



**HAL**  
open science

## Nanosized zeolite beta - Determining the safety of usage by zebrafish *Danio rerio* embryos

Ana Palčić, Sanja Babić, Aleksandra Maršavelski, Maja Galić, Natalija Topić  
Popović, Ivančica Strunjak Perović, Rozelindra Čož-Rakovac, Josip Bronić,  
Valentin Valtchev

### ► To cite this version:

Ana Palčić, Sanja Babić, Aleksandra Maršavelski, Maja Galić, Natalija Topić Popović, et al.. Nanosized zeolite beta - Determining the safety of usage by zebrafish *Danio rerio* embryos. *Microporous and Mesoporous Materials*, 2020, 299, pp.110103. 10.1016/j.micromeso.2020.110103 . hal-03034026

**HAL Id: hal-03034026**

**<https://normandie-univ.hal.science/hal-03034026>**

Submitted on 1 Dec 2020

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# 1 Nanosized zeolite beta - determining the safety of usage by zebrafish *Danio rerio* embryos

2  
3 Ana Palčić<sup>a,1,\*</sup>, Sanja Babić<sup>b,c,1,\*\*</sup>, Aleksandra Maršavelski<sup>d</sup>, Maja Galić<sup>b,c</sup>, Natalija Topić  
4 Popović<sup>b,c</sup>, Ivančica Strunjak Perović<sup>b,c</sup>, Rozelindra Čož-Rakovac<sup>b,c</sup>, Josip Bronić<sup>a</sup>, Valentin  
5 Valtchev<sup>e</sup>  
6

7 <sup>a</sup>Ruđer Bošković Institute, Division of Materials Chemistry, Laboratory for Synthesis of New  
8 Materials, Bijenička cesta 54, Zagreb, Croatia

9 <sup>b</sup>Ruđer Bošković Institute, Division of Materials Chemistry, Laboratory for Aquaculture  
10 Biotechnology, Bijenička cesta 54, Zagreb, Croatia

11 <sup>c</sup>Center of Excellence for Marine Bioprospecting (BioProCro), Ruđer Bošković Institute,  
12 Bijenička cesta 54, Zagreb

13 <sup>d</sup>University of Zagreb, Faculty of Science, Department of Chemistry, Horvatovac 102a, Zagreb,  
14 Croatia

15 <sup>e</sup>Normandie Univ, ENSICAEN, UNICAEN, CNRS, Laboratoire Catalyse et Spectrochimie, 6  
16 Boulevard Maréchal Juin, Caen, France  
17

18 <sup>1</sup>Equal contribution

19 \*Corresponding author

20 \*\*Corresponding author

21 E-mail addresses: ana.palcic@irb.hr (A. Palčić), sanja.babic@irb.hr (S. Babić)  
22  
23

## 24 **Keywords**

25 Nanozeolites; Organic structure directing agents; Nanotoxicology; Zebrafish embryotoxicity  
26 test; Molecular docking  
27  
28

## 29 **Highlights**

- 30 - Zeolite nanoparticles showed no impact on zebrafish embryonic development
- 31 - Tetraethylammonium cations tend to leach from the zeolite \*BEA framework
- 32 - Oxidative stress leads to hatching delay in zebrafish exposed to non-calcined zeolite  
33 samples and TEAOH  
34

## 35 **Abstract**

36 Zeolites are materials widely used in many fields of human activities. Furthermore, new  
37 potential applications constantly emerge, so understanding their possible impact on the  
38 environment is necessary. Within this study, the potential toxicity of nanosized particles (140  
39 and 600 nm) of a widely used zeolite beta was evaluated using zebrafish *Danio rerio* embryos.  
40 Embryotoxicity test, with an emphasis on sublethal changes, was performed on three  
41 concentrations of each nanosized zeolite sample (calcined and non-calcined). Toxicity of  
42 tetraethylammonium species (TEA) present in non-calcined zeolite samples was also  
43 investigated using experimental and computational approaches. The data suggest that non-  
44 calcined zeolites and tetraethylammonium hydroxide (TEAOH) itself caused hatching failure,  
45 but also initiated oxidative stress and apoptosis. Such observation confirmed certain TEA  
46 leaching from the zeolite framework, thus impacting embryonic development. Since molecular  
47 docking and molecular dynamics simulations did not show TEA inhibition of the hatching  
48 enzyme ZHE1 and the ROS formation was detected using fluorescence microscopy, it was  
49 concluded that oxidative stress is the major mechanism underlying the toxicity of non-calcined  
50 samples and TEAOH. Contrary to that, calcined zeolite nanoparticles, although having a strong  
51 interaction with the chorion and subsequently with the embryos, did not show a negative impact  
52 on zebrafish survival/development. Such a comprehensive study pinpointed zeolite  
53 nanoparticles as safe materials and opened the door for their application.

54

55

## 56 **1.0. Introduction**

57 Zeolites are crystalline microporous tectosilicate materials built of tetrahedra with central T  
58 atom (T=Si, Al, Ti, P, B, Ge, Ga, etc.) and surrounded by oxygen atoms at vertices [1].  
59 Depending on the arrangement of the tetrahedra, numerous tridimensional frameworks with the  
60 system of voids and channels of different sizes can get formed. Currently, there are over two  
61 million possible hypothetical zeolite structures and 234 approved zeolite frameworks found in  
62 nature and prepared in the laboratories [2,3]. Due to their particular properties such as tunable  
63 hydrophobicity, acidity, ion-exchange, morphology, size, and molecular sieving ability, zeolites  
64 are widely used in many industrial processes and everyday life [1,4,5]. The used materials range  
65 from all silica materials to low silica zeolites, from titanium-containing zeolites to  
66 silicoaluminophosphates. Synthetic zeolites are mainly used as catalysts in (petro)chemical  
67 industry, ion-exchangers in detergents and as molecular sieves in numerous separation  
68 processes. They are also employed in wastewater treatment, water purification, odor removal,

69 agriculture, medicine, solar cells, refrigeration, etc [6]. Furthermore, several studies reported a  
70 rise of zeolites application for biomedical purposes, i.e. as hosts for the encapsulation and  
71 delivery of anti-cancer drugs, but also for prevention of uncontrolled bleeding [7].

72 Zeolite beta is a member of the "Big Five" zeolites that dominate most of the commercial zeolite  
73 production for catalysis. It is applied as a catalyst in various industrial processes such as  
74 isomerization of waxes, Friedel Crafts reactions (alkylation and acylation), in the  
75 stereoselective Meerwein-Ponndorf-Verley reduction of ketones and for the  
76 tetrahydropyranlation of alcohols and phenols [8,9]. Zeolite beta is also employed as a catalyst  
77 in the cumene and ethylbenzene technologies at ENI [4]. Lately, Zr-, Hf- and Sn-beta zeolite  
78 materials are increasingly tested as catalysts for biomass valorization [10]. Similarly to other  
79 fields, there has been intensive development in the synthesis and application of nanosized  
80 zeolites [11,12]. Namely, nanosized zeolites have been shown to have good performance in  
81 water electrolysis [13], can act as ultraviolet shielding material [14], and sensors [15]. Due to  
82 their remarkable properties and the fact that they can be supplied in various forms (from  
83 colloidal suspensions, thin films, to membranes and self-supported morphologies), it can be  
84 assumed that nanosized zeolites will be involved in many scientific fields, industry, and  
85 consequently will become ubiquitous in many everyday products [6].

86 One of the most challenging issues in the field of nanotechnology is environmental health and  
87 safety, which is only achievable through consideration of the properties of engineered  
88 nanomaterials that could pose a hazard to the environment, but also to environmental organisms  
89 and human beings [16]. There are many articles dealing with the synthesis of nanosized zeolites  
90 [6,11], however, only a few studies investigated the impact of nanosized zeolites mainly by  
91 assessing zeolite cytotoxicity and neglecting a whole organism level [7,17,18]. Hence, it is  
92 necessary to carry out extensive studies that involve testing of different materials in terms of  
93 their chemical composition and framework type, as well as materials having different properties  
94 in terms of their surface area, crystal size and shape, porosity, hydrophobicity, acidity, and ion-  
95 exchange capacity. Furthermore, it is indispensable to establish the possible effects of nanosized  
96 zeolites on the specific ecosystems and living organisms.

97 With that goal, zebrafish *Danio rerio* - a promising small animal model that can be used in  
98 developmental, pharmacological, genetic and toxicological research, was employed as a model  
99 organism. Its small size, high fecundity, rapid development, optical transparency during the  
100 whole embryonic development, availability of genomic data and genetic similarity to humans,  
101 are just some of the reasons that enabled the use of an entire living organism (*in vivo*) in  
102 standardized *in vitro* format [19,20]. This ability to position zebrafish as a bridge between cell-

103 based tools and other *in vivo* models allows not only the extrapolation of the data across  
104 physiological targets and vertebrate taxa, but could also serve as a base of sustainable chemistry  
105 [19]. Nowadays, zebrafish are being used as *in vivo* platforms to study toxic effects and  
106 determine environmental risk assessment of pharmaceuticals [21], heavy metals [22], pesticides  
107 [23,24], microplastics [25,26], environmental samples [27–29], but also nanoparticles [30–32].  
108 Herein we provided the comprehensive insight into the impact of two nanosized zeolite beta  
109 particles sizes on zebrafish *Danio rerio* embryonic development. For the preparation of  
110 nanosized zeolite beta the presence of organic structure-directing agent (OSDA),  
111 tetraethylammonium hydroxide (TEAOH), is indispensable [5]. Namely, the TEA<sup>+</sup> cations  
112 assemble the porous zeolite network and thus are located within zeolite voids, i.e. distributed  
113 throughout the crystal. Prior to any application, OSDA has to be removed from the pores by  
114 calcination of the samples [33]. For this reason, both calcined and non-calcined zeolite materials  
115 have been investigated. Firstly, the physicochemical characterization of the prepared samples  
116 has been performed by a set of complementary techniques. Subsequently, *D. rerio* embryos  
117 were exposed to calcined and non-calcined nanosized zeolite samples, but also to the TEOH  
118 in concentrations corresponding to the ones present in zeolite samples. During zebrafish  
119 embryotoxicity test (ZET) special attention was given to sublethal effects, which were  
120 supplemented with molecular modeling in order to elucidate the mechanism of the observed  
121 effect of delayed hatching. The additional extent to the ZET test was done in terms of  
122 quantifying toxic effects at the cellular level by recording apoptotic cells and reactive oxygen  
123 species (ROS) formation. Taken together, multiple biological endpoints used in this study  
124 within one model organism proved to be a valuable and reliable basis for determining the impact  
125 of zeolite beta nanoparticles.

