Expansion Microscopy Revealed Specific Impacts of Nano Zinc Oxide on Early Organ Development in Fish

Mengyu Wang[1,2], Wen-Xiong Wang[1,2]*

1School of Energy and Environment, State Key Laboratory of Marine Pollution, City University of Hong Kong, Kowloon, Hong Kong, China

2Research Centre for the Oceans and Human Health, City University of Hong Kong Shenzhen Research Institute, Shenzhen 518057, China

*Corresponding author, Email: wx.wang@cityu.edu.hk

This Supporting Information contains 9 pages.

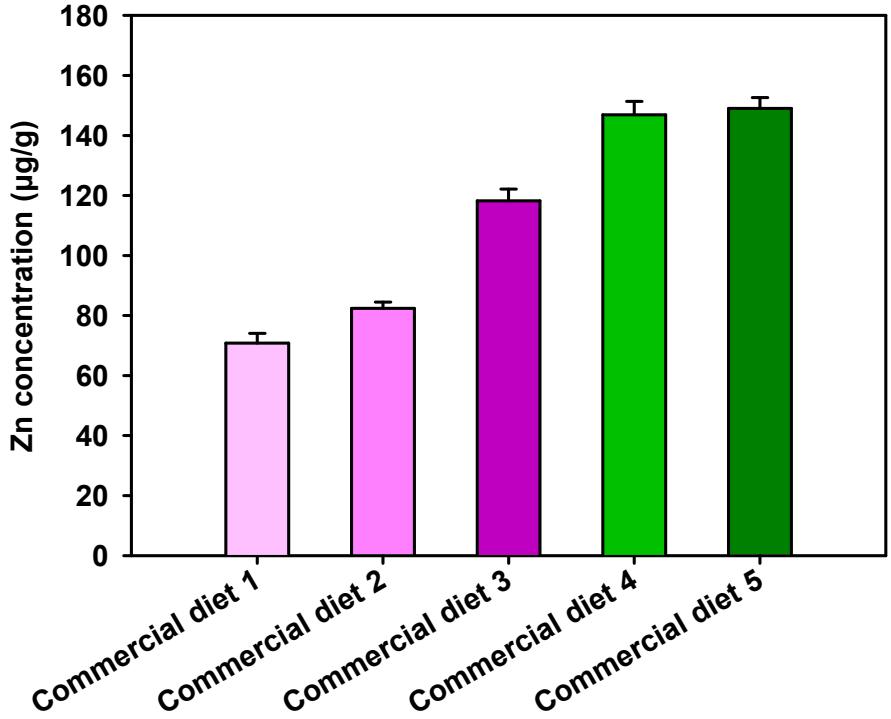

Figure S1. Zn concentration in 5 commercial fish diets purchased from the market.

Table S1. Composition of the basal diet.

Ingredients	g/kg
Casein	320
Gelatine	80
Dextrin	280
Cellulose	190
carboxymethylcellulose	20
Cod liver oil	30
Sunflower Seed Oil	30
Mineral premix	40
Vitamin premix	10

Where mineral premix included NaCl, 500; $MgSO_4 \cdot 7H_2O$, 7.5; $NaH_2PO_4 \cdot 2H_2O$, 12.5; KH_2PO_4, 16; Ca $(H_2PO_4) \cdot 2H_2O$, 100; $FeSO_4$, 125; $Ca(H_2PO_4)_2$, 44.6; $MnSO_4 \cdot 4H_2O$, 81; $CuSO_4 \cdot 5H_2O$, 2.5; $CoSO_4 \cdot 6H_2O$, 0.05; KI, 0.15 (g/kg).

Where mineral premix vitamin premix (mg/kg): thiamin, 20; riboflavin, 20;

pyridoxine, 20; cyanocobalamine, 2; folic acid, 5; calcium pantothenate, 50; inositol, 100; niacin, 100; biotin, 5; Vitamin A, 10; Vitamin D_3, 20; Vitamin E, 100; Vitamin K_3, 10; Choline chloride, 110 (g/kg).

Table S2. Concentrations and activities of aqueous inorganic species after Zn^{2+} (70 μg Zn/g diet) dissolution in SM7 medium at pH of 7.0 for 30 min (mol / L).

