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Mutual interaction between arsenic and biofilm in a mining impacted river

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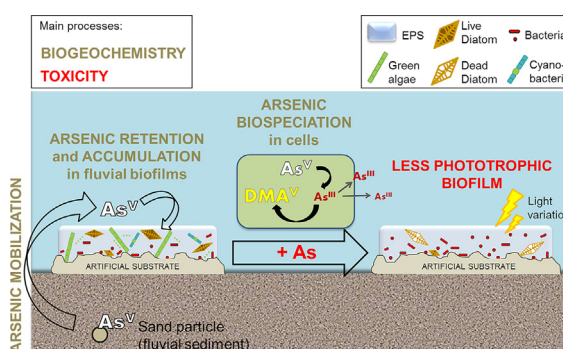
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HIGHLIGHTS

- Epilithic biofilms growing in a gold-mining impacted river accumulate high As concentrations.
- Arsenic inhibits algal growth and increases bacterial and dead diatom densities.
- Methylated As-species found intracellularly suggests As-detoxification by biofilms, even under eutrophic conditions.
- Nutrients, DOC, temperature or light availability must be considered when analyzing effects of As in freshwater ecosystems.

GRAPHICAL ABSTRACT



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ABSTRACT

Gold mining activities in fluvial systems may cause arsenic (As) pollution, as is the case at the Anllóns River (Galicia, NW Spain), where high concentrations of arsenate (As^V) in surface sediments (up to 270 mg kg^{-1}) were found. A 51 day-long biofilm-translocation experiment was performed in this river, moving some biofilm-colonized substrata from upstream (less As-polluted) to downstream the mine area (more As-polluted site), to explore the effect of As on benthic biofilms, as well as their role on As retention and speciation in the water-sediment interface. Eutrophic conditions (range: $0.07\text{--}0.38 \text{ mg L}^{-1}$ total phosphorus, TP) were detected in water in both sites, while sediments were not considered P-polluted (below 600 mg kg^{-1}). Dimethylarsenate (DMAV) was found intracellularly and in the river water, suggesting a detoxification process by biofilms. Since most As in sediments and water was As^V , the high amount of arsenite (As^{III}) detected extracellularly may also confirm As^V reduction by biofilms. Furthermore, translocated biofilms accumulated more As and showed higher potential toxicity (higher As/P ratio). In concordance, their growth was reduced to half that observed in those non-translocated, became less nutritive (less nitrogen content), and with higher bacterial and dead diatom densities. Besides the high As exposure, other environmental conditions such as the higher riparian cover at the more As-polluted site could contribute to those effects. Our study provides new arguments to understand the contribution of microorganisms to the As biogeochemistry in freshwater environments.

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1. Introduction

Microorganisms constitute the majority of all living matter on Earth, most of them living in the form of multicellular aggregates commonly

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referred to as biofilms, attached communities consisting especially of bacteria, algae, fungi and protozoa embedded within a polysaccharide matrix and generally located in close association with wet surfaces and interfaces (e.g., Mora-Gómez et al., 2016). Biofilm communities play a role in the environment both in maintaining and improving the ecological health of freshwater ecosystems (Romani, 2010; Battin et al., 2016). In rivers, biofilms are the first to interact with dissolved substances, such as pollutants, having a high capacity to accumulate heavy metals from the surrounding environment, and being able to actively influence their sorption, desorption and transformation (Behra et al., 2002; Guasch et al., 2010). Furthermore, they are also a site for biotransformation and/or transfer of chemicals to other aquatic organisms (e.g., Guasch et al., 2016). As a major component of benthic biofilms, diatoms (microscopic, unicellular brown algae) are considered good indicators of environmental conditions due to their quick response to environmental changes and their cosmopolitan distribution in aquatic systems, making them widely used in ecotoxicological studies (Morin et al., 2012; Luís et al., 2013, 2016). By carrying out ecotoxicological studies in the field, the effects of pollution may be evaluated under real exposure conditions, using a set of biofilm parameters (i.e. endpoints or biomarkers) together with the analysis of water chemistry and the prevailing environmental conditions (Guasch et al., 2010, 2016, 2017). For instance, *biofilm translocation* experiments in fluvial systems using biofilm developed on artificial substrates are considered an active biomonitoring approach to assess the effects of metal pollution on these natural communities (Bonet et al., 2014; Morin et al., 2016). Another interesting aspect of biofilms is that they allow the coexistence of microniches of different physiological requirements, allowing the simultaneous, but spatially separated occurrence of opposite redox processes in the same biofilm environment (e.g., Kulp et al., 2004; Huang, 2014). This characteristic contributes to the fact that biofilms play a major role in driving biogeochemical cycles (e.g., Huang, 2014). For instance, autotrophic and heterotrophic communities in biofilms may drive, directly or indirectly, a complex interplay of As mobilization, sequestration and transformation (speciation) processes that determine the fate of this metalloid in the environment (Huang, 2014; Wang et al., 2015).

Arsenic may be mobilized during gold mining activities since gold- and As-bearing minerals coexist (e.g., Garelick et al., 2009). In river systems, arsenic is predominantly bound to sediments, which may contain high amounts of this element, especially in mining areas (e.g., Drewniak and Skłodowska, 2013). In these areas, arsenic concentrations can reach up to hundreds or thousands of mg kg^{-1} in sediment (Smedley and Kinniburgh, 2002; Rubinos et al., 2011). Furthermore, mean As concentrations of $137 \mu\text{g L}^{-1}$, and even up to $7900 \mu\text{g L}^{-1}$, have been found in river waters close to mining areas (Smedley and Kinniburgh, 2002), clearly exceeding the concentration limit of chronic As exposure in freshwaters set at $150 \mu\text{g L}^{-1}$ (Criteria Continuous Concentration, CCC) by the Aquatic Life Criteria (USEPA, 2014).

Sediments may act as a sink for pollutants that can subsequently be released upon changes in environmental conditions (e.g., Magbanua et al., 2013). Particularly, changes in water chemistry, such as the introduction of high concentrations of phosphorous (P), may promote the release of As from solid phases through competitive ligand-exchange reactions (e.g., Rubinos et al., 2011).

Both the As total concentration and the As chemical form (As species) have to be considered in toxicological analyses, since a key factor in the As mobility and toxicity is its chemical speciation. In natural waters and soils, arsenic is mostly found in inorganic form (iAs) as arsenate (As^{V} , the thermodynamically stable state under oxic conditions) and arsenite (As^{III} , the predominant species in anoxic conditions) (Smedley and Kinniburgh, 2002; Oremland and Stolz, 2003; Sharma and Sohn, 2009). Both Eh (oxidation/reduction potential measurement) and pH are considered the most important abiotic factors controlling As speciation (Smedley and Kinniburgh, 2002). Usually, the form As^{III} is more toxic for the environment than As^{V} . In contrast, algae are more sensitive

to the As^{V} than to the reduced form As^{III} (Levy et al., 2005; Wang et al., 2015). Microorganisms contribute to the production of these As species and may perform other biotransformations, such as As methylation, resulting in organic products like monomethylarsenate (MMA^{V}) and dimethylarsenate (DMA^{V}) (e.g., Prieto et al., 2016a). Microorganisms may also produce more complex organic compounds, such as arsenosugars and arsenolipids (e.g., Huang, 2014). According to Hellweger et al. (2003), arsenic biotransformation in microalgae depends on P availability: in P-deficient conditions, As^{V} is uptaken by algae, reduced to As^{III} and further methylated as final products; in contrast, under P-enriched conditions, As^{V} is uptaken and reduced, but not all of it is methylated, resulting in As^{III} excretion into the environment. These transformations have an enormous impact on the environmental behavior of As, since the different chemical forms of As exhibit different toxicity towards higher organisms: usually methyl $\text{As}^{\text{III}} > \text{As}^{\text{III}} > \text{As}^{\text{V}} > \text{methyl As}^{\text{V}}$ (e.g., Huang, 2014).

In biofilms, arsenate competes with phosphate (PO_4^{3-}) assimilation (essential element for algal growth), due to the similarity of their chemical properties and structure (e.g., Prieto et al., 2013). This similarity leads to cell toxicity due to the replacement of phosphate by As^{V} in, for instance, phosphorylated compounds, which are vital for the cycling of ATP, inhibiting both photophosphorylation and oxidative phosphorylation (Levy et al., 2005; Rahman and Hassler, 2014; Wang et al., 2015). Due to the similar properties between As^{V} and PO_4^{3-} molecules, the estimation of biofilm As/P ratio allows a better predictability of the As toxicity in microorganisms (e.g., Wang et al., 2013).

The implications of As biogeochemistry on the ecology of freshwaters have been poorly addressed, despite having proved the key role of microorganisms, in particular microalgae and bacteria, on the As detoxification and fate in aquatic environments as in mining impacted rivers. With the aim of assessing the fate and effects of the As pollution in a freshwater ecosystem, we carried out a field study (translocation experiment) with benthic biofilms at the Anllóns River (Galicia, Spain), where high As concentrations in soils and river bed sediments caused by old gold-mining activities were found (Rubinos et al., 2003; Devesa-Rey et al., 2008; Rubinos et al., 2010; Costas et al., 2011). We also intended to study the influence of benthic biofilms on As retention, transformation and mobilization at the water-solid interface of this fluvial polluted-system. Most As in the sediments of the Anllóns River is associated to low-mobility phases (Devesa-Rey et al., 2008; Rubinos et al., 2011). However, previous laboratory experiments show that As biogeochemistry at the Anllóns River is also affected by the biological status of the river sediments, covered by biofilms (Devesa-Rey et al., 2009) mainly constituted by diatoms (Martíñá Prieto et al., 2016).

Therefore, we proceed from the assumption that the fluvial biofilm plays a crucial role in the mobilization of As from the sediment to other compartments. We expected to find a causal link between the presence of As in mobile forms in river sediments and their accumulation in the biofilm, causing changes in its structure and function. We also hypothesized that biofilms may transform and excrete into the water the bioadsorbed or bioaccumulated As by transforming it into other inorganic and organic As species (biospeciation), modulating As toxicity in the environment. A set of analyses was carried out to study the ecology of the Anllóns river in two sites, upstream and downstream the mine area, focusing on the relationship between environmental conditions, especially As exposure, and the structure and function of the biofilm community.

2. Material and methods

2.1. Study site

This experiment was carried out at the Anllóns River (Fig. 1), located in Galicia (Northwest Spain). The river is 71 km long, with a mean slope of 7.6%, and an average annual flow of $10.77 \text{ m}^3 \text{ s}^{-1}$. The basin covers an area of 516 km^2 mostly dedicated to forestry, agriculture and cattle

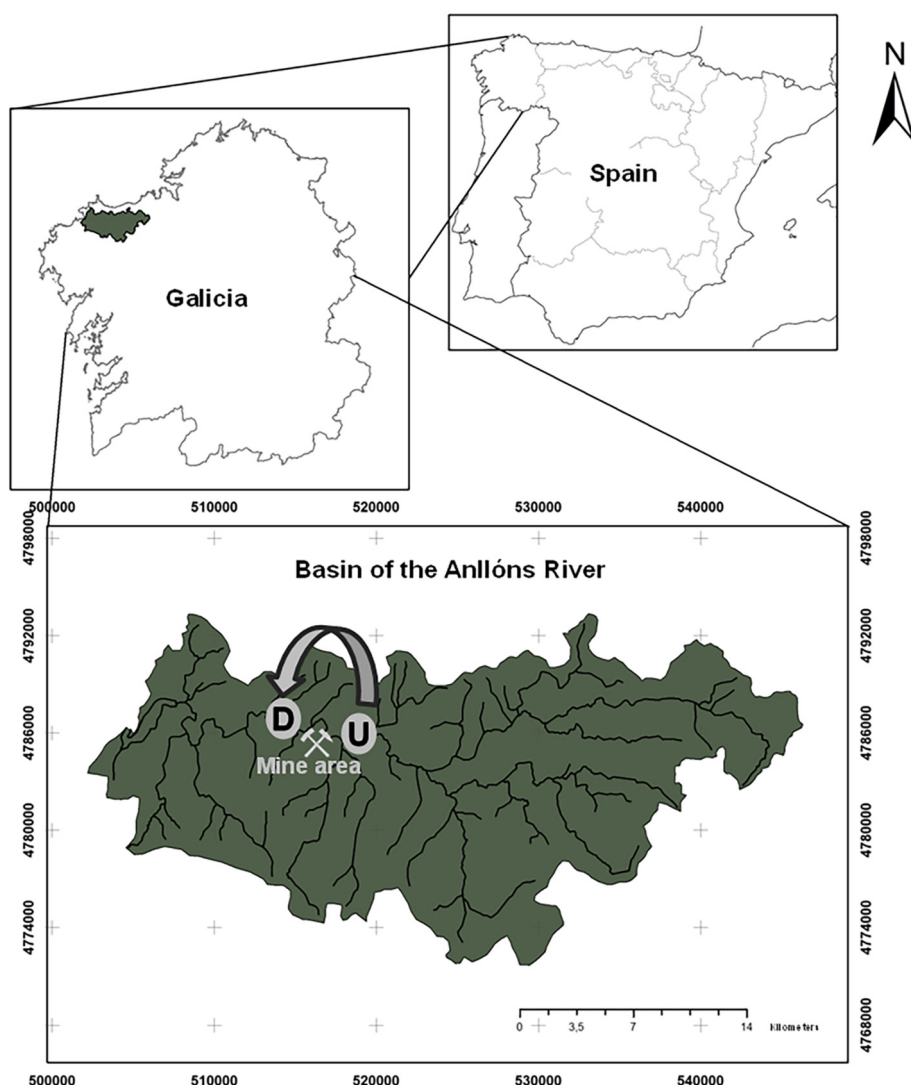


Fig. 1. Study site at the Anllóns River (Galicia, NW Spain). “U” (in gray circle) represents the Upstream sampling site (located upstream of the mining area); “D” (in grey circle) represents the Downstream sampling site (located downstream of the mining area). The grey arrow indicates the biofilm translocation from the Upstream to the Downstream sampling site.

