processes

MDPI

Article

Assessment of Advanced Oxidation Processes Using Zebrafish in a Non-Forced Exposure System: A Proof of Concept

Tamia Cabascango [1,2,†], Karol Ortiz [1,2,†], Christian Sandoval Pauker [3], Isabel Espinoza Pavón [1], Anuradha Ramoji [4,5], Jürgen Popp [4,5], Jady Pérez [1], C. Miguel Pinto [6], José Luis Rivera-Parra [7], Florinella Muñoz-Bisesti [1], María Belén Aldás [2], Cristiano V. M. Araújo [8] and Paul Vargas Jentzsch [1,*]

[1] Departamento de Ciencias Nucleares, Facultad de Ingeniería Química y Agroindustria, Escuela Politécnica Nacional, Ladrón de Guevara E11-253, 170525 Quito, Ecuador; tamia.cabascango12@gmail.com (T.C.); karol.ortiz.leon@gmail.com (K.O.); isabel.espinoza@epn.edu.ec (I.E.P.); jady.perez@epn.edu.ec (J.P.); florinella.munoz@epn.edu.ec (F.M.-B.)
[2] Departamento de Ingeniería Civil y Ambiental, Facultad de Ingeniería Civil y Ambiental, Escuela Politécnica Nacional, Ladrón de Guevara E11-253, 170525 Quito, Ecuador; maria.aldas@epn.edu.ec
[3] Laboratorio de Fisicoquímica, Departamento de Química, Universidad Técnica Federico Santa María, Avenida España 1680, 2390123 Valparaíso, Chile; christian.sandovalp@sansano.usm.cl
[4] Institut für Physikalische Chemie and Abbe Center of Photonics, Friedrich-Schiller-Universität Jena, Helmholtzweg 4, 07743 Jena, Germany; anuradha.ramoji@uni-jena.de (A.R.); juergen.popp@uni-jena.de (J.P.)
[5] Leibniz Institute of Photonic Technology, Albert-Einstein-Straße 9, 07745 Jena, Germany
[6] Observatorio de Biodiversidad, Ambiente y Salud (OBBAS), 170525 Quito, Ecuador; pinto@obbas.org
[7] Departamento de Petróleos, Facultad de Geología y Petróleos, Escuela Politécnica Nacional, Ladrón de Guevara E11-253, 170525 Quito, Ecuador; jose.riverap@epn.edu.ec
[8] Department of Ecology and Coastal Management, Institute of Marine Sciences of Andalusia (CSIC), Puerto Real, 11519 Cádiz, Spain; cristiano.araujo@icman.csic.es
* Correspondence: paul.vargas@epn.edu.ec; Tel.: +593-2-2976300 (ext. 4231)
† These authors contributed equally to this work.

Citation: Cabascango, T.; Ortiz, K.; Sandoval Pauker, C.; Espinoza Pavón, I.; Ramoji, A.; Popp, J.; Pérez, J.; Pinto, C.M.; Rivera-Parra, J.L.; Muñoz-Bisesti, F.; et al. Assessment of Advanced Oxidation Processes Using Zebrafish in a Non-Forced Exposure System: A Proof of Concept. *Processes* **2021**, *9*, 734. https://doi.org/10.3390/pr9050734

Academic Editor: Chunjiang An

Received: 16 March 2021
Accepted: 19 April 2021
Published: 22 April 2021

Abstract: Water bodies and aquatic ecosystems are threatened by discharges of industrial waters. Ecotoxicological effects of components occurring in untreated and treated wastewaters are often not considered. The use of a linear, multi-compartmented, non-forced, static system constructed with PET bottles is proposed for the quality assessment of treated waters, to deal with such limitations. Two synthetic waters, one simulating wastewater from the textile industry and the other one simulating wastewater from the cassava starch industry, were prepared and treated by homogeneous Fenton process and heterogeneous photocatalysis, respectively. Untreated and treated synthetic waters and their dilutions were placed into compartments of the non-forced exposure system, in which zebrafish (*Danio rerio*), the indicator organism, could select the environment of its preference. Basic physical–chemical and chemical parameters of untreated and treated synthetic waters were measured. The preference and avoidance responses allowed verification of whether or not the quality of the water was improved due to the treatment. The results of these assays can be a complement to conventional parameters of water quality.

Keywords: ecotoxicological assays; heterogeneous photocatalysis; Fenton; advanced oxidation processes; graphitic carbon nitride; *Danio rerio*

1. Introduction

The human population is growing every year and the obvious consequence is the rise in the global demand for food and goods, which leads to increasing pressure on water sources [1]. Overexploitation of water bodies is the consequence of both the increasing demand for freshwater for different purposes and climate change (droughts are increasing in many regions). According to Mekonnen and Hoekstra [2], approximately 4.0 billion people are under conditions of severe water scarcity for at least 1 month per year; it is reasonable to assume that water scarcity could affect more people in subsequent years as the effects of climate change gradually become more significant. To this already intricate scenario, complications related to the systematic pollution of surface water bodies due to human activities must be added. The adoption of strong policies to prevent the pollution of water bodies and efficient treatment of wastewater are crucial to avoid a crisis that could threaten mankind itself.

Treated, deficiently treated and untreated domestic and industrial wastewaters are usually discharged into receiving water bodies, leading to the introduction in these aquatic ecosystems of biodegradable and non-biodegradable organic matter, nutrients, pathogens, inorganic pollutants and toxic substances [3]. It is out of the question that pollutants introduced into a water body have impacts on organisms living in such ecosystems [4]. For example, the discharge of effluents with a high content of biodegradable organic matter into a receiving water body results, indirectly, in the consumption of dissolved oxygen, and fish mortality could take place [5]. The main strategy to protect water bodies usually applied by regional and national authorities in countries worldwide is the establishment of permissible limits for the discharge of (treated) wastewater of both domestic and industrial origin. The discharge of a liquid effluent to a river is allowed if the values of the parameters comply with permissible limits listed in the regulations. It is assumed that the discharged pollutants would be present in the water body at low concentrations, thus being easily eliminated by the self-purification processes in water. There are several processes involved in water self-purification that have been identified over time and they can be affected by human activities [6,7]. However, based only on ensuring compliance with the limits in regulations, is it possible to guarantee that water quality will not change sufficiently to trigger adverse effects in the aquatic ecosystem? Certainly, the answer is no. Since the assimilation capacity of a water body (e.g., a river) is variable, experimental and theoretical (models) approaches were proposed [8], as tools useful for water quality management. Bolinches et al. [9], based on their evaluation of the Manzanares river (Spain) scenario, highlighted the necessity for adjusting the quality of discharges to protected water bodies. Therefore, approaches focusing on the resulting water quality after the discharge must be prioritized.

It should be pointed out that, in some cases, water pollution may not necessarily result in mortality of aquatic species; variations in certain parameters could be noticeable by changes in the diversity and populations of organisms, and this is applied, for example, to assess water quality using benthonic macroinvertebrates [10]. This happens because species capable of resisting certain levels of pollution are selected and other species can die or move to other places with more favorable conditions. It was reported that some fish species can smell (detect) some pollutants [11,12]; therefore, changes in populations in affected zones by point- and nonpoint-source pollution have an explanation [13,14]. The behavior of living organisms in water in response to the presence of pollutants allowed the development of ecotoxicological strategies to evaluate the quality of water and even some trends. One strategy involves the non-forced exposition of organisms such as fish, tadpoles and shrimps to samples of water of different quality, allowing the organisms to move, avoiding contamination [15–17]. In this way, the tests do not result in the death of the organisms (the main difference with classical toxicological tests); instead, the organisms simply move to a specific compartment, allowing researchers to identify the water with the highest quality. This kind of test was used in the past to study the impact of anthropogenic activities over natural water bodies [18,19]. Although the conventional approach of measuring physicochemical parameters allows valuable information about the quality of water to be obtained, they likely do not provide a "big picture" of the impacts and/or implications on the environment of a discharged effluent. Therefore, such results should be combined with alternative ecotoxicological tests to reach broader interpretations [20,21]. The use of non-forced exposition systems, with well-known indicator organisms (e.g., zebrafish) for the assessment of water treatment methods, could be an innovative approach to complement traditional measurements.

Concerning wastewater treatment, advanced oxidation processes (AOPs) are a group of treatments that are effective for the removal of non-biodegradable and recalcitrant organic compounds such as pharmaceuticals, pesticides, solvents and synthetic dyes, among others [22–26]. They are based on the generation of highly reactive radicals such as hydroxyl radicals (•OH) [27]. These radicals can be produced by different strategies; among them, the Fenton processes were widely studied for decades [28–30]. Another attractive type of process is based on the photocatalytic generation of •OH with ultraviolet and visible radiation. In this sense, a great variety of solid semiconductors (suspended or supported) were proposed for water and wastewater treatment by heterogeneous photocatalysis [31–36]. To evaluate the efficiency of AOPs, the decrease in the concentration of a target pollutant, the chemical oxygen demand (COD) and the total organic carbon (TOC) are generally measured. There are limitations for this kind of evaluation; for instance, some toxic pollutants occurring at low concentrations in wastewater could be eliminated without important changes in the COD content, or the removal of the organic charge may be accompanied by the formation of degradation byproducts with toxic characteristics, leading to the underestimation of the toxicity of treated wastewater. Therefore, ecotoxicological strategies could be very useful to complement the evaluation of AOPs since they may overcome the drawbacks of traditional physical–chemical parameters. There are reports on the use of zebrafish to evaluate the quality of industrial wastewaters [37,38] and even with the application of non-forced avoidance assays [39]. However, there are no reports on the application of non-forced exposure systems to assess treatments based on AOPs.