126

## 127 **2.0. Materials and methods**

128

### 129 **2.1. Chemicals**

130 Tetraethylammonium hydroxide (35% water solution; TEOH), fumed silica (99.80%),  
131 aluminium isopropoxide (98%), Ludox HS-30, acridine orange (AO), 2',7'-dichlorofluorescein  
132 diacetate (DFC), ethyl 3-aminobenzoate methanesulfonate salt (MS-222), as well as calcium  
133 chloride dihydrate (p.a.), magnesium sulfate heptahydrate (98%) sodium bicarbonate (p.a.)  
134 were obtained from Sigma Aldrich (Germany). Potassium hydroxide (pellets, 85%) and sodium  
135 hydroxide (pellets, 98%) were obtained from Kemika (Croatia), while sodium aluminate (54%  
136 Al<sub>2</sub>O<sub>3</sub>) was purchased from Honeywell Riedel-de Haën AG (Germany). Artificial water was

137 prepared by dissolving 294.0 mg L<sup>-1</sup> CaCl<sub>2</sub> × 2 H<sub>2</sub>O, 123.3 mg L<sup>-1</sup> MgSO<sub>4</sub> × 7H<sub>2</sub>O, 63.0 mg L<sup>-1</sup>  
138 NaHCO<sub>3</sub>, and 5.5 mg L<sup>-1</sup> KCl (Sigma Aldrich, Germany) in deionized water.

139

## 140 **2.2. Preparation and characterization of zeolite nanoparticles**

141 Sample BEA-140 was prepared according to Landau et al. [34]. The needed amount of  
142 aluminium isopropoxide was dissolved in TEAOH. Freeze-dried Ludox HS-30 was added to  
143 TEAOH and stirred for 10 minutes by using a magnetic stirrer. Two components were mixed  
144 and stirred for 10 minutes and subsequently transferred into an oven preheated at 100°C. The  
145 final molar oxide composition of the synthesis mixture was 0.35Na<sub>2</sub>O:0.5Al<sub>2</sub>O<sub>3</sub>:25  
146 SiO<sub>2</sub>:9TEAOH:295H<sub>2</sub>O. The crystalline sample was recovered after 7 days of hydrothermal  
147 treatment. The solid phase has been washed with deionized water repeatedly until pH 7 by  
148 centrifugation and dried by freeze-drying. Organic structure-directing agent (TEAOH) was  
149 removed by calcination of the dried powder at 550°C for 6 h in static air (BEA-140-calc).

150 Sample BEA-600 was prepared by mixing of the suspension of fumed silica in a solution of  
151 KCl in H<sub>2</sub>O and TEAOH together with the solution of sodium aluminate in water and KOH.  
152 The molar oxide composition was 1.23K<sub>2</sub>O:1.23Na<sub>2</sub>O:1Al<sub>2</sub>O<sub>3</sub>:50SiO<sub>2</sub>:25TEAOH:745H<sub>2</sub>O  
153 [35]. After 10 minutes of agitation, the synthesis mixture was treated for 30 h at 140°C. The  
154 solid phase was centrifuged repeatedly in deionized water until pH 7 and dried by freeze-drying.  
155 Organic structure directing agent was removed by calcination of the dried powder at 550°C for  
156 6 h in static air. In this way, the sample BEA-600-calc was obtained.

157 Powder X-ray diffraction (XRD) data of the solid sample were collected on a Phillips PW3710  
158 diffractometer with CuK<sub>α</sub> source. Thermogravimetric measurements (TG) of the solid samples  
159 were performed using a Setaram Setsys TGA instrument, heating rate 5°C min<sup>-1</sup> in air. The size  
160 of the crystals was measured employing Malvern Zetasizer Nano ZS. The powders were  
161 dispersed both in deionized and artificial water and measured as such. Scanning electron  
162 micrographs were obtained by employing an FE-SEM JEOL JSM-7000F microscope (SEM).  
163 For transmission electron microscopy (TEM) imaging, a small amount of the sample was  
164 dispersed in ethanol. After being treated by ultrasonication, one drop of the sample mixture was  
165 taken from the ethanol solution and transferred to a copper grid covered by a holey carbon film.  
166 Transmission electron microscope JEOL JEM-3010 was used for TEM studies. A Gatan 794  
167 CCD camera was used for recording transmission electron microscopy images.

168 Zeolite suspensions were prepared in three concentrations: - 25, 50 and 100 µg L<sup>-1</sup> by dispersing  
169 in artificial water. Prior to embryotoxicity test samples were aerated to oxygen saturation.  
170 TEAOH solutions in artificial water having concentrations 4.5, 9 and 18 µg L<sup>-1</sup> were tested as

171 well. These concentrations correspond to the average amount of organic species within the  
172 zeolite material (18 wt% as measured by TG).

173

## 174 **2.3. Toxicity testing**

175

### 176 **2.3.1. Ethics statement**

177 Animal housing and spawning were performed in aquaria units approved by the Croatian  
178 Ministry of Agriculture and according to the Directive 2010/63/EU on the protection of animals  
179 used for scientific purposes [36]. All experiments in this study were conducted on the non-  
180 protected embryonal stages (up to 72 hpf), which do not require permission by animal welfare  
181 commissions [36].

182

### 183 **2.3.2. Zebrafish maintenance and egg production**

184 Zebrafish *D. rerio* (wildtype) were maintained under controlled laboratory conditions,  
185 described in detail in our previous works [27]. In the evening, males and females were  
186 sequentially added into the iSpawn-S Benchtop Size Breeding System (Techniplast, Italy) and  
187 were kept separated by a divider. The next day, the divider was removed and the spawning  
188 platform lifted to initiate the spawning. After spawning, eggs were collected within 20 min  
189 using 800 µm mesh and were rinsed with artificial water (AW) in order to remove the debris.

190

### 191 **2.3.3. Zebrafish embryotoxicity test**

192 Exposure was performed by the ZET test [37]. Fertilized eggs from 4- to 64- blastomeres were  
193 selected under a stereomicroscope (PRO-LUX, Croatia) and transferred individually into 24-  
194 well plates containing 1 mL of calcined and non-calcined BEA suspensions (25, 50, 100 µg  
195 mL<sup>-1</sup>) and TEAOH (4.5, 9 and 18 µg mL<sup>-1</sup>). The artificial water was used as negative control.  
196 Plates were kept at 27.0±0.5°C with a 14/10 h light/dark cycle in the Innova 42 incubator shaker  
197 (New Brunswick). Daily, 30% of the test sample volume (nano-zeolites, TEAOH, artificial  
198 water) was replaced in order to ensure constant conditions - minimize/prevent exposure  
199 concentration change during the assay, but also to preserve the optimal dissolved oxygen  
200 concentration necessary for zebrafish normal development. Prior to solution replacement, nano-  
201 zeolitesuspensions were dispersed by sonicator in order to prevent agglomeration (Bandelin  
202 Sonorex). The test was conducted with 10 embryos in three independent replicas. Up to 72  
203 hours post-fertilization (hpf) lethal and sub-lethal effects were estimated [27,37].

204

205 **2.3.3.1. Reactive oxygen species assay**

206 For identification of ROS induced by nano-zeolites, embryo staining with DCFDA was  
207 performed [38]. DCFDA is commonly used in detecting oxidative stress in zebrafish embryos  
208 because it is a cell-permeable and ROS-reactive reagent. After 72 hpf larvae exposed to the  
209 highest concentration of each tested sample (100 and 18  $\mu\text{g mL}^{-1}$  of nano-zeolite suspensions  
210 and TEAOH, respectively) were rinsed three times with AW and exposed to 10  $\mu\text{M}$  DCFDA in  
211 darkness. After 1 h of incubation, larvae were rinsed three times with AW and anesthetized  
212 with 0.03% MS-222 for 2 min. ROS formation was observed in DCFDA-stained fish using a  
213 fluorescence microscope (Olympus® BX51 light binocular microscope equipped with the  
214 Microsoft® AnalySIS Soft Imaging System Software) with a green fluorescent filter. Semi-  
215 quantitative analysis was performed using ImageJ software (n=10). In order to detect possible  
216 interferences of nano-zeolites with the ROS assay (e.g. binding of assay components,  
217 fluorescence interference due to the same wavelength of the assay dye and tested samples),  
218 interference controls were run in parallel [39].

219

220 **2.3. 3.2. Apoptosis assay**

221 To investigate the potential apoptosis in the whole zebrafish larvae, nucleic acid-selective dye,  
222 AO was used [38]. After 72-h exposure to nano-zeolite suspensions (100  $\mu\text{g mL}^{-1}$ ) and TEAOH  
223 (18  $\mu\text{g mL}^{-1}$ ), zebrafish larvae were rinsed three times with AW and incubated in AO (5  $\mu\text{g}$   
224  $\text{mL}^{-1}$  in AW) for 30 min in darkness. After the incubation period, fish were rinsed three times  
225 with AW. The AO-stained fish were anesthetized with 0.03% MS-222 for 2 min and observed  
226 under a fluorescence microscope (Olympus® BX51 light binocular microscope equipped with  
227 the Microsoft® AnalySIS Soft Imaging System Software) with a green fluorescent filter. Semi-  
228 quantitative analysis was performed using ImageJ software (n=10). In parallel with these  
229 experiments, interference controls were also tested [39].

230

231 **2.3.3.3. Thermogravimetric analysis of zebrafish**

232 To determine the accumulation of nanosized zeolite beta during the zebrafish embryonic  
233 development, samples were studied by TG analysis. After the estimation of previously  
234 mentioned endpoints at 72 hpf, the remaining larvae were rinsed three times with deionized  
235 water. Further, fish were sonicated for 2 min, rinsed three times with deionized water, sonicated  
236 for an additional 2 min and finally rinsed three times with deionized water. Sonication was  
237 conducted twice in order to remove particles that potentially remained at the surface of the fish  
238 body. Samples were then incubated at 60°C until a constant dry mass was recorded. Controls



239 on AW were run in parallel. Considering larvae low body mass, all dried fish per tested sample  
240 were transferred into an alumina crucible and heated till 800°C using a Setaram Setsys TGA  
241 instrument, heating rate 5°C min<sup>-1</sup> in air.

242

#### 243 **2.3.4. Docking**

244 AutoDock Vina [40] version 1.1.2 was used to explore potential binding sites for TEA  
245 (tetraethylammonium) cation on the zebrafish hatching enzyme ZHE1 whose structure was  
246 taken from the PDB data bank (PDB ID 3LQB) [41]. Atomic coordinates for TEA were also  
247 taken from the PDB (PDB ID 1A9X) [42]. Water molecules and cocrystal ligands (sulfate ion,  
248 1,2-ethanediol) were removed from the crystal structure of the hatching enzyme ZHE1,  
249 hydrogen and partial Gasteiger charges were added and the coordinates of the structure were  
250 saved in pdbqt format. TEA was also converted to a pdbqt file. SwissDock [43] was also used  
251 to dock TEA to ZHE1. SwissDock is based on the docking software EADock DSS and the  
252 calculations are performed using the CHARMM22/27 all-hydrogen force field [44]. The default  
253 parameters were used whereas the whole protein structure was considered as a target during  
254 docking.

