed an eleven-fold decrease of the chlorophyll *a* content in fluvial phototrophic biofilm exposed to high Zn concentration around 7.85 mg L^{-1} . These effects can be attributed to the substitution of Mg, which is the central atom of chlorophyll molecule, by dissolved Zn²⁺ or Cu²⁺, leading to the inhibition of photosynthesis. Interaction of chlorophyll with Cu and Zn showed that Cu forms preferentially Cu-chlorophyll link compare to Zn; in consequence, Zn effect is highly minor compared to Cu when both metals are present in the same concentration (Zvezdanović and Marković 2009).

Reactive oxygen species (ROS) production by aerobic organisms is frequently stimulated by stressors. When the cells cannot maintain a balance in ROS levels by protective antioxidant mechanisms, modifications in some metabolic processes may occurred (Gechev et al. 2006). An excess of Cu or Zn can promote the generation of ROS which can damage several cellular components, and/or promote a competitive binding to metal transporters inhibiting the uptake of other essential metals (Gaetke and Chow 2003; Nies 1999; Tottey et al. 2012). In consequence of ROS formation, Cu and Zn have been shown to cause adverse effects on the photosynthetic activity of green alga species (Cao et al. 2015; Perales-Vela et al. 2007), diatom species (Cid et al. 1995) and cyanobacterium species (Miao et al. 2009).

Miao et al. (2009) suggest that for similar concentrations, the toxic effects of copper on diatom, cyanobacterium and green alga microorganisms on growth rate and photosynthetic activity were higher than zinc. Surprisingly, our observations made on biofilm growth were not fully correlated with the measures of photosynthetic activity, while this parameter is supposed to be more sensitive than growth to detect deleterious effects (Dorigo and Le Boulanger 2001). The biomass accumulated by the biofilms resulted of the 16 days of metals exposure, while the photosystem II efficiency revealed the

instantaneous value of this parameter at time 16 days. The biomass growth is an integrative parameter whereas it seems that the photosystem efficiency is more a punctual vision of the physiology of the biofilm. It is possible that the Cu and Zn toxicity plays an important role in the first step of the growth and then becomes less important with the thickening of the biofilm, maybe due to the EPS production which becomes more preponderant with time.

Effects of Cu and Zn on EPS production and composition

In the present study, the three mono-species phototrophic biofilms have developed different responses face to metal contamination including modulation of their EPS matrix composition. In the presence of copper or zinc at high concentrations (Cu200 or Zn600), the amounts of extracted polysaccharides were lower compared to their controls for the eukaryotic *N. palea* and *U. confervicolum* species. On the opposite, the prokaryotic *P. autumnale* species was able to increase its polysaccharide production compared to the control. This might be considered as a protective mechanism towards metals toxicity. Cyanobacteria, known as producers of exopolysaccharides, have been considered as very promising chelating agents for the removal of positively charged metal ions from water solution (De Philippis et al. 2011).

As regards to the amount of proteins, the three phototrophic species are able to increase their proteins excretion in response to high concentrations of metals. In our experiments, the total amount of protein extracted was highest for Zn600 exposure than for Cu200, whatever the species considered. This may contribute to the lower toxicity of Zn compared to Cu, or conversely, Cu toxicity may impede the cellular activity and so the protein production. Although a fundamental role in cell tolerance towards cationic pollutants can be attributed to such released proteins, further investigations are needed to explore the involved mechanism and its specificity towards Zn cation.

To confirm global EPS production or consumption in the presence of high Cu and Zn concentrations, EPS obtained from the three biofilms after the CER step of the previously described multi-method extraction procedure (Loustau et al. 2018) were analysed by SEC. Absorbance monitoring at 210 nm clearly showed that only Zn exposure has triggered global EPS production for both *P. autumnale* and *N. palea* biofilms. In line with the colorimetric BCA assays, the overproduction of proteins by *P. autumnale* and by *N. palea* cells under Zn600 exposure was confirmed by the fluorescence profiles, but it was not the case for *U. confervicolum* despite an increase of the population of low molecular weight proteins. That can be explained by the fact that the amount of proteins extracted from the green alga biofilm is significantly lower than for the two other biofilms. In addition, SEC profiles revealed specific EPS molecular fingerprints in *N. palea*

and *P. autumnale* biofilms that have secreted different intermediate molecular weight protein-like polymers during their development in the presence of Zn600. These proteins were not detected in the presence of Cu200 although some increase in protein secretion was measured in *P. autumnale* biofilms exposed to Cu200. Possible interferences of non-proteic compounds such as pigments or polyphenols with the BCA reagent might lead to protein overestimation by the colorimetric assay.

Our results suggest that the presence of specific proteins in the extracellular matrix could play a significant role in the difference of species sensitivity to metals. Indeed, the diatom biofilm containing three intermediate molecular weight proteins seems to have the less sensitive photosynthesis process towards Zn exposure. Proteins and polysaccharides from EPS matrix may serve as a boundary between microbial cells and their immediate environment. Moreover, their overall anionic charges may be essential for sequestering metal cations (Cu^{2+} or Zn^{2+}) and reduce the metal income into the cell (García-Meza et al. 2005; Masmoudi et al. 2013; Kaplan et al. 1988). Further characterization of the secreted proteins and their capacity to adsorb metal cations *in vitro* will help to confirm such molecular mechanism.

To our knowledge, this is the first study investigating the quantitative and qualitative protein fingerprints produced by phototrophic biofilm under environmental metal exposure. In most of previous studies, authors limited their analyses to the polysaccharides fraction (Aslam et al. 2012; De Brouwer et al. 2002). Indeed, acidic sugars such as alginate-like exopolysaccharides are able to adsorb metal cations and protect microorganisms from the harsh environment. Polysaccharides characterisation is often preferred while in our study it would appear that proteins composing EPS biofilm play an important role for phototrophic cell tolerance under metal exposure. It will reinforce the main conclusion that the protein variation could serve as a fingerprint of the metal toxicity. It might be relevant to have a closer look at the anionic properties of the polymers (proteins and polysaccharides) composing the EPS of these three phototrophic biofilms and to analyse, *in vitro*, their capacity to sorb Cu and Zn at these concentrations. One interesting perspective would be to characterize the biochemical nature and physico-chemical properties of the polysaccharides produced by the cyanobacteria when exposed to cationic metals.

The negative effects of metals on biomass production and photosynthetic activity may have direct or indirect effects on the water ecosystem due to the role of microorganisms in the sorption of organic or inorganic nutrients (Fischer et al. 2002; Romani et al. 2004). The algal cell biomass recovery after exposure to contaminants is important for grazing invertebrates and therefore the ecosystem food web, a decrease affecting the transfer of energy towards higher trophic levels (Sheldon and Walker 1997; Schmitt et al. 1995). Knowledge on the tolerance and response of microalgae to metals is

important in order to determine the potential utility of microalgae for pollution assessment. In presence of copper and zinc in freshwater, the prediction of phototrophic biofilm modification allows to evaluate the changes in ecosystem functioning.

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