Electronic Supplementary Material

Nanoparticles induce dermal and intestinal innate immune system responses in zebrafish embryos

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Table S1 Primer sequences used for quantitative Real-Time PCR analysis.

Target gene	Primer Sequence (5' to 3')	Accession no.[a]
rpl13α	Forward: AGC TCA AGA TGG CAA CAC AG	NM_198143.1
	Reverse: AAG TTC TTC TCG TCC TCC GA	
il1β[b]	Forward: CAT TTG CAG GCC GTC ACA	NM_212844.2
	Reverse: GGA CAT GCT GAA GCG CAC TT	
tnfα[b]	Forward: ACC AGG CCT TTT CTT CAG GT	NM_212859.2
	Reverse: TGC CCA GTC TGT CTC CTT CT	
irg1l[b]	Forward: TCT CCA ATG AGG CTC ACA ACA TCC	NM_001077607.1[c]
	Reverse: AGC ACA GGC AGC AGC ACT AC	
socs3a	Forward: CCA ACA CGG GTC TTC TGT G	NM_199950
	Reverse: CGA GTC ACA TCC ATC GTC A	
ccl20a	Forward: TGC AGC TGT GTC GTG TTG C	NM_001136254.2[c]
	Reverse: ATT GCT TGC ACC TTC TCC CTC	
mucms1	Forward: CAC AGG ATT CAC TGC CAA C	XM_009293560.1[c]
	Reverse: GCA TGA TGT CGT GTT TTT GC	
trpv6	Forward: GGG ATG GAA TGA AAT GTT GG	NM_001001849.1[c]
	Reverse: TAA TGC AGG CTG CAT TGT TC	
apoa2	Forward: GCA CTC CAA GTG TCA GTG TGT	NM_001130586.1
	Reverse: TCT AGT GCT GGC TCA ACT GC	
arrdc3b	Forward: GAA AGA CCC GAA GCT CCA C	NM_001004605.1[c]

	Reverse: GGG GAT CAA CCT CAG AAT AC	
aox5	Forward: CTC CGC AGG GTG TTT TGT TG	NM_001278796.1[c]
	Reverse: GGG GTT CTC CAA TGC CCT TT	
try	Forward: CGC CAG TGG AAG CAA TTA C	NM_131708.2[c]
	Reverse: CTT GCC TCC CTC CAT AAA AC	

[a] GeneBank accession number (http://www.ncbi.nlm.nih.gov).

[b] Primer pairs used for expression analysis in intestinal tissue.

[c] self-designed primer pairs

Table S2 Speciation of 0.59 mg L^{-1} copper in egg water and egg water supplemented with 12.9 mg L^{-1} Suwannee river fulvic acid (SRHA) calculated using Visual Minteq.

Component	Without SRHA % of total concentration	Species name	With SRHA % of total concentration	Species name
Cu+2	49.391	Cu+2	9.215	Cu+2
	7.588	CuOH+	1.416	CuOH+
	0.1	Cu(OH)2 (aq)	0.019	Cu(OH)2 (aq)
	0.576	Cu2(OH)2+2	0.02	Cu2(OH)2+2
	25.552	CuCl+	4.767	CuCl+
	2.739	CuCl2 (aq)	0.511	CuCl2 (aq)
	0.024	CuCl3-	2.616	CuSO4 (aq)
	14.022	CuSO4 (aq)	13.845	FA1-Cu(6)(s)
			26.098	FA2-Cu(6)(s)
			5.641	FA1-Cu(7)(aq)
			35.843	FA2-Cu(7)(aq)
Na+1	83.56	Na+1	83.554	Na+1
	1.954	NaSO4-	14.485	NaCl (aq)
	14.486	NaCl (aq)	1.954	NaSO4-
K+1	82.896	K+1	82.888	K+1
	2.578	KSO4-	14.525	KCl (aq)
	14.526	KCl (aq)	2.578	KSO4-
Mg+2	44.56	Mg+2	44.549	Mg+2
	45.468	MgCl+	45.455	MgCl+
	9.971	MgSO4 (aq)	9.969	MgSO4 (aq)
Ca+2	51.908	Ca+2	51.881	Ca+2
	33.419	CaCl+	33.401	CaCl+
	14.673	CaSO4 (aq)	14.667	CaSO4 (aq)
			0.026	(6)Ca+2D(s)
			0.013	FA1-Ca(6)(s)
Sr+2	60.677	Sr+2	60.642	Sr+2
	24.348	SrCl+	24.333	SrCl+
	14.975	SrSO4 (aq)	14.968	SrSO4 (aq)
			0.03	(6)Sr+2D(s)
			0.013	FA1-Sr(6)(s)
Cl-1	81.745	Cl-1	81.748	Cl-1
	0.602	CaCl+	0.602	CaCl+

