1 **Supporting Information**

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3 **Synchrotron XRF analysis identifies cerium accumulation co-localized with pharyngeal**
4 **deformities in CeO_2 NP exposed *Caenorhabditis elegans***

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1. Method

1.1 Nanoparticle stock preparation and characterization

The CeO_2 NP (544841, Sigma Aldrich) stock suspensions were prepared by adding 10 mg L^{-1} CeO_2 NPs to 10 ml ddH_2O (15 MΩ·cm) with 0.2 % Tween 20 (P1379, Sigma Aldrich). The suspensions were vortexed at 3000 rotations/min (MS basic, IKA) for 5 minutes, before being alternately sonicated at 35 kHz (Sonorex RK106, Bandelin) for 5 minutes and vortexed for 10 seconds, for 1 hour. Three replicate working solutions were prepared by diluting 1 ml of the NP suspension 10 x in moderately hard reconstituted water (MHRW, pH 7.7) [1] with 0.2 % Tween 20 (MHRW-T). The aforementioned alternation of vertexing and sonication was repeated for the preparation of the MHRW-T. The working solutions were allowed to settle over night to remove larger aggregates from the suspensions. Aliquot samples were taken from the 5 ml mark, and immediately used for toxicity testing or characterization. For the purpose of comparison, the $Ce(NO_3)_3$ stock solution was prepared in MHRW + Tween 20.

Size distribution of the NPs was measured using Transmission electron microscopy (TEM, Morgagni 268, FEI), Nanoparticle tracing analysis (NTA, Nanosight LM10, Malvern Panalytical), and dynamic light scattering (DLS, Malvern PN3702 Zetasizer Nanoseries, Malvern Inc., Malvern, UK). Image analysis software ImageJ was used for maximum ferret diameter measurements of TEM micrographs. For electrophoretic mobility measurements of the NPs, particles suspensions were measured using dynamic light scattering (DLS, Malvern PN3702 Zetasizer Nanoseries). Size fraction and particle dissolution assessment was carried out at T-0 and 72 hrs of exposure by ultrafiltration (14,000 *g* for 30 min) of the exposure media using pre-conditioned <3 kDa Millipore Centrifugal filters (Amicon, Millipore, Billerica, MA) of low, medium, and a high $Ce(NO_3)_3$ or CeO_2 NP concentration, in triplicate. The contents of the whole exposure well was taken, to include all particles. Prior ultrafiltration samples were centrifuged (2000 g for 5 minutes) to remove the *Escherichia coli* and larger aggregates to avoid clogging of the filter units. Exposure concentrations and size fractions were determined using Inductively coupled mass spectroscopy (ICP-MS, ICP-MS Agilent 8800, Mississauga, Canada) measuring the Ce (140) isotope, at a detection limit of 0.0003 ppm. A house standard (1643H), prepared from the certified refence material 1640a with the addition of a 1 µg L^{-1} Ce from the CRM 71A, was used as a calibration check. A purity check of the CeO_2 NP stock solution showed 97.1% purity of the CeO_2 NPs in ddH_20.

58 1.2 Nematode Culture and Exposure

59 For synchronization of the nematode culture, nematodes previously kept in liquid cultures were
60 treated with hypochlorite solution for egg extraction. Eggs were allowed to hatch over night to
61 obtain synchronized L1 stage nematodes. Synchronized nematodes were exposed for 96 h, in
62 triplicate, in 24 well exposure plates, from L1 stage at 20°C in the dark, with continuous gentle
63 shaking (100 rotations/min). Each well contained 1 ml of *Escherichia coli* OP50 resuspended
64 in MHRW [1], and 11 ± 3 L1 stage nematodes. The N2 Bristol strain was exposed to seven
65 concentrations in the range of 0.5 – 34.96 mg Ce L^{-1}, or 12 concentrations in the range of 2.3
66 – 26 mg Ce L^{-1} of CeO_2 NPs or $Ce(NO_3)_3$, respectively (measured concentrations, see table
67 S1). For the termination of the N2 toxicity test at T-96 h, 500µl of Rose Bengal (616 µM) was
68 added to each well, before heating at 80°C for 10 minutes. For the assessment of growth,
69 fertility and reproduction [2], a stereo microscope (Leica M205C), equipped with digital
70 camera (MC170 HD, Leica), and a hand held tally counter were used.

71 To determine the uptake of Ce, nematodes were exposed in triplicate to either 2.3, 4.19, and
72 614, or 0.99, 8.03, and 34.96 mg Ce L^{-1} of either $Ce(NO_3)_3$ or CeO_2 NPs, respectively, for 72
73 hours. For total body burden nematodes were removed from the exposure and washed three
74 times in 1 ml MHRW (un-depurated nematodes). Parallel replicate nematodes were placed on
75 NGM agar plates seeded with *E. coli*, to assess the Ce fraction tightly bound to the nematodes.
76 Following 2 hours of depuration, nematodes were hand picked off the NGM plates and
77 transferred into 15 ml tubes (depurated nematodes). Supernatant from all samples was
78 evaporated prior ultrapure HNO_3 + 0.02% HF acid digestion at 90°C for 2 hours. To identify
79 possible transfer of Ce from the supernatant during transfer, blank samples containing no
80 nematodes were taken in addition. Samples were diluted to 10 vol % HNO_3 prior ICP-MS
81 analysis. For both, un-depurated and depurated nematode samples, exact numbers of
82 nematodes per individual tubes were recorded, for total Ce/nematode calculations.

83 For the *in vivo* assessment of the Sod-1, HyPer, and GRX strains, nematodes were sampled at
84 72 h of exposure. Samples were analyzed for fluorescence as previously described by Rossbach
85 et al. [3] and Maremonti et al. [4]. Briefly, nematodes were immobilized using 30 mM sodium
86 azide (NaN_3) and imaged on a fluorescent microscope (Leica DM6 B) equipped with a 405 nm
87 excitation and 535 emission filter. For the oxidized to reduced ration of the HyPer and GRX
88 biosensor strains, a second image at excitation 490 nm and emission 535 nm was taken. Ratio
89 calculations were conducted as described by Back et al. [5]. Approximately 10 nematodes were

90 images per triplicate exposure well. Additionally, nematodes were imaged in phase contrast
91 mode at 10x magnification to assess the total body length of the nematodes. For Quantification
92 of fluorescent measurements, the Leica Application Suit X image software was used, for pixel
93 based average intensity. To account for impacts of developmental stage on the SOD-1 signal
94 strength, SOD-1 average intensity was normalized to total body length [6].

95 To test the antioxidant properties of the CeO_2 NPs, the aforementioned Ce exposed GRX
96 nematodes were subjected to a further 10 min acute H_2O_2 (23613.366 VWR Chemicals)
97 exposure (107.7, 213.4, or 323.1 mM). Sampling was conducted as previously described.

