Electronic Supplementary Information

Exposure to biogenic phosphorus nano-agromaterials promotes early hatching and causes no acute toxicity in zebrafish embryos

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ESI Table 1. Checklist for Minimum Information for Publication of Quantitative Real-Time PCR Experiments[1]

ITEM TO CHECK	IMPORTANCE	REPORTED
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	E	YES
Number within each group	E	YES
Assay carried out by core lab or investigator's lab?	D	YES
Acknowledgement of authors' contributions	D	YES
SAMPLE		
Description	E	YES
Volume/mass of sample processed	D	YES
Microdissection or macrodissection	E	N/A
Processing procedure	E	YES
If frozen - how and how quickly?	E	YES
If fixed - with what, how quickly?	E	N/A
Sample storage conditions and duration (especially for FFPE samples)	E	YES
NUCLEIC ACID EXTRACTION		

Procedure and/or instrumentation	E	YES
Name of kit and details of any modifications	E	YES
Source of additional reagents used	D	N/A
Details of DNase or RNAse treatment	E	YES
Contamination assessment (DNA or RNA)	E	YES
Nucleic acid quantification	E	YES
Instrument and method	E	YES
Purity (A260/A280)	D	NO
Yield	D	NO
RNA integrity method/instrument	E	N/A
RIN/RQI or Cq of 3' and 5' transcripts	E	N/A
Electrophoresis traces	D	NO
Inhibition testing (Cq dilutions, spike or other)	E	YES
REVERSE TRANSCRIPTION		
Complete reaction conditions	E	YES
Amount of RNA and reaction volume	E	YES
Priming oligonucleotide (if using GSP) and concentration	E	N/A
Reverse transcriptase and concentration	E	YES
Temperature and time	E	YES
Manufacturer of reagents and catalogue numbers	D	YES
Cqs with and without RT	D*	N/A
Storage conditions of cDNA	D	YES
qPCR TARGET INFORMATION		
If multiplex, efficiency and LOD of each assay.	E	N/A
Sequence accession number	E	YES
Location of amplicon	D	NO
Amplicon length	E	YES
In silico specificity screen (BLAST, etc)	E	YES
Pseudogenes, retropseudogenes or other homologs?	D	N/A
Sequence alignment	D	NO
Secondary structure analysis of amplicon	D	NO
Location of each primer by exon or intron (if applicable)	E	N/A
What splice variants are targeted?	E	N/A
qPCR OLIGONUCLEOTIDES		
Primer sequences	E	YES
RTPrimerDB Identification Number	D	YES
Probe sequences	D**	N/A
Location and identity of any modifications	E	N/A
Manufacturer of oligonucleotides	D	YES
Purification method	D	YES
qPCR PROTOCOL		

Complete reaction conditions	E	YES
Reaction volume and amount of cDNA/DNA	E	YES
Primer, (probe), Mg++ and dNTP concentrations	E	YES
Polymerase identity and concentration	E	YES
Buffer/kit identity and manufacturer	E	YES
Exact chemical constitution of the buffer	D	N/A
Additives (SYBR Green I, DMSO, etc.)	E	YES
Manufacturer of plates/tubes and catalog number	D	YES
Complete thermocycling parameters	E	YES
Reaction setup (manual/robotic)	D	YES
Manufacturer of qPCR instrument	E	YES
qPCR VALIDATION		
Evidence of optimisation (from gradients)	D	NO
Specificity (gel, sequence, melt, or digest)	E	N/A
For SYBR Green I, Cq of the NTC	E	N/A
Standard curves with slope and y-intercept	E	NO
PCR efficiency calculated from slope	E	YES
Confidence interval for PCR efficiency or standard error	D	NO
r2 of standard curve	E	YES
Linear dynamic range	E	NO
Cq variation at lower limit	E	NO
Confidence intervals throughout range	D	NO
Evidence for limit of detection	E	NO
If multiplex, efficiency and LOD of each assay.	E	N/A
DATA ANALYSIS		
qPCR analysis program (source, version)	E	YES
Cq method determination	E	YES
Outlier identification and disposition	E	N/A
Results of NTCs	E	YES
Justification of number and choice of reference genes	E	YES
Description of normalisation method	E	YES
Number and concordance of biological replicates	D	YES
Number and stage (RT or qPCR) of technical replicates	E	YES
Repeatability (intra-assay variation)	E	YES
Reproducibility (inter-assay variation, %CV)	D	YES
Power analysis	D	NO
Statistical methods for result significance	E	YES
Software (source, version)	E	YES
Cq or raw data submission using RDML	D	NO

E: essential information; D: desirable information; FFPE: formalin-fixed, paraffin-embedded; RIN: RNA integrity number; RQI: RNA quality indicator; GSP: gene- specific priming; dNTP: deoxynucleoside triphosphate; LOD: limit of detection. N/A: Not Applicable

ESI Table 2. Efficiency (E%) and R^2 values for the reference and target genes.