255

##### 256 **2.3.4.1. Molecular dynamics simulation**

257 Three different complexes of ZHE1 + TEA were prepared according to three poses obtained by  
258 both docking simulations. TEA is described by the general Amber force field (GAFF) [45] with  
259 partial charges obtained through the standard restrained electrostatic potentials (RESP)  
260 calculations [46] at the HF/6-31G\* level of theory with the Gaussian09 simulation package  
261 [47]. Hatching enzyme ZHE1 was described with the Amber14SB force field. All complexes  
262 were solvated in truncated octahedral boxes of TIP3P water molecules, extending 10 Å from  
263 the protein with chloride anions added to neutralize the system. Minimization was conducted  
264 in three cycles by restraining different atoms with a force constant of 50 kcal mol<sup>-1</sup> Å<sup>-1</sup>. In the  
265 first cycle, restraint was applied on each protein atom while water, ions, and the substrate were  
266 allowed to move by using 500 steps of steepest descent minimization, followed by 1,000 steps  
267 of conjugate gradient minimization. In the second cycle, the whole substrates and the protein  
268 were fixed using restraint on backbone atoms only, while side chains of the protein, water, and  
269 ions were allowed to move using 500 steps of steepest descent, followed by 2,000 steps of  
270 conjugate gradient minimization. In the last cycle, the whole system was subjected to 5,000  
271 steps of minimization by applying 1,500 steps of steepest descent and 3,500 steps of conjugate  
272 gradient minimization with no applied constraints. Optimized systems were gradually heated

273 from 0 to 300 K and equilibrated during 50 ps using NVT conditions [constant number (N),  
274 volume (V), and temperature (T)]. The density of the system was then equilibrated during 150  
275 ps of the simulation under the NPT ensemble. The system was further equilibrated for 50 ps of  
276 the simulation and subjected to productive, unrestrained production simulations in NVT  
277 ensemble. Bonds involving hydrogen atoms were constrained using the SHAKE algorithm [48].  
278 The time step was 2 fs. The Particle Mesh Ewald method [49] was applied to calculate long-  
279 range electrostatic interactions. Nonbonded interactions were truncated at 10.0 Å. The 100 ns  
280 production runs were performed on the graphics processing unit (GPU; GeForce GTX 1080Ti)  
281 by using the pmemd.CUDA engine [50,51] of AMBER16 [52]. Simulations for each complex  
282 were conducted in triplicates resulting in 300 ns of simulations for each complex.

283

## 284 **2.5. Statistics**

285 All analysis was conducted using GraphPad Prism 6.01. (GraphPad Software Inc., USA).  
286 Statistical differences between nano-zeolite samples/TEOH and negative control (AW), as well  
287 as among nano-zeolite samples and TEAOH treatment group were evaluated by one-way  
288 analysis of variance (ANOVA) with Tukey's post hoc test. The results were expressed as means  
289  $\pm$  SD, and  $p \leq 0.05$  was used as a cutoff value of statistical significance. The results of the  
290 hatching rate were presented as box-plots. A line within the box represents the median value,  
291 while the boundaries of box-plot indicate 25<sup>th</sup> and 75<sup>th</sup> percentiles. Whiskers above and below  
292 the box indicate 10<sup>th</sup> and 90<sup>th</sup> percentiles.

293

## 294 **3. Results and discussion**

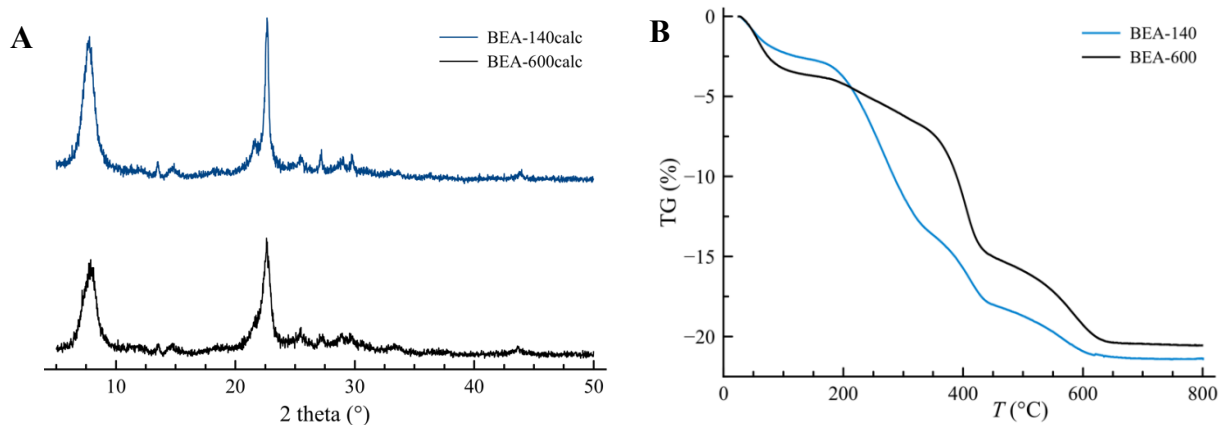
295

### 296 **3.1. Physico-chemical characterization of the tested samples**

297 The X-ray powder diffraction patterns (XRD) of two calcined samples are displayed in Fig. 1A.  
298 Both XRD patterns of the samples are typical of zeolite beta exhibiting broad peaks arising  
299 from the superposition of two systems of broadened reflections associated with polymorphs A  
300 and B of zeolite beta. TG analysis of the as-prepared zeolite beta samples was employed to  
301 measure the TEAOH content within the zeolite framework (Fig. 1B). Both samples exhibit four  
302 weight-loss steps. Two dehydration steps are ranged from room temperature to 180°C and  
303 events associated with TEA species release and degradation from 180 to 600°C. Consequently,  
304 the content of TEA species in the samples can be calculated from the TG curves. In the sample  
305 BEA-140, there is 20 wt% of organic structure-directing agent, while in the BEA-600 the

306 amount is 17 wt%. The average value of 18 wt% of organic species was taken for  
307 embryotoxicity tests.

308



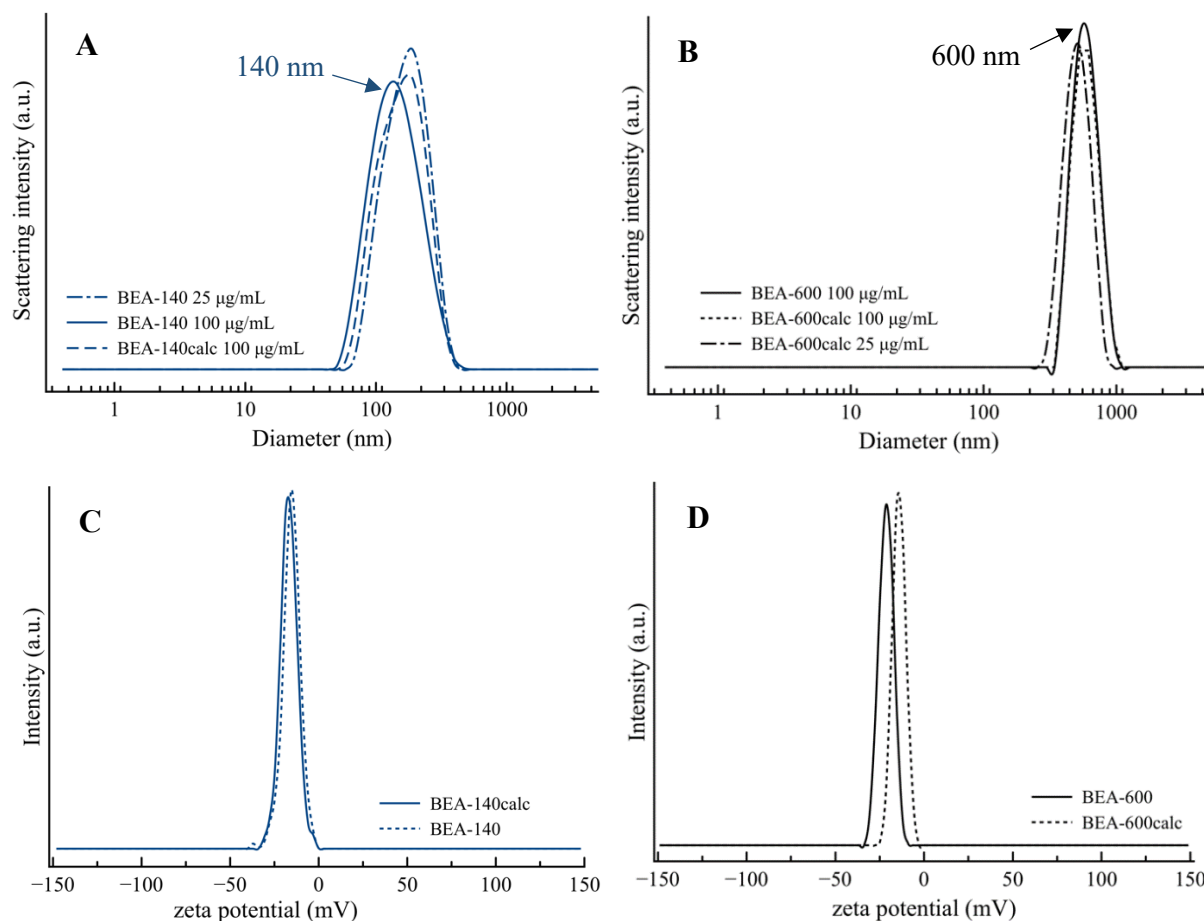
309

310 Figure 1. XRD patterns (A) of the calcined nano-zeolite beta samples used in this study and TG  
311 curves (B) of the as-prepared nano-zeolite beta samples.

312

313 Dynamic light scattering (DLS) measurements of all of the samples were performed using a  
314 concentration of  $100 \mu\text{g mL}^{-1}$ , dispersed both in deionized water and in AW. No difference in  
315 the size distribution has been observed with respect to the dispersant. Furthermore, for the sake  
316 of comparison, two samples having a concentration of  $25 \mu\text{g mL}^{-1}$  dispersed in AW were  
317 measured as well (Fig. 2). The maximum position of the DLS size distribution curve of the as-  
318 prepared BEA-140 having the concentration  $100 \mu\text{g mL}^{-1}$  is at 140 nm, whereas for the calcined  
319 BEA-140-calc ( $100 \mu\text{g mL}^{-1}$ ) and BEA-140 ( $25 \mu\text{g mL}^{-1}$ ) the maximum is at 160 nm. In the  
320 case of samples BEA-600 and BEA-600-calc ( $100 \mu\text{g mL}^{-1}$ ), the maximum is achieved at the  
321 hydrodynamic diameter of 600 nm. The sample BEA-600 ( $25 \mu\text{g mL}^{-1}$ ) exhibits a maximum at  
322 520 nm. Thus, considering the observed maximums in the DLS curves, the samples were  
323 labeled BEA-140 and BEA-600. The observed minor differences in the positions of the  
324 maximum of DLS curves in the studied samples can be attributed to slight fluctuations during  
325 the measurement due to the presence of different cations in the dispersant. Still, based on DLS  
326 data, it is evident that there is no irreversible aggregation of the zeolite beta particles during the  
327 calcination, as was observed previously [53].

