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Integrated Use of Bioaccumulation, Genotoxic, and Haematological Endpoints to Assess the Effect of Water Remediation Strategies on Fish Health: A Complementary Study

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Abstract: Biosorption successfully remediates saline water contaminated with legacy contaminants, but its effects on the health of marine organisms remain unclear. Therefore, our aim was to address this knowledge gap with data on the accumulation ability, as well as the cytogenetic and biochemical effects in turbot (*Scophthalmus maximus*). To this end, we exposed turbot for seven days to a mixture of remediated metals (Rem treatments: Cd, Hg, and Pb), with and without the presence of nanoparticles (NP), and compared them with the maximum allowable concentrations (MAC treatment) for effluent discharges. We determined the metal accumulation in the blood and kidney and evaluated haematological changes (red blood cell count, haemoglobin, and mean cell haemoglobin (MCH)) and genotoxicity (erythrocytic nuclear abnormalities assay) in the blood. The results showed that remediation with non-living macroalgae significantly reduced the metallic blood and kidney burdens in the Rem treatments. Furthermore, no genotoxic potential occurred in the Rem and MAC treatments in parallel with the reduction in MCH levels in the Rem treatments, which would reflect hematopoietic disturbances in the MAC. Our results validate biosorption remediation as we achieved a considerable reduction in metal loads while maintaining the health status of fish, highlighting the importance of testing water remediation methods in the biota.

Keywords: water quality; metallic mixtures; fish health; body burdens; haematological dynamics

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1. Introduction

Presently, the world is facing a global water crisis [1], which is intensified by water contamination and the increase in extreme climatic events. As such, water security and access to clean water are key points of the EU Green Deal and the United Nations Sustainable Development Goals, starting with SDG6 to "Ensure availability and sustainable management of water and sanitation for all". This global concern has led to the continuous

search for the best technologies to treat contaminated water, protecting humans and aquatic organisms, and increasing water reuse.

(Bio)sorption is a remediation methodology that has attracted the attention of the scientific community, and numerous studies evaluating the sorption capability of (bio)sorbents for a wide range of water contaminants are readily available in the literature [2–8]. However, most of these studies have several shortcomings, including the fact that the levels of contamination tested are usually unrealistically high. In most cases, contaminants have been studied and regulated separately on the basis of their individual toxicological profile, whereas both inorganic contaminants, recognized as legacy substances, and contaminants of emerging concern, namely engineered nanoparticles (NP), occur simultaneously in the environment. Generally, these studies only consider the final chemical status and rarely include a toxicological assessment of the water after the proposed treatment [7,9]. Depending on the functional groups and pore size, (bio)sorbents may display different affinities and selectivities for different contaminants, and the speciation of the latter in the reaction medium may influence their adsorption rate and toxicity [10,11].

Because of the global distribution of contaminants and the lack of information on their potential hazards, toxicological studies on combined exposures are even more important, as they may have additive, synergistic, or antagonistic effects [12]. Indeed, several studies have shown that exposure to multiple contaminants can have adverse effects on organisms [13,14]. However, few studies relate toxic effects and responses to mixtures of specific classes of contaminants, and the existent ones do it based on unrealistically high concentrations. In fact, the toxicity of mixtures within the legal thresholds for each contaminant is rarely assessed, reinforcing the importance of evaluating the effects of combined exposures to different contaminants within unhazardous limits and to predict their consequences to the environment, seafood, and human health [15].

Cadmium (Cd), mercury (Hg), and lead (Pb) are known as the "toxic trio" due to their toxic properties [16]. The first two are listed as PBT substances (Persistence, Bioaccumulation potential, and Toxicity) and lead is listed as a priority substance in European legislation-Directive 2008/105/EC [17]. These elements reach the aquatic ecosystems through natural (mainly soil erosion and volcanic activity) and human sources [18], which include untreated or inadequately treated wastewaters and industrial effluents [19]. The adverse effects of these elements in aquatic ecosystems will depend on the composition and volume of the effluent discharged, and in extreme scenarios may include the death of aquatic life, algal blooms, habitat destruction, debris, increased water flow, and other short- and long-term toxicity [19]. As these metals are persistent, they easily bioaccumulate and biomagnify throughout food chains, inducing a wide range of adverse effects both on wildlife and human health [20–26].

Silver (Ag) and gold (Au) NP are among the most widely used nanoparticles (NP) and are listed as priority substances by the OECD [27]. Despite this, information regarding Ag and Au NP's toxicity is still inconclusive. According to some studies, both NP can induce in vitro and in vivo toxic effects to humans and wildlife [23,28–34]. Nevertheless, these NP have been used in nano-remediation strategies of effluents and contaminated sites to eliminate or reduce the available potential of toxic elements [35,36]. Despite this attractive application, the fate of NPs when released into the environment, their interaction with other contaminants and biological systems, and thus their safety, are still largely unknown [37].

Under this scenario, the present study emerges as a complementary study to a previous one carried out by the same research group (Figueira and co-authors [8]), which demonstrated the biosorption capability of marine macroalgae on the removal of Hg, Cd, and Pb from saline waters. Based on the biosorption results obtained, and as a crucial following step, this study aimed to evaluate if the water remediation with marine macroalgae effectively contributed to improving water quality, with measurable gains on the the health of aquatic organisms. To accomplish this goal, we selected the sub-lethal contamination levels from the study by Figueira and co-authors [8], and assessed their impact on the health of turbot (*Scophthalmus maximus)*, a marine flatfish selected as a model organism.