	4.536	MgCl+	4.535	MgCl+
	0.262	KCl (aq)	0.262	KCl (aq)
	12.846	NaCl (aq)	12.845	NaCl (aq)
SO4-2	31.01	SO4-2	31.016	SO4-2
	39.275	NaSO4-	22.544	MgSO4 (aq)
	1.054	KSO4-	5.993	CaSO4 (aq)
	22.548	MgSO4 (aq)	0.114	SrSO4 (aq)
	5.996	CaSO4 (aq)	39.278	NaSO4-
	0.114	SrSO4 (aq)	1.054	KSO4-
(7)H+1D(aq)			0.164	(7)Sr+2D(aq)
			7.55	(7)Ca+2D(aq)
			1.162	(7)K+1D(aq)
			35.876	(7)Mg+2D(aq)
			57.623	(7)Na+1D(aq)
HFA1-(6)(s)			87.209	HFA1-(6)(s)
			1.216	FA1-H(6)(s)
			2.778	FA1-Ca(6)(s)
			1.694	FA1-Cu(6)(s)
			7.051	FA1-Mg(6)(s)
			0.052	FA1-Sr(6)(s)
HFA2-(6)(s)			14.955	HFA2-(6)(s)
			74.924	FA2-H(6)(s)
			10.099	FA2-Cu(6)(s)
			0.021	FA2-Mg(6)(s)
HFA1-(7)(aq)			85.851	HFA1-(7)(aq)
			0.995	FA1-H(7)(aq)
			1.88	FA1-Ca(7)(aq)
			1.289	FA1-Cu(7)(aq)
			9.95	FA1-Mg(7)(aq)
			0.035	FA1-Sr(7)(aq)
HFA2-(7)(aq)			15.339	HFA2-(7)(aq)
			74.51	FA2-H(7)(aq)
			10.116	FA2-Cu(7)(aq)
			0.033	FA2-Mg(7)(aq)

H+1D(s)(6)	14.278	(6)Ca+2D(s)
	1.832	(6)K+1D(s)
	27.14	(6)Mg+2D(s)
	59.036	(6)Na+1D(s)
	0.311	(6)Sr+2D(s)