328



329

330

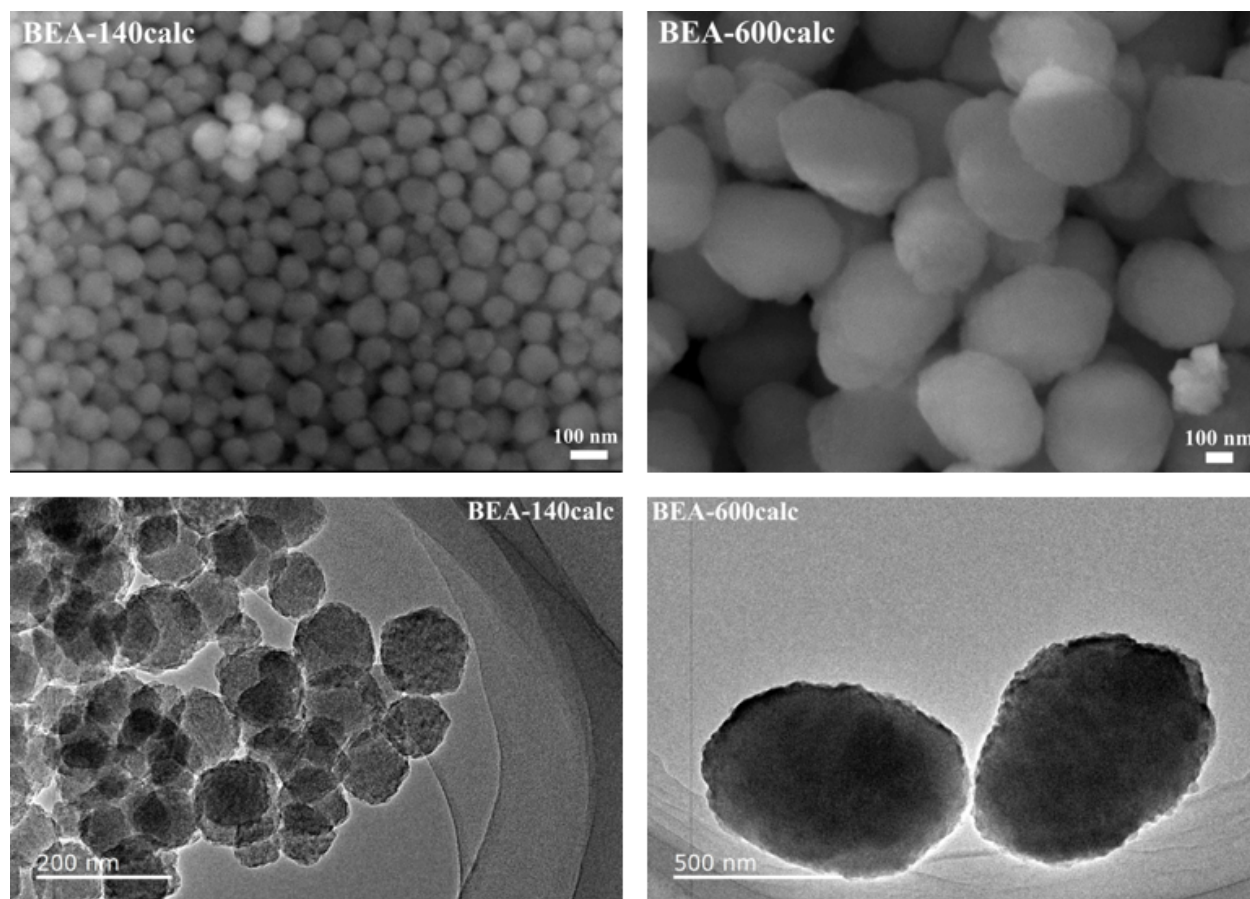
331 Figure 2. DLS size distribution curves of the studied samples BEA-140 and BEA-600 of  
 332 different concentrations (A and B) and the zeta potential curves of the nano-zeolite beta  
 333 suspensions having the concentration  $100 \mu\text{g mL}^{-1}$  (C and D).

334

335 Zeta potential measurements provide information on the colloidal stability of the samples. The  
 336 peaks of the highest studied concentration are positioned at  $-15.6 \text{ mV}$  and at  $-21.3 \text{ mV}$  for the  
 337 samples BEA-140 and BEA-600, respectively (Fig. 2). The suspensions of pristine samples  
 338 have more negative zeta potential than the calcined samples – the zeta potential maximum is at  
 339  $-13.7 \text{ mV}$  for BEA-140-calc and  $-12.9 \text{ mV}$  for BEA-600-calc. This is expected because of the  
 340 changing and condensing of the surface silanols of the zeolite beta particles during calcination.  
 341 In summary, all zeolite samples exhibit a negative zeta potential that prevents particle  
 342 aggregation but may have an impact on the interactions between the zeolite beta particles and  
 343 zebrafish.

344 SEM micrographs (Fig. 3, top) of the zeolite beta samples show that particles are uniform in  
 345 size and shape in both cases. Crystals in the sample BEA-140 are about  $150 \text{ nm}$  in size. The  
 346 particles are rounded and do not have well-defined edges. The crystals in the sample BEA-600  
 347 are oval in shape. Their size is around  $600 \text{ nm}$ . The TEM study (Fig. 3, down) confirms the

348 uniformity of the zeolite beta particle size and the morphological features observed by SEM.  
349 The average size of the particles is about 150 nm and 650 nm in the samples BEA-140 and  
350 BEA-600, respectively. In the sample BEA-140, the particles are isometric, while in the BEA-  
351 600 they are egg-like/ellipsoids. Thus, the micrographs of the tested samples corroborate the  
352 DLS findings.  
353



354  
355 Figure 3. SEM (the first row) and TEM (the second row) images of the studied calcined zeolite  
356 beta samples BEA-140calc and BEA-600calc.

357  
358

### 359 3.2. Embryotoxicity test

360 During 24, 48 and 72 h of zebrafish embryo exposure to the calcined and non-calcined zeolite  
361 beta suspensions and TEAOH, only a minor mortality rates ( $\leq 7\%$ ) were observed (Tbl. 1). Sub-  
362 lethal effects on all tested samples ( $< 8\%$ , Tbl. 1) revealed whether through yolk sac edema (Fig.  
363 S1, b) or blood accumulation at the yolk sac (Fig. S1, d). Based on the number of survived  
364 zebrafish, it can be asserted that all tested samples showed no toxicity or very low acute toxicity  
365 with small variances in the percentage of dead and/or abnormal embryos.

366

367 Table 1. Overview of endpoints measured at 72 h after *D. rerio* embryos exposure to the tested  
 368 samples.

		Observed endpoint					
		Dose ( $\mu\text{g mL}^{-1}$ )	Lethal embryos (%)	Affected embryos (%)	Heart beat rate (beats/15 sec)	Pigmentation formation (scored 1-3)	
Control	Artificial water	-	0.00 $\pm$ 0.00	3.33 $\pm$ 5.77	39.71 $\pm$ 1.25	2.93 $\pm$ 0.25	
	Zeolites	BEA- 140-calc	25	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	38.88 $\pm$ 1.96	2.90 $\pm$ 0.31
50			0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	37.81 $\pm$ 1.83	2.90 $\pm$ 0.31	
100			0.00 $\pm$ 0.00	3.33 $\pm$ 5.77	36.38 $\pm$ 1.50	2.93 $\pm$ 0.25	
BEA- 600-calc		25	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	37.44 $\pm$ 1.59	2.96 $\pm$ 0.18	
		50	0.00 $\pm$ 0.00	3.33 $\pm$ 5.77	39.57 $\pm$ 1.90	2.90 $\pm$ 0.31	
		100	6.66 $\pm$ 5.77	3.57 $\pm$ 5.77	35.00 $\pm$ 1.41	2.90 $\pm$ 0.31	
Zeolites with OSDA		BEA-140	25	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	38.02 $\pm$ 1.92	2.93 $\pm$ 0.25
			50	3.33 $\pm$ 5.77	3.83 $\pm$ 6.41	38.40 $\pm$ 3.13	2.96 $\pm$ 0.18
			100	3.33 $\pm$ 5.77	3.34 $\pm$ 5.77	38.42 $\pm$ 1.13	2.90 $\pm$ 0.31
	BEA-600	25	0.00 $\pm$ 0.00	3.33 $\pm$ 5.77	38.20 $\pm$ 1.90	2.96 $\pm$ 0.18	
		50	0.00 $\pm$ 0.00	3.33 $\pm$ 5.77	38.88 $\pm$ 0.83	2.93 $\pm$ 0.25	
		100	6.66 $\pm$ 5.77	3.97 $\pm$ 6.41	41.63 $\pm$ 1.19	2.90 $\pm$ 0.31	
OSDA	TEAOH	4.5	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	48.14 $\pm$ 1.68***	2.93 $\pm$ 0.25	
		9	6.66 $\pm$ 5.77	3.97 $\pm$ 6.41	46.71 $\pm$ 1.80***	2.90 $\pm$ 0.31	
		18	6.66 $\pm$ 5.77	7.54 $\pm$ 6.11	42.33 $\pm$ 1.50*	2.90 $\pm$ 0.31	

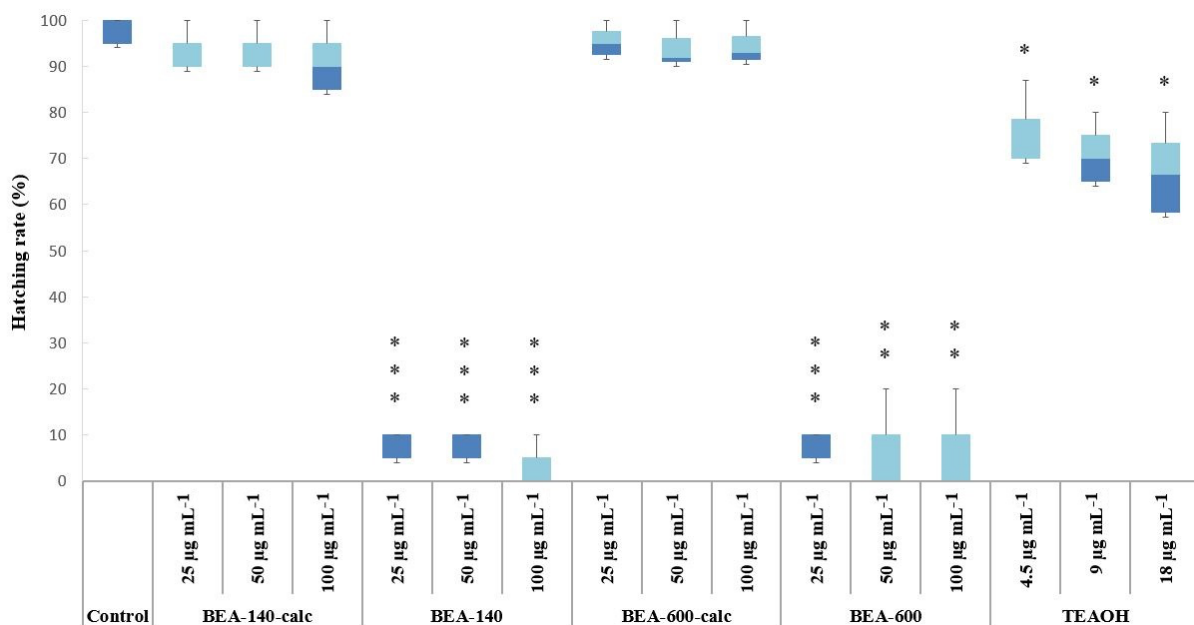
369 \*p<0.05, \*\*\*p<0.0001

370

371 As previously reported [7], nanoparticles with high positive zeta potential values are usually  
 372 cytotoxic, while neutral and negatively charged nanoparticles have no negative impact on the  
 373 cell. To support this theory Georgieva et al. [7] reported no impact of zeolite EMT nanoparticles  
 374 on human glioblastoma U87-MG and human kidney HEK-293T cell lines viability due to  
 375 exposure to negatively charged zeolite nanoparticles (10-30 nm; 50-400  $\mu\text{g mL}^{-1}$ ). Moreover,  
 376 Laurent et al. [17] showed that the viability of human cervical adenocarcinoma (HeLa) cells  
 377 was also not significantly affected after interaction with ultra-small LTL and EMT zeolites (8–  
 378 18 nm) free of organic templates (50-400  $\mu\text{g mL}^{-1}$ ). Based on the number of survived zebrafish,  
 379 it can be asserted that all tested samples showed no toxicity or very low acute toxicity with  
 380 small variances in the percentage of dead and/or abnormal embryos/larvae.