In this work, the use of a linear, multi-compartmented, non-forced, static system constructed for the assessment of the quality of treated water is proposed. Two synthetic waters, which were intended to simulate industrial wastewaters, were treated by AOPs. The preference (and avoidance) of zebrafish (*Danio rerio*) for compartments in non-forced exposure systems was evaluated taking into consideration the physical–chemical and chemical characteristics of the water. The applicability of these systems to complement the results of conventional water analysis is discussed.

2. Materials and Methods

2.1. Reagents and Synthetic Waters

Reactive Red 120 dye, RR-120 ($C_{44}H_{24}Cl_2N_{14}Na_6O_{20}S_6$, commercial purity), was purchased from Quifatex S.A. (Ecuador) and used as received. All other chemicals were of analytical grade. Hydrogen peroxide, H_2O_2 (30% w/w), was purchased from Fisher Scientific. Iron (II) sulfate heptahydrate, $FeSO_4 \cdot 7H_2O$ (>99%), sodium chloride, NaCl (≥99.0%), sodium hydroxide, NaOH (≥97.0%), sulfuric acid, H_2SO_4 (95.0–98.0%), urea, $CO(NH_2)_2$, were provided by Sigma-Aldrich, while calcium oxalate monohydrate, $Ca(COO)_2 \cdot H_2O$ (99%), was bought from VWR.

Two synthetic waters (i.e., aqueous solutions of "pollutants" simulating real wastewaters) were prepared: (1) an aqueous solution of RR-120 of a concentration of 80 mg/L, used to simulate wastewater from the textile industry, and (2) wastewater from the cassava starch industry. The synthetic water from the cassava starch industry was obtained by replicating, on a laboratory scale, processes taking place in this industry. For this, cassava (*Manihot esculenta*) tubers from the province of Manabí (Ecuador) were washed, weighed (100 g) and peeled. Cassava peels and associated residues were wasted. The peeled cassava was grated, mixed with 500 mL of distilled water for 5 min and then the coarse solids were separated by filtration through a piece of cotton fabric (muslin). Coarse solids were wasted and the filtrate was left still for 10 min to ensure the sedimentation of starch particles. The supernatant liquid was the synthetic water from the cassava starch industry.

2.2. Preparation of Supported g-C_3N_4 (g-C_3N_4 onto Calcium Carbonate)

The photocatalyst used to treat the synthetic water from the cassava starch industry was graphitic carbon nitride (g-C_3N_4), which is active with visible radiation. Since nanoparticles of g-C_3N_4 are difficult to remove from water and consequently could remain in the treated water, this photocatalyst was supported on larger particles of calcium carbonate (an insoluble salt). This material, consisting of g-C_3N_4 supported on calcium carbonate (called "supported g-C_3N_4" for purposes of this work), was prepared using the preparation method of g-C_3N_4 reported by Picho-Chillán et al. [32], based on the pyrolysis of urea. It should be noted that, in this study, the precursor was a solid mixture of urea and calcium oxalate monohydrate in a ratio of 2:1 (w/w) instead of only urea. Since the solid mixture was heated up to 600 °C, calcium oxalate was transformed to calcium carbonate [40], thus acting as a supporting material for g-C_3N_4. The supported g-C_3N_4 was disaggregated with a mortar and pestle and then washed with distilled water and sonicated to remove g-C_3N_4 nanoparticles that were not properly attached to the supporting material. For comparative purposes, urea and calcium oxalate monohydrate were pyrolyzed under the same conditions in separate experiments. The identity of species in the so-called "supported g-C_3N_4" was confirmed by infrared spectroscopic measurements. The infrared spectra of pyrolyzed urea (i.e., pure g-C_3N_4), pyrolyzed calcium oxalate monohydrate (i.e., calcium carbonate) and the supported g-C_3N_4 were recorded using a Varian 670 FT-IR spectrometer (Agilent Technologies, Santa Clara, CA, USA) with a liquid nitrogen-cooled mercury-cadmium-telluride (MCT)-based focal plane array (FPA) detector (field of view (FOV) of 211 μm × 211 μm and a lateral resolution of 3.3 μm × 3.3 μm). The spectral range was 3900 to 900 cm^{-1}, and the resolution was 2 cm^{-1}, with 16 scans.

2.3. Physical–Chemical and Chemical Characterization of Water

The temperature, pH and electrical conductivity of the water samples were measured with an Orion Star™ A320 multiparameter meter (Thermo Scientific™). Turbidity was measured with a Hach 2100P portable turbidimeter. Chemical oxygen demand (COD) and total organic carbon (TOC) were measured according to the standard methods 5220-D and 5310-B, respectively [41]. Temperature, pH, electrical conductivity, turbidity, COD and TOC were measured for both synthetic waters (untreated and treated). For the synthetic water from the cassava starch industry (untreated and treated), total cyanide in water was measured according to the standard methods 4500-CN C (distillation) and 4500-CN E (colorimetric method) [41].

The concentration of RR-120 was measured by UV–Vis spectrophotometry. For quantification purposes, a calibration curve was prepared using aqueous solutions of RR-120 of known concentrations (standard solutions). Both standard solutions and samples of the synthetic water containing the dye were filtered through syringe microfilters (pore size 0.22 μm, Millipore Millex-GV) before the spectrophotometric measurement. For the treated synthetic water, an additional filtration step with quantitative filter paper was included before the filtration through the syringe microfilter. The absorbance of the aqueous solutions was measured at a wavelength of 507 nm with a UV–Vis spectrophotometer (Hitachi U-1900).

To assess the quality of the dechlorinated tap water and verify that it was adequate for the requirements of zebrafish, basic physical–chemical and chemical characterization of this water was performed by the Centro de Investigación y Control Ambiental (CICAM) of the Escuela Politécnica Nacional, Quito, Ecuador.

2.4. Advanced Oxidation Processes

The synthetic water from the textile industry (i.e., water containing RR-120) was treated by a homogeneous Fenton process. As a reference, the pH of the solution, as well as the amounts of the reagents needed for the degradation of RR-120, were obtained from the work presented by Garófalo-Villalta et al. [42]. The Fenton reaction was carried out in a flat-bottom flask (containing 250 mL of the synthetic water) immersed in a water bath to guarantee a reaction temperature between 20 and 25 °C during the experimentation. An appropriate mixture of the reacting system was ensured by the use of a PTFE-coated magnetic stir bar. The pH value of the synthetic water was adjusted to 2.8 by the addition of H_2SO_4 1 M; then, 0.2 g of $FeSO_4 \cdot 7H_2O$ was added and the reaction started with the addition of H_2O_2 (30% *w*/*w*) in a sufficient amount to ensure a concentration of 17.7 mM. The total time of reaction was 60 min. After this time, the reaction was stopped by the addition of enough NaOH 1 M to reach a pH value of 12.0. Under this alkaline condition, the remaining H_2O_2 degraded and iron precipitated as a hydroxide [43]. The alkaline mixture stayed still for 10 min, and the liquid supernatant was separated and then treated with H_2SO_4 1 M to reach a pH value of 7.0. This resulting solution was considered the treated synthetic water from the textile industry and the remaining RR-120 was quantified by UV–Vis spectrophotometry.

The synthetic water from the cassava starch industry was treated using the supported g-C_3N_4 as the photocatalyst. A borosilicate crystallizing dish (95 × 55 mm) was used as a reactor for the photocatalytic treatment of the synthetic water. This reactor was placed into a cooling system based on the circulation of tap water. The content of the reactor was continuously mixed with a PTFE-coated magnetic stir bar. All these components were placed into a protecting wood box equipped with a Xe lamp (35 W). A similar experimental setup was reported by Picho-Chillán et al. [32]. For the treatment, 50 mL of the synthetic water from the cassava industry were mixed with 100 mg of supported g-C_3N_4 and placed into the reactor. Then, 125 µL of H_2O_2 30% (*w*/*w*) were added; the treatment started when the lamp was switched on. The total time of reaction was 120 min. After this time, the mixture was centrifuged with a M-SCEN-206 Lab centrifuge (LABEC) at 5500 rpm for 20 min. The collected liquid supernatant was then the treated synthetic water from the cassava starch industry.

2.5. Assay Organisms

Zebrafish (*Danio rerio*) were purchased from a local commercial supplier and acclimated for at least one week before the assays. The acclimation was performed by maintaining the fish in a 113.3-L aquarium filled with dechlorinated tap water and under continuous aeration. The dechlorination of tap water was achieved by bubbling air into water for 24 h. Approximately half of the aquarium water was changed every second day to eliminate residues derived from fish feeding. Fish were fed with artificial fish food once every 48 h, and the feeding was suspended 24 h before the tests. Zebrafish used in the assays was 3–4 months old and 1.5 ± 0.3 cm long.