The remediation methodology achieved overall efficiencies of up to 99% for Hg, 86% for Pb, and 20% for Cd [8], which at first sight suggests a considerable improvement in the overall health status of turbot. For an even more realistic approach, it is imperative to consider the impact of the co-occurrence of legacy and emergent contaminants in water. Thus, two of the most used metallic NP (Ag NP and Au NP) were added in each treatment. Given the potentially toxic effects of the selected metals and NP isolated [23,24,32,38–41], we evaluated the genotoxic potential and alteration in the haematological dynamics after fish co-exposure to the selected mixture of metals with and without NP. Plus, the accumulation profile of the tested contaminants was evaluated to establish a relationship between their body burdens and the cytogenetic and biochemical endpoints. These parameters have emerged as tools with great potential for environmental monitoring [42–44].

The results of this study will contribute to a better understanding of the biosorption processes described in the literature for metal contaminated waters; go beyond the improvement of the chemical status of the water, contributing to the overall health status of fish; and, in the final stage, minimize the impact of metal contamination on human health.

2. Material and Methods

2.1. Chemicals

All of the chemicals used were of analytical reagent grade. The standard stock solutions of Cd (1000 ± 2 mg/L), Hg (1001 ± 2 mg/L), and Pb (1000 ± 2 mg/L) were purchased from Merck (Damstadt, Germany). Gold NP (10 nm diameter, OD 1, stabilized suspension in 0.1 mM PBS, reactant free) and Ag dispersion NP (10 nm particle size, 0.02 mg/mL in aqueous buffer, with sodium citrate as stabilizer) were purchased from Sigma-Aldrich (Damstadt, Germany). Nitric acid (65%, Merck, Damstadt, Germany), hydrogen peroxide (30%, Scharlab, Spain), and the certified reference materials (CRM) BCR-634 and TORT-2 (LGC Standards, UK) were used for tissue digestion. Methanol (Merck, Damstadt, Germany) and Giemsa (Merck, Damstadt, Germany) were used in the genotoxicity essays and Drabkin's reagent (Merck, Damstadt, Germany) was used in the haemoglobin determination.

2.2. Scanning Transmission Electron Microscopy Analysis

The morphology of Ag NP (stock suspension) was checked by scanning transmission electron microscopy (STEM) using a Hitachi SU-70 with a Bruker Quantax 400 (B-U) EDS-microscope (Supplementary Materials).

2.3. Test Animals

Juvenile hatchery-brood turbot (*Scophthalmus maximus*) with an average wet weight of 23.4 ± 6.2 g and an average total length of 11.8 ± 1.0 cm were acquired from a local aquaculture (Acuinova, Mira, Portugal). At the laboratory, the animals were acclimated during 15 days in 25 L polyvinyl aquaria under a natural photoperiod, in aerated and recirculating artificial seawater (Tropic Marin), with salinity 33.8 ± 1.1, temperature 18.2 ± 3.4 °C, pH 8.0 ± 0.2, dissolved oxygen 8.5 ± 1.0 mg/L, and total ammonia 0.62 ± 1.99 mg/L. Fish were fed daily with commercial pellets, until apparent satiation.

2.4. Experimental Design and Exposure Conditions

The assay rationale was to test fish exposure to biosorption-remediated industrial effluent during a short period (7 days), mimicking an episode of industrial effluent discharge. For that, fish were co-exposed to metallic contaminants (Cd, Hg, and Pb) and NP (Ag and Au). Cadmium, Hg, and Pb were tested at two different concentrations each. The highest concentrations were chosen in accordance with the maximum allowable concentrations (MAC: 200 µg/L, 50 µg/L, and 1000 µg/L for Cd, Hg, and Pb, respectively) for effluent discharges, as defined in the European legislation (Directive 2009/90/EC [45]) (Figure 1). In addition to the MAC mixture, it was important to understand if water remediation processes were able to decrease the toxic potential of the mixture. Hence, the lowest

concentrations (Rem: 164 µg/L, 0.10 µg/L, and 108 µg/L for Cd, Hg, and Pb, respectively) corresponded to the values obtained after applying a methodology to remediate saline water with the same metallic contaminants, developed within the RemAS project [8] (Figure 1). As NP can influence both their behaviour (availability and toxicity) and that of other elements with which they are mixed [46], the MAC and Rem treatments were tested in the absence and presence of equal concentrations of Ag NP (5 µg/L) and Au NP (5 µg/L) (Figure 1). The concentrations of Ag NP and Au NP tested matched the lowest no effect concentration (NOEC) for Ag found in the marine biota [44].