381 Since there is a possibility of the TEA leaching from the zeolite channels, which may have an  
 382 impact on the results obtained for the pristine nano-sized zeolite beta sample, the toxicity of  
 383 TEA alone was tested as well. Upon exposure to 4.5, 9 and 18  $\mu\text{g mL}^{-1}$  TEAOH (corresponding  
 384 to the TEA concentrations present in 25, 50 and 100  $\mu\text{g mL}^{-1}$  of non-calcined BEA-140 and  
 385 BEA-600 samples, respectively) heart rate significantly increased (Tbl. 1, marked gray), which  
 386 was not manifested in accelerated movements. According to the results obtained during  
 387 exposure to BEA-140 and BEA-600, nano-sized zeolite beta samples prevented the cardiotoxic  
 388 effect of the TEA. Still, avoiding the presence of organic structure-directing agents is  
 389 indispensable for further practical uses of nanosized zeolites, especially for biomedical  
 390 applications. This claim has been pointed up by further observation of larvae hatching. Hatching  
 391 is a critical stage in zebrafish life since it represents the end of embryogenesis and the start of  
 392 their swimming life phase. Decreased hatching success can increase predation or lead to fish  
 393 death within the chorion. Moreover, hatching disruption can cause a negative impact at the  
 394 population level, affecting thus ecosystems [55]. Calcined zeolite nanoparticles tested within  
 395 this study did not affect zebrafish hatching, while on the other hand, strong inhibition of  
 396 hatching rate at 72 hpf was recorded on non-calcined BEA-140 and BEA-600 samples (Fig. 4).  
 397 The highest concentration of the sample BEA-140 caused the largest hatching rate reduction at  
 398 72 hpf (96.55% compared to the control values; Fig. 4). TEA caused also a statistically  
 399 significant decrease of hatching rate, but those values were lower (~65% of zebrafish hatched  
 400 during a 72-h exposure to 18  $\mu\text{g mL}^{-1}$  of TEAOH).

401



402

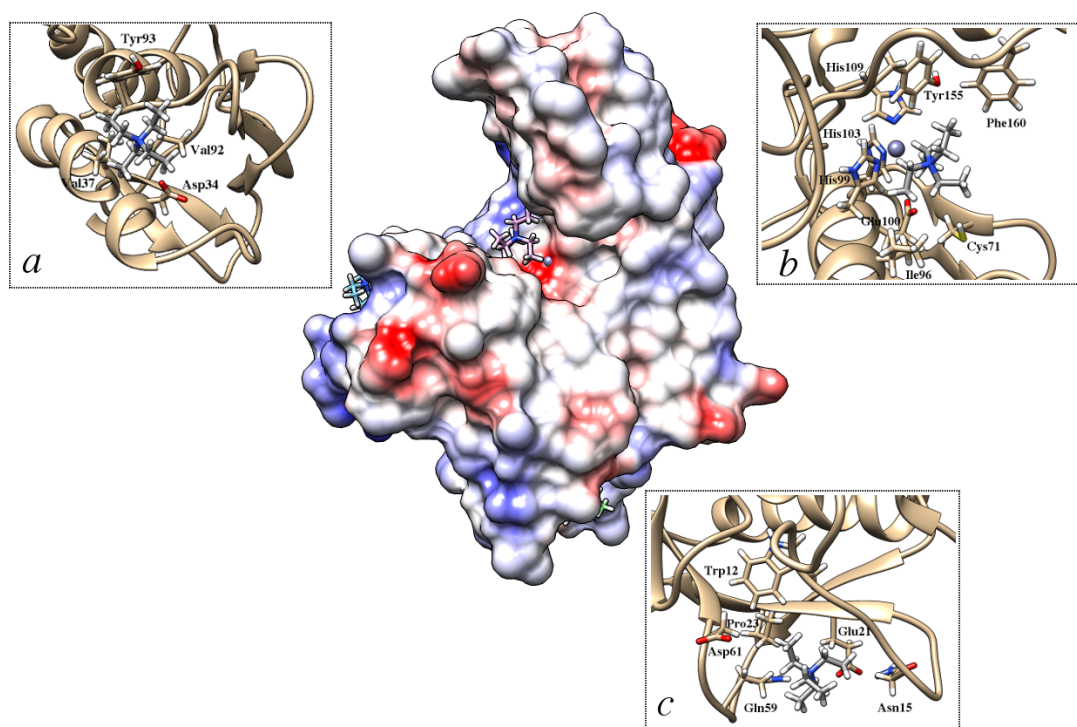
403 Figure 4. Hatching rate of 72 h old *D. rerio* embryos after exposure to the nano-zeolite beta  
404 suspensions and TEAOH. Statistical differences relative to the control group: \* $p < 0.05$ ,  
405 \*\* $p < 0.001$ , \*\*\* $p < 0.0001$ .

406

407 It is important to note that treated fish were not morphologically different from control fish,  
408 indicating that delayed hatching was not caused by slowed morphological development and  
409 thus by slowed maturation of the hatching gland. Since this option seems unfeasible, we  
410 investigated other possible mechanisms of the observed hatching inhibition. Hatching in  
411 zebrafish is regulated by exogenous factors such as light-dark cycles, oxygen levels, etc., but  
412 also endogenous factors such as muscle contractions, hormonal levels, the release of proteolytic  
413 enzymes from specialized cells [55]. Since the experiment was conducted in controlled  
414 conditions (light, temperature, oxygen level), the reason for decreased hatching was searched  
415 among endogenous factors. De la Paz et al. [55] reported that zebrafish hatching enzyme  
416 (ZHE1) expressed in the hatching gland is responsible for chorion degradation allowing  
417 zebrafish to hatch. Therefore, the inhibition of enzyme ZHE1 could lead to hatching retardation.  
418 Previous studies have shown that EDTA [56] and triazoles [55] inhibit hatching through  
419 affecting the hatching enzyme, either by complexing its  $Zn^{2+}$  ion or impairing the release of  
420 ZHE1 enzyme, respectively. To probe whether TEA directly binds to ZHE1 enzyme and  
421 interferes with the hatching process we performed docking simulations using two different  
422 docking programs AutoDock Vina and SwissDock. Molecular dynamics simulations were used  
423 to probe how specific are binding sites and poses found by docking simulations. Only those  
424 binding positions, found by both programs, were analyzed. Consequently, three different  
425 binding sites were identified (Fig. 5). Within the binding site *a*, TEA and Tyr93 interact through  
426 cation- $\pi$  interactions; Asp34 interacts with the TEA through electrostatic interactions, whereas  
427 ethyl groups of the TEA interact with Val37 and Val92 through van der Waals interactions.  
428 Within the binding site *b*, the TEA is bound within the enzyme active site. Cation- $\pi$  interactions  
429 are established between TEA and Tyr155 and Phe160, whereas Ile98 makes van der Waals  
430 interactions with ethyl groups of TEA. Glu100 makes electrostatic interactions with cationic  
431 TEA. Within the binding site *c*, cation- $\pi$  interactions are established between TEA and Trp12,  
432 electrostatic interactions are established with Glu21 and Asp61, whereas TEA makes  
433 hydrophobic interactions with Pro23.

434





435  
 436 Figure 5. Three binding sites *a*, *b* and *c* obtained using AutoDock Vina and SwissDock docking  
 437 software. Protein surface is colored according to electrostatic potential, where red stands for  
 438 negative, white for neutral and blue for positive potential. TEA is shown in grey.

439  
 440 To probe how specific these interactions are, we simulated three complexes for molecular-  
 441 dynamics simulations according to binding poses obtained using AutoDock Vina and  
 442 SwissDock (Fig. 5). Each complex was simulated in three independent replicas according to  
 443 the protocol described in Materials and methods. Our unconstrained simulations have shown  
 444 that binding of TEA is nonspecific. What was common for all three complexes is that after 20  
 445 ns of simulations TEA dissociated from ZHE1 into bulk water. The simulations were repeated  
 446 with all ligand atoms restrained by the harmonic potential with the force constant of 100 kcal  
 447 mol<sup>-1</sup> Å<sup>-1</sup> during optimization, equilibration and first 20 ns of MD simulation, after which force  
 448 constant was gradually decreased. This was then followed by unconstrained MD simulations.  
 449 However, in all three cases, TEA dissociated from the ZHE1 enzyme into bulk water again,  
 450 definitely referring to nonspecific binding of TEA to the ZHE1 enzyme. *In silico* study implies  
 451 that TEA does not bind to the ZHE1 enzyme which therefore excludes the inhibition of the  
 452 ZHE1 enzyme as a possible mechanism of hatching retardation.

453 It has been previously shown that tetraalkylammonium salts are reversible inhibitors of  
 454 cholinesterases [57], which was our rationale behind investigating whether TEA binds to and  
 455 inhibits ZHE1 enzyme. Another study has shown that organic cations, such as

456 tetramethylammonium (TMA) and TEA, can replace  $Mg^{2+}$  and  $Ca^{2+}$  ions in bacteriorhodopsin  
457 and maintain proton pumping ability [58]. In our case, displacement of  $Zn^{2+}$  ions with organic  
458 cation would inhibit the enzyme and consequently, the hatching process, because zebrafish  
459 hatching enzyme 1 is a Zn-protease needing  $Zn^{2+}$  as a catalytically active cation. This is in line  
460 with the previous study that demonstrated complete loss of enzyme activity in the presence of  
461 a chelating agent EDTA [56]. It has also been demonstrated that 4 metal oxide nanoparticles  
462 (CuO, ZnO,  $Cr_2O_3$ , and NiO) interfere with zebrafish hatching by shedding metal ions which  
463 then ligate to critical histidines in the ZHE1 active site [59]. In another study, the addition of  
464 the metal ion chelator, diethylene triamine pentaacetic acid (DTPA), reversed the hatching  
465 interference of the shed  $Cu^{2+}$ ,  $Zn^{2+}$ , and  $Ni^{2+}$  cations, proving that inhibition is indeed due to  
466 the shedding of metal ions [60]. Moreover, studies conducted on related zinc metalloprotease  
467 showed that substituting  $Zn^{2+}$  with  $Cu^{2+}$  or  $Ni^{2+}$  results in reduced or diminished enzyme  
468 activity [61,62]. Therefore, to investigate whether TEA could replace  $Zn^{2+}$  and stay stably  
469 bound, which would result in an inactive enzyme, we have additionally prepared another  
470 complex of ZHE1 with TEA, in which the organic cation occupies  $Zn^{2+}$  binding site. However,  
471 our three independent MD simulations showed that TEA dissociates from the enzyme into bulk  
472 water within the first 15 ns of MD simulations. Thus, TEA does not inhibit the enzyme by  
473 displacing the  $Zn^{2+}$  cation.

