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Acute Ecotoxicity Potential of Untreated Tannery Wastewater Release in Arequipa, Southern Peru

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Abstract: The centralized Rio Seco Industrial Park (RSIP) tannery collective in the Southern Peruvian city of Arequipa releases untreated tannery wastewater into a proximal creek that is a tributary of the Chili River. As industrial leather tanning wastewater contains high concentrations of metal(loid)s, salts, dyes, and organics, this complex mixture could exert a myriad of toxicological effects on the surrounding ecosystem. The RSIP effluent was analyzed to quantify the acute ecotoxicity and ecotoxicological status of this untreated industrial wastewater at multiple trophic levels with the following bioindicators: the floating macrophyte *Lemna minor*, invertebrates *Daphnia magna* and *Physa venustula*, and the amphibian *Xenopus laevis*. A physicochemical characterization of the RSIP effluent revealed a highly contaminated waste stream. In addition to chromium (10.4 ± 0.4 mg/L) and other toxic metals, the water harbored extremely high concentrations of total dissolved solids (67,770 ± 15,600 mg/L), biochemical oxygen demand (1530 ± 290 mg/L) and total nitrogen (490 ± 10 mg/L). The toxicological responses of certain bioindicator species tested were evaluated after exposure to 0, 1.5, 3.0, and 4.5% untreated tannery wastewater blended with dechlorinated tap water. *L. minor* experienced a significant decrease in the number of fronds, wet weight, and dry weight at the lowest blended wastewater of 1.5%. Bioassays with *D. magna* showed the effect on neonatal mortality with a calculated LC_{50} of 1.1% for 48 h. Bioassays with *P. venustula* embryos showed high sensitivity to diluted effluent with complete mortality at 3.0% wastewater and above. Finally, *X. laevis* showed a high sensitivity to the dilutions with an LC_{50} of 1.6 for embryos and 1.8% for tadpoles. Although RSIP wastewater contains many potentially toxic components, chromium and total dissolved solids, with a major contribution from sodium, are best correlated with acute toxicity variables. This suggests that conductivity or analogous measurements could provide a rapid and affordable forensic tool to query acute ecosystem pressures. Collectively, the results indicate that the release of untreated tannery wastewater from RSIP can exert pronounced acute impacts across trophic levels with the need for treatment or dilution to below 1% of total flow. As the assays addressed acute toxicity, the necessary treatment and/or dilution to mitigate chronic effects is likely much lower. In conclusion, untreated RSIP tannery wastewaters represent an ecological risk to downstream aquatic ecosystems; this needs to be addressed to prevent current and future environmental consequences.

Citation: Tejada-Meza, K.; Arenazas-Rodríguez, A.; Garcia-Chevesich, P.A.; Flores-Farfan, C.; Morales-Paredes, L.; Romero-Mariscal, G.; Ticona-Quea, J.; Vanzin, G.; Sharp, J.O. Acute Ecotoxicity Potential of Untreated Tannery Wastewater Release in Arequipa, Southern Peru. *Sustainability* **2023**, *15*, 15240. https://doi.org/10.3390/su152115240

Academic Editor: Subhasis Giri

Received: 14 September 2023
Revised: 19 October 2023
Accepted: 19 October 2023
Published: 25 October 2023

Keywords: industrial wastewater; *Daphnia magna*; *Lemna minor*; *Physa venustula*; *Xenopus laevis*; Latin America

1. Introduction

Approximately 2110 million square meters of leather are produced globally each year, generating more than 40 million liters of saline and chemically rich tannery wastewater [1,2]. The tanning process has a pronounced environmental footprint [3] and consumes approximately 63 m^3 of water per ton of product [4] bringing it into direct conflict with the environmental sustainability ambitions that are further amplified in arid climates. The process also results in highly concentrated industrial wastewater that in some cases is not adequately treated. The reaction with animal skins releases high amounts of total organic carbon (TOC) and increases biological oxygen demand (BOD) [5]. Tannery effluents commonly contain nitrate (NO_3^-), sulfate (SO_4^{2-}), chlorides (Cl^-), ammoniacal nitrogen (NH_4^+N), and microorganisms such as *Bacillus subtilis* and *Fusarium chlamydosporium*, as well as surfactants, organic dyes, and chromium [6,7]. Speciation is particularly important for the latter due to the carcinogenic and mobile nature of oxidized hexavalent chromium (Cr^{6+}) which is of clear concern in both tannery wastewater and other routes of environmental release [2,8]. Collectively, this combination of wastewater constituents can exert a variety of effects on receiving ecosystems if inadequately treated prior to discharge, such as water pollution [9], aquatic ecosystem damage [10], biodiversity loss [11], and even soil contamination [12] and the disruption of microbial communities [13], among many others.

The tannery industry plays an important economic role in developing countries that manufacture a wide variety of leather products such as footwear, bags, and clothing, and has a widely recognized environmental footprint [3]. Asia, Latin America, and Europe are considered the main leather producers, while Latin America and Africa show the highest annual growth rate in their production [14]. In the Latin American country of Peru, leather tanning activities are carried out mainly in the cities of Trujillo, Lima, and Arequipa. In the latter, much of the activity is centralized within the Rio Seco Industrial Park (RSIP), where approximately half of the country's leather industry is centralized [15]. Within this region, most processors do not implement adequate physical and chemical treatment before discharging effluent wastewater into the environment due to economic concerns [4,5]. In response, the Regional Government of Arequipa signed an agreement with local leather tanning companies to construct a treatment plant and six oxidation lagoons of which only two have been constructed (Figure 1). Of these, one lagoon was constructed without an underlying geomembrane, likely allowing for soil and groundwater contamination. Observations in April 2023 indicated that both lagoons were dry, suggesting that RSIP tannery wastewater bypasses the oxidation lagoons and is released into a proximal creek without treatment. This creek serves as a tributary of the Chili River (Figure 1) [16], likely polluting downstream aquatic ecosystems and local groundwater resources.

A variety of ecological health and diversity bioindicators representing different trophic levels have been used globally to assess the toxicity of individual and mixed pollutants in tannery-derived wastewater [17]. These include bacteria (*Vibrio fischeri* [18,19]), invertebrates (*Daphnia pulex* [20], *Daphnia magna* [21], and *Artemia salina* [22]), microalgae (*Scenedesmus obliquus* [23], *Pseudokirchneriella subcapitata* [24], and *Chlorella vulgaris* [25]), fish (*Carassius auratus* [26] and *Danio rerio* [27]), plants (*Lemna minor* [17,27] and *Hordeum vulgare* [28]), and certain mammals such as rats [29]. Due to their ecological distribution, diversity, and abundance characteristics, as well as their relevance in the food chain, freshwater organisms play an important role in the health of aquatic ecosystems, which is why they have been considered promising bioindicators and subject to biomonitoring [26].