	Concentration	Activity	Log Activity
$Ca(NO_3)_2$	1.7886E-31	1.7886E-31	-30.747
Ca^{2+}	8.7325E-09	8.7097E-09	-8.06
$CaCO_3$ (aq)	3.6842E-18	3.6842E-18	-17.434
$CaHCO_3^+$	6.0342E-17	6.0303E-17	-16.22
$CaNO_3^+$	2.221E-17	2.2195E-17	-16.654
$CaOH^+$	1.751E-14	1.7499E-14	-13.757
$CaSO_4$ (aq)	1.7608E-14	1.7608E-14	-13.754
CO_3^{2-}	2.5555E-13	2.5488E-13	-12.594
H^+	1.0007E-07	0.0000001	-7
H_2CO_3 (aq)	1.2227E-10	1.2227E-10	-9.913
HCO_3^-	5.4403E-10	5.4367E-10	-9.265
HSO_4^-	8.6298E-14	8.6242E-14	-13.064
K^+	1.2787E-09	1.2779E-09	-8.894
KNO_3 (aq)	6.6488E-19	6.6488E-19	-18.177
KOH (aq)	2.2361E-16	2.2361E-16	-15.651
KSO_4^-	7.9889E-17	7.9837E-17	-16.098
Mg^{2+}	2.0565E-08	2.0511E-08	-7.688
$Mg_2CO_3^{2+}$	4.1828E-25	4.1718E-25	-24.38
$MgCO_3$ (aq)	4.3484E-18	4.3484E-18	-17.362
$MgHCO_3^+$	1.1445E-16	1.1438E-16	-15.942
$MgOH^+$	7.8574E-13	7.8523E-13	-12.105
$MgSO_4$ (aq)	3.2939E-14	3.2939E-14	-13.482
Na^+	1.7399E-09	1.7388E-09	-8.76
$NaCO_3^-$	8.2578E-21	8.2524E-21	-20.083
$NaHCO_3$ (aq)	4.7378E-19	4.7378E-19	-18.324
$NaNO_3$ (aq)	3.9491E-19	3.9491E-19	-18.404
NaOH (aq)	2.2041E-16	2.2041E-16	-15.657
$NaSO_4^-$	8.4381E-17	8.4325E-17	-16.074

NO_3^-	8.0639E-10	8.0586E-10	-9.094
OH^-	1.0076E-07	1.0069E-07	-6.997
SO_4^{2-}	8.8482E-09	8.8251E-09	-8.054
$Zn(CO_3)_2^{2-}$	8.5094E-26	8.4871E-26	-25.071
$Zn(NO_3)_2$ (aq)	2.1311E-26	2.1311E-26	-25.671
$Zn(OH)_2$ (aq)	8.3577E-11	8.3577E-11	-10.078
$Zn(OH)_3^-$	2.663E-15	2.6612E-15	-14.575
$Zn(OH)_4^{2-}$	4.2582E-21	4.247E-21	-20.372
$Zn(SO_4)_2^{2-}$	9.7422E-21	9.7167E-21	-20.012
Zn^{2+}	6.5648E-08	6.5476E-08	-7.184
Zn_2OH^{3+}	4.3424E-17	4.3169E-17	-16.365
$ZnCO_3$ (aq)	9.6033E-16	9.6033E-16	-15.018
$ZnHCO_3^+$	1.1264E-15	1.1257E-15	-14.949
$ZnNO_3^+$	1.3263E-16	1.3254E-16	-15.878
$ZnOH^+$	6.5973E-10	6.593E-10	-9.181
$ZnSO_4$ (aq)	1.2642E-13	1.2642E-13	-12.898

Table S3. Concentrations and activities of aqueous inorganic species after Zn^{2+} (150 μg Zn/g diet) dissolution in SM7 medium at pH of 7.0 for 30 min (mol / L).