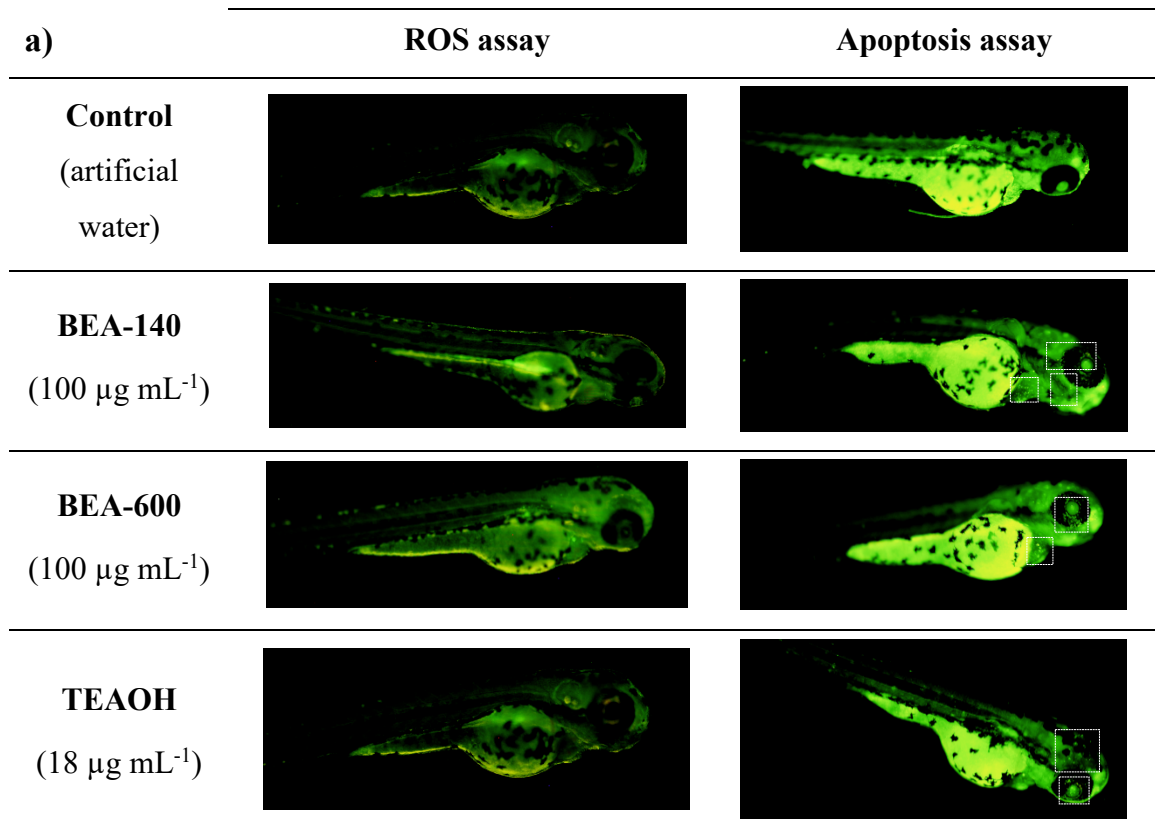
474

### 475 **3.3. ROS and apoptosis detection**

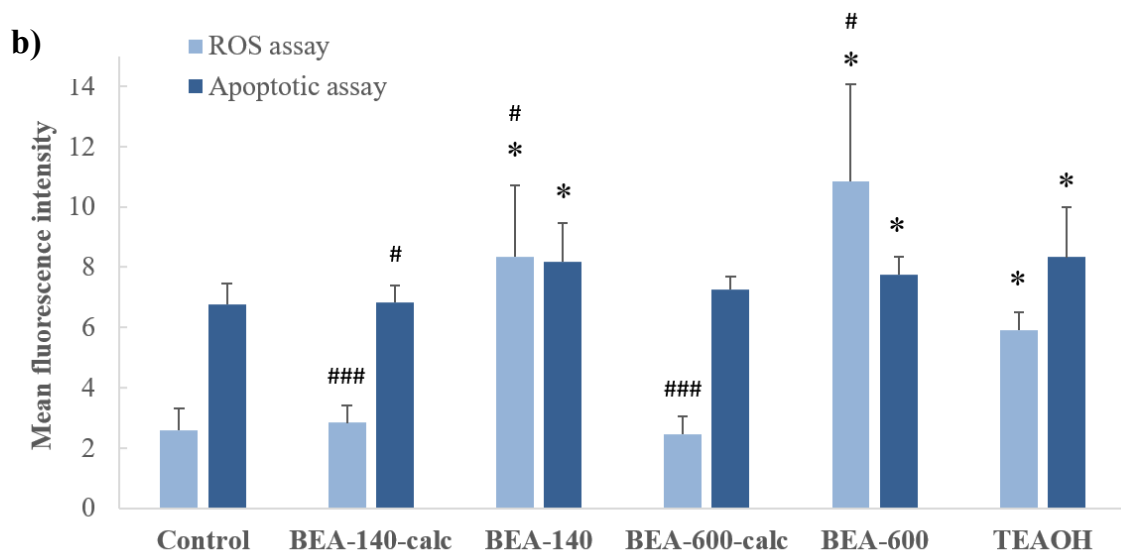
476 Generation of ROS and apoptosis are normal parts of the development and essential for normal  
477 cellular functioning. Despite, homeostatic cellular balance can be disrupted by exposure to e.g.  
478 toxicants, consequently resulting in ROS overproduction and cell death. Zebrafish exposure to  
479 pristine BEA-140 and BEA-600 samples, as well as TEAOH, resulted in a significant increase  
480 of the mean green fluorescence intensity (Fig. 6). Contrary, DCF and AO staining showed no  
481 significant ROS and/or apoptosis formation during exposure to calcined zeolite samples (Fig.  
482 6, b). The highest increase of ROS production in larvae exposed to BEA-600 and BEA-140,  
483 followed by TEAOH (4.21, 3.24 and 2.30 times increased compared to the control,  
484 respectively). Moreover, fish treated with BEA-140 and BEA-600 samples and stained with  
485 AO showed green fluorescent apoptotic spots on the heart, eye and head region (Fig. 6, a). Such  
486 finding was also observed during exposure to TEAOH. The results confirmed the dependence  
487 of cellular apoptosis with ROS induction in the whole zebrafish larvae, as is already recorded  
488 by Kumar et al. [63].

489

490  
491



492



493

494 Figure 6. (a) Fluorescent images of *D. rerio* larvae stained with ROS marker DCF and apoptosis  
495 marker AO after 72 h of exposure to tested nano-zeolite beta suspensions and TEAOH. (b) The  
496 bar graph represents the mean fluorescent intensity of DCF and AO in the whole larvae. The  
497 AO positive cells were indicated by dashed rectangles. Fluorescent intensity was calculated  
498 using Image J and the values were presented as the mean of fluorescence intensity  $\pm$  SD.

499 Statistical differences from the fluorescent intensity of untreated control larvae: \* $p < 0.05$ , and  
500 from the fluorescent intensity of TEAOH treatment group: # $p < 0.05$ , ### $p < 0.05$ .

501  
502 The absence of negative impact of calcined samples was in line with the study of Laurent et al.  
503 [17] which although confirmed internalization of ultra-small zeolites in HeLa cells, recorded  
504 neither oxidative stress nor abnormality in DNA replication. Considering the results obtained  
505 on non-calcined samples and TEAOH, we can conclude that OSDA leached to a certain extent  
506 from the zeolite, thus leading to ROS formation. To date, several authors emphasized that  
507 oxidative stress caused by ROS could lead to hatching delay [64,65]. For that reason, we  
508 propose that exactly oxidative stress is the major mechanism underlying the toxicity of non-  
509 calcined samples and TEAOH recorded within this study. Moreover, it is important to note that  
510 the TEA itself caused a significantly lower increase of the mean green fluorescence intensity  
511 (oxidative stress) than the as-prepared zeolite samples ( $p < 0.05$ ; Fig. 6). Regarding conducted  
512 interference controls, we did not observe any fluorescence, proving that nano-zeolite samples  
513 did not produce fluorescence that would lead to false-positive results.

514 In addition, microscopic pictures of the chorion (magnification 100x) were taken (Fig. S2). As  
515 can be seen, zeolite samples agglomerated at the surface of the zebrafish chorion. Considering  
516 that, if OSDA leaching occurred at the surface of the chorion, fish would be in direct and  
517 constant exposure, thus displaying higher oxidative stress than recorded during exposure to  
518 TEAOH itself.

519 Such observation of nanoparticle aggregation is not uncommon. Kashiwada [66] investigated  
520 the distribution of water-suspended fluorescent nanoparticles (solid latex solution) in the eggs  
521 and shown that nanoparticles in the range from 39.4 to 42.0 nm in diameter also aggregate and  
522 adsorb on the chorion of medaka eggs. Such aggregation at the surface of the chorion can  
523 negatively affect the nutrient absorption but also vitamin synthesis [67], impacting thus  
524 zebrafish embryonic development. Accordingly, limitations of chorion permeability for  
525 nanoparticles, as well as nanoparticle aggregation and adsorption on the chorion should be taken  
526 into account during the toxicity evaluation of nanoparticles on zebrafish embryos.

527

### 528 **3.4. TG analysis – interactions between zebrafish and zeolite nanoparticles**

529 Many studies emphasize the fact that the chorion represents a barrier of limited permeability,  
530 highlighting thus their importance during embryonic development of zebrafish [68,69]. The  
531 pore size of the chorion is estimated from 0.6 - 0.7  $\mu\text{m}$  [69], which is in theory larger than the  
532 size of zeolite nanoparticles used within this study. Despite nanoparticles are known to

533 aggregate and form larger agglomerates which complicate their interaction with the model  
 534 organism Kim and Tanguay [69] and Kashiwada [66] pointed out that such observation did not  
 535 prevent their accumulation in medaka fish. In fact, 474-nm water-suspended fluorescent  
 536 nanoparticles had the highest bioavailability to eggs, while particles of 39.4 nm in diameter  
 537 shifted into the yolk and gallbladder along with embryonic development [66]. To date, it is  
 538 entirely unknown whether zeolite beta nanoparticles can enter the embryos and whether they  
 539 are biocompatible. Thus, TG analysis of the dried fish was performed in order to determine if  
 540 tested zeolite samples interact with the chorion/fish (Tbl. 2). The weight fraction of the solid  
 541 residue for the control was 7.03 wt% of the initial mass of the dried larvae. TG analysis  
 542 confirmed that the larvae which were exposed to zeolite beta suspensions exhibit a higher  
 543 quantity of the solid phase remaining after the analysis, but differences are observed according  
 544 to the particle size.

