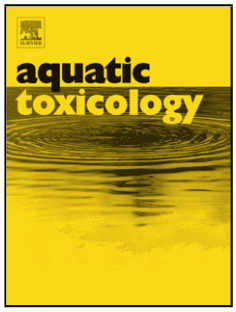

Effects of an hypersaline effluent from an abandoned potash mine on freshwater biofilm and diatom communities

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Effects of an hypersaline effluent from an abandoned potash mine on freshwater biofilm and diatom communities.

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Highlights

- Mining effluents from abandoned mines are a source of pollution to freshwaters.
- Freshwater biofilms were exposed to hypersaline mining effluents.
- Functional and structural impact on biofilms and diatom community were evaluated.
- Hypersaline effluents had short-term impacts on biofilm functioning.
- Diatom structure, size and biodiversity were affected by the hypersaline effluent.

ABSTRACT

Potash abandoned mines cause severe environmental damage to their bordering environment, with significant impacts on freshwater ecosystems mostly through uncontrolled discharge of hypersaline effluents. This study aimed to evaluate the ecological impact caused by a hypersaline effluent from an abandoned potash mine (Menteroda, Germany) on freshwater biofilms and, specifically, on diatom communities. Biofilm from a pristine stream was exposed under controlled conditions in microcosms to a mining effluent (ME), and its structural (algal biomass, community composition, diatom metrics) and functional (photosynthetic activity, nutrient uptake) responses were evaluated over time and compared with unexposed biofilms used as control. Biofilm

exposed to ME showed drastic functional responses after one day of exposure, with a significant decrease in photosynthetic efficiency and nutrient uptake, that were recovered over time. Biofilm exposed to ME showed a progressive increase in diatom metrics (abundance, density and growth rate) over time, compared to the control. However, a significant decrease in diatom species diversity, richness and cell size was also observed in biofilm exposed to ME. This study revealed that the ME affected the biofilm causing short-term functional responses, which were recovered simultaneously with a drastic diatom community structure shift.

Keywords: Aquatic biofilm, Diatoms, Hypersaline effluents, Freshwater salinization, Abandoned mines.

1. Introduction

Freshwater salinization has emerged as a topic of growing ecological concern (Cañedo-Argüelles et al. 2016; Kaushal et al. 2005). Salinity is considered as the total concentration of dissolved inorganic ions and it is an inherent component of all inland waters (Williams and Sherwood, 1994). Primary salinization is considered when natural processes, as rainfall, rock weathering, seawater intrusion or aerosol deposits, lead to the ion content increase of inland surface waters (Cañedo-Argüelles, Kefford, and Schäfer 2019). In contrast, the increase in ionic content of inland waters caused by human activities (i.e. construction activities, resource extraction or land cover changes increasing the transport of ions to surface), is considered as secondary salinization (Kaushal et al. 2018, Steffen et al. 2011, Cañedo-Argüelles, Kefford, and Schäfer 2019). Potash mining activities are considered an important driver of secondary salinization (Cañedo-Argüelles et al. 2012), especially when these mines are abandoned and uncontrolled polluted effluents are usually generated long afte the end of the mining activity (Younger 2000). In addition, the frequent lack of an effective regulatory framework to deal with abandoned mines in many regions contributes to perpetuate this legacy pollution. For example, despite the Mining Waste Directive (Directive

2006/21/EC) of the European Parliament provides measures, procedures and guidance to prevent or reduce any adverse effects on the environment, it does not clearly identify the administration that must deal with these effluents from abandoned mines, which frequently compromise the ecological status of the surrounding water bodies.

Freshwater salinization has an impact on freshwater communities, including biofilm communities (Cañedo-Argüelles et al., 2017). These impacts on aquatic organisms affect the ecosystem structure and functioning (i.e. phosphorous removal from water column; Sauer et al. 2016) and can change the ecosystem services provided by freshwater ecosystems (Iglesias 2020). In freshwater ecosystems, species richness decreases along a salinity gradient, and many freshwater species do not survive when a certain threshold of salinity is exceeded (Cañedo-Argüelles, Kefford, and Schäfer 2019). Indeed, freshwater organisms have different sensitivities towards salinity stress and hence it is expected that the salinity increase alters the composition of the freshwater communities (Berger, Frör, and Schäfer 2019).

Biofilms are microbial communities formed by bacteria, algae, fungi, and microfauna, embedded in an exopolysaccharide matrix and sticked to any surface in aquatic environments. Biofilms are complex microbial communities, rich in species that can occur over a wide variety of environmental conditions and can display a rapid response to any change in the environment (Besemer 2016). Furthermore, microbial biofilms maintain the functioning of the ecosystem and contribute significantly to the mechanisms of absorption and processing of nutrient and pollutants, which lead to the self-depuration of running water ecosystems (Sabater et al. 2007). In addition, the Water Framework Directive (WFD) (Directive 2000/60/EC, 2000) identifies biofilms and specifically diatoms as a biological compartment that should be targeted for assessing the ecological status of water bodies.

Biofilm responses to salinization can lead to a reduction in algal cell density, growth and photosynthetic activity, as well as a shift in the taxon dominance when it is exposed to

extremely high NaCl and $NaHCO_3$ concentrations in water (Entrekin et al. 2019). Regarding diatoms, high salt concentrations have been reported to have effects on diatom communities reducing its density and altering its external morphology (Trobajo et al. 2011). In addition, chronic exposures can increase the percentage of nuclear abnormalities in the diatom assemblage (Cochero, Licursi, and Gómez 2017). However, little is known about the consequences of extreme salinities on its functioning and whether and to what extent changes in the species community structure can affect the biofilm functionality, and thus, the ecosystem functioning.

The main objective of this study was to assess the effects of a hypersaline effluent from an abandoned potash mine (Menteroda, Germany) on the structure and functioning of freshwater biofilm, especially on diatom communities, under laboratory conditions. It was initially hypothesized that high salt concentrations would have significant effects on aquatic biofilm community structure and function, likely a decrease in the algal and cyanobacteria biomass, and hence, on the photosynthesis capacity and nutrient uptake rates. Additionally, it was predicted a shift in the diatom community composition, since some taxa would increase in response to salinity, whereas others would decrease.

2. Materials and methods

2.1. Experimental design

This experiment was carried out using a mining effluent from an abandoned potash mine located in Menteroda (51°19'01.4"N - 10°34'35.4"E, Germany). Extraction of potash in this mine started in 1906 and stopped in 1991. The wastes of the potash extraction were stored in a large dump next to the mine. The leaching waters from the dump are currently stored in a reservoir from which they can reach the surface waters by flowing through embankments or through the base of the tailing pile. This mining effluent is characterized by an extreme salinity, as evidenced by the high concentrations of dissolved inorganic ions: 78 200 mg L^{-1} Na^{+}, 6 700 mg L^{-1} K^{+}, 117 000 mg L^{-1} Cl^{-} and 1 210 mg L^{-1} Mg^{2+}.