farming. Gold mining activities were active in the area during the Roman Empire and between the years 1895 and 1910. Gold in this area is associated with pyrite and arsenopyrite (Nespereira, 1978). Concentrations of As up to $4000 \text{ mg As kg}^{-1}$ have been detected in superficial soil horizons due to the presence of arsenopyrite mineralization associated with gold ores in hydrothermal quartz veins (Boixet et al., 2007). High total As contents were also detected in the sediments of the Anllóns River, downstream of the gold-mining area (Rubinos et al., 2003), which were attributed to natural geogenic As enrichment exacerbated by mining activities (Devesa-Rey et al., 2008). Recently, the possibility of exploiting this mineralized zone again was considered, causing social concern among locals. With regard to water quality, there are two important sources of P pollution near the main town of Carballo, located upstream of the gold-mining area (at a distance of 14 km approximately): a wastewater treatment plant and a seafood canning factory (Rubinos et al., 2003). According to EU Directive 75/440/CE (Díaz-Fierros, 2003), the Anllóns River has been classified as A2 (potabilization requires physical and chemical treatments) with the exception of a stretch of the river, downstream of Carballo, which is classified as A3 (physical, intense chemical and disinfection treatments).

In this research, we carried out a translocation experiment between two sites with similar lithology (alkaline gneiss) and physicochemical

conditions, but with different As concentrations in the sediments. We selected one site upstream of the mining area referred to as Upstream site or Up site (coordinates $43.222149, -8.782352$); and a second one located just downstream of the mining area, with a higher As concentration, referred to as Downstream site or Down site (coordinates $43.230118, -8.831897$).

2.2. Experimental set-up

Small ($8.5 \times 2 \text{ cm}$) and large ($12 \times 9 \text{ cm}$) sandblasted glass tiles, fixed to cement slabs with neutral silicone sealant (Quilosa, Madrid, Spain), were placed horizontally on the riverbed at the Upstream site for natural biofilm colonization and growth (Fig. 2). The use of artificial glass substrates reduces the heterogeneity that occurs on natural substrates (Cattaneo et al., 1998), making quantitative sampling easier, and allows to control the maturity degree of the biofilm for the analyses. Translocation was carried out after 5 weeks of biofilm colonization: half of the artificial substrates were moved from the Up site to the Down site. The experiment started at that moment and several samplings were carried out in both sites to simultaneously assess the fate and effects of As on biofilms. The experiment lasted 51 days, from July 8th until August 28th 2014.

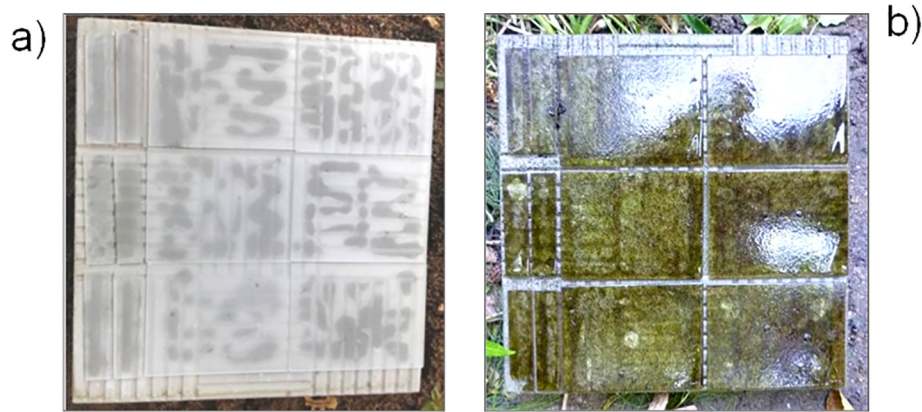


Fig. 2. Experimental setup at the Anllóns River. Artificial substrates before the biofilm colonization (a) and after 5 weeks of colonization (b), when the biofilm was translocated from the Upstream to the Downstream site.

2.3. Sampling

Biofilm and river water were sampled 5 times during the experiment: on days 2, 4, 7, 22 and 51 after translocation. Two samplings were carried out for dissolved As concentration: after 22 days of translocation at the Downstream site, and at the end of the experiment (day 51 after translocation) at both sampling sites. Sediments were sampled twice: on days 7 and 51 after translocation.

2.3.1. River water and sediment sampling and preparation

Triplicate water samples (1 L) were collected and immediately filtered using GF/F glass microfiber filters (Whatman, $\sim 0.7 \mu\text{m}$ of pore size) for nutrients analyses, including total dissolved phosphorus (TP), total dissolved nitrogen (TN) and total dissolved organic carbon (DOC). In the laboratory, some samples were filtered through $0.45\text{-}\mu\text{m}$ cellulose nitrate membrane filters NCS 045 47 BC (Albet LabScience, Dassel, Germany) for soluble reactive phosphorus (SRP); and some were filtered with sterile $0.45\text{-}\mu\text{m}$ Whatman Puradisc 25AS™ syringe filters (GE Healthcare Europe GmbH, Barcelona) for DOC determination. For the analysis of dissolved As, water was filtrated through $0.45 \mu\text{m}$ nylon membrane filters (Whatman). All river water samples were frozen until analysis. The GF/F filters were also kept for dry weight (DW) determination of river water suspended solids (SS).

Complex sediment samples were collected using a small plastic shovel from the top 5 cm of the riverbed sediment at various points from each site, and mixed in situ in hermetic plastic containers, topped up to prevent oxidation during the transport to the laboratory. There, sediment samples were centrifuged (3000 rpm, 15 min) and then solid sediment samples were freeze-dried and sieved ($<2 \text{ mm}$).

2.3.2. Biofilm sampling

Colonized artificial substrates were sampled at random and in triplicate in the field, and the biofilm was scraped into Falcon® tubes or glass vials (Anorsa, Spain). For bacterial density and diatom analyses (relative abundance and quantification of live diatom community), samples were preserved in glass vials with 5 mL of filtered river water with 37% formaldehyde (Panreac, Spain) (dilution 0.5:4.5), with 3 replicates for each one. All samples were kept in dark conditions in the fridge until they were analyzed in the laboratory (1 month for diatom analyses; 3 months for bacterial density). In turn, biofilm from large substrata was scraped into Falcon® 50 mL tubes and then some fresh samples were used to determine the concentration of Total bioaccumulated As and its species distribution in the different biofilm compartments, while other samples were freeze-dried to determine total biofilm dry weight biomass (DW) and elemental composition. Non-scraped samples from small artificial substrates were transported in triplicate to

the laboratory, in dark conditions and in an oxygenated box with river water, for in vivo fluorescence analyses.

2.4. Sample analysis

Most chemical analyses were done using certified reference material and following international and national standard methods (APHA, 1995, 2005; AENOR, 1994, 2012).

2.4.1. Environmental endpoints

2.4.1.1. Light measurements. Environmental light intensity ($\mu\text{mol photons s}^{-1} \text{ m}^{-2}$) was measured in situ with a light sensor (LICOR Inc., Lincoln, Nebraska, USA) at each sampling time at both sites. Light measurements were carried out on submerged biofilm in order to measure light reaching benthic biofilm, as well as below the riparian zone and below a nearby zone without vegetation to calculate the cover (%) of the sampled river sections.

2.4.1.2. River water analyses. Water temperature, dissolved oxygen, pH and electrical conductivity were measured in the field at each sampling time using multiparametric probes (WTW METERS, Weilheim, Germany). Redox potential was determined with a HANNA HI 9025 portable pH-Eh meter equipped with a Pt combination redox electrode (Hanna Instruments, Eibar, Spain). Eh values obtained with the Pt-Ag/AgCl electrode were corrected to refer them to the standard hydrogen electrode by adding 245 mV.

Suspended solids (SS) were analyzed in river water during the experiment (on days 2, 7, 22 and 51 after the translocation) in both sampling sites, according to APHA (1995). Dissolved phosphorus was determined by means of an acid digestion of the filtered samples ($\sim 0.7 \mu\text{m}$) with 1 mL of H_2SO_4 31% and 0.4 g of $(\text{NH}_4)_2\text{S}_2\text{O}_8$ at $121 \text{ }^\circ\text{C}$ during 30 min (APHA, 2005). Phosphorus in the extracts was determined using the phosphomolybdenum blue method described by Murphy and Riley (1962). Soluble reactive phosphorus was determined in $0.45 \mu\text{m}$ filtered samples following the same colorimetric method. Total dissolved nitrogen was determined by digestion of filtered samples ($\sim 0.7 \mu\text{m}$) with H_2SO_4 and 3% H_3PO_4 using the Kjeldhal method (UNE-EN 25663: AENOR, 1994). Dissolved organic carbon was determined in $0.45 \mu\text{m}$ filtered samples using a Total Organic Carbon Analyzer Model TOC-5000 (Shimadzu, Kyoto, Japan), by subtracting the inorganic carbon (IC) concentration from the total carbon (TC) concentration.

Arsenic concentration in river water was analyzed in samples taken at translocation day 22 and 51 following the method described by Prieto et al. (2016a). Total As concentration was determined in $0.45\text{-}\mu\text{m}$ filtered samples by Inductively Coupled Plasma Spectrometry (ICP-MS, Varian 820MS), equipped with collision reaction interface (CRI)

technology to reduce polyatomic interferences. For As speciation (As^{V} , As^{III} , DMA^{V} , MMA^{V} and Arsenobetaine, As-Bet), High-Performance Liquid Chromatography coupled with Inductively Coupled Plasma Spectrometry (HPLC-ICP-MS) was used. The detection limits under the experimental conditions were 2.8, 4.1, 2.9, 4.6 and 2.5 ng L^{-1} for As^{V} , As^{III} , MMA^{V} , DMA^{V} and As-Bet, respectively. The certified reference material EnvironMAT-Drinking Water HIGH EP-H-1 (Catog. number: 140-025-032, SCP Science) was used for quality control.

2.4.1.3. Sediment sample analysis. The pH and Eh were analyzed in situ with a HANNA HI 9025 portable pH-Eh meter equipped with a Pt combination redox electrode (Hanna Instruments, USA). Particle size distribution was determined by dry sieving (2, 1, 0.5, 0.25, 0.1 and 0.05 mm). A representative aliquot of the <2 mm fraction was milled and sieved (<50 μm) for specific analysis. Total phosphate was determined in previously ignited (450 °C for 1 h) samples (<50 μm), using the molybdenum blue method (Murphy and Riley, 1962) after acid digestion (concentrated HF + H_2SO_4 + HCl 10:1:10, 220 °C). Total Kjeldahl nitrogen was determined by wet digestion of samples (<50 μm) with H_2SO_4 , following the Kjeldahl method as described in Guitián and Carballas (1976). The determination of % OM was carried out following the UNE-EN 13039 standard (AENOR, 2012), by calcination at 450 °C for 2 h of 5 g dried samples. Total carbon was calculated using the equation $\%C = \%OM/1.724$.

Total As concentration in the sediment (<50 μm) was determined by X-ray fluorescence (XRF) spectrometry (custom built, equipped with a Philips high-voltage generator and a Mo anode of 2.2 Kw as X-ray source), following the considerations described by Devesa-Rey et al. (2008). The accuracy of the XRF measurement of total As was checked by using the certified reference material BCR CRM-277b. For this reference material, the As concentration (mg kg^{-1}) measured was 45.4 ± 4.1 (certified value 47.3 ± 1.6).

The easily-extractable As concentration (total As and As speciation) was obtained from freeze-dried sediment samples (<2 mm fraction) using a phosphate buffer, following the method of Gleyzes et al. (2002). The total As concentration as well as the As speciation (As^{V} , As^{III} , DMA^{V} , MMA^{V} and As-Bet) were determined by ICP-MS (Varian 820MS) and by HPLC-ICP-MS (Varian Prostar 230 HPLC-Varian 820MS), respectively. The detection limits were similar to those determined for water analysis.

Bioavailable concentrations of As at the Down site were measured twice (days 7 and 51 after translocation) using diffusive gradients in thin films (DGT) devices (DGT Research Ltd., Lancaster, UK) placed at the interface water-sediment, and following the procedures indicated by Prieto et al. (2016a). The mass of As in the resin gel (M), the time-averaged DGT concentrations (C_{DGT}) and the flux (F) of As measured by DGT were calculated according to Zhang and Davison (1995), and DGT® technical documentation.