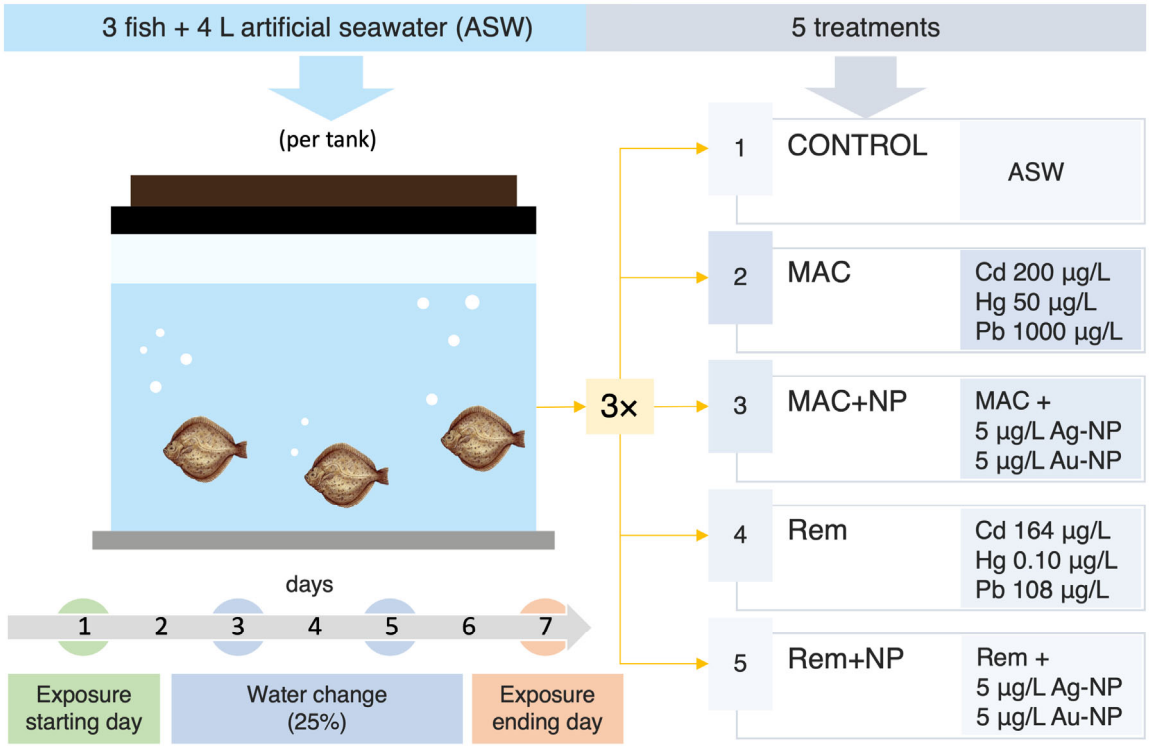

Figure 1. Schematic representation of the experimental design, treatments, and concentrations. MAC—maximum concentrations allowable for effluent discharges; Rem—MAC concentrations after remediation processes; MAC + NP—maximum concentrations allowable for effluent discharges plus Ag-NP and Au-NP; Rem + NP—MAC concentrations after remediation processes plus Ag-NP and Au-NP. ASW—artificial seawater; Cd—cadmium; Hg—mercury; Pb—lead.

The experimental assay, schematized in Figure 1, was performed in triplicate with three individuals per replicate in 4 L aerated seawater for 7 days. The natural photoperiod was maintained, and a 25% water change and re-dosing of the chemicals was carried out every other day. The physicochemical parameters of the water during the assay were salinity 32.4 ± 0.5, temperature 20.6 ± 0.6 °C, pH 8.04 ± 0.15, dissolved oxygen 7.99 ± 0.53 mg/L, and total ammonia 10.0 ± 0.0 mg/L.

The mixtures of metal contaminants were prepared fresh by diluting each element stock solution to the desirable concentration in artificial seawater, while nanoparticles were directly added to the fish tanks. The control condition was obtained by using only artificial sea salt.

Fish were sacrificed in accordance with the European Union guidelines concerning the protection and animal welfare (Directive 2010/63/EU [47]) and under the supervision of a member of staff authorized by the competent authorities.

2.5. Determination of Cd, Hg, Pb, and Ag in Blood and Kidney

At the end of the assay (7 days), the fish were sacrificed, and the blood and kidney were sampled and immediately frozen in liquid nitrogen, followed by storage at −80 °C. Blood

was collected from the posterior cardinal vein using heparinised Pasteur pipettes and stored in microtubes. Both the blood and kidneys were freeze-dried, ground to a fine powder, and stored until further processing for metal quantification. Dried blood, kidney, and CRM (BCR-634 and TORT-2 for blood and kidney, respectively) were microwave-digested in duplicate for the determination of Ag, Cd, and Pb using the following procedure: 500 µL of HNO_3 (63%) was added to the blood and kidney samples in Savillex PFA vials and placed in a hot plate for 5 h at 150 °C. After cooling, H_2O_2 (150 µL for blood samples; 100 µL for kidney samples) was carefully added to the samples and placed again in the hot plate at 150 °C for 2 h (blood) or 10 min (kidney). The digested samples were left to cool down overnight in the closed vials. Afterwards, the solution was diluted to 15 mL with ultra-pure water with 1% HNO_3 and then analysed by ICP-MS (Thermo X Series, Bremen, Germany). Gold was not determined due to analytical constraints of the methodology. The precision of the method ranged between 0% and 10%, with an extraction efficiency between 99.6% and 124% (determined with the CRM samples). The total Hg (T-Hg) in the dried blood and kidney was analysed by thermal decomposition atomic absorption spectrometry (TD AAS) with gold amalgamation, using an Advanced Mercury Analyser (AMA 254) (LECO®, Czech Republic) [48]. The accuracy and precision of the methodology were assessed by replicate analysis of CRM BCR-634 and TORT-2 for the blood and kidneys, respectively. The precision of the method was always better than 9% (n > 12), with recovery efficiencies between 109 and 116%.

2.6. Genotoxic Potential: Erythrocytic Nuclear Abnormalities (ENA) Assay

To evaluate the genotoxicity of the mixtures (MAC and Rem), the ENA assay was carried out in mature erythrocytes of turbot [49]. Blood smears were fixed with methanol for 10 min and stained with Giemsa (5%) for 30 min. From each smear, 1000 mature erythrocytes were scored under 1000× magnification (Olympus BX41, Tokyo, Japan) to determine the following nuclear lesions categories: kidney shaped nuclei (K), lobed nuclei (L), binucleate or segmented nuclei (S), vacuolated nuclei (V), and micronuclei (MN). Although the frequency (‰) of each nuclear abnormality category was individually reported, the results of the ENA assay were expressed as the sum of the frequencies for all of the categories considered (K + L+S + V + MN).