In a temperature-controlled chamber, six microcosms consisting of 6-L glass aquaria (length × width × height 26 × 15 × 17 cm) were used. Each microcosm contained 15 stream cobbles (previously scraped and autoclaved) used as substrates for natural biofilm colonization. The microcosms were filled with 3 L of artificial water, which was prepared to mimic a pristine stream as described in Ylla et al. (2009) by dissolving pure salts in distilled water, and creating the same chemical composition described in Vendrell-Puigmitja et al. (2020). A submersible pump (EDEN 105, Eden Water Paradise, Italy) was installed in each microcosm to ensure the recirculation of water. During the whole experiment, the water of each microcosm was completely renewed every 2 days to avoid nutrient depletion. Temperature and photoperiod in the chamber were set at 20°C and 12 h light: 12 h dark using LEDs (LENB 135-lm, LENB/14.97/11.98), respectively.

In each microcosm, 15 mL of natural biofilm suspension obtained by scraping cobbles collected from the Riera Major at Viladrau (a pristine stream located in the Natural Park of Montseny, in NE Spain), was inoculated to promote the cobbles colonisation. The biofilm suspension was added at the beginning of the experiment and at each water renewal during the colonization period to favor biofilm settlement.

After three weeks of colonization, the exposure period, which lasted for 16 days, started with the addition of water from the mining effluent. Two treatments were set: (i) the mining effluent from Menteroda abandoned mine (ME) and (ii) artificial water used as control (C). The exposure was set to mimic the realistic conditions of a river where a mining effluent of 4.30 $m^3 h^{-1}$ reaches a river of 100 $m^3 h^{-1}$. To achieve these conditions, 0.20 L of the mining effluent was added to 2.80 L of artificial water in the respective microcosms creating a salinity concentration of 15.0 g $L^{-}1$, which was maintained at each water renewal. Each treatment was performed in triplicate.

2.2. Physico-chemical conditions in microcosms

Dissolved oxygen concentration and saturation, temperature (YSI professional plus, YSI Incorporated, USA), pH (G-PH7-2 portable pH meter XS PH7 + DHS), and conductivity (G-COND7-2 conductivity-meter portable XS COND 7+) were measured directly at each microcosms with sensor probes at each water renewal.

Triplicate water samples for each treatment (one sample *per* microcosms) were collected and filtered through a 0.22 µm pore diameter glass microfiber filter (Prat Dumas Filter Paper, Couze-St-Front, France). Water samples were frozen immediately and kept at -20°C until analysis. NO_2^-, NO_3^-, NH_4^+ and PO_4^{3-} were analyzed following APHA (1992a, b), Reardon et al. (1966) and Murphy and Riley, (1962) respectively.

2.3. Biofilm sampling

Biofilms were sampled before (T0) and after 1, 8 and 16 days of the addition of the mining effluents one day after water renewal. At each sampling day, three random cobbles were collected from each microcosm. Immediately after collection, photosynthetic activity and photosynthetic community composition were measured directely with an amplitude modulated flurimeter (Mini-PAM fluorometer, Walz, Effeltrich, Germany) and BenthoTorch portable fluorimeter probe (bbe Moldaenke, Schwentinenta, DK) as defined in Vendrell-Puigmitja et al. (2020). Biofilms of the corresponding microcosm were then scraped using a toothbrush and suspended in water from the corresponding microcosm. Aliquots of 10 mL of biofilm suspension were then sub-sampled to analyze chlorophyll-a concentration, ash-free dry mass (AFDM) and diatom community (see below). The area of stones was measured by placing aluminum foil over the scratched surface and drawing the area of the stone and recalculating depending on the weight (Graham et al., 1988). Chlorophyll-a concentration and AFDM samples were stored at -20°C until analysis.

2.3.1. Chlorophyll-*a* concentration

A volume of 10 ml of scraped biofilm taken during the biofilm sampling at T0, T1, T8 and T16, was used to evaluate the chl-*a* concentration. The samples were prepared by doing an extraction using 90% acetone for 24 hours in the dark at 4 ˚C and then filtering through pore filter of 47 mm diameter and 1.2 µm pore filter (A0258855 Prat Dumas). Chlorophyll-a concentration was then determined by spectrophotometric measurements (NanoPhotometer™ P-360) following the method described in Jeffrey and Humphrey (1975).

2.3.2. Ash free dry mass

Total biofilm biomass was measured as ash free dry mass (AFDM). The biofilm suspension was filtered through pre-combusted (4h at 500°C, Carbolite muffle ELF 11/14B) and pre-weighted 47 mm GF/F Whatman glass-fiber filters (0.7 µm pore size), then dried for 72h at 60°C (Forced air oven, MEMMEERT IFE500), in order to calculate the dry mass (DM). Later on, samples were combusted at 500°C for 4h (Carbolite muffle ELF 11/14B) and weighted again. The differences in filter mass before and after drying for DM (72h at 60°C) and after combustion (4h at 500°C) subtracted from DM were calculated to obtain AFDM.

2.3.3. Photosynthetic efficiency and community composition in the biofilm

The photosynthetic efficiency (Yeff) was measured in Yield with the Mini-PAM (Pulse Amplitude Modulated) chlorophyll fluorimeter (Heinz Walz GmbH) that use light-emitting diodes that excite chlorophyll. The main photosynthetic groups' densities (diatoms, cyanobacteria and green algae) were measured by covering the colonized cobble with the BenthoTorch probe (BTo-09-048 GPS bbe Moldaenke).

2.3.4. Phosphorous uptake rate of biofilm

Biofilm phosphorus (PO_4^{3-}) uptake rate was determined at each sampling day directly on each microcosm with the remaining cobbles measuring PO_4^{3-} temporal decay after a controlled spike as described by Proia, Romaní and Sabater (2017). Previously, the

basal PO_4^{3-} was determined and it was added the corresponding concentration in each microcosms to quadruple the background concentration. Then, the biofilms were incubated for 240 min and sampled in 10 mL tubes at 1, 5, 15, 30, 60, 90, 120, 180 and 240 min after the addition through 0.22 µm nylon membrane filters (Prat Dumas Filter Paper, Couze-St-Front, France). The PO_4^{3-} uptake rate (U, µgP cm^{-2} h^{-1}) was calculated as:

$$U = \frac{P_0 - Pf}{A\ x\ Tf}$$

where P_0 = the initial mass of P after the spike (µg); Pf = the final mass of P at the end of incubation (µg); A = the area colonized by biofilm (cm^2) and Tf = the time of incubation end (h). The background concentration of PO_4^{3-} was quadruplated and water aliquots (10 mL) were then taken at 1, 5, 30, 60, 120, 180 and 240 min after the addition, filtered through 0.22 µm nylon membrane filters (GNWP04700) and stored at −20 °C until analysis.

2.3.5. Diatom metrics

A volume of 5 ml of scraped biofilm taken during the biofilm sampling at T0, T8 and T16 were placed in a 15 ml Falcon and preserved using 5 ml Ethanol 70%. Before the analysis, they were ultrasonicated to isolate the aggregated cells without damaging the frustules, and 125 µL of each sample was pipeted onto a Nageotte counting chamber to count the total number of diatom cells following Morin et al. (2010). Data were recorded as cells per samples substrate area (number of cells/cm^2). Two different types of counting were performed; empty cells considered as 'dead', and cells occupied by chloroplasts considered as 'alive' as described in Morin et al. (2010). According to Guillard (1973), the diatom community growth rates were determined (in cell divisions day^{-1}) by using live diatom counts (expressed in cells mL^{-1}).

