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1 **An integrated multilevel analysis profiling biosafety and**
2 **toxicity induced by Indium- and Cadmium-based**
3 **quantum dots *in vivo***

4

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37

38

39 **Abstract**

40 Indium phosphide quantum dots (QDs) have emerged as a new class of
41 fluorescent nanocrystals for manifold applications, from biophotonics to
42 nanomedicine. Recent efforts in improving the photoluminescence quantum
43 yield, the chemical stability and the biocompatibility turned them into a valid
44 alternative to well established Cd-based nanocrystals. *In vitro* studies
45 provided first evidence for the lower toxicity of In-based QDs. Nonetheless,
46 an urgent need exists for further assessment of the potential toxic effects *in*
47 *vivo*. Here we use the freshwater polyp *Hydra vulgaris*, a well-established
48 model previously adopted to assess the toxicity of CdSe/CdS nanorods and
49 CdTe QDs. A systematic multilevel analysis was carried out *in vivo*, *ex vivo*
50 and *in vitro* comparing toxicity endpoints of CdSe- and InP-based QDs,
51 passivated by ZnSe/ZnS shells and surface functionalized with penicillamine.
52 Final results demonstrate that both the chemical composition of the QD core
53 (InP *vs.* CdSe) and the shell play a crucial role for final outcomes.
54 Remarkably, in absence of *in vivo* alterations, cell and molecular alterations

55 revealed hidden toxicity aspects, highlighting the biosafety of InP-based
56 nanocrystals and outlining the importance of integrated multilevel analyses for
57 proper QDs risk assessment.

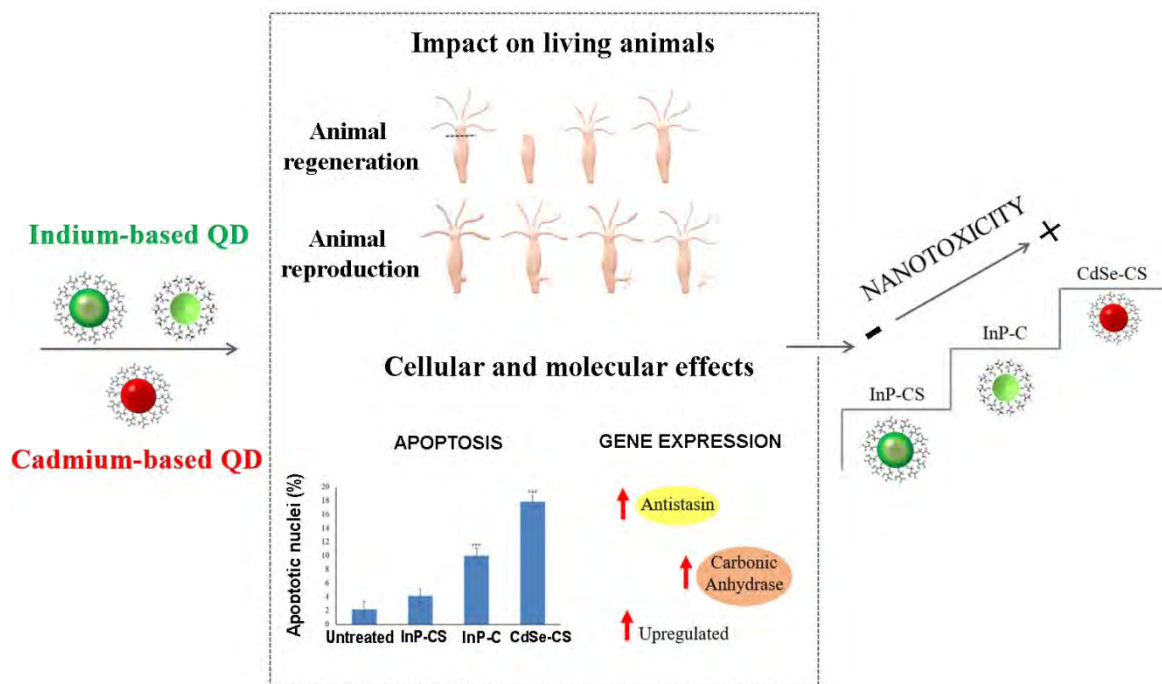
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60 **Table of Contents (TOC)**

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66 INTRODUCTION

67

68 Colloidal semiconductor nanocrystals (NCs), also called quantum dots (QDs),
69 are becoming established fluorophores in many applications, including
70 electronics, biology and medicine.¹ As a consequence the environmental
71 concerns related to QDs synthesis and use are growing up fast. Information
72 about the behaviour, transformation, fate, mode of actions and ecotoxicity of
73 QDs in aquatic environments provide evidences that QDs may threaten the
74 ecosystem at various trophic levels², even at very low concentrations (< mg
75 L⁻¹ in the case of cadmium-based QDs). In this respect, the most developed
76 systems so far are Cd-based QDs, which are characterized by an excellent
77 photoluminescence quantum yield (PLQY) approaching unity, narrow
78 photoluminescence (PL) line width, and a functionalizable surface, which can
79 be decorated in principle with any type of bioactive molecule.^{3, 4} These
80 properties make them very promising tools to address several issues: from
81 targeted drug delivery⁵ over multiplexed bioimaging,⁶ to monitoring electrical
82 signals in neurons⁷. On the other hand, due to their intrinsic toxicity the

83 potential of Cd-based QDs for real-life biological imaging and diagnostics
84 applications is very limited and their use *in vivo*, in humans, is obviously
85 precluded, even though environmental issues persist. In light of this, eco-
86 friendly QDs are attracting much attention over the last decade as alternative
87 nanocrystals.^{8, 9}

88 Among the possible semiconductor materials, indium phosphide (InP) NCs
89 were spotted as one of the most promising candidates.¹⁰ Their PL can be
90 tuned from the blue to the near infrared (NIR) region of the spectrum by
91 varying the NC size from around 2 to 6 nm due to the quantum confinement
92 effect. Furthermore, although very limited in number, toxicological studies
93 indicated a much lower intrinsic toxicity as compared to CdSe QDs.¹¹ While
94 the optical properties of InP QDs in terms of PLQY and line width are
95 inferior to those of CdSe QDs, significant progress in their chemical
96 synthesis has been made in the past decade.¹² A single-step synthesis of
97 InP/ZnS core/shell QDs reaching a PLQY of 30% was initially reported,^{13, 14}
98 and further improvements with values exceeding 80% were obtained by
99 growing a thin GaP interfacial layer between the InP core and the ZnS shell
100 or by using Zn(Se,S) graded shells.^{15, 16}