2.6. Free-Choice Non-Forced Exposure System

A linear, multi-compartmented, non-forced, static system, similar to the one reported by Araújo et al. [19], was constructed using PET bottles. Each compartment was 38 cm long and contained ~1000 mL of water. PET bottles were glued using white silicon (Sikaflex-11FC+) and the resulting compartments were connected at the mouths of the bottles. The system had seven compartments; therefore, the total length was 266 cm, as shown in Figure S1 (Supplementary Materials). For practical purposes, the connections between compartments were blocked with a piece of polystyrene to facilitate filling with water and avoid premature contact among waters of different qualities. The pieces of polystyrene were simultaneously removed to start the experiments.

Before the assays with fish, the capability of the system to maintain heterogeneity among the compartments was tested with aqueous solutions of NaCl. The compartments were filled with aqueous solutions of NaCl with concentrations in the following sequence: 100, 84, 66, 50, 34 and 17 mg/L. These solutions were prepared with dechlorinated tap water. The seventh compartment, after the compartment with the most diluted solution of NaCl, was filled with dechlorinated tap water. The initial electrical conductivity was measured in each compartment. Then, the pieces of polystyrene dividing the solutions in the compartments were removed, and the electrical conductivity of solutions in all compartments was measured every hour up to 6 h. In this manner, the degree of mixture of solutions in the compartments could be evaluated.

The assays with fish were carried out for the synthetic waters from the textile industry and the cassava starch industry. The experimental approaches to assess the quality of untreated and treated synthetic waters from the textile and cassava starch industries with the non-forced exposition system are summarized in Figure 1. Moreover, schemes showing the location of synthetic waters and their dilutions in the linear, multi-compartmented, non-forced, static system are depicted in Figure S2 (Supplementary Materials).

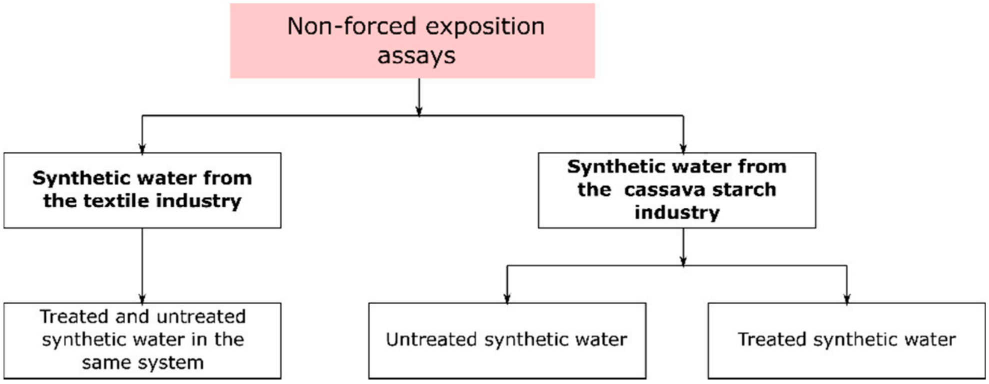

Figure 1. Summary of the non-forced exposition assays carried out to assess the quality of untreated/treated synthetic waters from the textile and cassava starch industries.

For the synthetic water from the textile industry, the first compartment was filled with untreated water (80 mg/L of RR-120). The next compartment was filled with the treated water and the four successive compartments were filled with dilutions of the treated water with dechlorinated tap water in the following ratios: (*v*/*v*) 1:2, 1:4; 1:8 and 1:16. The last compartment was filled with dechlorinated tap water.

It is well-known that wastewater from the cassava starch industry has variable COD contents that can be as high as 20,000 mg/L [44]. This value was reported for the cassava industry in Thailand. However, in South America, reported COD contents are lower; for example, the COD content of wastewater from the cassava industry in Brazil was 6014 mg/L [45]. In any case, such contents of organic matter are too high to be directly tested with fish. Therefore, for the synthetic water from the cassava starch industry, untreated and treated synthetic water were diluted with enough dechlorinated tap water to reach a COD content of 200 mg/L. These dilutions were prepared based on the measured COD of the untreated and treated synthetic water. Two different assays were carried out; in one assay, the first compartment contained the untreated synthetic water with a COD content of 200 mg/L, and, in the other assay, the first compartment contained the treated synthetic water, also with a COD content of 200 mg/L. For both assays, the subsequent compartments contained dilutions of the first solution (COD contents: 100, 50, 25, 10 and 5 mg/L) and the last compartment was filled with dechlorinated tap water.

Three organisms (zebrafish) were added into each compartment and then the pieces of polystyrene were removed to open the connection between compartments. Starting at this point, the number of organisms in each compartment was determined after 4 and 6 h. The assays were performed in a closed dark room, and a red light source was used for counting the organisms. All assays were performed in triplicate together with an assay with dechlorinated tap water in all compartments (blank).

2.7. Statistical Analysis

The distribution of organisms in the assays was evaluated with Fisher's exact test (one-sided *p*-value) using the GraphPad InStat 3 software. Comparisons (2×2) were performed between the distribution of expected (=9) and observed organisms in the control compartment with the distribution of the expected (=9) and observed fish in each treatment individually after 4 h and 6 h of exposure. The expected distribution was established as 9 fish in all cases, considering the initial number of organisms and no movement (absence of preference or avoidance) during the experiments. Comparisons were also performed, taking as a reference the treatment with the highest number of fish when it was superior to the control. Therefore, a possible relation between the number of expected organisms and the number of observed organisms in each compartment was statistically verified [15]. If $p < 0.05$, there was a statistically significant variation between the number of expected organisms and the number of observed organisms in each compartment. On the other hand, if $p > 0.05$, there was not a statistically significant variation between those numbers.

The number of expected organisms in each compartment c ($Expected_c$) was calculated with Equation (1).

$$Expected_c = \frac{\sum_{i=1}^{z} Observed_i}{z} \quad (1)$$

where i represents the number of each compartment, z is the total number of compartments, and $Observed_i$ is the number of organisms observed in each compartment at the end of the assay.

Fish preference (%) for water in each compartment c was calculated with Equation (2).

$$Preference\ (\%) = \frac{Observed_c - Expected_c}{Expected_c} * 100 \quad (2)$$

where $Observed_c$ is the number of organisms observed in each compartment c in a specific assay. If *Preference* (%) for a given compartment is positive (+), the organisms show a preference for this compartment. However, when this value is negative (−), the organisms tend to avoid this compartment [19]. These values allowed the assessment of the quality of water in the compartments.

For calculating preference/avoidance, the numbers of fish in the three replicates were pooled to minimize the statistical importance of each organism, as proposed by Araújo et al. [46]; therefore, multiple comparisons among means (statistically significant differences among compartments) could not be performed. Considering the formula to calculate avoidance, the escape of one fish from the most contaminated compartment would represent 33% (1 out of 3), which could lead to very high variability in the results if data were treated as replicates. Otherwise, the use of more fish in the experiments was prevented for two reasons: (i) meeting the 3R concepts for animal experimentation, reducing the number of fish, and (ii) avoiding the overpopulation effect in case all the fish moved to the control compartment (in this case, the maximum density of fish would be 21 fish/L: 3 fish per 7 compartments). It is important to consider that the stocking density in this case (21 fish/L) would already be above the OECD recommendation for ecotoxicological tests with juvenile zebrafish (between 5 and 10 fish/L; OECD guidelines 203 and 215 [47,48]), although for a very short period of time.

3. Results

3.1. Characterization of the Dechlorinated Tap Water

Before any experiments with zebrafish, the quality of dechlorinated tap water was verified. This was indispensable to ensure that this water had adequate physical–chemical and chemical characteristics; fish were maintained in this water to avoid any stressful conditions before the assays in the non-forced exposure system. Relevant parameters of water quality for the dechlorinated tap water used to maintain zebrafish and dilute the synthetic waters are presented in Table 1. Moreover, this table presents theoretical conditions of water that are appropriate for zebrafish life.

Table 1. Comparison of the appropriate theoretical conditions of water for zebrafish life and the physical–chemical and chemical properties of dechlorinated tap water (water used to maintain fish and dilute synthetic waters used in assays).

Parameter	Units	Theoretical Conditions		Dechlorinated Tap Water ***
		Lawrence *	Avbesh et al. **	
pH	-	7.0–8.0	6.8–7.5	7.23
Temperature	°C	24–30	26.0–28.5	21.5
Electrical Conductivity	µS/cm	-	300–1500	180.9
Alkalinity	mg $CaCO_3$/L	-	50–150	39.0
Hardness	mg $CaCO_3$/L	75–200	50–100	47.0
Dissolved oxygen	mg/L	7.8	>6.0	6.6
Turbidity	NTU	-	-	0.31
Biochemical oxygen demand	mg O_2/L	-	-	>2
Chemical oxygen demand	mg O_2/L	-	-	43

* Reference [49]. ** Reference [50]. *** Except for electrical conductivity, these parameters were measured by the CICAM. Additional results of the water analysis are presented in Table S1 (Supplementary Materials).

As expected, the dechlorinated tap water had a very low content of substances contributing to the biochemical oxygen demand (BOD_5) and chemical oxygen demand (COD). The analysis also showed a negligible residual free chlorine amount (see Table S1, Supplementary Materials).