2.4.2. Biological endpoints

2.4.2.1. In vivo fluorescence measurements. Several small biofilm-colonized artificial substrates ($8.5 \times 2 \text{ cm}^2$) were used to analyze in vivo fluorescence in the laboratory, using a MINI-PAM fluorometer (HeinzWalz, Effeltrich, Germany), at a constant temperature (20 °C) and from a constant distance between the light-emitting diode and the samples (5 mm). First, the minimum fluorescence yield (F_0) was given by the fluorometer in dark adapted samples and then a saturation pulse was applied to obtain the maximum PSII quantum yield (Y_{max}). After 15 min of light adaptation, a saturation pulse of actinic light was applied to the samples to obtain the effective PSII quantum yield (Y_{eff}). According to Corcoll et al. (2012a), the F_0 parameter can be used as an estimation of algal biomass. The Y_{max} is defined as a measurement of the photosynthetic capacity of the community, whereas the Y_{eff} is a measurement of the community photosynthetic efficiency.

2.4.2.2. Biofilm elemental composition. Total DW biomass was obtained by weighing freeze-dried samples of biofilm, from which nutrient stoichiometry (C, N, P content and molar ratios) was analyzed. Carbon and N biogenic elements were determined using an elemental analyzer (PerkinElmer 2400). In turn, TP biogenic element was determined after biofilm digestion in 4 mL of concentrated HNO_3 (65% Suprapure, Merck, Germany) and 1 mL of H_2O_2 (33% Suprapure Merck). Digestion transformed all organic P forms into inorganic forms and, then, total dissolved P content was obtained according to the method detailed in the SRP protocol. Quantification of the C, N and P elements of the benthic biofilm were performed on a dry weight basis, and C/N and N/P biofilm molar ratios were then calculated (Sterner and Elser, 2002; Muñoz et al., 2009; Scharler et al., 2015).

2.4.2.3. Bacterial density. Bacterial density was determined by flow cytometry, adapted from Amalfitano et al. (2009) and Perujo et al. (2016) for biofilm samples. Samples (1 mL) were placed in a glass vial for sonication with 4 mL of detaching solution (1/5 sample dilution) added to help separate cells and avoid their aggregation. After sedimentation of larger biofilm cells (and possibly some sediment particles), a sample purification process was carried out with Nycodenz® (Sigma-Aldrich, USA) to ensure the elimination of those larger particles and cells in the supernatant (1/10 diluted sample). Then, 400 μL of sample (1/10 diluted) were stained with Syto13 (4 μL Fisher, 5 μM solution) and incubated in dark conditions for 15–30 min (1/100 final sample dilution). To normalize fluorescence data, bead solution (10 μL of 10^6 beads mL^{-1} , Fisher 1.0 μm) was added to the samples in a known concentration. The fluorescence color of the beads was yellow-green (505/515), with maximum absorption and emission wavelengths at 495 nm and 505 nm, respectively. Bacterial density was measured using flow cytometry (FACSCalibur, Becton–Dickinson) with a selected size of $\sim 0.1 \mu\text{m}$ (corresponding to most mean heterotrophic bacterial size). Results are referred to as bacteria cm^{-2} .

2.4.2.4. Quantitative estimates of live diatom community. The quantitative estimates of live diatoms were carried out according to Morin et al. (2010), using a Nageotte counting chamber (Microgravure Precip, France) and a light microscopy at a $10\times$ magnification (photomicroscope Nikon Eclipse 80i, Nikon Co., Tokyo, Japan). Counting was separated into 2 types: empty cells that were considered 'dead' and cells occupied by chloroplasts were considered 'alive'. Data were recorded as cells per unit area of sampled substrate (cells cm^{-2}).

2.4.2.5. Relative abundances of the diatom species. Samples for diatom community identification were prepared in permanent slides as recommended by Leira and Sabater (2005), after digestion with 35% HCl and 30% H_2O_2 . Diatom identification to the lowest taxonomic level possible was carried out following standard references and recent nomenclature updates: i.e., Krammer and Lange-Bertalot (1986–1991) and Coste and Rosebery (2011). About 400 frustules were counted per slide, as far as possible, using a light microscope (Nikon E600, Tokyo, Japan) with Nomarski differential interference contrast optics at a magnification of $\times 1000$. Additionally, diatom species richness (S), Shannon-Wiener index of diversity (H), and species evenness (J) were calculated (Shannon and Weaver, 1949; Pielou, 1975).

2.4.2.6. Biofilm arsenic content. Extracellular and intracellular As extraction was carried out in translocated biofilms of the Down site on days 7 and 51 after translocation. For the extractions, 2 g of biofilm sample were used. As a first step, a rinse solution was obtained by washing the samples with river water to extract the soluble As slightly associated to the matrix of extracellular polymeric substances (EPS) and cell surfaces. Subsequently, a sequential extraction was performed following the procedure of Levy et al. (2005) for extracellular As, consisting in a phosphate extraction, and that of Miyashita et al. (2009) for intracellular As, consisting in its extraction from lysed cells with a methanol-

water solution. The details of the extraction procedure are indicated in Prieto et al. (2016a). The total As concentration, as well as the As speciation (As^{V} , As^{III} , DMA^{V} , MMA^{V} and As-Bet) were determined by ICP-MS (Varian 820MS) and by HPLC-ICP-MS (Varian Prostar 230 HPLC-Varian 820 MS), respectively. The detection limits were similar to those determined for water analysis.

Total amount of As in the biofilm was also analyzed, in freeze-dried biofilm samples, after digestion in 4 mL of concentrated HNO_3 (65% Suprapure, Merck, Germany) and 1 mL of H_2O_2 (33% Suprapure Merck), using a high performance microwave digestion unit (Milestone, Ethos Sel, Sorisole (BG), Italy). Then, samples were diluted to 15 mL with milli-Q water and dissolved total As concentration was measured using ICP-MS (7500c Agilent Technologies, Inc. Wilmington, Denmark).

2.5. Data analysis

Homogeneity of variances and normality of data were checked prior to statistical analyses, using SPSS v19.0 software. Before being included in the analysis, variables (except pH and Eh of the river water and the sediment were transformed: most of them were $\ln(x + 1)$ -transformed (sample size $n \geq 3$), including most of biofilm metrics (photosynthetic parameters, that is F_o , Y_{max} and Y_{eff} , live diatom quantification, diatom diversity indices, diatom relative abundance, bacterial density, biofilm DW, biofilm TP and TN content, total As and As-species accumulated in biofilms, and the light irradiance reaching biofilm communities); but proportions and percentages were “arcsine square root” transformed (%C, %N, %P, C/N, N/P and As/P all in biofilms, and % riparian cover). In the cases where sample size were too small ($n = 2$) only mean values were calculated (that is, in river waters: DOC, TP, TN, SRP, total As and As species; in sediments: TP, TN, TC, OM, total As by XRF and the easily extractable-As). The pH and Eh of water and sediment, as well as the water velocity, conductivity, temperature, and its content on oxygen and nutrients (TP, TN, SRP) were not transformed ($n = 1$).

Changes in biofilm biomass (F_o) during both colonization (5 weeks) and experiment period under As exposure (51 days) were fit to a 3-parameter log-normal curve using SigmaPlot v.11.0. We tested for differences between the estimated parameters using the following equation:

$$y = a e^{-0.5\left(\frac{x-x_0}{b}\right)^2}$$

where y represents the periphyton biomass, a is associated with the peak of the curve, b is the rate of inhibition after the peak, x_0 is the time required to reach the maximum or peak value, and x is time in days.

For other biofilm metrics and light measurements, a Two-Way Repeated Measures ANOVA was carried out with all the variables that were analyzed in triplicate every sampling day. That is, the riparian cover percentage, the light irradiance reaching biofilm communities, and the biofilm parameters, such as photosynthesis (F_o , Y_{max} and Y_{eff}), live diatom quantification, biogenic elements (%C, %N and %P) and their ratios (C/N and N/P), as well as the total As concentration accumulated and the As/P ratio in the biofilm. The time variable (expressed as *translocation days*) was the within-subject continuous variable, while the treatment (the Downstream and Upstream sites) was the between-subject variable. The As in different biofilm compartments (total As and As species) and bacterial density were checked by Two-Way ANOVA, since samples had been taken twice during the experiment, while One-Way ANOVA was applied to diatom diversity indices (S , H , J). Finally, post hoc Bonferroni's tests were applied to locate significant differences. All these analyses were carried out using SPSS v19.0 software.

Diatom relative abundance ($\geq 1\%$) of the last day (translocation day 51) was represented in a Non-Metric Multidimensional Scaling plot (NMDS), to show possible variations of community composition

between sites. Multi-response permutation procedures (MRPP) were used to test for inter-site versus intra-site heterogeneity in diatom community structure (Zimmerman et al., 1985) based on Bray Curtis distance (same as for the NMDS). PC-ORD software (version 6.0; McCune and Mefford, 2011) was used to perform the analyses.

The effect of the environmental factors on the biological responses was studied using redundancy data analysis (RDA), using variables taken at both sites (Up, Down) and every sampling day (2, 4, 7, 22 and 51). Previously, variables with a strong inter-correlation were eliminated to avoid collinearity, and then, two principal component analyses (PCA) were carried out to select the variables with the highest explained variance between sampling sites and time: one PCA using the explanatory variables (mainly physico-chemical) and the other one using the response variables (mainly biological variables). Finally, the RDA was carried out on the explanatory variables that best explained the variation of the response matrix (Borcard et al., 2011), corresponding to the light cover percentage, river water velocity and conductivity, river water nutrients (TP, TN, SRP), pH and Eh of sediment, and the biofilm As/P ratio. Biofilm As/P ratio was considered an explanatory variable due to the fact that it may determine the activity and metabolism of the biofilm, since it is the best proxy to estimate As toxicity in cells. The response variables comprised some biofilm metrics as DW, TP and TN content and C/N ratio, as well as photosynthetic parameters (F_o and Y_{eff}) and live diatom density. Correlation analyses were carried out using SPSS v19.0 software. The “vegan” package of R statistical software (version 3.2.2 for Windows; www.r-project.org) was used to carry out both PCAs and the final RDA.

The dynamics and distribution of As species (mean %) in the different environmental and biofilm compartments of the Downstream site (sediment, river water, biofilm rinse solution, extra- and intracellular biofilm) were represented in cumulative bar charts.

3. Results

3.1. Site conditions

3.1.1. Site physicochemical data

Physical and chemical conditions during the experiment are summarized in Tables 1a, 1b and 2. Riparian cover was high at both sites, but especially at the Down site (Table 1a), causing slightly differences in light conditions between both sampling sites. Low light irradiance in biofilms was detected on days 7 and 22 ($<50 \mu\text{mol photons s}^{-1} \text{m}^{-2}$), especially at the Down site on day 22 ($4.90 \pm 0.24 \mu\text{mol photons s}^{-1} \text{m}^{-2}$). In contrast, high irradiances ($>1000 \mu\text{mol photons s}^{-1} \text{m}^{-2}$) enough to cause photo-inhibition were measured on day 2, proving high and significantly temporal light variability. River water (Table 1a) showed a well oxygenated status, neutral pH and low mineralization. High concentrations of TN and TP were detected, especially at the end of the experiment, when high SRP concentrations were also observed. Conversely, higher concentrations of suspended solids were found at the beginning of the experiment. Low As concentrations were detected in all river water samples (ranging from 0.56 at the Up site to $1.83 \mu\text{g L}^{-1}$ at the Down site).

The sediments had neutral pH (ranging from 6.80 to 7.59) and Eh values corresponding to a suboxic state ($100 \text{ mV} < \text{Eh} < 400 \text{ mV}$) at this pH. A higher percentage of OM was observed in the sediments of the Up site and especially at the beginning of the experiment (in July), coinciding also with high TN concentrations. These values decreased during the experiment, resulting in high values of the C/N ratio, especially at the Up site at the end (Table 1b). Regarding the total amount of As in sediments, very high concentrations (ranging from 31 to 100 mg kg^{-1}) were detected at both sites, and especially at the Down site (Table 1b), where the highest concentrations of the easily-extractable As (ranging from 1.77 to 2.23 mg kg^{-1}) were also found (Table 1b). Moreover, the As time-averaged DGT concentration measured at the Down site is indicative of the average As concentration

Table 1a

Physico-chemical properties: environmental light measurements (riparian cover percentage and light reaching biofilms) and physico-chemical properties of river water in the Upstream (Up) and Downstream (Down) sampling sites of the Anllóns River, on days 2, 4, 7, 22 and 51 (corresponding to the last day of the experiment) after the translocation. Single values or mean values are shown ($n = 3$ for riparian cover, light irradiance reaching biofilm and total As on 22Down; $n = 2$ for DOC, nutrients and total As; $n = 1$ for pH, Eh, conductivity, oxygen and temperature). nd = no data. Statistical results of riparian cover for effects on time ($F = 151.741$, $p < 0.001$; degrees of freedom, $df = 4$) and sites ($F = 1593.701$, $p < 0.001$; $df = 1$) were achieved by Two-Way Repeated Measures ANOVA and Bonferroni's test, as well as results of light irradiance reaching biofilm for effects on time ($F = 1192.900$; $p < 0.001$; degrees of freedom, $df = 4$) and sites ($F = 7.093$; $p = 0.056$; $df = 1$). *Significant differences ($p \leq 0.05$) between sampling sites at a specific date (from Bonferroni's test). ^aValues of pH and Eh of sediments ($n = 1$) are indicated in brackets with the river water pH and Eh values, respectively.