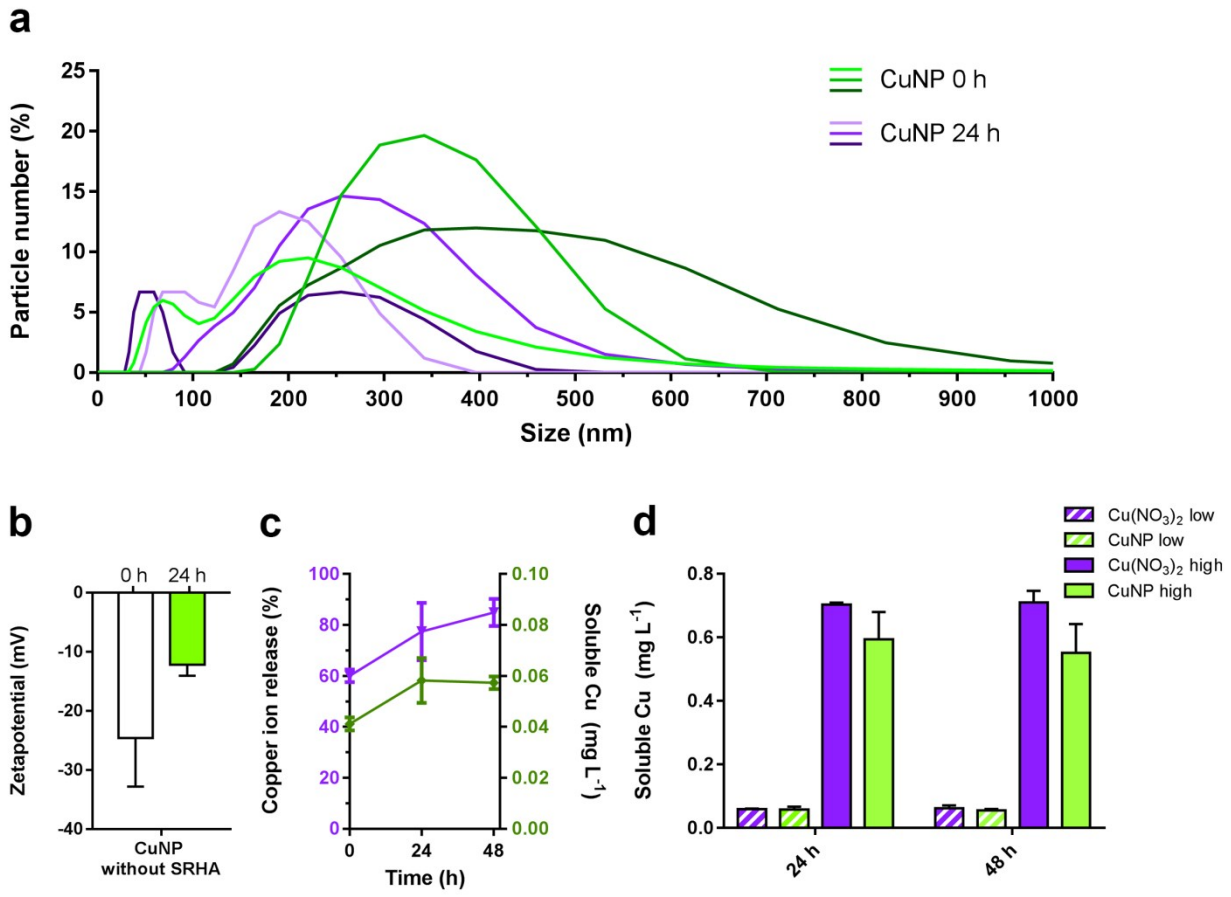

Figure S1. Characterisation of CuNP in medium with and without the addition of SRHA. (**a**) DLS profiles derived from the Malvern Zetasizer showing the distribution of the hydrodynamic diameter in relation to particle number (%) and (**b**) zeta potential of CuNP in egg water without SRHA after 0 h and 24 h. (**c**) Copper fraction (in %) and concentration of soluble copper (in mg L^{-1}) determined in the supernatant after centrifugation of 0.1 mg L^{-1} CuNP and (**d**) dissolution profile of Cu over time of 0.1 mg L^{-1} CuNP (low) and 1 mg L^{-1} CuNP (high) in egg water supplemented with SRHA mg L^{-1}. Error bars are + standard deviation (SD) of measured values for each exposure group consisting of 3 replicates.

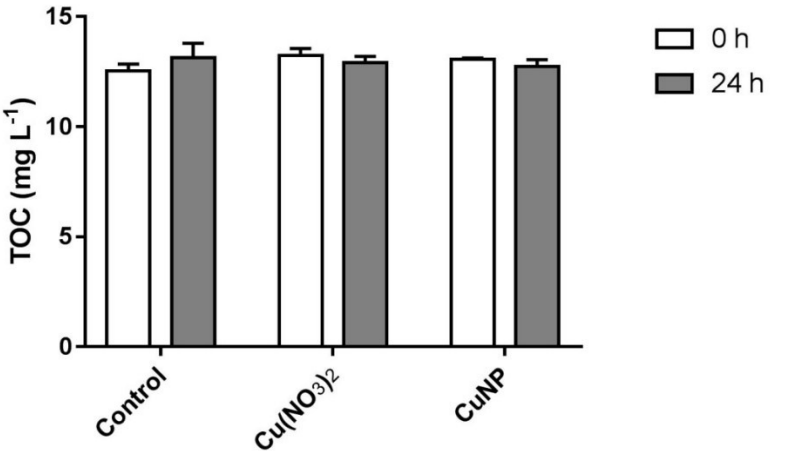

Figure S2. Total organic carbon (TOC) concentration in CuNP suspensions containing 30 mg L^{-1} SRHA (nominal) over time. Error bars are + standard deviation (SD) of measured values for each exposure group consisting of 3 replicates.