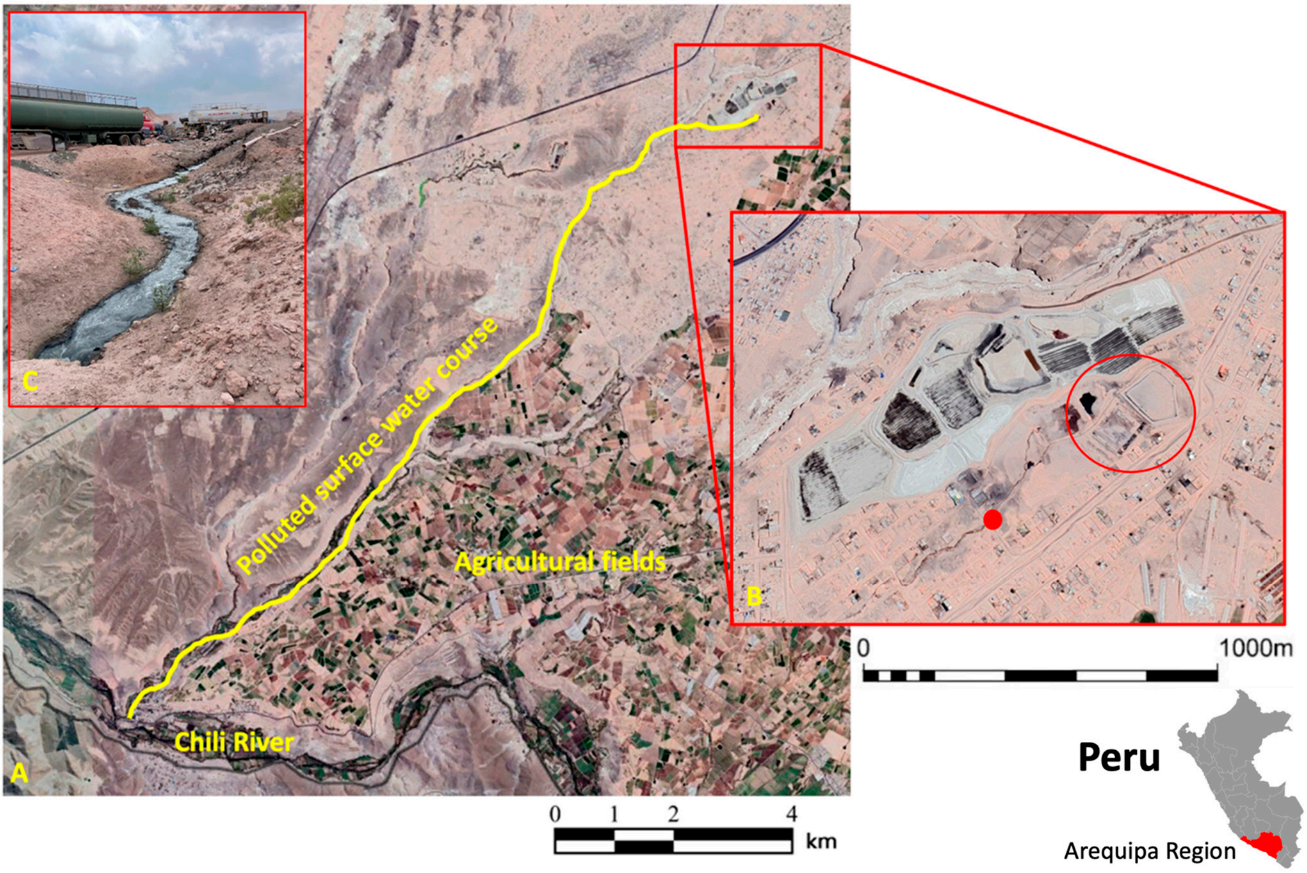

Figure 1. (**A**) Satellite image of the effluent-impacted creek contaminated by RSIP tannery wastewater in Arequipa, southern Peru. (**B**) Satellite view of the area where the two dry oxidation lagoons are located (circled). The red dot is the location of view depicted in (**C**) of the discolored and noxious smelling RSIP effluent within a creek that exits the complex in April 2023. Source: (**A**,**B**) Google Earth Pro (Maxar Technologies, 2023); (**C**) Authors' photo.

In Brazil, Monteiro et al. [30] investigated the possible mutagenic and histopathological effects of hexavalent chromium on *Lithobates catesbeianus* (tadpoles), concluding that the metal has a strong effect on the life cycle of the species. Likewise, Bhattacharya et al. [31] focused their work on a comparative evaluation of the toxic impacts of untreated tannery effluent and membrane-treated effluent using *Pila globosa* (snail) as an aquatic model in India, reporting significant effects of the former effluent type on the species. Moreover, Tisler et al. [32] investigated the toxicological effects of disinfecting agents in Slovenian tannery wastewater on several bioindicators, concluding that water fleas (*Daphnia magna*) were the most sensitive. Cooman et al. [33] evaluated the toxicity of Chilean tannery wastewaters on *Daphnia* spp., concluding that all tested waters had a toxic effect on the species. Finally, Arenazas [26] investigated the mortality of the bioindicator freshwater snail (*Physa venustula*) after exposure to (diluted) tannery effluents treated with oxidation lagoons at RSIP in Arequipa, concluding morphological alterations and lethal effects. In building upon these findings, the objective of this research was to take the novel experimental approach of evaluating the acute ecotoxicity of untreated, yet diluted Peruvian (RSIP) tannery wastewater discharges using a series of bioindicators from different trophic levels that included duckweed (*Lemna minor*), water fleas (*Daphnia magna*), freshwater snails (*Physa venustula*), and African frogs (*Xenopus laevis*). A series of dilutions was implemented for raw and untreated wastewater to better determine potential discharge levels when mixing with environmental waters, as well as to inform target ranges for acute effects. It was hypothesized that this environmentally relevant and complex mixture of untreated tannery effluent was sufficiently toxic to produce acute effects across these trophic levels even when present at several percents of bulk quantities.

2. Materials and Methods

2.1. Determination of Physicochemical Parameters for Tannery Wastewater

Three 10-L samples were collected directly from RSIP's untreated tannery wastewaters on 21 March 2022, in reinforced polyethylene containers, which were previously washed with distilled water and subsequently dried. For phosphorus and other sensitive analyses, samples were carried in glass containers following standard analytical procedures. After immediately measuring pH (electrometric method), the samples were placed in ice coolers, transported to the laboratory within hours, and refrigerated at 4 ± 0.5 °C, for no longer than two days before experimentation. The harvested water was analyzed within this window for the following parameters (and processing methods): turbidity (nephelometric method 2130), total dissolved solids (TDS) (method 2540-C), total suspended solids (TSS) (method 2540-D), chemical oxygen demand (COD) (open reflux method 5220-D), biochemical oxygen demand in five days (BOD_5) (Dilution method 5210-B), total nitrogen (Persulfate method 4500-PJ), total metals (ICP, using iCAP 7400 ICP-OES Spectrometer; Thermo Scientific, Shanghai, China; unfiltered and preserved with a few drops of nitric acid per the method), sulfate (ion chromatography 300 EPA), and chloride (argentometric determination 4500-Cl^-), all following standard methods as described by the American Public Health Association (Washington, DC, USA) [34].

2.2. Ecotoxicity Bioassays and Trophic Organisms

The organisms (Table 1) were collected from a local commercial aquarium and transported to the laboratory for maintenance, reproduction, and ecotoxicity tests. *Lemna minor* specimens were acclimatized in the laboratory for two weeks prior to experimentation in containers of dechlorinated (i.e., exposed to the environment for seven days) tap water and essential nutrients (weekly dose of 7.6 mL/L, using Plant Gro Nutrafin®). The bioassay consisted of placing 10 colonies of *Lemna minor* with two leaves of approximately the same size in polyethylene containers with a capacity of 250 mL. Tannery wastewater was diluted with dechlorinated tap water [35] and added to achieve final concentrations of 0, 1.5, 3.0, and 4.5% (concentrations selected arbitrarily after an initial assessment at higher dilutions resulted in near complete mortality as discussed in results) of the original tannery wastewater concentration. The test was conducted with four replicates at room temperature (23 ± 3 °C), under continuous light (white LED at 6000 Lux). The test lasted 168 h and the final quantified parameters were the total number of fronds, dry weight, and wet weight. *Lemna minor* is a macroscopic plant, and the effect can be detected by increases or decreases in growth, i.e., the number of fronds or leaves during the experimentation process; likewise, the weight was recorded using an analytical balance.

Table 1. Ecotoxicity experimental design matrix showing organisms, measured effects, and tested RSIP tannery effluent concentrations.

Organism	Effect	Effluent Concentrations (%)
Lemna minor	Number of fronds, dry and wet weight	0, 1.5, 3.0, 4.5
Daphnia magna	Mortality	0, 1.5, 3.0, 4.5
Physa venustula	Egg hatching, development delay	0, 1.5, 3.0, 4.5
Xenopus laevis embryos and larvae	Mortality and morphological effects	0, 1.5, 3.0, 4.5

Daphnia magna neonates (less than 24-h-old) were used for the corresponding ecotoxicity bioassays. The specimens were previously raised and fed with microalgae. *Daphnia magna* neonates were placed in glass petri dishes (60 × 15 mm), and subjected to 20 mL of the different dilutions of tannery residual water (0, 1.5, 3, and 4.5%) and four replicates per treatment, each with 10 individuals per plate. The tests were carried out at room temperature following the Organization for Economic Cooperation and Development (OECD) guidelines [36]. The bioassay lasted 48 h of acute exposure and daily mortality (absence of movement during 15 s of observation) was documented. For verification, a stereoscopic microscope was used where individuals were touched with a Pasteur pipette's tip to confirm a lack of movement corresponding to death.

