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Copper and Chromium toxicity is mediated by oxidative stress in *Caenorhabditis elegans*: The use of nanoparticles as an immobilization strategy

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ABSTRACT

Environmental contamination by heavy metals (HMs) has impelled searching for stabilization strategies, where the use of zero-valent iron nanoparticles (nZVI) is considered a promising option. We have evaluated the combined effect of Cu(II)-Cr(VI) on two *Caenorhabditis elegans* strains (N2 and RB1072 sod-2 mutant) in aqueous solutions and in a standard soil, prior and after treatment with nZVI (5% w/w). The results showed that HMs aqueous solutions had an intense toxic effect on both strains. Production of reactive oxygen species and enhanced expression of the heat shock protein Hsp-16.2 was observed, indicating increased HM-mediated oxidative stress. Toxic effects of HM-polluted soil on worms were higher for sod-2 mutant than for N2 strain. However, nZVI treatment significantly diminished all these effects. Our findings highlighted *C. elegans* as a sensitive indicator for HMs pollution and its usefulness to assess the efficiency of the nanoremediation strategy to decrease the toxicity of Cu(II)-Cr(VI) polluted environments.

1. Introduction

Growing concern in recent years about environmental contamination by heavy metals (HMs) and the associated risks to humans, animals and plants has led to a search for different HM stabilization strategies (Liu et al., 2018). HM pollution is caused by several factors, including the increasing anthropogenic release of HMs, cumulative behaviour, and little or no biodegradation of HMs (Fajardo et al., 2019a). HMs, including copper (Cu) and chromium (Cr), are introduced into the environment by anthropogenic contamination from several sources, such as mining, the chemical industry, atmospheric deposition and agriculture (Tosco et al., 2014; Xu et al., 2017). Cu is one of the most widely used metals in the world. Cu is an essential micronutrient for animals and plants but is toxic at high concentrations (Feng et al., 2018). Cu is introduced into the environment as an effluent from activities such as the use of wastewater for irrigation, mining, herbicides and inorganic fertilizers (Smith, 2009). Cr is a highly abundant element on Earth and is found in various oxidation states, although the most common species in natural environments are hexavalent Cr(VI) and trivalent Cr(III) (Wuana and Okieimen, 2011). Cr(VI) is the predominant form found in contaminated soils and is produced by activities such as electroplating, steelmaking, mining, tanning, cement production, metal processing, textile manufacturing, the production of paint pigments and dyes, or wood conservation (Dhal et al., 2013). In addition, Cr spreads easily beyond an initial contamination site through aquatic systems and groundwater (Dhal et al., 2013).

As stated above, HMs are highly toxic compounds that accumulate in the soil and can be harmful to the ecosystem and humans (Zhao et al., 2016; Xue et al., 2018; Alengebawy et al., 2021; Masri et al., 2021). In this context, both Cu and Cr are included in the United States Environmental Protection Agency Priority List. HMs have been reported to interfere with many physiological processes, resulting in excessive

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production of reactive oxygen species (ROS), enzymatic and cellular alterations, and damage to the cell membrane and DNA structure (Bruins et al., 2000). Furthermore, mixed HM contamination is typically found in environmental samples, and interactions between the different contaminants (and the respective matrix), creates potential synergistic effects that can increase toxicity (Moyson et al., 2019). For instance, Li et al. (2018) recently reported that the specific combination of Cu and Cr had synergistic toxic effects on the *Brassica napus* plant.

Altogether, this scenario has led to a search for different HM stabilization strategies to reduce their bioavailability and, consequently, their harmful effect on organisms. Nanoremediation has emerged among these techniques as a promising methodology for the detoxification of contaminated sites (Xue et al., 2018). Zero-valent iron nanoparticles (nZVI) have been one of the most widely used nanomaterials showing significant potential for soil and water remediation (Zou et al., 2016; Li et al., 2017; Jiang et al., 2018; Fajardo et al., 2019b, 2020a). HM immobilization mechanisms by nZVI are mainly based on sorption processes, the formation of surface complexes, or metal reduction (Fajardo et al., 2019b). Although the efficiency of this methodology has been assessed in several research studies (see Latif et al., 2020 for a detailed review), our nanotoxicological knowledge remains inadequate. Therefore, ecotoxicological studies are required to analyse the effects of both HM and HM-nZVI complexes on aqueous and terrestrial media to prevent undesirable consequences (Saccà et al., 2014).

Caenorhabditis elegans is a soil-dwelling bacterivorous nematode that is a very attractive model for ecotoxicological studies because of the following characteristics: a short life cycle (3 days at 20 °C from zygote to adult hermaphrodite), a short lifespan (approximately 20 days), a small size (1 mm) and easy culture and maintenance (Leung et al., 2008; Moyson et al., 2019). Furthermore, complete knowledge of the cell lineage of this nematode and its fully sequenced genome (Caenorharditis elegans Sequencing Consortium, 1998) allows for a large number of mutant and transgenic strains.

Several studies have proposed *C. elegans* as a sensitive indicator for pollution of HMs including Cu, Cr, Pb, Cd, Zn (Williams and Dusenbery, 1990; Donkin and Dusenbery, 1993; Wu et al., 2012; Saikia et al., 2014; Jiang et al., 2016; Fajardo et al., 2019b, 2020b). These studies suggested that the biosynthesis of defensive enzymes in response to oxidative stress has evolved as a specific mechanism by which exposed nematodes mitigate the damaging effects of HMs. However, the toxic effect of copper and chromium on *C. elegans* were individually analysed, and to the best of our knowledge, there is a lack of information regarding their combined effects on the nematode in both, aqueous solution, and soil.

In this study, we assessed the combined effect on the nematode *C. elegans* of Cu(II) and Cr(VI) polluted aqueous and terrestrial samples, before and after treatment with nZVI. Classical bioassays were conducted using two *C. elegans* strains: wild-type N2 (Bristol) and RB1072 (*sod-2* mutant). Moreover, possible oxidative damage in response to the toxicity of the HMs was evaluated by measuring ROS production and the expression of the heat shock protein Hsp-16.2 (using the transgenic strain TJ375 gpls1). Therefore, using *C. elegans* bioassays to perform a biological evaluation of the mixed toxicity effects of Cu(II)-Cr(VI) on polluted soil/water systems can provide useful knowledge on the efficiency and ecosafety of nZVI as a remediation strategy for HM-contaminated sites.