101 Since as-synthesized colloidal core/shell NCs are generally coated with
102 hydrophobic organic surfactants (i.e. long chain carboxylic acids), which make
103 them soluble exclusively in non-polar solvents, their phase transfer to
104 aqueous media is an essential fundamental step for their use in biology.
105 Ligand exchange procedures involving the aminoacid D-penicillamine (Pen)
106 have been recently demonstrated to efficiently work in the aqueous phase
107 transfer of InP-based NCs. Indeed, this small zwitterionic ligand was found to
108 guarantee the colloidal stability of InP/ZnS NCs in a wide range of
109 physiologically relevant pH values, and to promote weak interactions with
110 other biomolecules.¹⁷ Moreover, in contrast to more widely used cysteine
111 (Cys),¹⁸ Pen has a much lower propensity for dimerization under disulphide
112 formation due to two methyl groups in vicinity of the sulfhydryl functions. This
113 has important consequences on both the colloidal stability and PLQY of the
114 phase-transferred QDs, disulphides acting as efficient fluorescence
115 quenchers.¹⁷ Using this approach, stable and strongly luminescent Pen-
116 capped InP QDs could be conjugated with a specific antibody and
117 successfully used as probes in highly sensitive sandwich immunoassays
118 based on Förster resonance energy transfer (FRET).^{19, 20} Further examples of

119 biological applications of Pen-capped InP/ZnS QDs include multimodal
120 imaging probes achieved by grafting Gd-complexes on their surface^{21, 22} as
121 well as bi-luminescent probes obtained by sensitizing the emission of grafted
122 Eu or Yb complexes.²³

123 In this work, we carry out an exhaustive toxicological evaluation by using an
124 invertebrate model organism, *Hydra vulgaris*. Previously we showed that
125 *Hydra* is an amenable model to test the interaction of several nanomaterials,
126 including QDs, with biological systems.²⁴⁻³⁰ This small animal, shaped as a
127 polyp of a few millimetres with jellyfish consistency, has a very simple
128 anatomy, with no organs or biological fluids, but only two layers of epithelial
129 cells surrounding an empty cavity, and a crown of tentacles used for prey
130 capture. When *Hydra vulgaris* is exposed to nanoparticles, by the simple
131 soaking, a behavioural response (such as contractions, elongations, tentacle
132 writhing) or morphological damages (tissue disintegration, swelling) may be
133 elicited immediately, providing first toxicological indications. Going deeper into
134 mechanistic details, fluorescence microscopy can provide information on the
135 uptake mechanism (influenced by membrane interactions), while cell analysis
136 can indicate the induction of apoptosis (by looking at pyknotic nuclei and

137 membrane integrity). Finally, the molecular analysis by mean of gene
138 expression profiling may help to dissect the involvement of distinct molecular
139 pathways. A comprehensive toxicological analysis was performed in *Hydra* to
140 assess the overall effects of several Cd-based QDs. These studies revealed
141 a high degree of toxicity for aqueous synthesized CdTe QDs,^{25, 30,31} while
142 CdSe/CdS nanorods coated with polyethylene glycol (PEG),³² induced either
143 a behavioural response³³ or an active internalization²⁸ without signs of
144 morphological damages. The role of the surface charge was also recently
145 investigated and CdS/ZnS QDs coated with a positively charged polymer
146 were found to induce a massive gene deregulation together with evident
147 tissue damages.²⁴ All these findings depict *Hydra* as a model of choice to
148 analyze how chemical composition, size, surface charge and coating drive
149 QD toxicity.

150 Here we report a comparative study performed in *Hydra* between Pen-coated
151 InP-based QDs with different shell thicknesses, and we correlate the results
152 with those generated by Pen-coated CdSe/ZnS QDs. The experimental
153 design was conceived not only to test *in vivo* the biocompatibility of the Pen
154 organic coating, but also to directly compare QDs with different core

155 composition and same coating, and to dissect the contribute of metal
156 components to animal toxicity. Moreover, our study expands the current
157 toxicological knowledge of QDs, which is mainly focused on Cd-based
158 systems, and confirms the higher biosafety of InP-based QDs for biophotonic
159 and nanomedicine applications. Furthermore, it provides potential molecular
160 biomarkers to detect subtle adverse effects also in absence of evident
161 morphological alterations.

162

163 **Materials and Methods**

164 **QDs synthesis and aqueous phase transfer**

165 The detailed experimental procedures are given in Supporting Information.
166 InPZnS QDs (“InP-C”) consisting of an InPZnS alloy core and a thin ZnS
167 shell¹⁴ were synthesized following a reported procedure.¹³ To increase the
168 shell thickness, an additional, graded Zn(Se,S) shell was grown on the
169 surface of the InPZnS QDs (“InP-CS”), according to the procedure published
170 by Lim *et al.*³⁴ Hydrophobic CdSe/ZnS QDs (“CdSe-CS”) were purchased
171 from Life Technologies/Thermo Fisher Scientific (QD655). All types of QDs
172 were transferred from the organic (chloroform) to the water phase by means

173 of a biphasic ligand exchange reaction using penicillamine in presence of
174 TCEP at pH 9.¹⁷ Phase transfer is accomplished within 2 h and the aqueous
175 phase is purified by gel chromatography using Sephadex NAP-5™ columns
176 and distilled water for elution.

177

178 **Inductively Coupled Plasma (ICP) elemental analysis**

179 ICP elemental analysis was carried out via inductively coupled plasma optical
180 emission spectroscopy (ICP-OES) using an iCAP 6300 DUO ICP-OES
181 spectrometer (Thermo Fisher Scientific). All chemical analyses performed
182 were affected by a systematic error of about 5%. 150 polyps were incubated
183 for 24 h with 70 nM dispersion of InP-C, InP-CS or Cd-CS QDs. After
184 extensive washes with Hydra culture medium, the polyps were digested by
185 the addition of concentrated acid (HCl/HNO₃ 3:1 (v/v) mixture). The
186 supernatant was diluted using Milli-Q water and analysed, without any further
187 operations. The estimation of the intracellular Indium and Cadmium content
188 was normalized to the number of test animals.

189

190 ***Hydra* culture**

12

191 *Hydra vulgaris* were cultured in *Hydra* medium comprising 1 mM calcium
192 chloride and 0.1 mM sodium hydrogen carbonate at pH 7.³⁵ The animals
193 were fed on alternate days with *Artemia* nauplii at 18°C with a 12:12 h light:
194 dark regime. Polyps from homogeneous populations, adult polyps without
195 bud, were selected for the experiments.

196

197 **Determination of *in vivo* toxicity endpoints**

198 Morphology - Toxicity tests were carried out on groups of 20 polyps, placed
199 into plastic multiwells maintained at a temperature of 18°C. A range of
200 nominal concentrations was selected for InP- and Cd-based QDs, to assess
201 the progressive effects on the morphology and physiology of individual
202 polyps. QD chronic exposure was carried out for 24, 48 and 72 h, refreshing
203 the test solution every 24 h. Effects were monitored and recorded by
204 microscopic examination of each polyp at 24 h intervals. A numerical score,
205 previously introduced by Wilby³⁶ was used to indicate progressive
206 morphological alterations induced by the toxicant, ranging from 10 to indicate
207 a normal morphology, down to 0 to indicate polyp disintegration. Median
208 score values were determined and used for statistical analysis. TraceCERT

209 standards (Sigma - Aldrich) for Indium and Cadmium were employed as
210 references for ICP.