Physa venustula eggs were produced in the laboratory at pH of 6.5–8.5 [37]. Petri dishes (60 × 15 mm) were used with four repetitions per treatment at room temperature. Two masses of eggs containing similar total egg numbers (between 15 and 62 eggs) were placed in each Petri dish; the actual egg count was documented for each sample. Subsequently, the groups of eggs were subjected to nine days of experimentation against different tannery wastewater dilutions (0, 1.5, 3, and 4.5% effluent solution). The organisms were monitored daily using a stereoscopic microscope, evaluating and documenting hatching percentages.

The *Xenopus laevis* (albinos) specimens were acclimatized and reproduced in an 80-L capacity aquarium. For embryo studies, eighty eggs in the gastrulation stage (stages 8–11 according to the scale used by Nieuwkoop et al. [38]) were used, with five eggs per Petri dish (60 × 15 mm) and 20 mL of diluted RSIP solution (0, 1.5, 3, and 4.5%, four repetitions by dilution of effluent), at room temperature for 96 h. Morphological alterations in development and mortality were documented daily using a stereoscopic microscope. For analogous larvae studies, eighty larvae (six-day-old tadpoles, previously reared in the laboratory) were placed in polyethylene containers with 200 mL of diluted RSIP tannery wastewater (five for each container), with four replicates per treatment at room temperature for 96 h. The organisms were monitored daily with a stereoscopic microscope, documenting mortality and morphological abnormalities.

2.3. Quantification of Acute Toxicity

The toxicity indices LC_{50} (lethal concentration for 50% of the population) and LOEC (lowest observable effect concentration) were determined. ATU (acute toxicity units) of the tannery effluent was determined using Equation (1) as previously applied by Saldaña et al. [39].

$$ATU = \left(\frac{1}{LC_{50}}\right) * 100 \tag{1}$$

where LC_{50} represents the mean lethal concentration according to the classification used by Saldaña et al. [39], reflected as "very toxic" (ATU > 4 and LC_{50} < 25%), "toxic" (ATU between 2 and 4, and LC_{50} between 25 and 50%), "moderately toxic" (ATU between 1.33 and 1.99, and LC_{50} between 50 and 75%), and "slightly toxic" (ATU < 1.33 and LC_{50} > 75%).

Additionally, a classification was made based on percent survival and the LC_{50} index expressed as percent dilution, following the ranges proposed by Roig et al. [40]: "highly toxic" (LC_{50} < 10 and % survival < 10), "moderately toxic" (LC_{50} between 20 and 10, and % survival between 10 and 20), "marginally toxic" (LC_{50} between 60 and 21, and % survival between 21 and 50), "slightly toxic" (LC_{50} between 61 and 100, and % survival between 50 and 80), and "nontoxic" (LC_{50} > 100 and % survival > 80).

2.4. Statistical Analyses

The experimental design was implemented in 4 × 4 random blocks. To evaluate the assumptions of normality and homogeneity of the data variances, the Shapiro-Wilk and Levene tests were carried out, respectively. When needed, the non-parametric Kruskall Wallis test was used to detect differences among groups, together with the one-way Analysis of Variance (ANOVA), followed by the Tukey's test ($\alpha = 0.05$). All statistical processing and analyses were performed in the Statistics 12 software (Statsoft, Inc., Tulsa, OK, USA).

The analysis for each group of results obtained from each bioassay and the determination of the LC_{50} for the residual was performed with the Probit method (e.g., [41]), performed in the Microsoft Excel® program for Microsoft 365 MSO (version 2201 build 16.0.14827.20158). All statistical confidence limits were set at 95% and the SigmaPlot 14 software (academic version) was used to generate charts.

Finally, the experimental design of completely randomized blocks was specified, where the independent variable corresponds to the dilutions or treatments of the tannery effluent (0, 1.5, 3.0, and 4.5%) and the dependent variable are the sublethal effects evaluated in *Lemna minor* (number of fronds, dry and wet weight), *Daphnia magna* (mortality,) *Physa venustula* (egg hatching and delay in the development of head and shell formation), and *Xenopus laevis*'s embryos and larvae (mortality and morphological effects). The intervening variables were room temperature, relative humidity, photoperiod (hours of light and darkness), volume of the diluted effluent, and exposure time to the effluent.

3. Results

3.1. Physicochemical Parameters of the Harvested RSIP Effluent Used in Toxicology Experiments

The collected RSIP tannery effluent was a highly concentrated electrolyte-, organic-, and nutrient-rich industrial waste stream (see Tables 2 and 3) that appears to undergo minimal treatment before environmental release (Figure 1). It is of particular note that the total dissolved solids (67,770 ± 15,600 mg/L) was almost twice that of seawater and had a low ratio of suspended-to-dissolved solids. A large fraction of that salinity could be attributed to sodium, chloride, and other base cations including magnesium (Mg), potassium (K), and calcium (Ca). Phosphorus (6.8 ± 0.3 mg/L) was approximately twice that present in typical wastewater treatment plants and 6–7 times above US discharge standards (1 mg/L) [42]. Tannery effluent contains protein, hair, skin, and emulsified fats that are removed from the hides and released in the effluent, increasing the total solids, nitrogen, and organic carbon contents [43]. Total nitrogen (490 ± 10 mg/L) was 10–20 times higher than typical of US municipal wastewater (MPLs of up to 20 mg/L) (e.g., [44]) and approximately 1500 times higher than Peruvian wastewater discharge standards (MPL of 0.315 mg/L) (DS 004-2017-MINAM). While nitrogen speciation was not conducted, other authors [5,45,46] have found similar high total nitrogen loading in other analyzed tannery wastewaters. Biochemical oxygen demand (1530 ± 290 mg/L), which is a surrogate of organic loading, was 5–10 times higher than typically found in US municipal wastewater treatment plants and more than 100 times above permitted wastewater discharge standards of 20 to 30 mg/L [47,48]. Biological demand comprised about half of the total chemical oxidation demand, suggesting a high initial degree of biological recalcitrance. As listed in Table 3, RSIP effluent also contained metals such as chromium (10.4 ± 0.4 mg Cr/L) and copper (0.062 ± 0.013 mg Cu/L) at potentially toxic concentrations in addition to metalloids of agricultural concern such as boron (10.4 ± 0.4 mg B/L) that exceeded the maximum admissible values (MAV) for non-domestic wastewater discharges into the sanitary sewer system (DS 004-2017-MINAM).

Table 2. Select physiochemical properties of the RSIP tannery wastewater (n = 3).

Parameter	Unit	Result ± SD
pH	-	8.6 ± 0.4
Total dissolved solids (TDS)	mg/L	67,700 ± 15,600
Total suspended solids (TSS)	mg/L	650 ± 190
Total nitrogen	mg/L	490 ± 10
Biochemical oxygen demand	mg/L	1530 ± 290
Chemical oxygen demand	mg/L	3030 ± 50
Sulfate	mg/L	4850 ± 430
Turbidity	NTU	180 ± 10
Chlorides	mg/L	12,300 ± 400

Table 3. Metal(loid)s cations present in the harvested RSIP tannery wastewater (*n* = 3).

Metal	Concentration Values ± SD (mg/L)	Metal	Concentration Values ± SD (mg/L)
Aluminum	0.68 ± 0.05	Lithium	2.1 ± 0.4
Antimony	0.003 ± 0.001	Magnesium	230 ± 50
Arsenic	0.42 ± 0.10	Manganese	0.24 ± 0.05
Barium	0.12 ± 0.03	Molybdenum	0.021 ± 0.004
Bismuth	0.0004 ± 0.0001	Nickel	0.016 ± 0.003
Boron	7.2 ± 1.5	Lead	0.032 ± 0.007
Cadmium	0.0012 ± 0.0003	Potassium	183 ± 6
Calcium	470 ± 120	Rubidium	1.2 ± 0.3
Cerium	0.0009 ± 0.0001	Silica	99 ± 26
Cesium	0.017 ± 0.003	Silicon	46 ± 12
Cobalt	0.014 ± 0.002	Sodium	26,000 ± 5500
Copper	0.062 ± 0.013	Thallium	0.001 ± 0.0001
Chromium	10.4 ± 0.4	Titanium	0.33 ± 0.00
Strontium	5.0 ± 1.1	Uranium	0.0016 ± 0.0003
Phosphorous	6.8 ± 0.3	Vanadium	0.020 ± 0.003
Gallium	0.16 ± 0.03	Tungsten	0.0007 ± 0.0001
Germanium	0.013 ± 0.002	Zinc	0.49 ± 0.10
Iron	1.7 ± 0.4	Zirconium	0.0007 ± 0.0002

Note: Other compounds quantifiable by ICP, specifically silver, beryllium, hafnium, mercury, lanthanum, lutetium, niobium, selenium, tin, tantalum, tellurium, and thorium returned values below detectable limits.