2. Material And methods

2.1. Caenorhabditis elegans strains and culture

Three strains of *C. elegans* were used in this study: a Bristol N2 (wild-type) strain, a RB1072 [*sod-2 (ok1030) I*] strain that carries the mutant allele *sod-2*, and a transgenic strain TJ375 gpls1 (*hsp-16.2p::GFP*) with an *hsp-16.2* promoter gene fused to a GFP reporter that possesses inducible fluorescence. The strains were obtained from the *Caenorhabditis* Genetic Center (University of Minnesota, St. Paul, MN, USA), which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440).

All the strains were placed in plates containing a nematode growth medium (NGM, 17 g agar, 3 g L^{-1} NaCl, 2.5 g peptone, 1 mL 1 M $CaCl_2$, 1 mL of 5 mg mL^{-1} cholesterol, 1 mL 1 M $MgSO_4$, and 25 mL 1 M potassium phosphate buffer at pH 6), seeded with the *Escherichia coli* strain OP50 as a food source for the nematodes and maintained at 20 °C (Brenner, 1974). Agar chunks from plates with large numbers of larvae in the dauer stage (a stage of the life cycle that appears during periods of food scarcity or overpopulation) were transferred to fresh plates with NGM seeded with OP50. After three days, numerous adult hermaphrodites were found on the plates, as well as large numbers of larvae in the juvenile stages L1-L2. Worm synchronization was performed by washing the plates and filtering the worms with an M9 medium (Na_2HPO_4, KH_2PO_4, NaCl, and $MgSO_4{\cdot}7\ H_2O$ in deionized water plus cholesterol at 5 μg mL^{-1}). A 10-μm nylon mesh filter (Merck Millipore Ltd. Cork, Ireland) was used that retained all the nematodes except those in juvenile stages L1-L2. The initial length of 100 L1 larvae randomly selected from both strains was measured (N2: 254.3 ± 3.0 μm, *s.e.m.*; RB1072: 229.2 ± 5.1 μm, *s.e.m.*) to evaluate worm growth.

2.2. Zero-valent iron nanoparticles (nZVI)

NANOFER 25 S iron nanoparticles (nZVI) were synthesized and commercially supplied by NANO IRON s.r.o. (Rajhrad, Czech Republic) as an aqueous dispersion of stabilized nZVIs (coated with sodium polyacrylate, 3%). According to the supplier, the nZVIs are 70–90% Fe(0) and 10–30% iron oxides. Additional details on the physical and chemical characteristics of nZVIs are available at www.nanoiron.cz. Size of NPs determined using high-resolution transmission electron microscopy (HR-TEM; JEM-2011; Jeol, Japan, operating at 200 keV) showed a particle size range between 61 and 71 nm in diameter. A drop of the nZVI (0.2% w/v) suspension was loaded on a TEM grid and air-dried prior to observation.

2.3. Ecotoxicity analysis in aqueous solutions

A solution of $CuSO_4.5\ H_2O$ and $K_2Cr_2O_7$ (Sigma-Aldrich, Spain) was prepared in the M9 medium at final concentrations of 12.5 mM Cu(II) and 4.5 mM Cr(VI) for use in the assays. These values represent the maximum concentrations of Cu and Cr for urban soils according to Spanish regulations (BOCM, 2006). The stock solution was used to prepare dilutions in M9 to obtain solutions at 6.3–2.2, 3.2–1.1, 1.3–0.4, 0.12–0.04, and 0.01–0.004 mM Cu(II)-Cr(VI). The toxicity of these nominal concentrations to *C. elegans* was tested. The metal toxicity to *C. elegans* after nZVI treatment was assessed in parallel. nZVI (5% w/w) was added to each HM solution and incubated at 120 rpm in the dark for 72 h at room temperature. This nZVI concentration was selected according to the ranges employed by previous environmental remediation studies and considering it showed no effect on *C. elegans* (survival, reproduction or growth endpoints), both in aqueous solution or when added to different soil samples (Saccà et al., 2014; Fajardo et al., 2019b). Afterwards, the samples were centrifuged at 12000 rpm for 20 min, and the supernatants were collected to analyse the residual toxicity of Cu (II)-Cr(VI).

2.3.1. Acute toxicity (24 h)

The acute toxicity of the Cu(II)-Cr(VI) solutions (12.5–4.5, 6.3–2.2, 3.2–1.1, 1.3–0.4, 0.12–0.04, and 0.01–0.004 mM, untreated and treated with nZVI) was assessed from *C. elegans* survival at 24 h of exposure (Yang et al., 2016). The experiments were carried out using 12-well plates (BioLite, Thermo Fisher Scientific, Rochester, NY, USA). A volume of 0.5 mL of each Cu(II)-Cr(VI) solution, 0.5 mL of *E. coli* OP50 (2.5×10^{10} cell mL^{-1}, M9) and 10 L4 larvae were added to each well. Wells containing 0.5 mL of M9, 0.5 mL of OP50 and 10 L4 larvae were used as controls. The plates were then incubated at 20 °C in the dark for 24 h.