2.3.6. Identification, size and relative abundances of diatom species

To identify the species level, hydrogen peroxide (30%) was used to clean diatoms samples of all sampling times (T0, T8 and T16) to remove the organic material and dissolve calcium carbonates according to Leira and Sabater (2005). Cleaned frustules were mounted on permanent glass slides using Naphrax (Brunel Microscopes Ltd, UK; RI=1.74). Using standard references and recent nomenclature updates listed in Coste et al. (2009) and Prygiel and Coste (2000), about 400 random frustules per slide were counted at 1,000x magnification, and diatoms were measured and identified to the lowest taxonomic level feassible. Relative abundances of species (percentage of the total abundance) were estimated and diversity was calculated using the Shannon-Weaver index (H′) (Shannon and Weaver, 1963). From species relative abundances and their individual biovolumes, a mean cell length (cm^2) of the community was calculated as described in Barral-Fraga et al. (2016).

2.4. Data Analysis

Differences in physico-chemical variables, biofilm functional responses (photosynthetic efficiency and phosphorous uptake rate), and diatom metrics (diatom abundance, density, cell growth rate, size and species richness) between treatments were evaluated using one-way ANOVA for each sampling day. The effects of exposure throughout time were evaluated using one-way repeated measures analysis of variance (rm-ANOVA). Both analyses were carried out with SPSS Statistics software (version 21). One-way ANOSIM tests (using Bray-Curtis similarity coefficients) were carried out with Bonferroni correction on relative abundances of the biofilm community composition and diatom taxa in Past3 version 3.23. A principal component analysis (PCA) using R Studio software (version 3.6.0) was conducted to analyze the relationships between the biofilm parameters tested at the exposure period (T16). Statistical significance for all tests conducted was set at $p < 0.05$.

3. Results

3.1. Physico-chemical conditions of the microcosms

During the colonization period, the oxygen, temperature, conductivity and pH remained stable with no significant deviations among microcosms (Table 1). Nutrients concentration decreased slightly between water renewals and nutrient depletion was prevented by the periodical water replacement every 2 days. Specifically, phosphate decreased from 120 (± 9) µg/L to 114 (± 8) µg/L on average at each water renewal, and the whole average nitrogen (DIN) decreased from 647 (± 47) µg/L to 509 (± 38) µg/L at each water renewal. Once the exposure period started, dissolved oxygen, water temperature and pH remained stable with no significant deviations, but the conductivity changed significantly in the ME treatment microcosms, increasing up to 13200 (± 268) µS cm^{-1} on average during the exposure period (Table 2).

Table 1. Physico-chemical conditions on each treatment (3 microcosms per treatment) during the colonisation (n= 9) and exposure (n= 4) period (mean ±SD). C= control, ME=mining effluent.

		Physico-chemical parameters					
Period	**Treatment**	**Oxygen (mg L^{-1})**	**Oxygen (%)**	**Conductivity (µS cm^{-1})**	**Temperature (°C)**	**pH**	**PO_4^{3-} (µg L^{-1})**
Colonization	**C**	7.80 *(±0.10)*	88.0 *(±0.57)*	200 *(±3.17)*	21.1 *(±0.20)*	7.00 *(±0.15)*	120 *(± 9.17)*
	ME	7.60 *(±0.08)*	87.5 *(±1.03)*	196 *(±7.80)*	21.1 *(±0.25)*	7.00 *(± 0.39)*	119 *(± 8.56)*
Exposure	**C**	7.30 *(±0.20)*	81.0 *(±2.01)*	215 *(±9.20)*	20.4 *(±0.62)*	6.20 *(±0.34)*	122 *(± 7.45)*
	ME	7.50 *(±0.09)*	83.0 *(±3.50)*	13200 *(±268)*	19.8 *(±0.55)*	6.70 *(±0.33)*	110 *(± 9.38)*

3.2. Biofilm community composition and functioning

3.2.1. Chlorophyll-*a* concentration and AFDM

Chlorophyll-*a* concentration in the biofilm increased progressively during the exposure period in C and ME, being slightly higher in ME than C at the end of the exposure (Table 2). Just before the exposure period started (T0), the chlorophyll-*a* was 1.76 (±0.72) µg cm^{-2}, whereas at the end of the exposure period (T16) it was 4.44 (±0.42) µg cm^{-2} forC and 5.13 (± 0.09) µg cm^{-2} for ME. AFDM reached its maximum at T8 in both cases, being 0.58 (±0.71) mg cm^{-2} in C and 1.15 (± 1.78) mg cm^{-2} in ME, without significant differences between them (one-way ANOVA F = 0.7, p = 0.570). At the end of the experiment (T16),

both treatments showed a significant decrease in the AFDM from T8, but without significant differences between them (rm- ANOVA F = 2.5, p = 0.100).

3.2.2. Photosynthetic community composition

The photosynthetic community composition of the biofilm over time was mainly formed by diatoms on both treatments. ME exposure leaded to a significant change in community structure, and diatoms were significantly affected over time (Table 2). Specifically, diatoms abundance resulting significantly higher in ME-exposed biofilms particularly from T8 (Table 2) until the end of the exposure (T16) when its abundance doubled that of C biofilms (Figure 1, Table 2).

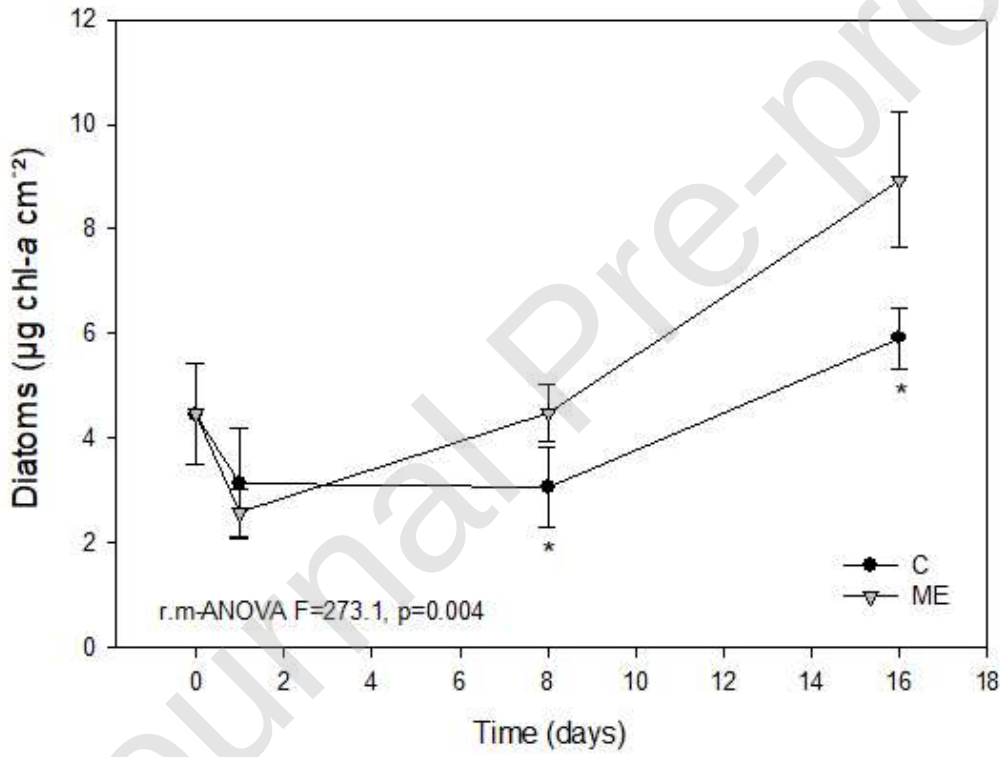

Figure 1. Diatom abundance (µg chl-*a* cm^{-2}) (mean ± SD), n=3. *:significant difference ($p<0.05$).