3.2. *Acute Mortality and Ecotoxic Indices for Model Organisms Exposed to Dilluted RSIP Tannery Wastewater*

Preliminary experiments determined that raw RSIP water was "highly toxic" with mortality well above LC_{50} parameters and/or pronounced effects for all experimental blends above 10% RSIP water. An experimental dilution series of 4.5% RSIP water or less was selected across the chosen organisms to better understand toxicological responses during environmental dilution associated with release.

3.2.1. *Lemna Minor*

Three response variables were evaluated in the ecotoxicological tests carried out with the freshwater floating plant *Lemna minor* (duckweed): number of fronds, wet weight, and dry weight after an exposure time of 168 h (7 days). Figure 2 shows the effect on frond development, wet weight, and dry weight of *Lemna minor* against three dilution levels (plus control). Significant differences were observed when comparing each selected dilution with the control sample, as shown in Table S1, and the lowest consistent observable effect levels (LOEL) of exposure corresponded to the lowest tested value of 1.5% tannery wastewater. While mortality tests such as LC_{50} were not achieved for *Lemna minor*, there was a 50% or greater reduction in these response variables after exposure to more than 3% tannery wastewater. Further reduction was insignificant when increasing tannery wastewater exposure from 3% to 4.5%.

3.2.2. *Daphnia Magna*

The mortality of the invertebrate *Daphnia magna* (water flea) exhibited a pronounced effect after wastewater exposure (see Figure 3 and Table S2). Statistically significant differences were observed between the dilutions and the control for both timeframes (see Table S2 for details). After 48 h of exposure, the net mortality was 65.0 ± 12.9% for the lowest tested tannery wastewater (1.5%). The 4.5% dilution approached a 97.5 ± 5.0% mortality rate, and mortality between 3% and 4.5% tannery waste was statistically indistinguishable. Although limited concentration points were used, which limits the precision of measurements, this resulted in an LC_{50} of 2.5% and 1.1% effluent exposure for 24 and 48 h, respectively (see Table S2).

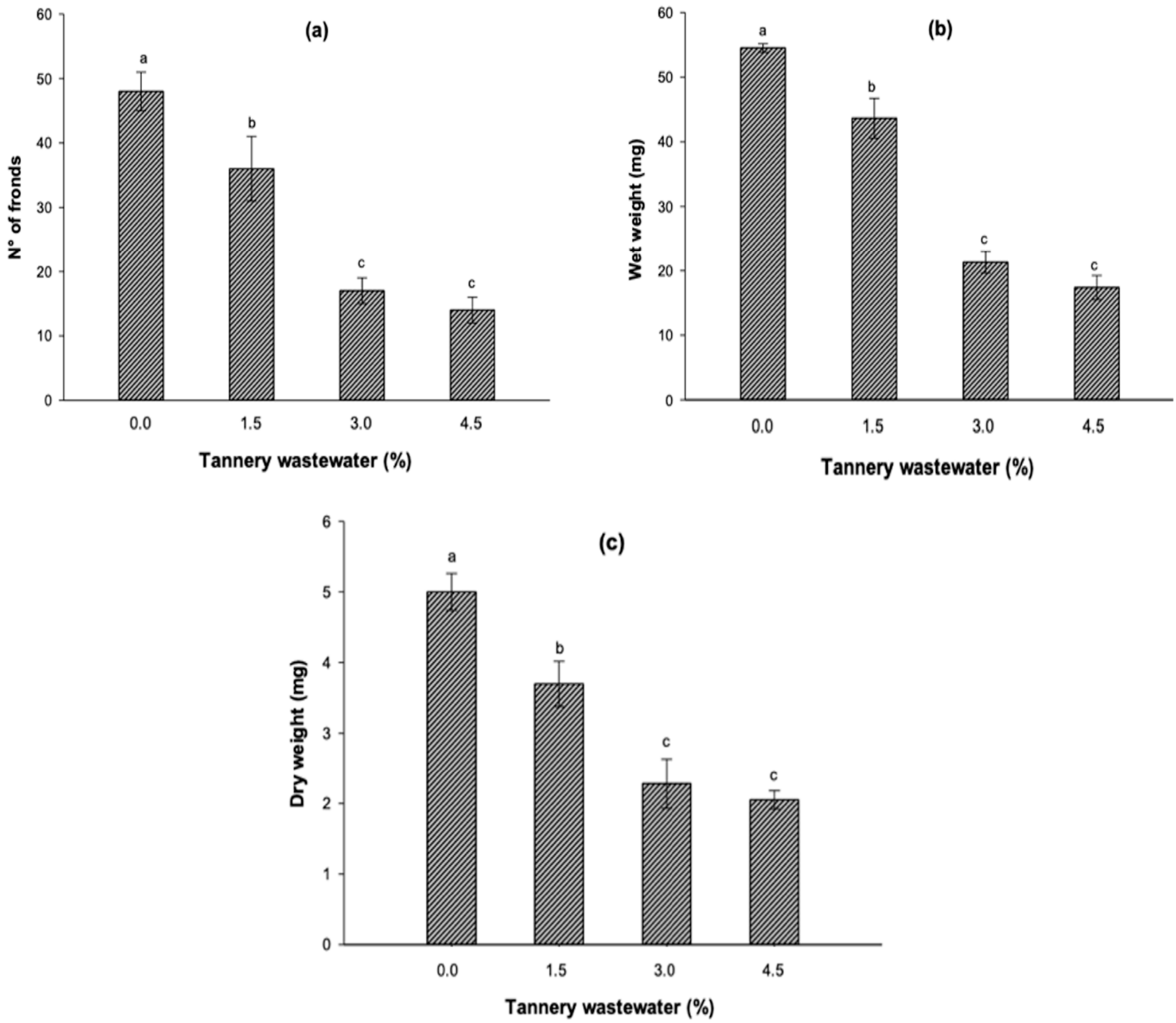

Figure 2. Effect of diluted RSIP tannery wastewater exposure on (**a**) number of fronds, (**b**) wet weight, and (**c**) dry weight of *Lemna minor*. The error bars represent the SD of four repetitions, while the letters convey statistical differences across treatments, as determined by Tukey's test.

3.2.3. *Physa Venustula*

The freshwater snail *Physa venustula* (see Figure 4 and Table S3) experienced pronounced reductions in egg hatching after 216 h (9 days) of exposure to tannery wastewater. While the control experienced some variability in egg hatching (86.3 ± 15.5%), there was a dramatic reduction after exposure to 1.5% tannery wastewater (39.6 ± 9.7%). No *Physa venustula* eggs hatched at exposures greater than 3% tannery wastewater. Experimental limitations in sufficient exposure points below these values limited the quantification of an LC_{50}, which visually appeared near or below 1.5% exposure. Furthermore, a delay in the development of head and shell formation was observed in the 1.5% dilution treatment (Figure 5b), some appearing with a much smaller size compared to the control. Figure 5 shows photographs that highlight the negative effects of dilution on the normal development of *Physa venustula* embryos.

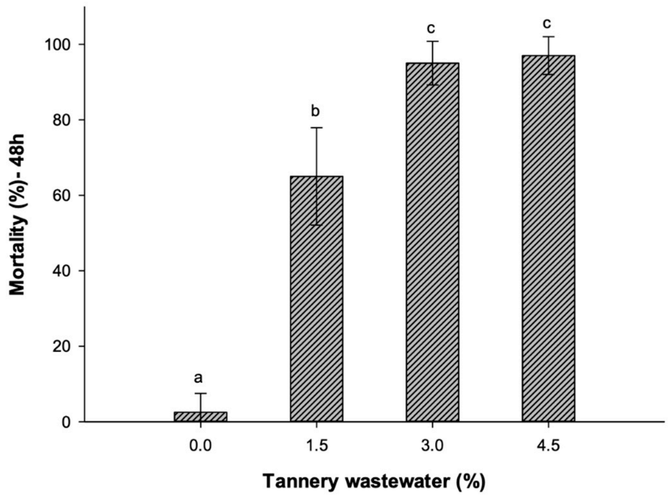

Figure 3. Effect on mortality of *Daphnia magna* after exposure to different percentages of tannery wastewater at 48 h of exposure. The error bars represent the SD of four replicates. The letters represent statistically different groupings, as determined by Tukey's test.