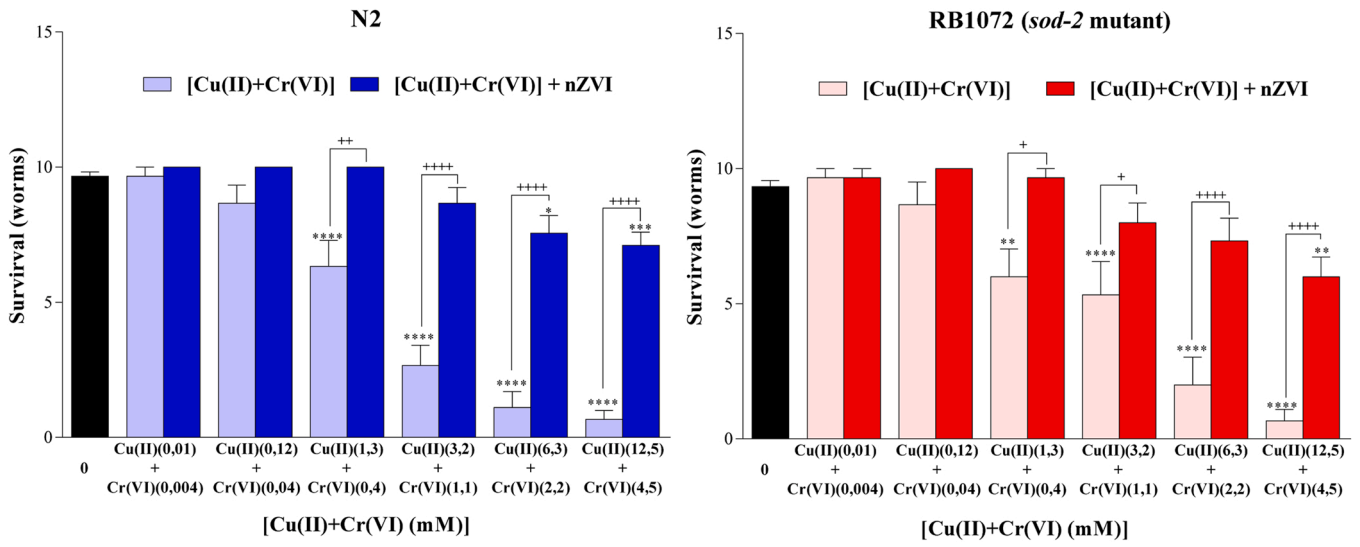

Fig. 1. Acute toxicity (24-h exposure) of aqueous Cu(II)-Cr(VI) solutions (untreated and treated with 5% w/w nZVI) on worm survival of N2 (left) and RB1075 strains (right); data are expressed as mean ± standard error of the mean (n = 4); * ** * $P < 0.0001$; * * $P < 0.01$, compared to result of control (0); $^{++++}$ $P < 0.0001$; $^{++}$ $P < 0.01$; $^{+}$ $P < 0.05$, compared to result for untreated sample.

After this period, the live nematodes in each well were counted using a stereoscopic microscope. Worms that moved when pushed with a fine wire (50 μm) were considered alive. Nematodes of the N2 and RB1072 strains were used in the experiments.

2.3.2. Toxicity (96 h)

We assessed the effects of Cu(II)-Cr(VI) solutions (12.5–4.5, 6.3–2.2, 3.2–1.1, 1.3–0.4, 0.12–0.04, and 0.01–0.004 mM, untreated and treated with nZVI) for 96 h on three *C. elegans* ecotoxicological endpoints: growth, reproduction and survival (Höss et al., 2009; ISO 2, 1087, 2010). Worms of N2 and RB1072 strains and 12-well plates were used, as described above. Then, 0.5 mL of each HM solution, 0.5 mL of *E. coli* OP50 (2.5×10^{10} cells mL^{-1}, M9) and 10 L1 larvae were added to each well. Wells containing 0.5 mL of M9, 0.5 mL of *E. coli* OP50 and 10 L1 larvae were used as controls. The plates were incubated at 20 °C in the dark for 96 h, after which 0.5 mL of Rose Bengal solution (0.4 g L^{-1}) were added to each well to stain the worm cuticle. The plates were exposed to 80 °C for 10 min to terminate the experiment. Worm growth, reproduction and survival were determined using a stereomicroscope. Growth (μm) was determined by subtracting the initial mean length of the nematodes (see above) from the final length after incubation. Reproduction (the number of offspring per adult) was assessed as the counted number of juvenile offspring (L1) divided by the number of introduced worms. Survival was evaluated as the counted number of exposed test organisms that were recovered.

2.4. Measurement of intracellular reactive oxygen species (ROS) in C. elegans

The intracellular levels of ROS were measured using a 2′, 7′ dichlorofluorescein-diacetate probe (H_2DCF-DA, Sigma Spain) according to Yoon et al. (2018) with slight modifications. In brief, L1 larvae of N2 or RB1072 strains were obtained by filtration as a suspension of 50 L1 per 10 μL in the M9 medium. Black plates with 96 wells each (Nunclon Delta Surface, Nunc, Thermo Fisher Scientific, Roskilde, Denmark) were used. Forty microlitres of each Cu(II)-Cr(VI) solution (12.5–4.5, 6.3–2.2, 3.2–1.1 mM, untreated and treated with 5% w/w nZVI), 50 μL of H_2DCF-DA (final concentration of 50 μM) and 10 μL of the L1 suspension were transferred to the wells. The control wells contained 50 μL of M9 and 50 μL of H_2DCF-DA. The experiment was performed in duplicate. Fluorescence was measured (at a 485-nm excitation and a 535-nm emission) on a Tecan Genios plate reader (Tecan Group Ltd., Männedorf, Switzerland). Six readings were taken at hourly intervals. The results were expressed as a percentage of the fluorescence (relative fluorescence units, RFU) emitted by paraquat (5 mM), an intracellular free radical generating compound (Castello et al., 2007).

2.5. Measurement of Hsp-16.2 expression in C. elegans

The TJ375 gpls1[*hsp-16.2p::GFP*] transgenic strain was used to visualize the expression of *hsp-16.2* by the fluorescence of the GFP reporter using published protocols (Pastuhov et al., 2017). Worms were collected in 1.5-mL vials from plates to obtain a suspension of 1000 worms per mL in the M9 medium. The vials were centrifuged (2700 rpm, 2 min), the supernatant was discarded, and the vials were washed three times with 1 mL of M9. Then, 10 μL of the pellet with the worms was added to a 1.5-mL tube containing 500 μL of 6.3–2.2 mM Cu(II)-Cr(VI) solution (untreated or treated with nZVI) and rotated at room temperature (2 h). The tube was centrifuged (2700 rpm, 2 min), and the supernatant was discarded except for ~ 50 μL, which was transferred to an NGM plate. A platinum loop was used to transfer several worms onto a slide with a 2% agarose pad at the centre and 10 mM sodium azide as an anaesthetic for the worms. The expression of Hsp-16.2 was assessed by observing the fluorescence of the GFP reporter protein with an epifluorescence microscope (Leica, Wetzlar, Germany) using a suitable filter (for a 480-nm excitation and a 510-nm emission).

