

Supplementary Information

Prompt response of estuarine denitrifying bacterial communities to copper nanoparticles at relevant environmental concentrations

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Supplementary Experimental

Selection of primers targeting nitrite and nitrous oxide reductase genes

Both nitrite (*nir*) and nitrous oxide (*nosZ*) reductase genes have been shown to exhibit phylogenetic distinct clades and to harbour taxonomically diverse microorganisms.¹⁻⁴ This has posed challenges to the design of primers that are both unambiguous and comprehensive of the denitrifier diversity. Common primers for nitrite reductase *nirK* gene are F1aCu/R3Cu⁵ and nirK2F/nirK5R;⁶ for *nirS* gene are cd3aF/R3cd^{7,8} and nirS2F/nirS4R.⁶ At the time these were designed the sequences available were mostly from Proteobacteria strains, which

narrowed the diversity of these genes to this taxon. Nowadays they are still commonly used but advances in culture-independent methods to investigate the microbial community composition allowed the identification of *nir* genes across diverse taxonomic groups. A recent revision grouped *nir* genes into four clusters for *nirK* gene and three clusters for *nirS*.² The previously described primers only target *nirK* and *nirS* in cluster I, and to address this issue the authors have designed specific primers for each cluster.²

The *nosZ* gene primers nosZ2F/nosZ2R⁹ have been designed to be as universal as possible, and in spite of its potential bias against gram-positive bacteria they have been widely used ever since. More recently *nosZ* have been grouped into two different clades. Clade I mostly consisted of Proteobacteria sequences following the species phylogeny as previously observed, and clade II consisted of sequences found among a diverse range of bacterial and archaeal phyla.⁴ Moreover, *nosZ* in clade II has since been recognized as one comprising atypical nitrous oxide reducers found in various ecosystems.¹⁰

In order to choose the primers with the widest coverage for our study we carried out an *in silico* analysis based on a microbial diversity assessment (16S rRNA gene amplicons) previously performed at the same sampling site of Douro estuary.¹¹ Prokaryotic OTUs were categorized based on the presence of the functional genes within genomes that coded for proteins in the denitrification pathway, as compiled and reported before for *nir*² and *nosZ*.⁴

The analysis showed bacterial OTUs predominantly affiliated with genera showing *nosZ* in clade I (Table S1). For this reason the primer pair nosZ2F/nosZ2R was chosen for this study, based on the intuition that the primer set yielding the greater diversity would be the most suitable one.⁹ OTUs affiliated with Bacteria genera showing *nirK* in cluster I and II were predicted, however, for *nirS* only OTUs affiliated with Bacteria genera showing *nirS* in cluster I could be predicted (Table S1). The most commonly used primers were designed for *nirS* and *nirK* in cluster I because for several environments they are the most ubiquitous and abundant. This was the case for *nirS*, and preliminary tests in our samples showed that using primers nirSC1F/nirSC1R for *nirS* in cluster I resulted in amplification (assessed by loading the PCR product in an agarose gel). Therefore, these primers were chosen for this study. Contrary to what was expected, at Douro estuary *nirK* in cluster II was more abundant than in cluster I. To confirm this assumption we amplified our samples using primers nirKC1F/nirKC1R and nirKC2F/nirKC2R and *nirK* in cluster II was the only for which we could see amplification (assessed by loading the PCR product in an agarose gel). Therefore, we chose primers nirKC2F/nirKC2R for this study.

The Archaea *Nitrosopumilus* sp. possibly containing *nirK* homologous genes,¹² was found at Douro estuary, however, Archaea globally had a very low abundance and represented only 0.7 % of the OTUs. Specific primers have been designed for targeting the archaeal *nirK*, and although its transcription potential has been shown, no significant increase in transcript copy number was observed with increased denitrifying activity.¹² For these reasons we chose not to include these primers in the present study.

