**Cerium oxide nanoparticles induce oxidative stress in the sediment dwelling amphipod, *Corophium volutator***

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**Supplementary Information**

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**Full Experimental Methods**

*General procedures and materials for exposures*

All acids for sample manipulation were prepared from quartz distilled AnalaR 6 M HCl and 15.4 M HNO3, diluted with 18 MΩ H2O (Millipore) if necessary. All glassware and HDPE was pre-cleaned in AnalaR 3M HNO3 for 24 h and triple rinsed in 18 MΩ H2O. Ultra-clean Teflon vials were used at all other times to minimize Ce blank contribution. Artificial seawater (Tropic Marine Salt ‘ASW’; pH = 8 ± 0.4, salinity = 25 ± 0.4 PSU, at 12 ± 0.7 ºC) was used in all exposures. Individual stock exposure solutions of CeO2 NPs and bulk scale CeO2 (100 ml) were prepared immediately prior to exposure, using ASW and sonicated using an Ultrasonic probe (Cole-Parmer® 130-W, 20 kHz 100% amplitude) for 20 seconds on ice using standard operation protocols developed by the National Physics Laboratory (<http://www.nanotechia-prospect.org>). These stocks then diluted for exposure studies and particle characterisation (stock also made in ultrapure deionized water (DI, 18.2 MΩ-cm).

*Particle Characterisation*

The NPs selected for the CeO2 study was provided by NM-211 Antaria (~10 nm) and the bulk scale counterpart (NM-213) (<5 µm) were obtained from the Joint Research Council, Ispra. Particle types were uncoated and characterised in ultrapure deionized water (DI, 18.2 MΩ-cm) and artificial seawater (ASW; pH 8.4, 25 PSU) at the Facility for Environmental Nanoscience Analysis and Characterisation (FENAC) at the University of Birmingham and at the National Physics Laboratory (London).

# Polydispersity, hydrodynamic diameter and zeta-potential of the particles were determined on a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd. Malvern, UK) operating with a He-Ne laser at a wavelength of 633 nm using back scattered light. Samples were held at 12ºC for 2 mins prior to analysis to allow for particle stabilisation. Ten replicate measurements were made on each sample and data are reported as means and error limits as 95% confidence interval calculated from the standard deviations of the replicates. For some samples, one or more replicate measurements were discarded where data quality were poor (signal too close to the background or the polydispersity was too high).

Centrifugal sedimentation was used to determine particle size distribution (CPS Disc Centrifuge Model DC 2000 instrument, Analytic Ltd, UK). The centrifuge was brought up to speed by partially filling the disc with a sucrose gradient fluid and dodecane cup fluid. Equilibrium of the disc centrifuge occurs for 1 hour at 6000 rpm. 50 mg L-1 (NPs or bulk scale CeO2) of sample was then injected into the disc, with a calibration standard injected after every 3 samples to ensure the correctness of the equipment. Acquisition and processing of the data was undertaken on the Disc Centrifuge Control System software ([Fabrega et al. 2011](#_ENREF_4)).

X-ray diffraction measurements were obtained using a Siemens D5000 diffractometer. This consisted of a thetatheta goniometer and an NPL specimen stage. Cu- K α X-ray (40 kV, 30 mA) was used as the source for these measurements filtered using a Ni filter to remove the Cu- Kβ component of the X-ray. The X-ray optics consisted of a 0.6mm anti scatter slit, a 1mm collimation slit and a 1mm detector slit. The diffraction measurement was conducted using coupled theta-theta drives in standard Bragg- Brentano geometry. Data were collected over a 2-theta range of 5 to 150° using a step size of 0.010° and a count time of 1.5 s/step. Diffracted data were collected electronically and stored on a PC. Having collected the full diffraction trace the Scherrer equation was used to evaluate the crystallite size ([Fabrega et al. 2011](#_ENREF_4)).