2.7. Haematological Dynamics

2.7.1. Erythrocytic Maturity Index (EMI) and Immature Erythrocytes (IE) Frequency

EMI was adapted by Castro and co-authors [43], providing information on the progressive route to cell maturity and complementing the data obtained by the frequency of immature erythrocytes (IE). Briefly, 10 fields randomly selected per slide (one slide per fish; the same slides used for ENA and IE assays) were photographed at 40× magnification (Olympus BX41, Tokyo, Japan). Afterwards, digital microphotographs were analysed in ImageJ software and the minor axis of the nucleus and the major axis of the cell were measured in 25 erythrocytes per field. EMI was calculated by dividing the minor axis of the nucleus and the major axis of cell, and then each cell was classified in 1 of the 10 maturity classes, defined as follows: class 1 [0.0; 0.1[; class 2 [0.1; 0.2[; class 3 [0.2; 0.3[; class 4 [0.3; 0.4[; class 5 [0.4; 0.5[; class 6 [0.5; 0.6[; class 7 [0.6; 0.7[; class 8 [0.7; 0.8[; class 9 [0.8; 0.9[; class 10 [0.9; 1.0]. The erythrocyte maturity decreased from class 1 (higher maturity) to class 10 (lower maturity). For each experimental group, the average values for the frequency (%) of cells observed in each maturity class were represented.

For IE scoring, 1000 erythrocytes (mature and immature) were scored per smear, under 1000× magnification (Olympus BX41, Tokyo, Japan). Immature erythrocytes had a bluish-grey cytoplasm and a rounder and larger nucleus than the mature erythrocytes, which had an elliptical and smaller nucleus [50]. The frequency of IE was calculated according to the following expression [51]:

$$\text{IE frequency } (‰) = \frac{\text{IE}}{(\text{ME} + \text{IE})} \times 1000$$

2.7.2. Red Blood Cell (RBC) Count, Haemoglobin (Hb) Concentration, and Mean Cell Haemoglobin (MCH)

The RBC count and Hb were immediately estimated after blood sampling. The RBC count was carried out using a haemocytometer chamber (Neubauer chamber) and the results were expressed as 10^6 cells/mm^3. Haemoglobin was estimated using the cyanmethaemoglobin method [52], using Drabkin's reagent. The intensity of the colour is proportional to Hb concentration and was measured spectrophotometrically at 540 nm (SpectraMax 190 plate reader, Avantor, Ireland). MCH represents the absolute amount of Hb in each RBC, and was expressed as picograms (pg) per cell and calculated by the following:

$$\text{MCH (pg)} = \frac{\text{Hb (g/dL)} \times 10}{\text{RBC} \times 10^6 \text{ (1/µL)}}$$

2.8. Statistical Analysis and Software

Statistical analyses were performed using IBM.SPSS®, version 27.0, and all of the tests were considered significant when $p < 0.05$. The effects of the different treatments on the dependent variables (T-Hg, ENA, EMI, IE, RBC, Hb, and MCH) were evaluated through one-way ANOVA, followed by multiple comparisons tests. To verify ANOVA assumptions, graphical validation tools were used. Whenever assumptions were not fulfilled, data were evaluated with the equivalent non-parametric test. The description of the statistical results followed the suggestions of Muff and co-authors [53]. Graphical figures were made in Microsoft PowerPoint (version 2302) and the bar graph was obtained using Prism-GraphPad (trial version).

3. Results

3.1. Accumulation of Hg, Cd, Pb, and Ag in the Blood and Kidneys

The total Hg, Cd, Pb, and Ag accumulation in turbot blood and kidneys after 7 days of exposure are depicted in Table 1.

Table 1. Mean concentrations (µg/g, fresh weight) of total mercury (T-Hg), cadmium (Cd), lead (Pb), and silver (Ag) in the blood and kidneys of turbot (*S. maximus*) exposed to different metallic mixtures, with and without nanoparticles, for 7 consecutive days. T-Hg data are represented by mean ± standard deviation and different lower-case letters denote significant differences ($p < 0.05$). MAC—maximum concentrations allowable for effluents discharges; Rem—MAC concentrations after remediation processes; MAC + NP—maximum concentrations allowable for effluent discharges plus Ag-NP and Au-NP; Rem + NP—MAC concentrations after remediation processes plus Ag-NP and Au-NP; LOD—limit of detection.

	Treatments	T-Hg	Cd	Pb	Ag
Blood	Control	0.053 ± 0.020 [a]	0.050	0.081	0.050
	MAC	0.23 ± 0.08 [b]	0.20	0.27	0.010
	MAC + NP	0.28 ± 0.08 [b]	0.25	0.16	0.004
	Rem	0.038 ± 0.011 [a]	0.097	0.44	<LOD
	Rem + NP	0.041 ± 0.005 [a]	0.12	0.036	0.028
Kidney	Control	0.019 ± 0.009 [a,c]	0.45	0.075	0.028
	MAC	0.04 ± 0.01 [a,c]	0.73	0.52	0.033
	MAC + NP	0.04 ± 0.02 [a,c]	0.78	0.60	<LOD
	Rem	0.015 ± 0.002 [a]	0.46	0.18	<LOD
	Rem + NP	0.016 ± 0.003 [a]	0.66	0.16	0.007

As a result of restrictions regarding sample mass, replicate analyses were only performed for T-Hg. However, it was possible to infer an accumulation trend for Cd, Pb, and Ag. After 7 days of exposure, there was very strong evidence that the MAC treatments with and without NP (MAC and MAC + NP) accumulated more T-Hg in the blood compared

with the other mixtures and the control (F = 32.916; p = 0.000). The kidneys accumulated less T-Hg than the blood, with very strong evidence of no significant differences between the mixtures and control, but highlighting that MAC treatments accumulated more T-Hg than Rem treatments (F = 9.788; p = 0.000). The Cd accumulation trend in the blood was MAC ≈ MAC + NP > Rem ≈ Rem + NP > control, and in the kidneys it was MAC ≈ MAC + NP ≈ Rem + NP > Rem ≈ control. In general, the trend of Pb accumulation in the blood was Rem > MAC > MAC + NP > C > Rem + NP suggesting lower levels than their equivalents without NPs. Lead in the kidneys was similar to that of Cd in the blood. Silver burdens were very low in both the blood and kidneys, with similar levels across treatments. Contrary to T-Hg, the kidneys were more prone to Cd and Pb accumulation than the blood.