211

212 Reproduction efficiency - Animals (5 *Hydra* with one bud) were treated with
213 70 nM of QDs for 24 h. After QD exposures the animals were washed five
214 times by adding an excess of fresh Hydra medium and kindly pipetting for
215 30 seconds to remove residual nanocrystals, afterward the polyps were
216 placed in 3.5 cm petri dishes (1 *Hydraldish*). Control polyps at the same
217 developmental stage were not treated. Both treated and untreated *Hydra*
218 were fed daily for 14 days. Population growth rates were determined by
219 considering the growth rate of an exponentially growing group of 5 founder
220 animals as $\ln(n/n_0) = kt$ where k is the constant growth, n is the number of
221 animals at time t and n_0 the number of animal at t_0 .³⁷ Three independent
222 experiments were performed.

223 Regeneration efficiency - Groups of 20 polyps were incubated in presence of
224 70 nM QDs for 24 h, washed and then bisected in the upper gastric region
225 and allowed to regenerate in fresh medium. The regenerating heads were
226 classified as stage 0 (wound closure), stage 1 (tentacle bud) and stage 2

227 (tentacle length reaching $\frac{1}{4}$ of final length). Developmental stages were
228 monitored through a stereomicroscope every 24 h and relative percentages
229 estimated at each time point for all QD conditions compared to untreated
230 polyps.

231

232 ***In vitro* toxicity endpoints**

233 Assessment of apoptosis - Apoptotic cell rate was evaluated by 4'-6-
234 Diamidino-2-phenylindole (DAPI) nuclear staining. Both QD-treated (50 nM, 24
235 h incubation) and untreated polyps were macerated into single cell
236 suspensions, using a solution composed of acetic acid, glycerol and H₂O in
237 a 1:1:13 (v/v) ratio.³⁸ Dissociated cells were fixed with 4% paraformaldehyde
238 and spread on slides, then washed with PBS (NaCl 137 mM, KCl 2.7 mM,
239 Na₂HPO₄ 10 mM, KH₂PO₄ 1.8 mM) and finally stained with DAPI for 2 min. Two
240 independent cell macerations were analysed and more than 300 cells were
241 counted for each treatment in order to determine the percentage of apoptotic
242 nuclei. Imaging was performed using an inverted microscope (Axiovert 100,
243 Zeiss, MA, USA) equipped with a digital colour camera (Olympus, DP70).

244 The software system Cell F (Olympus) was used for imaging acquisition and
245 analysis.

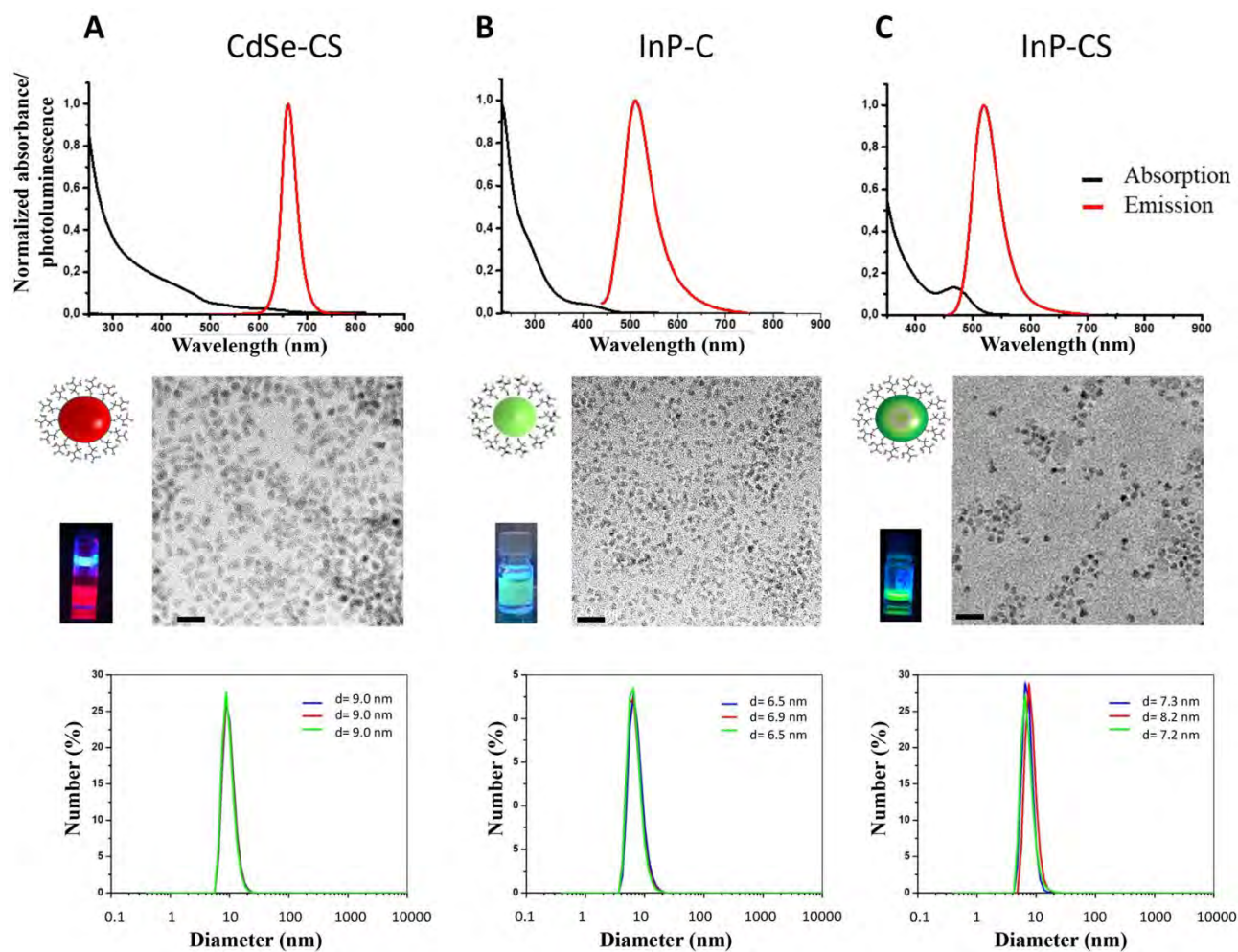
246 Gene expression analysis - Differences in gene expression profiles induced
247 by QD treatment were assessed by quantitative real time polymerase chain
248 reaction (qRT-PCR). For each experimental condition, RNA was extracted
249 from groups of 25 animals by purification in Trizol Reagent (Life
250 Technologies) according to the manufacturer's instructions. RNA was
251 quantified and quality checked by SmartSpec plus spectrophotometer (Biorad,
252 CA, USA) and agarose gel electrophoresis, respectively. RNA samples were
253 treated with DNaseI (*Amplification Grade, Invitrogen*) according to supplier's
254 instructions. The first-strand cDNA was synthesized by High Capacity cDNA
255 Reverse Transcription Kit (Applied Biosystem) using 0.5 µg of DNA-free RNA
256 in a final volume of 10 µL. qRT-PCR was performed in 25 µL of reaction
257 mixture consisting of 1x Express SybrR GreenER qPCR SuperMix with
258 premixed ROX (Invitrogen), serial cDNA dilutions and 0.3 µM each primer.
259 The reactions were processed using the StepOne Real-Time PCR System
260 (Applied Biosystem) according to the following thermal profile: 95°C for 2
261 min, one cycle for cDNA denaturation; 95°C for 15 sec and 60°C for 1 min,

262 40 cycles for amplification. Specific primers of *Hydra* homologues genes of
263 antistasin (*XM_002158823*), and carbonic anhydrase (*XM_002154950.1*) were
264 designed using Primer3 software (<http://frodo.wi.mit.edu/primer3/>) and are
265 listed in table S1. Gene expression was evaluated in QD treated and
266 untreated polyps; three technical replicates from three independent
267 experiments were carried out. The expression profiles were analysed by
268 applying the $\Delta\Delta C_t$ method³⁹ where the values of the gene of interest were
269 normalized for the values of reference control gene *Hydra Elongation Factor*
270 *1 α* (Z68181.1).