X-ray photoelectron spectroscopy analysis measurements were obtained under ultra-high vacuum using a Kratos AXIS Ultra DLD (Kratos Analytical, UK) instrument fitted with a monochromated Al Kα source, operated at 15kV and 5mA emission. Photoelectrons from the surface (X nanometres) were detected in the normal emission direction over an analysis area of approximately 700 x 300 micrometres. Spectra in the binding energy range 1400 to –10 eV and a step size of 1 eV, using pass energy of 160eV, were acquired from selected areas of each sample. The peak areas were measured after removal of a Tougaard background. The manufacturer’s intensity calibration and commonly employed sensitivity factors were used to determine the concentration of the elements present. High resolution narrow scans of some of the peaks of interest were acquired with a step size of 0.1 eV and 20 eV pass energy.

The energy scale was calibrated according to ISO 15472 Surface chemical analysis – X-ray photoelectron spectrometers – Calibration of energy scales. However, the charge neutraliser was used when acquiring the spectra, which shifted the peaks, by several eV The C 1s hydrocarbon peak (285 eV binding energy) was used to determine the shift for identifying the peaks. Samples were prepared using carbon adhesive tape to affix them to 1 cm copper squares ([Fabrega et al. 2011](#_ENREF_4)).

Samples for TEM were prepared by ultracentrifugation, spinning 9 mL of 10 mg L-1 suspensions at 30 000 rpm for 1 hour onto 300 mesh Cu TEM-grids with carbon/formvar film. The TEM-grids were thereafter immersed in Milli-Q water and dried overnight. Micrographs were thereafter acquired using a JEOL 1200EX transmission electron microscope at 80 keV, and particle size distributions were manually measured using the software Digital Micrograph (Gatan Inc.). Additional measurements were performed on a JEOL 7000F in SEM/STEM mode for bulk samples and size observations noted when using the HAADF-STEM EELs with the Joel 2100F (Cs corrected, CEOS, Germany).

EDX (Oxford Inca) measurements were made on a JEOL 7000F to confirm Ce as a major element in the particles. High angle annular darkfield scanning transmission electron microscopy (HAADF-STEM, Cs corrected Joel 2100F) with a *Gatan Enfina* Electron Energy Loss spectrometer was used to determine the components of the nanoparticles using the methodology described previously ([Baalousha et al. 2015](#_ENREF_2), [Merrifield et al. 2013](#_ENREF_7)).The Ce(III) and Ce(IV) oxidation states have a different M5/M4 ratio (Ce(IV) ~0.75 and Ce(III) ~1.2). The measurement of the peaks used the integrated signal of the peaks measured after a 2nd differential filter to remove the step wedge form of the spectra as described previously ([Merrifield et al. 2013](#_ENREF_7)).

The rate and extent of dissolution for both bulk and NPs CeO2 in ASW was established by equilibrium dialysis. For this, pre-rinsed 10 cm dialysis cells (1000 Da molecular weight cut-off) were filled with 18 MΩ water and placed in either bulk or NPs CeO2 – ASW stock exposure suspensions (12.5 mg L-1 CeO2, 2 L), and stirred for 10 days. Membranes were removed at 0 h, 24 h, 120h and 240h, and aliquots of the media outside the membranes were collected at 0 and 240h. All samples were then acidified with 15.4 M HNO3 (2%) and analysed with ICPMS or ICPOES.