Positive controls for the genes of interest

Bacteria *Roseobacter denitrificans* (obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures GmbH) was used as positive control for *nirS* and *nosZ* in cluster I, and *Fulvivirga imtechensis* AK7 (obtained from the Japan Collection of Microorganisms) was used as positive control for *nirK* in cluster II. These strains were chosen after an *in silico* PCR was performed by retrieving the sequences of the genes of interest annotated for these organisms in UniProtKB (<https://www.uniprot.org/>) and uploading it in Serial Cloner 2.6 (http://serialbasics.free.fr/Serial_Cloner.html) to align with the primers. Both strains were grown in marine agar medium (3 %) for 2 - 3 days, at room temperature. DNA was extracted with the E.Z.N.A. Bacterial DNA Kit (Omega Bio-tek) and the genes were amplified in a PCR reaction on a Veriti Thermal Cycler (Applied Biosystems) performed with 12.5 μ L of DreamTaq PCR Master Mix (Thermo Scientific), with primers at a concentration of 1 μ mol L⁻¹ and 25 - 50 ng of DNA template, in a final volume of 25 μ L. The primers are described in Table S2 and the thermal cycler conditions were as described in previous works for *nirK*,² *nirS*,¹³ and *nosZ*.⁹ PCR products of duplicate reactions were pooled together and the bands with the expected size - 457 bp for *nirK*, 425 bp for *nirS*, 267 bp for *nosZ* - were excised from an agarose gel (1.5 %), under UV light, with the QIAquick Gel Extraction Kit (QIAGEN). The excised bands were sequenced with Sanger sequencing technology at STAB VIDA (Porto, Portugal). The *nirS* gene was sequenced only in the forward direction and the remaining genes were sequenced in both directions. Forward and reverse sequences were assembled with the CAP3 software,¹⁴ embedded into the Unipro UGENE software (Linux 64-bit version 1.32).¹⁵ The consensus sequences were then confirmed by blast searches against UniprotKB (accessed on 24/11/2019) using the blastx algorithm (<http://www.ncbi.nlm.nih.gov>).¹⁶

Absolute and relative quantification of genes

Reverse transcription quantitative real-time PCR (RT-qPCR) was performed using 6 ng of cDNA for *16S rRNA*, *nirS* and *nosZ* in a StepOnePlus real-time PCR System (Applied Biosystems) using the Power SYBR Green PCR Master Mix (ThermoFisher Scientific). For relative quantification a

serial dilution of cDNA pooled from eight microcosms samples was used as standard to allow calculating the slope and correlation coefficients of the standard curve and the PCR efficiency (Table S2). For *nirK* the same conditions resulted in the inhibition of the PCR reaction, in spite of the fact that the primers and thermal programs had been successful in amplifying *nirK* in cluster II of *F. imtechensis*, as described above. Different conditions were tested in order to overcome the inhibition (varying thermal cycler conditions, varying cDNA concentrations) and the amplification of DNA by PCR was proven successful. For that reason 6 ng of the product was employed as a template in qPCR to determine *nirK* relative expression. For absolute quantification *nirS* and *nosZ* PCR products were obtained from *R. denitrificans* as described above. Additionally, *R. denitrificans* was also used to obtain PCR products of the *16S rRNA* gene using the primers described in Table S2 and thermocycler conditions described in a previous work.¹⁷ PCR products were cloned using the TOPO-TA cloning kit with pCR 2.1-TOPO and One Shot TOP10 Chemically Competent *E. coli* (Invitrogen – Thermo Fisher). Colonies developed for 10 h in solid medium (40 g L⁻¹ LB Broth, ampicillin 50 µg ml⁻¹ and X-gal 100 mmol L⁻¹) at 37 °C, and then selected plasmid-inserted colonies were grown overnight in liquid medium. Plasmids were isolated from this culture using the GenElute Plasmid MiniPrep kit (Sigma-Aldrich). Confirmation of the correct gene fragments was done by Sanger sequencing and genes were amplified in a StepOnePlus real-time PCR System, as described above. A standard curve was constructed by plotting the quantification cycle (Cq) versus the logarithm of the number of copies of the gene, assuming for standard DNA 660 g mol⁻¹ and 6.022 x 10²³ bp mol⁻¹.¹⁸ A correction for ploidy was introduced for *16S rRNA* following a search in *rrnDB* database¹⁹ and considering an average of three *16S rRNA* operon copy numbers per genome. For *nirS* and *nosZ* a conservative approach (1 copy per organism) was considered, following a previous work listing organisms harbouring these genes, with the respective copy numbers.²⁰