271 RESULTS AND DISCUSSION

272 Two types of InP-based QDs differing in their passivating zinc chalcogenide
273 shell were synthesized in order to test their colloidal stability in aqueous
274 solutions and overall behaviour during *in vivo* experiments: alloyed InPZnS
275 QDs containing a thin ZnS shell (termed "InP-C") prepared in a single-step
276 one-pot reaction¹³ and InPZnS/ZnSe/ZnS QDs (termed "InP-CS") obtained by
277 growing an additional Zn(Se_{0.1}S_{0.9}) gradient shell on the surface of the latter.
278 The phase transfer of both types of samples to water was achieved by

279 ligand exchange, mixing a dispersion of QDs in chloroform and an aqueous
280 solution at pH 9 containing penicillamine (Pen), in presence of the mild
281 reducing agent TCEP (tris-(2-carboxyethyl)-phosphine (see also SI). Basic pH
282 promotes the deprotonation of the thiol group of Pen, which results in
283 stronger binding to the surface of the different ZnS-coated QDs. On the
284 other hand, the reducing agent TCEP prevents the formation of penicillamine
285 disulfide which may reduce the colloidal stability and the PLQY of the final
286 systems.¹⁷ Commercial CdSe/ZnS core/shell QDs (CdSe-CS) were
287 functionalized with Pen using the same approach. Structure, net-charge and
288 optical properties of the different QDs are shown in Figure 1, SI Figure S1,
289 S2 and Table S2. The sizes of QDs measured in Hydra medium were 9 nm,
290 6.6 and 7.6 nm for CdSe-CS, InP-C and InP-CS, respectively. Slight or no
291 modifications were observed among the mean diameters measured in water
292 (Figure S1), suggesting that the testing medium does not promote QD
293 aggregation and/or precipitation.



294

295

Figure 1. Physical characterization of QDs. Optical properties (upper panel),

296

schematic structure and TEM images (middle panel) and DLS measurements

297

in *Hydra* medium (lower panel) of the different types of QDs: A) CdSe-CS B)

298

InP-C and C) InP-CS. The UV-Vis (black line) and PL (red line) spectra of

299

the QDs have been taken after aqueous phase transfer with penicillamine.

300

Scale bar: 50 nm in all images.

301

302 Considering that these nanocrystals present similar physico-chemical features
303 (size, shape, charge and coating), we evaluated their biological impact by
304 normalising their toxicity on QD concentration. To study the interactions of
305 QDs with *Hydra*, living polyps were exposed to different concentrations of
306 QDs (from 25 nM to 100 nM) in their culture medium. This range of
307 concentration was selected on the basis of our previous works carried out
308 with different types of nanocrystals ^{24-27, 30}.

309 After 24 h the animals were thoroughly rinsed to remove QDs that adhere to
310 the external epithelium and inspected by fluorescence microscopy. InP-C
311 treated animals did not show any fluorescence, which suggests that QDs
312 have either a PLQY too low for detection (SI Figure S2 and Table S2)
313 and/or that PL quenching occurred during their internalization/incubation
314 period.

315 On the other hand, polyps incubated with InP-CS and CdSe-CS (at a
316 concentration of at least 50 nM) showed fluorescent foci uniformly dispersed
317 throughout the whole animal body, as reported in Figure 2. This labelling
318 pattern reflects the QDs accumulation into intracellular granular-like storage
319 structures, and it may depend on macropynocytosis, i.e. the internalization of

320 a large fraction of external fluids, as observed in our previous works.^{28, 40}

321 The fluorescence staining appeared clear and much stronger in CdSe-CS

322 treated animals, and this might be due to a difference in their uptake

323 efficiency, in their PLQYs, or/and photostability within *Hydra* tissues.

324 In *Hydra*, as in other eukaryotic systems, the net surface charge of

325 nanomaterials influences the uptake efficiency, and the positive charge

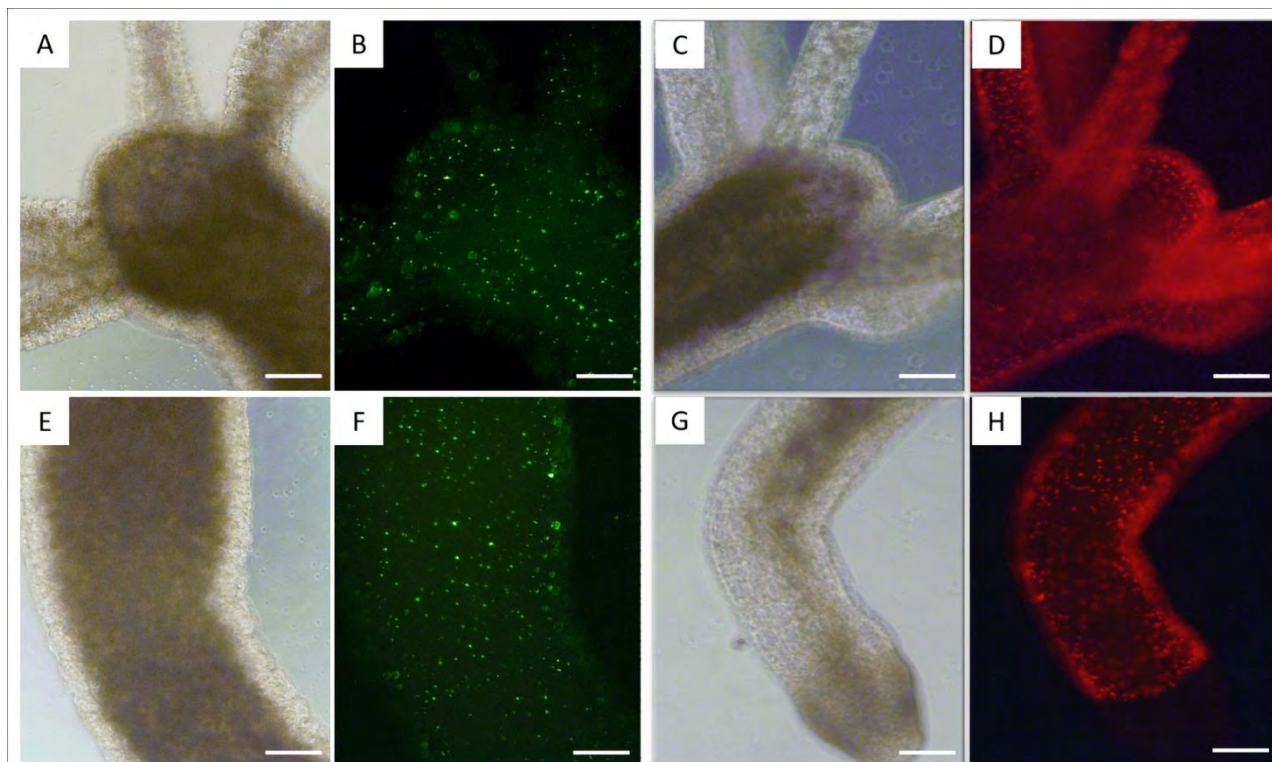
326 greatly enhances the internalization rate because of the electrostatic