545 Considering the results of TG analysis and the fact that fish exposed to BEA-140-calc hatched  
 546 normally, while specimens exposed to BEA-140 stayed within the chorion at 72 hpf (Fig. S2),  
 547 we speculate that the crystals of BEA-140-calc and BEA-140 (particle size 140 nm) passed  
 548 through the chorion and accumulated inside the model organism. The value of the difference  
 549 obtained by TG analysis between the BEA-140-calc and BEA-140 represents the amount of the  
 550 zeolite beta sample that was bonded to/accumulated in larvae (+4.19 and +4.86 wt%,  
 551 respectively) (Tbl. 2). Further research is needed to confirm this interaction and accumulation  
 552 of nanosized zeolites in/on the hatched larvae. The increase of the mass of the solid residue  
 553 after TG measurement for the samples BEA-600-calc and BEA-600 is lower than for the  
 554 samples comprising smaller particles, +2.94 and + 3.38 wt%, respectively (Tbl. 2), yet still  
 555 notable. The collected data strongly suggest not only that zeolite beta particles have strong  
 556 interaction with the chorion and subsequently with the embryos, but also that they remained  
 557 firmly bonded even after several cycles of ultrasonication.

558

559 Table 2. TG analysis of the washed and dried *D. rerio* larvae exposed to nanosized zeolite beta  
 560 suspensions for 72 h.

<b>Sample</b>	<b>Fraction of the solid residue</b>	<b>Difference to control</b>
Control	7.03 wt%	-
BEA-140	11.89 wt%	+4.86 wt%
BEA-140calc	11.22 wt%	+4.19 wt%
BEA-600	9.97 wt%	+2.94 wt%
BEA-600calc	10.41 wt%	+3.38 wt%

561  
562  
563  
564  
565  
566  
567  
568  
569  
570  
571  
572  
573  
574  
575  
576  
577  
578  
579  
580  
581  
582  
583  
584  
585  
586  
587  
588  
589  
590  
591  
592  
593  
594

#### **4. Conclusion**

Stable suspensions of pristine and calcined zeolite beta nanoparticles (140 and 600 nm) in AW have been prepared and their potential toxicity towards zebrafish *Danio rerio* embryos was evaluated. The results indicate that non-calcined beta zeolites containing TEA caused zebrafish hatching inhibition accompanied by oxidative stress. A similar effect was observed with TEAOH. The assumption that the observed effects are due to the TEA interactions with the zebrafish hatching enzyme ZHE1 has been validated by molecular docking and molecular dynamics simulations. However, the computational investigation points out that the TEA does not bind to the ZHE1 enzyme thus excluding the ZHE1 enzyme inhibition as a potential cause of the hatching reduction. Hence, the observed hatching delay of the non-calcined zeolite suspensions and TEAOH was, as detected using fluorescence microscopy, attributed to the oxidative stress. This is further supported by the finding that the zebrafish embryos developed normally in the presence of the calcined zeolite nanoparticles despite the strong interaction with the chorion and subsequently with the embryos. In addition, the necessity to decrease the quantity of the organic structure-directing agents in zeolite synthesis reaction mixtures was shown. Finally, the obtained results have shown that selected model organisms could improve our ability to understand the mechanism of the toxicity of aluminosilicates, and should be incorporated in nanoparticle toxicity monitoring and risk assessment studies in materials science in general.

#### **Declaration of competing interest**

All authors have no potential sources of conflict of interest.

#### **Acknowledgments**

The financial support from the Croatian Academy of Science and experiment.com platform is gratefully acknowledged. This study was partially supported by the Scientific Centre of Excellence for Marine Bioprospecting – BioProCro, a project co-financed by the Croatian Government and the European Union through the European Regional Development Fund - the Competitiveness and Cohesion Operational Programme (KK.01.1.1.01). A.M. would like to thank the Zagreb University Computing Centre (SRCE) for granting computational resources on the ISABELLA cluster.

595 References

- 596 [1] V. Valtchev, L. Tosheva, Chem. Rev. 113 (2013) 6734-6760.  
597 <https://doi.org/10.1021/cr300439k>
- 598 [2] <http://www.hypotheticalzeolites.net> (accessed January 31, 2020)
- 599 [3] <http://www.iza-structure.org/databases> (accessed January 31, 2020)
- 600 [4] G. Bellussi, R. Millini, P. Pollesel, C. Perego, New J. Chem. 40 (2016) 4061-4077.  
601 <https://doi.org/10.1039/c5nj03498a>
- 602 [5] S.E. Lehman, S.C. Larsen, Environ. Sci.: Nano 1 (2014) 200-213.  
603 <https://doi.org/10.1039/c4en00031e>
- 604 [6] S. Mintova, M. Jaber, V. Valtchev, Chem. Soc. Rev. 44 (2015) 7207-7233.  
605 <https://doi.org/10.1039/c5cs00210a>
- 606 [7] V. Georgieva, C. Anfray, R. Retoux, V. Valtchev, S. Valable, S. Mintova, Micropor.  
607 Mesopor. Mater. 232 (2016) 256-263. <https://doi.org/10.1016/j.micromeso.2016.06.015>
- 608 [8] M. Koehle, R.F. Lobo, Catal. Sci. Technol. 6 (2016) 3018-3026.  
609 <https://doi.org/10.1039/c5cy01501d>
- 610 [9] W.J. Roth, P. Nachtigall, R.E. Morris, J. Čejka, Chem. Rev. 114 (2014) 4807-4837.  
611 <https://doi.org/10.1021/cr400600f>
- 612 [10] H.Y. Luo, J.D. Lewis, Y. Román-Leshkov, Annu. Rev. Chem. Biomol. Eng. 7 (2016)  
613 663-92. <https://doi.org/10.1146/annurev-chembioeng-080615-034551>
- 614 [11] H. Awala, J.P. Gilson, R. Retoux, P. Boullay, J.M. Goupil, V. Valtchev, S. Mintova,  
615 Nature Mater. 14 (2015) 447-451. <https://doi.org/10.1038/nmat4173>
- 616 [12] L. Kyu, L.B., Huong, P.T., Geun LD. Kyu, Method for Removing Carbon Dioxide Using  
617 Nano-Zeolite Supported with Fe Ion., (2016) Pat. KR20170142691.
- 618 [13] M. Nishihara, Y. Terayama, T. Haji, S.M. Lyth, S. Satokawa, H. Matsumoto, eXPRESS  
619 Polymer Lett. 12, (2018) 256-264. <https://doi.org/10.3144/expresspolymlett.2018.23>
- 620 [14] Q. Li, G. Liao, S. Zhang, L. Pang, H. Tong, W. Zhao, Z. Xu, Appl. Surf. Sci. 427 (2018)  
621 437-450. <https://doi.org/10.1016/j.apsusc.2017.08.024>
- 622 [15] G.S. Wang J, Zhang J, Triethylamine Fluorescence Sensor for Detecting e.g.  
623 Triethylamine Gas, Comprises Nano-L-Type Zeolite and Rare-Earth Beta-Diketone  
624 Complex Obtained by Reacting Rare-Earth Ion and  $\beta$ -Diketone Ligand., (2017) Pat.  
625 CN107089905-A.
- 626 [16] C.R. Thomas, S. George, A.M. Horst, Z. Ji, R.J. Miller, J.R. Peralta-Videa, T. Xia, S.  
627 Pokhrel, L. Mädler, J.L. Gardea-Torresdey, P.A. Holden, A.A. Keller, H.S. Lenihan, A.E.  
628 Nel, J.I. Zink, ACS Nano 5 (2011) 13-20. <https://doi.org/10.1021/nm1034857>

- 629 [17] S. Laurent, E.P. Ng, C. Thirifays, L. Lakiss, G.M. Goupil, S. Mintova, C. Burtea, E.  
630 Oveisi, C. Hébert, M. De Vries, M.M. Motazacker, F. Rezaee, M. Mahmoudi, *Toxicol.*  
631 *Res.* 2 (2013) 270-279. <https://doi.org/10.1039/c3tx50023c>
- 632 [18] L.C.J. Thomassen, D. Napierska, D. Dinsdale, N. Lievens, J. Jammaer, D. Lison, C.E.A.  
633 Kirschhock, P.H. Hoet, J.A. Martens, *Nanotoxicology* 6 (2012) 472-485.  
634 <https://doi.org/10.3109/17435390.2011.587901>
- 635 [19] P.D. Noyes, G.R. Garcia, R.L. Tanguay, *Green Chem.* 18 (2016) 6410-6430.  
636 <https://doi.org/10.1039/c6gc02061e>
- 637 [20] D.H. Pham, B. De Roo, X.B. Nguyen, M. Vervaele, A. Kecskés, A. Ny, D. Copmans, H.  
638 Vriens, J.P. Locquet, P. Hoet, P.A.M. De Witte, *Sci. Rep.* 6 (2016) 37145.  
639 <https://doi.org/10.1038/srep37145>
- 640 [21] A.P. Deveau, V.L. Bentley, J.N. Berman, 45, (2017) 1-9.  
641 <https://doi.org/10.1016/j.exphem.2016.09.012>
- 642 [22] D.D. Nabinger, S. Altenhofen, P.E.R. Bitencourt, L.R. Nery, C.E. Leite, M.R.M.R.  
643 Vianna, C.D. Bonan, *Sci Total Environ.* 624 (2018) 1623-1633.  
644 <https://doi.org/10.1016/j.scitotenv.2017.10.057>
- 645 [23] D. Bridi, S. Altenhofen, J.B. Gonzalez, G.K. Reolon, C.D. Bonan, *Toxicology.* 392  
646 (2017) 32-39. <https://doi.org/10.1016/j.tox.2017.10.007>
- 647 [24] H.C Liu, T.Y. Chu, L.L. Chen, W.J. Gui, G.N. Zhu, *Environ. Pollut.* 231 (2017) 1093-  
648 1103. <https://doi.org/10.1016/j.envpol.2017.05.072>
- 649 [25] G. Malafaia, A.M. de Souza, A.C. Pereira, S. Gonçalves, A.P. da Costa Araújo, R.X.  
650 Ribeiro, T.L. Rocha, *Sci. Total Environ.* 700 (2020) 1348673.  
651 <https://doi.org/10.1016/j.scitotenv.2019.134867>
- 652 [26] L. Qiang, J. Cheng, *Ecotoxicol. Environ. Safety* 176 (2019) 226-233.  
653 <https://doi.org/10.1016/j.ecoenv.2019.03.088>
- 654 [27] S. Babić, J. Barišić, H. Višić, R. Sauerborn Klobučar, N. Topić Popović, I. Strunjak-  
655 Perović, R. Čož-Rakovac, G. Klobučar, *Water Research* 115 (2017) 9-21.  
656 <https://doi.org/10.1016/j.watres.2017.02.049>
- 657 [28] M.A.G. Barbosa, R. Capela, J. Rodolfo, E. Fonseca, R. Montes, A. André, A. Capitão,  
658 A.P. Carvalho, J.B. Quintana, L.F.C. Castro, M.M. Santos, *Ecotoxicol. Environ. Safety*  
659 182 (2019) 1094062. <https://doi.org/10.1016/j.ecoenv.2019.109406>
- 660 [29] L. Wu, Y. Jiang, L. Zhang, L. Chen, H. Zhang, *Environ Sci Pollut Res Int.* 21 (2014)  
661 2663-2676. <https://doi.org/10.1007/s11356-013-2193-9>