3.2. Advanced Oxidation Processes

The synthetic water from the textile industry was treated through the homogeneous Fenton process. The degradation of RR-120 led to decolorization of the solution, as can be observed in Figure S3 (Supplementary Materials). However, the degradation of the dye did not ensure an improvement in water quality since degradation byproducts may possess toxic characteristics. Both untreated and treated synthetic waters from the textile industry were characterized and the results are shown in Table 2.

Table 2. Physical–chemical and chemical characteristics of the untreated and treated synthetic water from the textile industry.

Parameter	Units	Untreated Synthetic Water	Treated Synthetic Water
pH	-	5.91	7.44
Temperature	°C	20.0	16.6
Electrical conductivity	µS/cm	64.5	1224.0
Turbidity	NTU	0.61	8.32
Chemical oxygen demand	mg O_2/L	53.0	16.5
Total organic carbon	mg C/L	25.6	22.5
Concentration of RR-120	mg/L	80.00	2.38

By examining the results of the treated synthetic water, the drop in the concentration of RR-120 is evident. The electrical conductivity rises due to the treatment and this has an explanation in the formation of weak acids (typical degradation byproducts resulting from oxidative processes) and inorganic ions such as nitrate and sulfate resulting from mineralization of the dye. The decrement in values of COD and total organic carbon (TOC) due to the treatment indicates that part of the RR-120 was mineralized. The achieved mineralization percentage based on TOC is 12.1%.

The heterogeneous photocatalytic process used for the treatment of the synthetic water from the cassava starch industry required the photocatalyst g-C_3N_4. To facilitate the separation of g-C_3N_4 after the treatment, a supported g-C_3N_4 was prepared. The grayish appearance of the material resulting from the pyrolysis of the mixture of urea and calcium oxalate monohydrate suggested the formation of graphitic carbon nitride. This material was disaggregated, cleaned with distilled water and dried. Then, the infrared spectrum was recorded and the occurrence of $CaCO_3$ and g-C_3N_4 was confirmed. The infrared spectrum of the supported g-C_3N_4 is shown in Figure S4 (Supplementary Materials). In this figure, for comparative purposes, the spectra of pyrolyzed urea and calcium oxalate monohydrate are also shown. The infrared spectrum of the supported g-C_3N_4 shows three bands at 713, 873 and 1405 cm^{-1}, which can be attributed to typical vibrations of $CaCO_3$ [51]. Weak signals can be observed at 1255, 1568 and 1635 cm^{-1}, which could be assigned to vibrational modes of g-C_3N_4 [52].

The synthetic water from the cassava starch industry was treated through a heterogeneous photocatalytic process with the supported g-C_3N_4. Both untreated and treated synthetic waters were characterized and the results are shown in Table 3.

Table 3. Physical–chemical and chemical characteristics of the untreated and treated synthetic water from cassava starch industry.

Parameter	Units	Untreated Synthetic Water	Treated Synthetic Water
pH	-	6.81	8.24
Temperature	°C	18.6	19.4
Electrical conductivity	µS/cm	1268.5	1188.5
Turbidity	NTU	9.48	4.94
Chemical oxygen demand	mg O_2/L	5980.0	5510.0
Total organic carbon	mg C/L	2972.8	2731.0
Total cyanide	mg/L	1.36	0.05

The first aspects that can be observed from the data are the low COD and TOC removal values, 7.85 and 8.13%, respectively. The contents of organic matter in the treated synthetic water are still too high for a discharge into a receiving water body. The turbidity of water was improved and the electrical conductivity changed slightly. Perhaps the most important improvement in water quality attributable to the treatment is the decrement in the content of total cyanide. The cassava plant produces two cyanogenic glucosides, linamarin and lotaustralin [53]. Processing of the roots results in the release of these substances into wastewater, and natural degradation processes taking place in water can produce hydrogen cyanide (HCN). Cyanogenic glucosides coming from cassava can act as precursors of HCN [54] and the high toxicity of this compound could affect aquatic organisms. Our measurements confirmed the occurrence of cyanide-related substances that were measured as total cyanide. The content of total cyanide in the untreated synthetic water was 1.36 mg/L and this value dropped to 0.05 mg/L in the treated synthetic water. This represents the removal of more than 96% of the content of total cyanide. It is likely that this is the most important benefit of the proposed treatment and could facilitate further complementary treatments.

3.3. Water Selection by the Zebrafish

The first step was a test to verify the maintenance of heterogeneity between compartments. For this, aqueous solutions containing different concentrations of NaCl were placed into the compartments. The initial electrical conductivity of solutions in all compartments was measured; then, the polystyrene pieces between the compartments were removed and the electrical conductivity of all solutions was measured again after 4 h and 6 h. The results, shown in Table S2 (Supplementary Materials), indicate that the mixture of solutions of adjacent compartments is minimal and concentration gradients can be maintained. The coefficient of variation (CV) for the electrical conductivity reached a maximum of 2.74%.

The assays performed only with dechlorinated tap water allowed us to confirm that the organisms had no special preference for any compartment. The results of these assays are presented in Tables S3 and S4 (Supplementary Materials).

The preference and avoidance responses of zebrafish exposed to untreated, treated and dilutions of treated synthetic waters from the textile industry are plotted in Figure 2.

After 4 h of exposition, zebrafish already showed a preference for dechlorinated tap water and diluted solutions of the treated synthetic water, as shown in Figure 2a. This trend was confirmed after 6 h of exposition (Figure 2b); organisms not only avoided the untreated synthetic water but also seemed to tolerate very well the most diluted solution of the treated synthetic water. It is possible that, for this very diluted solution, the concentration of RR-120 was too low to repel the organisms and/or the degradation byproducts of RR-120 did not possess toxic characteristics affecting zebrafish.

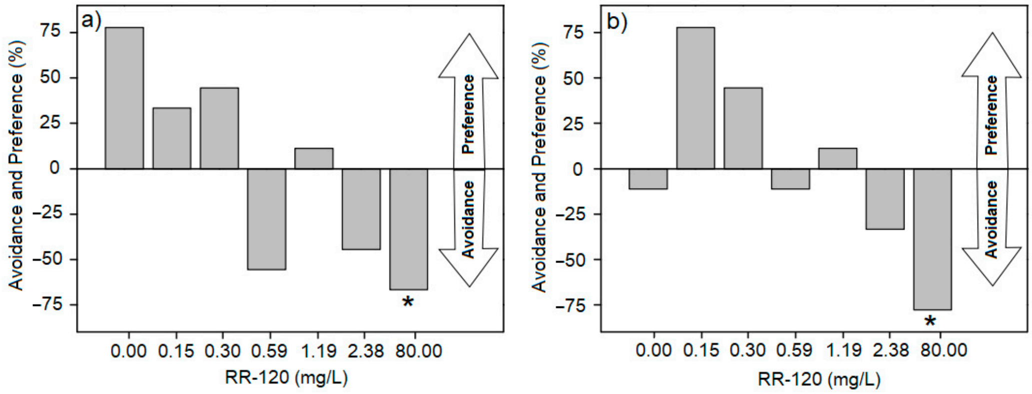

Figure 2. Preference and avoidance responses (%) of zebrafish exposed to untreated, treated and dilutions of treated synthetic waters from the textile industry: (**a**) after 4 h of exposition, and (**b**) after 6 h of exposition. Data were pooled from 3 replicates; therefore, the standard deviation is not presented. Asterisks represent statistically significant differences ($p < 0.05$) in the distribution of organisms when compared with the distribution in control (Fisher's exact test (2×2); see Tables S5 and S6 in Supplementary Material).

The preference and avoidance responses of zebrafish exposed to dilutions of untreated synthetic water from the cassava starch industry are plotted in Figure 3. After 4 h of exposition (Figure 3a), the organisms were already repelled by solutions with the highest COD content and preferred the dechlorinated tap water. After 6 h, the trend was confirmed and, additionally, the preference for the most diluted untreated synthetic water (COD content of 5 mg/L) increased, as shown in Figure 3b.

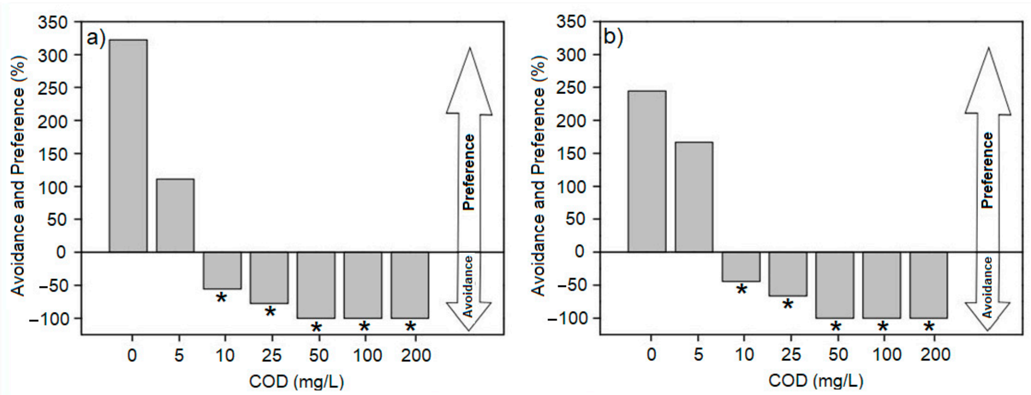

Figure 3. Preference and avoidance responses (%) of zebrafish exposed to dilutions of untreated synthetic water from the cassava starch industry: (**a**) after 4 h of exposition, and (**b**) after 6 h of exposition. Data were pooled from 3 replicates; therefore, the standard deviation is not presented. Asterisks represent statistically significant differences ($p < 0.05$) in the distribution of organisms when compared with the distribution in control (Fisher's exact test (2×2); see Tables S7 and S8 in Supplementary Material).