2.6. Ecotoxicity analysis in soil

Standard Lufa 2.2 soil (LUFA Speyer, Germany) was used in the experiments. The basic characteristics of this sandy loam soil are as follows: total organic carbon, 0.63%; nitrogen, 0.05%; pH 5.6; cation exchange capacity, 8.5 meq/100 g; and water holding capacity, 43.3 g/100 g (values referred to dry matter).

Five hundred grams of Lufa 2.2 soil was artificially contaminated with 12.5–4.5, 6.3–2.2, 3.2–1.1, 1.3–0.4, 0.12–0.04, and 0.01–0.004 mM Cu(II)-Cr(VI). The HMs were added to the soil as aqueous solutions and mixed by hand. The humidity was adjusted to 22% (w/w) in all the samples, which were then stored in the dark at room temperature for 45 days. After this period, 150 g of each soil sample was weighed, and nZVI (5% w/w) was added; each sample was then manually mixed and stored in the dark for 72 h.

Bioassays were performed to assess the effect of polluted soil samples (untreated and treated with nZVI) on the worms. The toxicity at 96 h

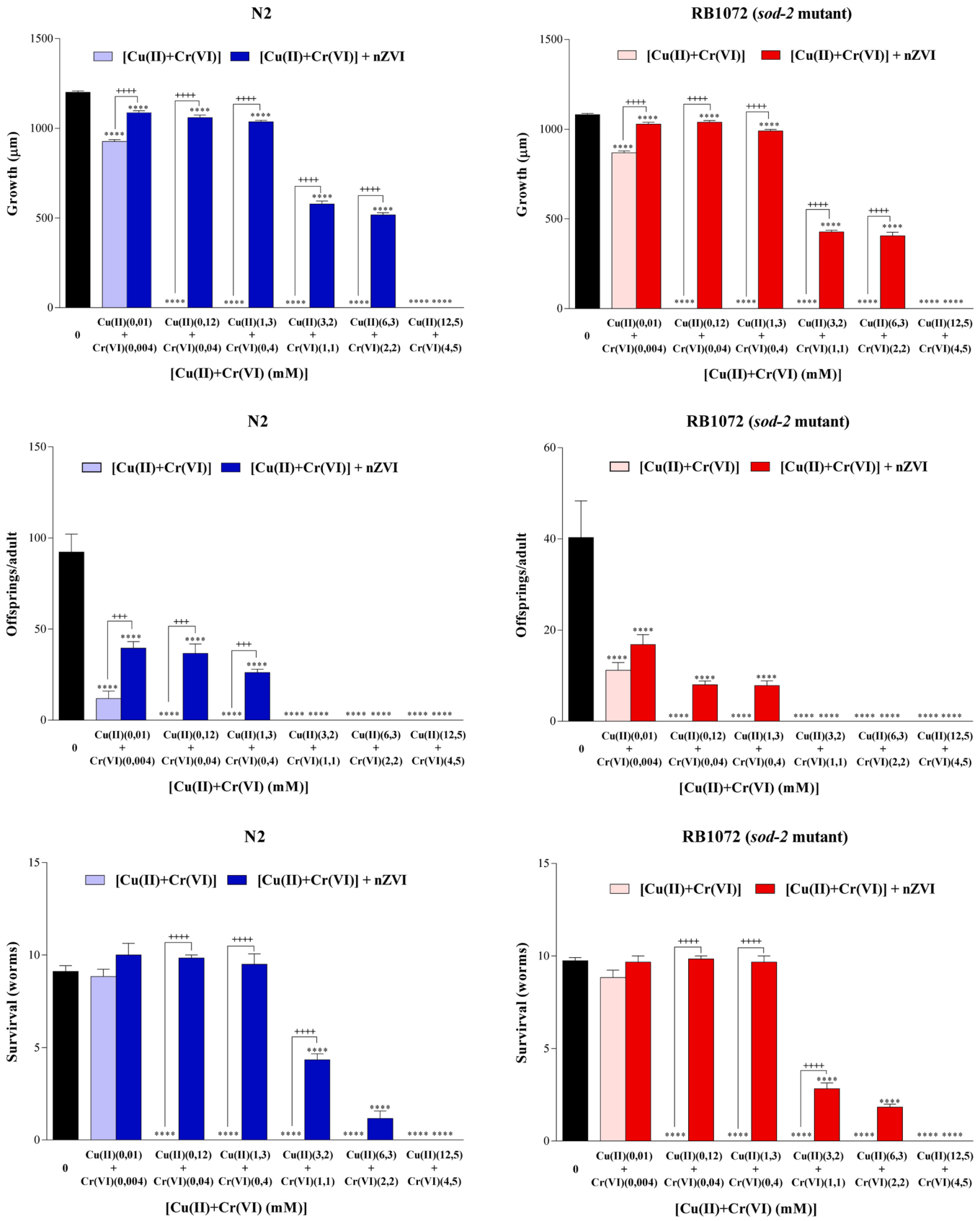

Fig. 2. The 96-h effect of aqueous Cu(II)-Cr(VI) solutions (untreated and treated with 5% w/w nZVI) on worm growth, reproduction and survival of N2 (left panel) and RB1075 strains (right panel); data are expressed as mean ± standard error of the mean (n = 4); * *** * $P < 0.0001$, compared to result of control (0); $^{++++}$ $P < 0.0001$, compared to result for untreated sample.