3.2.3. Diatom metrics

During the experiment, an increase in the diatom growth rate (GR) was observed for both C and ME-exposed biofilms. Nevertheless, at the end of the experiment the GR was significantly higher in ME-exposed biofilms than in the C (Figure 2-a, Table 2). In

addition, after 8 days of exposure, and until the end of the experiment, an increase of diatom cell density was observed in ME-exposed biofilms ($1.65 \cdot 10^7 \pm 5.14 \cdot 10^4$ cell div mL^{-1}) being higher than in the C at the end of the experiment (Figure 2-b, Table 2). By constrast, a significant decrease in the average cell size was observed in ME-exposed biofilms (9.42 ± 0.71 µm) compared to the C (30.1 ± 3.14 µm) (Figure 2-c, Table 2).

Over 44 diatom taxa were identified from the different biofilm samples collected in all sampling times. Species richness (*S*) and diversity index (*H'*) were significantly different between the C and the ME at the end of the experiment (Table 2). Under the influence of the mining effluent (ME), a loss of species richness (S_{ME} = 9, S_C = 42) and diversity (H'_{ME} = 1.15, H'_C = 2.79) of diatom communities within biofilms were observed at T16. Taxonomic composition and relative abundance of diatom species were markedly different between C and ME-exposed biofilms (Figure 3, Table 2). ME-exposed diatom communities were characterized by significantly smaller species (Table 2) such as *Fragilaria crotonensis* Kitton (FCRO), *Navicula reichardtiana* Lange-Bertalot (NRCH), *Nitzschia intermedia* Hantzsch ex Cleve & Grunow (NINT), *Rossithidium pusillum* (Grunow) Round & Bukhtiyarova (RPUS) and *Rhoicosphenia abbreviata* (C.Agardh) Lange-Bertalot (in less than the 2%) (Figure 3). For the C biofilms, the predominant taxa were *Fragilaria crotonensis* (FCRO), *Navicula reichardtiana* (NRCH), *Nitzschia tropica* Hustedt (NTRO), *Ulnaria biceps* (Kützing) Compère (UBIC) and *Cocconeis euglypta* Ehrenberg (CEUG) (Figure 3).

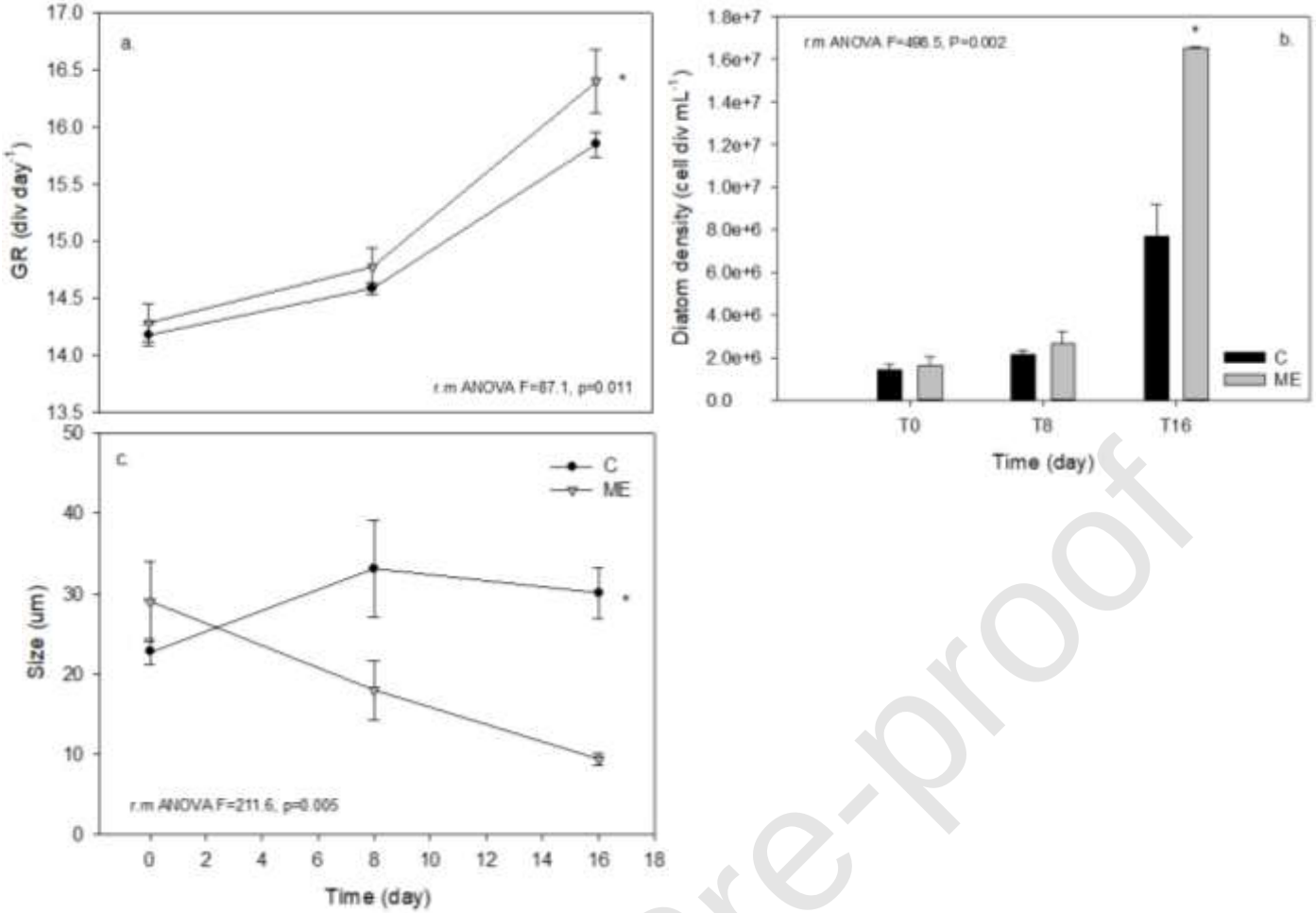

Figure 2. a. Diatom growth rate (div day^{-1}) (mean ± SD), n=3, **b**. diatom density (cell division mL^{-1}) (mean ± SD), n=3 and **c**. average diatom cell size (µm^2) (mean ± SD), n=3**.** C=control and ME= Mining effluent during the exposure period. *: significant difference ($p<0.05$).