	Concentration	Activity	Log Activity
$Ca(NO_3)_2$	1.7186E-17	1.7199E-17	-16.765
Ca^{2+}	0.00031565	0.00024655	-3.608
$CaCO_3$ (aq)	5.9132E-09	5.9175E-09	-8.228
$CaHCO_3^+$	1.0303E-07	9.6859E-08	-7.014
$CaNO_3^+$	3.8952E-08	3.6619E-08	-7.436
$CaOH^+$	5.2689E-10	4.9533E-10	-9.305
$CaSO_4$ (aq)	0.000034203	0.000034228	-4.466
CO_3^{2-}	1.8515E-08	1.4462E-08	-7.84
H^+	1.0637E-07	0.0000001	-7
H_2CO_3 (aq)	6.9328E-06	6.9379E-06	-5.159
HCO_3^-	0.000032814	0.000030848	-4.511
HSO_4^-	6.2992E-09	5.922E-09	-8.228
K^+	0.000049785	0.000046803	-4.33
KNO_3 (aq)	1.4182E-09	1.4193E-09	-8.848
KOH (aq)	8.1836E-12	8.1896E-12	-11.087
KSO_4^-	2.1358E-07	2.0079E-07	-6.697

Mg^{2+}	0.00046024	0.0003595	-3.444
$Mg_2CO_3^{2+}$	9.3093E-12	7.2715E-12	-11.138
$MgCO_3$ (aq)	4.3212E-09	4.3244E-09	-8.364
$MgHCO_3^+$	1.2099E-07	1.1374E-07	-6.944
$MgOH^+$	1.4639E-08	1.3762E-08	-7.861
$MgSO_4$ (aq)	0.000039614	0.000039643	-4.402
Na^+	0.000039866	0.000037478	-4.426
$NaCO_3^-$	1.0736E-11	1.0093E-11	-10.996
$NaHCO_3$ (aq)	5.7902E-10	5.7944E-10	-9.237
$NaNO_3$ (aq)	4.9574E-10	4.961E-10	-9.304
NaOH (aq)	4.7473E-12	4.7508E-12	-11.323
$NaSO_4^-$	1.3276E-07	1.2481E-07	-6.904
NO_3^-	0.000049959	0.000046967	-4.328
OH^-	1.071E-07	1.0069E-07	-6.997
SO_4^{2-}	0.00077581	0.00060599	-3.218
$Zn(CO_3)_2^{2-}$	6.3117E-16	4.9301E-16	-15.307
$Zn(NO_3)_2$ (aq)	1.3051E-16	1.3061E-16	-15.884
$Zn(OH)_2$ (aq)	1.5068E-10	1.5079E-10	-9.822
$Zn(OH)_3^-$	5.1071E-15	4.8012E-15	-14.319
$Zn(OH)_4^{2-}$	9.8091E-21	7.6619E-21	-20.116
$Zn(SO_4)_2^{2-}$	1.0583E-10	8.2666E-11	-10.083
Zn^{2+}	1.5125E-07	1.1814E-07	-6.928
Zn_2OH^{3+}	2.4501E-16	1.4053E-16	-15.852
$ZnCO_3$ (aq)	9.8244E-11	9.8316E-11	-10.007
$ZnHCO_3^+$	1.2259E-10	1.1525E-10	-9.938
$ZnNO_3^+$	1.4825E-11	1.3938E-11	-10.856
$ZnOH^+$	1.2653E-09	1.1895E-09	-8.925
$ZnSO_4$ (aq)	1.5651E-08	1.5662E-08	-7.805

Table S4. Concentrations and activities of aqueous inorganic species after *n*ZnO (70 µg Zn/g diet) dissolution in SM7 medium at pH of 7.0 for 30 min (mol / L).

	Concentration	**Activity**	**Log Activity**
$Ca(NO_3)_2$	1.7888E-31	1.7888E-31	-30.747
Ca^{2+}	8.7325E-09	8.7103E-09	-8.06
$CaCO_3$ (aq)	3.6845E-18	3.6845E-18	-17.434
$CaHCO_3^+$	6.0347E-17	6.0308E-17	-16.22
$CaNO_3^+$	2.2211E-17	2.2197E-17	-16.654