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99 ## 2. Results

100 CeO_2 NP characterization

101 Several particle characteristics, including electrophoretic mobility and aggregation, are
102 sensitive to small changes in pH, conductivity, or media composition, and may affect the
103 stability of the particles in the exposure [7]. Therefore, to explain uptake, retention, and toxic
104 effects of NPs, detailed characterization of the particles in their pristine state and in the
105 exposure media is of importance [8].

106 To monitor the behavior of cerium during the exposure, concentrations in the exposure media
107 were quantified by ICP-MS at T - 0 and 72 h (Table S1). Size distributions were relatively
108 constant at 0 and 72 hr with the majority of the Ce being found in the particulate fraction,
109 assumed to be associated with the *E. coli* bacterial cells. The sharp increase in the suspended
110 fraction at 34.96 mg/l is most likely due to a sampling error. Results confirmed no significant
111 difference between measured and nominal concentrations for the $Ce(NO_3)_3$ exposure at the
112 start (T-0) of the exposure (Table S1). However, a 35 – 80 % reduction in total Ce concentration
113 was measured at 72 h in the $Ce(NO_3)_3$ exposure. In comparison, CeO_2 NP concentrations
114 remained relatively stable with a 0.4 – 22 % reduction over the course of the 72 hours exposure
115 period (Table S1). The high degree of Ce removal from the $Ce(NO_3)_3$ exposure is hypothesized
116 to be the results of adhesion of the Ce to the exposure well surface.

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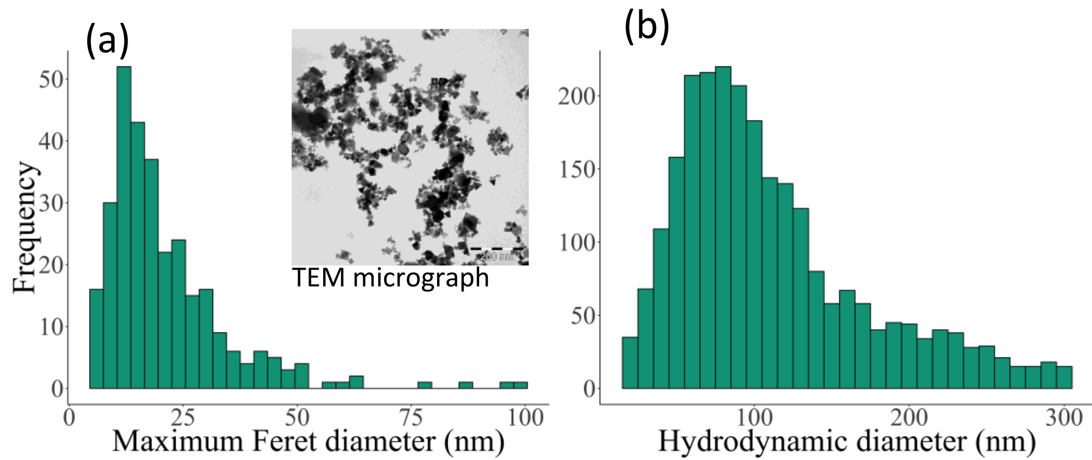

118 **Figure S1:** Histograms of the size distribution of CeO_2 NPs measured using TEM (a) and NTA
119 (b). Note the different range of x-values for the two graphs. The maximum Ferret diameters in
120 (a) are measured from N=300 NPs from 5 TEM micrographs. The hydrodynamic diameters in
121 (b) are measured from 5 videos of 60 seconds, and an upper cut-off at 300 nm was set when
122 plotting the results.

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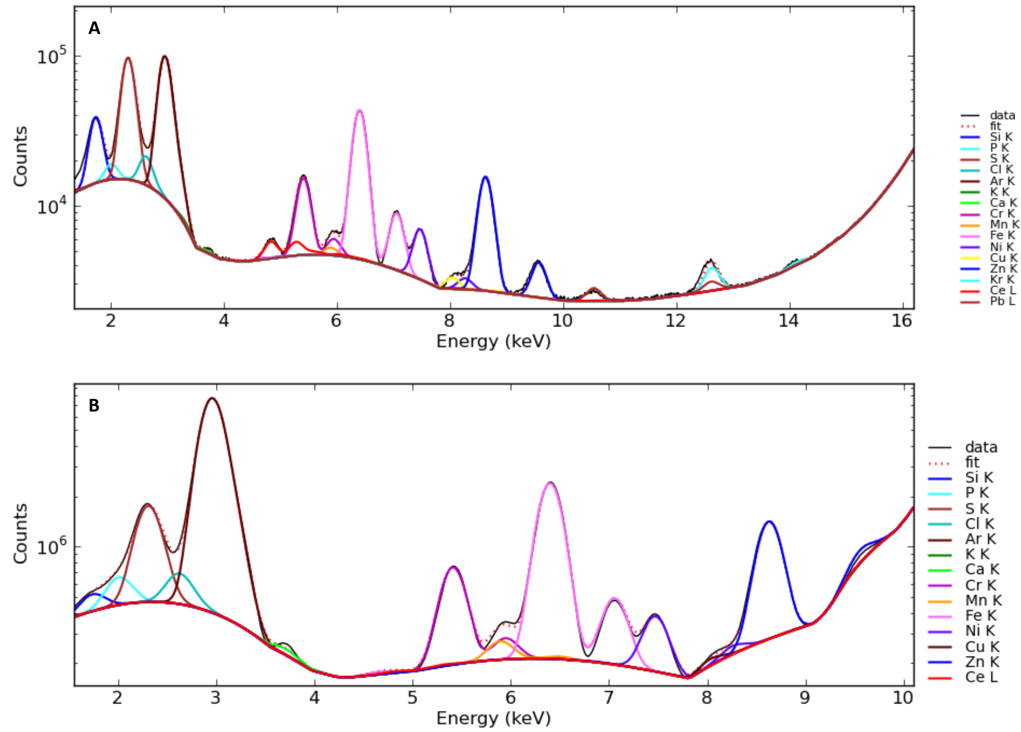

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128 **Figure S2:** Sum XRF spectra of maps of whole intact *Caenorhabditis elegans* exposed to either
129 $Ce(NO_3)_3$ (A) or CeO_2 NPs (B) obtained at the microprobe end-station of the P06 Hard X-ray
130 Micro/Nano-Probe beamline of the PETRA III storage ring of the DESY facility (Hamburg,
131 Germany).

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Table S1: Speciation data for $Ce(NO_3)_3$ or CeO_2 NPs in MHRW + Tween at the start (T – 0) and the end (72 hours) of exposure. Total, suspended, and <3 kDa fraction were analyzed using ICP-MS, while the particulate fraction was calculated as the difference between the total and suspended fraction.