3.2. Genotoxic Potential: Erythrocytic Nuclear Abnormalities (ENA)

The results of the ENA assay are depicted in Table 2, scored as individual abnormalities and total ENA.

Table 2. Mean frequency (‰) of each nuclear abnormality category (mean ± standard deviation) in the peripheral erythrocytes of turbot (*S. maximus*) exposed to different metallic mixtures, with and without nanoparticles, for 7 consecutive days. Values (‰) concern the sum of frequencies for all of the nuclear abnormality categories scored (ENA frequency) and the frequency of each abnormality category individually. MAC—maximum concentrations allowable for effluents discharges; Rem—MAC concentrations after remediation processes; MAC + NP—maximum concentrations allowable for effluents discharges plus Ag-NP and Au-NP; Rem + NP—MAC concentrations after remediation processes plus Ag-NP and Au-NP.

Treatments	Individual Nuclear Abnormalities					Total ENA
	Kidney (k)	Lobed (L)	Segmented (S)	Vacuolated (V)	Micronuclei (MN)	
Control	3.3 ± 3.1	2.7 ± 1.5	4.0 ± 5.0	0 ± 0	0 ± 0	10 ± 6
MAC	4.0 ± 2.0	5.3 ± 4.4	4.2 ± 4.5	0.2 ± 0.4	0 ± 0	14 ± 9
MAC + NP	4.3 ± 2.6	2.3 ± 3.8	3.5 ± 3.7	0 ± 0	0 ± 0	10 ± 9
Rem	3.8 ± 1.9	5.7 ± 3.2	3.3 ± 2.5	0 ± 0	0 ± 0	13 ± 6
Rem + NP	3.0 ± 3.0	4.2 ± 3.3	2.2 ± 1.6	0 ± 0	0 ± 0	9.3 ± 6.9

There was no evidence that the different treatments influenced individual or total ENA frequency, either compared to the control or between treatments (F = 0.115, p = 0.976; H = 5.284, p = 0.259; F = 0.166, p = 0.954, F = 0.267, p = 0.897 for K, L, S, and total ENA, respectively) (Table 2).

3.3. Haematological Dynamics

3.3.1. Erythrocytic Maturity Index (EMI) and Immature Erythrocytes Frequency (IE)

The EMI of turbot was identified up to the seventh class of erythrocytic maturity (Figure 2).