Translocation date & Site	Environmental light		River water ^a											
	Riparian cover (%)	Light reaching biofilm ($\mu\text{mol photons s}^{-1} \text{m}^{-2}$)	pH	Eh (mV)	Electrical cond. ($\mu\text{S cm}^{-1}$)	O ₂ (mg L ⁻¹)	T (°C)	Water velocity (m s ⁻¹)	SS (mg L ⁻¹)	DOC (mg L ⁻¹)	TP (mg L ⁻¹)	SRP (mg L ⁻¹)	TN (mg L ⁻¹)	Total As ($\mu\text{g L}^{-1}$)
2Up	90.09 ± 0.81*	1023.00 ± 113.32*	7.07 (7.59)	415 (215)	141	8.88	17.60	0.98	3.60	nd	0.07	0.03	1.07	nd
2Down	98.62 ± 0.14*	1454.67 ± 32.96*	7.16 (7.16)	493 (232)	142	9.28	17.70	0.38	12.60	nd	0.08	0.06	0.81	nd
4Up	83.82 ± 0.19*	156.20 ± 3.40*	7.05 (7.24)	431 (240)	145	9.41	16.50	0.93	nd	nd	0.08	0.03	1.03	nd
4Down	95.00 ± 0.64*	40.90 ± 1.44*	7.19 (7.24)	470 (241)	145	9.02	17.80	0.35	nd	nd	0.09	0.05	1.09	nd
7Up	80.89 ± 2.22*	31.01 ± 0.49*	7.00 (7.38)	431 (191)	149	8.77	17.60	0.87	0.60	nd	0.08	0.04	0.50	nd
7Down	97.66 ± 0.24*	42.69 ± 0.58*	7.22 (7.30)	461 (329)	146	8.98	18.70	0.34	3.10	nd	0.11	0.04	0.36	nd
22Up	93.30 ± 0.95*	45.02 ± 0.34*	6.81 (7.55)	420 (224)	153	8.53	19.10	0.67	1.90	4.45	0.09	0.01	3.65	nd
22Down	91.53 ± 0.26*	4.90 ± 0.24*	7.06 (7.13)	459 (250)	152	8.92	19.20	0.55	1.90	3.39	0.09	0.05	1.94	1.83
51Up	71.20 ± 2.51*	155.43 ± 7.21*	6.83 (6.80)	519 (253)	158	8.00	18.90	0.49	1.20	2.92	0.38	0.07	2.15	0.56
51Down	88.38 ± 1.12*	52.51 ± 2.13*	7.13 (6.86)	464 (281)	161	8.86	18.70	0.20	0.50	2.64	0.19	0.06	3.15	0.79

released from the sediment, was of $0.36 \mu\text{g L}^{-1}$ and, although the total As retained by DGT devices increased with time ($0.05 \mu\text{g}$ on day 7 and $0.25 \mu\text{g}$ on day 51), the As time-averaged concentration decreased over the experiment ($0.44 \pm 0.00 \mu\text{g L}^{-1}$ after 7 days of translocation; $0.29 \pm 0.00 \mu\text{g L}^{-1}$ after 51 days, at the end of the experiment). Finally, sediment samples showed grain size differences between sites (Table 2), with a higher percentage of fine sand at the Down than at the Up site.

3.1.2. Benthic biofilm analyses

The evolution of the biofilm after the translocation of artificial substrates and until the end of the experiment is reflected in Table 3, showing significant differences between the translocated and non-translocated biofilms for the following biofilm metrics: the minimum fluorescence yield (F_0), the density of live diatoms and total bacteria, the amount of accumulated As and the As/P ratio, as well as the elemental composition (% C, % N and % P) and the C/N and N/P ratios.

In the non-translocated biofilms (Upstream site), F_0 was higher (mean value 345.95 ± 145.90) than in the translocated ones (195.14 ± 45.70). Furthermore, the F_0 parameter was significantly fitted to a curve (Fig. 3) showing how the growth of the translocated biofilms located at the Downstream site reached half of those non-translocated (Up site). The percentages of C, N and P were generally higher in the

non-translocated biofilms (Up site, with mean values of 23.27 ± 4.35 for % C, 3.97 ± 0.83 for % N and 0.19 ± 0.03 for % P), than in those translocated at the Down site (showing values of 18.27 ± 5.99 for % C, 2.68 ± 0.89 for % N and 0.12 ± 0.03 for % P). However, C/N and N/P molar ratios were higher in the translocated biofilms (7.08 ± 0.84 of C/N and 22.09 ± 5.41 of N/P) than in those non-translocated (5.97 ± 0.17 of C/N and 20.50 ± 2.64 of N/P).

Live diatom density was, in accordance with the F_0 parameter, higher in the non-translocated biofilms ($492.99 \times 10^3 \pm 193.35 \times 10^3$ cells cm^{-2}) than in those translocated ($4.92 \times 10^3 \pm 2.75 \times 10^3$ cells cm^{-2}). In contrast, a higher density of bacteria was found in the translocated biofilms (Down site), especially at the end of the experiment.

During the experiment, the As concentration was generally higher ($p = 0.017$) in the translocated biofilms (mean values of $15.20 \pm 6.85 \mu\text{g As g}^{-1}$ biofilm at the Down site) than in the non-translocated ones (mean values $8.82 \pm 2.24 \mu\text{g As g}^{-1}$ biofilm at the Up site). Differences were more apparent in the As/P ratio ($p < 0.001$), reaching mean values of $8.75 \times 10^{-3} \pm 5.10 \times 10^{-3}$ in the translocated biofilms, while $2.27 \times 10^{-3} \pm 1.17 \times 10^{-3}$ were found in the non-translocated biofilms.

Summarizing, the biofilms at the Up site (non-translocated biofilms) contained higher nutrient content (lower C/N and C/P ratios), as well as a higher algal biomass (F_0) and had more live diatoms than at the Down

Table 1b

Physico-chemical properties of sediments in the Upstream (Up) and Downstream (Down) sampling sites of the Anllóns River, on day 7 after the translocation and day 51 (the end of the experiment). Mean values are shown ($n = 2$). Values of pH and Eh are shown in Table 1a.

Translocation date & Site	Sediment						
	OM (%)	TC (mg kg^{-1})	TN (mg kg^{-1})	C/N	TP (mg kg^{-1})	Total As (XRF) (mg kg^{-1})	Easily-extractable As (mg kg^{-1})
7Up	4.91	28.50×10^3	1169.73	24	363.25	31	1.32
7Down	2.71	15.70×10^3	604.21	25	381.75	54	2.23
51Up	2.33	13.50×10^3	327.22	41	402.80	51	0.66
51Down	2.13	12.40×10^3	379.68	32	253.10	110	1.77

Table 2
Grain size distribution (%) of the sediments of the Anllóns River, in the Upstream and Downstream sampling sites, on 7 and 51 days after the translocation.

Grain size fraction	Upstream		Downstream	
	7 days	51 days	7 days	51 days
<50 µm	11.9	8.1	6.9	4.5
50–100 µm	2.1	2.5	3.4	2.9
100–250 µm	22.4	18.4	46.6	42.4
250–500 µm	15.0	13.6	37.0	41.7
500–1000 µm	16.1	18.6	5.0	5.7
1–2 mm	32.5	38.8	1.1	2.8

site, where the biofilms had a higher bacterial density. The As content of the biofilms and the As/P ratio were also higher at the Down site, evidencing higher potential As toxicity.

3.1.3. Diatom community composition

At the end of the experiment, 34 species of diatoms were identified, and 4 more only at genus rank (see the Supplementary table in the on-line version). The most abundant species identified were *Cocconeis placentula* Ehrenberg var. *placentula* (55.37%) and *Mayamaea atomus* (Kützing) var. *atomus* (18.34%). Regarding sampling sites, *C. placentula* var. *placentula* (67.60%) and *Cocconeis pediculus* Ehrenberg (14.16%) were the most abundant species at the Up site (non-translocated biofilms), whereas *M. atomus* (33.48%) dominated in the samples of the Down site (translocated biofilms) together with *C. placentula* var. *placentula* (43.21%). No significant differences were found between the translocated and non-translocated biofilms in the diatom diversity indices at the end of the experiment (*S*, *H* and *J*). The NMDS ordination showed slight differences in the diatom assemblages between the

samples at this moment (Fig. 4), suggesting natural variability between communities of the sampled sites (inter – replicates). However, less variability of species composition was found within than between sites (MRPP value: $A = 0.14$, $p = 0.12$).

3.1.4. The influence of environmental parameters on the biofilm responses

Redundancy Analysis (RDA) plot (Fig. 5) shows the influence of environmental parameters (in grey) on the responses attributed to the biofilm (parameters in italics and black), in every sampling day (2, 4, 7, 22 and 51) and sampled site (D for Down site, U for Up site). Based on this analysis, 72.17% of the total variance attributed to all physico-chemical parameters is by the riparian cover, the SRP, TP and TN of the river water, the conductivity and velocity of the river water, the pH and Eh of sediments, and the biofilm As/P ratio as a parameter of potential toxicity.

Almost half of the total variance (49.16%) is expressed by the first axis, which arranges the sampling sites according to the N and P contents, live diatoms and algal biomass (*Fo*), all of which are higher in the biofilms located at the Up site, where environmental conditions are described by higher water TN, TP and electrical conductivity. In turn, the C/N ratio was higher in the translocated biofilms at the Down site, where a higher As/P biogenic ratio was also detected, as well as the highest riparian cover. Therefore, sampling site distribution on axis 1 followed a gradient related to canopy cover, water chemistry and As pollution, separating most Downstream (on the left) from Upstream samples (on the right of the plot).

The second axis explains 23.01% of the total variance and shows an event that happened on day 7 in both sampling sites, related to an increase in water velocity and water turbidity, causing an increase in

Table 3
Biomass metrics of the non-translocated and translocated biofilms located Upstream (Up) and Downstream (Down) of the mine area, respectively, sampled at several times after the translocation: on days 2, 4, 7, 22 and 51 (corresponding to the last day of the experiment). Value units may refer to the surface of the colonized artificial-glass substrates (cm²) or the biofilm freeze-dried mass (g). Mean values ± standard deviations (n = 3) are shown. nd = no data. Except for Bacteria density, all statistical results (F and p) for effects on time (degrees of freedom, $df = 4$) and treatment ($df = 1$) were achieved by Two-Way Repeated Measures ANOVA and Bonferroni's test. For Bacteria density, statistical results (F and p) for effects on time ($df = 1$) and treatment ($df = 1$) were achieved by Two-Way ANOVA and Bonferroni's test. Significant differences in time or site effects are set at $p \leq 0.05$. *Significant differences ($p \leq 0.05$) between sampling sites at a specific date (from Bonferroni's test). (*) Marginal significance ($0.05 < p \leq 0.1$) between sampling sites at a specific date (from Bonferroni's test).