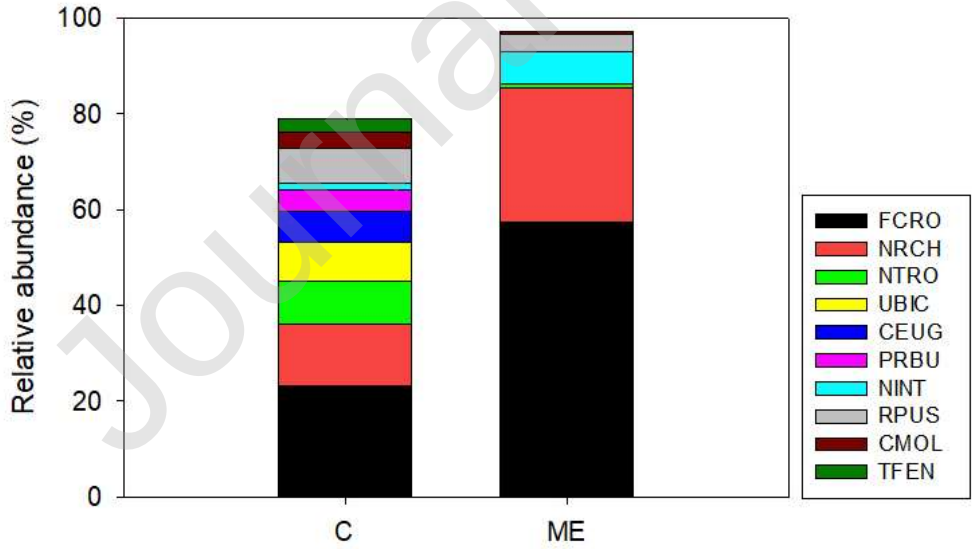

Figure 3: Relative abundance (mean value, n=3) of the 10 major diatom species (>3%) within diatom communities collected in the microcosms (C and ME) at t16. Where *Fragilaria crotonensis* (FCRO), *Navicula reichardtiana* (NRCH), *Nitzschia tropica* (NTRO), *Ulnaria biceps* (UBIC), *Cocconeis euglypta* (CEUG), *Planothidium robustius* (Hustedt) Lange-Bertalot (PRBU), *Nitzschia intermedia* (NINT), *Rossithidium*

pusillum (RPUS), *Caloneis molaris* (Grunow) Krammer (CMOL) and *Tabellaria fenestra* (Lyngbye) Kützing (TFEN).

3.2.4. Photosynthetic efficiency (Yeff)

The biofilm photosynthetic efficiency (Yeff) during the exposure period was stable over time in the C, but decreased significantly after one day of exposure (T1) in the ME treatment (Table 2, Figure 4-a). After 8 days of exposure (T8) the Yeff of ME biofilms were still significantly lower than in C microcosms (Table 2, Figure 4). Finally, the Yeff values of ME biofilms were recovered at the end of the exposure period (T16) being not significantly different from the C biofilms (Figure 4-a). Overall, the repeated measures analysis of variance confirmed that ME exposure significantly affected the photosyntheic efficiency of treated biofilms whith respect to C (Table 2).

3.2.5. Phosphorous uptake rate

The biofilm P-uptake rate was affected by ME, decreasing significantly after one day of exposure (0.17 ± 0.02 µgP $h^{-1}cm^{-2}$) compared to the C (0.31 ± 0.06 µgP $h^{-1}cm^{-2}$) (Figure 4-b, Table 2), but recovered from T8 until the end of the exposure. Overall,significant differences were observed over time between treatments (Figure 4-b, Table 2).

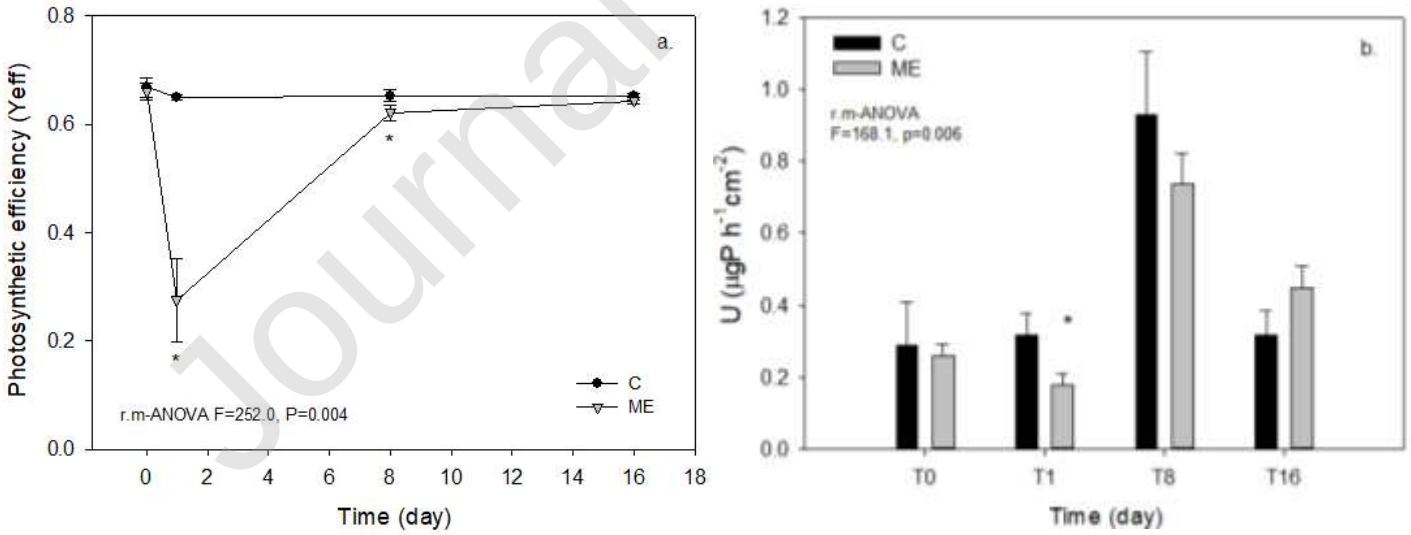

Figure 4. a. Photosynthetic efficiency (Yeff) (Yield) (mean ± SD), n=3, and **b.** P-uptake rate (µgP h^{-1} cm^{-2}) of the biofilm during the exposure period, (mean ± SD), n=3. C= control, ME= mining effluent. * significant difference ($p<0.05$).

3.2.6. Overall biofilm responses

The principal component analysis (PCA) revealed a clear effect of ME exposure on the overall biofilm response at the end of the experiment (T16) (Figure 5). The first axis separates the ME treatment with biofilms showing the highest diatom growth rate (GR), diatoms abundance and more chlorophyll-a, with respect to the control (C). PCA ordination highlighted that higher diatoms density and GR (found in ME exposed biofilms) corresponded to lower cell size and biodiversity (*H'*) of diatom species of the respective community. Photosynthetic efficiency was lower under ME whereas the phosphorous uptake rate was higher.