Genes	Efficiency (E%)	R^2 values
Reference gene: Beta actin (*β- actin*)	94	0.998
Reference gene: Elongation factor alpha (*elf-1α*)	92.2	0.994
Target gene: Activin type 2 receptor (*acvr 2 α*)	96.3	0.976
Target gene: Zebrafish hatching enzyme 1 (*zhe1*)	102.9	0.996
Target gene: Superoxide dismutase 1 (*sod 1*)	96.3	0.996
Target gene: Catalase (*cat*)	91.3	0.999

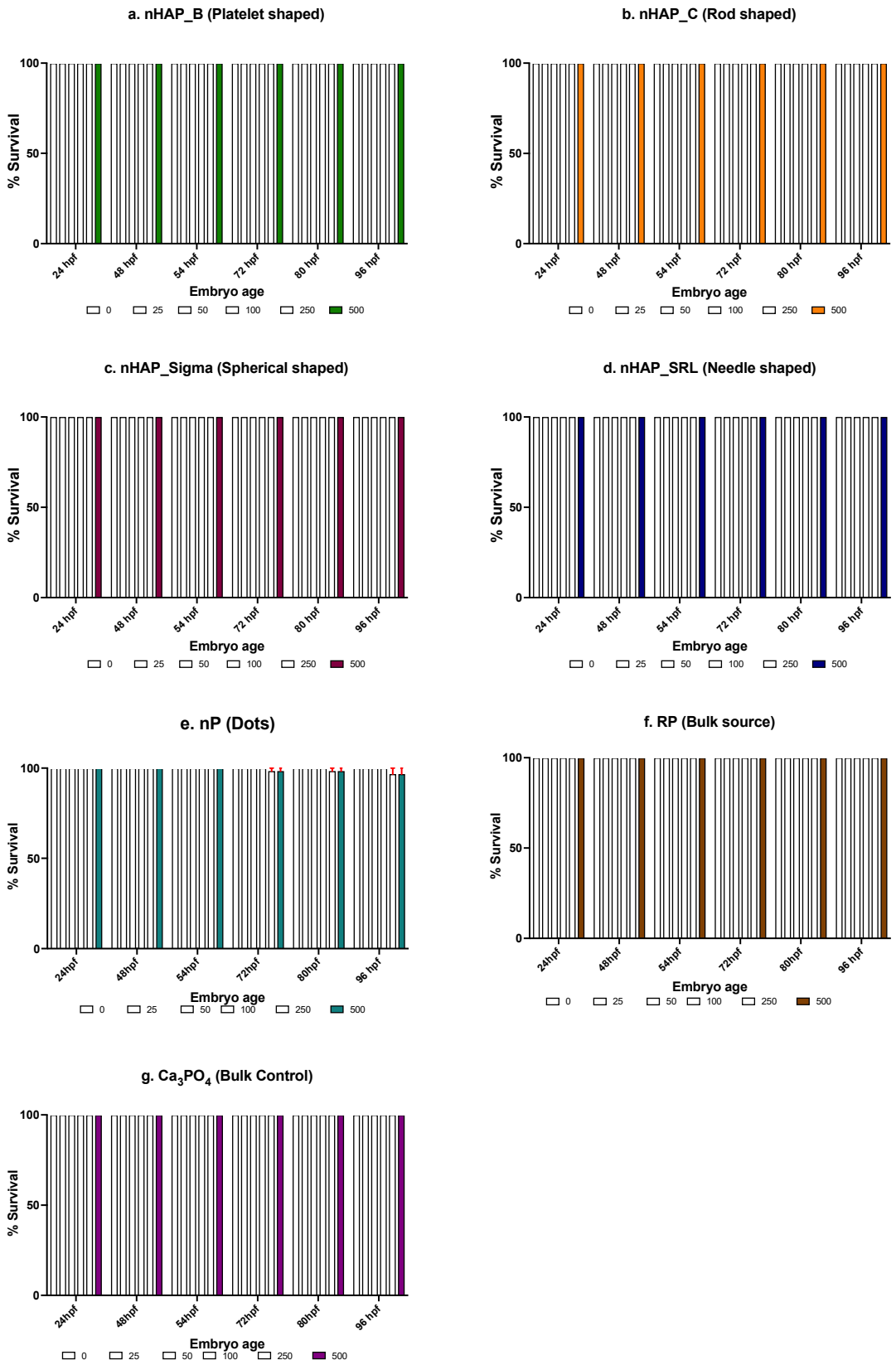
ESI Table 3. Reference gene stability using geNorm[2], NormFinder[3], and BestKeeper[4] algorithm

Gene stability by GeNorm at 48 hpf		
Gene name	Stability value	
β- actin | elf-1α	0.46	
Gene stability by NormFinder t 48 hpf		
Genes	Geomean of ranking values	
β- actin	1.41	
elf-1α	1.19	
Pearson correlation coefficient (r) by BEST KEEPER at 48 hpf		
	β- actin	**elf-1α**
n	72	72
geo Mean [CP]	18.82	19.04
AR Mean [CP]	18.82	19.05
min [CP]	17.79	18.18
max [CP]	20.24	21.54
std dev [+/- CP]	0.48	0.34
CV [% CP]	2.54	1.77
min [x-fold]	-2.04	-1.82
max [x-fold]	2.68	5.65
std dev [+/- x-fold]	1.39	1.26
Pearson correlation coefficient (r) by BEST KEEPER		
	β- actin	**elf-1α**