Translocation date & Site	Biofilm													
	<i>Fo</i>	<i>Ymax</i>	<i>Yeff</i>	Live Diatoms ($\times 10^3$ cells cm ⁻²)	Bacteria ($\times 10^6$ cells cm ⁻²)	DW (mg cm ⁻²)	C (%)	N (%)	P (%)	C/N	N/P	As (µg g ⁻¹)	As/P	
2Up	271 ± 112.46	0.46 ± 0.14	0.28* ± 0.05	4.96 ± 1.27	0.12 ± 0.04	0.40 ± 0.31	13.96 ± 4.26	2.30 ± 0.79	0.13 ± 0.02	6.13 ± 0.24	17.39 ± 3.23	10.42 ± 2.27	0.004* ± 0.002	
	179.33 ± 55.89	0.48 ± 0.28	0.35* ± 0.18	9.97 ± 8.21	0.08 ± 0.04	1.24 ± 1.25	12.84 ± 5.98	1.48 ± 0.38	0.10 ± 0.03	8.36 ± 1.96	16.04 ± 7.07	17.73 ± 6.43	0.013* ± 0.006	
4Up	147.67* ± 15.31	0.54 ± 0.06	0.40 ± 0.02	7.66 ± 10.96	nd	0.66 ± 0.29	27.23 ± 8.32	4.70*(*) ± 1.60	0.21 ± 0.08	5.85* ± 0.25	23.11 ± 1.77	7.76 ± 2.16	0.002 ± 0.001	
	228.00* ± 18.73	0.58 ± 0.04	0.34 ± 0.03	2.06 ± 1.24	nd	1.23 ± 0.61	14.87 ± 5.52	2.21*(*) ± 0.93	0.09 ± 0.03	6.80* ± 0.30	23.92 ± 2.93	24.51 ± 18.55	0.014 ± 0.010	
7Up	354.33 ± 206.36	0.28 ± 0.14	0.23 ± 0.13	0.95* ± 0.28	nd	3.30 ± 1.51	16.22 ± 4.41	2.57 ± 0.74	0.14 ± 0.04	6.34 ± 0.17	18.66 ± 3.37	7.89 ± 3.42	0.002 ± 0.002	
	306.67 ± 34.65	0.25 ± 0.19	0.22 ± 0.17	1.88* ± 0.45	nd	1.10 ± 1.29	20.12 ± 2.71	3.20 ± 0.48	0.14 ± 0.02	6.3 ± 0.19	23.21 ± 4.18	12.60 ± 3.16	0.005 ± 0.002	
22Up	434.33 ± 84.05	0.47 ± 0.06	0.30 ± 0.08	12.20* ± 3.62	nd	1.10 ± 0.46	26.49 ± 0.40	4.51 ± 0.15	0.25 ± 0.01	5.87 ± 0.11	17.91 ± 0.85	8.78 ± 1.56	0.002 ± 0.000	
	231.67 ± 103.16	0.51 ± 0.06	0.40 ± 0.06	5.79* ± 1.12	nd	1.43 ± 0.56	25.26 ± 9.74	3.81 ± 1.78	0.15 ± 0.03	6.88 ± 0.94	25.18 ± 7.46	8.80 ± 1.07	0.004 ± 0.001	
51Up	522.43* ± 311.31	0.48 ± 0.08	0.40 ± 0.04	2439.22* ± 950.64	0.11* ± 0.16	1.47 ± 0.54	32.44 ± 4.36	5.75*(*) ± 0.87	0.23 ± 0.00	5.65* ± 0.09	25.43 ± 3.97	9.26 ± 1.78	0.001* ± 0.001	
	179.20* ± 16.04	0.55 ± 0.02	0.47 ± 0.01	27.41* ± 6.35	1.97* ± 1.24	1.15 ± 1.08	23.45 ± 9.11	3.77*(*) ± 1.61	0.15 ± 0.04	6.34* ± 0.41	23.91 ± 4.88	12.38 ± 5.05	0.008* ± 0.006	
Time effect	F = 1.449	F = 2.984	F = 2.735	F = 35.045	F = 2.942	F = 1.630	F = 4.669	F = 5.711	F = 5.170	F = 3.547	F = 2.547	F = 0.888	F = 1.826	
	p = 0.264	p = 0.051	p = 0.066	p < 0.001	p = 0.125	p = 0.215	p = 0.011	p = 0.005	p = 0.007	p = 0.030	p = 0.080	p = 0.494	p = 0.173	
Site effect	F = 10.936	F = 0.063	F = 0.312	F = 8.323	F = 6.312	F = 2628.636	F = 7.357	F = 17.822	F = 25.761	F = 8.129	F = 17.413	F = 15.417	F = 145.397	
	p = 0.030	p = 0.814	p = 0.606	p = 0.045	p = 0.036	p = 0.0127	p = 0.053	p = 0.013	p = 0.007	p = 0.046	p = 0.014	p = 0.017	p < 0.001	

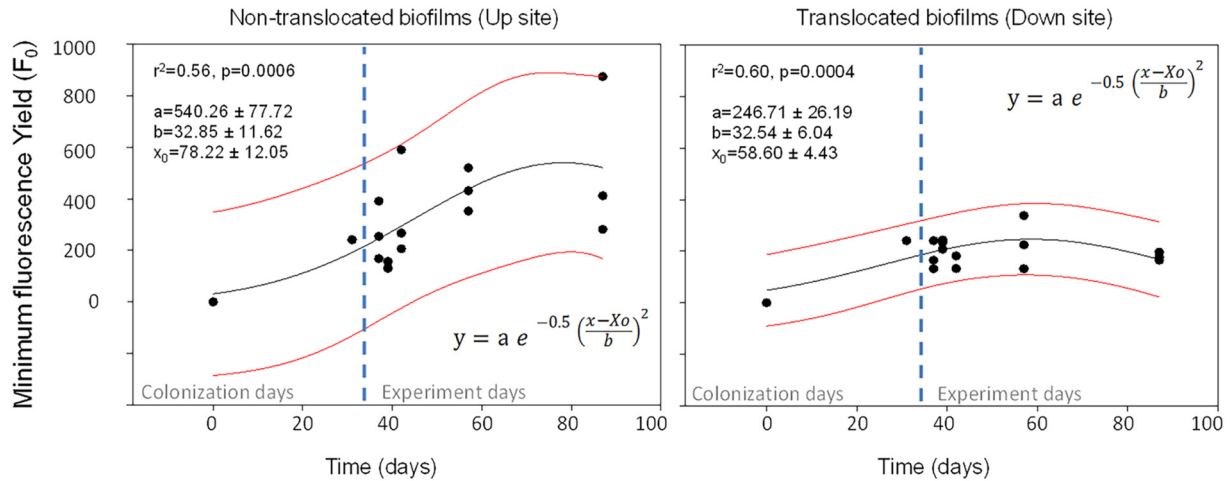


Fig. 3. Changes in biofilm biomass during the “biofilm colonization days” versus the “experiment days” at the Upstream site (plot on the left) and the Downstream site (plot on the right). Fitting of F_0 parameter over time (days) follows a 3-parameter log-normal curve, assuming growth inhibition at the end of the experiment, and where the a parameter is associated with the peak of the curve; the b parameter is the rate of inhibition after the peak; the x_0 parameter is the time required to reach the maximum or peak value and x is time in days. Vertical dotted lines indicate the translocation day (before that time, all samples were located exclusively in the Upstream site, corresponding to the colonization period). Differences between the parameters for each sampling site were tested with two-way ANOVA in order to check if changes in the growth of the communities were statistically significant. Significance was set at $p \leq 0.05$. Red lines indicate 95% confidence band.

biofilm DW and a decrease in the Y_{eff} , and illustrating the effects of a previous rain event.

3.2. Arsenic speciation

In river water (Table 4), arsenate was the dominant species (99% at the Down site on day 22 and 72.5% on day 51; and almost 89% at the Up site on day 51). At the end of the experiment, As^{III} , DMA^V and $As\text{-Bet}$ percentages were higher at the Down site, where these species represented all together >27% of the total As, but only 11% at the Up site. In contrast, only inorganic As species were found in the sediment samples:

As^V was the predominant As species in both sites, and especially at the Down site, where it represented >86% of the total As (Table 4).

As was previously mentioned, the translocated biofilms accumulated more total As than the non-translocated biofilms. For this reason, arsenic speciation was focused on the translocated biofilms (Down site), in the extracellular and intracellular biofilm compartments (Table 5). The total As in the rinse solution, which contains soluble As slightly associated to cell surfaces and EPS matrix, showed the same proportion of As^V and As^{III} on day 7, but As^V dominated at the end of the experiment. A low amount of DMA^V was also detected in this rinse solution (around 2% on day 7 and 0.6% on day 51). Arsenic in the extracellular compartment, where it is adsorbed to cell surfaces and EPS matrix, showed a species distribution very similar to that of the rinse solution, but with higher DMA^V concentrations at the end of the experiment. Finally, the total As concentration in the intracellular compartment at the end of the experiment was dominated by As^V species, followed by an important amount of DMA^V (around 25% on day 7 and 18% on day 51) and As^{III} . Low percentages of $As\text{-Bet}$ (ranging from 0.13 to 1.35%) appeared in all the compartments at the end of the experiment. No MMA^V was detected in any sample. In summary, while in river water and sediment As^V was the predominant species, other As species were relevant in the biofilm, namely, As^{III} in the rinse solution and extracellular compartment (particularly on day 7), and also DMA^V in the intracellular fraction.

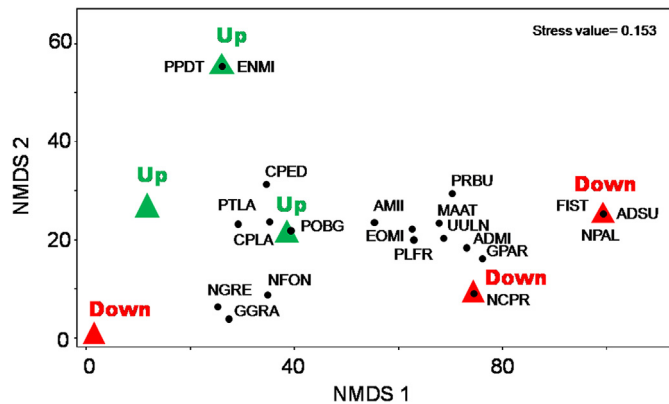


Fig. 4. Nonmetric dimensional scaling (NMDS) plot showing sampling sites ordination (Up, in green; Down, in red) according to their diatom species composition at the end of the experiment (day 51) after biofilm translocation to the Downstream site). Species abbreviation: ADMI, *Achnanthydium minutissimum* (Kützing) Czarnecki; AMII, *Achnanthydium minutissimum* (Kützing) Czarnecki f. *inconspicuum* (Østrup) Compère & Riaux-Gobin; ADSU, *Achnanthydium subatomus* (Hustedt) Lange-Bertalot; CPED, *Cocconeis pediculus* Ehrenberg; CPLA, *Cocconeis placentula* Ehrenberg var. *placentula*; ENMI, *Encyonema minutum* (Hilse in Rabhenhorst) D.G. Mann in Round, Crawford & Mann; EOMI, *Eolimna minima* (Grunow) Lange-Bertalot; GGRA, *Gomphonema gracile* Ehrenberg; GPAP, *Gomphonema parvulum* (Kützing) Kützing var. *parvulum* f. *parvulum*; MAAT, *Mayamaea atomus* (Kützing) Lange-Bertalot var. *atomus*; NCPR, *Navicula capitatoradiata* Germain; NGRE, *Navicula gregaria* Donkin; NFON, *Nitzschia fonticola* Grunow in Van Heurck; NPAL, *Nitzschia palea* (Kützing) W. Smith var. *palea*; PLFR, *Planothidium frequentissimum* (Lange-Bertalot) Lange-Bertalot; PTLA, *Planothidium lanceolatum* (Brebisson ex Kützing) Lange-Bertalot; PPDT, *Planothidium pseudotanense* (Cleve-Euler) Lange-Bertalot; PRBU, *Planothidium robustius* (Hustedt) Lange-Bertalot; POBG, *Psammothidium oblongellum* (Østrup) Van de Vijver; UULN, *Ulnaria ulna* (Nitzsch) Compère; FIST, *Fistulifera* sp. Species representing $\geq 1\%$ in at least one sample.

4. Discussion

4.1. Site characterization

Several environmental factors measured in the sediments and river water were similar at both sampling sites, attributed in particular to the similar lithology and intense farming activities in the basin. Thus, eutrophic conditions were detected in the water in both sites during the whole experiment (experimental ranges: 0.07–0.38 mg TP L⁻¹ and 0.36–3.15 mg TN L⁻¹), especially at the end, as most values correspond to eutrophic conditions set at 0.075 mg TP L⁻¹ and 1.5 mg TN L⁻¹ by Dodds et al. (1998). Furthermore, high concentrations of SRP, commonly analyzed as a measurement of the immediately available orthophosphate (PO₄⁻³) in water and effectively used for predicting algal production (Dodds, 2006; Allan and Castillo, 2007), confirmed these eutrophic conditions (experimental range: 0.01–0.07 mg SRP L⁻¹, usually 0.01–0.03 mg SRP L⁻¹, according to Mainstone and Parr, 2002).

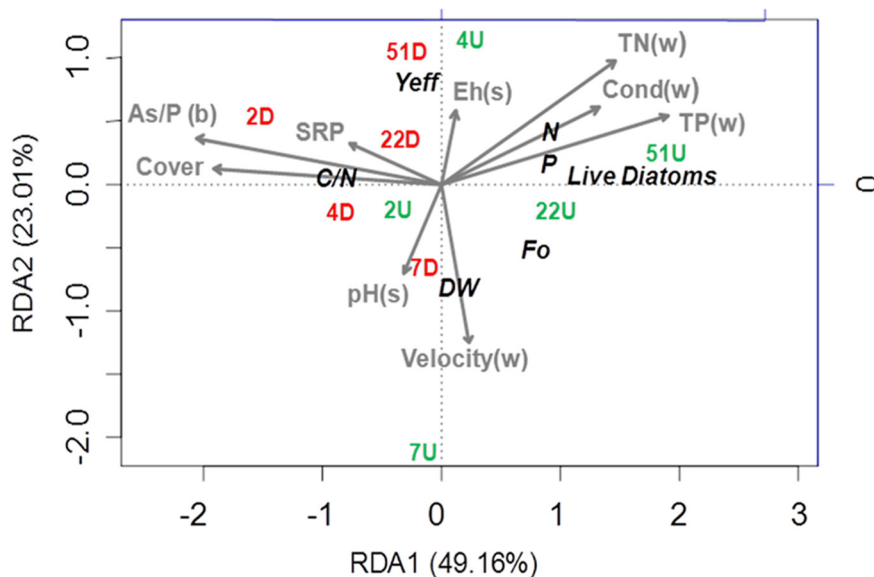


Fig. 5. Plot of redundancy data analysis (RDA) to explain the influence of the environmental factors (in grey) on the biological responses (in black) at every sampling day (2, 4, 7, 22, 51) and sampled site (U: Up site, in green; D: Down site, in red). The explanatory variables that best explained the variation of the response are represented in the plot. The different compartments (biofilm, water or sediment) where variables were measured are specified in parentheses (b, w and s, respectively).