		T-0							72 hours						
		Total		**Suspended**		**<3k Da**		**Particulate**	**Total**		**Suspended**		**<3k Da**		**Particulate**
Exposure	**Nominal concentration (mg/L)**	conc (mg/L)	SD	conc (mg/L)	SD	conc (mg/L)	SD	Calculated (mg/l	conc (mg/L)	SD	conc (mg/L)	SD	conc (mg/L)	SD	Calculated (mg/l)
Control		0,00019	0,00004	0,00000	0,00000	0,00000	0,00001	0,00019	0,00025	0,00009	0,00022	0,00021	0,00014	0,00015	0,00003
$Ce(NO_3)_3$	**2**	2,29809	0,30279	0,03584	0,00459	0,00008	0,00004	2,26225	1,48625	1,05547	0,00569	0,00201	0,00022	0,00020	1,48056
	4	4,18789	0,12378	0,05911	0,05964	0,00002	0,00001	4,12878	N/A	N/A	0,02037	0,01334	0,00058	0,00078	N/A
	6	6,14161	0,28008	0,58100	0,52821	0,00006	0,00002	5,56061	2,08925	0,85947	0,23719	0,29517	0,00008	0,00008	1,85206
CeO_2 NPs	**1.03**	0,99128	0,01215	0,12702	0,01455	0,00003	0,00005	0,86425	1,0144	0,01288	0,01107	0,00076	0,00027	0,00024	0,09037
	8.25	8,02740	0,12549	0,64659	0,08292	0,00007	0,00003	7,38082	6,3242	0,07758	0,11131	0,02129	0,00003	0,00003	0,52110
	33	34,95596	0,42262	1,57788	0,11983	0,00005	0,00006	33,37808	34,83600	1,96401	20,54884	0,74131	0,35360	0,49998	14,28715

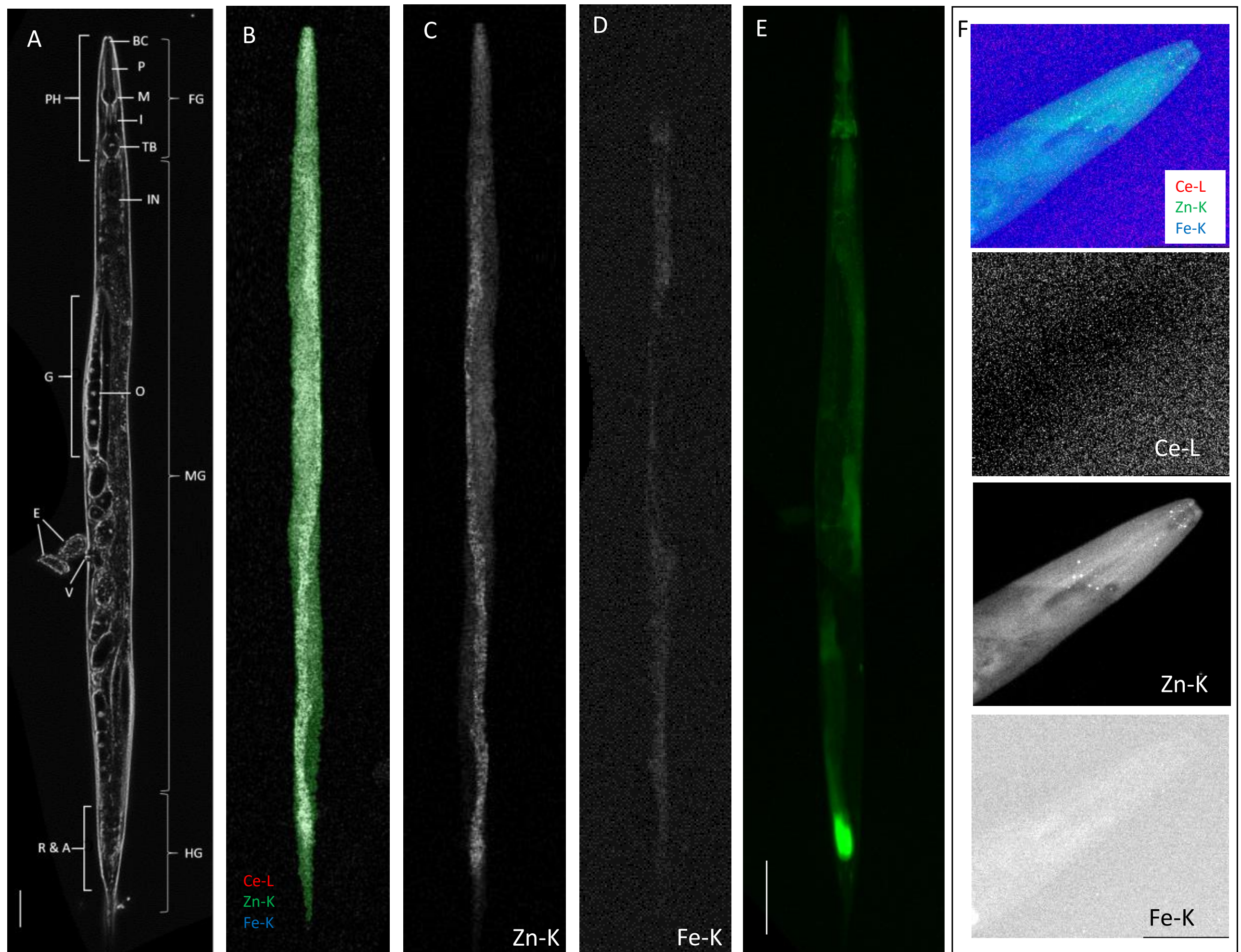

Figure S3: Imaging of an unexposed nematode (control). (A) Phase contrast (PH) microscopy image showing nematode anatomy. The foregut (FG), midgut (MG), hindgut (HG), buccal cavity (BC), procorpus (P), metacorpus (M), isthmus (I), terminal bulb (TB), pharynx (PH), intestine (IN), gonads (G), oocytes (O), vulva (V), rectum (R), anus (A), and eggs (E). (B - D) 2D XRF map (19,5 keV, 1 x 1 µm^2, step size, 7 msec exposure/pt), elemental maps; (B) Ce, Zn, and Fe (composition map), (C) Zn and (D) Fe. (E) *sod-1* gene expression of an unexposed control nematode. (F) 2-D XRF elemental maps of the nematode pharynx showing the nerve ring. Scale bars represent 100 µm.