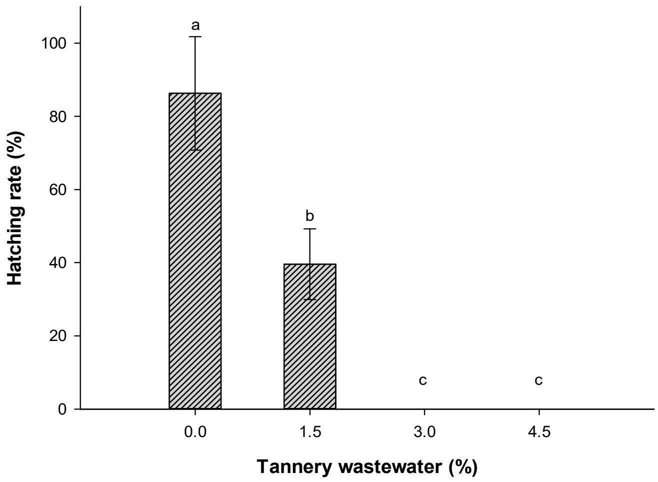

Figure 4. Effects of diluted tannery effluent on the hatching of *Physa venustula* eggs after a 216 h incubation. The error bars represent the SD of four repetitions, while the letters indicate the statistical differences between each treatment, determined by the Tukey test.

3.2.4. *Xenopus Laevis* (Embryos and Larvae)

Significant repression as a function of a 96-hour incubation in the presence of tannery wastewater was documented for *Xenopus laevis* (African frog) embryos and larvae (tadpoles) (see Figure 6 and Table S4). Statistically significant differences were observed between all dilutions and the control, and a statistical difference was also observed between each of the dilutions. Moreover, the difference between the control and the first dilution was very large, where exposure to 1.5% tannery wastewater resulted in 50 $\pm$ 12% mortality for embryos and 45 $\pm$ 10% for tadpoles, with a calculated LC_{50} of 1.6 and 1.8% exposure, respectively. Exposure to 4.5% wastewater approached complete mortality in both assays. Morphological effects on embryos such as gastrula arrest, presence of exogastrules, and reduced pigmentation of the eye were observed from the 1.5% dilution and above (Figure 7a–d). Morphological effects in tadpoles included folded tail and hyperactivity. No edema or fin reduction was observed (Figure 7e–h).

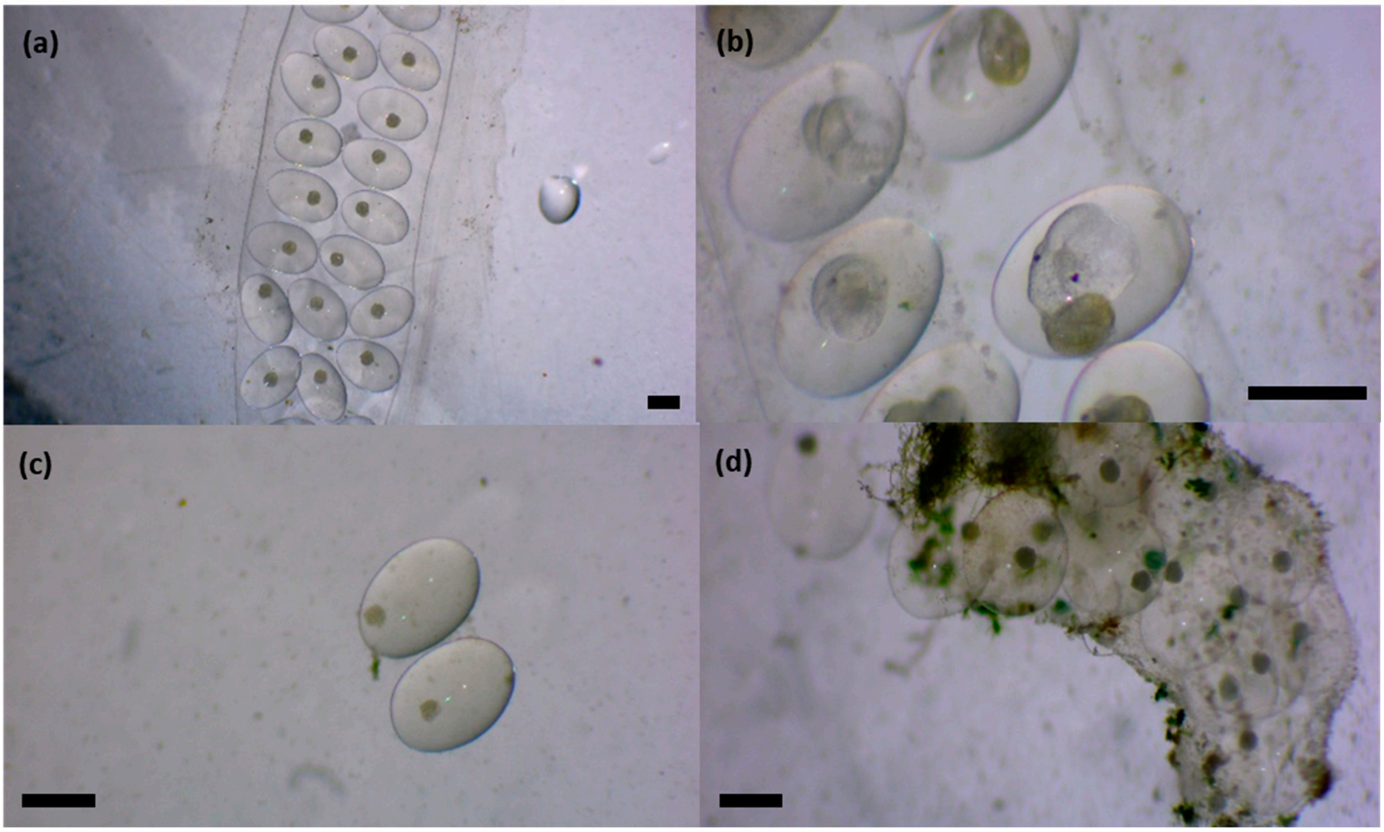

Figure 5. Effects of diluted RSIP tannery wastewater on the development of ovigerous masses of *Physa venustula*: (**a**) embryo in morula stage (0 days)—control; (**b**) hippo stage larvae (9 days)—1.5% dilution; (**c**) blastula stage embryos (9 days)—3% dilution; (**d**) set of dead embryos embedded in organic matter (9 days)—4.5% dilution (scale = 500 um).

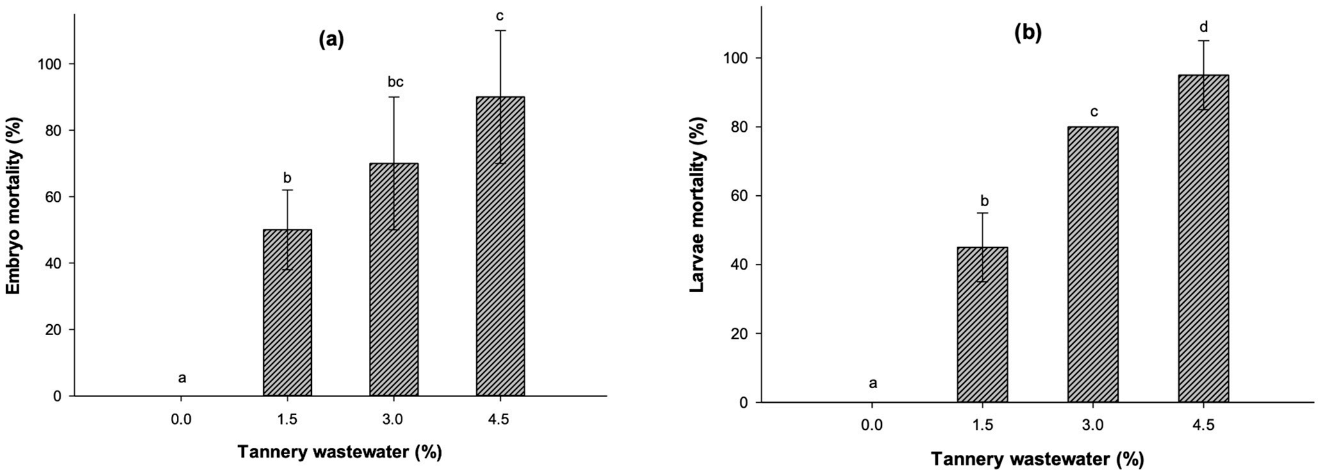

Figure 6. Effect of Tannery wastewater on the mortality of (**a**) embryos and (**b**) larvae of *Xenopus laevis*, at 96 h of exposure. The error bars represent the SD of four repetitions, while the letters indicate the statistical differences between each treatment, determined by the Tukey's test.