Supplementary Tables

Table S1. Predicted OTU-containing *nir* and *nosZ* in the microbial community of Douro estuary sediment and respective relative abundance

Clade	OTU	Kingdom	Phylum	Class	Order	Family	Genus	2014	2015	2014	2015
								Jun	Jun	Dec	Dec
<i>nirK I</i>	C2_1511	Bacteria	Proteobacteria	Rhizobiales	Bradyrhizobiales	Alphaproteobacteria	Bradyrhizobium	0.00000	0.00000	0.00998	0.00000
<i>nirK I</i>	C2_4712	Bacteria	Proteobacteria	Rhizobiales	Bradyrhizobiales	Alphaproteobacteria	Bradyrhizobium	0.00000	0.00000	0.00998	0.00249
<i>nirK II</i>	C2_975	Bacteria	Proteobacteria	Burkholderiales	Comamonadales	Betaproteobacteria	Acidovorax	0.08072	0.11641	0.44897	0.23670
<i>nirS I</i>	A1_10994	Bacteria	Proteobacteria	Pseudomonadales	Pseudomonadales	Gammaproteobacteria	Pseudomonas	0.01883	0.03004	0.00998	0.01246
<i>nirS I</i>	A1_13628	Bacteria	Proteobacteria	Pseudomonadales	Pseudomonadales	Gammaproteobacteria	Pseudomonas	0.01883	0.04506	0.03991	0.05481
<i>nirS I</i>	A1_1863	Bacteria	Proteobacteria	Rhodocyclales	Rhodocyclales	Betaproteobacteria	Azoarcus	0.05919	0.03380	0.04989	0.06229
<i>nirS I</i>	A1_2344	Bacteria	Proteobacteria	Pseudomonadales	Pseudomonadales	Gammaproteobacteria	Pseudomonas	0.02153	0.03755	0.01995	0.02990
<i>nirS I</i>	A1_7747	Bacteria	Proteobacteria	Rhodocyclales	Rhodocyclales	Betaproteobacteria	Azoarcus	0.06727	0.07886	0.14966	0.09966
<i>nirS I</i>	A1_827	Bacteria	Proteobacteria	Pseudomonadales	Pseudomonadales	Gammaproteobacteria	Pseudomonas	0.82333	0.87123	1.27706	1.16604
<i>nirS I</i>	C2_1511	Bacteria	Proteobacteria	Rhizobiales	Bradyrhizobiales	Alphaproteobacteria	Bradyrhizobium	0.00000	0.00000	0.00998	0.00000
<i>nirS I</i>	C2_4712	Bacteria	Proteobacteria	Rhizobiales	Bradyrhizobiales	Alphaproteobacteria	Bradyrhizobium	0.00000	0.00000	0.00998	0.00249
<i>nirS I</i>	C2_9	Bacteria	Proteobacteria	Rhodocyclales	Rhodocyclales	Betaproteobacteria	Dechloromonas	0.00538	0.00751	0.00998	0.01495
<i>nirS I</i>	DA2_28640	Bacteria	Proteobacteria	Pseudomonadales	Pseudomonadales	Gammaproteobacteria	Pseudomonas	0.01345	0.02253	0.01995	0.05980
<i>nosZ I</i>	A1_10994	Bacteria	Proteobacteria	Pseudomonadales	Pseudomonadales	Gammaproteobacteria	Pseudomonas	0.01883	0.03004	0.00998	0.01246
<i>nosZ I</i>	A1_13628	Bacteria	Proteobacteria	Pseudomonadales	Pseudomonadales	Gammaproteobacteria	Pseudomonas	0.01883	0.04506	0.03991	0.05481
<i>nosZ I</i>	A1_1635	Bacteria	Proteobacteria	Alteromonadales	Alteromonadales	Gammaproteobacteria	Marinobacter	0.15606	0.13519	0.16961	0.20181
<i>nosZ I</i>	A1_1863	Bacteria	Proteobacteria	Rhodocyclales	Rhodocyclales	Betaproteobacteria	Azoarcus	0.05919	0.03380	0.04989	0.06229
<i>nosZ I</i>	A1_2344	Bacteria	Proteobacteria	Pseudomonadales	Pseudomonadales	Gammaproteobacteria	Pseudomonas	0.02153	0.03755	0.01995	0.02990
<i>nosZ I</i>	A1_23555	Bacteria	Proteobacteria	Rhodobacterales	Rhodobacterales	Alphaproteobacteria	Roseovarius	0.08879	0.11266	0.07982	0.10464
<i>nosZ I</i>	A1_25707	Bacteria	Proteobacteria	Rhodobacterales	Rhodobacterales	Alphaproteobacteria	Roseovarius	0.03767	0.02629	0.01995	0.03737

