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Assessment of the Environmental Impact of Acid Mine Drainage on Surface Water, Stream Sediments, and Macrophytes Using a Battery of Chemical and Ecotoxicological Indicators

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Citation: Alvarenga, P.; Guerreiro, N.; Simões, I.; Imaginário, M.J.; Palma, P. Assessment of the Environmental Impact of Acid Mine Drainage on Surface Water, Stream Sediments, and Macrophytes Using a Battery of Chemical and Ecotoxicological Indicators. *Water* **2021**, *13*, 1436. <https://doi.org/10.3390/w13101436>

Academic Editor: Massimo Zacchini

Received: 30 April 2021

Accepted: 19 May 2021

Published: 20 May 2021

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Abstract: Mining activities at the Portuguese sector of the Iberian Pyrite Belt (IPB) have been responsible for the pollution of water, sediments, and biota, caused by the acid mine drainage (AMD) from the tailing deposits. The impact has been felt for years in the rivers and streams receiving AMD from the Aljustrel mine (SW sector of the IPB, Portugal), such as at the Água Forte stream, a tributary of the Roxo stream (Sado and Mira Hydrographic Region). To evaluate the extent of that environmental impact prior to the remediation actions, surface water, sediments, and the macrophyte *Scirpus holoschoenus* L. were sampled at the Água Forte and the Roxo streams, upstream and downstream from the confluence. The surface water and the sediments were extremely acidic at the Água Forte stream (pH ranges 2.22–2.92 for the water and 2.57–3.32 for the sediment), with high As, Cu, Pb, and Zn concentrations of 2.1, 120, 0.21, and 421 mg kg⁻¹, respectively, in the water, and 661, 1746, 539, and 1994 mg kg⁻¹, respectively, in the sediment, in the location closer to the mine. Two aquatic bioassays evidenced the high ecotoxicity of the Água Forte water at that site, with very low EC₅₀ values for *Vibrio fischeri* luminescence inhibition (<3.1% v/v) and *Daphnia magna* 48-hour immobilization/mortality assays (<6.3% v/v). The impact of the AMD was also evident in the sediments of the Roxo stream, but not so marked in the water, with circa neutral pH and lower As, Cu, Pb, and Zn concentrations. Consistently, the ecotoxicological response was only felt in the sampling point closer to the confluence of the Água Forte with the Roxo stream, with an EC₂₀ of 27.0% (v/v) towards the *V. fischeri*. One of the dominant and well adapted macrophytes, *S. holoschoenus* L., presented low bioaccumulation factors for Cu (0.04) and Zn (0.15) in their emerging parts, and very low concentrations for As and Pb, making this plant a potential candidate to be used in phytoremediation actions to treat and control AMD in the IPB.

Keywords: acid mine drainage (AMD); Iberian Pyrite Belt; trace elements; water; ecotoxicity; sediments; macrophytes; *Scirpus holoschoenus* L.

1. Introduction

Mining activities in Portugal are ancient, having contributed to its economic development at different times. However, the oldest explorations were not planned in a way to minimize the environmental impacts inherent to their operation, thus, affecting the quality of the abiotic and biotic compartments in the areas of influence of these mining activities [1].

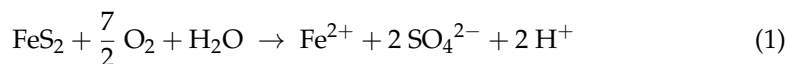
The Aljustrel mining area (Portugal), located in the SW sector of the Iberian Pyrite Belt (IPB), constitutes one paradigmatic example of this reality [1–3]. The exploitation started

before the Roman occupation of the Iberian Peninsula [4], evident by the vestiges of intense mining activity which were found together with the Roman spoils [5] and was one of the most important mining explorations during that period. The intensive mining activity started again in 1849, with the concession being given to different foreign and national companies over the years, for example, Transtagana [6], Société Anonyme Belge des Mines d'Aljustrel [7], Pirites Alentejanas S.A., and more recently, Almina, Minas do Alentejo S.A. During its intense exploitation, which took place during the twentieth century, the pyrite at Aljustrel was processed by a wet treatment, which began by the roasting or calcination of the ore, in which the copper sulfide was transformed into copper sulphate, followed by the dissolution of the sulphate in water. The solution was put in contact with iron, leading to the reduction of the metallic cations (e.g., copper) to their zerovalent state, and then were separated from the contaminated processing waters, which were discarded [8]. The legacy of thousands of years of pyrite exploitation at Aljustrel are evident in the area, with the deposition of high volumes of waste tailings composed by Roman slag, pyrite ore, and volcano-sedimentary complex host rocks, mainly at Algares industrial area and the São João sector [1,9].

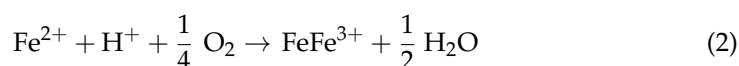
One of the biggest problems associated with mines such as Aljustrel is the production of acidic effluents (acid mine drainage, AMD) with high concentrations of metals and metalloids, which originated in the mining process and also from the oxidation of sulfides present in those waste deposits [10]. Sulfides are minerals composed of sulfur (S) in its lowest natural oxidation state (S^{2-}), metals (e.g., iron (Fe)), and/or semi-metals (e.g., arsenic (As)) [11]. When oxidized, they generate very acidic environments that promote the solubilization of the remaining constituents of these minerals (metals and/or semi-metals). In the case of the Aljustrel mine, the most abundant sulfide is pyrite, FeS_2 , in which the S is in the form of disulfide ion, S_2^{2-} [8].

The chemical process that represents the decomposition of pyrite is well known, consisting of the following steps [8,11–15]:

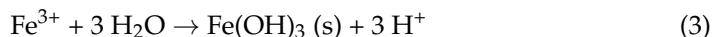
- (1) It starts with the production of free Fe(II) ion, sulfate (SO_4^{2-}), and hydrogen (H^+) ions:



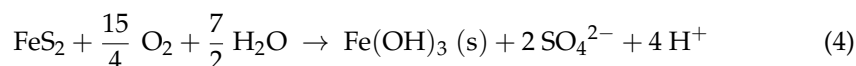
- (2) The products of this reaction remain in solution, but, if the environment is sufficiently oxidizing, the Fe(II) ion is transformed into the Fe(III) ion:



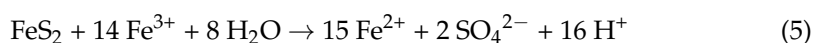
- (3) The Fe(III) ion precipitates as a hydroxide, very insoluble, leaving very little ion in solution:



- (4) These reactions can be represented as one reaction, which explains the production of acidity and the Fe oxyhydroxide precipitate in the riverbanks, which has a very characteristic ochreous color ("yellow boy", [16]):



- (5) The Fe(III) ion that remains in solution has the capacity to oxidize more pyrite, simultaneously producing more hydrogen ions that contribute to increase the acidity of the medium:



It should be noted that the natural oxidation process of pyrite is not exclusively chemical, but rather biochemical, with some bacteria playing a very important role in

the process of pyrite oxidation, namely *Acidithiobacillus ferrooxidans* (a strain named first as *Thiobacillus ferrooxidans*), *Acidithiobacillus thiooxidans*, and *Leptospirillum ferrooxidans*, chemolithotrophic bacteria, able to obtain energy from the oxidation of iron and sulfur compounds [11–13,17]. The action of these bacteria can significantly increase the rate of these reactions [15,18].

Figure 1 represents a simplified scheme that illustrates the pollution sources resulting from the tailings deposited in the area, containing large contents of pyrite, prone to the reaction with oxygen and water in the exposed layers. Rainwater circulates and percolates easily over and through the deposited materials, causing the production of AMD, which reaches the downstream areas affecting the quality of surface water, groundwater, and stream sediments.

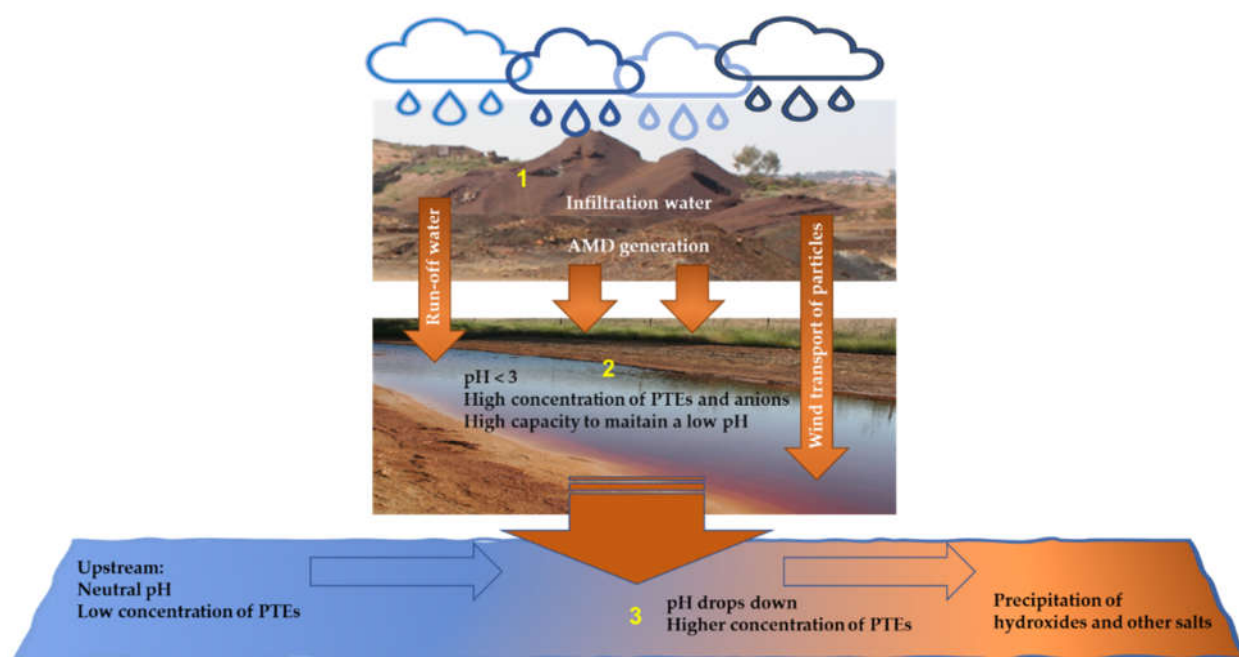


Figure 1. Pollution scheme caused by mining activity in the surrounding environment. (1) Decomposition of pyritic environments; (2) acid effluents with high concentrations in solubilized trace elements (PTEs); (3) dilution in water courses. Adapted from [8,19].