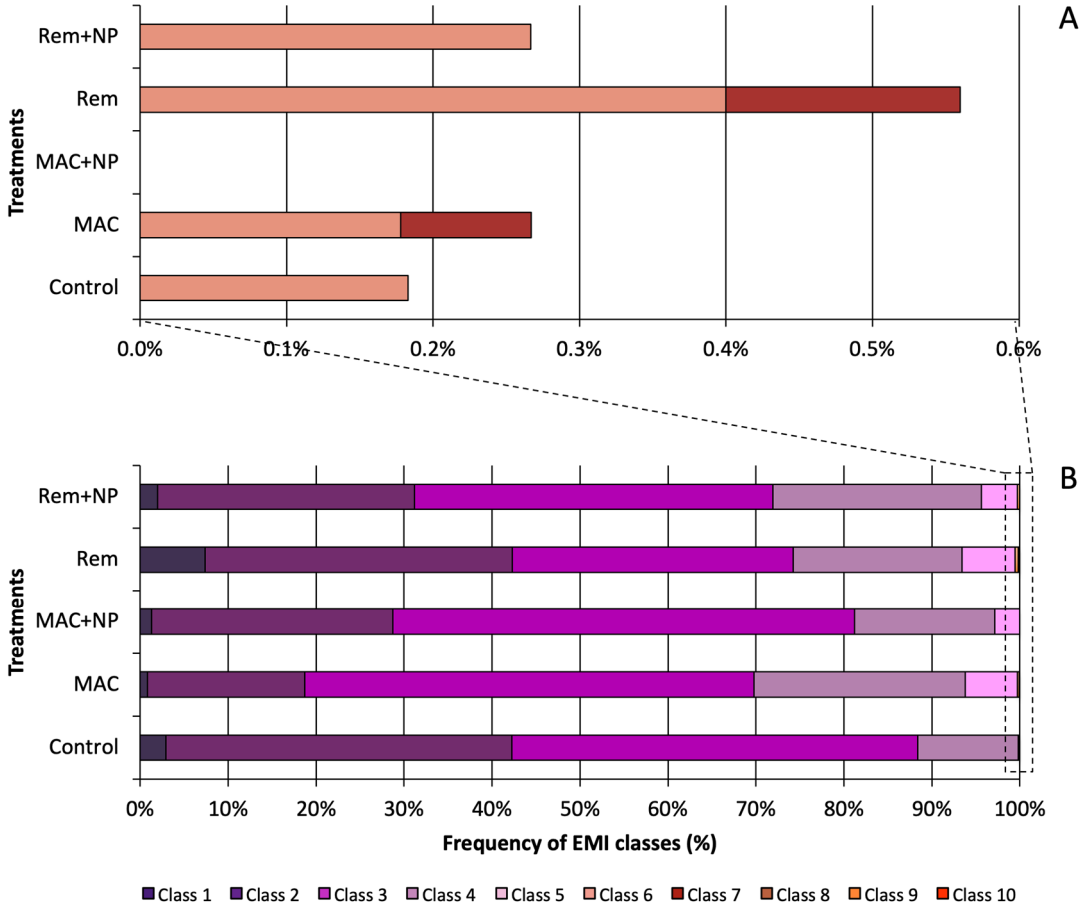

Figure 2. Erythrocytic maturity index (EMI) in *S. maximus* exposed to different metallic mixtures, with and without nanoparticles, for 7 consecutive days. (**A**) Mean values for the frequency (%) of cells observed in each maturity class (maturity decreases from class 1 to class 7) are represented. (**B**) Detailed representation of the frequency (%) of EMI classes 6 and 7. MAC—maximum concentrations allowable for effluents discharges; Rem—MAC concentrations after remediation processes; MAC + NP—maximum concentrations allowable for effluent discharges plus Ag-NP and Au-NP; Rem + NP—MAC concentrations after remediation processes plus Ag-NP and Au-NP.

There was also no evidence that the different treatments influenced the EMI ($H = 5.385$, $p = 0.250$; $F = 0.815$, $p = 0.527$; $F = 0.895$, $p = 0.481$; $H = 1.228$, $p = 0.873$; $F = 0.487$, $p = 0.745$; $F = 0.808$, $p = 0.531$; $F = 1.629$, $p = 0.197$, from class 1 to 7). Yet, class 7 (lower maturity) was absent in the control and NP treatments (MAC-NP and Rem-NP), while MAC + NP also lacked class 6. Similar to the frequency of ENA, there was little evidence that the different treatments affected the frequency of IE after 7 days of exposure ($H = 5.464$, $p = 0.243$) (Figure 3A).

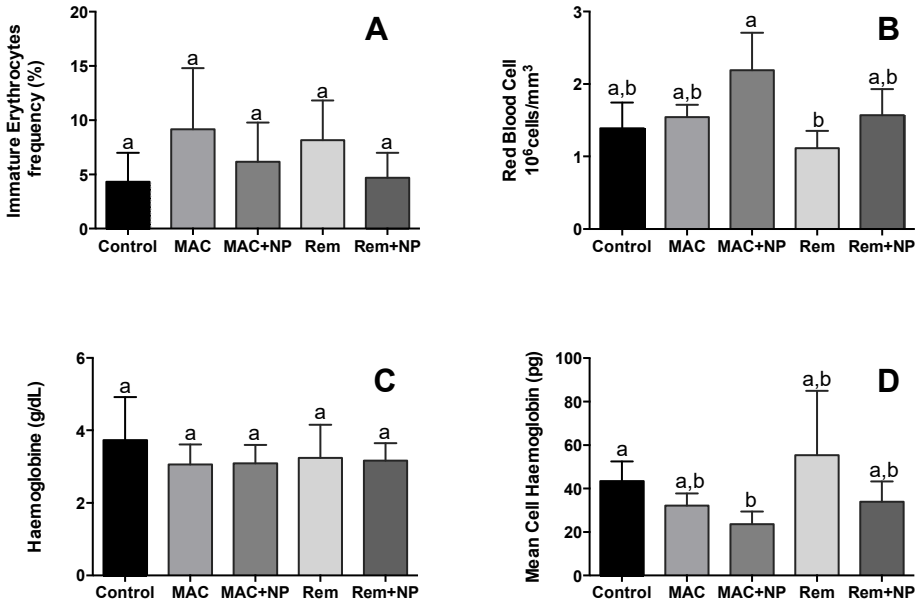

Figure 3. Haematological dynamics in *S. maximus* exposed to different metallic mixtures, with and without nanoparticles, for 7 consecutive days. (**A**) Immature erythrocyte frequency (%), (**B**) red blood cell count (10^6 cells/mm^3), (**C**) haemoglobin (g/dL), and (**D**) mean cell haemoglobin (pg). MAC—maximum concentrations allowable for effluents discharges; Rem−MAC concentrations after remediation processes; MAC + NP—maximum concentrations allowable for effluent discharges plus Ag-NP and Au-NP; Rem + NP—MAC concentrations after remediation processes plus Ag-NP and Au-NP. Different lower-case letters denote significant differences ($p < 0.05$). Columns correspond to mean values and error bars represent the standard deviation.

3.3.2. Red Blood Cell (RBC) Count, Haemoglobin (Hb) Concentration, and Mean Cell Haemoglobin (MCH)

There was very strong evidence that RBC increased only in MAC + NP relative to Rem ($H = 14.561$, $p = 0.006$; Figure 3B), while the influence of the different treatments on the Hb concentration showed weak evidence of differences relative to the control or among treatments ($F = 0.769$, $p = 0.0555$; Figure 3C). In contrast, there was strong evidence that the MCH decreased in MAC + NP against the control ($H = 13.498$, $p = 0.009$) (Figure 3D).