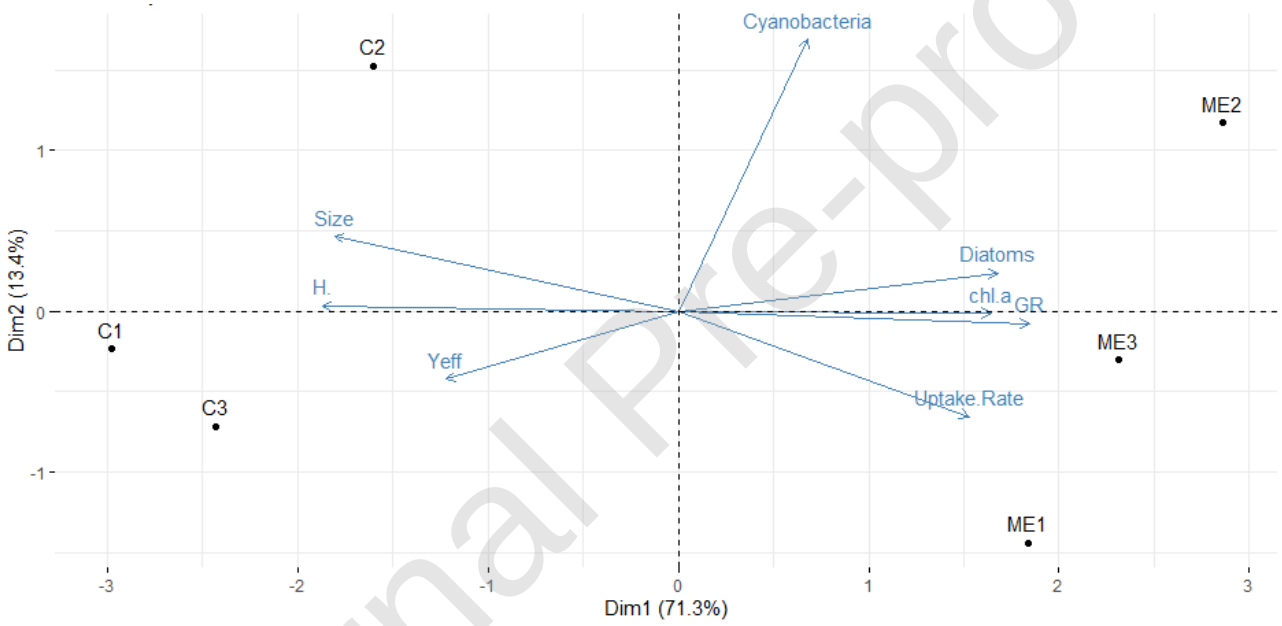

Figure 5. Principal component analysis (PCA). Plotted vectors indicate the correlation scores between the community composition, photosynthetic efficiency (Yeff), phosphorous uptake rate, chlorophyll-a (chl-a) and diatom metrics (growth rate, size, Shannon index H') at the end of the experiment (T16).

Table 2: *F* and *p* values of one-way ANOVA,one-way repeated measures ANOVA, and one-way ANOSIM test performed during the exposure period. Non significant differences were found at T0 (just before the exposure) in any of the parameters evaluated. The most responsive endpoints are reported.

	one-way ANOVA						**Repeated-measures (ANOVA)**	
	T1		**T8**		**T16**			
	F	*p*	F	*p*	F	*p*	F	*p*
Conductivity (µS cm^{-1})	1760.8	<0.001	966.4	<0.001	805.4	<0.001	31.1	0.031
Diatoms:								
Abundance (µg chl-*a* cm^{-2})	n.s		6.3	0.045	7.2	0.002	273.1	0.004
GR (div day^{-1})	-		n.s		3.4	0.037	87.1	0.011
Density (cell div ml^{-1}l)	-		n.s		103.3	<0.001	498.5	0.002

Cell size (µm)	-		22.0	<0.001	66.6	<0.001	211.6	0.005
H'	-		38.4	0.003	403.2	<0.001	98.6	0.010
Biofilm:								
Chl-a (µg cm^{-2})	n.s		n.s		7.9	0.048	311.3	0.003
Yeff	69.8	<0.001	8.7	0.050	n.s		252.0	0.004
Phosphorus uptake (µg P h^{-1} cm^{-2})	4.5	0.015	n.s		n.s		168.1	0.006
one-way ANOSIM								
	R	*p*	R	*p*	R	*p*		
Photosynthetic community composition (µg cm^{-2})	n.s		n.s		0.3	0.011	-	
Diatom taxa	-		n.s		0.6	0.040	-	

4. Discussion

Our study revealed alterations on the biofilm structure and functioning caused by the hypersaline mining effluent from an abandoned potash-mine. Freshwater salinization, induced by the abandoned potash-mine, impacted firstly on the biofilm functioning, causing a strong decrease of photosynthetic efficiency and a slight reduction of P-uptake capacity after only one day of exposure that was recovered throughout time. Thereafter, alterations also occurred at the biofilm structure level, with a significant increase of the diatoms growth rate associated with a decrease of its cell size, species richness and diversity.

In agreement with what was previously described by Cañedo-Argüelles et al. (2017), the hypersaline mining effluent did not influence the dominance among algal groups whithin biofilms during the whole experiment in our study. Previous studies suggested that the threshold for detecting steady effects of freshwater salinization on freshwater biofilm communities (i.e. drastic reductions in abundance and/or the number of surviving taxa) may occur around 3 g L^{-1} of salinity (Cañedo-Argüelles et al. 2017). However, the tolerable threshold that biofilm communities can resist has not been determined yet. Our study revealed that freshwater salinization, induced by an effluent from potash mining with a salinity concentration of 15 g L^{-1} created in each microcosm, caused a drastic shift on the biofilm structure and functioning, although the latter was able to recover over time.

Biofilm community structure may be affected by freshwater salinization (Loureiro et al. 2013; Venâncio et al. 2017). Our results showed that, salinization caused a shift in the diatom community of the exposed biofilms, favoring salt-tolerant diatom species that occupied the niches left by the sensitive ones. Previous works report high abundances of extremely salt-tolerant diatom species (i.e. *Rhoicosphenia abbreviata)* in biofilms exposed to hypersaline mining effluents under conductivities between 10 to 16 mS cm^{-1} (Rovira et al., 2012). Indeed, salinization can cause drastic changes in freshwater diatom communities, affecting the diatom stability and leading to a transition to a new stable state (Herbert et al. 2015). Some of these changes may be related to salinity effects on diatoms valve morphology, which may be taxon-specific (Trobajo Pujadas et al. 2009). In this regard, we observed a direct effect of salinity on cell size probably as a consequence of the energy allocated to osmoregulation processes under high ion concentrations (Entrekin et al. 2019). The elongation of the diatom is driven by the osmotic pressure which breaks down siliceous components of the cell wall. The diatoms, at high salt concentrations, may not be able of producing the necessary intracellular osmolarity to produce the same pressure as at low salt concentrations (Entrekin et al. 2019). Consequently, if cell elongation is less efficient under hypersaline conditions, cell size can decrease faster than under non-polluted conditions (Mitra et al., 2012). Indeed, in our study we observed that the smaller and salt-tolerant taxa occupied the space left by larger diatom species. Moreover, cell size is a key trait determining a species' ability to recover after disturbance due to the fact that smaller cells have typically higher growth rates that confer greater resilience (Lange, Townsend, and Matthaei 2015). In addition, in those communities exposed to the hypersaline mining effluent, a decrease in the diatom species richness was observed. In the ME microcosms, higher densities but smaller cell sizes of the species *Fragilaria crotonensis*, *Navicula reichardtiana* and *Nitzschia intermedia* were observed, whereas higher densities of *Fragilaria crotonensis*, *Navicula reichardtiana*, *Nitzschia tropica*, *Ulnaria biceps* and *Cocconeis euglypta* were found in the control biofilms. Rovira et al. (2012) report that diatoms react to Cl^- changes

as low as 100 mg L^{-1}. Ziemann et al. (2001) report a shift in the diatom composition of river Wipper after salinization and established that it should not exceed a maximum chloride concentration of 400 mg L^{-1} to preserve diatoms diversity. In the salinized section of the River Werra (27 000 mg L^{-1} Cl), a decrease in species richness is also observed (Bäthe and Coring 2011). Our study demonstrated that, despite diatom dominance within the biofilm assemblages increased in response to freshwater salinization, the diversity and species richness of these communities tended to decrease during the exposure.