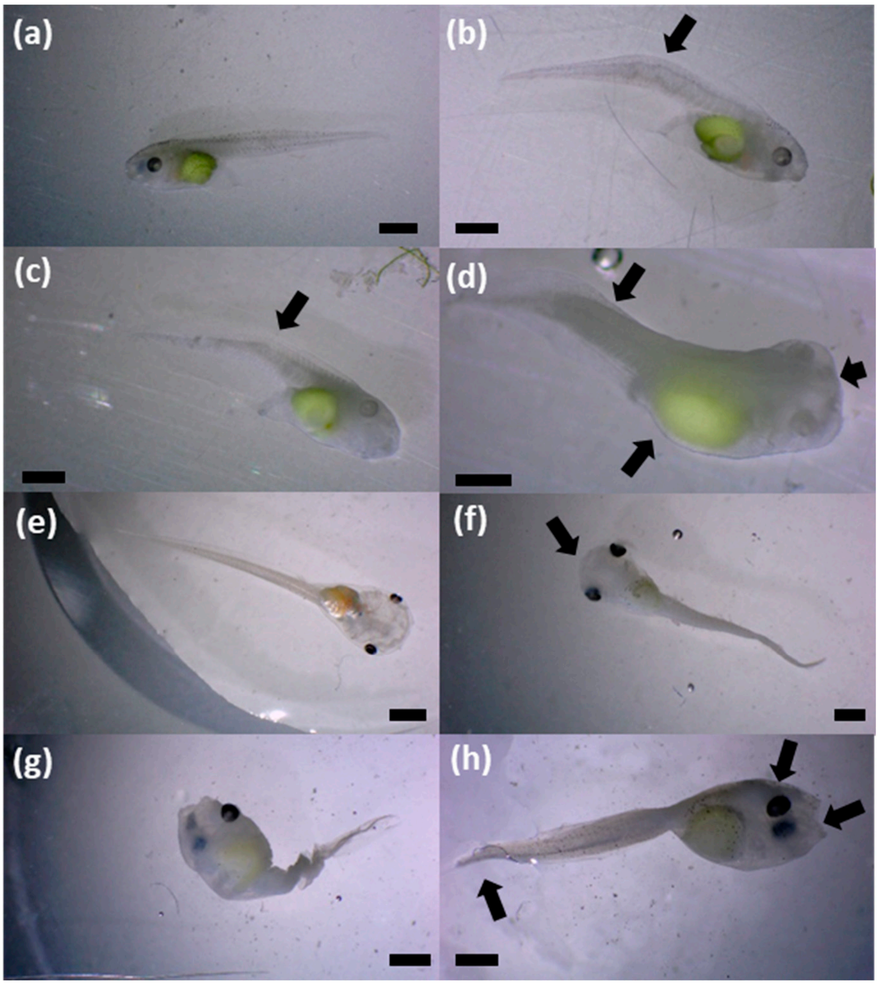

Figure 7. Effects of tannery wastewater on the development of *Xenopus laevis* embryo and larvae: (**a**) stage 21 (according to the criteria used by Nieuwkoop et al. [38])-control; (**b**) spinal cord injury—1.5% dilution; (**c**) partial pigmentation of the eye and yolk sac degeneration—3.0% dilution; (**d**) degeneration of caudal vertebrae, yolk sac and mouth—4.5% dilution; (**e**) healthy larvae-control; (**f**) degeneration of caudal vertebrae, bent tail—1.5% dilution; (**g**) total alteration of the organism (post 46 stage)—3.0% dilution; (**h**) alteration of head, eyes, mouth—4.5% dilution. (Scale = 1 mm).

3.3. Determination of Acute Toxicity Units (ATU) and Toxicological Classification of Tannery Wastewater

The criteria for determining ATU [39] and ecotoxicological classification [40] were applied using the LC_{50} values calculated with the data corresponding to the highest dilution of the effluent (i.e., 4.5%) from the bioassays. Obtained results are shown in Table 4.

Table 4. Acute toxic units and ecotoxicological status of the tannery effluent studied in four organisms.

Organism	ET (h)	LC_{50}	%S	ATU	CS	CR
Lemna minor	168	-	-	-	-	-
Daphnia magna hatchlings	48	1.1	2.5	89	Very toxic	Highly toxic
Physa venustula embryos	216	-	0	-	-	Highly toxic
Xenopus laevis embryos	96	1.6	10	63	Very toxic	Highly toxic
Xenopus laevis larvae	96	1.8	5	60	Very toxic	Highly toxic

ET: Exposure time; LC_{50}: Median lethal concentration; %S: percent survival; ATU: Acute toxicological units; CS: Classification according to Saldaña et al. [39]; CR: Classification according to Roig et al. [40].

Regarding *Lemna minor*, it was not possible to determine an LC_{50} because in this organism only sublethal effects were identified. However, it was possible to calculate the LC_{50} for *Daphnia magna,* and, therefore, a value of 89 ATU was determined for 48 h of exposure, respectively. For *Physa venustula* embryos, the LC_{50} could not be calculated due to experimental constraints; as discussed above, it appears to be below the LOEC of 1.5% dilution obtained in this study and hence corresponds to a highly toxic CR. For the embryos and larvae of *Xenopus laevis*, an LC_{50} of 1.6 and 1.8% fits into a very toxic CS and a highly toxic CR (see Table 4).

4. Discussion

4.1. Physicochemical Parameters

The RSIP tannery wastewater used in these experiments has extremely high quantities of dissolved solids, organics, and nutrients. These values are consistent with past measurements from the facility and other raw or minimally treated tannery wastewaters highlighting the impact of this contaminated industrial waste stream. It is common for the skins to be preserved with NaCl in the slaughterhouse and then transported to the tanneries to obtain the leather. Subsequent salt removal by soaking generates about 12–15 L/kg of wastewater with high TDS in the range of 21,000–57,000 mg/L [49] as well as sodium and chloride [50]. The waters used in this experiment fell in the upper range of previous reports with a TDS value of 67,700 ± 15,600 (Table 2). A large fraction of this could be attributed to sodium at 26,000 ± 5500 mg/L and chlorides at 12,300 ± 400 mg/L (Table 2). Other authors have reported highly variable chloride values, between 160 and 6400 mg/L [45,47,51–54], while Villalobos–Lara et al. [55] (Mexico) reported chloride content of 16,000 ± 1600 mg/L that was more consistent with the present analysis of RSIP effluent.

The TSS in the RSIP effluent waters (650 ± 190 mg/L, Table 2) was lower than many prior reports [8,51–53], where TSS ranged from 12,500 to 35,200 mg/L. This could be the result of RISP factories having filters that retain suspended solids prior to discharge. However, other measurements from this investigation suggest that treatment does not adequately address soluble contaminants. Haruna et al. [56] developed classifications based on TSS, where "weak" (TSS < 100 mg/L), "medium" (100 < TSS < 220 mg/L), and "strong" (>220 mg/L) classifications suggest that TSS pollution in the tannery wastewater generated at the RSIP is strong. Typically, TSS levels link to high turbidity, and the turbidity of the studied wastewater at 178 ± 8 NTU (Table 2) was consistent with reports for a location in Cameroon [52] but generally lower than other reports that range from 700 to 3785 NTU [50,52,55] which could be attributed to the presence or absence of filtration-based treatment.

The high quantities of boron found in this investigation (7.2 ± 1.5 mg/L, Table 2) are likely associated with the use of boric acid as a preservative for hides [57,58] and exceed the MAV by approximately 80%. Nitrogen is an important parameter that is regulated in part due to concerns about eutrophication pressures associated with release, and several authors report the values as ammoniacal nitrogen [45,54,59–65]. In this study, 490 ± 10 mg total N/L was obtained using the persulfate method (Table 2), which is in between what was reported by Vo et al. [45] in China and that from Villaseñor–Basulto et al. [5] in Mexico, who obtained values of 350 and 850 mg N/L, respectively, for tannery wastewater using the same method applied in this study. While the present study did not differentiate between species, the literature precedent suggests that it is dominated by ammonia, which has more pronounced direct acute toxic pressures in aquatic organisms than nitrate (e.g., [66]).