The spread of slag when the mine was exploited by the Romans marks the beginning of pollution in the Aljustrel area. In maps prior to 1700, Ribeira do Roxo (purple stream, in Portuguese) was already marked with that name, which surely indicates that the waters were already polluted at that time, noting its reddish color [20]. Indeed, names such as Roxo, Ribeira da Água Forte (strong water stream, in Portuguese), and Ribeira da Água Azeda (sour water stream, in Portuguese) provides a safe indication of the characteristics of the surface water of the hydrological system in the Aljustrel area. An identical example is the Rio Tinto (red or dark river), in the Spanish sector of IPB, whose water is also heavily polluted by the AMD from the abandoned mines in the region, i.e., the Peña del Hierro and Rio Tinto mines, located in the river’s headwaters [15,21].

Lobato [7] reported a description made in 1892 by Pery about the rivers and streams of the Aljustrel municipality as follows:

“(. . .) neither the water from Água Forte stream nor Roxo stream, after its confluence, are usable for agriculture, because they are contaminated by the drainage waters of the Aljustrel cupriferous pyrite mine, poisoned by salts of iron, copper, arsenic, and whose unfortunate influence is exerted until the Sado river, preventing fish farming and destroying the vegetation of the banks.”

Indeed, the Água Forte stream received, for years, the drainage water from the Algarves industrial area. This was an area of about 58 ha where deposits of minerals, ancient mountains of cementation, and Roman scoria were found (Figure 2a). The flow from there was, of course, variable, and proportional to the rainfall over this area (Figure 2b). The company carried out works that conveyed the run-off waters to a dam where they were stored, the so-called Água Forte Dam (Figure 2c). During the rainy season, the Água Forte Dam sent the supernatant to the Água Forte stream, and the Algarves AMD was also sometimes thrown there directly, without going through the evaporation circuit. The entire riverbed of the Água Forte stream, and the land next to its banks, thus had some characteristics similar to the Algarves area (Figure 2d). This area was outside the company's concession and was, during centuries, an uncontrolled source of pollution [8].

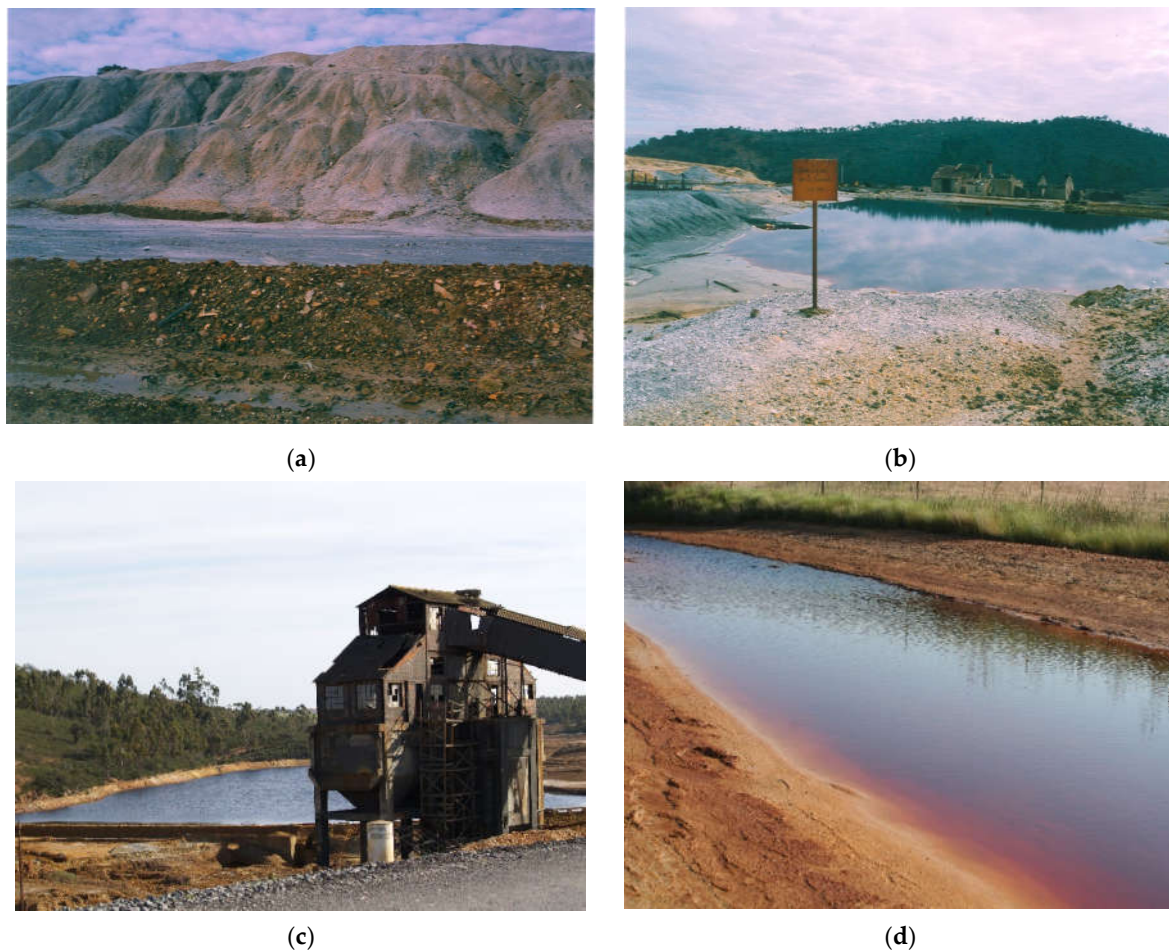


Figure 2. (a) Tailings deposited at Algarves, in the Aljutrel mining area, prior to the rehabilitation performed by EDM (Empresa de Desenvolvimento Mineiro); (b) Overview of the draining area; (c) Água Forte dam; (d) Água Forte stream, evidencing the reddish color of the water.

Several studies have documented these impacts in rivers and streams, in Aljutrel [3,9,22–24], and also in other mines from the IPB, both in Portugal, for example, the Lousal mine, affecting the Corona stream [18,25,26], São Domingos, affecting the São Domingos stream, a tributary to the Chança river [10,27] and, in Spain, mainly in Tinto and Odiel river basins, affected by different mines, for example, Rio Tinto, Tharsis, and Sotiel-Coronado [15,17,21,28–33], in the Oraque and Meca rivers (Odiel tributaries), affected by the Tharsis mine [33], also affecting the Chanza and Guadiamar rivers [32]. In fact, in specific hydrogeochemical conditions, the environments affected by AMD can be characterized by extreme conditions, for example, negative pH values [33], similar to those found in the Cobica River basin, affected by AMD-polluted waters coming from the Lagunazo mine

and the Herrerias mine, where, in addition to the high concentrations of toxic elements, the mean pH value was negative (pH = -1.56) [34]. Despite these extreme conditions, some of these environments are characterized by a high level of microbial diversity, mainly eukaryotic, the so-called extremophiles, as reported in the Rio Tinto (SW Spain) [35].

Despite the deleterious impact in the water and sediments affected by AMD, several authors have reported that the effects tended to decrease with the distance from the pollution source. For instance, the geochemical characteristics of the AMD discharged to the Corona stream from the tailings deposited at the Lousal mine, as well as the extent of pollution in the water and sediments at the stream, were evaluated by Ferreira da Silva et al. [18]. The AMD evidenced low pH values (1.9–2.9) and a high concentration of SO_4^{2-} and of some metals (Fe, Mn, Al, Zn, Cu, Cd, and Co), with some seasonal variations, which were also present in high concentrations in the water and sediments of the Corona stream [18]. However, the severity of the contamination decreased downstream, and turned negligible at 4 km from the pollution source, a consequence of two effects:

- (i) the dilution with fresh waters, lowering the metals concentrations and neutralizing the acidity and
- (ii) the reduced solubility of most trace elements, removed them from solution by a co-precipitation or adsorption to the Fe and aluminium (Al) precipitates [18].