4. Discussion

Water is a vital resource, but life needs more than water: it needs safe water. Water was once viewed as an inexhaustible resource with an infinite capacity to purify and dilute everything, but that perspective has been changing. The need to ensure clean water for all is mandatory, and, among others, has led to the search for new methodologies able to remediate contaminated water. Accordingly, we assessed if a proposed biosorption remediation strategy could improve the health of a marine organism. To do this, we focused on a whole mixture approach, including both legacy and emerging contaminants, and assessed the potentially toxic effects of realistic concentrations of chemicals commonly found in effluent discharges (Cd, Hg, and Pb, and Ag-NP and Au-NP), and then compared the whole mixture after remediation. This approach is recommended for a preliminary evaluation of an effluent mixture as it can adequately demonstrate the coexisting exposure that organisms face in the environment [54]. The whole-mixture approach has the advantage of including all of the interactions that a component-based approach may have missed. However, it is not possible to generalise the information obtained to other mixtures or to identify the component(s) responsible for the effects [54]. Our results showed that the proposed remediation strategy was highly efficient at reducing the metal body burden

from fish blood and kidney. However, this improvement was not evident in terms of the cytogenetic and biochemical endpoints that indicate the health status of the organism.

4.1. Metals in Fish Blood and Kidneys

Blood is a suitable indicator of metallic environmental concentrations for several organisms, such as turtles, fish, and humans [25,43,55–57]. Although blood is not a tissue with a high capacity for accumulation, but rather a vehicle for other organs [57], it can still reflect the immediate uptake from the environment [43,56,58]. Alissa and Ferns [59] found that some human blood constituents can bind metals, showing that RBC can capture 98-99% of the Pb and 95% of Hg. The works of Ley-Quiñónez and co-authors [55] in turtles and of Janicka and co-authors [25] in humans also demonstrated the ability of blood to reflect Cd, Pb, and Hg accumulation. Castro et al. [43] stressed blood suitability to evaluate Hg bioaccumulation. Similar to these studies, we found that blood reflected the metal concentrations tested in the different mixtures, confirming its suitability as a short-term indicator of environmental conditions. Plus, the higher T-Hg levels found in the blood compared with the kidneys reinforced the efficacy of blood to reflect 7 days of exposure. Silver NP and Au NP are known to spread rapidly from the blood to the tissues [60,61]; notwithstanding the lack of information for Au NP in this work, overall Ag levels were in the same range for all of the treatments.

The accumulation trend of Cd and Pb in the kidneys highlights it as one of the main targets for metal accumulation and reinforces its role as a detoxifying organ [24,57,62,63]. Some authors found that Cd accumulates in fish kidneys 48 h after intraperitoneal injection of sub-lethal levels [62] and that the kidneys were the main target for Pb accumulation after 4 weeks of exposure via diet [24]. For T-Hg, the kidneys showed lower levels than the blood and did not show the high accumulation capacity observed in other studies (e.g., [56,57]). However, this difference may be related to the element kinetics. The presence of high levels of xenobiotics in the kidney could induce toxic effects in haematopoiesis and the elimination of xenobiotics [57,62,63]. Therefore, the levels of Cd, Pb, and Ag, and to a lesser extent, Hg, in the kidneys may influence the haematological dynamics of turbot, mainly due to the function of the head−kidney as an organ forming erythroid lineages.

Although the MAC treatments were in line with the allowable concentrations found in industrial effluents, Cd in both the blood and kidneys exceeded the maximum levels allowed for fish muscle meat (0.05 μg/g [64]), except for the blood in the control fish. Similarly, Pb levels were above the maximum permitted levels for fish muscle meat (0.3 μg/g [64]) in the mixtures without NP in the case of blood, and in MAC treatments for the kidney. This evidence confirms the high bioaccumulation ability reported for these elements, and that fish exposed to MAC treatments have unsafe levels of these metals.

The reported interaction between NPs and the other chemicals in a mixture suggests that NP may affect their uptake and that of other chemicals [46]. Yet, in the present study, the presence of NP did not seem to affect accumulation, as the differences found were only between metal concentrations (MAC vs. Rem) and not attributable to the presence of NP (as verified for T-Hg and the trend observed for Cd in blood, and Pb and Ag in the kidney). The high levels found in Rem + NP in the kidney should be carefully interpreted, given the small number of samples.

Overall, it can be highlighted that the chosen remediation methodology promoted a reduction in blood and kidney metal loads in the remediated treatments compared with the MAC treatments (T-Hg: 6.8×, 2.5×; Cd: 2× and Pb: 1.7×, 3×, for blood and kidney, respectively, and Ag in the kidney: 3.7×). The reduction in these metallic body burdens may promote an improvement in the health status of fish (even beyond the assessed parameters) and considerably decrease the risk for human health by fish ingestion. This is especially evident for Cd and Pb, whose levels in MAC treatments were above the legal thresholds.

4.2. Genotoxicity and Alteration of Haematological Parameters

Genotoxicity endpoints such as DNA and chromosomal damage successfully demonstrated the toxic potential of several chemicals, particularly those whose mechanism of action is through oxidative stress-mediated processes such as Hg, Cd, Pb, Ag NP, and Au NP [31,38,39,41,51]. Nevertheless, in the present study, no significant increases in ENA frequency (total and individual) occurred in any treatment, particularly in the MAC treatments, which may indicate either no genotoxic potential of the mixture after a short-term exposure or an efficient protective response by the fish. Guilherme and co-authors [51] suggested precaution when evaluating the genotoxic potential in the absence of ENA as other types of DNA damage might occur, rather than the ability to cope with the damage. Udroi (2006) pointed out that this phenomenon could result from the inhibition of DNA synthesis, time of maturation of erythrocytes, and erythropoiesis inhibition [65]. Other authors have advocated that increasing erythrocytic catabolism could promote the erythropoietic rate, which can lead to the absence of ENA [49,50]. Pacheco and Santos (2002) suggested that under a constant erythropoiesis rate, fast elimination of the abnormal erythrocytes from the spleen can occur, and thus no ENA can be detected [66]. For these reasons, it is crucial to complement ENA assessment with erythrocytic kinetics, assessed by the frequency of IE and EMI. The frequency of IE provides knowledge of the haematological dynamics, reflecting the balance between several factors, such as immature cells input versus splenic cells removal and the cell maturation rate. In the present study, similar to the pattern observed with ENA, no increase in the frequency of IE was observed, indicating no changes in the haemodynamic profile, which may suggest that MAC treatments both with and without NPs are not cytotoxic.