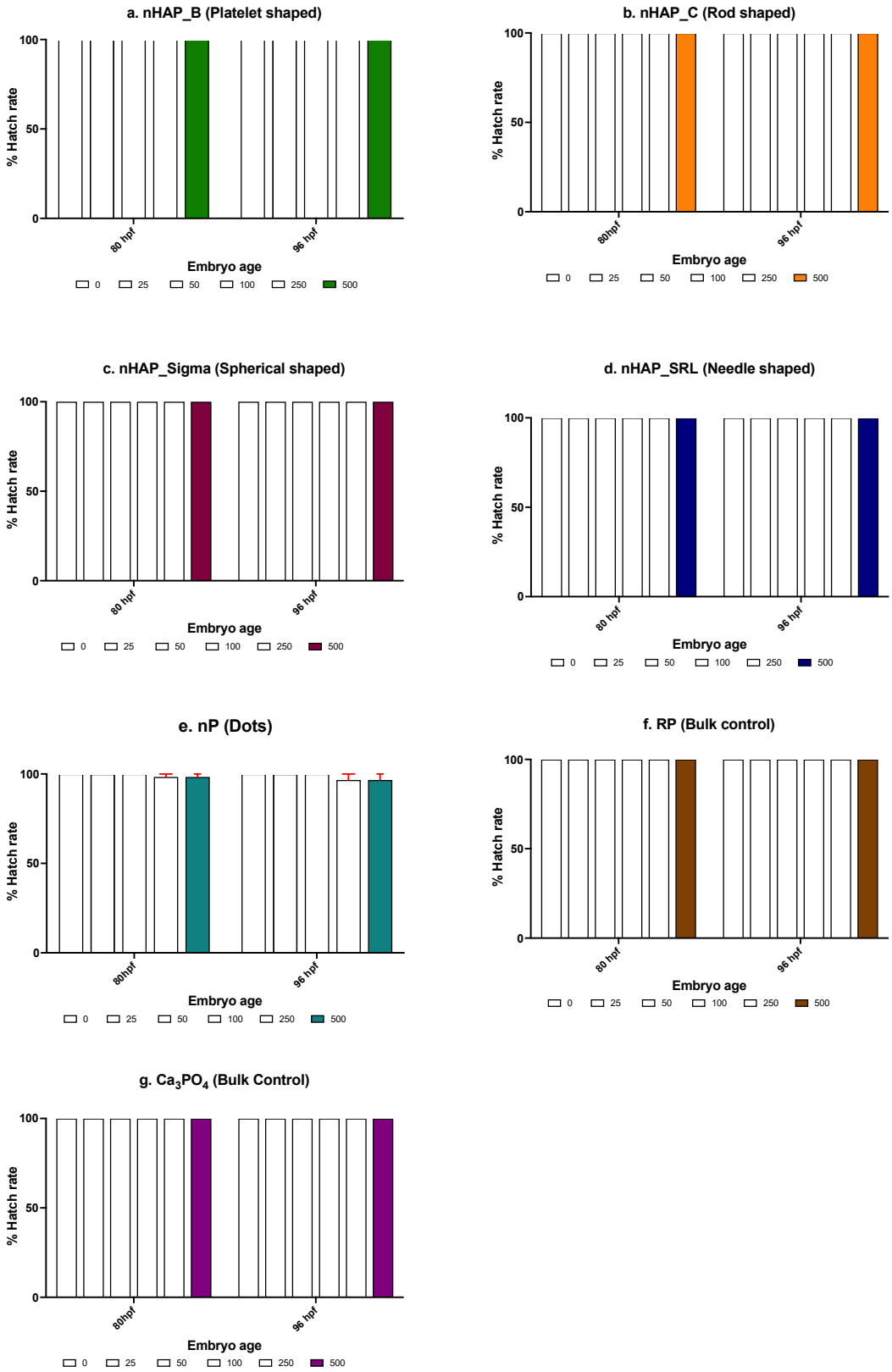
Elf1a	0.67	-
p-value	0.001	-
Pearson correlation coefficient (r)		
BestKeeper vs.	**β- actin**	**elf-1α**
coeff. of corr. [r]	0.935	0.89
p-value	0.001	0.001
Ranking Order (Better--Good--Average) at 48 hpf		
Method	1	2
Delta CT	**elf-1α**	β- actin
BestKeeper	**elf-1α**	β- actin
Normfinder	**β- actin**	elf-1α
Genorm	**β- actin | elf-1α**	
Recommended comprehensive ranking	**elf-1α**	β- actin

Gene stability by GeNorm at 96 hpf		
Gene name	Stability value	
β- actin | elf-1α	0.224	
Gene stability by NormFinder at 96 hpf		
Genes	Geomean of ranking values	
β- actin	1.414	
elf-1α	1.189	
Pearson correlation coefficient (r) by BEST KEEPER at 96 hpf		
	β- actin	**elf-1α**
n	72	72
geo Mean [CP]	18.36	19.33
AR Mean [CP]	18.36	19.33
min [CP]	17.44	18.53
max [CP]	19.25	20.21
std dev [+/- CP]	0.37	0.28
CV [% CP]	2.01	1.46
min [x-fold]	-1.89	-1.74
max [x-fold]	1.86	1.84
std dev [+/- x-fold]	1.29	1.22
Pearson correlation coefficient (r) by BEST KEEPER		
	β- actin	**elf-1α**
Elf1a	0.886	-
p-value	0.001	-
Pearson correlation coefficient (r)		
BestKeeper vs.	**β- actin**	**elf-1α**
coeff. of corr. [r]	0.98	0.961