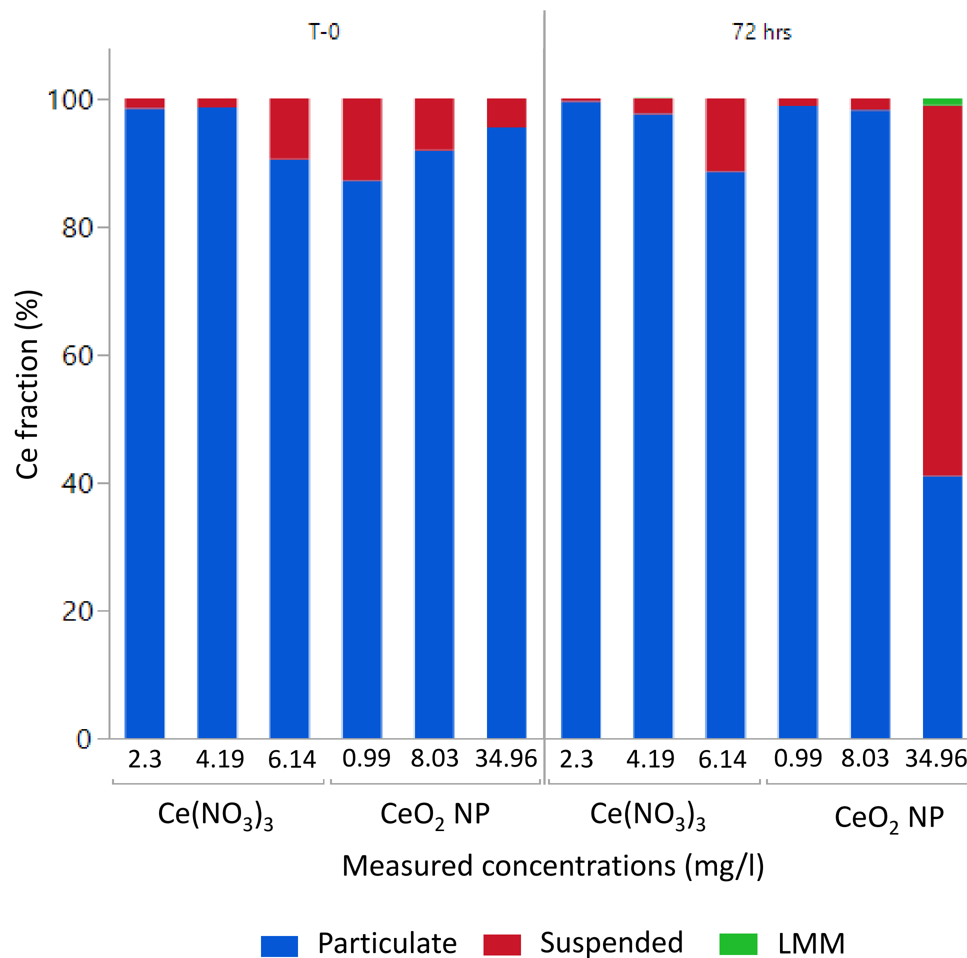

Figure S4: Particulate, suspended, and low molecular mass (LMM, <3 kDa) Ce fraction in the exposure media containing either $Ce(NO_3)_3$ or CeO_2 NPs at T-0 or 72 hours in MHRW containing *E. coli*. For the suspended fraction *E. coli* was removed via centrifugation.

Table S2: Measured Ce concentrations in undepurated and depurated nematodes (n = 21 ± 9) for either $Ce(NO_3)_3$ or CeO_2 NPs exposed for 72 hours in MHRW*.

	Ce concentrations exposure media		Ce concentrations in nematodes	
Exposure	Nominal (mg/l)	Measured (mg/l)	Undepurated (µg/nematode)	Depurated (µg/nematode)
$Ce(NO_3)_3$	2	2.3 ± 0.3	0.1 ± 0.05	0.01 ± 0.004
	4	4.19 ± 0.12	0.09 ± 0.04	0.06 ± 0.004
	6	6.14 ± 0.28	0.09 ± 0.02	0.04 ± 0.01
CeO_2 NPs	1.03	0.99 ± 0.12	0.003 ± 0.0007	<LOD
	8.25	8.03 ± 0.13	0.04±0.01	0.02 ± 0.02
	33	34.96 ± 0.42	0.04 ± 0.02	0.004 ± 0.0008

*LOD = limit of detection = 0.0003 ppm.