<i>nosZ I</i>	A1_29855	Bacteria	Proteobacteria	Rhodobacterales	Rhodobacteraceae	Alphaproteobacteria	Roseovarius	0.01883	0.07135	0.04989	0.04236
<i>nosZ I</i>	A1_7747	Bacteria	Proteobacteria	Rhodocyclales	Rhodocyclaceae	Betaproteobacteria	Azoarcus	0.06727	0.07886	0.14966	0.09966
<i>nosZ I</i>	A1_7862	Bacteria	Proteobacteria	Alteromonadales	Alteromonadaceae	Gammaproteobacteria	Marinobacter	0.01614	0.01127	0.00000	0.00747
<i>nosZ I</i>	A1_788	Bacteria	Proteobacteria	Rhodobacterales	Rhodobacteraceae	Alphaproteobacteria	Roseovarius	0.08610	0.14270	0.19954	0.09468
<i>nosZ I</i>	A1_827	Bacteria	Proteobacteria	Pseudomonadales	Pseudomonadaceae	Gammaproteobacteria	Pseudomonas	0.82333	0.87123	1.27706	1.16604
<i>nosZ I</i>	C2_1511	Bacteria	Proteobacteria	Rhizobiales	Bradyrhizobiaceae	Alphaproteobacteria	Bradyrhizobium	0.00000	0.00000	0.00998	0.00000
<i>nosZ I</i>	C2_4712	Bacteria	Proteobacteria	Rhizobiales	Bradyrhizobiaceae	Alphaproteobacteria	Bradyrhizobium	0.00000	0.00000	0.00998	0.00249
<i>nosZ I</i>	C2_7831	Bacteria	Proteobacteria	Rhodocyclales	Rhodocyclaceae	Betaproteobacteria	Thauera	0.00269	0.00000	0.00000	0.00000
<i>nosZ I</i>	C2_975	Bacteria	Proteobacteria	Burkholderiales	Comamonadaceae	Betaproteobacteria	Acidovorax	0.08072	0.11641	0.44897	0.23670
<i>nosZ I</i>	DA1_5583	Bacteria	Proteobacteria	Rhodobacterales	Rhodobacteraceae	Alphaproteobacteria	Roseovarius	0.02960	0.04506	0.02993	0.04485
<i>nosZ I</i>	DA2_28640	Bacteria	Proteobacteria	Pseudomonadales	Pseudomonadaceae	Gammaproteobacteria	Pseudomonas	0.01345	0.02253	0.01995	0.05980
<i>nosZ II</i>	A1_116	Bacteria	Bacteroidetes	Flavobacteriales	Flavobacteriaceae	Flavobacteriia	Maribacter	0.15337	0.16523	0.14966	0.15447
<i>nosZ II</i>	A1_4166	Bacteria	Bacteroidetes	Flavobacteriales	Flavobacteriaceae	Flavobacteriia	Maribacter	0.04305	0.02629	0.01995	0.05980

Data are from a previous survey at Douro estuary¹¹ and corresponds to four samplings at the same site in December and June of 2014 and 2015; taxonomic classification was obtained with SILVA SSU Ref dataset (v128; <http://www.arb-silva.de>). Sequences are deposited at the European Nucleotide Archive under the accession number PRJEB38114.