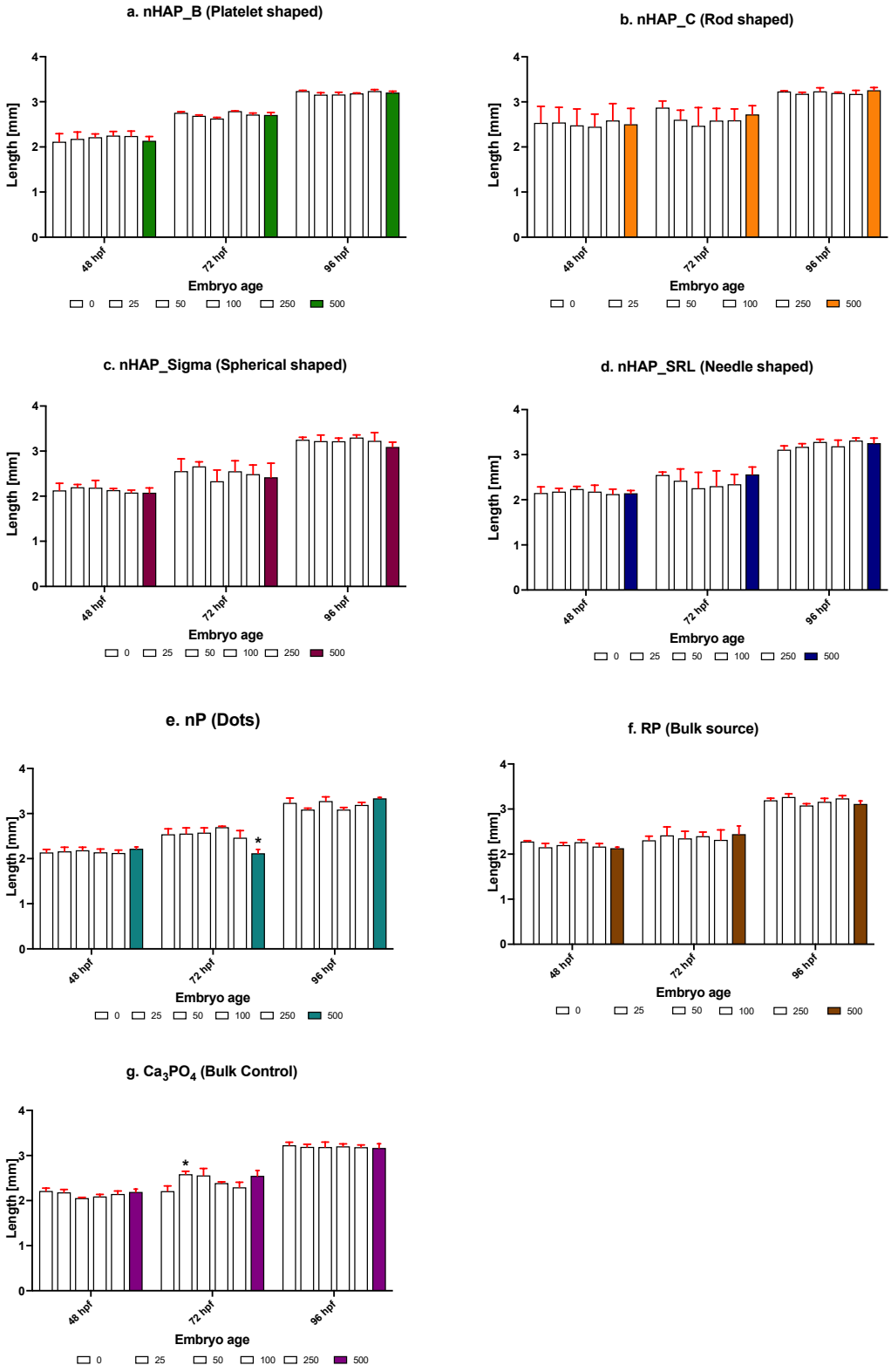
p-value	0.001	0.001
Ranking Order (Better--Good--Average) at 96 hpf		
Method	1	2
Delta CT	**elf-1α**	β- actin
BestKeeper	**elf-1α**	β- actin
Normfinder	**β- actin**	elf-1α
Genorm	**β- actin | elf-1α**	
Recommended comprehensive ranking	**elf-1α**	β- actin



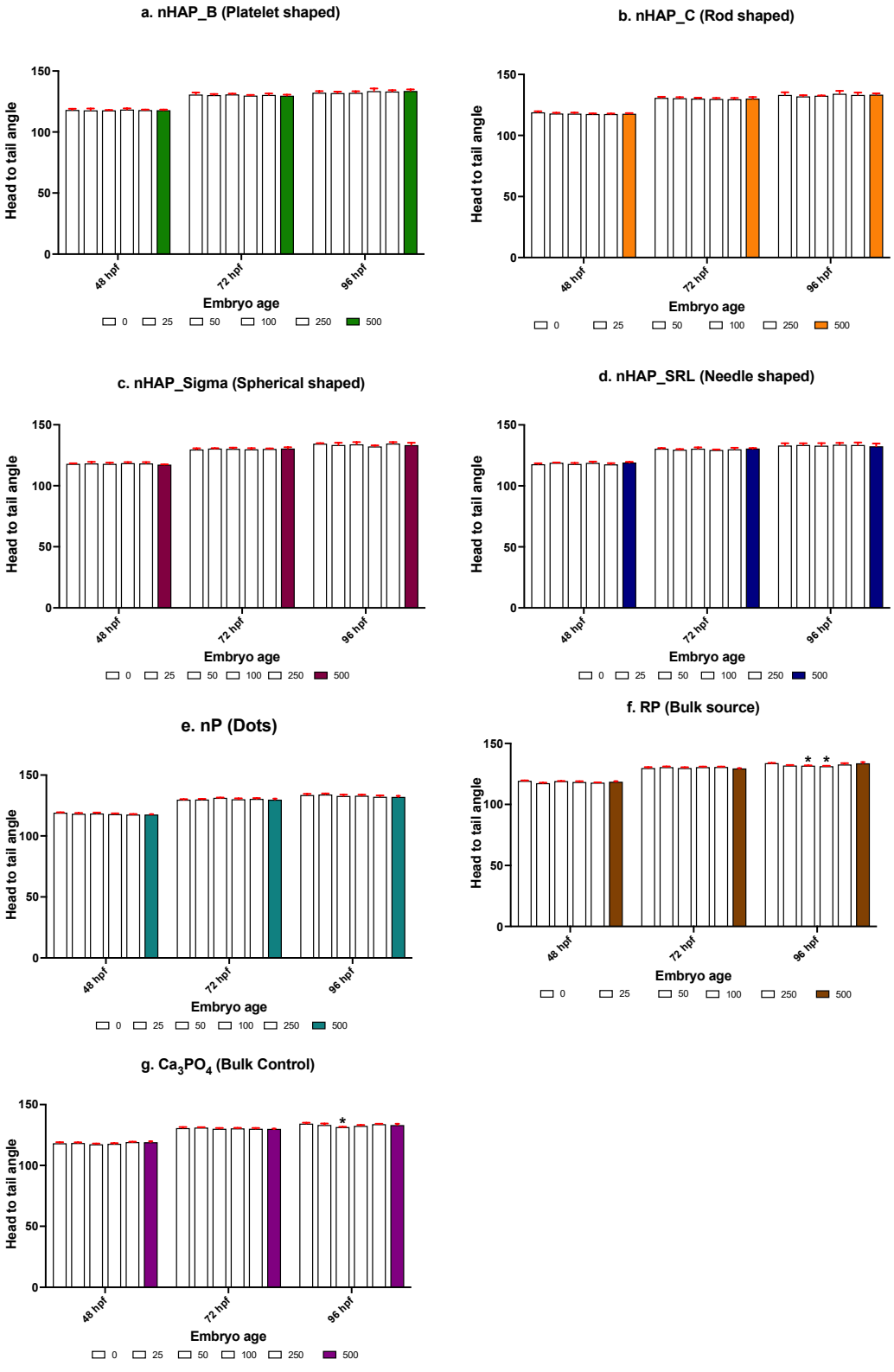
ESI Fig. 1. Effect of a. biologically synthesized nHAP (nHAP_B), b. chemically synthesized nHAP (nHAP_C), c. Sigma Aldrich nHAP (nHAP_Sigma), d. SRL nHAP (nHAP_SRL), e. nanophosporous (nP), f. rock phosphate bulk control (RP), and g. calcium phosphate bulk control (Ca_3PO_4) at increasing concentrations (0, 25, 50, 100, 250 and 500 $\mu g.mL^{-1}$) on survival of zebrafish embryos at after 96 hpf. Values reported as mean ± S.E.M.