The preference and avoidance responses of zebrafish exposed to dilutions of treated synthetic water from the cassava starch industry are plotted in Figure 4. It must be pointed out that the COD contents of the dilutions of treated synthetic water were the same as the dilutions of untreated synthetic water; therefore, the content of organic matter was not a variable affecting the behavior of zebrafish in these two assays. The organisms in the assay with dilutions of treated synthetic water were attracted to solutions with the highest COD content.

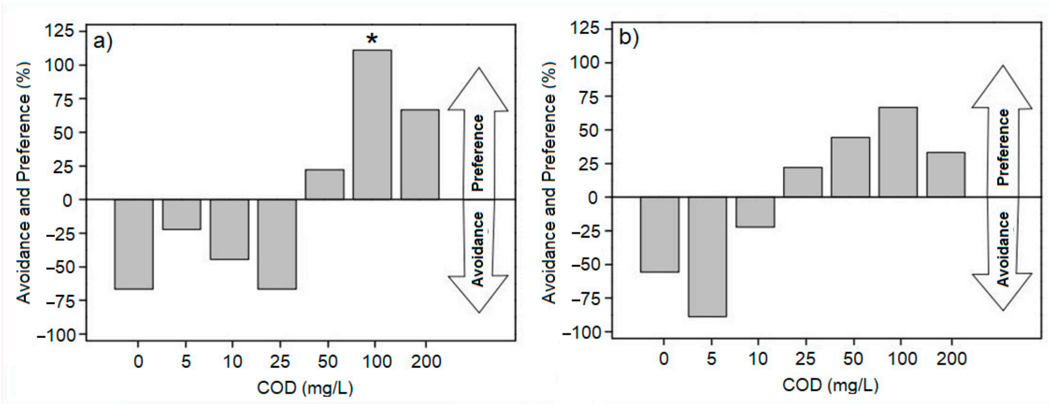

Figure 4. Preference and avoidance responses (%) of zebrafish exposed to treated synthetic water from the cassava starch industry: (**a**) after 4 h of exposition, and (**b**) after 6 h of exposition. Data were pooled from 3 replicates; therefore, the standard deviation is not presented. Asterisks represent statistically significant differences ($p < 0.05$) in the distribution of organisms when compared with the distribution in control (Fisher's exact test (2×2); see Tables S9 and S10 in Supplementary Material).

Changes in the distribution of organisms per compartment could be related to the content of total cyanide in water. The relation of the distribution of organisms per compartment and the content of total cyanide for the assays with untreated and treated synthetic waters from the cassava starch industry is depicted in the plots of Figure 5. Certainly, the removal of cyanide seems to have a strong influence on the preference of zebrafish.

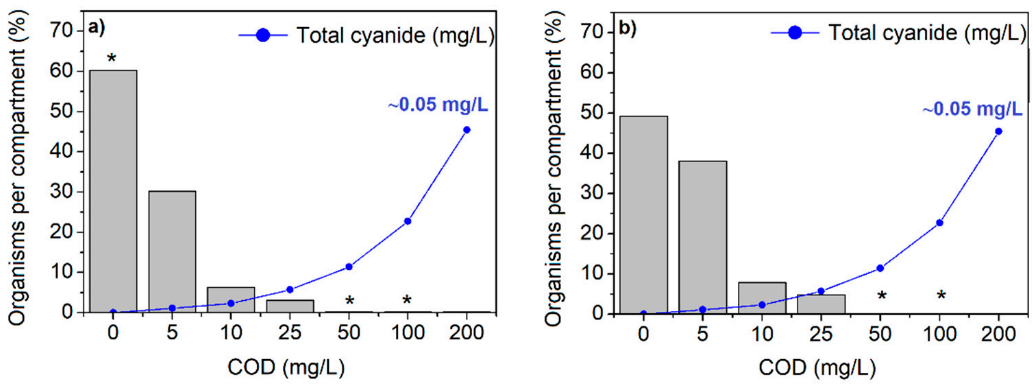

Figure 5. *Cont.*

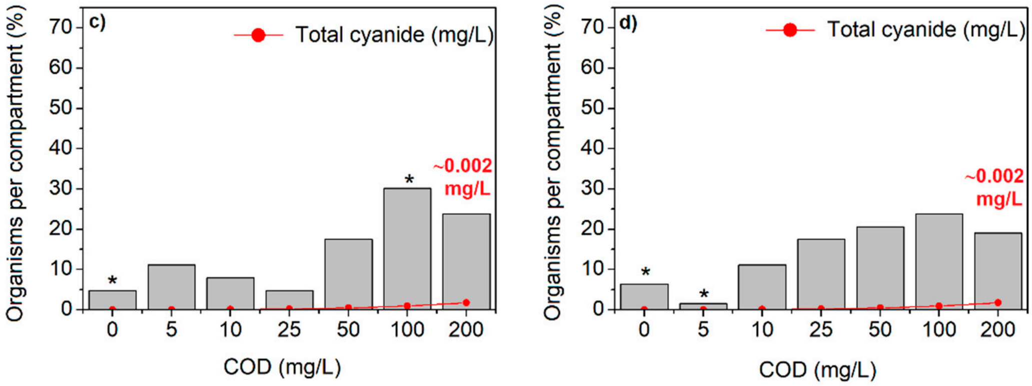

Figure 5. Relation of the distribution of organisms per compartment and the content of total cyanide for (**a**) dilutions of the untreated synthetic water after 4 h of exposition; (**b**) dilutions of the untreated synthetic water after 6 h of exposition; (**c**) dilutions of the treated synthetic water after 4 h of exposition; and (**d**) dilutions of the treated synthetic water after 6 h of exposition. The asterisk (*) indicates values that show significant and very significant statistical variations with respect to the expected number of organisms. Data of organisms per compartment were pooled from 3 replicates; therefore, the standard deviation is not presented.

4. Discussion

The synthetic water from the textile industry contained a dye which is a pollutant usually found in low concentrations in real industrial wastewaters. Even in low concentrations, dyes can affect aquatic flora and fauna [55], thus interfering in ecosystems. On many occasions, the treatments applied to waters containing dyes are effective only in decolorization, and aspects concerning the fate of degradation byproducts are often underestimated. In this regard, the application of non-forced exposure systems with zebrafish can provide a more accurate assessment of the quality of treated water and the potential to repel organisms. This was confirmed with the results of assays with untreated and treated synthetic water from the textile industry. Zebrafish showed a preference for treated water, especially in a higher dilution (see Figures 2–4). It makes sense that organisms avoid the untreated water because, according to an earlier report, zebrafish embryos exposed to an azo dye (congo red dye) presented inner damages and also larvae hatching was stopped [56]. For the specific case of RR-120, it seems that the degradation byproducts resulting from the treatment with the Fenton process did not possess toxic characteristics, at least not with the dilutions considered in this study. Moreover, since most of the dye is degraded due to the treatment, the photosynthesis of phytoplankton and aquatic plants would not be affected, as happens in the presence of the dye, which alters the transmission of light through the water [57]. Both the physical–chemical characteristics of water and the behavior of the organisms indicate that the treatment improved the quality of water, at least compared to the untreated water.

The synthetic water from the cassava starch industry contained a high organic charge that is biodegradable [44,45]. Unlike wastewaters from the textile industry, the biodegradability of the organic charge contained in wastewater from the cassava starch industry is not the problem. The problem lies in the contamination potential associated with very high values of COD; therefore, the uncontrolled discharge of this wastewater could easily lead to the consumption of dissolved oxygen in a water body, thus triggering negative effects on aquatic life. For this reason, authorities focus on the establishment of permissible limits for the discharge into water bodies. The assays with synthetic water from the cassava starch industry reported in this paper show an important limitation concerning this criterion broadly applied by regulators worldwide. Certain pollutants occurring in wastewaters in a very low concentration may have strong impacts on the behavior of organisms, as observed for precursors of hydrogen cyanide present in cassava tubers. The heterogeneous photocatalytic process applied to treat this synthetic water showed poor performance in the removal of the organic charge; however, it was highly efficient in the removal of precursors of hydrogen cyanide and, potentially, other unknown pollutants that were not observed in this study. When these "trace" pollutants were removed, the behavior of organisms was different; fish were attracted to solutions with the highest COD content. The reason for such behavior could be the absence of at least precursors of hydrogen cyanide, which were present in the untreated synthetic water. Of course, the occurrence of other trace components contributing to the toxicity of the untreated water cannot be ruled out; however, the behavior of organisms would suggest their effective removal due to the treatment. Furthermore, since most of the organic charge in this kind of synthetic water can be attributed to (soluble) starch, the observed movement of the organisms to the concentrated waters may be related to the search for food. In fact, the presence of high organic load can unmask the potential risk of the discharges as the sensory stimuli of chemicals could be confounded with food [58–61].