However, the geology of the river basin is of paramount importance to the transference of acidity and toxic elements, controlling, to a larger extent, the neutralizing capacity of water [17,36]. Consequently, in some cases, as in the Odiel and Tinto Rivers in Spain, pollution persists for more than 50 km, all the way to the river mouth, affecting estuarine sediments at the Ría de Huelva estuary and the Gulf of Cadiz [17,29,36,37], or in the Guadiana Estuary [38]. Of major importance is the impact on water reservoirs which receive AMD-affected waters, and which are used for human consumption, irrigation, and recreational use, a situation reported by Grande et al. [32] in the river network of the Spanish sector of the IPB, emphasizing the need to adopt urgent remediation measures to improve the quality of the input waters [16,33].

Recently, the Aljustrel area affected by the abandoned mining works was rehabilitated by EDM (Empresa de Desenvolvimento Mineiro), the public company responsible for the Portuguese mines' rehabilitation program, and it is expected that the situation documented by this study has changed steadily. The rehabilitation project implemented by EDM, since 2006, comprised:

- (i) the removal of pyrite deposits, mining waste, and contaminated soil dispersed in the mining areas and their confinement in Algarves;
- (ii) the superficial sealing of the deposits created with clay and vegetal soil;
- (iii) the construction of channels on the slopes and banks to collect/deviate the non-contaminated surface waters and reduce the volume of acidic waters produced; and
- (iv) planting artificial riparian zones to treat the residual contamination of the waters from the mine area.

The results reported in this study, and in other studies that have assessed the environmental impact in the Aljustrel mining area [2–4,9,22,23] can be used to evaluate the benefits of the remediation measures at the site, documenting the “ground zero” situation. Unfortunately, remediation measures are not always effective to counterbalance years of AMD impact, and, for instance, some remediation actions took by the regional Government of Andalusia in the Spanish part of the IPB, both active and passive, have been revealed as highly ineffective for reducing the environmental impact of AMD waters, due to chemical and climatic constraints [17].

To evaluate the effectiveness of remediation actions, it is important to consider the physicochemical characteristics of the abiotic compartments affected by AMD, as well as the response of living organisms, i.e., inhabitants of those compartments, that can be integrated as biomonitors of AMD effects [9,22,23,26,34]. In line with this idea, the impact of the contaminants in the surface water affected by AMD in the IPB has been evaluated using

diatom communities (Bacillariophyceae), a successful algal group in aquatic media, which can evidence the response to the metal pollution through the size reduction or teratological forms of the organisms [9,22,23,26,34]. Other target species, such as the fish (*Leuciscus cephalus*) has also been used to assess the impact of AMD in rivers [36]. In addition to this approach, it would be important to assess the water's and/or sediment's ecotoxicity using standardized bioassays, easily reproducible in the lab, to evaluate the effectiveness of the remediation measures, to compare the ecotoxicological responses overtime, and to assess the impact of AMD polluted tributaries in the receiving streams. That approach was used with the freshwater clam, *Corbicula fluminea*, known as the Asian clam [31], in a weight-of-evidence methodology to evaluate the sediment toxicity in the Odiel River basin, but, to our best knowledge, the ecotoxicological characterization of water and sediments in the Aljustrel mine affected streams has not been made.

In this context, the aims of the study were:

- (i) to assess the pollution load at the Água Forte and Roxo streams, potentially affected by the AMD from the Aljustrel mining area, considering the water physicochemical parameters;
- (ii) to assess the ecotoxicity of these streams to the aquatic species *Vibrio fischeri* and *Daphnia magna*;
- (iii) to evaluate the physicochemical characteristics of the sediments collected in the same sampling sites;
- (iv) to assess the As, Cu, Pb, and Zn total concentrations and partitioning in the sediments, using a sequential extraction procedure; and
- (v) to analyze the behavior of the most abundant macrophyte, *Scirpus holoschoenus* L., collected along the stream banks, regarding the trace element bioaccumulation.

Three sampling sites were selected at the Água Forte stream and four sampling sites at the Roxo stream, two upstream from the confluence of the Água Forte stream and two downstream from that confluence.

2. Materials and Methods

2.1. Study Area and Characterization of the Sampling Sites

Aljustrel is located in the Portuguese sector of the Iberian Pyrite Belt (IPB, Alentejo region, South of Portugal), which extends from near Seville, in Spain, to Grândola, occupying an area of 230 km long by 30 km wide [5]. The area is characterized by a Mediterranean mesothermic humid climate, with hot and dry summers, and with low pluviosity (annual average rainfall of 500–650 mm) [39]. It represents an important metallogenetic province of volcanic-hosted massive sulfide deposits, one of the world's most important [3,9,10]. These massive sulfide deposits are mainly constituted by pyrite (FeS_2), the dominant ore (>90%) [4], and variable amounts of sphalerite ((Zn,Fe)S), galena (PbS), and chalcopyrite (CuFeS_2). Other minerals, such as arsenopyrite (FeAsS), are less representative, while silver (Ag) and gold (Au) are important subproducts (Aljustrel's geological setting was extensively described by, e.g., [3–5,9,22,23]). The extraction of pyrite has been mainly responsible for the pollution observed in soils, tailings, superficial water, and sediments [1]. After a period of interruption in the mining works, exploration was reestablished in 2009 by the company ALMINA—Minas do Alentejo, S.A., using the best available technologies and environmental practices, and the above-described mine-activity-affected areas begun to be rehabilitated by EDM, the state company which is responsible for the environmental liabilities in mine areas [4,9].

Samples of water, sediment, and the aerial part of a macrophyte (*Scirpus holoschoenus* L.) were collected from three sampling sites in the Água Forte stream (Figure 3), approximately 10 km long (Site 1, 37°53'00.7" N 8°08'37.0" W; Site 2, 37°53'45.9" N 8°08'13.3" W; and Site 3, 37°55'17.2" N 8°08'51.8" W), and in four sites at the Roxo stream, two upstream (Site 4, 37°55'47.6" N 8°04'58.4" W, right after the Roxo Dam and Site 5, 37°56'42.2" N 8°08'51.3" W, right before the confluence of the Água Forte stream) and two downstream from the confluence of the Água Forte stream (Site 6, 37°56'43.6" N 8°09'03.8" W, right

after the confluence of Água Forte in Roxo stream and Site 7, $37^{\circ}55'55.9''$ N $8^{\circ}10'56.6''$ W, Figure 1).

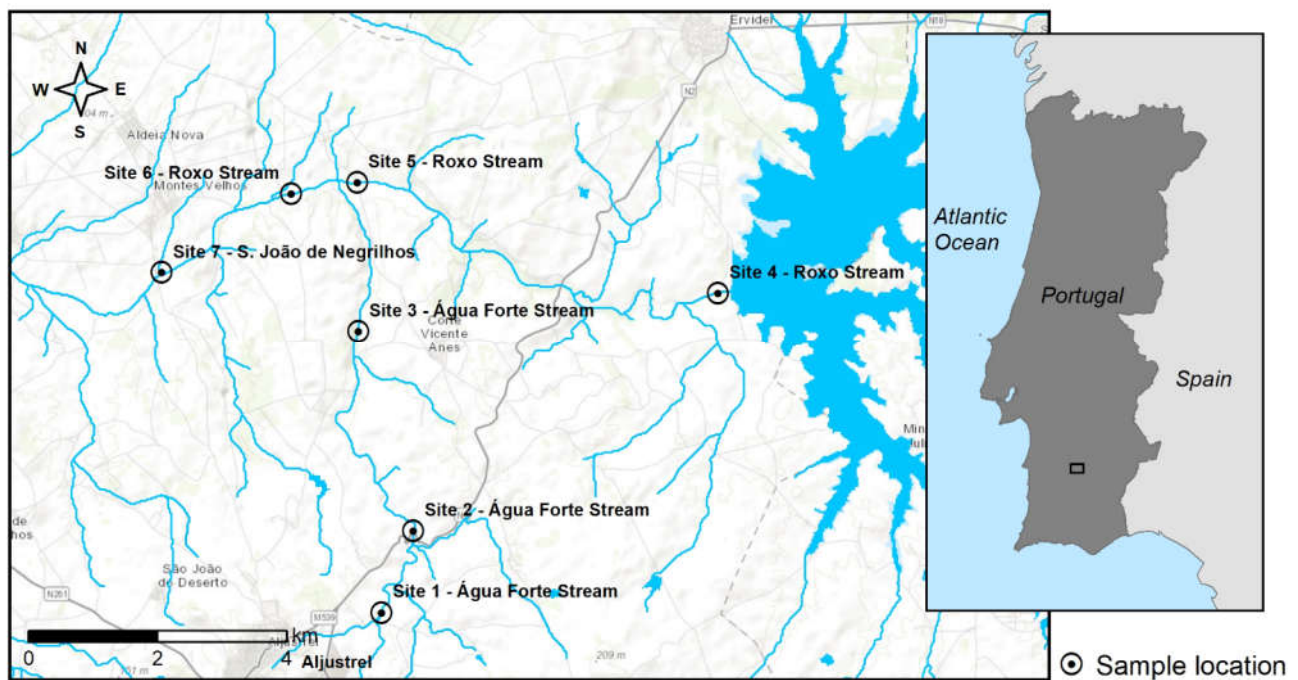


Figure 3. Map evidencing the different sampling sites along the Água Forte and the Roxo streams (design credits to Dr. Selma Pena, LEAF, ISA, University of Lisbon).