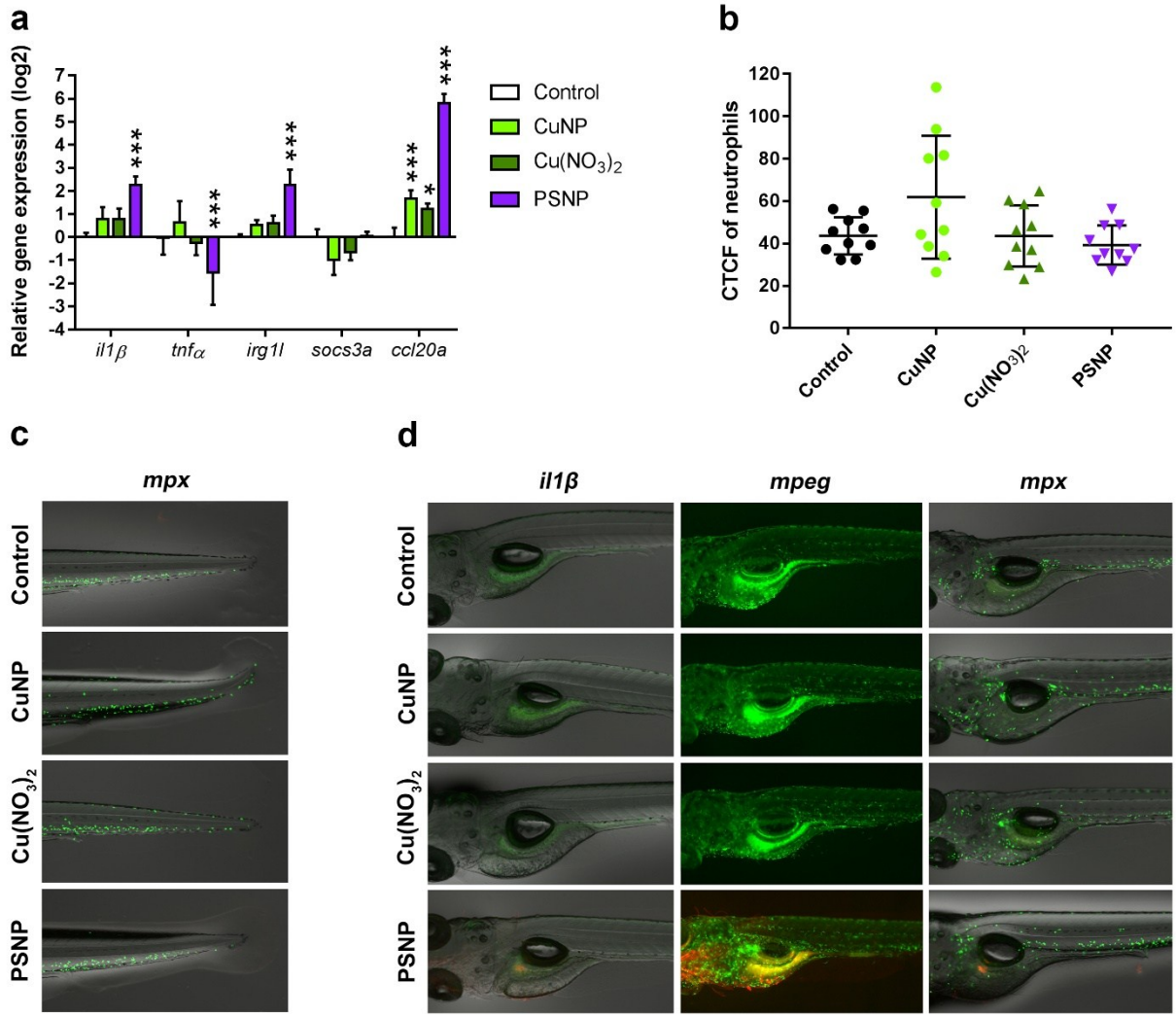

Figure S3. Immune response in zebrafish embryos after waterborne exposure to CuNP, corresponding $Cu(NO_3)_2$, and PSNP. (**a**)Transcriptional alterations of immune response related genes (*il1β, tnfα, irg1l, socs3a, ccl20a*) in whole zebrafish embryos exposed to 1 mg L^{-1} CuNP, corresponding $Cu(NO_3)_2$ concentration and 10 mg L^{-1} PSNP from 72-120 hpf. Relative expression levels were normalized to *rpl13α*, calculated relative to expression levels in control embryos. Asterisks indicate significant differences to controls (*p < 0.05, **p < 0.01, and ***p < 0.001). Values are presented as mean ± SD (n = 5). (**b**) Corrected total cell fluorescence (CTCF) of the tail area from *Tg(mpx:eGFP)* zebrafish embryos, in which GFP is expressed in neutrophils, exposed to 0.1 mg L^{-1} CuNP, corresponding $Cu(NO_3)_2$ concentration and 10 mg L^{-1} PSNP. Tha Analysis is based on images as depicted in c (n = 10). Embryos were exposed from 96-120 hpf. The tendency of increased neutrophil recruitment in the tail can be observed for CuNP. (**c**) Representative