The results regarding preference/avoidance confirm that these responses are appropriate to be applied as a proof of concept in environmental risk studies. In this regard, three important advantages could be highlighted: (i) innovation of a realistic exposure approach, in which organisms are exposed to different dilutions of the rivers and the discharges into them; (ii) application of a simple but ecologically relevant response to identifying the level of repellence of the contaminants, and (iii) the assessment of the ability of the river to self-depurate the chemical discharge and how the efficiency in this process could affect the distribution of the biodiversity. As initially described by different authors [15,18,62], the avoidance test in a non-forced exposure system allows the simulation of different scenarios, in which contaminants are discharged into the aquatic ecosystems and, then, the effects on the spatial distribution of organisms can be measured. The non-forced exposure approach allows the spatial integration of habitats that are commonly treated as independent and isolated areas in the classical exposure approaches [17,19]. The importance of considering that habitats are connected relies on understanding how the effect observed in an ecosystem could affect other undisturbed adjacent ones [63,64] under a meta-ecosystem approach.

Secondly, the use of an approach based on repellence, instead of toxicity, provides a novel view of the risks that contaminants can present for organisms and ecosystems [21,65,66]. This approach makes clear that the loss of biodiversity caused by the discharges of the effluents is not exclusively attributed to the toxicity and consequent death of organisms, but also the repellence of the chemical present triggering an avoidance response in many populations [21,65,67]. The results of the current study showed that the discharge of effluents into a river has the potential to trigger an avoidance response, which could bring about serious consequences for the ecosystems. However, our results showed also the importance of the efficient treatment of effluents to minimize this potential repellence once effluents are discharged.

It is important to remark that both AOPs proposed to treat the synthetic waters had limited efficacy in the removal of pollutants. This was intended and allowed us to verify an improvement in the ecotoxicological quality of treated waters (compared to untreated waters), although the measured physical–chemical parameters indicated poor mineralization and low removal of COD for the synthetic waters. Especially interesting is the fact that, for waters from the cassava starch industry with the same COD content, the preference and avoidance responses of zebrafish were remarkably different when the untreated and treated water were used in the assays. In a study using dairy wastewater to assess the ability of self-depuration of a river and the consequences for the spatial distribution of fish, Silva et al. [39] showed that a total COD of 30 mg/L was attractive to fish despite the potential toxicity that it could exert. Such a result demonstrates that conventional parameters may not be sufficient to assess water quality, and ecotoxicological strategies could be an important complement.

Finally, the proof of concept here applied helps to elucidate the resistance or vulnerability of the aquatic ecosystem after receiving treated or untreated effluents. As shown by Silva et al. [39] and by our studies, not only the capacity of the river to self-depurate the effluent but also an efficient treatment to reduce the chemical load is crucial for the dynamics of migration that the discharge can cause. We encourage the use of this approach from the perspective of assessing wastewater treatment and the ability to self-depurate the rivers.

5. Conclusions

Two synthetic waters intended to simulate industrial wastewaters were treated by AOPs. Two experimental approaches using a linear, multi-compartmented, non-forced, static system to assess the quality of the untreated and treated synthetic waters were applied.

The synthetic water from the textile industry contained a dye, and this water was treated by a Fenton process. Zebrafish showed a preference for treated water and this is evidence of the improvement of water quality due to the treatment. In this experimental approach, a comparison between the untreated and treated synthetic waters was performed by placing both waters in the same system.

The synthetic water from the cassava starch industry contained a high organic charge, and this water was treated by a heterogeneous photocatalytic process with visible radiation. The achieved COD and TOC removal values were below 10%; however, the content of total cyanide decreased by more than 96%. Zebrafish showed a marked preference for the treated water while they avoided the untreated water. Such behavior was attributed mostly to the removal of precursors of hydrogen cyanide due to the treatment.

The importance of applying ecotoxicological assays for the assessment of water quality is evident. Limitations of conventional parameters usually used for monitoring purposes can be overcome if they are combined with assays such as those proposed in this work. These assays can be applied to evaluate the efficiency of complex wastewater treatments. Future works should focus on the assessment of treatments based on other advanced oxidation processes. There are several promising variations of these oxidative treatments and their applicability is dependent on the impacts (positive and negative) that they can have on the environment.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/pr9050734/s1, Figure S1: Linear, multi-compartmented, non-forced, static system constructed with PET bottles, Figure S2: Schemes showing the location of synthetic waters (and/or their dilutions) in the linear, multi-compartmented, non-forced, static system, Table S1: Results of the water analysis of dechlorinated tap water performed by CICAM, Figure S3: Untreated and treated synthetic water from the textile industry, Figure S4: Infrared spectra of the supported g-C_3N_4, and pyrolyzed urea and calcium oxalate monohydrate, Table S2: Electrical conductivity of aqueous solutions of NaCl in the compartments for the test of heterogeneity, Table S3: Distribution of organisms in the compartments filled with dechlorinated water after 4 h, Table S4: Distribution of organisms in the compartments filled with dechlorinated water after 6 h, Table S5: Number of organisms observed after 4 h in each compartment for the assays with the untreated and treated synthetic waters from the textile industry, Table S6: Number of organisms observed after 6 h in each compartment for the assays with the untreated and treated synthetic waters from the textile industry, Table S7: Number of organisms observed after 4 h in each compartment for the assays with the untreated synthetic water from the cassava starch industry, Table S8: Number of organisms observed after 6 h in each compartment for the assays with the untreated synthetic water from the cassava starch industry, Table S9: Number of organisms observed after 4 h in each compartment for the assays with the treated synthetic water from the cassava starch industry, Table S10: Number of organisms observed after 6 h in each compartment for the assays with the treated synthetic water from the cassava starch industry.

Author Contributions: Conceptualization, C.M.P., C.V.M.A., M.B.A., F.M.-B. and P.V.J.; methodology, C.S.P., I.E.P., C.V.M.A. and P.V.J.; investigation, T.C. and K.O.; resources, C.M.P., A.R. and J.P. (Jürgen Popp); data curation, J.L.R.-P.; writing—original draft preparation, T.C., K.O. and P.V.J.; writing—review and editing, I.E.P., J.P. (Jady Pérez), J.L.R.-P., C.M.P., M.B.A., A.R., J.P. (Jürgen Popp), C.V.M.A., and F.M.-B.; supervision, C.S.P., C.V.M.A., M.B.A. and P.V.J.; project administration, J.P. (Jady Pérez) and F.M.-B.; funding acquisition, F.M.-B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Escuela Politécnica Nacional (Ecuador), Project PIS-17-13. The APC was funded by Vicerrectorado de Investigación y Proyección Social of Escuela Politécnica Nacional (Ecuador).

Institutional Review Board Statement: Ethical review and approval were waived for this study, due to local regulations. No protocol of an institutional animal care and use committee (IACUC) was required for this research because Escuela Politécnica Nacional does not have an IACUC. The methods used in this study for the manipulation and euthanizing of zebrafish are in agreement with the Guidelines for the Use of Fishes in Research of the American Fisheries Society (2014), thus ensuring the ethical treatment of zebrafish.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated or analyzed during this study are included in this published article (and its Supplementary Materials). Specific details concerning this study are available from the corresponding author on reasonable request.

Acknowledgments: The authors acknowledge the Department of Nuclear Sciences (Escuela Politécnica Nacional) in Quito, Ecuador, for their support of this research work. C.V.M.A. received the Ramón y Cajal contract (RYC-2017-22324) from the Spanish Ministry of Science and Innovation.