The BOD of this organic-rich wastewater was 1530 ± 290 mg O_2/L (Table 2), a value that agrees with reports from other countries [55,65]. The chemical oxygen demand (COD) as an indicator of total oxidizable potential was almost double the BOD at 3030 ± 50 mg/L (Table 2) and is similar to that reported by others [45,46,60,63,65]. This indicates a high degree of recalcitrance in the balance between COD and BOD. Large amounts of sodium sulfate and ammonium sulfate are used in the tanning, pickling, and deliming stages of leather processing [67]. The wastewater under study contained sulfate at 4850 ± 430 mg/L (Table 2), which is higher than that reported by other international studies [45,61,62].

The pH of this wastewater was 8.6 ± 0.4 (Table 2), which was close to the values reported by other studies [40,41] in tannery wastewaters sampled in Arequipa and Brazil, respectively, and consistent with reported values in tannery wastewaters from other regions ranging from 8.3 to 9.2 [42,44,45]. In the unhairing process, it is common to use bases such as sodium hydroxide and calcium hydroxide, or slaked lime ($Ca(OH)_2$). The amount of slaked lime is commonly between 30 and 45 kg per cubic meter of water [67], which is likely why tannery wastewater at RSIP has large amounts of calcium and sodium (see Table 3). Likewise, the calcium values found in this study are slightly above the 50–440 mg/L range reported [68] in India. In addition to sodium and chloride, other particularly high quantities of ions were calcium (472 ± 118 mg/L), magnesium (227 ± 48 mg/L), and potassium (183 ± 6 mg/L). The high quantities of magnesium (Table 3) could be due to the use of magnesium salts to precipitate chrome from the effluent of the chrome tanning process [69,70]. Specifically, magnesium phosphates and oxides are good precipitating agents for the removal and recovery of chromium from tanning wastewater [71]. In the RSIP's chrome tanning processing, salts such as sodium bicarbonate and potassium bicarbonate are used [67], which may be the source of potassium documented in the analysis (Table 3).

Chromium is of particular concern in tannery wastewater and is contributed by the addition of trivalent chromium (Cr^{3+}) salts during the tanning processes [72]. Only 60 to 70% of the total chromium salts react with hides and about 30–40% of chromium remains in solid and liquid waste (especially used tanning solutions) [73]. Total chromium values reported by different authors in other countries vary between 29 and 258 mg/L [1,50,51,53,59,62,64,74,75]. The chromium concentration found in this RSIP analysis (10.4 ± 0.4 mg/L, Table 3) was below what is commonly reported but exceeds maximum admissible concentrations (D.S. 004-2017-MINAM); however, it agrees with the 8.1 ± 4.8 mg/L reported previously [76] in Arequipa and the 11.5–14.3 mg/L range reported in Thailand [77]. Measurements from the present study were limited to total chromium and did not differentiate between Cr^{3+} and Cr^{6+}; the oxidized species Cr^{6+} is a notable concern in the tanning industry containing acute and chronic toxicity, neurotoxicity, dermatotoxicity, genotoxicity, carcinogenicity, and hepatotoxicity, and seriously endangers the physical health of humans and experimental animals [53,78].

4.2. Determination of Accumulated Mortality and Ecotoxic Indices in Model Organisms Due to the Effect of Exposure to Dilluted RSIP Tannery Wastewater

4.2.1. *Lemna Minor*

According to the results of the physicochemical parameters (Table 3), one of the elements with the highest concentrations is sodium. The calculated concentrations in the 1.5, 3.0, and 4.5% dilutions are 390, 780, and 1170 mg/L, respectively. Huber and Sankhla [79] (Germany) revealed the harmful effects of sodium and chloride ions on *Lemna minor*, highlighting the enzymatic alterations that ultimately trigger a negative effect on the rate of photosynthesis with measurable effects recorded over an experimental range from 990 to 4970 mg/L. Additionally, another study concluded that *Lemna minor* is sensitive to magnesium (EC_{50}: 200 mg/L) [80]; however, after estimating its values in the experimental treatments (approximately 3, 7, and 10 mg/L of magnesium in the 1.5, 3.0, and 4.5%, respectively, as shown in Table 3) this seems unlikely to exert a strong effect. Calcium had calculated quantities of 7, 14, and 21 mg/L in the 1.5, 3.0, and 4.5% dilutions, respectively (Table 3). Tkalec et al. [81] (Croatia) demonstrated the inhibition in the development of fronds and dry weight of *Lemna minor* against 2% calcium chloride and calcium bromide concentrations for two weeks.

One of the more toxic metals present in this effluent is chromium, with calculated quantities of 0.16, 0.31, and 0.47 mg/L in the 1.5, 3.0, and 4.5% dilutions, respectively. Both Reale et al. [82] (Italy) and Augustynowicz et al. [83] (Poland) reported adverse effects on the development of *Lemna minor* from Cr concentrations as low as 0.175 mg/L; the authors documented more intense effects at higher concentrations; both concentrations and trends are consistent with results from the present study and suggest a potential acute pressure in *Lemna minor* that may come from chromium, sodium, and perhaps other components of this complex mixture.

4.2.2. *Daphnia Magna*

For this bioindicator, the LC_{50} at 24 h was 2.5% and at 48 h it was 1.1% (Figure 3 and Table S2), indicating acute toxicity at dilutions lower than those evaluated in this investigation. Sodium has been shown to have ecotoxic effects on *Daphnia magna* [84], at concentrations as low as 7.6 mg/L (NaCl). Calculated sodium in the experiments carried out in the present study (390, 780, and 1170 mg/L in the 1.5, 3.0, and 4.5% dilutions, respectively) greatly exceed these values, which supports the idea that sodium is either responsible for or correlates across these experiments for ecotoxic effects on water fleas.

Conversely, maximum calculated quantities for calcium, magnesium, and potassium all fell below published toxicity thresholds. Specifically, they were about one-half that published for calcium and approximately one-tenth that found for Mg and K (LC_{50} of 52 mg/L, 140 mg/L, and 93 mg/L for calcium, magnesium, and potassium, respectively, as reported by Biesinger and Christensen [85]). These values were obtained by dosing with $CaCl_2$, $MgCl_2$, and KCl in *Daphnia magna* for 48 h without feeding, conditions equal to those used in this study, suggesting that the calcium, magnesium, and potassium present in the treatments would not be directly responsible for the documented high mortality rates.

Past reports suggest that concentrations of 0.3 mg/L of chromium can cause 70% mortality in *Daphnia magna* [86]. In the present study, a 65% mortality was determined with approximately 0.16 mg/L of total chromium (corresponding to the 1.5% RSIP water). The percentage of mortality increases when chromium concentrations increase, suggesting that chromium may also play a role in *Daphnia magna*'s acute toxicity.

4.2.3. *Physa Venustula*

Past reports highlight the mortality of invertebrate embryos such as freshwater snails, in response to the presence of heavy metals such as nickel, zinc [87], and chromium [88]. Several authors have similarly reported low-to-null levels in the hatching of snails when exposed to less than 0.0032 mg/L of copper [89] and 0.026 mg/L of chromium [90]. Both of these values were exceeded in the present study's 1.5% and higher percent RSIP waters. Changes in some biochemical parameters and genotoxic effects were also observed in freshwater snails after treatment with tannery effluents [26]. Arenazas [26] previously reported a 70 $\pm$ 3% mortality of *Physa venustula* embryos against a 10% dilution of residual water within the RSIP lagoons when they were in operation. In the present study, only 39.6 $\pm$ 9.7% of eggs hatched with the 1.5% diluted effluent, and 100% mortality (or 0% hatching) was recorded for the 3.0 and 4.5% dilutions (Figure 4 and Table S3), highlighting potential toxicity attenuation that was achieved when these lagoons were in operation. While the quantity of chromium in the study by Arenazas [26] was higher than for this harvested effluent, the concentrations of sodium, calcium, and magnesium were much higher in the present study. Prior reports have revealed the toxic effects of sodium on insects [91], *Daphnia magna* [85], and amphibians [92], suggesting a parallel for snails.