Although IE frequency provides indirect information on the erythrocyte catabolism and erythropoiesis, thus complementing the ENA assay, it does not provide information regarding the different stages of erythrocyte maturity. Fish erythrocytes exhibit different cell stages simultaneously [67], which may hinder the interpretation of data such as the frequency of IE. Hence, as shown by Castro and co-authors, EMI emerges as a suitable tool to assess erythrocytes' maturity, establishing an erythron profile [43]. Nonetheless, EMI seems species-specific: a study performed using eels (*Anguilla anguilla*) showed the presence of 10 maturity classes [43], in contrast with the seven classes obtained in turbot. In this work, classes 6 and 7 showed a low frequency in all of the conditions, with class 7 absent from the control and MAC and Rem treatments with NP. Class 6 was missing from MAC + NP treatments, which might suggest a lower erythropoiesis rate within this mixture. The EMI and IE results suggest no relevant alterations of erythropoiesis.

The alteration of haematological parameters such as RBC, Hb, and MCH also provides information regarding fish health after exposure to contaminants [42,68–70]. In our study, the RBC count was within the range of values previously found in farmed turbot and generally less-active species (usual ranges from 0.5 to 1.5 $\times$ $10^6/\mu L$) [71]. Changes in the number of RBCs are usually associated with changes in oxygen supply induced by chemical stressors and other factors [72]. Overall, the RBC count was similar between all of the treatments and the control, denoting no effects from the mixtures on these target cells. However, there was an increase in erythrocyte counts in MAC + NP compared with Rem, suggesting an increase in oxygen demands in MAC + NP, which demonstrates the adaptive responses of fish to challenging conditions. Witeska (2005) linked the increase in RBC to fish adaptive responses after short-term exposure to metals [72], which shows their ability to maintain the uptake of oxygen from the environment [73]. Similar to RBC, Hb levels were constant among treatments. Despite the fact that haematological parameters are reliable for assessing fish health, they display different responses depending on the stressor, fish species, and exposure [72,74,75]. For instance, the common carp (*Cyprinus carpio*) showed increasing levels of RBC and Hb after exposure to several metals, including Cd and Pb [74], as did tench (*Tinca tinca*) after short-term sub-lethal exposure to Hg [76]. Decreasing levels of RBC and Hb were reported for grey mullet (*Mugil cephalus*) after exposure to Cd and Pb [75], while the common carp showed no changes in RBC [72]. The presence of Ag NP

induced a decrease in RBC and Hb in the Caucasian scrapper (*Capoeta capoeta*) [77], but the Ag NP concentration tested was 1000 times higher than that used in this work, suggesting that the low levels tested were unlikely to induce changes in these parameters.

Contrary to RBC and Hb, MCH was decreased in MAC + NP treatments relative to the control. Low MCH levels suggest a limitation in Hb synthesis and thus lower Hb levels at each RBC, which does not reflect the total Hb concentration as the body responds with a slight increase in RBC. The increase in RBC counts at MAC + NP may indicate a mechanism through which fish compensate for poor oxygen uptake. Some authors found that the exposure of fish to metals (e.g., Cd) reduced the haematopoietic ability of the kidney [75,78]. In general, the metallic burden of the kidney was higher in MAC conditions relative to the control, which might compromise this organ's performance.

Overall, the MAC treatments did not induce significant changes in the haematological dynamics of turbot, which could be due to their realistic levels and/or the short-term exposure. In addition, we only assessed a few of the several parameters that could provide an early warning for fish health impairment following exposure to chemical stressors. The changes in MCH levels suggest that the effects of these stressors on the kidney should be further investigated.

5. Conclusions

The biosorption remediation process using non-living and non-chemically treated powders of *Ulva lactuca* and *Fucus vesiculosus* proposed by Figueira and co-authors [8] was efficient in the removal of potential toxic metals (Hg, Pb) from saline waters, with removal efficiencies of up to 99% for Hg and up to 86% for Pb. This improvement in the chemical status of the water efficiently reduced the bioaccumulation potential of the tested metallic mixture, evidenced by the significant decrease of these elements in the blood and kidney of turbot. Although this remediation strategy prevented the bioaccumulation potential of the non-remediated treatments, the improvement in the turbot health status was not so evident. Overall, the non-remediated treatments (MAC) showed no genotoxicity or marked cytotoxic potential. The slight changes in haematological dynamics suggest that prolonged exposure to this mixture (MAC) may induce changes in kidney and overall erythropoiesis, while maintaining optimal conditions in the remediated treatments. The main reason for not being able to signal improvements in the physiological status of the fish is that MAC treatments (pre-remediation levels) showed no significant effects, suggesting that the currently set effluent limits should be adjusted to maintain fish health status. This study highlights that the validation of new remediation methods should comply with their ability to reduce body burdens and maintain or improve the health of aquatic organisms. Furthermore, it highlights that the evaluation of remediation methods should weigh process costs against the benefits to organisms, which, as this work shows, is not an easy equation to establish, despite the advocated benefits of the many strategies described in the literature.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15081564/s1, Figure S1: Scanning transmission electron microscope (STEM) images of silver nanoparticles (Ag NP) dispersion (stock supension, scale 150 nm).

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