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

References

1. Gheewala, S.H.; Silalertruksa, T.; Nilsalab, P.; Mungkung, R.; Perret, S.R.; Chaiyawannakarn, N. Water footprint and impact of water consumption for food, feed, fuel crops production in Thailand. *Water* **2014**, *6*, 1698–1718. [CrossRef]
2. Mekonnen, M.M.; Hoekstra, A.Y. Four billion people facing severe water scarcity. *Sci. Adv.* **2016**, *2*, e1500323. [CrossRef]
3. Yotova, G.; Lazarova, S.; Kudłak, B.; Zlateva, B.; Mihaylova, V.; Wieczerzak, M.; Veneli-nov, T.; Tsakovski, S. Assessment of the Bulgarian wastewater treatment plants' impact on the receiving water bodies. *Molecules* **2019**, *24*, 2274. [CrossRef] [PubMed]
4. Pereda, O.; Solagaistua, L.; Atristain, M.; de Guzmán, I.; Larrañaga, A.; von Schiller, D.; Elosegi, A. Impact of wastewater effluent pollution on stream functioning: A whole-ecosystem manipulation experiment. *Environ. Pollut.* **2020**, *258*, 113719. [CrossRef] [PubMed]
5. von Sperling, M. *Biological Wastewater Treatment Series*; IWA Publishing: London, UK, 2007; Volume 1, p. 78.
6. Ostroumov, S.A. On some issues of maintaining water quality and self-purification. *Water Resour.* **2005**, *32*, 337–346. [CrossRef]
7. Ostroumov, S.A. Water quality and conditioning in natural ecosystems: Biomachinery theory of self-purification of water. *Russ. J. Gen. Chem.* **2017**, *87*, 3199–3204. [CrossRef]
8. Hashemi Monfared, S.A.; Dehghani Darmian, M.; Snyder, S.A.; Azizyan, G.; Pirzadeh, B.; Moghaddam, M.A. Water quality planning in rivers: Assimilative capacity and dilution flow. *Bull. Environ. Contam. Toxicol.* **2017**, *99*, 531–541. [CrossRef]
9. Bolinches, A.; De Stefano, L.; Paredes-Arquiola, J. Adjusting wastewater treatment effluent standards to protect the receiving wa-ters: The case of low-flow rivers in central Spain. *Environ. Earth Sci.* **2020**, *79*, 446. [CrossRef]
10. Poikane, S.; Johnson, R.K.; Sandin, L.; Schartau, A.K.; Solimini, A.G.; Urbanič, G.; Arbačiauskas, K.; Aroviita, J.; Gabriels, W.; Miler, O.; et al. Benthic macroinvertebrates in lake ecological assessment: A review of methods, intercalibration and practical recommendations. *Sci. Total Environ.* **2016**, *543*, 123–134. [CrossRef] [PubMed]
11. Tierney, K.B.; Baldwin, D.H.; Hara, T.J.; Ross, P.S.; Scholz, N.L.; Kennedy, C.J. Olfactory toxicity in fishes. *Aquat. Toxicol.* **2010**, *96*, 2–26. [CrossRef]
12. Lari, E.; Bogart, S.J.; Pyle, G.G. Fish can smell trace metals at environmentally relevant concentrations in freshwater. *Chemosphere* **2018**, *203*, 104–108. [CrossRef] [PubMed]
13. Tawari-Fufeyin, P.; Ekaye, S.A. Fish species diversity as indicator of pollution in Ikpoba River, Benin City, Nigeria. *Rev. Fish Biol. Fish.* **2007**, *17*, 21–30. [CrossRef]
14. Kruk, A.; Penczak, T. Natural regeneration of fish assemblages in the Pilica river after a reduction of point-source pollution. *River Res. Appl.* **2013**, *29*, 502–511. [CrossRef]
15. Moreira-Santos, M.; Donato, C.; Lopes, I.; Ribeiro, R. Avoidance tests with small fish: Determination of the median avoidance concentration and of the lowest-observed-effect gradient. *Environ. Toxicol. Chem.* **2008**, *27*, 1576–1582. [CrossRef]
16. Araújo, C.V.M.; Shinn, C.; Moreira-Santos, M.; Lopes, I.; Espíndola, E.L.G.; Ribeiro, R. Copper-driven avoidance and mortality in temperate and tropical tadpoles. *Aquat. Toxicol.* **2014**, *146*, 70–75. [CrossRef] [PubMed]
17. Vera-Vera, V.C.; Guerrero, F.; Blasco, J.; Araújo, C.V.M. Habitat selection response of the freshwater shrimp *Atyaephyra desmarestii* experimentally exposed to heterogeneous copper contamination scenarios. *Sci. Total Environ.* **2019**, *662*, 816–823. [CrossRef]
18. Lopes, I.; Baird, D.J.; Ribeiro, R. Avoidance of copper contamination by field populations of *Daphnia longispina*. *Environ. Toxicol. Chem.* **2004**, *23*, 1702–1708. [CrossRef]

19. Araújo, C.V.M.; Griffith, D.M.; Vera-Vera, V.; Vargas Jentzsch, P.; Cervera, L.; Nieto-Ariza, B.; Salvatierra, D.; Erazo, S.; Jaramillo, R.; Ramos, L.A.; et al. A novel approach to assessing environmental disturbance based on habitat selection by zebra fish as a model organism. *Sci. Total Environ.* **2018**, *619–620*, 906–915. [CrossRef] [PubMed]
20. Clements, W.H.; Hickey, C.W.; Kidd, K.A. How do aquatic communities respond to contaminants? It depends on the ecological context. *Environ. Toxicol. Chem.* **2012**, *31*, 1932–1940. [CrossRef] [PubMed]
21. Araújo, C.V.M.; Laissaoui, A.; Silva, D.C.V.R.; Ramos-Rodríguez, E.; González-Ortegón, E.; Espíndola, E.L.G.; Baldó, F.; Mena, F.; Parra, G.; Blasco, J.; et al. Not only toxic but repellent: What can organisms' responses tell us about contamination and what are the ecological consequences when they flee from an environment. *Toxics* **2020**, *8*, 118. [CrossRef] [PubMed]
22. Ikehata, K.; Naghashkar, N.J.; El-Din, M.G. Degradation of Aqueous Pharmaceuticals by Ozonation and Advanced Oxidation Processes: A Review. *Ozone Sci. Eng.* **2006**, *28*, 353–414. [CrossRef]
23. Wols, B.A.; Hofman-Caris, C.H.M. Review of photochemical reaction constants of organic micropollutants required for UV advanced oxidation processes in water. *Water Res.* **2012**, *46*, 2815–2827. [CrossRef] [PubMed]
24. Asghar, A.; Raman, A.A.A.; Daud, W.M.A.W. Advanced oxidation processes for in-situ production of hydrogen peroxide/hydroxyl radical for textile wastewater treatment: A review. *J. Clean. Prod.* **2015**, *87*, 826–838. [CrossRef]
25. Gautam, P.; Kumar, S.; Lokhandwala, S. Advanced oxidation processes for treatment of leachate from hazardous waste landfill: A critical review. *J. Clean. Prod.* **2019**, *237*, 117639. [CrossRef]
26. Cuerda-Correa, E.M.; Alexandre-Franco, M.F.; Fernández-González, C. Advanced oxidation processes for the removal of antibiotics from water. An overview. *Water* **2020**, *12*, 102. [CrossRef]
27. Miklos, D.B.; Remy, C.; Jekel, M.; Lindend, K.G.; Drewes, J.E.; Hübner, U. Evaluation of advanced oxidation processes for water and wastewater treatment-A critical review. *Water Res.* **2018**, *139*, 118–131. [CrossRef] [PubMed]
28. Babuponnusami, A.; Muthukumar, K. A review on Fenton and improvements to the Fenton process for wastewater treatment. *J. Environ. Chem. Eng.* **2014**, *2*, 557–572. [CrossRef]
29. Bello, M.M.; Raman, A.A.A.; Asghar, A. A review on approaches for addressing the limitations of Fenton oxidation for recalcitrant wastewater treatment. *Process. Saf. Environ. Prot.* **2019**, *126*, 119–140. [CrossRef]
30. Espinoza, I.; Sandoval-Pauker, C.; Ramos Guerrero, L.; Vargas Jentzsch, P.; Muñoz Bisesti, F. Fenton process combined with precipitation for the removal of Direct Blue 1 dye: A new approach. *J. Serb. Chem. Soc.* **2020**, *85*, 547–558. [CrossRef]
31. Sandoval, C.; Molina, G.; Vargas Jentzsch, P.; Pérez, J.; Muñoz, F. Photocatalytic degradation of azo dyes over semiconductors supported on polyethylene terephthalate and polystyrene substrates. *J. Adv. Oxid. Technol.* **2017**, *20*, 20170006. [CrossRef]
32. Picho-Chillán, G.; Dante, R.C.; Muñoz-Bisesti, F.; Martín-Ramos, P.; Chamorro-Posada, P.; Vargas-Jentzsch, P.; Sánchez-Arévalo, F.M.; Sandoval-Pauker, C.; Rutto, D. Photodegradation of Direct Blue 1 azo dye by polymeric carbon nitride irradiated with accelerated electrons. *Mater. Chem. Phys.* **2019**, *237*, 121878. [CrossRef]
33. Gangu, K.K.; Maddila, S.; Jonnalagadda, S.B. A review on novel composites of MWCNTs mediated semiconducting materials as photocatalysts in water treatment. *Sci. Total Environ.* **2019**, *646*, 1398–1412. [CrossRef] [PubMed]
34. Li, M.; Liu, Y.; Zeng, G.; Liu, N.; Liu, S. Graphene and graphene-based nanocomposites used for antibiotics removal in water treatment: A review. *Chemosphere* **2019**, *226*, 360–380. [CrossRef] [PubMed]
35. Altamirano Briones, A.; Cóndor Guevara, I.; Mena, D.; Espinoza, I.; Sandoval-Pauker, C.; Ramos Guerrero, L.; Vargas Jentzsch, P.; Muñoz Bisesti, F. Degradation of meropenem by heterogeneous photocatalysis using TiO_2/fiberglass substrates. *Catalysts* **2020**, *10*, 344. [CrossRef]
36. Coronel, S.; Sandoval Pauker, C.; Vargas Jentzsch, P.; de la Torre, E.; Endara, D.; Muñoz-Bisesti, F. Titanium dioxide/copper/carbon composites for the photocatalytic degradation of phenol. *Chem. Chem. Technol.* **2020**, *14*, 161–168. [CrossRef]
37. Galus, M.; Jeyaranjaan, J.; Smith, E.; Li, H.; Metcalfe, C.; Wilson, J.Y. Chronic effects of exposure to a pharmaceutical mixture and municipal wastewater in zebrafish. *Aquat. Toxicol.* **2013**, *132–133*, 212–222. [CrossRef]
38. García-Cambero, J.P.; Beltrán, F.J.; Encinas, A.; Rivas, F.J.; Oropesa, A.L. The added value of a zebrafish embryo-larval model in the assessment of wastewater tertiary treatments. *Environ. Sci. Water Res. Technol.* **2019**, *5*, 2269–2279. [CrossRef]
39. Silva, D.C.V.R.; Queiroz, L.G.; Marassi, R.J.; Araújo, C.V.M.; Bazzan, T.; Cardoso-Silva, S.; Silva, G.C.; Müller, M.; Silva, F.T.; Montagner, C.C.; et al. Predicting zebrafish spatial avoidance triggered by discharges of dairy wastewater: An experimental approach based on self-purification in a model river. *Environ. Pollut.* **2020**, *266*, 115325. [CrossRef]
40. Freeman, E.S.; Carroll, B. The application of thermoanalytical techniques to reaction kinetics. The thermogravimetric evaluation of the kinetics of the decomposition of calcium oxalate monohydrate. *J. Phys. Chem.* **1958**, *62*, 394–397. [CrossRef]
41. American Public Health Association; American Water Works Association; Water Environment Federation. *Standard Methods for the Examination of Water and Wastewater*, 23rd ed.; American Public Health Association: Washington, DC, USA, 2017.
42. Garófalo-Villalta, S.; Medina-Espinosa, T.; Sandoval-Pauker, C.; Villacis, W.; Ciobotă, V.; Muñoz Bisesti, F.; Vargas Jentzsch, P. Degradation of Reactive Red 120 dye by a heterogeneous Sono-Fenton process with goethite deposited onto silica and calcite sand. *J. Serb. Chem. Soc.* **2020**, *85*, 125–140. [CrossRef]
43. Laverde-Cerda, E.; Altamirano-Briones, A.; Zárate-Pozo, P.; Sandoval-Pauker, C.; Ramos-Guerrero, L.; Muñoz-Bisesti, F.; Vargas-Jentzsch, P. Iron removal for kinetic studies on Fenton treatments: A new approach based on ferrocyanide. *Rev. Mex. Ing. Quim.* **2020**, *19*, 159–164. [CrossRef]
44. Jiraprasertwong, A.; Maitriwong, K.; Chavadej, S. Production of biogas from cassava wastewater using a three-stage upflow anaerobic sludge blanket (UASB) reactor. *Renew. Energy* **2019**, *130*, 191–205. [CrossRef]