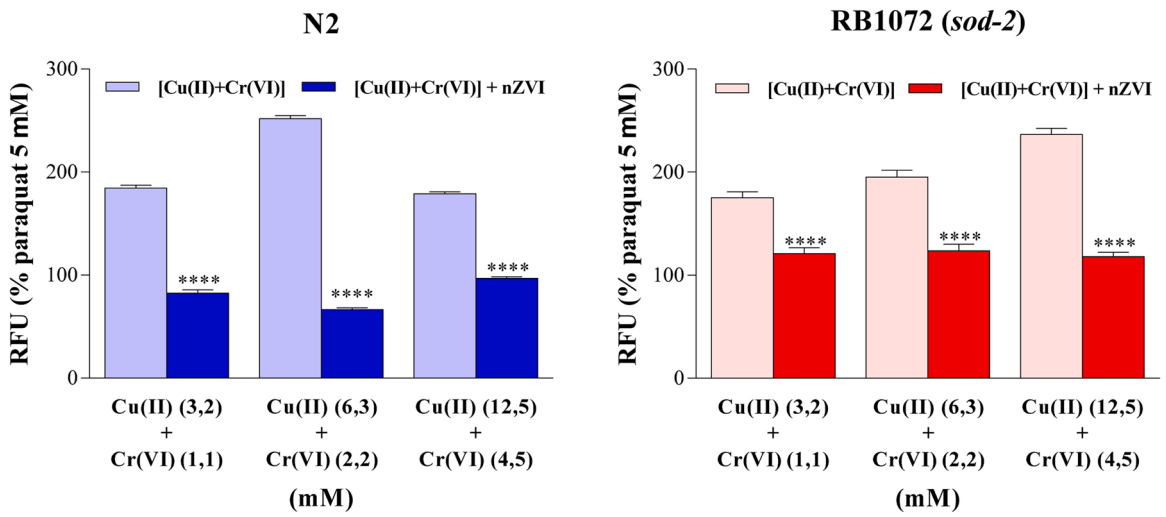

Fig. 3. Effect of aqueous Cu(II)-Cr(VI) solutions (untreated and treated with 5% w/w nZVI) on reactive oxygen species (ROS) accumulation in *C. elegans* N2 and RB1072 (*sod-2* mutant) strains, where results are expressed as relative fluorescence units (RFU) and as a percentage of the fluorescence emitted by paraquat (5 mM); two independent experiments were performed with four replicates per treatment (n = 50); data are shown as mean ± standard error of the mean; * ** * P < 0.0001, compared to result for untreated sample.

was measured according to published protocols (ISO, 2010; Fajardo et al., 2015). Worms of N2 and RB1072 strains were used. Plate wells containing 0.3 g of soil, 0.2 mL M9, 0.5 mL *E. coli* OP50 (2.5×10^{10} cell mL^{-1} M9) and 10 L1 larvae each were incubated at 20 °C in the dark for 96 h. After this period, 0.5 mL of Rose Bengal solution (0.4 g L^{-1}) was added to each well, and the test was terminated by heat killing the worms at approximately 80 °C for 10 min. The nematodes were recovered from the soil using tap water and a Pasteur pipette (ISO, 2010). Briefly, the soil was extracted from each well by washing with 5 mL of water. The suspension was transferred to a centrifuge tube, mixed vigorously, and centrifuged (2900 rpm, 5 min). The supernatant was discarded, and the pellet containing soil and nematodes was resuspended in 2 mL of a mixture (1:2) of colloidal silica (Ludox TM50, Sigma, Spain) and deionized water. The tube was shaken vigorously and centrifuged (2900 rpm, 5 min) to remove the nematodes. The supernatants from three extractions per tube (6 mL) were pooled to perform the worm analysis. The ecotoxicological endpoints of growth, reproduction and survival were quantified as previously described.

2.7. Statistical analysis

All the data are expressed as the mean ± standard error of the mean (*s.e.m.*). A statistical analysis was performed using GraphPad Prism 5 software (San Diego, Ca, USA). Significant differences between groups were determined using an analysis of variance (one-way ANOVA) followed by the Tukey test of multiple comparisons. $P < 0.05$ was considered significant.

3. Results and discussion

3.1. Toxicity of aqueous Cu-Cr solutions on C. elegans. Impact of nZVI treatment

Adults worms exposed to the highest Cu(II)-Cr(VI) concentrations, in aqueous solution, showed significant acute toxicity manifested through a pronounced inhibitory effect on *C. elegans* survival in both the N2 and RB1072 strains after 24 h of exposure (Fig. 1). This negative effect almost disappeared at Cu-Cr concentrations equal to or lower than 0.12–0.04 mM (Fig. 1). Likewise, the effect of aqueous HMs solutions (untreated and treated with nZVI) was studied after 96 h of exposure, and clear negative responses were found (Fig. 2). Even at concentrations as low as 0.12 mM Cu(II) and 0.04 mM Cr(VI), HM completely eliminated growth, reproduction and survival in both strains of worms. Therefore, the results showed that combined Cu(II)-Cr(VI) pollution in aqueous samples produced significant toxic effects on the two *C. elegans* strains studied, and this negative impact was observed over a wide range of concentrations. These findings are consistent with literature data on the negative impact of Cu and Cr, when considered individually, on different ecotoxicological parameters (Shen et al., 2009; Jiang et al., 2016), and the probable synergistic effect of mixed HMs on *C. elegans*.

By contrast, treating the Cu(II)-Cr(VI) solutions for 72 h with nZVI (5% w/w) significantly changed the effects of HMs on both strains of *C. elegans*. A decrease in acute toxicity and a highly significant reversal of worm survival compared to the untreated solutions was observed in both the N2 and RB1072 strains (Fig. 1). The results were even more evident after 96 h of exposure (Fig. 2); the pronounced toxic effect of the untreated solutions (except at the lowest Cu(II)-Cr(VI) concentration tested) on worm growth and survival was reversed by all the nZVI-treated solutions (except at the highest Cu(II)-Cr(VI) concentration assessed), even reaching the control values in worms from both strains. However, the effects on worm reproduction were not reversed for most HM-concentrated samples (12.5–4.5, 6.3–2.2, and 3.2–1.1 mM Cu(II)-Cr (VI)). This result can be explained considering that the observed increase in the nematode growth in these solutions did not reach the value (~1000 μm, L4-adult) required for reproductive capacity.