Regarding DOC, the values corresponded to typical concentrations in running water (Thurman, 1985; Allan and Castillo, 2007) in both sites. For the heterotrophic component of the biofilms, DOC is usually a major C source, and particularly under low light conditions (Romání et al., 2004; Allan and Castillo, 2007). With respect to sediments, and regarding nutrient concentrations, they were not considered polluted (the values detected were below the threshold values of 600 mg TP kg⁻¹ and 550 mg TN kg⁻¹, according to Persaud et al., 1993), except for TN at the beginning of the experiment and especially at the Up site. More organic matter (% OM and C concentration) was also found in the sediments of the Up site. High C/N ratio values in the sediments suggested an allochthonous origin of this organic matter, probably coming from terrestrial plants, since C/N ratios >12 are indicative of OM rich in lignin and cellulose, and are attributable to terrestrial origin (Lamb et al., 2006). Insufficiently treated water from the collectors of the wastewater and sewage treatment plant, as well as effluents from the canning factory, both located upstream in the town of Carballo, might also contribute to the allochthonous OM, and also TP and TN as well (Rial, 2007; Devesa-Rey et al., 2009).

With regard to As concentrations, the river water was not contaminated in any of the sampling sites, since concentrations were far below 150 µg As L⁻¹, which is the limit for a chronic exposure in freshwater systems (Aquatic Life Criteria, USEPA, 2014). However, sediment As

concentrations were high. According to the Canadian Sediment Quality Guidelines (Severe Effect Level set at 33 mg kg⁻¹ by Persaud et al., 1993), the sediments in both sites may be considered heavily As-polluted and, thus, likely to affect the sediment-dwelling organisms, especially at the Down site. In this site, the generic reference level for soils in this region, set at 50 mg kg⁻¹ by Vázquez and Anta (2009), was exceeded twice on day 51. Arsenic was also more easily extractable at the Down site, indicating that it can be easily transferred from sediments to other compartments as water and biota, and, consequently, be more available. Inputs of dissolved phosphate may favor As mobilization, as shown by Rubinos et al. (2010, 2011).

4.2. Arsenic fate

In keeping with the higher As concentration and mobility in the sediments at the Down site, the bioaccumulated As concentration and As/P ratio were always higher in the translocated biofilms than in the non-translocated ones. Focusing on As distribution and speciation at the Down site, biofilms accumulated high As concentrations, which were equally distributed among the intracellular and extracellular compartments. The total As concentration in the biofilms exceeded the easily-extractable As in the sediments, thus suggesting As accumulation over time and confirming that biofilm is a major sink for arsenate (Lopez et al., 2016). This indicates that biofilms growing on rock surfaces and granular sediments are able to accumulate the As released from the sediment, which is the main source of As since the water concentration is very low (below the environmental limits established). The As time-averaged DGT concentration measured at the Down site supports these results. Average As concentration accumulated in this device was below the As concentration in the river water in this polluted site, suggesting that there is a contribution of As from the sediment.

With regard to As mobility, interchanges between the water and sediment may be modified due to inputs of dissolved phosphate (Rubinos et al., 2011), leading to As release from the sediment to the water column. In this scenario, As retention and biotransformation in fluvial biofilms might have important implications for the ecosystem health. Previous laboratory studies which analyze As mobilization from As-polluted sediments from the Anllóns River have also shown As accumulation by biofilms and similar As distributions among intracellular and extracellular compartments (Prieto et al., 2016b). Biofilm

Table 4
Percentages of As speciation (referring to the total arsenic concentration analyzed) in samples of sediment and river water from the Upstream and Downstream site are shown (n = 2). n.d. = not detected.

Translocation date & Site	Arsenic speciation in river water (% of Total As)				
	As ^V	As ^{III}	DMA ^V	MMA ^V	As-Bet
22Down	99.39	n.d.	0.61	n.d.	n.d.
51Up	88.80	2.45	2.98	n.d.	5.77
51Down	72.50	8.40	7.00	n.d.	12.10
	Arsenic speciation in sediment (% of Total As)				
	As ^V	As ^{III}	DMA ^V	MMA ^V	As-Bet
7Up	61.11	38.90	n.d.	n.d.	n.d.
7Down	88.14	11.86	n.d.	n.d.	n.d.
51Up	64.65	35.35	n.d.	n.d.	n.d.
51Down	86.06	13.95	n.d.	n.d.	n.d.

Table 5

Total As concentration and percentage of As species in translocated biofilms (in the rinse solution, the extracellular and the intracellular compartments) on 7 and 51 days after translocation at the Downstream site (Down). Mean values \pm standard errors are shown ($n = 3$ in samples on day 7; $n = 5$ in samples on day 51). Statistical results (F and p) for effects on time (degrees of freedom, $df = 1$) and biofilm compartment ($df = 2$) were achieved by two-way ANOVA. Significant differences are set at $p \leq 0.05$. n.d. = not detected.

Biofilm compartment	Total arsenic ($\mu\text{gAs g}^{-1}$)	Arsenic speciation in biofilm (% of total As)		DMA ^V	MMA ^V	As-Bet
		As ^V	As ^{III}			
Rinse solution						
7Down	0.97 \pm 0.35	48.33 \pm 3.89	49.66 \pm 4.15	2.01 \pm 0.40	n.d.	n.d.
51Down	1.48 \pm 0.63	78.06 \pm 5.36	19.97 \pm 4.46	0.62 \pm 0.62	n.d.	1.35 \pm 0.83
Extracellular						
7Down	1.96 \pm 0.60	49.48 \pm 4.90	50.52 \pm 4.90	n.d.	n.d.	n.d.
51Down	1.99 \pm 0.63	83.73 \pm 5.88	11.61 \pm 4.14	4.54 \pm 1.68	n.d.	0.13 \pm 0.13
Intracellular						
7Down	1.18 \pm 0.52	60.27 \pm 9.70	14.39 \pm 7.62	25.34 \pm 4.21	n.d.	n.d.
51Down	2.91 \pm 0.55	77.95 \pm 6.66	3.54 \pm 2.01	18.26 \pm 4.58	n.d.	0.25 \pm 0.25
Biofilm compartment effect	F = 1.564 p = 0.237	F = 0.403 p = 0.674	F = 18.585 p < 0.001	F = 30.522 p < 0.001		F = 7.526 p = 0.004

also retained As from As-polluted waters, but in this case it was mostly accumulated in the extracellular compartment (Prieto et al., 2016a).

4.3. Biofilm responses

The most significant differences in the biological properties between the sampling sites were the inhibition of algal growth (although photosynthesis was not clearly affected), the increase of diatom mortality, the development of higher bacterial density, and the loss of nutritional elements (biofilm with less N and more C content). The higher amount of DOC at the Down site would contribute to the development of a less nutritive biofilm, which would be reflected in the strong decrease in live diatoms and the increase in bacterial density. These changes in the structure of biofilm may be attributed to the As toxicity, since some of them were already detected in previous As-exposed biofilms (e.g., Rodriguez Castro et al., 2015; Tuulaikhuu et al., 2015; Barral-Fraga et al., 2016). A selective decrease in diatom abundance, measured as % Fo for brown algae, was already detected after chronic As exposure in biofilms developed on artificial substrates in channels including a sediment compartment (Tuulaikhuu et al., 2015).

However, effects caused by other environmental factors, mainly light availability, have to be considered. Light is the first energy source for the autotrophic component of biofilm, modulating not only biofilm structure and its function but also biofilm response to pollutants as metals (Corcoll et al., 2012b; Guasch et al., 2016). At the Down site, the higher riparian cover could contribute to a lower algal growth and, in combination with DOC availability, may favor the growth of heterotrophic bacteria (Romaní et al., 2004; Allan and Castillo, 2007). Turbidity, favored by the finer particle size of the sediment and evidenced by the higher SS in water, may influence biofilm communities (Magbanua et al., 2013), enhancing heterotrophic organisms at the Down site. Light regime is usually strongly variable for fluvial biofilms in space and time (e.g., Guasch and Sabater, 1998), and high irradiances are considered when $>1000 \mu\text{mol photons s}^{-1} \text{m}^{-2}$ (Hill, 1996). Light intensity controls photosynthesis, and many authors have reported that there is a range of intensities over which photosynthesis is highly efficient ($30\text{--}400 \mu\text{mol s}^{-1} \text{m}^{-2}$) with an inhibitory effect above $500 \mu\text{mol s}^{-1} \text{m}^{-2}$ (Villeneuve et al., 2010). In this study, the light available to the biofilms was very variable during the experiment. Particularly at the Down site, light may have been a limiting factor, especially during the last days of the experiment, but may also have caused photoinhibition at the beginning, when very high irradiance was detected in both sites. Effects of light stress in combination with metal toxicity were already found in biofilms for which metal exposure caused a magnification of light inhibitory effects on algae and a reduction in relative contribution of diatoms (Corcoll et al., 2012b). Taking into account the higher As amount at the Down site and the higher potential As toxicity in these translocated biofilms ($>\text{As/P}$), this magnification of light

inhibitory effects could partially explain the observed lower algal growth and higher diatom mortality. Therefore, arsenic may affect algal growth, but a direct or combined effect of light conditions should not be dismissed.

4.4. Evidence of arsenic speciation by biofilm

Arsenate was the predominant species in all the studied compartments (Fig. 6), particularly in the river water, as predicted by the chemical theory and observed by Prieto et al. (2016a, 2016b) for this river. Arsenite was very scarce in water but increased in sediment and also in the rinse solution and extracellular compartment of the biofilm, to again diminish in the intracellular compartment (Fig. 6).

Our results point to an effect of the biofilm on As speciation by, first, reducing As^V to As^{III}. This biotransformation may occur extracellularly (adsorbed As^V), or intracellularly (uptaken As^V) followed by As^{III} excretion (Oremland and Stolz, 2003; Rahman and Hassler, 2014). Excretion of As^{III}, which typically occurs under P-enriched conditions (Hellweger et al., 2003), does not seem to be relevant in this case, as only low amounts of As^{III} were detected in the water. Inside the cells, As^{III} might be transformed into the less toxic methylated species (DMA^V), which were then also excreted, suggesting active As detoxification by the biofilm. Although As-methylation was reported to occur mainly under P-limiting conditions regarding the model proposed by Hellweger et al. (2003), this transformation took place in the apparent eutrophic conditions of this study. Accordingly, an intensification of this biomethylation process in eutrophic conditions was also previously detected (Baker and Wallschläger, 2016; Yan et al., 2016).

The As-Bet detected in the river water was unexpected, since this species is almost absent in freshwater organisms (e.g., Caumette et al., 2012). It was tentatively attributed to the contribution of the seafood canning factory located upstream of the sampled sites. This type of pollution was not considered in the design of this study, thus requiring further investigations.

Overall, the results point to a relevant contribution of benthic biofilms to As biogeochemistry in freshwater environments. The mutual interactions between As and biofilm are tentatively represented in Fig. 7. The As^V is deposited in the sediments, where it is available for microorganisms (epipsammon), which may transform and excrete As into the water-sediment interface of the river. Moreover, the fate of arsenic (mainly As^V) in the Anllóns River system is very dependent on the inputs of dissolved phosphate, acting as an As-displacing ligand in the sediments (Rubinos et al., 2011), which promotes the exchanges between the water and sediment. This dissolved As^V can then be available for microorganisms, namely for the benthic biofilms growing on rock surfaces (epilithic biofilms composed by bacteria and, mostly, microalgae), represented in this work by those developed on artificial substrates. Once on the biofilm surface, As^V may accumulate in the

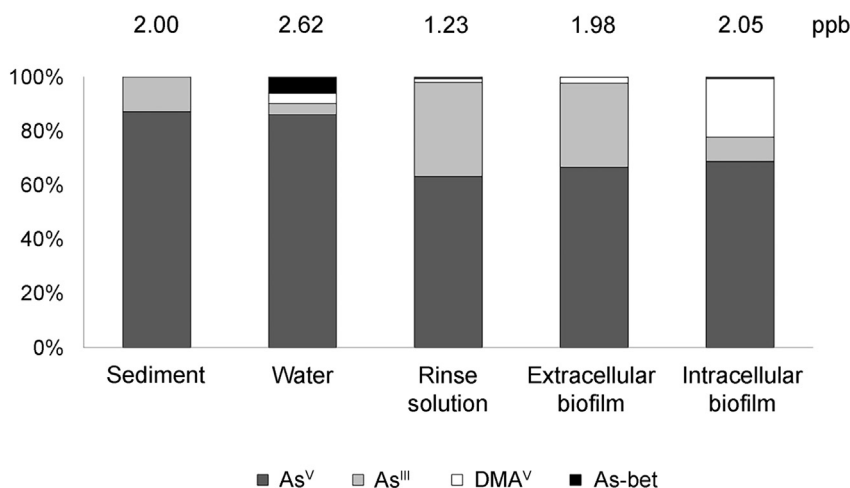


Fig. 6. Arsenic speciation at the Downstream site. Stacked bar plots show the evolution of As speciation at the Downstream site through the different analyzed compartments: sediment (the easily-extractable As), river water, and biofilm (rinse solution, extracellular and intracellular fractions), with mean values calculated from samplings on day 7 after translocation (except for river water, which was on day 22) and at the end of the experiment (51 days after translocation). Total mean As concentrations are also shown above each bar expressed in ppb (concretely, in $\mu\text{g As g}^{-1}$ for sediment and biofilm; while in $\mu\text{g As L}^{-1}$ for river water). As^V = arsenate; As^{III} = arsenite; DMA^V = dimethylarsenate; As-Bet = arsenobetaine.