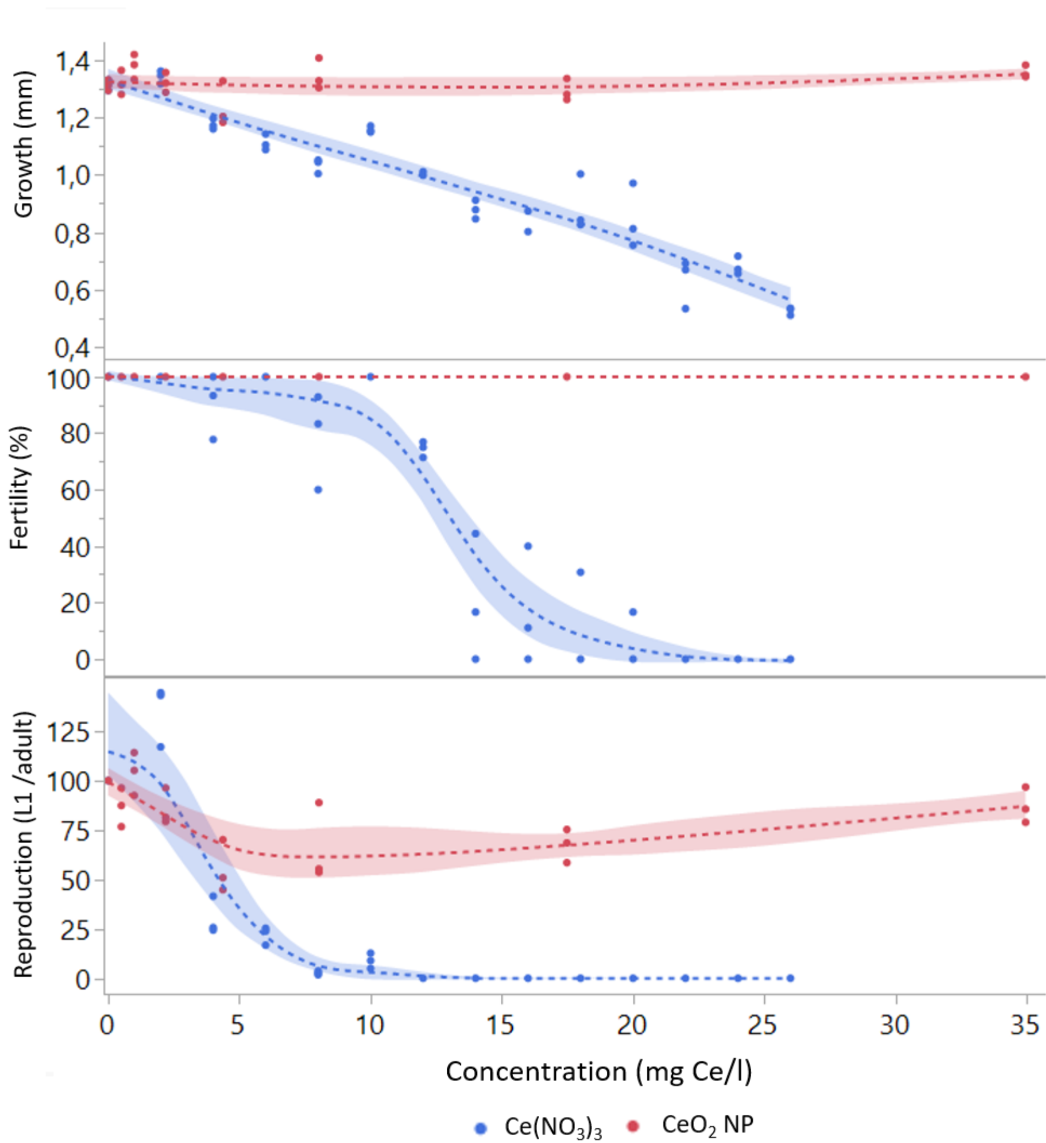

Figure S5: Growth, fertility and reproduction of *C. elegans* following the exposure to either $Ce(NO_3)_3$ (a) or CeO_2 NPs (b) (mean ± SD) in MHRW containing *E. coli* in a standard 96 hr toxicity test.

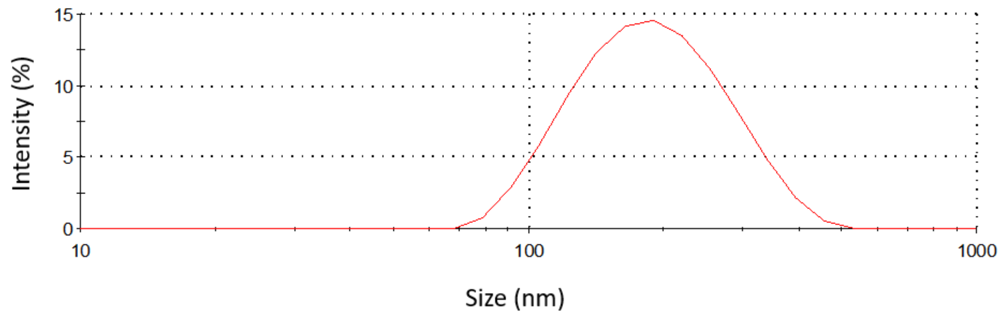

Figure S6: Size distribution of the CeO_2 NPs in the stock suspension (MHRW-T), as measured by dynamic light scattering (5 measurements, 10 runs each).

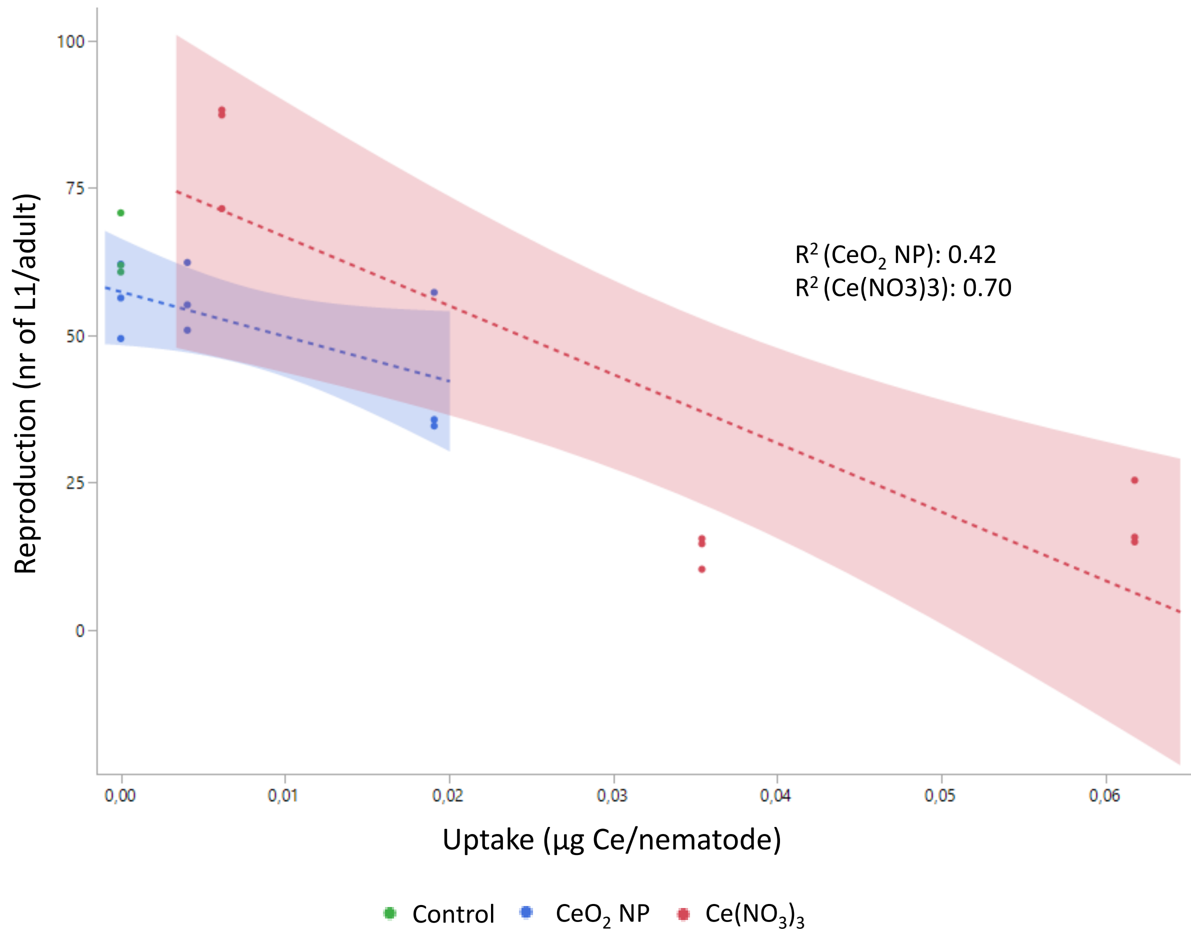

Figure S7: Measured reproduction (nr of L1s/hermaphrodites) as a function of Ce uptake (retained Ce/nematode follow depuration) with lines of best fit.