Therefore, the decreased toxicity found in the samples treated with nZVI showed that the bioavailability of the HMs in the tested samples may have been efficiently reduced. Similar results have recently been reported, showing that nZVI can immobilize metals, such as lead, cadmium, and zinc, in aqueous solution (Fajardo et al., 2019b). The removal mechanisms of HMs by nZVI were reported to be mainly based on the metal's redox potential (Chen et al., 2016). Sorption to nZVI surface, or complex formation, was responsible for immobilization of HMs with a standard potential (E0) lower than elemental iron (−0.41 V); in addition to sorption, nZVI can reduce and thus precipitate HMs with an E0 higher than that of elemental iron. Thus, the nZVI-mediated reduction of Cu(II) and Cr(VI) ions is the most likely removal mechanism, because both Cu and Cr are clearly more electropositive than Fe.

3.2. HM-induced oxidative stress in C. elegans. Impact of nZVI treatment

The oxidative stress defines any condition in biological systems where there is an excessive production of ROS or an insufficient antioxidant defence that produces oxidative damage. The main ROS are the

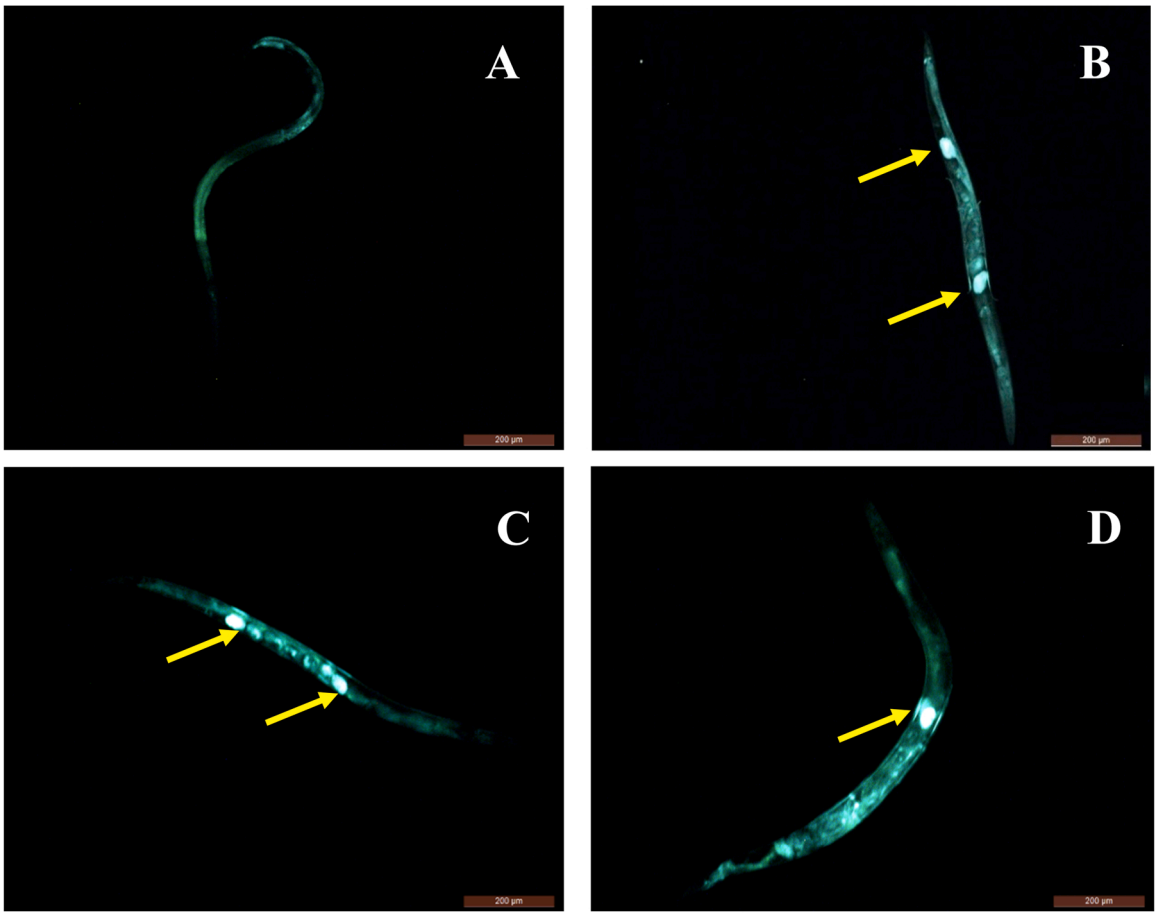

Fig. 4. Effect of Cu(II)-Cr(VI) aqueous solutions before and after treatment with 5% w/w nZVI on localization of Hsp-16.2 protein in worms of *C. elegans* transgenic strain TJ375 gpls1 (*hsp-16.2p:: GFP*): A) control (without HM); B) paraquat (5 μM); C) Cu (6.3 mM)-Cr (2.2 mM); D) Cu (6.3 mM)-Cr (2.2 mM) treated with nZVI; arrows indicate Hsp-16.2 expression sites.

superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\cdot}$) (Labuschagne and Brenkman, 2013). High concentrations of HMs increase the ROS and, consequently, oxidative damage (Feng et al., 2018; Mahmud et al., 2018).

In this study, we analysed whether Cu(II)-Cr(VI) solutions before and after treatment with nZVI generate ROS and, therefore, oxidative stress in the *C. elegans* strains N2 and RB1072 (*sod-2* mutant). Experiments were performed using a 2´,7´-dichlorofluorescein diacetate (H_2DCFDA) probe, which is oxidized inside a cell in the presence of ROS to produce a highly fluorescent compound (Labuschagne and Brenkman, 2013; Yoon et al., 2018). Fig. 3 shows that the most concentrated HM solutions (12.5–4.5, 6.3–2.2 and 3.2–1.1 mM Cu(II)-Cr(VI)) caused ROS generation in worms from both the N2 and RB1072 strains. These results indicated that the toxic effects of Cu(II)-Cr(VI) on the *C. elegans* ecotoxicological endpoints were significantly mediated by the generation of ROS, in accordance with the results of Zhou et al. (2017), showing that mixed toxicities of various HMs evoked the generation of intracellular ROS. Fig. 3 shows a decrease in ROS when the Cu(II)-Cr(VI) solutions were treated with nZVI, indicating that metal immobilization by nZVI translated into a significant decrease in the oxidative stress.