*Modified Static System*

Sediment and *C. volutator* organisms were collected from an intertidal area of the Otter estuary, south Devon (grid reference: SY065820). Animals were acclimated at 12°C in sediment and 5 mm overlaying 25 PSU artificial seawater (ASW), in a 12:12h light: dark cycle (Fabrega et al. 2011). Sediment was sieved through 300 µm mean diameter using reference seawater to exclude residual benthic organisms and stored in the dark at 4°C prior to use. An acute 10 day water exposure of CeO2 NPs was performed on *C. volutator*, based on a modified static system with an additional 2 day depuration period to allow voiding of the gut (Scarlett et al. 2007). Animals were not fed throughout the exposure. Briefly, 2 L beakers were filled with 160 ml of sieved natural sediment and left for 24 h to settle at 12°C. The beakers were then filled gently to the 1200 ml mark with ASW (25 PSU) containing CeO2 NPs at concentrations of 0, 6.5, 12.5, 25, 50, 100 mg L-1 (3 beakers per concentration) and housed 20 animals. Exposure waters were made for each concentration tested by adding the relevant amount of stock solution (500 mg L-1) to 4 L of ASW (a sufficient amount for the required replicates). On the day of exposure, adult organisms (4-7 mm) were harvested by passing the upper 3 cm of holding tank through a 300 µm nominal pore sieve. Animals were not fed during the exposure. Beakers were randomly allocated a position in the exposure room to eliminate any possible differences in temperature across the room, that might have occurred (albeit that these differences were likely to be less than 1°C). Glass pipette tips on the end of an airline provided gentle aeration to each test vessels. Water parameters were monitored on day 1, 5 and 10 of exposure (Table S2) and conformed to the Standard Guide for Conducting 10-day Static Sediment Toxicity Tests with Marine and Estuarine Amphipods (ASTM E1367-99).

## Post exposure sample preparation for ICP-MS/OES

Water samples were collected by syringe, transferred to a Teflon beaker and acidified with 15.4 M HNO3 (2%). Sediment cores were removed with a plastic corer, from the base of the same tanks and digested in a mixture of 1:4 of H2O2:HNO3 in a microwave system (Ethos EZ, Milestone Inc, Shelton). *C. volutator* were sifted from the sediment, the number of live and dead organisms were recorded and separated for subsequent analyses. Whole organism and tissue samples were dried using a heating block at 60ºC and digested in 1 ml 15.4 M HNO3 After 3 days, filter papers used to collect faecal pellets were dried for 24 h at 60ºC and digested in 2 ml 15.4 M HNO3. A blank filter paper digest was also sent for background analysis

*Instrument and quality control for ICP-MS and ICP-OES*

All samples for ICP were analysed at the University of Plymouth. The Varian 725-ES ICP-OES (Stockport, UK) operating parameters used were power, 1400 W, plasma, auxiliary and nebulizer flows, 13, 1.5 and 0.68 L min-1 and the instrument stabilization and replication read time was 10 and 4 s respectively. The Thermo Scientific X Series 2 ICP-MS (Hemel Hempstead, UK) operating parameters were power, 1400 W, coolant, auxiliary and nebulizer flows, 13, 0.7 and 0.84 L min-1, the dwell time was 10 ms per isoptope and the number of sweeps were 50 For the ICP-MS a collision cell with 7% hydrogen in helium was used at a flow rate of 3.5 ml min-1 to decrease the amount of CeO2 formed in the plasma. Both pieces of equipment used a V-groove nebuliser and a Sturman-Masters spray chamber.

Calibration for both machines were conducted using two Ce independent standards, the first obtained from Sigma-Aldrich (995 mg L-1) and the second from Aristar® (998 mg L-1), both of which were plasma emission grade solutions. All samples and standards were sonicated for 15 min and then immediately vortex mixed before analysis.