EPS matrix, adsorb to cells or even be uptaken by microorganisms, which in turn may modify As speciation (through arsenate reduction and methylation) and excrete it into the water as the more toxic As^{III} and/or as organic species such as DMA^V (and, less uncertain, As-Bet), thus becoming available to the aquatic food web.

5. Conclusions

In this experiment, we have demonstrated the mutual interaction between As and benthic biofilms in a mining impacted river, where the toxicant was mostly associated with sediments. Biofilms located at the site with higher As concentrations in sediments, accumulated more As than those of the site with lower concentrations. This As exposure may partially explain the inability of algae to grow and the increase

in bacterial and dead diatom densities. Methylated As-species (DMA^V) were found in the intracellular biofilm compartment and even under eutrophic conditions, suggesting that detoxification processes (methylation) occurred within the biofilm. Our study provides valuable information to understand the contribution of benthic biofilms to As biogeochemistry in freshwater environments and, specifically, in the water-benthic biofilm interface. Furthermore, this study contributes to highlight the importance of biofilms as indicators of the quality of freshwater ecosystems, supporting the recognition by the Water Framework Directive (European Commission, 2000) as “biological quality elements” (BQEs) for the assessment of the ecological status of aquatic systems.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2018.04.287>.

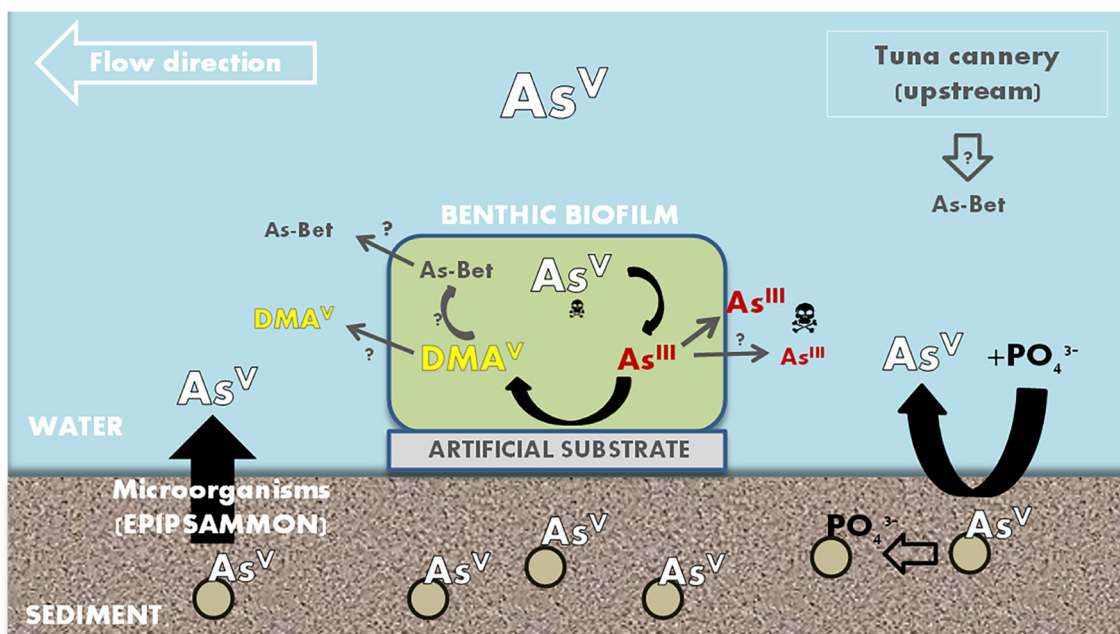


Fig. 7. Hypothesized scenario of As fate at the Downstream site, based on obtained results and literature, focusing on the contribution of the benthic biofilms to the As biogeochemistry in this freshwater environment. Arsenate (As^V) may be mainly released from the sediment to the water by epipsammic biofilm (see it on the left side) or by exchange with phosphate (PO₄³⁻), coming from external inputs (see it on the right side). Once in the water, arsenate may be uptaken by the benthic biofilm, which may also transform it to other As-species (arsenite, As^{III}; and dimethylarsenate, DMA^V) mainly by reduction and further methylation reactions. More complex organoarsenicals were found in water, specifically the arsenobetaine (As-Bet), which could come from the seafood factory (tuna cannery) located upstream the sampling sites, or be produced by the biofilm itself, what would be less probable.

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Competing financial interests

The authors declare no competing financial interests.

References

- AENOR (Asociación Española de Normalización y Certificación), 1994. Water Quality. Determination of Kjeldahl Nitrogen. Method After Mineralization With Selenium (Norma Española UNE-EN 25663. (ISO 5663:1984). Madrid).
- AENOR (Asociación Española de Normalización y Certificación), 2012. Soil Improvers and Growing Media - Determination of Organic Matter Content and Ash (Norma Española UNE-EN 13039. Madrid).
- Allan, J.D., Castillo, M.M., 2007. Primary producers. Stream Ecology: Structure and Function of Running Waters. Springer, Netherlands, pp. 105–134.
- Amalfitano, S., Fazi, S., Puddu, A., 2009. Flow cytometric analysis of benthic prokaryotes attached to sediment particles. J. Microbiol. Methods 79, 246–349.
- APHA, 2005. Standard Methods for the Examination of Water and Wastewater. 21 ed. American Public Health Association, American Water Works Association, Water Environment Federation.
- APHA A., 1995. WEF, 1995 Standard Methods for the Examination of Water and Wastewater. 1.
- Baker, J., Wallschläger, D., 2016. The role of phosphorus in the metabolism of arsenate by a freshwater green alga, *Chlorella vulgaris*. J. Environ. Sci. 49, 169–178.
- 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. Environ. Sci. Pollut. Res. 23 (5), 4257–4270.
- Battin, T.J., Besemer, K., Bengtsson, M.M., Romani, A.M., Packmann, A.L., 2016. The ecology and biogeochemistry of stream biofilms. Nat. Rev. Microbiol. 14 (4), 251.
- Behra, R., Landwehrjohann, R., Vogel, K., Wagner, B., Sigg, L., 2002. Copper and zinc content of periphyton from two rivers as function of dissolved metal concentration. Aquat. Sci. 64, 300.
- Boixet, L., Gleeson, C.F., García, J., 2007. The Corcoesto gold deposit. 23rd International Applied Geochemistry Symposium (IAGS 2007). Congress Proceedings of Oviedo, pp. 14–19.
- Bonet, B., Corcoll, N., Tlili, A., Morin, S., Guasch, H., 2014. Antioxidant enzyme activities in biofilms as biomarker of Zn pollution in a natural system: an active bio-monitoring study. Ecotoxicol. Environ. Saf. 103, 82–90.
- Borcard, D., Gillet, F., Legendre, P., 2011. Introduction. In: Legendre, P., Legendre, L. (Eds.), Numerical Ecology with R. Springer, New York, pp. 1–7.
- Cattaneo, A., Asiola, A., Comoli, P., Manca, M., 1998. Organisms' response in a chronically polluted lake supports hypothesized link between stress and size. Limnol. Oceanogr. 43, 1938–1943.
- Caumette, G., Koch, I., Reimer, K.J., 2012. Arsenobetaine formation in plankton: a review of studies at the base of the aquatic food chain. J. Environ. Monit. 14 (11), 2841–2853.
- Corcoll, N., Ricart, M., Franz, S., Sans-Piché, F., Schmitt-Jansen, M., Guasch, H., 2012a. The use of photosynthetic fluorescence parameters from autotrophic biofilms for monitoring the effect of chemicals in river ecosystems. In: Guasch, H., Ginebreda, A., Geislinger, A. (Eds.), Handbook of Environmental Chemistry. Emerging and Priority Pollutants in Rivers Vol. 19. Springer, Heidelberg, pp. 86–114.
- Corcoll, N., Bonet, B., Leira, M., Montuelle, B., Tlili, A., Guasch, H., 2012b. Light history influences the response of fluvial biofilms to Zn exposure. J. Phycol. 48 (6), 1411–1423.
- Costas, M., Prego, R., Filgueiras, A.V., Bendicho, C., 2011. Land-ocean contributions of arsenic through a river-estuary-ria system (SW Europe) under the influence of arsenopyrite deposits in the fluvial basin. Sci. Total Environ. 412, 304–314.
- Coste, M., Rosebery, J., 2011. Guide iconographique pour la mise en oeuvre de l'Indice Biologique Diatomée 2007. Action 14: Développement et optimisation des méthodes de bioindication pour les cours d'eau. Cemagref groupement de Bordeaux, Cestas Gazinet, p. 236.
- Devesa-Rey, R., Paradelo, R., Díaz-Fierros, F., Barral, M.T., 2008. Fractionation and bioavailability of arsenic in the bed sediments of the Anllóns River (NW Spain). Water Air Soil Pollut. 195 (1–4), 189–199.
- Devesa-Rey, R., Moldes, A.B., Díaz-Fierros, F., Barral, M.T., 2009. Study of phytopigments in river bed sediments: effects of the organic matter, nutrients and metal composition. Environ. Monit. Assess. 153 (1–4), 147–159.
- DGT® Technical Documentation. DGT-for Measurements in Waters, Soils and Sediments <http://www.dgtresearch.com/dgtresearch/dgtresearch.pdf>.
- Díaz-Fierros, F., 2003. Las aguas superficiales. (coord). In: Casares, J.J. (Ed.), Reflexiones sobre el medio ambiente en Galicia. Consellería de Medio Ambiente, pp. 253–276 Xunta de Galicia.
- Dodds, W.K., 2006. Nutrients and the “dead zone”: the link between nutrient ratios and dissolved oxygen in the northern Gulf of Mexico. Front. Ecol. Environ. 4, 211–217.
- Dodds, W.K., Jones, J.R., Welch, E.B., 1998. Suggested classification of stream trophic state: distributions of temperate stream types by chlorophyll, total nitrogen, and phosphorus. Water Res. 32, 1455–1462.
- Drewniak, L., Sklodowska, A., 2013. Arsenic-transforming microbes and their role in biomineral processes. Environ. Sci. Pollut. Res. 20 (11), 7728–7739.
- EU, 2000. Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for community action in the field of water policy. Off. J. Eur. Communities L 327 (1), 1–73.
- Garelick, H., Jones, H., Dybowska, A., Valsami-Jones, E., 2009. Arsenic pollution sources. Reviews of Environmental Contamination. Volume 197. Springer New York: pp. 17–60. https://doi.org/10.1007/978-0-387-79284-2_2.
- Gleyzes, C., Tellier, S., Astruc, M., 2002. Chapter 4: Sequential extraction procedures for the characterisation of the fractionation of elements in industrially-contaminated soils. In: Ph. Quevauviller (Ed.), Methodologies for Soil and Sediment Fractionation Studies. Royal Society of Chemistry, Cambridge, pp. 66–104.
- Guasch, H., Sabater, S., 1998. Light history influences the sensitivity to atrazine in periphytic algae. J. Phycol. 34 (2), 233–241.
- Guasch, H., Serra, A., Corcoll, N., Bonet, B., Leira, M., 2010. Metal ecotoxicology in fluvial biofilms: potential influence of water scarcity. Water Scarcity in the Mediterranean. Springer Berlin Heidelberg, pp. 41–53.
- Guasch, H., Artigas, J., Bonet, B., Bonnineau, C., Canals, O., Corcoll, N., Foulquier, A., et al., 2016. The use of biofilms to assess the effects of chemicals on freshwater ecosystems. In: Romani, A.M., Guasch, H., Balaguer, M.D. (Eds.), Aquatic Biofilms: Ecology, Water Quality and Wastewater Treatment. Caister Academic Press, Norfolk (UK ISBN: 978-1-910190-17-3).
- Guasch, H., Bonet, B., Bonnineau, C., Barral, L., Duran, R., Cagnon, Ch., 2017. Microbial biomarkers. In: Cravo-Laureau, C., Lauga, B. (Eds.), Microbial ecotoxicology. Springer, pp. 251–281.
- Guitián, F., Carballas, T. (Eds.), 1976. Técnicas de Análisis de Suelos. Pico Sacro, Santiago de Compostela.
- Hellweger, F.L., Farley, K.J., Lall, U., Di Toro, D.M., 2003. Greedy algae reduce arsenate. Limnol. Oceanogr. 48 (6), 2275–2288.
- Hill, W.R., 1996. Effects of Light. In: Stevenson, R.J., Bothwell, M.I., Lowe, R.L. (Eds.), Algal Ecology: Freshwater Benthic Ecosystems. San Diego, California. Academic Press, pp. 121–148.
- Huang, J.H., 2014. Impact of microorganisms on arsenic biogeochemistry: a review. Water Air Soil Pollut. 225 (2):1–25. <https://doi.org/10.1007/s11270-013-1848-y>.
- Krammer, K., Lange-Bertalot, H., 1986–1991. Bacillariophyceae 1. Teil: Naviculaceae. 876 p.; 2. Teil: Bacillariaceae, Epithemiaceae, Surirellaceae, 596 p.; 3. Teil: Centrales, Fragilariaceae, Eunotiaceae, 576 p.; 4. Teil: Achnantheaceae. Kritische Ergänzungen zu Navicula (Lineolatae) und Gomphonema. 437 p. vol Band 2/1–4. Süßwasserflora von Mitteleuropa. Gustav Fischer Verlag, Stuttgart.
- Kulp, T.R., Hoef, S.E., Oremland, R.S., 2004. Redox transformations of arsenic oxyanions in periphyton communities. Appl. Environ. Microbiol. 70 (11), 6428–6434.
- Lamb, A.L., Wilson, G.P., Leng, M.J., 2006. A review of coastal palaeoclimate and relative sea-level reconstructions using $\delta^{13}\text{C}$ and C/N ratios in organic material. Earth Sci. Rev. 75 (1–4), 29–57.
- Leira, M., Sabater, S., 2005. Diatom assemblages distribution in catalan rivers, NE Spain, in relation to chemical and physiological factors. Water Res. 39, 73–82.
- Levy, J.L., Stauber, J.L., Adams, M.S., Maher, W.A., Kirby, J.K., Jolley, D.F., 2005. Toxicity, bio-transformation, and mode of action of arsenic in two freshwater microalgae (*Chlorella* sp. and *Monoraphidium arcuatum*). Environ. Toxicol. Chem. 24 (10), 2630–2639.
- Lopez, A.R., Hesterberg, D.R., Funk, D.H., Buchwalter, D.B., 2016. Bioaccumulation dynamics of arsenate at the base of aquatic food webs. Environ. Sci. Technol. 50 (12), 6556–6564.
- Luís, A.T., Coelho, H., Almeida, S.F.P., Ferreira da Silva, E., Seródio, J., 2013. Photosynthetic activity and ecology of benthic diatom communities from streams affected by Acid Mine Drainage (AMD) in pyritic mines. Fundam. Appl. Limnol. 182 (1), 47–59.
- Luís, A.T., Durães, N., Almeida, S.F.P., Ferreira da Silva, E., 2016. Integrating geochemical (surface waters, stream sediments) and biological (diatoms) approaches to assess environmental impact in a pyritic mining area: Aljustrel (Alentejo, Portugal). J. Environ. Sci. 42, 215–226.
- Magbanua, F.S., Townsend, C.R., Hageman, K.J., Lange, K., Lear, G., Lewis, G.D., Matthaie, C.D., 2013. Understanding the combined influence of fine sediment and glyphosate herbicide on stream periphyton communities. Water Res. 47 (14), 5110–5120.
- Mainstone, C.P., Parr, W., 2002. Phosphorus in rivers - ecology and management. Sci. Total Environ. 282–283, 25–47.
- Martiñá Prieto, D., Devesa-Rey, R., Paradelo, R., Penalta-Rodríguez, M., Díaz-Fierros, F., Barral, M.T., 2016. Monitoring benthic microflora in river bed sediments: a case study in the Anllóns River (Spain). J. Soils Sediments 16 (6):1825–1839. <https://doi.org/10.1007/s11368-016-1395-4>.
- McCune, B., Mefford, M.J., 2011. PC-ORD. Multivariate Analysis of Ecological Data. Version 6. MjM Software. Gleneden Beach, Oregon, U.S.A.
- Miyashita, S., Shimoya, M., Kamidate, Y., Kuroiwa, T., Shikino, O., Fujiwara, S., Kaise, T., 2009. Rapid determination of arsenic species in freshwater organisms from the