In response to HM-induced ROS production, organisms activate defence mechanisms to reduce oxidative damage, including oxidative enzyme induction (Song et al., 2014). The first enzymatic defence against ROS increase is superoxide dismutase (SOD), which reduces the superoxide anion ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) (Labuschagne and Brenkman, 2013). Increased SOD activity in response to high HM concentrations has been previously observed in the *C. elegans* nematode (Song et al., 2014, 2019). Five genes encoding SOD have been found in *C. elegans*: two (*sod-1*, *sod-5*) in the cytoplasm, two (*sod-2*, *sod-3*) in the mitochondria, and one (*sod-4*) extracellular gene (Van Raamsdonk and Hekimi, 2012).

Thus, it could be inferred that the deletion of genes encoding any SODs in *C. elegans* will increase oxidative damage; the responses of worms containing the deletion will be affected by a decrease in SOD-mediated defence mechanisms. In this study, we used strain RB1072, a *sod-2* mutant, to evaluate stress-mediated molecular responses to Cu-Cr exposure. Our results indicated significantly lower growth and reproduction in the mutant *sod-2* strain than in the N2 strain, suggesting that weaker enzymatic defence mechanisms than those of the wild types resulted in higher sensitivity to both metals (Fig. 2). Surprisingly, worm survival was not significantly different between strains, suggesting that *sod-2* deletion increases oxidative damage but does not affect the lifespan, as previously reported by Van Raamsdonk and Hekimi (2009).

Increased ROS enhances the production of antioxidant enzymes and other molecules, such as heat shock proteins (Hsps). Hsps are induced when organisms encounter hostile environments which enable cells to maintain normal physiological activities (Song et al., 2019). The overexpression of genes encoding Hsp has been reported to occur as a general defensive response to the environmental oxidative stress generated by HMs (Kumar et al., 2015). Among the considered Hsps, Hsp-16.2 is an anti-stress reporter protein that plays a key role in longevity and is strongly expressed in the intestine and pharynx of *C. elegans* under thermal or environmental stress (Shen et al., 2009; Saikia et al., 2014). In this study, worms of the transgenic strain TJ375 gpls1 (*Hsp-16.2p:: GFP*) were used to monitor the stress response to Cu(II)-Cr(VI) solutions (untreated and treated with nZVI) in terms of Hsp-16.2 expression. The