Table S2. Real-time PCR conditions and respective sequencing primers

Gene	Primer	Sequence	Fragment size (bp)	Annealing temp (°C)	Standard curves real-time PCR ^d					
					qPCR			RT-qPCR		
					Standard (ng/μL) ^e	R ² / Efficiency(%)	Tm (°C)	Standard (ng/μL) ^f	R ² / Efficiency(%)	Tm (°C)
<i>nirK</i>	nirKC2F/nirKC2R ²	TGCACATCGCCAACGGNATGTWYGG/ GGCGCGGAAGATGSHRTGRTCAC	457	56 ^a	0.01 – 5	0.994 / 95	80.8	N/A	N/A	N/A
<i>nirS</i>	nirSC1F/nirSC1R ²	ATCGTCAACGTCAARGARACVGG/ TTCGGGTGCGTCTTSABGAASAG	425	56 ^a	0.01 - 25	0.942 / 110	88.4	0.01 – 10	0.937 / 103	86.7
<i>nosZ</i>	nosZF2/ nosZR2 ⁹	CGCRACGGCAASAAGGTSMSST/ CAKRTGCAKSGCRTGGCAGAA	267	60 ^b	0.01 - 25	0.980 / 106	85.4	0.01 – 10	0.950 / 94	86.2
<i>16S rRNA</i>	341F/ 534R ²¹	CCTACGGGAGGCAGCAG/ ATTACCGCGGCTGCTGG	193	57 ^c	0.01 - 25	0.981 / 99	82.8	0.01 – 25	0.965 / 99	82.6

^a Thermal cycling conditions consisted of an initial denaturation step of 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, annealing at 56 °C for 30 s and 72 °C for 30 s, with a final extension step of 10 min at 72 °C.

^b Thermal cycling conditions consisted of an initial denaturation step of 95 °C for 10 min, followed by 6 cycles of denaturation at 95 °C for 15 s, annealing at 65 °C for 30 s, and extension at 72 °C for 30 s; followed by 40 cycles where the annealing temperature was changed to 60 °C; and a final extension step at 72 °C for 10 min.

^c Thermal cycling conditions consisted of an initial denaturation step of 95 °C for 10 min, followed by 8 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s, and extension at 72 °C for 30 s; followed by 27 cycles where the annealing temperature was changed to 57 °C; and a final extension step at 72 °C for 10 min.

^d Reaction mixture (20 μl): 10 μl of Power SYBR Green PCR Master Mix (ThermoFisher Scientific), 0.6 μM of primers and 1 μl of template.

^e Standards consisted of linearized plasmid DNA obtained after cloning conventional PCR products for each gene. Standard curves were made with 5 serial dilutions and the ranges are presented for each gene.

^f Standards consisted of cDNA pooled from eight microcosms samples. Standard curves were made with 5 serial dilutions and the ranges are presented for each gene.

N/A - not applicable.

Table S3. *p*-values of the regressions of pH and NO₃⁻ concentration in water over time for microcosms exposed to Cu NPs < 50 nm (EXP50) and exposed to Cu NPs < 150 nm (EXP150), and *p*-values for the *t*-test comparing the slopes of both EXP

	pH	NO₃⁻ water
EXP50	1.68E-04	9.59E-07
EXP150	1.04E-03	1.63E-02
EXP50 vs EXP150	6.86E-01	7.40E-05