At Sites 5 and 7 it was impossible to collect plant and sediment samples, due to accessibility problems (the banks were too sloping), and therefore the water samples were collected from the bridge over the watercourse.

2.2. Water Physicochemical Characterization

Composite water samples were collected at each sampling site, after the wet season, selecting spots with sufficient depth to avoid sediment perturbation. All samples were transported to the laboratory in polyethylene bottles, in a refrigerated thermal cooler box with ice packs, where they were conserved and stored following the requisites for each parameter to be analyzed [40].

The water physicochemical parameters were determined using officially recommended methods of analysis [40] as follows: Water pH and electrical conductivity (EC, mS cm^{-1} , 20°C) were measured by potentiometry and conductimetry, respectively; total dissolved solids (TDS, mg L^{-1}) and total suspended solids (TSS, mg L^{-1}) were determined gravimetrically in the fractions obtained after water filtration of a known volume of water through a glass fiber membrane, oven-dried at 105°C until constant weight; total carbon (C) (TC, mg L^{-1}) and total organic C (TOC, mg L^{-1}) were determined with a TOC Analyzer (LABOTec, Ho Chi Minh City, Vietnam), by photo-chemical oxidation with UV and chemical oxidation with a sodium persulphate/phosphoric acid solution; dissolved anions (fluoride (F^{-}), chloride (Cl^{-}), nitrate (NO_3^{-}); sulphate (SO_4^{2-}), mg L^{-1}) were analyzed by ionic chromatography using a Metrohm model 761 ionic chromatographer, with a Metrosep Anion Dual 2 column (METROHM, Herisau, Switzerland), after filtration through a $0.2\ \mu\text{m}$ cellulose acetate membrane; and the trace element total concentrations (As, Cu, Pb, and Zn), were analyzed by atomic absorption spectrometry, with flame or electrothermal atomization, with a Varian equipment (SpectrAA 220FS, 220Z, and 110Z, Scientific instruments (Agilent Technologies), Palo Alto, California), after the digestion of the samples with concentrated nitric acid (5 mL HNO_3 :100 mL water, on a hot plate).

2.3. Water Ecotoxicological Characterization

Water ecotoxicological characterization was performed using two standardized bioassays: the luminescence inhibition of the bacteria *Vibrio fischeri* [41] and a 48-hour immobilization/mortality assay with the freshwater crustacean *Daphnia magna* [42].

Inhibitory effects of the water samples, and of their dilutions with a non-toxic control (2% w/v NaCl solution), 3.1, 6.2, 12.5, 25.0, and 50.0% (v/v), on the light emission of *V. fischeri* (NRRL B-11177) were determined according to ISO 11348-2 [41]. All measurements were carried out in duplicate. A decrease in the luminescence was measured after 30 min of contact using a LUMISTox 300 Bench Top Luminometer (HACH-LANGE GmbH, Hørsholm, Denmark). The sensitivity of *V. fischeri* organisms was tested with a reference substance, potassium dichromate ($K_2Cr_2O_7$) from Merck® (Darmstadt, Germany).

The *D. magna* acute immobilization test was performed according to the standardized method ISO 6341 [42], with water samples and their dilutions (50, 25, 12.5, and 6.3% v/v). Holding and dilution water was prepared according to [42], and this solution was also used as the negative control. Five juveniles, aged less than 24 h at the start of the test, were exposed to 20 mL of water sample, at different concentrations, for a period of 48 h. Tests were conducted in environmental chambers at 20 ± 2 °C, with a 16 h light/8 h dark cycle, using three replicates per treatment. Immobilization was recorded, by visual observation, after 48 h exposure and compared with the control. The sensitivity of the organisms was tested with a reference substance, potassium dichromate ($K_2Cr_2O_7$), as a positive control.

Whenever possible, the EC₂₀ or EC₅₀ values (water concentration, % v/v, which promoted a toxic effect on 20% or 50% of the exposed organisms, considering the selected endpoint) were calculated.

2.4. Sediment Physicochemical Characterization and Risk Analysis

Approximately 1 kg of sediment (surface layer, up to 5 cm depth) was collected at each sampling location, using a polypropylene spatula, and transferred into a polypropylene bottle to be transported. At the laboratory, the sediment samples were oven-dried at 60 °C, sieved through a 2 mm sieve, and subjected to general physicochemical characterization.

Sediment pH was determined in a sediment to deionized water suspension of 1:2.5 (m/v); total oxidizable organic carbon was determined after the method proposed by Walkley and Black [43], and converted to organic matter content (OM, % w/w) by multiplying by a factor of 1.72; total nitrogen was analyzed by the Kjeldahl method (N_K , % w/w); total trace element concentrations (As, Cu, Pb, and Zn, mg k^{-1}) in the sediment were determined after digestion of the samples with aqua regia [44]. Samples were digested with a mixture of HCl (37%) and HNO₃ (70%) in a ratio of 3:1 (v/v) at room temperature for 16 h, and after, at 130 °C, for 2 h under reflux conditions. Then, each suspension was filtered, diluted to 100 mL with HNO₃ 0.5 M, and stored at 4 °C until analysis. Atomic absorption spectrometry (AAS) was used in the analysis, with flame or electrothermal atomization, with a Varian equipment (SpectrAA 220FS, 220Z and 110Z). All concentrations were reported on a dry weight basis.

To evaluate the degree of pollution of the sediments with PTEs, their total concentrations were compared with freshwater sediment quality values (SQVs), which are established considering the toxic responses of a wide range of organisms. These guidelines provide two threshold values, the threshold effect concentration (TEC, value below which adverse biological effects are expected to occur rarely), and the probable effect concentration (PEC, value above which adverse effects are expected to occur) [31]. The quotient of the concentration of an element in the sediment to its PEC value is called the PEC quotient (PEQ_q) and was used to evaluate the toxicity level at that site promoted by a specific element [18,26]. To evaluate the toxicity promoted by the combined effect of all the chemicals in the sediment, all calculated PEQ_q were summed, and the sum was normalized by dividing by the number of PEQ_q, i.e., calculating the mean value considering all calculated PEQ_q, the mean PEQ_q [45]. The degree of pollution of the sediment and its toxicity was predicted and compared considering the value of the mean PEQs obtained, and adapting

the ranges proposed by MacDonald et al. [45], i.e., not toxic samples, when the mean PEQ_q are <0.1 ; with low toxicity, mean PEQ_q between 0.1 and 0.5; with medium toxicity, mean PEQ_q between 0.5 and 1.5, and with high predicted toxicity when the mean PEQ_q are >1.5 .

The three first steps of the European Community Bureau of Reference (BCR) sequential extraction procedure, used for trace element speciation, were performed as described by Rauret et al. [46]. Despite the numerous sequential extraction methods, which differ in the reagents, experimental conditions, and number of steps, for example [28,47], the BCR sequential extraction procedure is considered to be a simple, well described, and standardized procedure, and has been previously used by other authors to evaluate the environmental impact of AMD affected environments [10,38]. The sequential steps of the extraction scheme and the operational fractions recovered can be summarized as: Step 1, extraction of sediment samples with acetic acid 0.11 mol L^{-1} to liberate exchangeable/acid-extractable elements; Step 2, solubilization of the trace elements associated with the reducible phases, using hydroxylamine hydrochloride; and Step 3, extraction of the elements released by oxidation with hydrogen peroxide in 0.1 mol L^{-1} ammonium acetate. The fractions obtained were compared with the total concentration obtained through the aqua regia digestion. All concentrations were reported on a dry weight basis.

2.5. Trace Element Concentrations in *Scirpus holoschoenus* L.

Emerging material of the macrophyte *S. holoschoenus* L. (composite sample of five different plants of the same species at each sampling site) were collected and analyzed for their PTEs concentration. Samples were stored in polyethylene bags and transported to the laboratory where they were thoroughly washed with tap water to remove any attached particles and rinsed three times with deionized water. After these washing operations, the samples were dried for 48 h, at $60 \text{ }^\circ\text{C}$, and ground in an electric mill. Dried samples were stored in polyethylene bags, in the dark, until analysis. Approximately 2 g of dried plant sample were ashed in a muffle furnace at $500 \text{ }^\circ\text{C}$, for 6 h, dissolved with 10 mL of HCl 3 M, and evaporated to near dryness twice, dissolved again with the same acid solution, filtered (Whatman[®] 40, Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), and adjusted to a volume of 100 mL with Milli-QTM ultra-pure water. Plant digested samples were analyzed for total As, Cu, Pb, and Zn concentration by flame or electrothermal atomic absorption spectrometry, using a Varian apparatus (SpectrAA 220FS, 220Z, and 110Z). All concentrations were reported on a dry weight basis.

Whenever possible, bioaccumulation factors (BF) for each specific element were calculated, as the quotient between the element concentration in the emerging plant material to the element total concentration in the sediment:

$$BF = \frac{[\text{trace element concentration in the plant}]}{[\text{trace element concentration in the sediment}]} \quad (6)$$

2.6. Quality Control

To avoid contamination, the materials used in the sampling campaign and in the lab for the trace element quantification were rinsed with a solution of HNO_3 0.5 M. The solutions were prepared with Milli-QTM ultra-pure water (Merck KGaA, Darmstadt, Germany), the reagents had analytical grade, meeting the ACS (American Chemical Society) requirements, and the stock standard solutions were Merck Certificate AA standards.