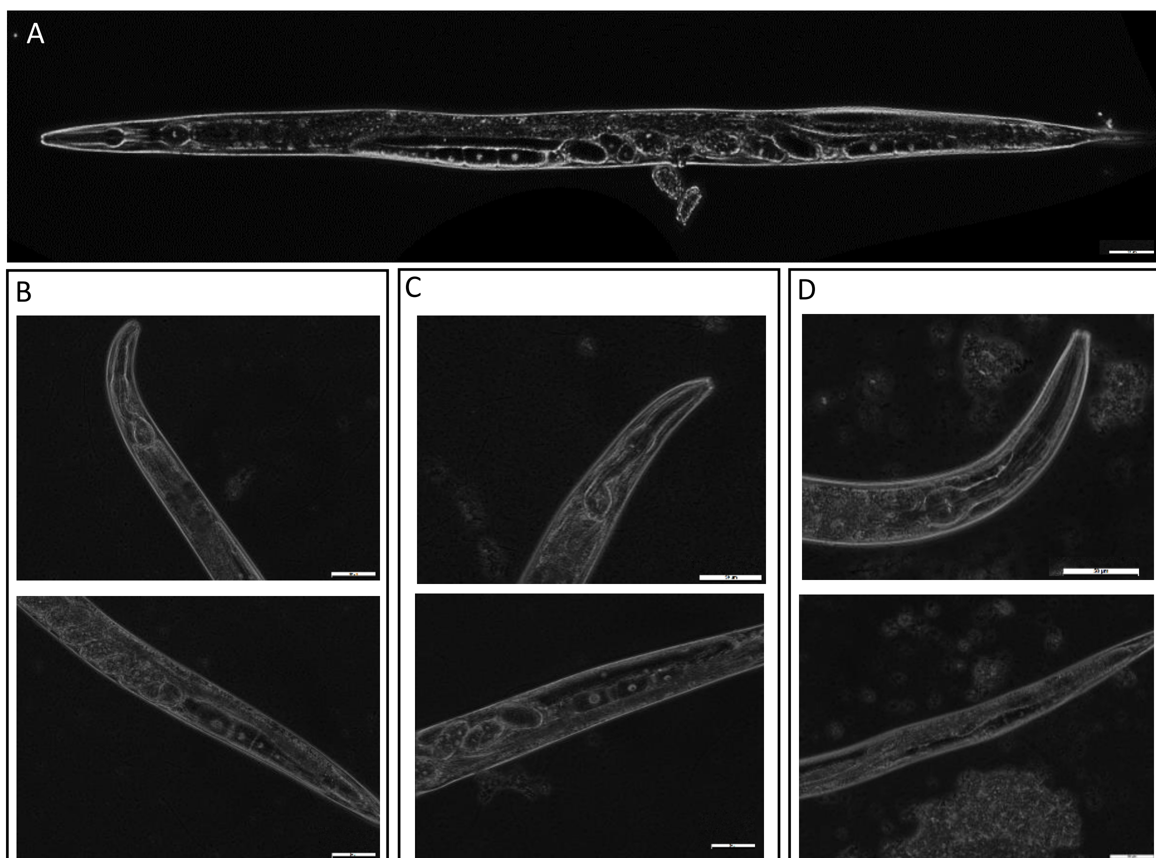

Figure S8: (A) Histological analysis of whole body control, or pharynx and posterior gonads, eggs, and intestine of (B) 17.48 mg/l or (C) 8.03 mg/l CeO_2 NP, or (D) 6.14 mg/l $Ce(NO_3)_3$ exposed *C. elegans.* All scale bars represent 50 µm.

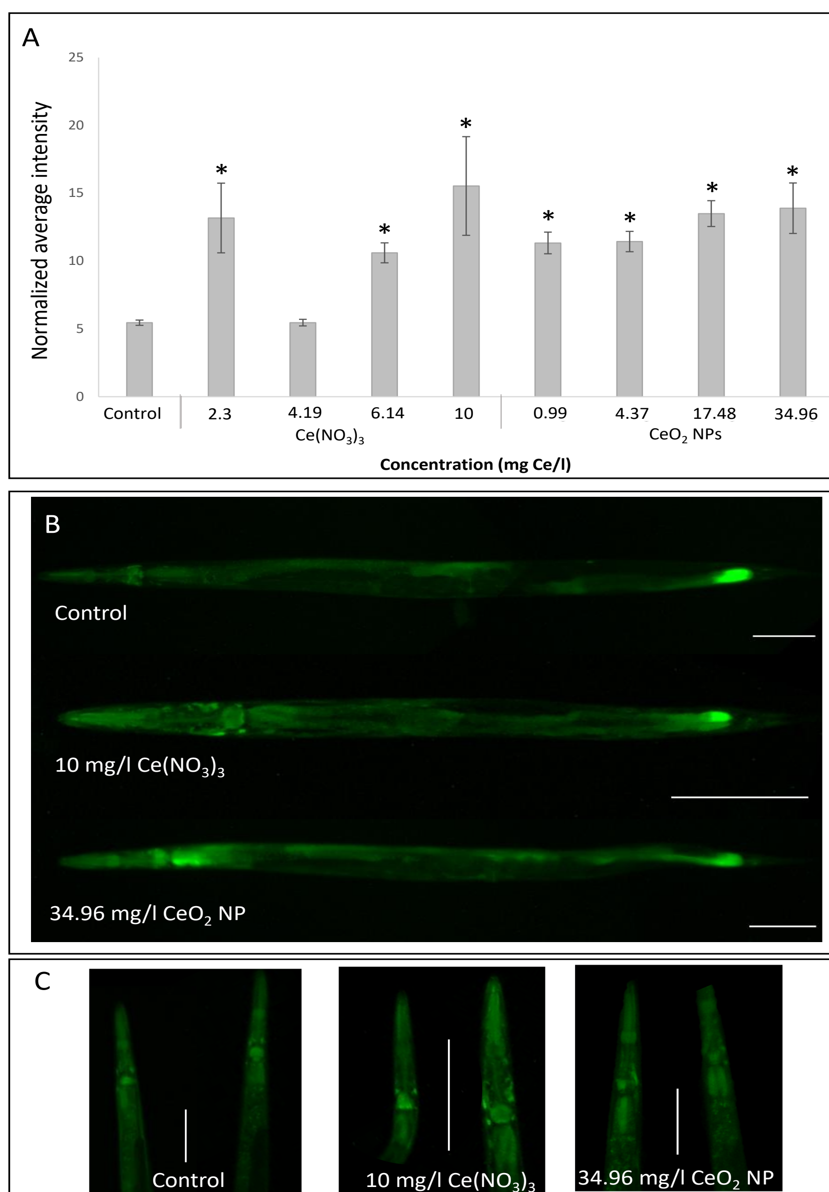

Figure S9: (A) Normalized average intensity measurements from the Sod-1 nematode strain after exposure to either $Ce(NO_3)_3$ or CeO_2 NPs, and (B) representative Sod-1 nematode images, with (C) close up images of the pharynx for two individuals from 3 different treatments. Scale bars represent 100 µm. Asterisk (∗) indicate a significant difference ($p < 0.05$ students t-test) between the exposure group and control.