- arsenic-rich Hayakawa River in Japan using HPLC-ICP-MS. *Chemosphere* 75 (8), 1065–1073.
- Mora-Gómez, J., Freixa, A., Perujo, N., Barral-Fraga, L., Romani, A.M., 2016. Limits of the biofilm concept and types of aquatic biofilms. In: Guasch, H., Balaguer, M.D. (Eds.), *Aquatic Biofilms: Ecology, Water Quality and Wastewater Treatment* In Press. (ISBN: 978-1-910190-17-3).
- Morin, S., Proia, L., Ricart, M., Bonneau, C., Geislinger, A., Ricciardi, F., Guasch, H., Romani, A., Sabater, S., 2010. Effects of a bactericide on the structure and survival of benthic diatom communities. *Vie Milieu* 60 (2), 107–114.
- Morin, S., Cordonier, A., Lavoie, I., et al., 2012. Consistency in diatom response to metal-contaminated environments. In: Guasch, H., Ginebreda, A., Geislinger, A. (Eds.), *Handbook of Environmental Chemistry. Emerging and Priority Pollutants in Rivers* vol. 19. Springer, Heidelberg:pp. 117–146. https://doi.org/10.1007/978-3-642-25722-3_5.
- Morin, S., Gómez, N., Tornés, E., Licursi, M., Rosebery, J., 2016. Benthic diatom monitoring and assessment of freshwater environments: standard methods and future challenges. In: Romani, A.M., Guasch, H., Balaguer, M.D. (Eds.), *Aquatic Biofilms: Ecology, Water Quality and Wastewater Treatment*. Caister Academic Press, Norfolk, UK (ISBN: 978-1-910190-17-3).
- Muñoz, I., Romani, A.M., Rodrigues-Capítulo, A., Esteban, J.G., García-Berthou, E., 2009. Relaciones tróficas en el ecosistema fluvial. *Conceptos y técnicas en ecología fluvial*, pp. 347–366 Fundación BBVA.
- Murphy, J., Riley, J., 1962. A modified single solution method for the determination of phosphate in natural waters. *Anal. Chim. Acta* 27, 31–36.
- Nespereira, X., 1978. Síntesis sobre los yacimientos auríferos gallegos. *Braña* 1, 18–49.
- Oremland, R.S., Stolz, J.F., 2003. The ecology of arsenic. *Science* 300 (5621), 939–944.
- Persaud, D., Jaugumagi, R., Hayton, A., 1993. Guidelines for the Protection and Management of Aquatic Sediment Quality in Ontario: Report. Water Researchources Branch, Ontario Ministry of the Environment.
- Perujo, N., Freixa, A., Vivas, Z., Gallegos, A.M., Butturini, A., Romani, A.M., 2016. Fluvial biofilms from upper and lower river reaches respond differently to wastewater treatment plant inputs. *Hydrobiologia* 765 (1), 169–183.
- Pielou, E.C., 1975. *Ecological Diversity*. John Wiley and Sons, New York.
- Prieto, D.M., Devesa-Rey, R., Rubinos, D.A., Díaz-Fierros, F., Barral, M.T., 2013. Arsenate Retention by Epipsammic Biofilms Developed on Streambed Sediments: Influence of Phosphate. *BioMed Research International* 2013. Article ID 591634, 10 p. <https://doi.org/10.1155/2013/591634>.
- Prieto, D.M., Rubinos, D.A., Piñeiro, V., Díaz-Fierros, F., Barral, M.T., 2016a. Influence of epipsammic biofilms on the biogeochemistry of arsenic in freshwater environments. *Biogeochemistry* 129 (3):291–306. <https://doi.org/10.1007/s10533-016-0232-6>.
- Prieto, D.M., Martín-Liñares, V., Piñeiro, V., Barral, M.T., 2016b. Arsenic transfer from arsenic-rich sediments to river water in the presence of biofilms. *J. Chemother.* 2016 (Article ID 6092047).
- Rahman, M.A., Hassler, C., 2014. Is arsenic biotransformation a detoxification mechanism for microorganisms? *Aquat. Toxicol.* 146, 212–219.
- Rial, M.E., 2007. Investigación dos procesos que regulan o caudal e a calidade das augas na bacía do Río Anllóns. Doctoral dissertation. Universidade de Santiago de Compostela, Santiago de Compostela.
- Rodríguez Castro, M.C., Urrea, G., Guasch, H., 2015. Influence of the interaction between phosphate and arsenate on periphyton's growth and its nutrient uptake capacity. *Sci. Total Environ.* 503:122–132. <https://doi.org/10.1016/j.scitotenv.2014.06.094>.
- Romani, A.M., 2010. Freshwater biofilms. In: Dürr, S., Thomason, J.C. (Eds.), *Biofouling*. Blackwell Publishing Ltd Oxford, pp. 137–153.
- Romani, A.M., Guasch, H., Munoz, I., Ruana, J., Vilalta, E., Schwartz, T., Emtiazi, F., Sabater, S., 2004. Biofilm structure and function and possible implications for riverine DOC dynamics. *Microb. Ecol.* 47, 316–328.
- Rubinos, D., Barral, M.T., Ruiz, B., Ruiz, M., Rial, M.E., Álvarez, M., Díaz-Fierros, F., 2003. Phosphate and arsenate retention in sediments of the Anllóns River (northwest Spain). *Water Sci. Technol.* 48 (10), 159–166.
- Rubinos, D., Iglesias, L., Devesa-Rey, R., Díaz-Fierros, F., Barral, M.T., 2010. Arsenic release from river sediments in a gold-mining area (Anllóns River basin, Spain): effect of time, pH and phosphorous concentration. *Eur. J. Mineral.* 22 (5), 665–678.
- Rubinos, D.A., Iglesias, L., Díaz-Fierros, F., Barral, M.T., 2011. Interacting effect of pH, phosphate and time on the release of arsenic from polluted river sediments (Anllóns River, Spain). *Aquat. Geochem.* 17 (3), 281–306.
- Scharler, U.M., Ulanowicz, R.E., Fogel, M.L., Wooller, M.J., Jacobson-Meyers, M.E., Lovelock, C.E., et al., 2015. Variable nutrient stoichiometry (carbon: nitrogen: phosphorus) across trophic levels determines community and ecosystem properties in an oligotrophic mangrove system. *Oecologia* 179 (3), 863–876.
- Shannon, C.E., Weaver, W., 1949. *The Mathematical Theory of Communication*. University of Illinois Press.
- Sharma, V.K., Sohn, M., 2009. Aquatic arsenic: toxicity, speciation, transformations, and remediation. *Environ. Int.* 35 (4):743–759. <https://doi.org/10.1016/j.envint.2009.01.005>.
- Smedley, P.L., Kinniburgh, D.G., 2002. A review of the source, behaviour and distribution of arsenic in natural waters. *Appl. Geochem.* 17 (5):517–568. [https://doi.org/10.1016/S0883-2927\(02\)00018-5](https://doi.org/10.1016/S0883-2927(02)00018-5).
- Sterner, R.W., Elser, J.J., 2002. *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton University Press.
- Thurman, E.M., 1985. *Organic Geochemistry of Natural Waters*. Martinus Nijhoff/Dr. W. Junk Publishers, Dordrecht.
- Tuulaikhuu, B.-A., Romani, A.M., Guasch, H., 2015. Arsenic toxicity effects on microbial communities and nutrient cycling in indoor experimental channels mimicking a fluvial system. *Aquat. Toxicol.* 166:72–82. <https://doi.org/10.1016/j.aquatox.2015.07.005>.
- US EPA, 2014. National Recommended Water Quality Criteria: Aquatic Life Criteria. Environmental Protection Agency Web <http://water.epa.gov/scitech/swguidance/standards/criteria/aqlife/index.cfm> (Accessed 21 March 2016).
- Vázquez, F.M., Anta, R.C., 2009. Niveles genéricos de metales pesados y otros elementos traza en suelos de Galicia. *Santiago de Compostela, Xunta de Galicia*.
- Villeneuve, A., Montuelle, B., Bouchez, A., 2010. Influence of slight differences in environmental conditions (light, hydrodynamics) on the structure and function of periphyton. *Aquat. Sci.* 72, 33–44.
- Wang, N.-X., Li, Y., Deng, X.-H., Miao, A.-J., Ji, R., Yang, L.-Y., 2013. Toxicity and bioaccumulation kinetics of arsenate in two freshwater green algae under different phosphate regimes. *Water Res.* 47:2497–2506. <https://doi.org/10.1016/j.watres.2013.02.034>.
- Wang, Y., Wang, S., Xu, P., Liu, C., Liu, M., Wang, Y., Ge, Y., 2015. Review of arsenic speciation, toxicity and metabolism in microalgae. *Rev. Environ. Sci. Biotechnol.* 14 (3): 427–451. <https://doi.org/10.1007/s11157-015-9371-9>.
- Yan, C., Che, F., Zeng, L., Wang, Z., Du, M., Wei, Q., Zhen, Z., 2016. Spatial and seasonal changes of arsenic species in Lake Taihu in relation to eutrophication. *Sci. Total Environ.* 563, 496–505.
- Zhang, H., Davison, W., 1995. Performance characteristics of the technique of diffusion gradients in thin-films (DGT) for the measurement of trace metals in aqueous solution. *Anal. Chem.* 67, 3391–3400.
- Zimmerman, G.M., Goetz, H., Mielke, P.W., 1985. Use of an improved statistical method for group comparisons to study effects of prairie fire. *Ecology* 66 (2), 606–611.