## Radical production assay

We assessed the oxidative function of bulk and NPs CeO2 in exposure media using the chromagenic probe. ABTS reacts with OH. to produce a stable oxidised product that is then measured at an absorbance maximum of 420 nm (ε = 3.6 × 104 M–1 cm–1) at 12°C in a Tecan Infinite® 200 PRO series (Männedorf, Switzerland)([Yim et al. 1993](#_ENREF_10)) . Reactions were buffered by Tris (100 μM), pH 7.0, and UV-visible spectra (200-900 nm) were recorded once every minute for 10 min. Nanoparticle and bulk CeO2 were prepared in DI water or ASW (25 PSU) and then added to the system to give final concentrations in the range of 0-100 mg L-1 in the presence of 88 mM H2O2 and 100 μM ABTS. Data presented are the results from at least three independent reactions. An additional experiment was undertaken to understand whether bromide ions could quench OH.. This was undertaken by the addition of 0.6 mM of bromide in the form of chlorine free hypobromous acid (HOBr) to the reaction mixture of NPs in DI water (0-100 mg L-1), 88 mM H2O2 and 100 μM ABTS. Reactions were buffered by Tris (100 μM), pH 7.0, and UV-visible spectra (200-900 nm) were recorded once every minute for 10 min. The concentration of 0.6 mM was chosen as this is approximated to the concentration found in ASW used.

*Plasmid Relaxation Experiments*

Supercoiled plasmid pBR322 (Promega UK, Southhampton) was used to probe for Fenton-like production of OH.. Covalent changes and damage to DNA was measured via changes in migration speed of DNA on agarose gel electrophoresis, according to the methods of Heckert *et al.,* 2008([Heckert et al. 2008](#_ENREF_6)). Plasmids were produced in Escherichia coli strain JM109 and purified by the alkaline lysis method (Strataprep EF plasmid kit, Stratagene, La Jolla, California). The CeO2 preparations were prepared in an identical manner to those used for the ABTS experiments. Reactions containing bulk and NPs CeO2(12.5 mg L-1)in 100 μM Tris, pH 7.0, 88 mM H2O2 and 1 μg of plasmid either in DI water or ASW and were incubated at 37°C for 60 min. Reactions were stopped by addition of excess EDTA (10 mM) and the nicking of supercoiled DNA was resolved by electrophoresis on 0.7% agarose gels containing Sybr Safe in Tris-Acetate-EDTA (TAE) buffer.

*Assessments of sub-lethal effects on C.volutator*

DNA damage in *C.volutator* was assessed using the Comet assay in which DNA fragmentation is quantified. Animals were homogenised on ice in 500 µl phosphate buffered saline (pH7.4), cell suspensions were then spun gently (15 seconds, 0.5 x g) and the supernatant removed. An aliquot of each supernatant (~1 x 106 cells) was mixed with 1% low melting point agarose and placed onto 1% high melting point agrose-coated slides. These samples were then subject to 1 h lysis, followed by 45 min denaturation in electrophoresis buffer (0.3 M NaOH and 1 mM EDTA),and electrophoresis for 30 min at 25 V and 300 mA. Samples on the slides were then subjected to neutralisation and stained with 20 µg ml-1 ethidium bromide and examined using a fluorescent microscope (excitation: 420–490 nm; emission: 520 nm). The Olive Tail Moment in 100 cells per preparation was quantified using Kinetic V COMET Software ([Galloway et al. 2010](#_ENREF_5)). Olive Tail Moment is defined as the product of the tail length and the fraction of total DNA in the tail. Tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed / broken pieces (represented by the intensity of DNA in the tail).

Superoxide dismutase (SOD) activity was determined by inhibition of nitroblue tetrazolium (NBT) reduction with xanthine-xanthine oxidase used as a superoxide generator using a spectrophotometer (UV-2401PC, Shimadzu, Milton Keynes, UK) at 560 nm for 10 minutes. The substrate solution contained 0.1 mM xanthine, 0.1 mM EDTA, 0.05 mg ml-1 BSA and 25 µM NBT in phosphate buffer (0.1 M: pH 7.2), with xanthine oxidase (XO) at 6.25 mU with 33µl of tissue homogenate (as above). A standard curve of purified SOD was run (10-0.01U ml-1) and % inhibition calculated and plotted against SOD concentration.([Van Der Oost et al. 2005](#_ENREF_9))