Supplementary Figures

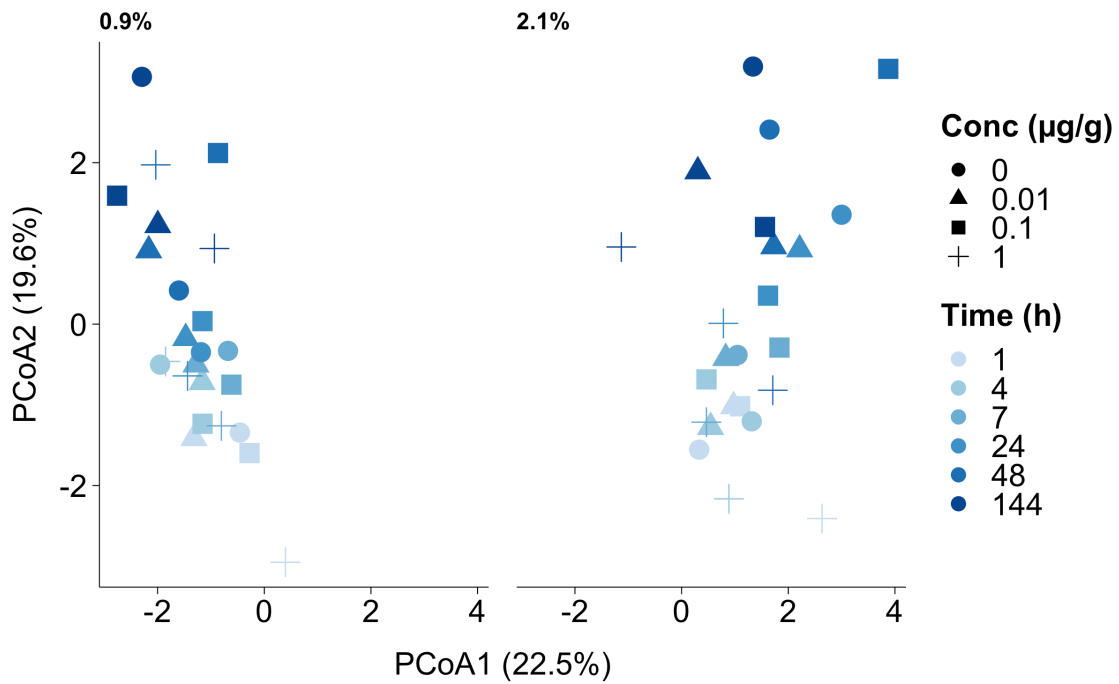


Fig. S1 Microcosms response throughout the exposure period under different salinities (0.9 % and 2.1 %). Principal coordinates analysis was carried out on euclidean distances of the standardized data (z-score) from DIN and metal environmentally available fraction and shows the microcosms amended with Cu NPs < 50 nm behaving similarly under the two salinity regimes.