images of tail fluorescence. (**d**) Representative images of control, 0.1 mg L^{-1} CuNP, corresponding $Cu(NO_3)_2$, and 10 mg L^{-1} PSNP waterborne exposed *Tg(il1b:GFP-F)*, *Tg(mpeg1:eGFP)*, *Tg(mpx:eGFP)* zebrafish embryos. Embryos were exposed from 96-120 hpf. Transgenic reporter zebrafish line *il1β* expresses interleukin 1 beta, *mpeg1:eGFP* marks macrophages, *mpx:eGFP* marks neutrophils. Fluorescent red labeled PSNP show accumulation in the gut and on the skin, however, the particles are not co-localized with increased expression or phagocytic cell recruitment.

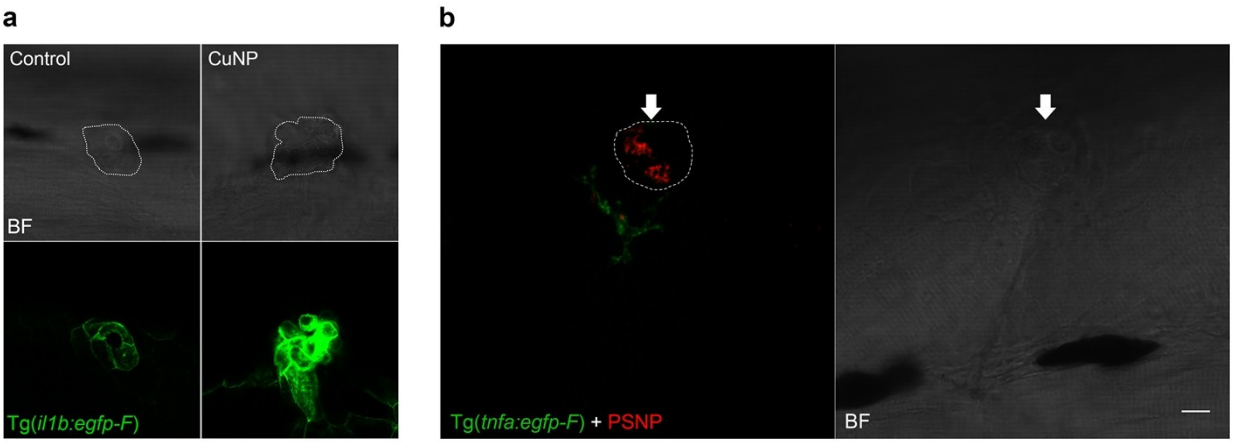

Figure S4. PSNP around neuromast engulfed by a macrophage and damage of cellular structure by CuNP. (**a**) Neuromast structure with healthy hexagonal cells in the control and cellular neuromast structure affected by CuNP treatment as shown by the expression of *il1β* (green). (**b**) PSNP accumulation (red) in neuromast (white arrow) is delineated by white lines and below PSNP (red) is phagocytosed by a *tnfα* positive macrophage (green). Scale bar = 10 μm.