$CaOH^+$	1.7511E-14	1.75E-14	-13.757
$CaSO_4$ (aq)	1.7611E-14	1.7611E-14	-13.754
CO_3^{2-}	2.5554E-13	2.5488E-13	-12.594
H^+	1.0006E-07	0.0000001	-7
H_2CO_3 (aq)	1.2228E-10	1.2228E-10	-9.913
HCO_3^-	5.4403E-10	5.4368E-10	-9.265
HSO_4^-	8.6303E-14	8.6248E-14	-13.064
K^+	1.2787E-09	1.2779E-09	-8.894
KNO_3 (aq)	6.6491E-19	6.6491E-19	-18.177
KOH (aq)	2.2361E-16	2.2361E-16	-15.651
KSO_4^-	7.9895E-17	7.9845E-17	-16.098
Mg^{2+}	2.0565E-08	2.0513E-08	-7.688
$Mg_2CO_3^{2+}$	4.1831E-25	4.1725E-25	-24.38
$MgCO_3$ (aq)	4.3488E-18	4.3488E-18	-17.362
$MgHCO_3^+$	1.1446E-16	1.1439E-16	-15.942
$MgOH^+$	7.8578E-13	7.8528E-13	-12.105
$MgSO_4$ (aq)	3.2944E-14	3.2944E-14	-13.482
Na^+	1.7399E-09	1.7388E-09	-8.76
$NaCO_3^-$	8.2579E-21	8.2526E-21	-20.083
$NaHCO_3$ (aq)	4.738E-19	4.738E-19	-18.324
$NaNO_3$ (aq)	3.9493E-19	3.9493E-19	-18.403
NaOH (aq)	2.2042E-16	2.2042E-16	-15.657
$NaSO_4^-$	8.4387E-17	8.4333E-17	-16.074
NO_3^-	8.0639E-10	8.0587E-10	-9.094
OH^-	1.0076E-07	1.0069E-07	-6.997
SO_4^{2-}	8.8482E-09	8.8257E-09	-8.054
$Zn(CO_3)_2^{2-}$	7.4116E-26	7.3927E-26	-25.131
$Zn(NO_3)_2$ (aq)	1.8563E-26	1.8563E-26	-25.731
$Zn(OH)_2$ (aq)	7.2798E-11	7.2798E-11	-10.138
$Zn(OH)_3^-$	2.3195E-15	2.318E-15	-14.635
$Zn(OH)_4^{2-}$	3.7087E-21	3.6993E-21	-20.432
$Zn(SO_4)_2^{2-}$	8.4864E-21	8.4648E-21	-20.072
Zn^{2+}	5.7177E-08	5.7032E-08	-7.244
Zn_2OH^{3+}	3.294E-17	3.2752E-17	-16.485

$ZnCO_3$ (aq)	8.3649E-16	8.3649E-16	-15.078
$ZnHCO_3^+$	9.8116E-16	9.8053E-16	-15.009
$ZnNO_3^+$	1.1552E-16	1.1545E-16	-15.938
$ZnOH^+$	5.7464E-10	5.7427E-10	-9.241
$ZnSO_4$ (aq)	1.1012E-13	1.1012E-13	-12.958

Table S5. Concentrations and activities of aqueous inorganic species after *n*ZnO (150 µg Zn/g diet) dissolution in SM7 medium at pH of 7.0 for 30 min (mol / L).

	Concentration	**Activity**	**Log Activity**
$Ca(NO_3)_2$	1.787E-31	1.787E-31	-30.748
Ca^{2+}	8.7325E-09	8.7044E-09	-8.06
$CaCO_3$ (aq)	3.6815E-18	3.6815E-18	-17.434
$CaHCO_3^+$	6.0308E-17	6.0259E-17	-16.22
$CaNO_3^+$	2.2196E-17	2.2179E-17	-16.654
$CaOH^+$	1.7502E-14	1.7488E-14	-13.757
$CaSO_4$ (aq)	1.7587E-14	1.7587E-14	-13.755
CO_3^{2-}	2.5567E-13	2.5485E-13	-12.594
H^+	1.0008E-07	0.0000001	-7
H_2CO_3 (aq)	1.2226E-10	1.2226E-10	-9.913
HCO_3^-	5.4404E-10	5.436E-10	-9.265
HSO_4^-	8.6258E-14	8.6189E-14	-13.065
K^+	1.2787E-09	1.2777E-09	-8.894
KNO_3 (aq)	6.6468E-19	6.6468E-19	-18.177
KOH (aq)	2.2357E-16	2.2357E-16	-15.651
KSO_4^-	7.984E-17	7.9776E-17	-16.098
Mg^{2+}	2.0565E-08	2.0499E-08	-7.688
$Mg_2CO_3^{2+}$	4.1797E-25	4.1663E-25	-24.38
$MgCO_3$ (aq)	4.3453E-18	4.3453E-18	-17.362
$MgHCO_3^+$	1.1438E-16	1.1429E-16	-15.942
$MgOH^+$	7.8539E-13	7.8475E-13	-12.105
$MgSO_4$ (aq)	3.2899E-14	3.2899E-14	-13.483
Na^+	1.7399E-09	1.7385E-09	-8.76
$NaCO_3^-$	8.2567E-21	8.2501E-21	-20.084
$NaHCO_3$ (aq)	4.7365E-19	4.7365E-19	-18.325