The analytical data was validated by routine quality control procedures implemented at the lab for all the methods, including the use of standard operating procedures, analysis of reagent blanks and control standards in parallel, and the use of a minimum of three independent analytical replicates per sample. A geological certified reference material, Buffalo river sediment (NIST[®] RM 8704, NIST, National Institute of Standard and Technology, Gaithersburg, MD, USA), was extracted with aqua regia to validate the analytical procedures for the trace element quantification. The results showed good agreement between the obtained and the certified values for the trace elements which were analyzed (As, Cu, Pb, and Zn) (mean relative errors $< 10\%$).

2.7. Statistical Analysis

All data were checked for homogeneity of variance and normality (Kolmogorov–Smirnov test) and, when possible, subjected to one-way ANOVA. Whenever significant differences were found ($p < 0.05$) a post hoc Tukey HSD test was used to further elucidate differences among means ($p < 0.05$). All statistical analysis was carried out with the software STATISTIC 6.0 Software™ (StatSoft, Inc., Tulsa, OK, USA) [48].

For the *V. fischeri* bioluminescence inhibition test, the EC₂₀ and EC₅₀ values were determined using the LUMISsoft 4 Software™ (HACH-LANGE GmbH, Hørsholm, Denmark), while for the *D. magna* bioassay the EC₅₀ values were determined applying a nonlinear regression model with a four-parameter logistic curve, using the software SigmaPlot 10.0 (Systat Software, San Jose, CA, USA) [49].

3. Results and Discussion

3.1. Water Physicochemical Characterization

The influence of the AMD from the Aljustrel mine on the characteristics of the Água Forte stream was obvious from the extreme acidity of the water, with pH values ranging from 2.20 at Site 1, the nearest to the mine, to 2.77 at Site 3 (Table 1). Water sampled at Site 1 was, as expected, the most affected by the AMD from the Aljustrel mine, which was evident by its extreme acidity as well as by other parameters, namely the high EC and TDS, indicators of a high soluble salt content (highly mineralized), and by parameters characteristic of AMD affected waters, namely the high SO₄²⁻ concentration (± 10 times higher than at Site 2), and the high concentrations of As (± 100 times higher than at Site 2), and of other PTEs, such as Cu, Pb, and Zn (Table 2). The concentrations found for Cu, Pb, and Zn at Site 1 were higher than the range of values reported by Luís et al. [9] for streams in the surroundings of the Aljustrel mining area; only the As concentration was below the upper limit of the range of concentrations reported by those authors. Considering the pH, the SO₄²⁻, and the As, Cu, Pb, and Zn concentrations, the values at Site 1 were within the range of concentrations found in the most impacted AMD surface waters in the Aljustrel area characterized by Luís et al. [22].

Surface water collected at Site 1 was also affected by another anthropic source of contamination, which was the wastewater discharge from the Aljustrel's wastewater treatment plant (WWTP). Although the sampling point was selected at a site upstream from the discharge point, the turbulence of the mixing may have influenced the characteristics of the water upstream from this site. This was obvious from the high NO₃⁻, TC, and TOC concentrations, with the TOC representing 96.7% of the TC at Site 1 (Table 2). It is interesting to note that the representativeness of the organic C to the total C is not so marked in the sampling Sites 6 and 7, Roxo upstream, that had a TC content that was not statistically different from that at Site 1, but with a significant lower TOC content (<50% of the TC). That higher TC content at sampling Sites 6 and 7 may be a consequence of the fine soil particle transport by the run-off from the agricultural fields, a phenomenon already reported in the area [50], which can also account for the slight increase in nitrates (NO₃⁻) concentration. The effect of the mixing with the high pH, reductive, organic-rich waters from the WWTP in the Água Forte stream was evaluated by Maia et al. [3] who found two different effects, dependent on the season: in the rainy season, the mixing of the waters is effective, due to the continuous flux of acid drainage from the mine, leading to an immediate precipitation of the Fe from the mine, while, in the hot season, the intermittency of the flux may lead to the emergence of periods when the flux is interrupted, and the WWTP discharge prevails, leading to the reductive dissolution of Fe hydroxides and hydrosulfates from the sediments, releasing significant amounts of metals into solution. Since the sampling campaign reported in this study refers to the end of the rainy season, the flux in the Água Forte stream was continuous and the gradient of pH and PTE concentrations in the surface water were not influenced by the later effect. This was also evident by the surface water pH, with lower values than those reported by Maia et al. [3] downstream from this mixing point (4.0–4.5), evidencing a stronger effect of the AMD from mine dumps

at Aljustrel in the samples analyzed in this study. Nevertheless, other authors in what they have classified as “a year with atypical weather conditions” have observed that the location of the sampling site (i.e., its distance from the mine contaminated area) seemed to exert a higher influence on the hydrogeochemical data in the Aljustrel area than seasonality [22], which applies to the results found in this study for the surface water, with a clear gradient for pH (increase) and PTE concentrations (decrease) with the distance from the mine.

Table 1. Water physicochemical characteristics in the Água Forte stream (Sites 1, 2, and 3), and along the Roxo stream, upstream (Sites 4 and 5) and downstream from the confluence of the Água Forte stream (Sites 6 and 7) (mean values \pm standard deviation, $n = 3$). Values in a column marked with the same letter are not significantly different (Tukey HSD test, $p > 0.05$).

Sites	pH	EC _w (mS cm ⁻¹)	TDS (mg L ⁻¹)	TSS (mg L ⁻¹)	TC (mg L ⁻¹)	TOC (mg L ⁻¹)
1	2.20 \pm 0.03 a	8.59 \pm 0.03 e	14.010 \pm 104 d	80 \pm 2 b	18.4 \pm 0.1 c	17.8 \pm 0.6 e
2	2.96 \pm 0.04 c	2.15 \pm 0.05 d	1317 \pm 15 c	27 \pm 15 a	11.3 \pm 0.7 b	9.5 \pm 0.2 d
3	2.77 \pm 0.04 b	2.17 \pm 0.06 d	1414 \pm 6 c	18 \pm 16 a	4.0 \pm 0.3 a	3.3 \pm 0.4 a
4	7.86 \pm 0.06 e	0.89 \pm 0.01 a	493 \pm 21 a	9 \pm 9 a	11 \pm 2 b	7.7 \pm 0.2 c
5	7.98 \pm 0.06 ef	1.02 \pm 0.01 b	576 \pm 6 a	17 \pm 1 a	9.1 \pm 0.4 b	5.3 \pm 0.3 b
6	8.10 \pm 0.06 f	1.05 \pm 0.01 b	606 \pm 12 a	218 \pm 26 c	20.3 \pm 0.8 c	9.5 \pm 0.1 d
7	7.26 \pm 0.06 d	1.44 \pm 0.01 c	857 \pm 23 b	29 \pm 5 a	18.5 \pm 0.2 c	5.1 \pm 0.1 b

EC, electrical conductivity; TDS, total dissolved solids; TSS, total suspended solids; TC, total carbon; TOC, total organic carbon.

Table 2. Anions, metalloids, and metals concentrations in the Água Forte stream (Sites 1, 2, and 3), and along the Roxo stream, upstream (Sites 4 and 5) and downstream from the confluence of the Água Forte stream (Sites 6 and 7) (mean values \pm standard deviation, $n = 3$). Values in a column marked with the same letter are not significantly different (Tukey HSD test, $p > 0.05$).

Sites	F ⁻ (mg L ⁻¹)	Cl ⁻ (mg L ⁻¹)	NO ₃ ⁻ (mg L ⁻¹)	SO ₄ ²⁻ (mg L ⁻¹)	As (μ g L ⁻¹)	Cu (mg L ⁻¹)	Pb (μ g L ⁻¹)	Zn (mg L ⁻¹)
1	29 \pm 5 c	202 \pm 11 a	349 \pm 20 d	11.959 \pm 1127 c	2079 \pm 110 b	120 \pm 4 c	210 \pm 10	421 \pm 36 b
2	n.a.	262 \pm 11 c	31 \pm 7 bc	1025 \pm 163 ab	24 \pm 22 a	0.61 \pm 0.03 a	<DL	8.2 \pm 0.4 a
3	8 \pm 1 b	194 \pm 11 a	36 \pm 14 c	1399 \pm 221 b	17 \pm 5 a	4.8 \pm 0.2 b	<DL	23.1 \pm 0.2 a
4	0.39 \pm 0.02 a	202.3 \pm 0.6 a	1.0 \pm 0.3 a	37.2 \pm 0.3 a	4 \pm 1 a	<DL	<DL	0.05 \pm 0.03 a
5	0.35 \pm 0.02 a	208 \pm 9 ab	6.3 \pm 0.2 ab	44.09 \pm 0.03 a	6.1 \pm 0.4 a	<DL	<DL	0.05 \pm 0.02 a
6	0.34 \pm 0.02 a	226 \pm 2 b	4.0 \pm 0.4 ab	46.3 \pm 0.7 a	37 \pm 2 a	0.069 \pm 0.006 a	<DL	0.16 \pm 0.02 a
7	0.37 \pm 0.02 a	304.2 \pm 0.3 c	14.9 \pm 0.1 abc	181.0 \pm 0.6 ab	8.7 \pm 0.4 a	0.029 \pm 0.007 a	<DL	0.72 \pm 0.06 a

n.a., not analyzed; DL, detection limit; DL(Cu) = 0.017 mg L⁻¹; DL(Pb) = 170 μ g L⁻¹.