The biofilm functioning was also affected by the hypersaline effluent, in conjunction with the structural changes observed, particularly in the diatom community. After one day of exposure significant inhibition of autotrophic activity was observed, followed by a rapid recovery. It is well known that short-term exposure to high salt concentration immediately reduces the biofilm photosynthetic efficiency (Cook and Francoeur. 2013). The decrease in photosynthetic efficiency was caused by the photoinhibitory stress induced by the high salt concentration *per se.* It has been described that a drastic increase in the cellular sodium (Na) content can inhibit the CO_2 assimilation processes causing that the cells may down-regulate their light harvesting capacity to acclimatate their low carbon metabolic capacity (Lu and Zhang 2000). The subsequent recovery can be explained by two key factors: i) the rapid ions extrusion from the cells, which is probably associated with the sodium proton antiporter (Lu and Zhang 2000) and ii) the salt-tolerant species' functional redundancy that continued to sustain its activity under stress (Rovira et al. 2012). On the other hand, at the end of the experiment, the phosphorous uptake capacity of ME biofilms was also affected, being significantly lower than the C just after one day of the exposure, and recovered along time at the end of the experiment. Despite the poorly described effects on nutrient uptake capacities under salt exposures (Entrekin et al. 2019), it is known that chemical stressors can affect the ability of biofilms to assimilate PO_4^{3-} since they can be less effective in removing it from the water column (Proia,

Romaní, and Sabater 2017). In this study, the increase in diatom abundance along with the reduction in size could have caused an increase, above the control of the PO_4^{3-} uptake capacity since the species with high surface:volume ratios (i.e the small ones) are characterized by higher nutrient uptake rates per unit biomass (Den Haan et al., 2016). Therefore smaller cells have higher nutrient uptake rates relative to the larger ones (Lange, Townsend, and Matthaei 2015).

We investigated biofilm structure and functioning related to processes that can be relevant at the ecosystem scale, such as photosynthetic efficiency (for the energy supply) or P-uptake (for the self-depuration capacity).The effects of the hypersaline mining effluent did compromise the functioning of the biofilm only in the short-term, though a significant decrease was appreciated in the diatoms richness, highlighting the potential long-term effects of salinization on biofilm biodiversity. In this context, it would be relevant to investigate the effects on the biodiversity of the other microbial components conforming the biofilm. To quantify the overall effects of freshwater salinization on ecosystem services related to biofilm-driven processes, temporal data and more variables (i.e. organic matter breakdown or community metabolism) should also be considered. Furthermore, given that biofilms are the basal level of the stream food webs, further research is needed to assess the direct and indirect effects of freshwater salinization on higher trophic levels.

5. Conclusion

The present study indicates that freshwater salinization from potash mining effluents has the potential to significantly alter the photosynthetic communities of receiving streams. The high salt concentration created by the hypersaline effluent addition in our microcoms, resulted in a significant decline in diatom biodibersity, leading to a community dominated by tolerant species, and an immediate decrease in the photosynthetic efficiency and nutrient uptake capacity that were recovered throughout the experiment. Hence, freshwaterr salinisation threatens not only aquatic biodiversity,

but also ecosystem functioning and services (Estévez et al. 2019). Thus, this is an issue that requires further investigation since the expected increase in water scarcity and desertification in many regions of the world, which would be induced by climate change, is expected to increase the amount of salinized rivers around the world, exacerbating the biological degradation and ultimately supposing a risk to human health (Rotter et al. 2013; Kaushal 2016).

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Lidia Vendrell-Puigmitja: Conceptualization, Methodology, Formal analysis, Investigation, Writting – original draft, Visualization. **Laia Llenas**: Conceptualization, Validation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition. **Lorenzo Proia**: Conceptualization, Methodology, Investigation, Writing - Review & Editing. **Sergio Ponsa**: Writing - Review & Editing, Project administration, Funding acquisition. **Carmen Espinosa**: Conceptualization, Methodology, Investigation, Writing - Review & Editing. **Soizic Morin**: Methodology, Investigation, Writing - Review & Editing. **Meritxell Abril**: Conceptualization, Methodology, Investigation, Validation, Writing - Review & Editing, Supervision, Project administration.

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References

APHA (American Public Health Association), 1992a. Method 4500 - NO2-. Nitrogen (Nitrite)*. Standard Methods for the Examination of Water and Wastewater, 18th ed. Washington, D.C.

APHA (American Public Health Association), 1992b. Method 4500-NO3-. Nitrogen (Nitrate)*. tandard Methods for the Examination of Water and Wastewater, 18th ed. Washington, D.C.

Barral-Fraga, L., Morin, S., Rovira, M. D., Urrea, G., Magellan, K., & Guasch, H. (2016). Short-term arsenic exposure reduces diatom cell size in biofilm communities. Environmental Science and Pollution Research, 23(5), 4257-4270.

Bäthe, J., & Coring, E. (2011). Biological effects of anthropogenic salt-load on the aquatic fauna: a synthesis of 17 years of biological survey on the rivers Werra and Weser. Limnologica, 41(2), 125-133.

Berger, E., Frör, O., & Schäfer, R. B. (2019). Salinity impacts on river ecosystem processes: a critical mini-review. Philosophical Transactions of the Royal Society B, 374(1764), 20180010..

Besemer, K. (2015). Biodiversity, community structure and function of biofilms in stream ecosystems. Research in microbiology, 166(10), 774-781.

Cañedo-Argüelles, M., Hawkins, C. P., Kefford, B. J., Schäfer, R. B., Dyack, B. J., Brucet, S., ... & Coring, E. (2016). Saving freshwater from salts. Science, 351(6276), 914-916.

Cañedo-Argüelles, M., Grantham, T. E., Perrée, I., Rieradevall, M., Céspedes-Sánchez, R., & Prat, N. (2012). Response of stream invertebrates to short-term salinization: a mesocosm approach. Environmental Pollution, 166, 144-151.

Cañedo-Argüelles, M., Brucet, S., Carrasco, S., Flor-Arnau, N., Ordeix, M., Ponsá, S., & Coring, E. (2017). Effects of potash mining on river ecosystems: An experimental study. Environmental Pollution, 224, 759-770.

Cañedo-Argüelles, M., Kefford, B., & Schäfer, R. (2019). Salt in freshwaters: causes, effects and prospects-introduction to the theme issue.Cochero, J., Licursi, M., & Gómez, N. (2017). Effects of pulse and press additions of salt on biofilms of nutrient-rich streams. Science of the Total Environment, 579, 1496-1503.