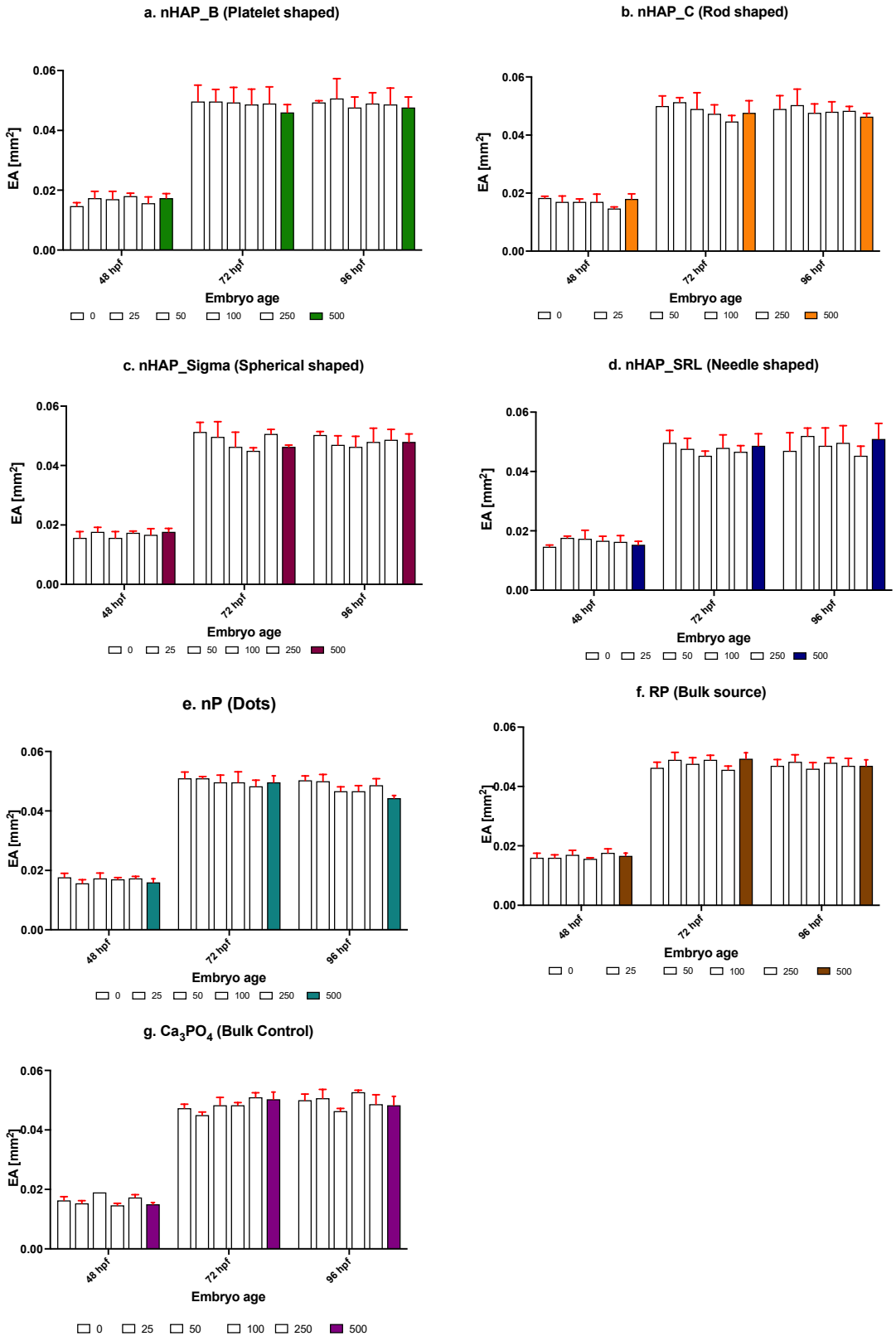
ESI Fig. 2. Effect of a. biologically synthesized nHAP (nHAP_B), b. chemically synthesized nHAP (nHAP_C), c. Sigma Aldrich nHAP (nHAP_Sigma), d. SRL nHAP (nHAP_SRL), e. nanophosphorus (nP), f. rock phosphate bulk control (RP), and g. calcium phosphate bulk control (Ca_3PO_4) at increasing concentrations (0, 25, 50, 100, 250 and 500 $\mu g.mL^{-1}$) on hatch rate of zebrafish embryos at 80 and 96 hpf. Values reported as mean ± S.E.M.