327 interaction with the negatively charged biological membranes.²⁸ Herein, we

328 observed that negatively charged QDs (as reported in SI Table S2) were

329 also taken up by *Hydra* polyps, although at a minor extent.

330



331

332 **Figure 2.** Biodistribution of InP- and CdSe-based QDs in *Hydra*. Bright-field
333 and fluorescence images of *Hydra* treated with InP-CS QDs (two left
334 columns) and CdSe -CS QDs (two right columns). A spotted fluorescence
335 pattern is uniformly distributed from head (B, D) to foot regions (F, H) of
336 treated animals. A, C, E, G show the corresponding images in bright field.

337 Scale bar: 200 μm .

338

339 Due to the intrinsic lower fluorescence of the InP QDs, to investigate
340 whether or not the fluorescence difference was due to differences in the
341 uptake efficiency, elemental analyses by mean of ICP were conducted on

342 large number of polyps (around 150) treated with each QD type (70 nM, 24
343 h). Results showed a greater Indium content for InP-C treated animals
344 compared to InP-CS treatment (47,2 ng of In/Hydra and 17,7 ng of In/Hydra,
345 respectively) indicating that the lower photoluminescence of InP-C compared to
346 InP-CS is responsible for the absence of fluorescence in InP-C treated
347 polyps, rather than a low rate of internalization. For CdSe-CS QD treated
348 polyps Cadmium content equal to 30,4 ng/Hydra was detected, which is in
349 the same range of the two others.

350

351

352 ***In vivo* evaluation of QD toxicity endpoints**

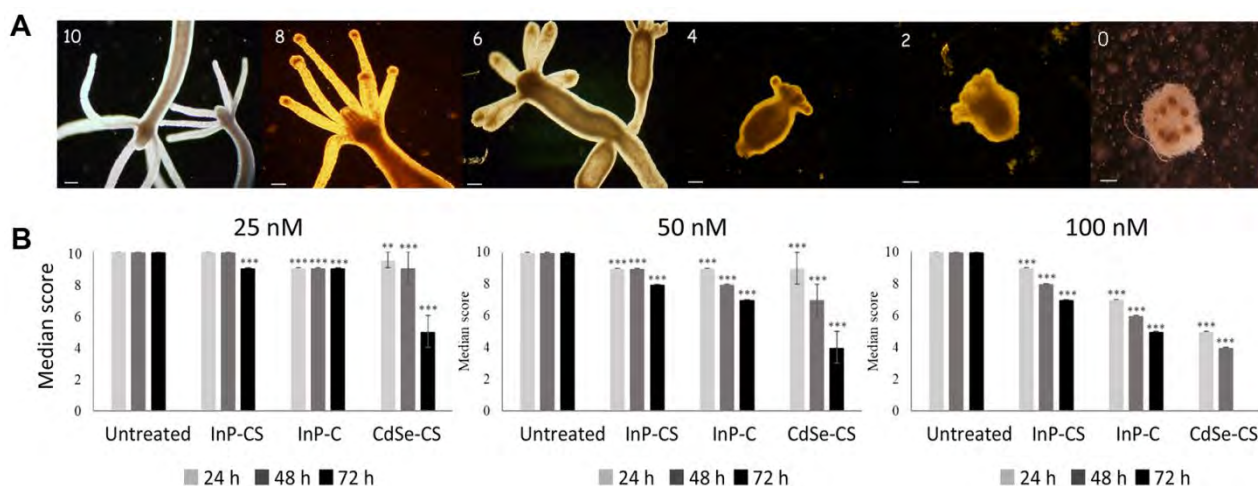
353 The high sensitivity to heavy metals makes *Hydra* an amenable tool for
354 assessment of inorganic nanoparticle's toxicity in natural ecosystems,
355 modelling biological barriers for their absorption, distribution, and persistency
356 in the food chain. Potential adverse effect of QDs here synthesised were
357 investigated by soaking living *Hydra* polyps with increasing doses of InP-C,
358 InP-CS and CdSe-CS QDs (from 25 nM to 100 nM) and inspected after 24,
359 48 and 72 h of incubation. Morphological alterations were quantified by using

23

360 a methodology assigning numerical values to morphological damages,^{25, 36, 41}
361 and visually displayed in Figure 3A and SI Table S3. Median scores
362 recorded at each QD test concentration (Figure 3B) indicate that In based
363 QDs are characterized by a “safer” profile (higher numerical values) with
364 respect to Cd based QDs. Polyps exposed to InP-CS QDs exhibited
365 negligible morphological alterations even at the highest test concentration
366 (100 nM), while InP-C revealed a substantial safe profile up to 50 nM.
367 Conversely, animals exposed to CdSe-CS displayed evident morphological
368 changes, leading to death at 72 h time point (Figure 3B). These data are in
369 line with our previous works on cadmium telluride (CdTe) QDs,²⁵
370 demonstrating high toxicity of Cd-based QDs, independently from the organic
371 coating.^{24, 42} Conversely, In-based QDs produce reversible effects, similar to
372 those reported for non-toxic nanomaterials (e.g. carbon nano-onions and
373 silica NPs) tested in *Hydra* at same doses (50-100 nM).^{26, 27} Comparison of
374 InP-C and InP-CS QDs effects on *Hydra* morphology demonstrates that the
375 inorganic zinc chalcogenide shell attenuates the Indium effect. On the other
376 hand, it does not counteract CdSe-CS QDs toxicity, suggesting either partial
377 degradation in animal tissues or leaking of free heavy metals out the inner

378 core. This also indicates that the toxicity of the QDs is mainly driven by the
 379 core components.