$NaNO_3$ (aq)	3.9479E-19	3.9479E-19	-18.404
NaOH (aq)	2.2038E-16	2.2038E-16	-15.657
$NaSO_4^-$	8.4329E-17	8.4261E-17	-16.074
NO_3^-	8.0639E-10	8.0574E-10	-9.094
OH^-	1.0077E-07	1.0069E-07	-6.997
SO_4^{2-}	8.8481E-09	8.8196E-09	-8.055
$Zn(CO_3)_2^{2-}$	1.8759E-25	1.8699E-25	-24.728
$Zn(NO_3)_2$ (aq)	4.695E-26	4.695E-26	-25.328
$Zn(OH)_2$ (aq)	1.8418E-10	1.8418E-10	-9.735
$Zn(OH)_3^-$	5.8694E-15	5.8647E-15	-14.232
$Zn(OH)_4^{2-}$	9.3896E-21	9.3594E-21	-20.029
$Zn(SO_4)_2^{2-}$	2.1456E-20	2.1387E-20	-19.67
Zn^{2+}	1.4476E-07	1.4429E-07	-6.841
Zn_2OH^{3+}	2.1117E-16	2.0965E-16	-15.679
$ZnCO_3$ (aq)	2.1161E-15	2.1161E-15	-14.674
$ZnHCO_3^+$	2.4824E-15	2.4805E-15	-14.605
$ZnNO_3^+$	2.9227E-16	2.9204E-16	-15.535
$ZnOH^+$	1.4541E-09	1.4529E-09	-8.838
$ZnSO_4$ (aq)	2.7842E-13	2.7842E-13	-12.555

Supplementary Note

Expansion Microscopy Protocol.[1]

Medaka larvae were first treated with 4% PFA (paraformaldehyde) at 4 °C and permeated with 0.1% (w/w) Triton X-100 in 1× PBS (phosphate-buffered saline, finalizing the PBST). Then, the medaka larvae were incubated in 0.1 mg/mL AcX (SE (6-((acryloyl)amino)hexanoic acid) (Acryloyl-X)) in PBST at 4 °C. After washing with PBST, the medaka was immersed in a gelation solution including 8.625% sodium acrylate, 2.5% acrylamide, 0.15% BIS (N, N'-methylenebisacrylamide), 0.2% 2,2'-azobis[2-(2-imidazoline-2-yl) propane] dihydrochloride [VA-044], 0.05% Triton X-100, 2 M NaCl, 1× PBS. Afterward, each sample was placed in a 0.5mm thick silicone sheet with the hollowed-out center and then filled with a fresh gelation solution in the middle of the sheet.

After gelation, the samples were embedded in the hydrogel. Gel samples need to be digested to allow transparency and full expansion. The gels were then gently placed in a 12-well plate with a paintbrush in a pre-warmed digestion buffer for pre-digestion. The digestion buffer consisted of 1 mM EDTA (Ethylenediaminetetraacetic acid), 50

mM Tris-HCl [pH 8.0], 0.5% [w/w] Triton X-100, 1 M NaCl, and 16 U/mL proteinase K. Each digestion lasted 8 hours and was repeated three times at 37 °C. After washing with in staining base buffer (0.1% Triton X-100, 1 M NaCl, 1× PBS), gels were stained in Alexa Fluor 488 NHS ester, ATTO 647N NHS ester. Then, gels were repeated in the previous pre-digestion to complete the post-digestion. Post-digested gels were incubated with a decalcification solution (10% [w/w] EDTA, 1 M NaCl, 1× PBS) to elute calcium ions. The gels were immersed in decreased concentrations of NaCl and finally in sufficient deionized water overnight for full absorption and expansion.

1. Sim, J.; Park, C. E.; Cho, I.; Min, K.; Eom, M.; Han, S.; Jeon, H.; Cho, H.-J.; Cho, E.-S.; Kumar, A., Nanoscale resolution imaging of the whole mouse embryos and larval zebrafish using expansion microscopy. *BioRxiv* **2021**.