Considering only the pH, surface water at the Roxo stream was, apparently, not affected by the confluence of the Água Forte stream, with values which were not significantly different at Sites 5 and 6, sampled before and after, respectively, the confluence of the acid waters from the Água Forte stream, both with pH >7 (Table 1). Water samples at Sites 4 and 5, the Roxo upstream, presented lower concentrations of SO₄²⁻ (similar to the values reported for the Roxo Dam [50]) as well as As and Zn. Copper and Pb concentrations at those sites were even below the detection limit of the method. Arsenic, Cu, and Zn concentrations increased in the sampling Sites 6 and 7, downstream from the Água Forte confluence, but that increase was not statistically significant, relative to the water sampled upstream. The same was true for SO₄²⁻ concentration, which allowed us to conclude that, considering these water quality parameters, the Roxo stream was not affected by the AMD. Other authors have noticed this reduction in the pollution load of the water, in the transition zone between acidic and circum-neutral pH values, associated with a rapid precipitation of Fe, Al, and Mn oxyhydroxides and the removal of trace metals and metalloids by sorption and/or coprecipitation [36]. This involves a natural attenuation process that, unfortunately, is not permanent and, under specific conditions (e.g., changes in redox conditions, temperature, and pH) can be reversed, and the PTEs desorption may occur,

triggering the transference of the elements from the particulate phase to the solution [36]. An example of this phenomena can be expected to happen occasionally in the Água Forte stream, because of the mixing with the WWTP discharge, as discussed by Maia et al. [3].

Considering Pb, its concentration in the surface water was only detectable at Site 1 and diminished at Sites 2 and 3 to non-detectable values, irrespective of the fact that the stream at those sites was still AMD affected. Durães et al. [24] explained the low concentrations found for Pb in waters affected by AMD by its retention in Fe oxyhydroxides or sulphates, with a greater sorption capacity for Pb than for Cu and Zn in waters with low pH, an explanation that can be applied in this case.

As for the other analyzed anions (Table 2), fluoride, F^- , was also found at a significantly higher concentration at Site 1, a value that decreased at Site 3, and from there to the Roxo stream, where the F^- concentrations were lower and not statistically different in any of the sampling sites at that stream, therefore, not affected by the Água Forte stream. Chloride (Cl^-) was, apparently, not related to the AMD phenomena, with concentrations ranging from 194 to 304 $mg\ Cl^- L^{-1}$, similar to those reported by Alexandre et al. for the irrigation water from the Roxo Dam [50].

3.2. Water Ecotoxicological Characterization

Water from the Água Forte stream was very toxic, both for *V. fischeri* and *D. magna*, with very low EC_{20} and EC_{50} values (Table 3). Considering the different responses, their relative ecotoxicity was site 1 > site 3 > site 2, in agreement with the water physicochemical characteristics. In fact, considering the results which were discussed in Section 3.1, water at Site 1 had the worse characteristics for all the parameters, affected both by the AMD and by the wastewater treatment plant discharge, while for the other two sampling sites at the Água Forte stream, Site 3 presented a slightly lower pH value, and higher SO_4^{2-} , Cu, and Zn concentrations than at Site 2 (but only significantly different in the case of pH and Cu). Therefore, toxicity at sampling Site 3 was slightly higher than at Site 2 towards the two bioindicators. This fact, collecting water with worse characteristics at a sampling point further away from the mine, can be explained by the reduced water flow at the Água Forte stream, leading to the formation of ponds with stagnant water at some sites, with shallow water, mainly where the distance between stream banks is higher. At these sites, the equilibrium between the water and the sediment layer is different, increasing the effects of the AMD.

Table 3. Results of the ecotoxicological bioassays (mean values, $n = 3$, for the *D. magna* immobilization and $n = 2$ for the *V. fischeri* luminescence inhibition, (95% confidence interval)).

Site	<i>V. fischeri</i> Luminescence Inhibition		<i>D. magna</i> Immobilization
	EC_{20} (30 min) (% v/v)	EC_{50} (30 min) (% v/v)	EC_{50} (48 h) (% v/v)
1	<3.1	<3.1	<6.3
2	12.0 [8.9; 15.2]	41.3 [38.1; 44.4]	12.4 [10.0; 15.1]
3	5.6 [5.0; 6.2]	28.4 [27.8; 29.0]	< 6.3
4	n.t.	n.t.	n.t.
5	n.t.	n.t.	n.t.
6	27.0 [24.1; 29.9]	n.t.	n.t.
7	n.t.	n.t.	n.t.

EC_{20} and EC_{50} , water concentration, % v/v, which promoted a toxic effect on 20% or 50% of the exposed organisms, considering the selected endpoint; n.t., no toxic response.

Water at the Roxo stream was not toxic towards the planktonic freshwater crustacean *D. magna*, and the toxicity to the bacteria *V. fischeri* was also small, only quantifiable at Site 6, right after the confluence with the AMD affected stream, with an EC_{20} of 27% v/v (Table 3). Considering these results, it is possible to state that the dilution effect of the Roxo stream was sufficient to overcome the effect caused by the mixing with the Água Forte stream, highly affected by the AMD from the Aljustrel mining area, evident by the chemical characteristics of the surface water and by its ecotoxicological characteristics.

3.3. Sediment Physicochemical Characterization and Risk Analysis

Sediments collected at the Água Forte stream were severely affected by the AMD from the Aljustrel mine (Table 4): they presented acid pH values, with values ranging from 2.57 to 3.32 at Sites 2 and 1, respectively, and very high concentrations for As, Cu, Pb, and Zn. Although for Pb, there was an apparent gradient of decreasing concentrations with increasing distance from the mine, the same was not true for the others. For instance, Cu and Zn presented significantly higher concentrations at Site 3 relative to Site 2, in accordance with the results previously discussed for the water (Table 3).

Table 4. Composition of the sediments in the Água Forte stream (Sites 1, 2, and 3), and along the Roxo stream, upstream (Site 4) and downstream from the confluence of the Água Forte stream (Site 6) (mean values \pm standard deviation, $n = 3$), and freshwater sediment quality values (mg kg^{-1}), from the Canadian Sediment Quality Guidelines for the Protection of Aquatic Life [51]. Values in a column marked with the same letter are not significantly different (Tukey HSD test, $p > 0.05$). All concentrations reported on a dry weight basis.

Sites	pH	OM (%)	N _K (%)	As (mg kg^{-1})	Cu (mg kg^{-1})	Pb (mg kg^{-1})	Zn (mg kg^{-1})	Mean-PEC _q
1	3.32 \pm 0.01 b	24.4 \pm 0.1 e	0.8 \pm 0.1 d	661 \pm 39 bc	1746 \pm 24 d	539 \pm 9 e	1994 \pm 132 d	15.0
2	2.57 \pm 0.09 a	14.6 \pm 0.1 c	0.09 \pm 0.01 a	3335 \pm 361 d	652 \pm 71 b	411 \pm 36 d	350 \pm 38 a	51.3
3	3.29 \pm 0.03 b	6.8 \pm 0.2 a	0.10 \pm 0.01 a	1064 \pm 138 c	1093 \pm 280 c	259 \pm 11 c	674 \pm 103 c	18.3
4	7.30 \pm 0.10 d	16.7 \pm 0.3 d	0.41 \pm 0.01 c	30 \pm 2 a	28 \pm 2 a	25.9 \pm 0.8 a	411 \pm 32 ab	0.9
6	4.58 \pm 0.10 c	12.0 \pm 0.1 b	0.25 \pm 0.01 b	542 \pm 48 b	883 \pm 51 bc	160 \pm 3 b	572 \pm 41 bc	10.0
TEC	-	-	-	5.9	35.7	35.0	123	-
PEC	-	-	-	17	197	91.3	315	-

TEC, threshold effect concentration, assumed as the Interim Sediment Quality Guideline (ISQG) [51]; PEC, probable effect concentration; mean PEC_q, mean value of all calculated PEC_q, the quotient of the concentration of an element in the sediment to its PEC value; OM, organic matter; N_K, Kjeldahl nitrogen.

To evaluate the degree of pollution of the sediments with PTEs, their total concentrations should be compared with freshwater sediment quality values (SQVs), which have been established considering the toxic responses of a wide range of organisms. Therefore, these values intend to protect the environment but, indirectly, they also protect public health [31,45]. Despite the fact that SQVs should be site specific, in their absence, SQVs established by the environmental authorities from other countries are often used [31], and several authors have compared and evaluated them to reach consensus based SQVs [45]. All guidelines usually provide two different values, the threshold effect concentration and the threshold effect level (TEC and TEL are values below which adverse biological effects are expected to occur rarely), and the probable effect concentration and probable effect level (PEC and PEL are values above which adverse effects are expected to occur [31]). Both values are intended to be used to evaluate the degree to which adverse biological effects are likely to occur because of exposure to the element. In this study, the total concentrations of the PTEs in the sediments were compared with the Canadian Sediment Quality Guidelines for the Protection of Aquatic Life [51] (Table 4), very similar to the SQVs from the United States Environmental Protection Agency (USEPA) [31].