4.2.4. *Xenopus Laevis*

Morphological disorders, delayed development, and death in the embryos of *Xenopus laevis* were documented after exposure to diluted RSIP tannery effluent (Figure 7a–d and Table S4). The studied RSIP effluent presents a mixture of several heavy metals and other elements that, under individual and combined actions, could have affected the normal development of the embryos (see Tables 2 and 3). Exposure to metals such as zinc, copper, lead, and cadmium has previously been shown to reduce tadpole survival before metamorphosis [84], as well as other negative effects on the development of embryos and larvae of *Xenopus laevis* in South Africa [93]; in comparison with the present investigation, the concentrations of these metals are lower in the tested dilutions than for those other studies. Other investigations [94,95] report that metals such as sodium, calcium, potassium, and manganese can generate lethal effects and morphological alterations in the development of amphibian embryos, findings similar to the results obtained in this research.

Tornabene et al. [96] (United States) described the negative effects of NaCl concentrations up to 8000 mg/L, with recorded LC_{50} values between 4100 and 4834 mg/L reached for amphibian species *Pseudacris maculata* and *Rana pipiens*, respectively. Chlorides, also present in the studied wastewater, can have synergistic effects with other contaminants. Studies with *Rana sylvatica* and *Pseudacris maculata* in the United States highlight the potential for interactive effects between Cl^- and other contaminants because survival was lower than in individuals exposed to only chlorides Cl^- [97]. Morphological alterations such as the folded tail (Figure 7e–h) are widely reported in studies of heavy metal toxicity in amphibians [98]. Moreover, alterations in the eyes and yolk sac have also been shown to be caused by Zn in larvae of *Xenopus laevis* [99].

The mutagenic and histopathological effects of hexavalent chromium on *Lithobates catesbeianus* larvae have already been studied by Monteiro et al. [30]. The authors exposed tadpoles to potassium dichromate concentrations (1.4, 4.2, and 12.7 mg/L of chromium), concluding that all treatments resulted in mutagenic effects including anti-mitotic activity and induced histopathological alterations in the liver and kidney. The tested RSIP effluent had a chromium concentration of 10.4 ± 0.4 mg/L (Table 3) before dilution, highlighting its acute potential, however, after dilution, samples were below those tested for Monteiro et al. [30]. As that test did not establish the lowest observable adverse effect level, there is uncertainty in further extrapolation.

4.3. Determination of Acute Toxicity Units (ATU) and Toxicological Classification of Tannery Wastewater

While the present study was not sufficiently mechanistic or deconstructionist to determine which chemical parameters impact toxicity within and across the studied trophic levels, it is important to recognize that the percent dilutions studied have negative effects on the tested bioindicators. This highlights the toxicity of RSIP effluent during and after environmental release and provides evidence for potential acute toxicity effects that could result at these lower dilutions when the RSIP effluent is received by the downstream Chili River. More broadly, the studied wastewater is categorized as "Very toxic" (according to Saldaña et al. [39]) and "Highly toxic" (according to Roig et al. [40]) (Table 4) across the studied organisms. This is consistent with prior investigations that characterized tannery wastewater elsewhere [100–102], indicating that during the leather production process tannery effluents cause serious contamination of surface waters, affecting aquatic ecosystems.

The order of affectation to the evaluated organisms is, firstly, *Physa venustula* embryos, followed by *Daphnia magna* hatchlings and, finally, *Xenopus laevis* larvae and embryos. Oxygen availability has previously been speculated to play a role in these types of effects, as evidenced by effluent concentrations that can deplete the oxygen content of the media [100]. While this was not directly quantified, the calculated oxygen demand for batch incubations after dilution would be in the tens of mg/L, which could exceed oxygen reintroduction during incubation and exert this resource limitation.

In short, this industrial outflow poses an acute toxicological risk for the environment with all bioindicators exhibiting negative acute effects at the lowest tested 1.5% dilution. This could represent a real scenario when mixing these wastewaters with natural water bodies, as they are currently mingling with the Chili River in Arequipa (see Figure 1) with minimal treatment. For example, an untreated and stable tannery wastewater flow of approximately 30 L/s (0.03 m^3/s), combined with Chili River's average flows between 23 m^3/s (low flow) and 170 m^3/s (high flow), results in the total dilution of the polluted waters between 0.13% and 0.02%. Typical low-flow seasons last from April through December, conceptually resulting in less wastewater dilution for the majority of the year. Further research should evaluate the presence and development of native bioindicators upstream, downstream, and in the vicinity of where these toxic effluents are currently being discharged into the Chili River to assess the environmental relevance of these laboratory findings. Testing should also include lower dilutions than those evaluated in this investigation to better constrain low and no effect levels in a controlled setting. Studies should further extend to chronic toxic effects, which were not explored here, as well as a more targeted inquiry into the effects of identified compounds of concern in the effluent such as Cr^{6+} and total dissolved solids. Though the observed toxic flow rate is constant, further studies should sample RSIP's tannery effluent at different times while tanneries are normally operating, to account for possible variations in the chemistry of the aqueous solution. Importantly, these results highlight the relevance of treating RSIP wastewater effluents prior to environmental release to minimize environmental risk and pollution of critical water resources in this arid region.

5. Conclusions

Collectively, the results indicate that the release of untreated tannery wastewater from the RSIP can exert pronounced acute impacts across trophic levels with the need for treatment or dilution to below 1% of total flow. As the assays addressed acute toxicity, the necessary treatment and/or dilution to mitigate chronic effects is likely much lower. Additional temporal and spatially resolved monitoring of the waste stream or receiving water bodies is warranted.

The work also provides guidelines to mitigate acute adverse ecological effects that result from the discharge of this complex mixture to the receiving water bodies as well as to query if these acute effects are currently exerted on impacted flora and fauna. The bioassays with *Lemna minor* demonstrated a decrease in the number of fronds, wet weight, and dry weight as the effluent dilution increased. *Daphnia magna*, *Physa venustula*, and *Xenopus laevis* embryos and larvae had high sensitivity and could be extrapolated to other amphibian species endemic to lotic ecosystems near the contaminated area. Although RSIP wastewater contains many potentially toxic components and mechanisms of toxicity were not biochemically queried, when merged with prior literature and the chemical analyses reported herein (Tables 2 and 3), these findings suggest that chromium and total dissolved solids, with a major contribution from sodium, are best correlated with acute toxicity variables. Hence, management protocols that target these constituents could improve ecotoxicity outcomes. Furthermore, readily available chemical analysis tools such as handheld or remote conductivity could provide a rapid and affordable forensic tool to query acute ecosystem pressures and provide real-time feedback. More broadly, untreated RSIP tannery wastewaters represent an ecological risk to downstream aquatic ecosystems; this needs to be addressed to prevent current and future environmental consequences.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su152115240/s1, Table S1: Sublethal effects evaluated in the bioassay with *Lemna minor* exposed (for 168 h) to different dilutions of tannery effluent. Table S2: Mortality evaluated in the bioassay with *Daphnia magna* exposed (24 and 48 hours) to different dilutions of tannery effluent. Table S3: Effects on the hatching percentage of *Physa venustula* eggs exposed to different dilutions of tannery effluent after a 216 h incubation. Table S4: Effects of different dilutions of tannery effluent (for 96 h) on the mortality of *Xenopus laevis* embryos and larvae.

Author Contributions: Conceptualization, K.T.-M., A.A.-R. and C.F.-F.; methodology, K.T.-M., A.A.-R., P.A.G.-C. and C.F.-F.; validation, K.T.-M., A.A.-R., P.A.G.-C., C.F.-F., L.M.-P., G.R.-M., J.T.-Q., G.V. and J.O.S.; formal analysis, K.T.-M., A.A.-R., P.A.G.-C. and C.F.-F.; investigation, K.T.-M., A.A.-R. and C.F.-F.; resources, L.M.-P. and J.O.S.; data curation, K.T.-M., A.A.-R. and C.F.-F.; writing—original draft preparation, K.T.-M., A.A.-R., P.A.G.-C., C.F.-F., L.M.-P., G.R.-M., J.T.-Q., G.V. and J.O.S.; writing—review and editing, K.T.-M., A.A.-R., P.A.G.-C., C.F.-F., L.M.-P., G.R.-M., J.T.-Q., G.V. and J.O.S.; visualization, A.A.-R. and P.A.G.-C.; supervision, P.A.G.-C. and J.O.S.; project administration,

L.M.-P. and J.O.S.; funding acquisition, L.M.-P. and J.O.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Center for Mining Sustainability, a joint effort between the Universidad Nacional de San Agustín de Arequipa (Peru) and the Colorado School of Mines (United States). Funding for this Center comes from Peru's Mining Cannon.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank Jim Ranville, who provided insightful edits during manuscript preparation.

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

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