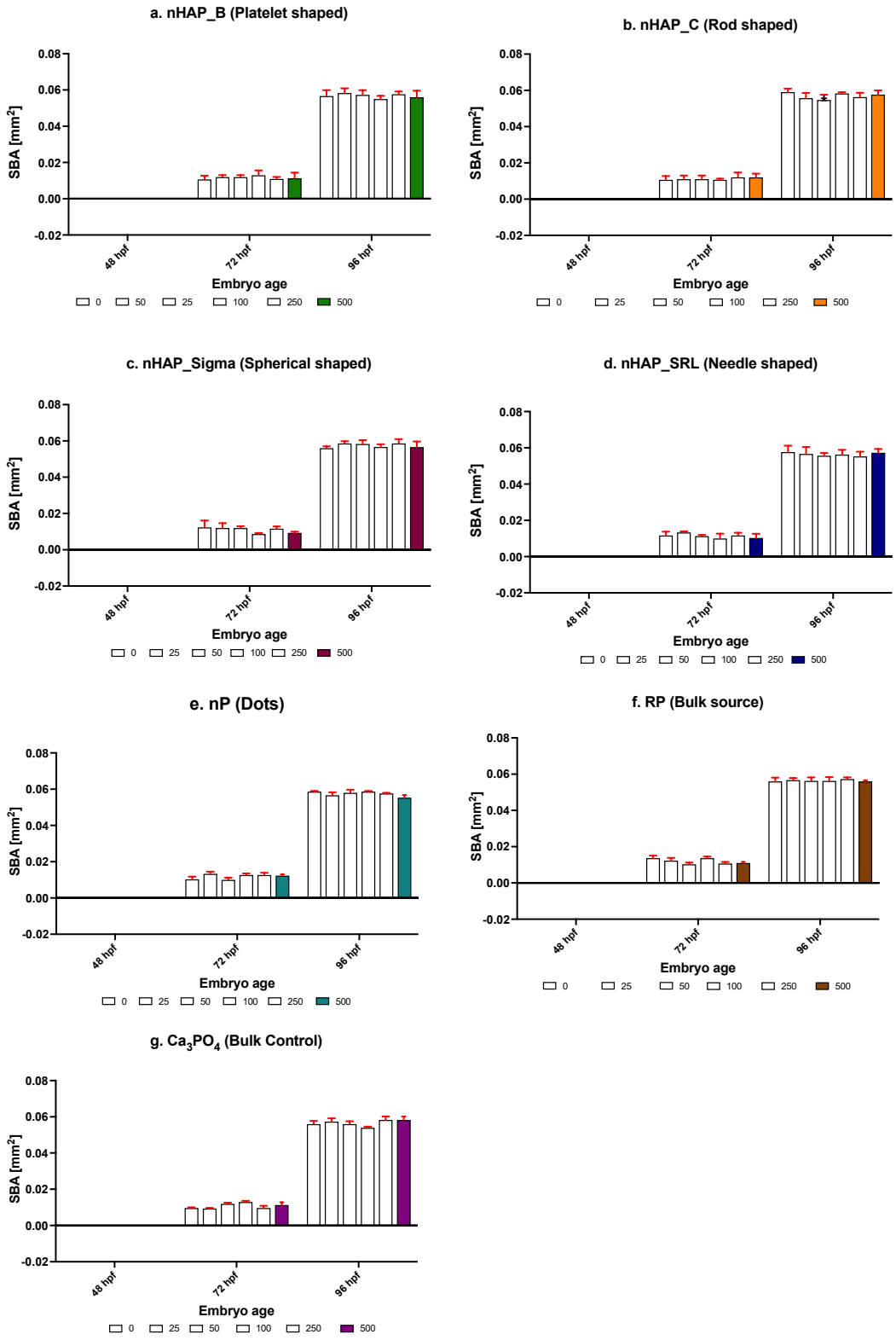
ESI Fig. 3. Effect of a. biologically synthesized nHAP (nHAP_B), b. chemically synthesized nHAP (nHAP_C), c. Sigma Aldrich nHAP (nHAP_Sigma), d. SRL nHAP (nHAP_SRL), e. nanophosphorus (nP), f. rock phosphate bulk control (RP), and g. calcium phosphate bulk control (Ca_3PO_4) at increasing concentrations (0, 25, 50, 100, 250 and 500 $\mu g.mL^{-1}$) on embryo length of zebrafish embryos at 48, 72 and 96 hpf. Values reported as mean ± S.E.M.