380 Next, the modulations of two important physiological processes were chosen
 381 as additional endpoints, *i.e.* the reproduction and regeneration efficiencies,
 382 known to be harshly impaired by chemical pollutants. For both experiments,
 383 we exposed the animals to 70 nM QDs. This intermediate dose was selected
 384 according to the outcomes obtained by the morphological analyses (Figure
 385 3B), showing that concentrations higher than 50 nM (for InP QDs) and lower
 386 than 100 nM (for CdSe QDs) may ensure induction of a robust, but not
 387 lethal effect for both QD types



388

389 **Figure 3.** Morphological changes and associated numerical score system

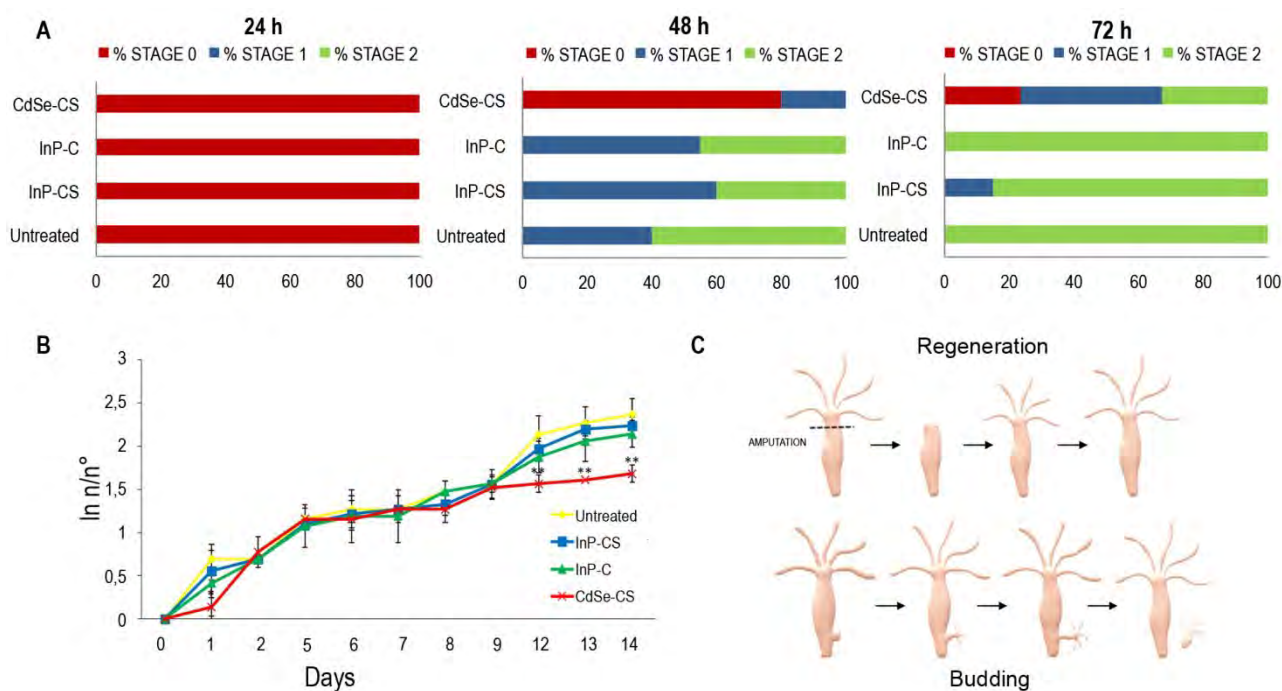
390 employed for toxicological analysis. A) *Hydra* polyps respond to

391 environmental stimuli and nano-insults through a broad range of
392 morphological changes, which range from tentacle contractions, then body
393 contraction and swelling, up to tentacle loss and whole tissue disintegration.
394 These morphological phenotypes are quantified on a large number of animals
395 by associating each phenotype to a numerical score, ranging from 10
396 (healthy animal) to zero (animal death). B) Dose-responses histograms of
397 *Hydra* polyps exposed to different QDs. The median score value is reported
398 for each QD type, QD concentration and treatment duration. Three
399 independent experiments were carried out. Asterisks denote significant
400 difference between untreated and treated polyps (*, $P < 0.05$; **, $P < 0.01$,
401 ***, $P < 0.005$) according to a post-hoc Mann Whitney U Test. Scale bars,
402 100 μm

403
404 Owing to the superb plasticity of the *Hydra* cells and tissues, adult polyps
405 can regenerate body parts after amputation and restore morphogenetic
406 processes. Normally these processes start a few minutes post amputation
407 and culminate in the formation of the missing parts of the body, namely the
408 head or the foot in three/four days. Any stress applied during this period

409 may result in abnormal regeneration or may slow down and even arrest the
410 process. In this work, healthy polyps were bisected in the upper gastric
411 region and allowed to regenerate (Figure 4A) in presence of 70 nM QDs or
412 in the normal medium. Animal stumps were daily inspected for viability, and
413 regeneration stages were recorded 24, 48 and 72 h post amputation. Stage
414 0 indicates a complete inhibition of regeneration (0 tentacle), stage 1
415 indicates heads with emerging tentacle (one or two), while stage 2 denotes a
416 normal regeneration (tentacle length reaching $\frac{1}{4}$ of final length). Polyp
417 exposure to InP-CS QDs slightly impaired the regeneration patterning during
418 the first 48 h, as shown by the lower percentage (40%) of polyps present in
419 stage 2 compared to untreated polyps (60%). This inhibitory effect was totally
420 transient as at 72 h the polyps paralleled untreated animals. A similar
421 behavior was induced by InP-C QDs, while CdSe-CS QDs caused complete
422 inhibition of regeneration in 80% of treated animals and this effect could not
423 be recovered (60% of polyps still in stage 0 and stage 1 at 72 h).

424



425

426 **Figure 4.** Influence of InP-based QDs on *Hydra* regeneration and

427 reproduction. A) Impact of QDs (70 nM) on regeneration. The regenerating

428 polyps were observed through a stereomicroscope and grouped in three

429 stages according to their developmental stage. Histograms are representative

430 of a single experiment. Three independent experiments were carried out. B)

431 Impact of the QDs on reproduction. Five animals with one bud were treated

432 with 70 nM InP-C and InP-CS QDs for 24 h and the following day each

433 animal was washed and placed in a well to monitor progeny. Asterisks

434 denote a significant difference between untreated and CdSe-CS treated

435 polyps (**, $P < 0.01$) according to ANOVA test. C) Schematic representation436 of *Hydra* regeneration (upper panel) and budding (lower panel) processes.

437

438 We also estimated the reproductive capability of QDs treated polyps. *Hydra*
439 reproduces asexually by budding (schematically represented in Figure 4C),
440 which is sustained by continuous cell proliferation and migration. This
441 process takes approximately 3 days³⁷. Environmental factors, such as the
442 presence of aquatic pollutants or the feeding regime may affect significantly
443 cell viability and tissue growth and in turn compromise the normal bud
444 growth and detachment.

445 Thus, the population growth rate is an excellent index to monitor the
446 transgenerational response of *Hydra* to environmental threats. Five polyps
447 (founders) were exposed to 70 nM for 24 h, then extensively washed and
448 allow to originate five independent *Hydra* populations over 14 days. The
449 growth curve shown in Figure 4B indicate a slight delay of the reproduction
450 rate induced by CdSe-CS QDs but not by InP QDs exposure, showing long
451 term effects of Cd-based QD.