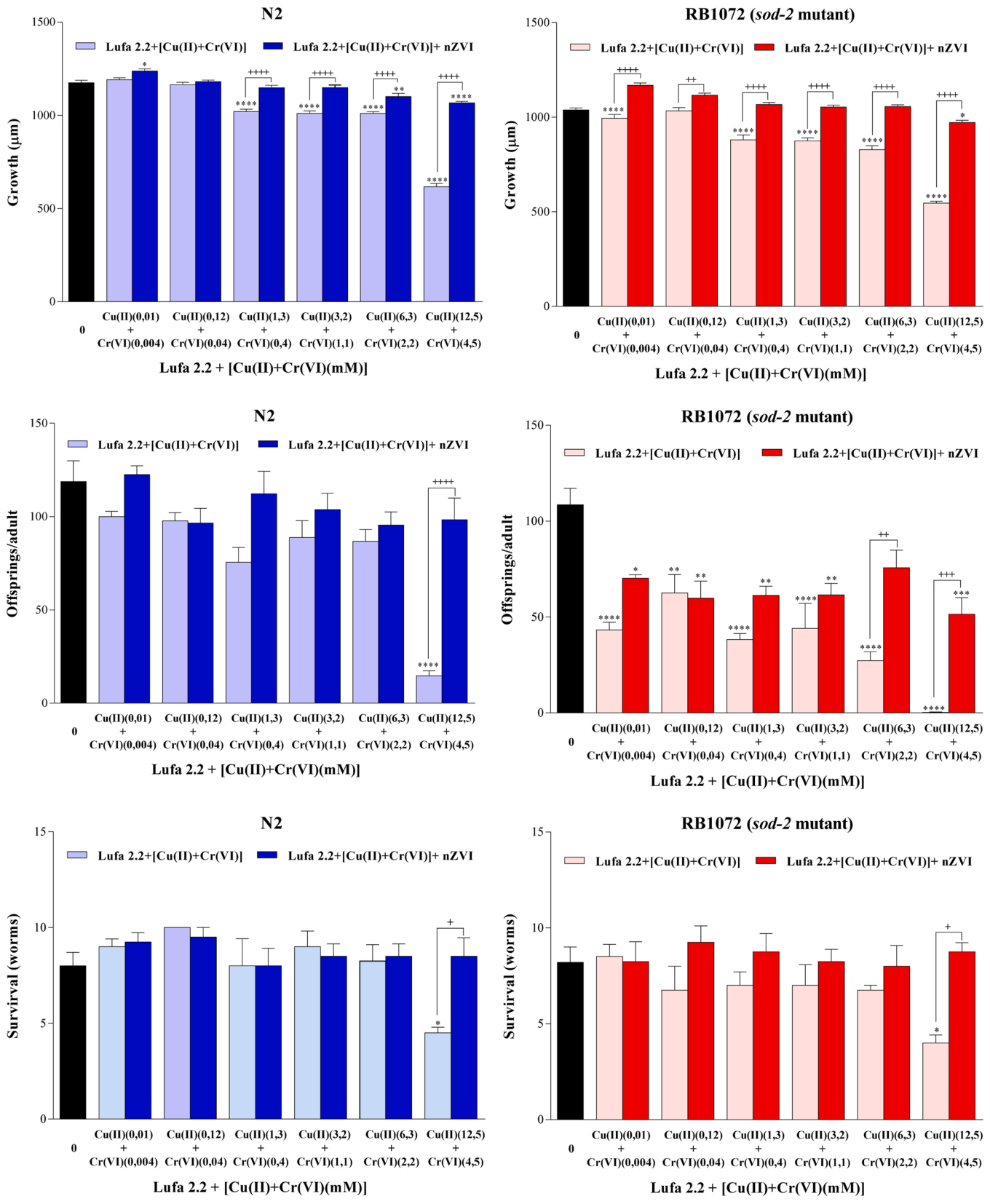

Fig. 5. Effect of Cu(II)-Cr(VI) polluted soil (untreated and treated with 5% w/w nZVI) on growth, reproduction and survival of *C. elegans* strains N2 (left panel) and RB1072 (*sod-2* mutant) (right panel); data are expressed as mean ± standard error of the mean (n = 4); * ** * $P < 0.0001$; * * $P < 0.01$; * $P < 0.05$, compared to result for control (0); $^{++++}$ $P < 0.0001$; $^{++}$ $P < 0.01$; $^{+}$ $P < 0.05$, compared to result for untreated sample.

results were compared with those of worms exposed to paraquat (5 mM) used as a positive control. Fig. 4 shows greater fluorescence and thus, enhanced expression of Hsp-16.2, in the pharynx and intestine of nematodes exposed to a high Cu-Cr concentration. The fluorescence did not disappear when the worms were exposed to Cu(II)-Cr(VI) solutions treated with nZVI but was localized to the intestine (Fig. 4).

Altogether, the obtained results clearly showed that mixtures of Cu (II)-Cr(VI) in aqueous media had a significant toxic effect on *C. elegans*, in which ROS increase plays a fundamental role. Contaminant removal after nZVI treatment may reduce toxic responses, which a priori suggests the potential utility of environmental nanotechnology for HM decontamination. However, the ecotoxicity of interactions between these pollutants, nZVI, and more complex matrices, such as soil, is an important subject in evaluating nanoremediation for environmental restoration.

3.3. Ecotoxicological response of *C. elegans* in Cu(II)-Cr(VI) polluted soil. Impact of nZVI treatment

The Fig. 5 shows the effects of Cu-Cr-polluted soil, before and after nZVI treatment, on the *C. elegans* ecotoxicological response. Note that the dramatic toxic effects of aqueous solutions with equivalent HM contents on the nematode growth, reproduction, and survival were less evident within the soil environment; any tested soil sample abolished the endpoints considered. Thus, the physicochemical characteristics of the edaphic matrix would exert a "protective" action diminishing the deleterious effects of HM contamination. HMs can be adsorbed to soil particles, coprecipitated or complexed with organic matter (Wang et al., 2010), whereas only the small fraction of soluble and bioavailable metals produce a toxic effect on exposed organisms. Boyd and Williams (2003) reported LC50 values for Cu from 193 to 818 mg kg^{-1} soil, depending on the soil type, which are within the range used in this study. The *C. elegans* strains exhibited different sensitivities towards Cu (II)-Cr(VI) (Fig. 5). Although the growth of worms from the N2 strain was significantly affected by several concentrations of HMs in the soil environment, only the highest concentrations affected reproduction and survival. By comparison, the *sod-2* mutant strain showed greater sensitivity to HMs in terms of both growth and reproduction. This finding supports the important role of ROS-defensive enzymes, such as *sod*, in mitigating the effect of heavy metals.

The soil treatment with nZVI may immobilize the tested metals (Cu-Cr) thereby reversing their deleterious effects (Fig. 5). These findings are in accordance with previous reports of a protective effect of nZVI against the negative impact of Pb, Cd and Zn on soil organisms (Fajardo et al., 2019b). As above-mentioned, the standard redox potential of Cu and Cr suggested that the nZVI mode of action was likely the heavy metals reduction, decreasing their content in the most available soil fraction. Thus, the efficient removal and decreased bioavailability is associated to the reduced HM toxicity towards *C. elegans* within the soil environment. This fact makes nZVI treatment a method of choice for the remediation of soils (at least in the short term) contaminated by Cu(II)-Cr(VI).

Moreover, these results are in agreement with those obtained for the HM-polluted aqueous solutions, indicating that nZVI can immobilize Cu-Cr in both soil and water systems under the experimental conditions of this study. The nZVI treatment, through its interaction with Cu(II) and Cr(VI), reduced HM-induced toxicity and oxidative stress in *C. elegans* improving growth, reproduction and survival of the nematode.

4. Conclusions

In this study, wild and transgenic strains of *C. elegans* were used to conduct bioassays to assess the environmental stress from co-mingled contamination (Cu-Cr) in aquatic and terrestrial systems. The results obtained indicated that Cu(II)-Cr(VI) mixtures in the soil environment and, particularly, in aqueous solutions had a notably toxic effect on the nematode *C. elegans*, which was related to increased oxidative damage from ROS production. However, the addition of nZVI significantly reversed the toxic effects of Cu-Cr, suggesting these nanomaterials possess a remarkable capacity to immobilize these metals, at least in the short term. Therefore, the ecotoxicologically obtained data provide useful knowledge on the efficiency and ecosafety of nZVI treatment as a remediation strategy for water/soil systems.

Our study highlighted that *C. elegans* is a useful in vivo model for assessing environmental toxicity, particularly for the reliable biomonitoring of the extent of HM pollution. Moreover, ROS-mediated toxicity makes oxidative stress-responsive biomolecules (genes and proteins) promising biomarkers to evaluate the usefulness of nanoremediation in multiple-HM-contaminated environments.

CRediT authorship contribution statement

Carmen Fajardo: Investigation, Writing – original draft. **Carmen Martín:** Investigation, Writing – original draft. **Elena Garrido:** Investigation. **Sebastián Sánchez-Fortún:** Investigation, Writing – original draft. **Mar Nande:** Investigation. **Margarita Martín:** Investigation, Supervision, Writing – original draft. **Gonzalo Costa:** Conceptualization, Investigation, Supervision, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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