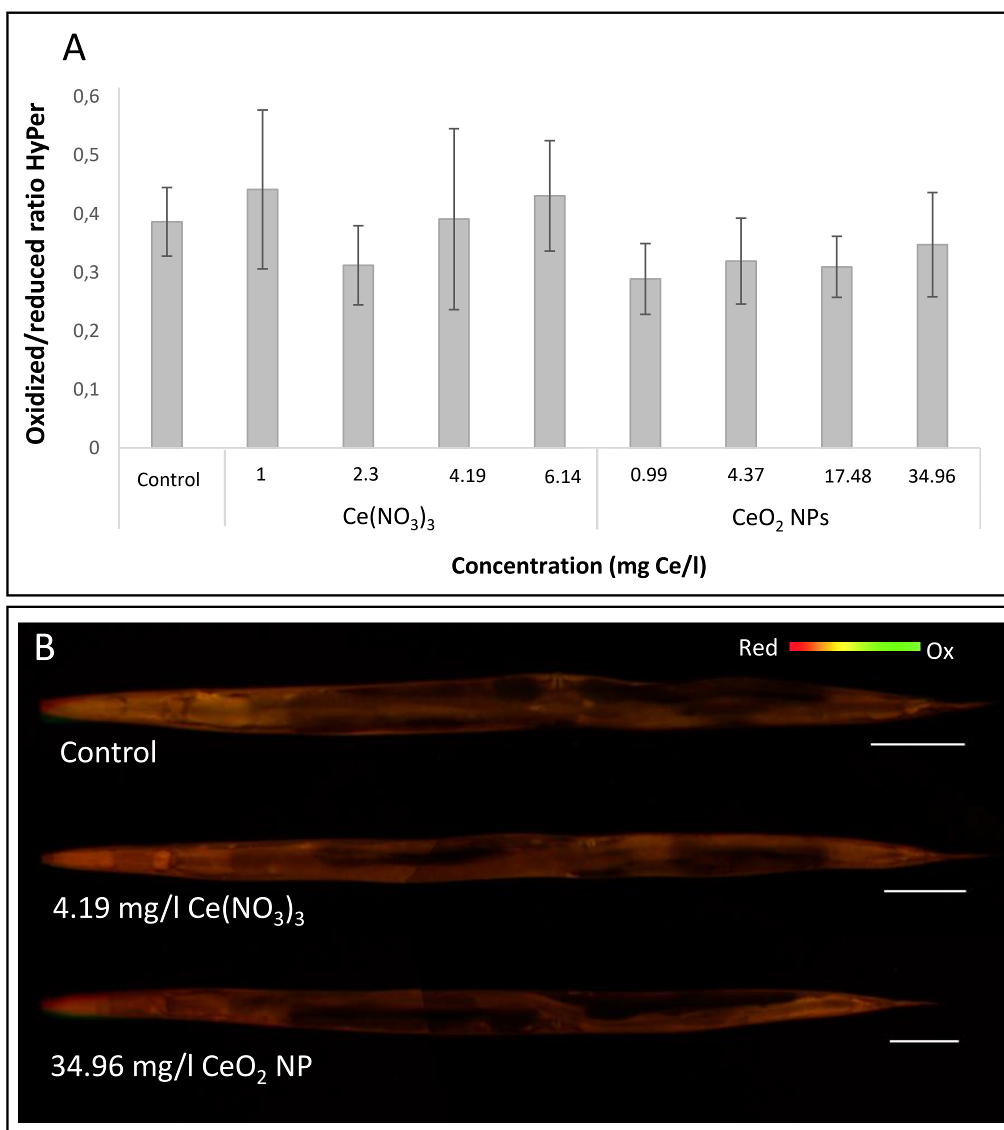

Figure S10. (A) Oxidized to reduced ratios of the hydrogen peroxide biosensor HyPer measured i*n vivo* in response to the exposure to either $Ce(NO_3)_3$ or CeO_2 NPs (n = 10 per concentration), with (B) corresponding representative images (n = 10 per concentration). Scale bars represent 100 µm.