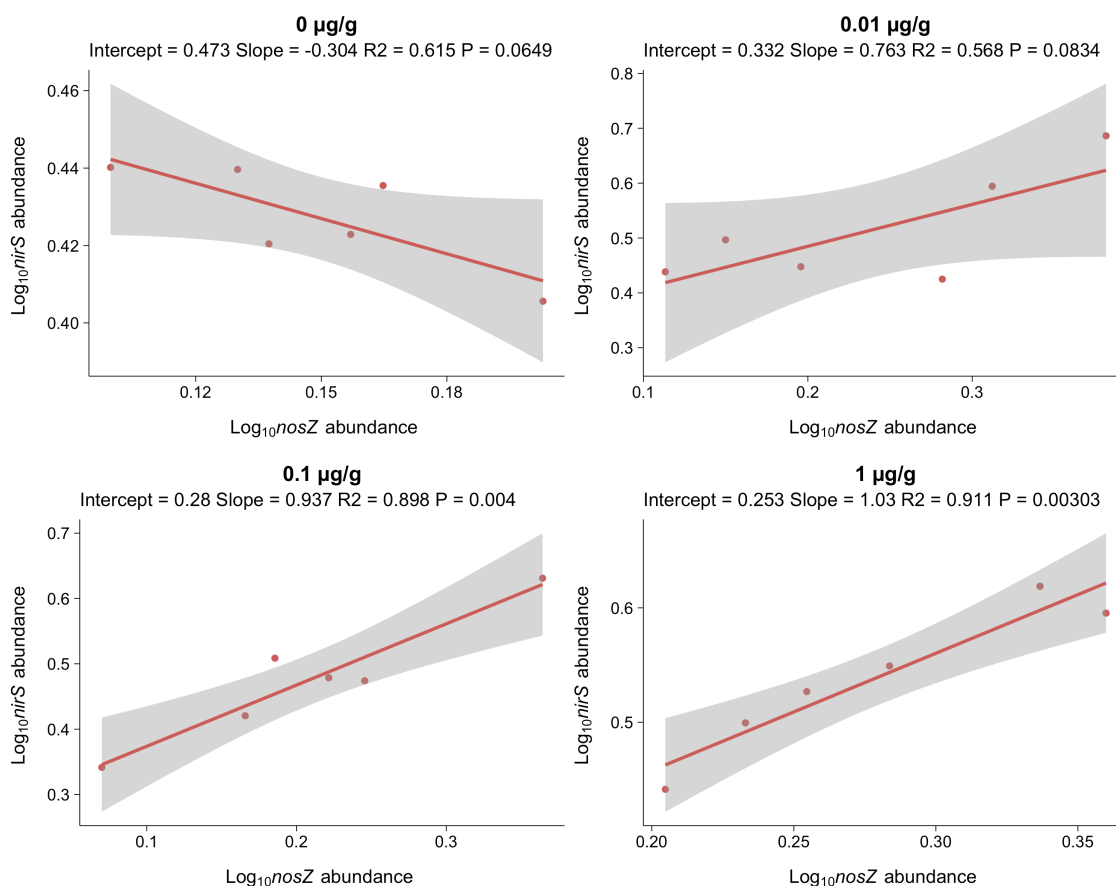


Fig. S2 Correlation between *nirS* and *nosZ* abundances in microcosms exposed to Cu NPs < 50 nm. Gene copy numbers *per gram* dry sediment were log10 transformed and *nirS* and *nosZ* values were normalized by the *16S rRNA* gene. Data are the average of two replicates, with a RSD between 9 and 24 % and the linear regression is shown with 95 % confidence interval.

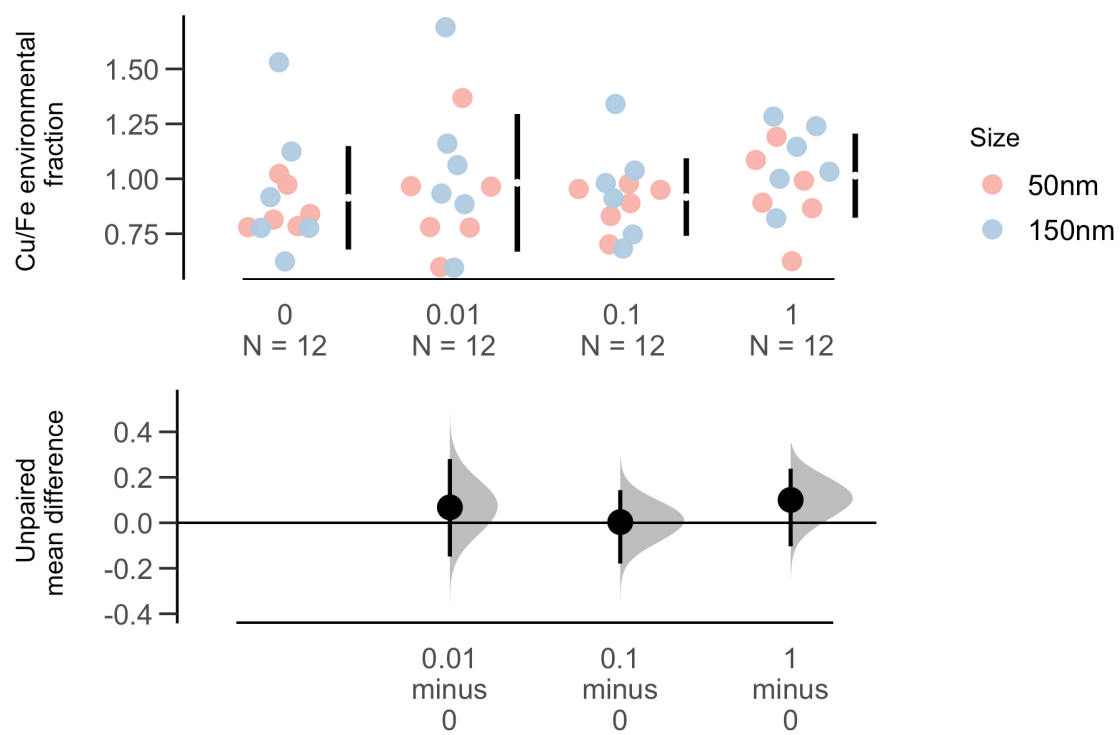


Fig. S3 Estimation plot of Cu environmental fraction normalised to Fe. Results are reported for microcosms exposed to Cu NPs 0, 0.01, 0.1 and 1 $\mu\text{g g}^{-1}$. Gapped lines to the right of each concentration group are mean value \pm standard deviation and the effect size, with 95 % confidence intervals, is plotted below.

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