Oxidative damage of polyunsaturated lipids in cell membranes in the form of tissue lipid peroxidation (LPO) was assessed using a modified method of thiobarbituric acid reacting substances (TBARS) ([Camejo et al. 1998](#_ENREF_3)). *C.volutator* homogenate (as above, 40 µl) was added to 96-well microtitreplates (in triplicate) containing 1 mol L-1 butylated hydroxytoluene (2,6-Di-O-*tert*-butyl-4-methylphenol), 50% (w/v) trichloroacetic acid and 1.3% (w/v) thiobarbituric acid (dissolved in 0.3% (w/v) NaOH). The plate was incubated at 60ºC for 1 h, cooled on ice and read at 530 nm at 25°C in a Tecan Infinite® 200 PRO series (Männedorf, Switzerland). Results were measured as malondialdehyde equivalents (MDA) determined against a standard curve using 1,1,3,3-tetraethoxypropane (0–24 μM), and expressed per mg protein.

Results

There was no statistically significant difference (two-way ANOVA p<0.05) in the production of the ABTS radicals when the CeO2 NPs were dispersed in ASW or in DI water with the addition of 0.6mM chlorine free hypobromous acid (HOBr) at all concentration tested (see figure S1). Since bromide was able to quench the production of free radicals in this system, these results suggest OH. may be reacting rapidly with reductants in seawater, such as bromide.



Figure S1. *In vitro* experiments undertaken in DI water with the addition of 0.6 mM chlorine free hypobromous acid (HOBr) and ASW with CeO2 NPs. Increases in ABTS radical production were dependent on increasing concentrations of NPs. ABTS radical was followed spectrophometrically at 430 nm for 10 mins, as described in methods. Open circles represent CeO2 NPs in ASW and open squares represents CeO2 NPs in DI water with the addition of 0.6 mM chlorine free hypobromous acid (HOBr). There was no statistically significant difference (two-way ANOVA p<0.05) in ABTS radical production between the two groups.