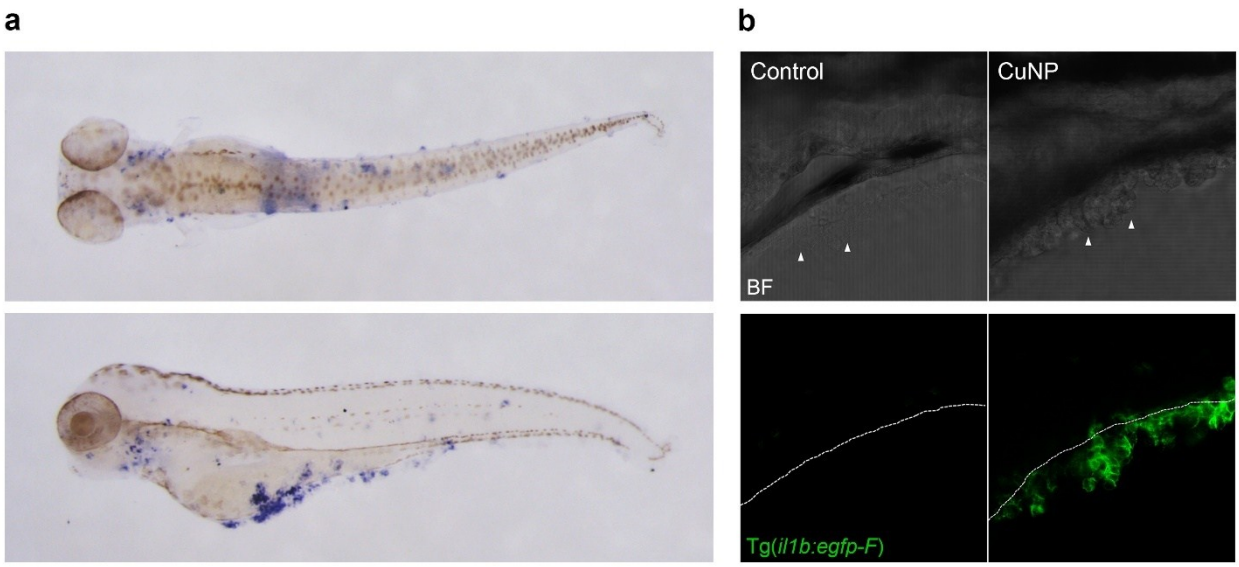

Figure S5. Expression of *irg1l* and *il1β* in skin cells. (**a**) Images of *in situ* hybridization showing the expression of *irg1l* mRNA on the outer epidermis in zebrafish embryos at 120 hpf after exposure to 1 mg L^{-1} CuNP. (**b**) Skin cells towards the distal end of the yolk sack. Control cells with normal hexagonal structures and CuNP exposed embryos with structurally damaged cells expressing *il1β* (green).