It is possible to see that the total concentrations for all the evaluated PTEs in the sediment were very high, above the TEC and the PEC values at Sites 1, 2, 3, and 6. These sites, where both TEC and PEC values are surpassed, can be classified as having the highest level of toxicity (Level 4, in a four levels grade), as proposed by other authors [9,45]. For the elements As and Zn, the SQVs were surpassed at Site 4, upstream from the AMD of the Aljustrel mine.

The quotient of the concentration of an element in the sediment to its PEC value is called by some authors as the PEC quotient (PEQ_q) and used to evaluate the toxicity level at that site promoted by a specific element [18,26,45]. In the most polluted sites for each individual element, the PEC_q was 196.2 for As at Site 2, and 8.9, 5.9, and 6.3 for Cu, Pb, and Zn, respectively, at Site 1, which can be considered to be very high PEC_q.

However, the combined effect of the toxicity promoted by all the chemicals in the sediment is more important than the evaluation of the toxicity at each site. This evaluation is possible by calculating each individual PEQ_q , summing them, and normalizing the sum to the number of PEQ_q , i.e., calculating the mean value considering all calculated PEQ_q , the mean PEQ_q [45] (Table 4). Considering that the sediment samples should be predicted with high toxicity when mean PEQs exceed 1.5 (one of the limits proposed by MacDonald et al. [45]), it is possible to conclude that the sediments in all sampling sites at the Água Forte Stream are extremely toxic (Site 2 > Site 3 > Site 1), and that the toxicity prevails in the Roxo stream, at Site 6 (Table 4).

Considering the results for the sediment pH at the Roxo stream, as well as the concentrations for As, Cu, and Pb, there was a clear distinction between the samples collected upstream from the confluence of Água Forte stream (Site 4) and downstream from that confluence (Site 6) (Table 4), with a reduction in the pH from 7.30 to 4.58, and a significant increase of those PTEs concentrations measured at both sites. This means that, for the sediment compartment, the effects of the AMD, coming through the Água Forte stream, in the Roxo stream were evident. If the most upstream site was considered to be a reference, indicative of what could be considered to be the background concentrations for these PTEs in the sediments, the enrichment factors for those elements at Site 6 were 18.1 for As, 31.5 for Cu, 6.2 for Pb, and 1.39 for Zn.

It was also possible to note the effect of the WWTP discharge on the sediment compartment at Site 1, already evident in the surface water characteristics. At that site, the OM and N_K content of the sediments at the Água Forte stream were statistically higher than at the other sampling sites (Table 4).

The modified BCR sequential extraction (three-step) procedure [46] was used to characterize the different extractable PTEs fractions and compare them with the total concentrations (Table 5). The results from these fractionations are very important, because the chemical state in which the elements are bound in the sediment (easily exchangeable ions, metals carbonates, oxides, sulphates, organometallic compounds, and ions in crystal lattices of minerals) markedly affect the equilibrium in sediment-water interface, their mobility, bioavailability, and toxicity [10,28,38], contributing to the risk assessment of PTEs release to the aquatic environment.

From the results, the different behaviors of the elements were obvious. Lead was the element with the lower exchangeable fraction in the sediment compartment (Table 5), relative to the total, which explains the low concentrations found in the water, only quantifiable in the most heavily contaminated site, the closest to the mine (Table 2). In the Água Forte stream, Pb in the reducible fraction (second step) and in the oxidizable fraction (third step) were also low, <10% of the total, a behavior that changed in the Roxo stream, not because the concentrations in those fractions were higher, but because the degree of contamination with Pb was lower (lower total Pb content).

Arsenic was also characterized by its low extractable fraction representativeness; <1% in the exchangeable fraction and <10% in the reducible and oxidizable fractions, without a clear tendency to any of those fractions (Table 5). This is a good indicator of the water quality, because at Site 6, Roxo downstream, the total concentration of As in the sediment is still very high, 18 times higher than in the sediment of that watercourse upstream, evidencing an effect of the AMD from the Aljustrel mine in the sediments at that site. That fact was also true for Pb, with a 6.2-times increase in Pb total concentration between those two sites. Therefore, the low percentages of the exchangeable fractions for As and Pb in the sediments (low mobility) can be considered to be an indication of low risk for the superficial water. Delgado et al. [38], when evaluating the risk of the sediments of the Guadiana Estuary, potentially affected by AMD from the IPB, also identified As and Pb as the elements with the lower potential hazard, considering their low relative mobility (% extracted by the first step).

Table 5. Trace element concentrations in the sediment, after fractionation by the BCR procedure (mean \pm SD, $n = 3$), and its percentage relative to the total concentration; 1st step (exchangeable fraction/acid-extractable metals), 2nd step (reducible fraction/associated with Fe–Mn oxides), 3rd step (oxidizable fraction/bound to organic matter or sulphides).

Element	Site	1st Step		2nd Step		3rd Step		Total (mg kg ⁻¹)
		(mg kg ⁻¹)	% of Total	(mg kg ⁻¹)	% of Total	(mg kg ⁻¹)	% of Total	
As	1	2.6 \pm 0.2	0.40	55 \pm 4	8.36	3.5 \pm 0.2	0.54	661 \pm 39
	2	0.36 \pm 0.03	0.01	0.6 \pm 0.1	0.02	1.9 \pm 0.5	0.06	3335 \pm 361
	3	1.9 \pm 0.3	0.18	7 \pm 2	0.67	0.20 \pm 0.02	0.02	1064 \pm 138
	4	0.213 \pm 0.005	0.71	3.0 \pm 0.4	9.88	1.4 \pm 0.6	4.82	30 \pm 2
	6	1.3 \pm 0.2	0.24	7.2 \pm 0.8	1.34	0.25 \pm 0.08	0.04	542 \pm 48
Cu	1	710 \pm 12	40.7	215 \pm 26	12.3	205 \pm 31	11.8	1746 \pm 24
	2	21 \pm 2	3.3	9 \pm 2	1.4	5 \pm 4	0.8	652 \pm 71
	3	156 \pm 20	14.2	219 \pm 58	20.0	165 \pm 82	15.1	1093 \pm 280
	4	<DL	–	<DL	–	10 \pm 1	34.7	28 \pm 2
	6	98 \pm 8	11.1	229 \pm 14	26.0	26 \pm 2	2.9	883 \pm 51
Pb	1	<DL	–	22.8 \pm 1.0	4.2	10.4 \pm 0.5	1.9	539.3 \pm 9.1
	2	<DL	–	9.6 \pm 0.7	2.3	9.9 \pm 0.3	2.4	411.1 \pm 36.2
	3	<DL	–	19.4 \pm 5.2	7.5	9.8 \pm 0.3	3.8	259.3 \pm 10.7
	4	<DL	–	15.3 \pm 0.7	59.1	14.8 \pm 0.6	57.0	25.9 \pm 0.1
	6	<DL	–	53.4 \pm 4.9	33.3	10.2 \pm 0.2	6.4	160.5 \pm 3.4
Zn	1	1961 \pm 295	98	75 \pm 3	4	145 \pm 16	7	1994 \pm 132
	2	106 \pm 6	30	5 \pm 1	2	12 \pm 3	3	350 \pm 38
	3	344 \pm 62	51	152 \pm 32	23	154 \pm 60	23	674 \pm 103
	4	17 \pm 1	4	203 \pm 11	49	134 \pm 3	33	411 \pm 32
	6	132 \pm 11	23	129 \pm 12	22	114 \pm 3	20	572 \pm 41

DL(Cu) = 2 mg kg⁻¹ DW; DL(Pb) = 6,7 mg kg⁻¹ DW.

A completely different behavior can be ascribed to Zn, whose liability is very high, at least at the sites affected by AMD; at Site 1, 98% of the Zn total concentrations corresponds to the exchangeable fraction, 30% at Site 2, and 51% at Site 3. At the Roxo stream, upstream from the confluence of the Água Forte stream, the reducible Zn fraction was the most representative (49%), followed by the oxidizable fraction (33%), a behavior that changes again in the downstream sampling site (Site 6), increasing again the Zn fraction in the exchangeable fraction. The fact that Zn was mainly found in the exchangeable fraction, characterized by its high mobility, has been corroborated by other authors, which have alerted for the potential impact that this can cause [38].

Copper, at Site 1, also had a great mobility, 40.7% of the Cu total concentration was in an exchangeable fraction, a value that declined at Site 3 (14.2%) and Site 6, at the Roxo stream (11.1%), evidenced by an increase in the reducible fraction at those sites. In accordance with the lower concentration found for Cu in water at Site 2 (Table 2), the Cu exchangeable concentration in the sediment at that site was also low, and the same was true for the other two extracted fractions. The predominance of Cu and, especially, Zn in the exchangeable and acid-soluble fraction at some sites meant that these elements were easily released by desorption and ion-exchange reactions, indicating their high mobility and associated risk [28,38].

3.4. Trace Element Concentrations in *Scirpus holoschoenus* L.

The dominant macrophyte on the banks of both streams is the species *S. holoschoenus* L. which seems to be very well adapted to the high concentrations of PTEs and to the acidity of the water and sediments of some of the sites, with no visible symptoms of toxic effects. The presence of this perennial rhizomatous plant in mining areas had already been identified by Otones et al. [52], claiming its preference for acid soils in humid areas.