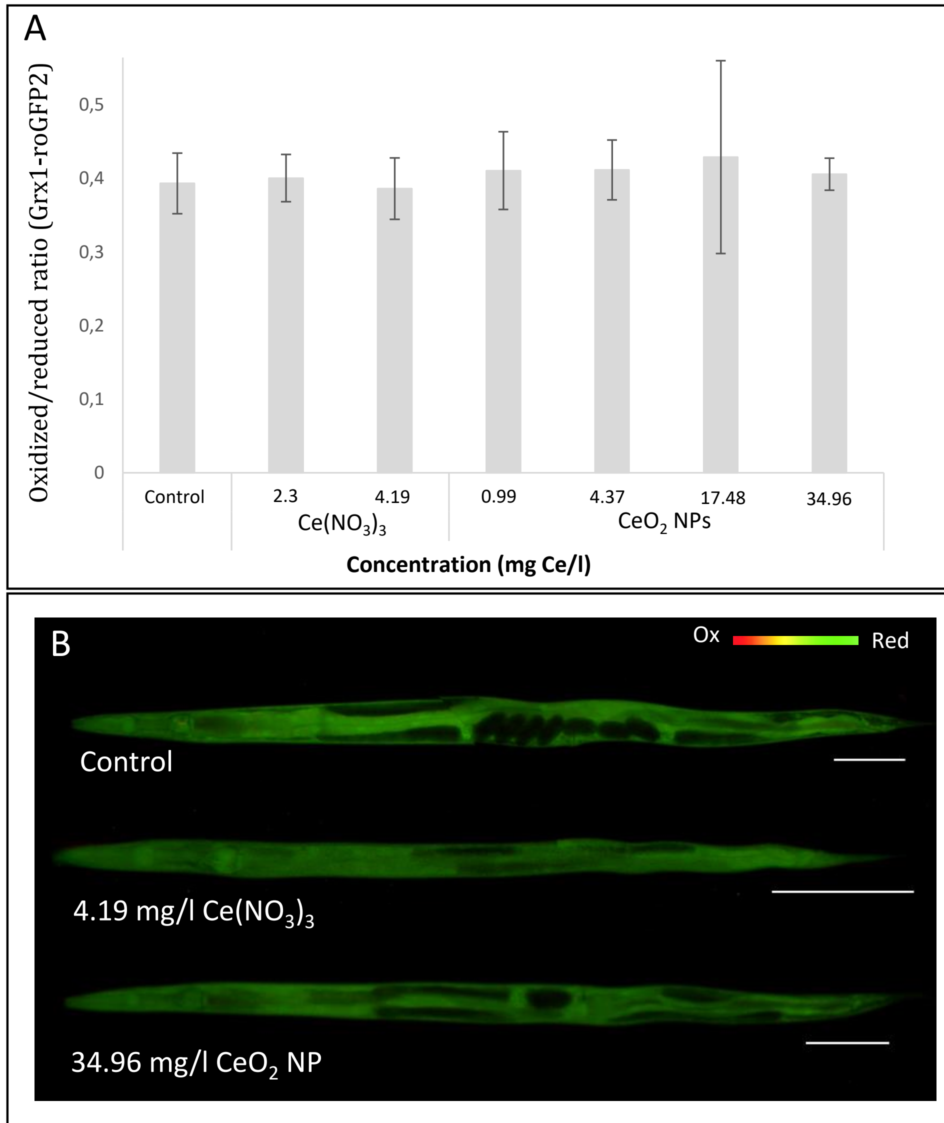

Figure S11: Oxidized to reduced ratios of the biosensor Grx1-roGFP2 (A) with corresponding representative images (B), measured *in vivo*, in response to the exposure to either $Ce(NO_3)_3$ or CeO_2 NPs (n = 10 per concentration). Scale bars represent 100 µm.

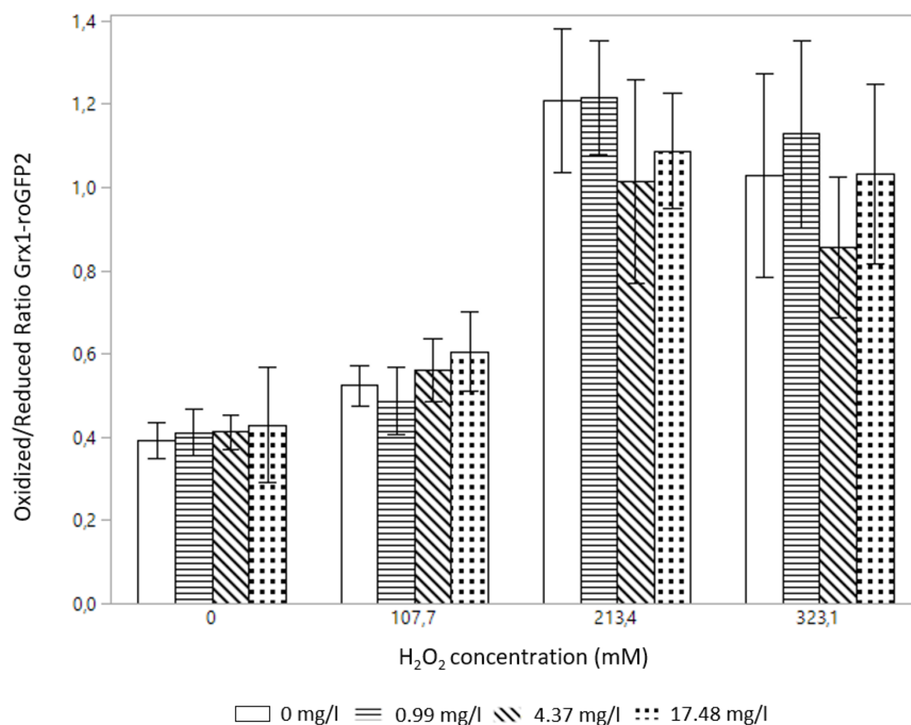

Figure S12: Oxidized to reduced ratios of the biosensor Grx1-roGFP2 measured *in vivo,* in response to a chronic 72 hour CeO_2 NP (0, 0.99, 4.37, or 17.48 mg Ce/l) exposure with a subsequent acute (6 min) exposure to H_2O_2 (0, 107.7, 213.4, or 323.1 mM) (n = 10 nematodes per concentration).

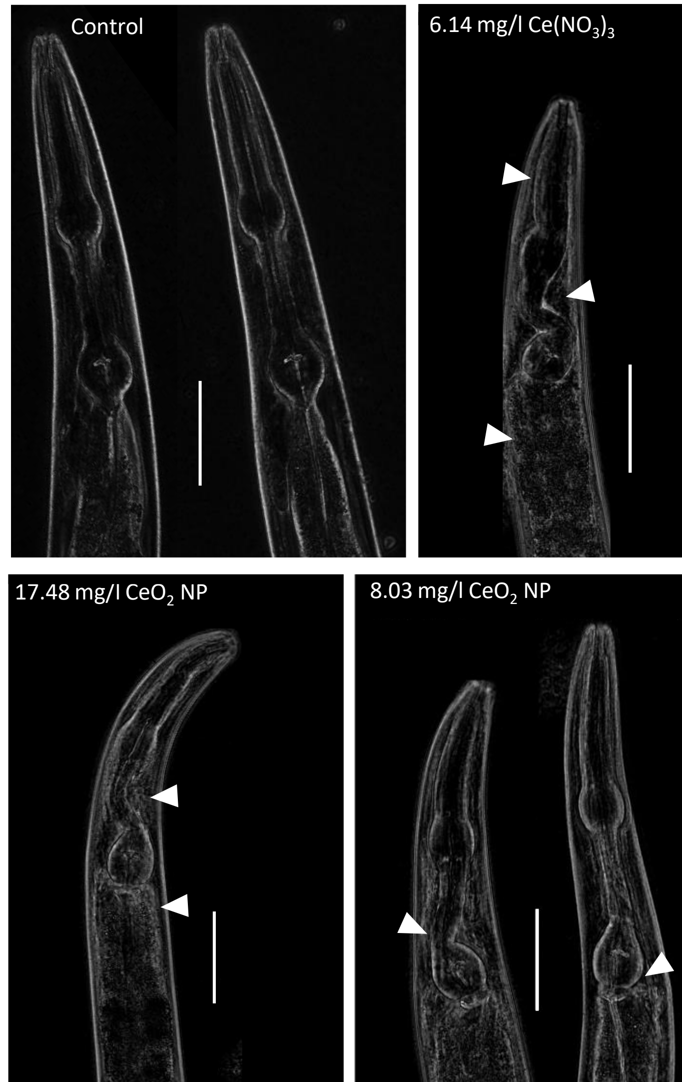

Figure S13: Pharyngal deformities of N2 strain *C. elegans* exposed to either $Ce(NO_3)_3$ or CeO_2 NPs for 72 hours in MHRW. Arrows indicate deformities observed in nematodes exposed to either $Ce(NO_3)_3$ or CeO_2 NPs. Scale bars represent 100 µm.

References:

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