45. Fleck, L.; Tavares, M.H.F.; Eyng, E.; de Andrade, M.A.d.M.; Frare, L.M. Optimization of anaerobic treatment of cassava processing wastewater. *Eng. Agríc.* **2017**, *37*, 574–590. [CrossRef]
46. Araújo, C.V.M.; Shinn, C.; Mendes, L.B.; Delello-Schneider, D.; Sanchez, A.L.; Espíndola, E.L.G. 2014. Avoidance response of *Danio rerio* to a fungicide in a linear contamination gradient. *Sci. Total Environ.* **2014**, *484*, 36–42. [CrossRef] [PubMed]
47. OECD. *Test No. 203: Fish, Acute Toxicity Test, OECD Guidelines for the Testing of Chemicals, Section 2*; OECD Publishing: Paris, France, 1992. [CrossRef]
48. OECD. *Test No. 215: Fish, Juvenile Growth Test, OECD Guidelines for the Testing of Chemicals, Section 2*; OECD Publishing: Paris, France, 2000. [CrossRef]
49. Lawrence, C. The husbandry of zebrafish (*Danio rerio*): A review. *Aquaculture* **2007**, *269*, 1–20. [CrossRef]
50. Avdesh, A.; Chen, M.; Martin-Iverson, M.T.; Mondal, A.; Ong, D.; Rainey-Smith, S.; Taddei, K.; Lardelli, M.; Groth, D.M.; Verdile, G.; et al. Regular Care and Maintenance of a Zebrafish (*Danio rerio*) Laboratory: An Introduction. *J. Vis. Exp.* **2012**, *69*, e4196. [CrossRef]
51. Bosch Reig, F.; Gimeno Adelantado, J.V.; Moya Moreno, M.C.M. FTIR quantitative analysis of calcium carbonate (calcite) and silica (quartz) mixtures using the constant ratio method. Application to geological samples. *Talanta* **2002**, *58*, 811–821. [CrossRef]
52. Liu, J.; Zhang, T.; Wang, Z.; Dawson, G.; Chen, W. Simple pyrolysis of urea into graphitic carbon nitride with recyclable adsorption and photocatalytic activity. *J. Mater. Chem.* **2011**, *21*, 14398–14401. [CrossRef]
53. Cumbana, A.; Mirione, E.; Cliff, J.; Bradbury, J.H. Reduction of cyanide content of cassava flour in Mozambique by the wetting method. *Food Chem.* **2007**, *101*, 894–897. [CrossRef]
54. Zuk, M.; Pelc, K.; Szperlik, J.; Sawula, A.; Szopa, J. Metabolism of the cyanogenic glucosides in developing flax: Metabolic analysis, and expression pattern of genes. *Metabolites* **2020**, *10*, 288. [CrossRef]
55. Bulgariu, L.; Escudero, L.B.; Bello, O.S.; Iqbal, M.; Nisar, J.; Adegoke, K.A.; Alakhras, F.; Kornaros, M.; Anastopoulo, I. The utilization of leaf-based adsorbents for dyes removal: A review. *J. Mol. Liq.* **2019**, *276*, 728–747. [CrossRef]
56. Hernández-Zamora, M.; Martínez-Jerónimo, F. Congo red dye diversely affects organisms of different trophic levels: A comparative study with microalgae, cladocerans, and zebrafish embryos. *Environ. Sci. Pollut. Res.* **2019**, *26*, 11743–11755. [CrossRef]
57. Chu, J.-H.; Kang, J.-K.; Park, S.-J.; Lee, C.-G. Application of magnetic biochar derived from food waste in heterogeneous sono-Fenton-like process for removal of organic dyes from aqueous solution. *J. Water Process. Eng.* **2020**, *37*, 101455. [CrossRef]
58. Engström-Öst, J.; Karjalainen, M.; Viitasalo, M. Feeding and refuge use by small fish in the presence of cyanobacteria blooms. *Environ. Biol. Fish.* **2006**, *76*, 109–117. [CrossRef]
59. Tierney, K.B. Chemical avoidance responses of fish. *Aquat. Toxicol.* **2016**, *174*, 228–241. [CrossRef]
60. Araújo, C.V.M.; Rodríguez, E.N.V.; Salvatierra, D.; Cedeño-Macias, L.A.; Vera-Vera, V.C.; Moreira-Santos, M.; Ribeiro, R. Attractiveness of food and avoidance from contamination as conflicting stimuli to habitat selection by fish. *Chemosphere* **2016**, *163*, 177–183. [CrossRef]
61. Ruuskanen, S.; Rainio, M.J.; Kuosmanen, V.; Laihonen, M.; Saikkonen, K.; Saloniemi, I.; Helander, M. Female preference and adverse developmental effects of glyphosate-based herbicides on ecologically relevant traits in Japanese quails. *Environ. Sci. Technol.* **2020**, *54*, 1128–1135. [CrossRef]
62. Araújo, C.V.M.; Moreira-Santos, M.; Ribeiro, R. Active and passive spatial avoidance by aquatic organisms from environmental stressors: A complementary perspective and a critical review. *Environ. Int.* **2016**, *92–93*, 405–415. [CrossRef]
63. Fleeger, J.W.; Carman, K.R.; Nisbet, R.M. Indirect effects of contaminants in aquatic ecosystems. *Sci. Total Environ.* **2003**, *317*, 207–233. [CrossRef]
64. Moe, S.J.; De Schamphelaere, K.; Clements, W.H.; Sorensen, M.T.; Van Den Brink, P.J.; Liess, M. Combined and interactive effects of global climate change and toxicants on populations and communities. *Environ. Toxicol. Chem.* **2013**, *32*, 49–61. [CrossRef]
65. Hansen, J.A.; Woodward, D.F.; Little, E.E.; DeLonay, A.J.; Bergman, H.L. Behavioral avoidance: Possible mechanism for explaining abundance and distribution of trout species in a metal-impacted river. *Environ. Toxicol. Chem.* **1999**, *18*, 313–317. [CrossRef]
66. Jutfelt, F.; Sundin, J.; Raby, G.D.; Krang, A.-S.; Clark, T.D. Two-current choice flumes for testing avoidance and preference in aquatic animals. *Methods Ecol. Evol.* **2017**, *8*, 379–390. [CrossRef]
67. Moreira-Santos, M.; Ribeiro, R.; Araújo, C.V.M. What if aquatic animals move away from pesticide-contaminated habitats before suffering adverse physiological effects? A critical review. *Crit. Rev. Environ. Sci. Technol.* **2019**, *49*, 989–1025. [CrossRef]