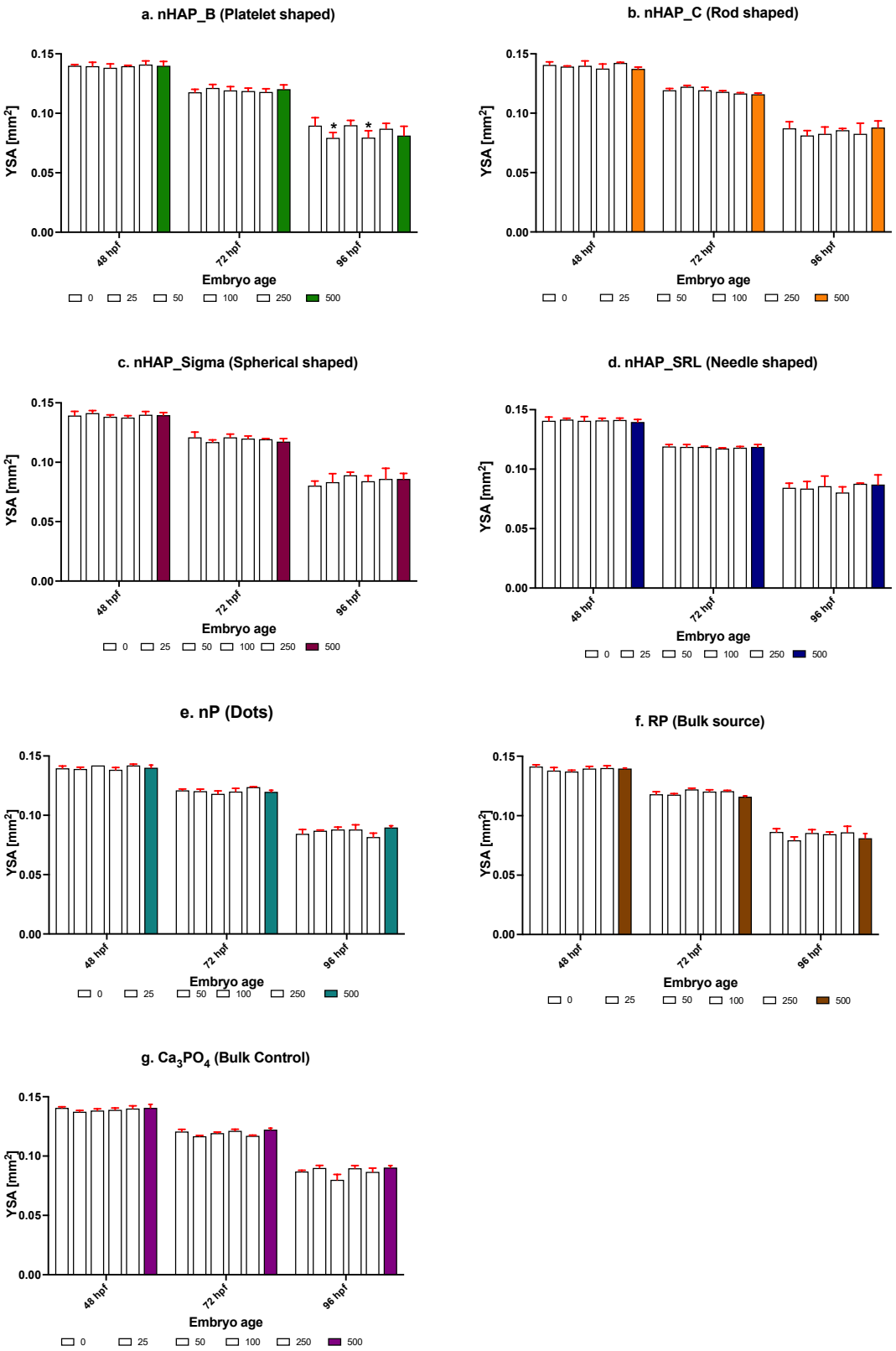
ESI Fig. 4. Effect of a. biologically synthesized nHAP (nHAP_B), b. chemically synthesized nHAP (nHAP_C), c. Sigma Aldrich nHAP (nHAP_Sigma), d. SRL nHAP (nHAP_SRL), e. nanophosphorus (nP), f. rock phosphate bulk control (RP), and g. calcium phosphate bulk control (Ca_3PO_4) at increasing concentrations (0, 25, 50, 100, 250 and 500 $\mu g.mL^{-1}$) on head to tail angle of zebrafish embryos at 48, 72 and 96 hpf. Values reported as mean ± S.E.M.



ESI Fig. 5. Effect of a. biologically synthesized nHAP (nHAP_B), b. chemically synthesized nHAP (nHAP_C), c. Sigma Aldrich nHAP (nHAP_Sigma), d. SRL nHAP (nHAP_SRL), e. nanophosphorus (nP), f. rock phosphate bulk control (RP), and g. calcium phosphate bulk control (Ca_3PO_4) at increasing concentrations (0, 25, 50, 100, 250 and 500 $\mu g.mL^{-1}$) on eye area of zebrafish embryos at 48, 72 and 96 hpf. Values reported as mean ± S.E.M.