452

453 ***In vitro* assessment of QD toxicity**

454 QDs can negatively affect cell proliferation, differentiation and viability.

455 Moreover, the exposure of cells to QDs may trigger distinct cell death

456 mechanisms. While necrosis often occurs as a result of the direct injury of

457 the cell structure, apoptosis is a programmed cell death, finely concerted by

458 endogenous players. Cells dying by apoptosis undergo evident morphological

459 changes, *i.e.* collapses of the cytoskeleton, disassembling of the nuclear

460 envelop, condensation of chromatin (pyknotic nuclei) followed by breaking up

461 into fragments forming so-called apoptotic bodies. In multicellular organisms,

462 apoptosis occurs physiologically during the growth, development, and

463 maintenance of multicellular organisms and in response to environmental

464 stressors.⁴³ In *Hydra*, apoptosis regulates many physiological processes such

465 as the oogenesis and spermatogenesis, starvation, and head regenerating

466 tips.^{44,45} We recently demonstrated that the programmed cell death may467 occur in *Hydra* as a defence mechanism in the presence of sub-lethal

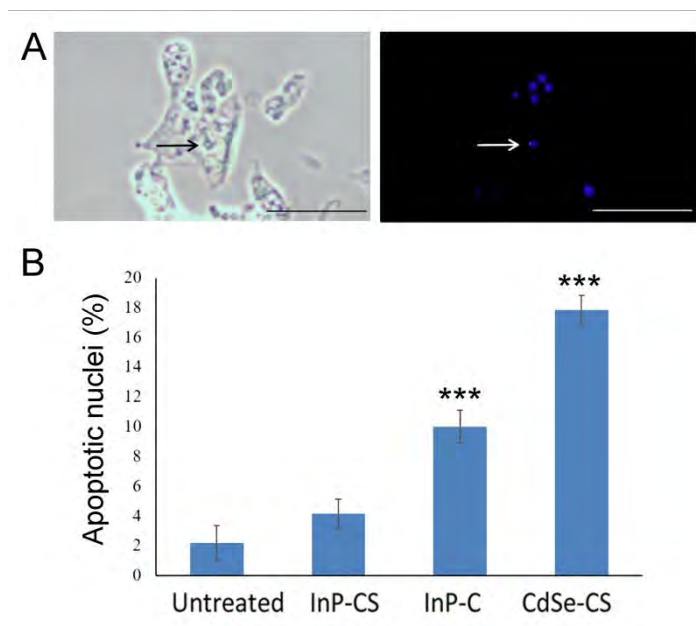
468 concentrations of nanomaterials, even in the absence of detectable

469 morphological alterations, demonstrating the interest of this type of evaluation
470 in case of QD exposure.^{25, 26, 46}

471 To this aim *Hydra* were incubated with 50 nM QD solutions for 24 h, then
472 macerated into fixed single cell suspensions³⁸ and inspected by fluorescent
473 microscopy. Typically, condensed chromatin forms pyknotic nuclei, which are
474 phagocytised by both the ectodermal and the endodermal epithelial cells.^{47, 48}

475 These structures are easily distinguishable by fluorescence microscopy upon
476 40-6-Diamidino-2-phenylindole (DAPI) staining (Figure 5A). Counts of pyknotic
477 nuclei reported in Figure 5B show that CdSe-CS QDs dramatically increase
478 the apoptotic rate (18%); milder effects were induced by the exposure to In-
479 C QDs even though the apoptotic cell number was significantly higher (10%)
480 compared to untreated animals. Conversely, the InP-CS QDs solution had
481 marginal effects with a percentage of damaged nuclei only slightly higher
482 than control animals, which as mentioned above, normally show a
483 physiological rate of apoptosis. These data confirm a safe profile of
484 InPZnS/(ZnSe,S) QDs in *Hydra*, highlighting two important aspects: the
485 protective role of the shell in reducing the effect of InP-based QDs, and the
486 high toxicity of Cd-based QDs, even protected by the same inorganic shell.

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491 **Figure 5.** Effect of QDs on *Hydra* apoptotic rate. A) Microscope image of
492 single cells prepared from QD treated *Hydra*. Left, an ectodermal epithelial
493 cell in bright field. Right, fluorescence imaging following DAPI staining
494 shows a pyknotic nucleus (white arrow), typical apoptosis hallmark in the
495 epithelial cell. Scale bars: 20 μm . B) Quantitative assessment of apoptosis
496 induction. Following 24 h incubation with 50 nM QDs, polyps were
497 macerated in single cells and the percentage of apoptotic cells was
498 determined by counting the DAPI-stained fragmented nuclei. Asterisks

499 denote a significant difference between untreated and QD- treated polyps
500 (***, $P < 0.05$) according to one-way ANOVA test.

501

502

503 ***Genotoxic effects***

504 Over the last decade, nanotoxicology approaches explored the impact of
505 novel nanomaterials at the molecular level with the aim to unravel genome,
506 epigenome, transcriptome and proteome changes in response to
507 nanostressors.⁴⁹ *Hydra* genome sequencing disclosed genetic features finely
508 conserved across Metazoan evolution.⁵⁰ Very recently, the conservation of the
509 key regulatory genetic pathways in *Hydra* allowed us to dissect *Hydra*
510 transcriptome modulation in response to different nanomaterials and identify
511 nanotoxicity inducible genes. More precisely, we proved by RNAseq analyses
512 that silica nanoparticles, with a safe profile on animal tests, produced only
513 minor modifications on the gene expression landscape.²⁷ Conversely,
514 transcriptome profiling of *Hydra* polyps exposed to CdSe/ZnS QDs revealed
515 that the expression of hundreds of genes was radically affected. In particular,
516 two highly overexpressed genes, namely carbonic anhydrase and the serine