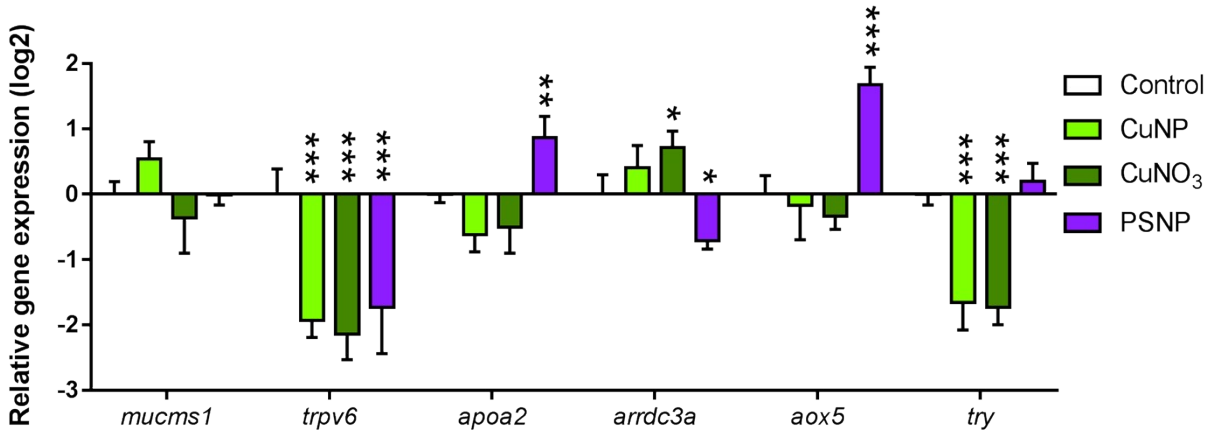

Figure S6. Gene expression in zebrafish embryos after waterborne exposure to CuNP, corresponding $Cu(NO_3)_2$, and PSNP. Transcriptional alterations of mucin (*mucms1*), transient receptor potential cation channel (*trpv6*), apolipoprotein (*apoa2*), arrestin domain containing 3a (*arrdc3a*), aldehyde oxidase 5 (*aox5*), and trypsin (*try*) in zebrafish embryos exposed to 1 mg L^{-1} CuNP, corresponding $Cu(NO_3)_2$ concentration and 10 mg L^{-1} PSNP from 72-120 hpf. Relative expression levels were normalized to *rpl13α*, calculated relative to expression levels in control embryos. Asterisks indicate significant differences to controls (*p < 0.05, **p < 0.01, and ***p < 0.001). Values are presented as mean ± SD (n = 5).

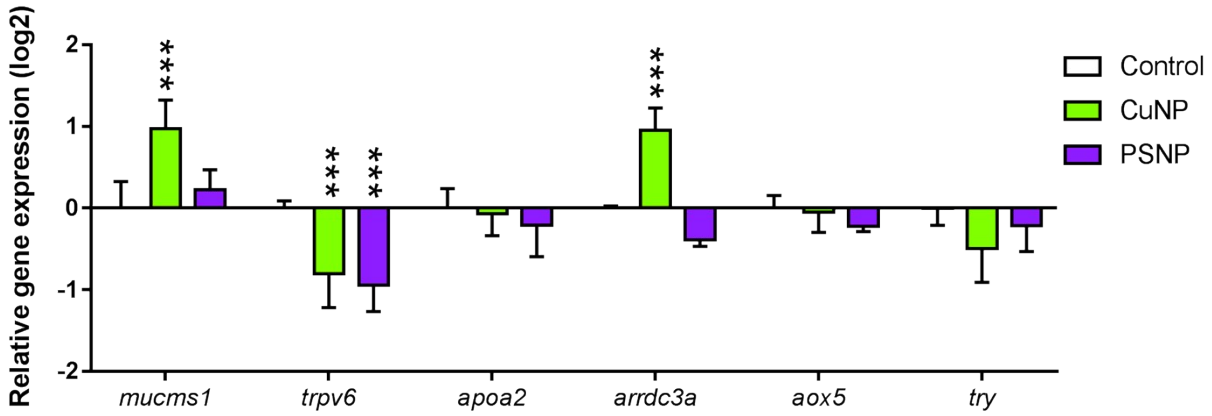

Figure S7. Gene expression in zebrafish embryos after injection CuNP and PSNP. Transcriptional alterations of mucin (*mucms1*), transient receptor potential cation channel (*trpv6*), apolipoprotein (*apoa2*), arrestin domain containing 3a (*arrdc3a*), aldehyde oxidase 5 (*aox5*), and trypsin (*try*) in zebrafish embryos exposed to 1 mg L^{-1} CuNP, corresponding $Cu(NO_3)_2$ concentration and 10 mg L^{-1} PSNP from 72-120 hpf. Relative expression levels were normalized to *rpl13α*, calculated relative to expression levels in control embryos. Asterisks indicate significant differences to controls (*p < 0.05, **p < 0.01, and ***p < 0.001). Values are presented as mean ± SD (n = 5).