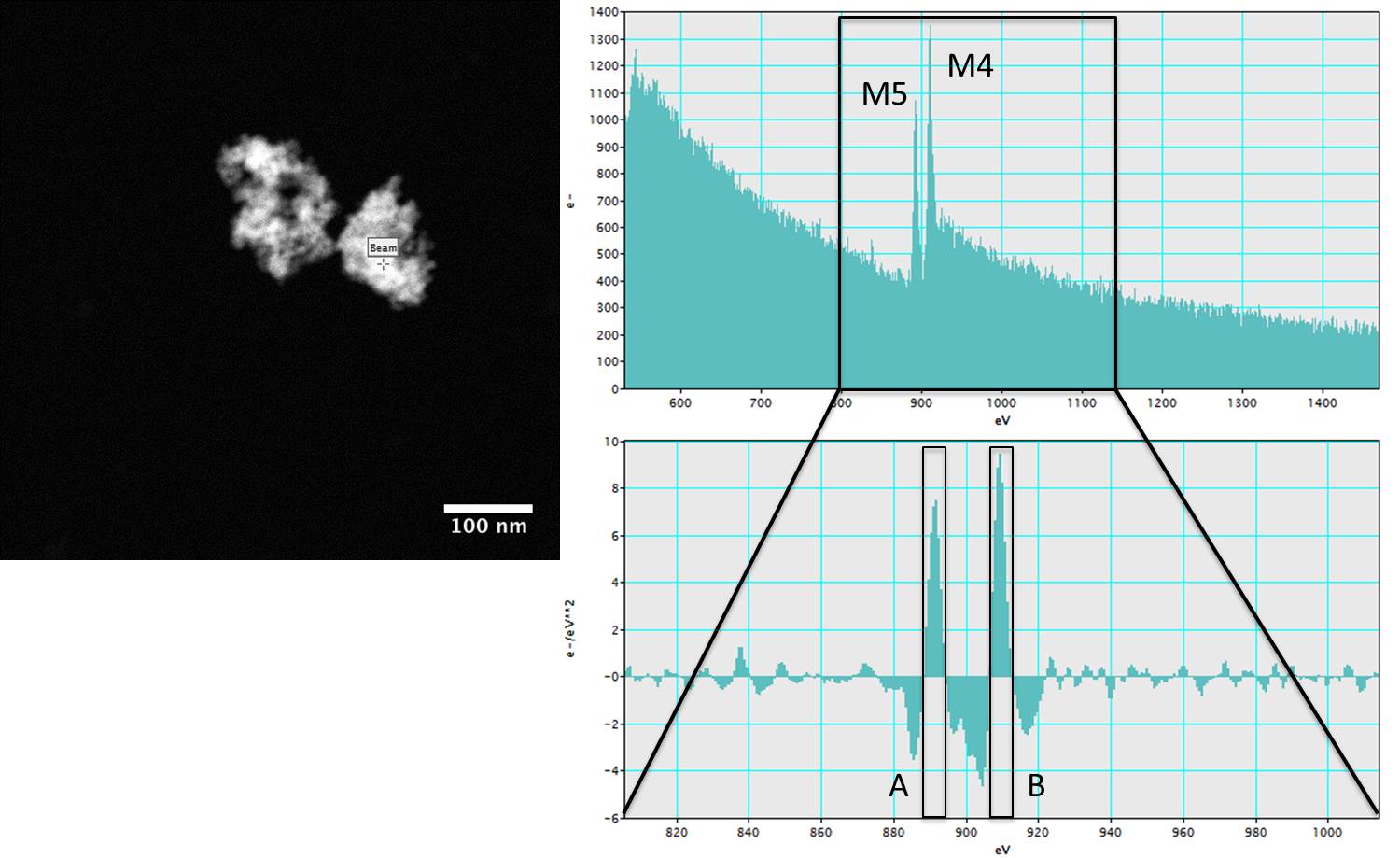


Figure S2. Example of EELs analysis of CeO2 NPs in Milli-Q water. An image was taken, then a point spectra (position ‘beam’), the M5 and M4 value is found from the integrated intensity (boxed area A and B) after a second differential filter is used. M5/M4 gives the valance of CeOx (around 0.8 for IV and 1.2 for III).

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| --- | --- | --- | --- | --- |
| **Characterisation Parameter** | **CeO2 NPs in DI water** | **CeO2 NPs in ASW** | **Bulk CeO2 in DI water** | **Bulk CeO2 in ASW** |
| Size by DLS*A* (FENAC) | 483 ± 47 nm | 870 ± 177 nm | 757 ± 380 nm | 1339 ± 1215 nm |
| Zeta potential (FENAC) | 24.5 ±1.9 mV | -14.8 ± 1.7 mV | -8.72 ± 1.4 mV | -12.6 ± 2.8 mV |
| Size by CPS*B* disc centrifugation (NPL)([Tantra et al. 2012](#_ENREF_8)) | 340 ± 50 nm | 520 ± 90 nm | 570 ± 80 nm | 650 ± 80 nm |
| Additional CPS information (NPL)([Tantra et al. 2012](#_ENREF_8)) | Mass appeared to be dominated by smaller aggregates for CeO2 NPs, with 90% (D90) of the aggregate particles being >163 ± 14 nm | D90 = 130 ± 60 nm | D90 = 158 ± 12 nm | D90 = 210 ± 20 nm |
| X-ray diffraction (XRD) (NPL)([Tantra et al. 2012](#_ENREF_8)) | Dry powder -10.3 nm | | Dry powder – 33.3 nm | |
| Size by TEM*C* (FENAC) (n = >80) | 390 ± 130 nm | 470 ± 240 nm | ND | ND |
| Size by SEM*D* (FENAC) (n = >80) | N/A | N/A | 2200 ± 900 nm | 1340 ± 600nm |
| Discrete particle size (FENAC) | 8.54 ± 2.42 nm | 8.58 ± 3.82 nm | ND | ND |
| EELS*E* (M5:M4) (FENAC) (n = >5) | 0.86 ± 0.08 ( Ce (IV)) | 1.21 ± 0.1 ( Ce (III)) | 0.79 ± 0.1 ( Ce (IV)) | 0.83 ± 0.09 ( Ce (III)) |