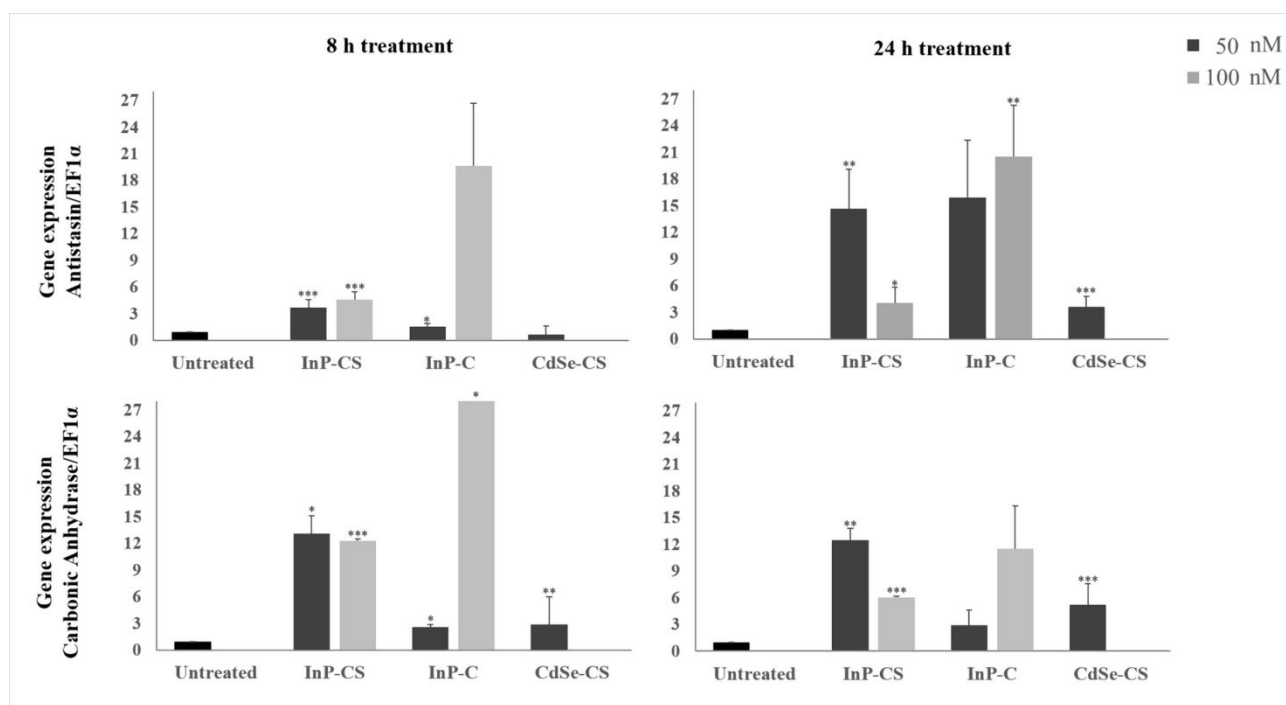
517 protease inhibitor antistasin, showed an impressive up-regulation upon 8 h
518 and 24 h QD exposure and might therefore be considered gene signatures
519 of heavy metal-based QDs exposure in *Hydra*. The exploitation of biomarkers
520 based on gene expression is a well-established methodology to provide
521 reliable outcomes about the early molecular response evoked by an
522 exogenous stimulus.⁵¹ *Hydra* polyps were incubated with 50 nM and 100 nM
523 of InP-CS and InP-C QDs and with 50 nM CdSe-CS QDs, for 8 h and 24 h
524 and the biomarker expression was investigated by qRT-PCR (Figure 6). Two
525 distinct concentrations of QD were investigated in order to profile the dose -
526 response fluctuations of biomarkers. In the case of CdSe-CS QDs, we did
527 not test the concentration 100 nM due to the significant presence of necrotic
528 cells. Overall, QD treatments up-regulate the expression of both genes. The
529 highest activation of carbonic anhydrase expression (fold change >25) was
530 detected at 8 h by 100 nM InP-C together with robust overexpression of
531 antistasin at 8 and 24 h (fold change >18). As expected, significant up-
532 regulation of these biomarkers was induced also by CdSe-CS, especially at
533 24 h. Surprisingly, a robust increase of biomarker expression was induced by
534 InP-CS QDs, which is in contrast with the low effect caused on morphology

535 and biological processes. We suggest that carbonic anhydrase and antistasin
536 may be symptomatic of a more general pollutant exposure, and show the
537 possibility to use them as excellent and sensitive biomarkers of exposure
538 able to probe aquatic pollutants, even at very low concentrations. Further
539 experiments will clarify their use to predict possible harmful effects on
540 animals, which are very difficult to be estimated, as several biological
541 responses are measurable only after protracted exposure.

542 The functional role of these genes as biomarker for nanotoxicity needs to be
543 established. Antistasin is a 17kDa serine protease inhibitor originally isolated
544 from the salivary glands of the Mexican leech (*Haemantaria officinalis*). It has
545 been shown to be a potent anticoagulant activity and to prevent
546 metastases.⁵² Antistasin-like gene is strongly expressed in gland and mucous
547 cells and may be involved in protecting gastric tissues from autodigestion.⁵³
548 Although functional studies are demanded, the up-regulation of the protease
549 inhibitor antistasin may represent an attempt to prevent protein degradation
550 likely activated by nanoparticle exposure in *Hydra*. This protective role may
551 also explain the gradual increase of antistasin gene expression observed
552 after a 24 h QD exposure compared to 8 h incubation.

553 Carbonic anhydrase (CA) is a zinc-containing metallo-enzyme, which
554 catalyzes the reversible hydration of carbon dioxide. It contributes to fixation
555 of atmospheric CO₂ and participates in many other physiological processes
556 such as pH homeostasis, electrolyte transport and many biosynthetic
557 reactions.⁵⁴ The role of CA as environmental biomarker has been
558 investigated over the recent years.^{55 56} Many works demonstrated that heavy-
559 metal exposure may inhibit CA activity possibly through their capability to
560 bind and displace the native cofactor, producing non-functional CA
561 metallovariants in several species. Conversely, enhanced activity of CA has
562 been described as well in *Chlamydomonas reinhardtii*⁵⁷, *Thalassiosira*
563 *weissflogii*,^{58 59} indicating that alternative cofactors (e.g. cadmium) may
564 successfully substitute Zn²⁺ without impairing the catalytic activity. Despite the
565 wealth of data on CA activity, little is known about gene expression
566 modulation in response to chemical pollutants. Although its biological role as
567 biomarker needs to be further elucidated, higher overexpression after 8 h
568 with respect to 24 h treatment suggests that CA may find application as
569 early expression biomarker of nanotoxicity.

570



571

572 **Figure 6.** Gene expression profiles of selected stress responsive genes in

573 QDs-treated polyps analyzed by qRT-PCR. The Elongation factor 1-alpha

574 (EF-1 α) was used as reference gene. The data represent mean of three

575 biological replicates. The value of each biological replicate is the average of

576 three technical repeats.

577

578 Taken together, our findings suggest that the choice of InP-based QDs

579 appears to be a valid alternative to CdSe-based QDs for manifold

580 applications. Since aquatic species, including freshwater invertebrates, are

581 natural targets for nanoparticles, which possibly contaminate natural waters

582 through sewage effluent and landfill leakages, our results suggest a safety

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583 profile of Pen-capped InPZnS/Zn(Se,S) QDs in aquatic organisms, at least at
584 the tested doses. Therefore, considering that *Hydra* is very susceptible to
585 aquatic pollutants, these QDs may not represent a concrete risk for
586 environmental health. Our extensive assessment of toxicity endpoints and
587 comparative analyses may drive future research on other types of eco-
588 friendly Cd-free QDs to monitor aquatic environments, preventing endogenous
589 species health, or estimating bioaccumulation of heavy metals in the food
590 chain up to humans.

591

592

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601

602 **Supporting Information.** S1: Synthesis of InPZnS alloy nanocrystals. S2: Surface
603 modification of QDs with Pen, S-3. Characterization of the QDs. Table S1: Primer
604 sequences used in qRT-PCR analyses. Table S2: Hydrodynamic diameter, zeta-potential
605 and QY values of the used In-and Cd-based QDs. Table S3. Morphological
606 changes and associated scores for toxicological assessment in *Hydra*.

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