Cook, L. J., & Francoeur, S. N. (2013). Effects of simulated short-term road salt exposure on lotic periphyton function. Journal of Freshwater Ecology, 28(2), 211-223.

Coste, M., Boutry, S., Tison-Rosebery, J., & Delmas, F. (2009). Improvements of the Biological Diatom Index (BDI): Description and efficiency of the new version (BDI-2006). Ecological indicators, 9(4), 621-650.

Den Haan, J., Huisman, J., Brocke, H. J., Goehlich, H., Latijnhouwers, K. R., Van Heeringen, S., ... & Hoitinga, L. (2016). Nitrogen and phosphorus uptake rates of different species from a coral reef community after a nutrient pulse. Scientific reports, 6(1), 1-13.

Entrekin, S. A., Clay, N. A., Mogilevski, A., Howard-Parker, B., & Evans-White, M. A.

(2019). Multiple riparian–stream connections are predicted to change in response to salinization. Philosophical Transactions of the Royal Society B, 374(1764), 20180042.

Estévez, E., Rodríguez-Castillo, T., González-Ferreras, A. M., Cañedo-Argüelles, M., & Barquín, J. (2019). Drivers of spatio-temporal patterns of salinity in Spanish rivers: a nationwide assessment. Philosophical Transactions of the Royal Society B, 374(1764), 20180022.

Mining Waste Directive (2006) Directive 2006/21/EC of the European Parliament and of the Council of 15 March 2006 on the management of waste from extractive industries and amending Directive 2004/35/EC

Graham, A. A., McCaughan, D. J., & McKee, F. S. (1988). Measurement of surface area of stones. Hydrobiologia, 157(1), 85-87.

Herbert, E. R., Boon, P., Burgin, A. J., Neubauer, S. C., Franklin, R. B., Ardón, M., ... & Gell, P. (2015). A global perspective on wetland salinization: ecological consequences of a growing threat to freshwater wetlands. Ecosphere, 6(10), 1-43.

Iglesias, M. C. A. (2020). A review of recent advances and future challenges in freshwater salinization. Limnetica, 39(1), 185-211.

Jeffrey, S. T., & Humphrey, G. F. (1975). New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. Biochemie und physiologie der pflanzen, 167(2), 191-194.

Kaushal, S. S., Groffman, P. M., Likens, G. E., Belt, K. T., Stack, W. P., Kelly, V. R., ... & Fisher, G. T. (2005). Increased salinization of fresh water in the northeastern United States. Proceedings of the National Academy of Sciences, 102(38), 13517-13520.

Kaushal, S. S. (2016). Increased salinization decreases safe drinking water.

Kaushal, S. S., Likens, G. E., Pace, M. L., Utz, R. M., Haq, S., Gorman, J., & Grese, M. (2018). Freshwater salinization syndrome on a continental scale. Proceedings of the National Academy of Sciences, 115(4), E574-E583.

Lange, K., Townsend, C. R., & Matthaei, C. D. (2016). A trait-based framework for stream algal communities. Ecology and Evolution, 6(1), 23-36.Leira, M., & Sabater, S. (2005). Diatom assemblages distribution in catalan rivers, NE Spain, in relation to chemical and physiographical factors. Water Research, 39(1), 73-82.

Lu, C., & Zhang, J. (2000). Role of light in the response of PSII photochemistry to salt stress in the cyanobacterium Spirulina platensis. Journal of Experimental Botany, 51(346), 911-917.

S. Morin, L. Proia, M. Ricart, C. Bonnineau, A. Geiszinger, F. Ricciardi, et al. Effects of a bactericide on the structure and survival of benthic diatom communities. Life Environ, 60 (2010), pp. 107-114

Proia, L., Romaní, A., & Sabater, S. (2017). Biofilm phosphorus uptake capacity as a tool for the assessment of pollutant effects in river ecosystems. Ecotoxicology, 26(2), 271-282.

Rotter, S., Heilmeier, H., Altenburger, R., & Schmitt-Jansen, M. (2013). Multiple stressors in periphyton–comparison of observed and predicted tolerance responses to high ionic loads and herbicide exposure. Journal of applied ecology, 50(6), 1459-1468.

Rovira, L., Trobajo, R., Leira, M., & Ibáñez, C. (2012). The effects of hydrological dynamics on benthic diatom community structure in a highly stratified estuary: the case of the Ebro Estuary (Catalonia, Spain). Estuarine, Coastal and Shelf Science, 101, 1-14.

Sabater, S., Guasch, H., Ricart, M., Romaní, A., Vidal, G., Klünder, C., & Schmitt-Jansen, M. (2007). Monitoring the effect of chemicals on biological communities.

The biofilm as an interface. Analytical and bioanalytical chemistry, 387(4), 1425-1434.

Sauer, F. G., Bundschuh, M., Zubrod, J. P., Schäfer, R. B., Thompson, K., & Kefford, B. J. (2016). Effects of salinity on leaf breakdown: dryland salinity versus salinity from a coalmine. Aquatic Toxicology, 177, 425-432.

Schäfer, R. B., Bundschuh, M., Rouch, D. A., Szöcs, E., Peter, C., Pettigrove, V., ... & Kefford, B. J. (2012). Effects of pesticide toxicity, salinity and other environmental variables on selected ecosystem functions in streams and the relevance for ecosystem services. Science of the Total Environment, 415, 69-78.

Steffen, W., Grinevald, J., Crutzen, P., & McNeill, J. (2011). The Anthropocene: conceptual and historical perspectives. Philosophical Transactions of the Royal Society A: Mathematical, Physical and Engineering Sciences, 369(1938), 842-867.

Rovira Torres, L. (2013). The ecology and taxonomy of estuarine benthic diatoms and their use as bioindicators in a highly stratified estuary (Ebro Estuary, NE Iberian Peninsula): a multidisciplinary approach= L'ecologia i la taxonomia de les diatomees bentòniques estuarianes i el seu ús com a bioindicadors en un estuari altament estratificat (l'estuari de l'Ebre, NE Península Ibèrica): un estudi multidisciplinari.

Trobajo Pujadas, R. (2003). Ecological analysis of periphytic diatoms in Mediterranean coastal wetlands (Empordà wetlands, NE Spain). Universitat de Girona.Trobajo,

R., Rovira, L., Mann, D. G., & Cox, E. J. (2011). Effects of salinity on growth and on valve morphology of five estuarine diatoms. Phycological Research, 59(2), 83-90.

Vendrell-Puigmitja, L., Abril, M., Proia, L., Angona, C. E., Ricart, M., Oatley-Radcliffe, D. L., ... & Llenas, L. (2020). Assessing the effects of metal mining effluents on freshwater ecosystems using biofilm as an ecological indicator: Comparison

between nanofiltration and nanofiltration with electrocoagulation treatment technologies. Ecological Indicators, 113, 106213.

Ylla, I., Borrego, C., Romaní, A. M., & Sabater, S. (2009). Availability of glucose and light modulates the structure and function of a microbial biofilm. FEMS microbiology ecology, 69(1), 27-42.

Younger, P. L. 2000. Holistic Remedial Strategies for Short- and Long-Term Water Pollution from Abandoned Mines. Institution of Mining and Metallurgy. Transactions. Section A: Mining Technology 109(SEPT/DEC).