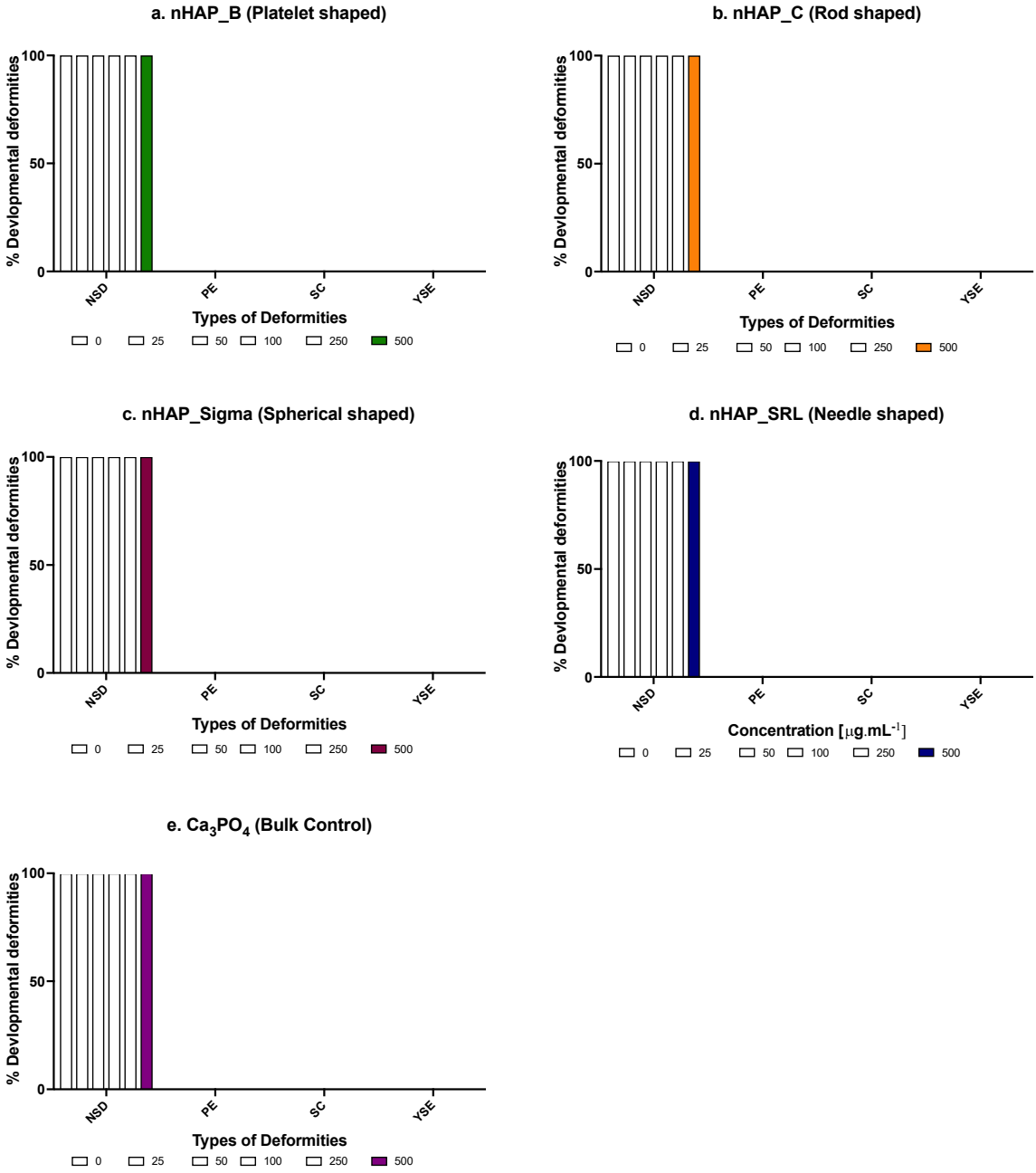


ESI Fig. 6. Effect of a. biologically synthesized nHAP (nHAP_B), b. chemically synthesized nHAP (nHAP_C), c. Sigma Aldrich nHAP (nHAP_Sigma), d. SRL nHAP (nHAP_SRL), e. nanophosphorus (nP), f. rock phosphate bulk control (RP), and g. calcium phosphate bulk control (Ca_3PO_4) at increasing concentrations (0, 25, 50, 100, 250 and 500 $\mu g.mL^{-1}$) on swim bladder area of zebrafish embryos at 48, 72 and 96 hpf. Values reported as mean ± S.E.M.



ESI Fig. 7. Effect of a. biologically synthesized nHAP (nHAP_B), b. chemically synthesized nHAP (nHAP_C), c. Sigma Aldrich nHAP (nHAP_Sigma), d. SRL nHAP (nHAP_SRL), e. nanophosphorus (nP), f. rock phosphate bulk control (RP), and g. calcium phosphate bulk control (Ca_3PO_4) at increasing concentrations (0, 25, 50, 100, 250 and 500 $\mu g.mL^{-1}$) on

yolk sac area of zebrafish embryos at 48, 72 and 96 hpf. Values reported as mean ± S.E.M.



ESI Fig. 8. No structural deformities (NSD) in zebrafish embryos after exposure to a. biologically synthesized nHAP (nHAP_B), b. chemically synthesized nHAP (nHAP_C), c. Sigma Aldrich nHAP (nHAP_Sigma), d. SRL nHAP (nHAP_SRL), and e. calcium phosphate bulk control (Ca_3PO_4) at increasing concentrations (0, 25, 50, 100, 250 and 500 $\mu g.mL^{-1}$) at 72 hpf. PE: pericardial edema, SC: spinal curvature, YSE: Yolk sac edema.

References:

1. Bustin, S. A.; Benes, V.; Garson, J. A.; Hellemans, J.; Huggett, J.; Kubista, M.; Mueller, R.; Nolan, T.; Pfaffl, M. W.; Shipley, G. L., The MIQE Guidelines: M inimum I nformation for Publication of Q uantitative Real-Time PCR E xperiments. Oxford University Press: 2009.
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3. Andersen, C. L.; Jensen, J. L.; Ørntoft, T. F., Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer research* **2004,** *64* (15), 5245-5250.
4. Pfaffl, M. W.; Tichopad, A.; Prgomet, C.; Neuvians, T. P., Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. *Biotechnology letters* **2004,** *26* (6), 509-515.