**Table S1.** Characteration of CeO2 NPs and bulk CeO2 in both ultrapure deionized (DI, 18.2 MΩ-cm) water and artifical seawater (25 PSU), as assessed by various techniques. ND = not determined. *A* Dynamic light scattering, *B* CPS disc centrifugation, *C* transmission electron microscopy, *D* scanningelectorn microscopy and *E* electron energy-loss spectrscopy. All characterisation was undertaken at the National Physical Laboratory (NPL) or Facility for Environmental Nanoscience Analysis and Characterisation (FENAC).

**Table S2.** Average of dailywater parameters measured over the 10 day exposure.

|  |  |
| --- | --- |
| **Water Parameters** | **Mean ±SE** |
| Salinity (PSU) | 25 ± 0.5 |
| pH | 8.1 ± 0.2 |
| Dissolve Oxygen (%) | 99.7 ±0.4 |
| Temperature (°C) | 12 ± 0.3 |

**Table S3.** Elemental Composition of ASW (Tropic Marine; 25 PSU). Major cations were measured with a Perkin Elmer Atomic Absorption Spectrophotometer. The major anions were measured with a 2010 Dionex in chromatograph with AS-4 column. Remaining elements were measured by ICP-MS ([Atkinson and Bingman 1997](#_ENREF_1)).

|  |  |
| --- | --- |
| **Major Cations (mmol kg-1)** |  |
| Na+ | 338.54 |
| K+ | 7.25 |
| Mg+2 | 36.64 |
| Ca+2 | 7.25 |
| Sr+ | 0.06 |
|  |  |
| **Major Anions (mmol kg-1)** |  |
| Cl- | 380.67 |
| SO4-2 | 16.08 |
| TCO2 | 0.84 |
| TB | 0.28 |
|  |  |
| **Trace (µmol kg-1)** |  |
| Li | 22.21 |
| Si | 10.72 |
| Mo | 1.91 |
| Ba | 0.25 |
| V | 2.14 |
| Ni | 1.30 |
| Cr | 5.82 |
| Al | 176.16 |
| Cu | 1.46 |
| Zn | 0.42 |
| Mn | 0.54 |
| Fe | 0.18 |
| Cd | 0.18 |
| Pb | 1.76 |
| Co | 1.00 |
| Ag | 2.07 |
| Ti | 0.47 |

**Table S4.** Elemental Aqua Regia leachable concentrations in Otter Estuary sediments. Data as published from our laboratory ([Fabrega et al. 2011](#_ENREF_4)).

|  |  |
| --- | --- |
| **Element** | **mg kg-1** |
| Al | 6230 |
| As | 7.3 |
| Ba | 65.6 |
| Ca | 7350 |
| Cd | 0.097 |
| Cr | 14.7 |
| Cu | 14.9 |
| Fe | 12900 |
| Hg | 0.103 |
| K | 2720 |
| Li | 16.7 |
| Mg | 4120 |
| Mn | 265 |
| Na | 9680 |
| Ni | 8.89 |
| P | 491 |
| Pb | 21.9 |
| S | 1080 |
| Sr | 28.4 |
| V | 18.4 |
| Zn | 49.5 |

**Author Contributions**

Study conception and funding (YD, CRT, TSG); Study design (YD, CRT, TSG); Animal exposure and post-exposure sample collection and animal dissections (YD, TSG) were performed at the University of Exeter; Particle characterization in the exposure media (BS, CE, KA) were executed the FENAC, directed by EVJ and JRL.

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