Arsenic and Pb concentrations in the emerging parts of the plant were below the detection limits (DL) for both elements, invalidating their quantification (DL(As) = 0.417 mg kg⁻¹ and DL(Pb) = 41.7 mg kg⁻¹). That fact was somehow understandable, given the low ex-

changeable concentrations of both trace elements in the sediment, as evaluated by the BCR sequential extraction procedure (Table 5).

As for the concentrations of Cu and Zn in the plants collected along the Água Forte stream (Sites 1, 2, and 3 (Figure 4)), they were significantly higher than those found in the same macrophyte sampled at the Roxo stream. Moreover, by comparing the Cu and Zn concentrations in the plants sampled downstream to the concentrations in the plants sampled upstream (Figure 4), it is possible to see that there is a significant difference in both values (at least a four-fold increase in the concentration downstream). That increase in Cu and Zn concentrations in the plant seems to be a response to the increase in the concentrations of both elements downstream, in the water (Table 2) and in the sediment (exchangeable and total concentrations, Table 5).

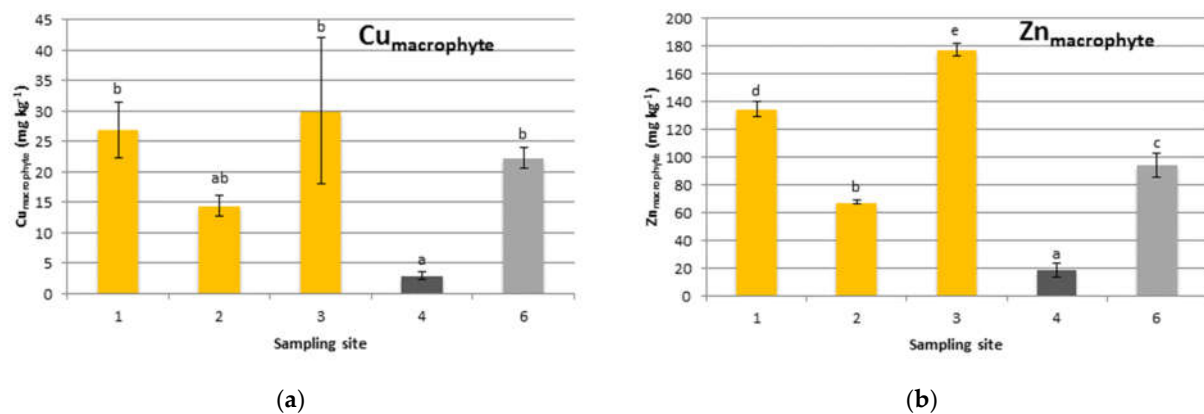


Figure 4. Copper (a) and Zn (b) concentrations in *S. holoschoenus* L. collected in the Água Forte stream (Sites 1, 2, and 3), and along the Roxo stream, upstream (Site 4) and downstream from the confluence of the Água Forte stream (Site 6) (mean values \pm standard deviation, $n = 3$). Values in bars marked with the same letter are not significantly different (Tukey HSD test, $p > 0.05$).

However, to understand the behavior of the studied plant regarding those PTEs, bioaccumulation factors (BF) were calculated (Table 6), and the values which were obtained were low (mean BF(Cu) = 0.04 and mean BF(Zn) = 0.15, $n = 15$), indicating that *S. holoschoenus* L. does not accumulate these elements in its emerging parts, on the contrary, it behaves as an excluder for those elements. This characteristic makes *S. holoschoenus* L. a potential candidate to be used in the revegetation of areas affected by AMD, namely those in the IPB, with a low risk of uptake of PTEs. This possibility has already been identified by other authors [52], that have studied the behavior of different plants in As-contaminated mine areas and have acknowledged *S. holoschoenus* L. as a potential candidate to be used in phytostabilization strategies, evidencing high root bioaccumulation factor values in relation to the labile As contents and low translocation factor values, behaving as an As excluder. In fact, according to Karathanasis and Johnson [53], plant selection criteria for high metal load wetlands should be mainly driven by their metal tolerance and rhizosphere surface area, rather than by their metal bioaccumulation potential, because their main contribution to the AMD treatment is based on substrate stabilization, microbial attachment, and rhizosphere oxidation, rather than on their metal-uptake ability.

In addition, considering that it is an emerging macrophyte, well adapted to AMD affected streams, it would be interesting to assess the potential of *S. holoschoenus* L. to be used in artificial wetlands to treat AMD affected surface waters, or to explore the possibility of its use in floating beds of macrophytes [54].

Table 6. Trace element bioaccumulation factors in *S. holoschoenus* L. (mean, maximum, and minimum values, $n = 15$). BF, bioaccumulation factor = (element concentration in the emerging plant material/element concentration in the sediment).

	BF (Cu)	BF (Zn)
Mean	0.04	0.15
Maximum	0.11	0.26
Minimum	0.02	0.05

4. Conclusions

The surface water and the sediments were extremely acid at the Água Forte stream, with high SO_4^{2-} , As, Cu, Pb, and Zn concentrations, evidencing a high impact of the AMD from the Aljustrel mining area. The sampling site closer to the mine area also had some contamination from the WWTP discharge, alerting for the need to introduce improvements in the treatment for reducing organic matter and nutrients discharged into the Água Forte stream. Two aquatic bioassays evidenced high ecotoxicity responses for the Água Forte surface water, i.e., very low EC_{50} values for *V. fischeri* luminescence inhibition (<3.1–41.3% v/v) and for *D. magna* 48-h immobilization/mortality (<6.3–12.4% v/v).

The sediments contamination at the Água Forte stream was confirmed by the fact that the concentrations for As, Cu, Pb, and Zn surpassed the freshwater SQVs in all sampling sites, both for the TEC and the PEC values, ranking the sediments at that stream with the highest level of toxicity. The high level of toxicity in the sediments was highlighted by combining the toxic effects promoted by As, Cu, Pb, and Zn, assessed by the calculation of the mean PEQ_s values. Using these indices (TEC, PEC, and mean PEQ_s), it was possible to see that toxicity prevails in the Roxo stream, in the sediments sampled after the confluence of the acid waters from the Água Forte stream.

The impact of the AMD in the surface water at the Roxo stream was not as marked as in the sediment, with circa neutral pH for the water and lower As, Cu, Pb, and Zn concentrations. Arsenic, Cu, and Zn concentrations increased in the samples collected downstream from the Água Forte confluence, but that increase was not statistically significant relative to the water sampled upstream. The same was true for SO_4^{2-} concentration, which allowed us to conclude that, considering these water quality parameters, the Roxo stream was not affected by the AMD. This reduction in the pollution load of the water occurred in the transition zone between acidic and circum-neutral pH values and can be considered to be a natural attenuation process, associated with a rapid precipitation of Fe, Al, and Mn oxyhydroxides and the removal of trace metals and metalloids by sorption and/or coprecipitation.

The response of the ecotoxicological bioassays were consistent with these results, evidencing that the water samples collected at the Roxo stream were not toxic towards the planktonic freshwater crustacean *D. magna*, and the toxicity towards the bacteria *V. fischeri* was also small, only quantifiable at the sample collected right after the confluence with the AMD affected stream, with an EC_{20} of 27% v/v , evidencing that the dilution effect of the Roxo stream was sufficient to overcome the effect caused by the mixing with the Água Forte stream.

One of the dominant macrophytes on the banks of both streams, *S. holoschoenus* L., does not bioaccumulate Cu and Zn in its emerging material (low bioaccumulation factors), and presented very low concentrations for As and Pb (below quantification limits). The adaptability of this macrophyte to the high concentrations of PTEs and acidity of the environment, with no visible toxicity effects, makes *S. holoschoenus* L. a potential candidate to be used in phytoremediation actions to be applied in the IPB, or in constructed wetlands to treat AMD.

The rehabilitation project carried out by EDM in the Aljustrel mining area (briefly described in the Introduction), had the merit of reducing the volume of acidic waters impacting the hydrological system in the Aljustrel area. The physicochemical and eco-

toxicological assessment of the waters and sediments carried out in this study should be repeated, to assess the merits of the actions carried out. Nevertheless, the effectiveness of the remediation measures and the ecological status of the water and sediments could be significantly improved if the soils in the mining area were submitted to a phytoremediation process, which was not the case.

Author Contributions: Conceptualization, P.A.; methodology, P.A., P.P., I.S., and M.J.I.; validation, P.A., P.P., I.S., and M.J.I.; formal analysis, P.A., P.P., I.S., and M.J.I.; investigation, N.G., I.S., and M.J.I.; resources, I.S. and M.J.I.; writing—original draft preparation, P.A.; writing—review and editing, P.A. and P.P.; visualization, P.A.; supervision, P.A. and P.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Polytechnic Institute of Beja, LEAF (Linking Landscape, Environment, Agriculture and Food Research Unit), funded by FCT (UID/AGR/04129/2020) and ICT (UIDB/04683/2020) projects with the reference POCI-01-0145-FEDER-007690.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors acknowledge the support given by Selma Pena (LEAF, ISA, University of Lisbon), in the design of the map provided in Figure 3.

Conflicts of Interest: The authors declare no conflict of interest.

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