- 662 [30] N.B. Abramenko, T.B. Demidova, E. V. Abkhalimov, B.G. Ershov, E.Y. Krysanov, L.M.  
663 Kustov, J. Hazard. Mater. 347 (2018) 89-94.  
664 <https://doi.org/10.1016/j.jhazmat.2017.12.060>
- 665 [31] J. Duan, Y. Yu, Y. Li, Y. Li, H. Liu, L. Jing, M. Yang, J. Wang, C. Li, Z. Sun,  
666 Nanotoxicology 10 (2016) 575-585. <https://doi.org/10.3109/17435390.2015.1102981>
- 667 [32] M. Ghobadian, M. Nabiuni, K. Parivar, M. Fathi, J. Pazooki, Ecotoxicol. Environ. Safety  
668 122 (2015) 260-267. <https://doi.org/10.1016/j.ecoenv.2015.08.009>
- 669 [33] O. Larlus, V.P. Valtchev, Chem. Mater. 17 (2005) 881-886.  
670 <https://doi.org/10.1021/cm048799r>
- 671 [34] M. V. Landau, L. Vradman, V. Valtchev, J. Lezervant, E. Liubich, M. Talianker, Ind.  
672 Eng. Chem. Res. 42 (2003) 2773–2782. <https://doi.org/10.1021/ie020899o>
- 673 [35] M.A. Camblor, J. Pérez-Pariente, Zeolites 11 (1991) 202-210.  
674 [https://doi.org/10.1016/S0144-2449\(05\)80220-9](https://doi.org/10.1016/S0144-2449(05)80220-9)
- 675 [36] European Parliament and Council of the European Union, Directive 2010/63/EU of the  
676 European Parliament and of the Council of 22 September 2010 on the protection of  
677 animals used for scientific purposes, 2013.  
678 [https://doi.org/doi:10.3000/19770677.L\\_2013.124.eng](https://doi.org/doi:10.3000/19770677.L_2013.124.eng)
- 679 [37] OECD, Test No. 236: Fish Embryo Acute Toxicity (FET) Test., OECD Guidelines for  
680 the Testing of Chemicals, Section 2, OECD Publishing. (2013).  
681 <https://doi.org/10.1787/9789264203709-en>
- 682 [38] W.S. Lee, H.J. Cho, E. Kim, Y.H. Huh, H.J. Kim, B. Kim, T. Kang, J.S. Lee, J. Jeong,  
683 Nanoscale 11 (2019) 3173-3185. <https://doi.org/10.1039/c8nr09321k>
- 684 [39] C. Schimpel, B. Rinner, M. Absenger-Novak, C. Meindl, E. Fröhlich, A. Falk, A.  
685 Zimmer, E. Roblegg, E. NanoTox-Lett. 6 (2015) 1-14. <https://doi.org/10.1515/entl-2015-0006>
- 686
- 687 [40] O. Trott, A.J. Olson, J. Comput. Chem. 31 (2010) 455-461.  
688 <https://doi.org/10.1002/jcc.21334>
- 689 [41] A. Okada, K. Sano, K. Nagata, S. Yasumasu, J. Ohtsuka, A. Yamamura, K. Kubota, I.  
690 Iuchi, M. Tanokura, J. Mol. Biol. 402 (2010) 865-878.  
691 <https://doi.org/10.1016/j.jmb.2010.08.023>
- 692 [42] J. Wang, R.M. Wolf, J.W. Caldwell, P.A. Kollman, D.A. Case, J. Comput. Chem. 25  
693 (2004) 1157-1174. <https://doi.org/10.1002/jcc.20035>
- 694 [43] A. Grosdidier, V. Zoete, O. Michielin, Nucleic Acids Res. 39 (2011) W270–W277.  
695 <https://doi.org/10.1093/nar/gkr366>

- 696 [44] B.R. Brooks, C.L. Brooks, A.D. Mackerell, L. Nilsson, R.J. Petrella, B. Roux, Y. Won,  
697 G. Archontis, C. Bartels, S. Boresch, A. Caflisch, L. Caves, Q. Cui, A.R. Dinner, M. Feig,  
698 S. Fischer, J. Gao, M. Hodoseck, W. Im, K. Kuczera, T. Lazaridis, J. Ma, V. Ovchinnikov,  
699 E. Paci, R.W. Pastor, C.B. Post, J.Z. Pu, M. Schaefer, B. Tidor, R.M. Venable, H.L.  
700 Woodcock, X. Wu, W. Yang, D.M. York, M. Karplus, *J. Comput. Chem.* 30 (2009) 1545-  
701 614. <https://doi.org/10.1002/jcc.21287>
- 702 [45] J. Wang, R.M. Wolf, J.W. Caldwell, P.A. Kollman, D.A. Case, *J. Comput. Chem.* 25  
703 (2004) 1157-1174. <https://doi.org/10.1002/jcc.20035>
- 704 [46] P. Cieplak, W.D. Cornell, C. Bayly, P.A. Kollman, *J. Comput. Chem.* 16 (1995) 1357-  
705 1377. <https://doi.org/10.1002/jcc.540161106>
- 706 [47] M.J. Frisch, G.W. Trucks, H.B. Schlegel, G.E. Scuseria, M.A. Robb, J.R. Cheeseman, et  
707 al. Gaussian 09 Revision D.01., Gaussian Inc., Wallingford CT. (2010).
- 708 [48] J.P. Ryckaert, G. Ciccotti, H.J.C. Berendsen, *J. Comput. Phys.* 23 (1977) 327-341.  
709 [https://doi.org/10.1016/0021-9991\(77\)90098-5](https://doi.org/10.1016/0021-9991(77)90098-5)
- 710 [49] T. Darden, D. York, L. Pedersen, *J. Chem. Phys.* 98 (1993) 10089.  
711 <https://doi.org/10.1063/1.464397>
- 712 [50] A.W. Götz, M.J. Williamson, D. Xu, D. Poole, S. Le Grand, R.C. Walker, *J. Chem.*  
713 *Theory Comput.* 8 (2012) 1542-1555. <https://doi.org/10.1021/ct200909j>
- 714 [51] R. Salomon-Ferrer, A.W. Götz, D. Poole, S. Le Grand, R.C. Walker, *J. Chem. Theory*  
715 *Comput.* 9 (2013) 3878-3888. <https://doi.org/10.1021/ct400314y>
- 716 [52] D. Case, R.M. Betz, D.S. Cerutti, T.E. Cheatham III, T.A. Darden, R.E. Duke, et al.,  
717 Amber 2016, University of California, San Francisco. (2016).
- 718 [53] M. Smaïhi, E. Gavilan, J.O. Durand, V.P. Valtchev, *J. Mater. Chem.* 14 (2004) 1347-  
719 1351. <https://doi.org/10.1039/b400521j>
- 720 [54] S. Laurent, E.P. Ng, C. Thirifays, L. Lakiss, G.M. Goupil, S. Mintova, C. Burtea, E.  
721 Oveisi, C. Hébert, M. De Vries, M.M. Motazacker, F. Rezaee, M. Mahmoudi, *Toxicol.*  
722 *Res.* 2 (2013) 270-279. <https://doi.org/10.1039/c3tx50023c>
- 723 [55] J.F. De La Paz, N. Beiza, S. Paredes-Zúñiga, M.S. Hoare, M.L. Allende, *Int. J. Mol. Sci.*  
724 18 (2017) 710. <https://doi.org/10.3390/ijms18040710>
- 725 [56] K. Sano, K. Inohaya, M. Kawaguchi, N. Yoshizaki, I. Iuchi, S. Yasumasu, *FEBS J.* 275  
726 (2008) 5934-5946. <https://doi.org/10.1111/j.1742-4658.2008.06722.x>
- 727 [57] J. Stojan, M. Golicnik, M.T. Froment, F. Estour, P. Masson, *Eur. J. Biochem.* 269 (2002)  
728 1154-1161. <https://doi.org/10.1046/j.1432-1033.2002.02749.x>

- 729 [58] E.H.L. Tan, D.S.K. Govender, R.R. Birge, *J. Am. Chem. Soc.* 1996 (1996) 2752-2753.  
730 <https://doi.org/10.1021/ja953491+>
- 731 [59] S. Lin, Y. Zhao, Z. Ji, J. Ear, C.H. Chang, H. Zhang, C. Low-Kam, K. Yamada, H. Meng,  
732 X. Wang, R. Liu, S. Pokhrel, L. Mädler, R. Damoiseaux, T. Xia, H.A. Godwin, S. Lin,  
733 A.E. Nel, *Small* 9 (2013) 1776-1785. <https://doi.org/10.1002/sml.201202128>
- 734 [60] S. Lin, Y. Zhao, T. Xia, H. Meng, Z. Ji, R. Liu, S. George, S. Xiong, X. Wang, H. Zhang,  
735 S. Pokhrel, L. Mädler, R. Damoiseaux, S. Lin, A.E. Nel, *ACS Nano* 5 (2011) 7284-7295.  
736 <https://doi.org/10.1021/nn202116p>
- 737 [61] K.M. Fukasawa, T. Hata, Y. Ono, J. Hirose, *J. Amino Acids* 2011 (2011) 574816.  
738 <https://doi.org/10.4061/2011/574816>
- 739 [62] F.X. Gomis-Ruth, F. Grams, I. Yiallouros, H. Nar, U. Kusthardt, R. Zwillig, W. Bode,  
740 W. Stocker, *J. Biol. Chem.* 269 (1994) 17111-17117. <https://doi.org/10.2210/pdb1iaa/pdb>
- 741 [63] P. Kumari, P.K. Panda, E. Jha, K. Kumari, K. Nisha, M.A. Mallick, S.K. Verma, *Sci.*  
742 *Rep.* 7 (2017) 16284. <https://doi.org/10.1038/s41598-017-16581-1>
- 743 [64] T.H. Chen, C.C. Lin, P.J. Meng, *J. Hazard. Mater.* 277 (2014) 134-140.  
744 <https://doi.org/10.1016/j.jhazmat.2013.12.030>
- 745 [65] X. Zhu, J. Wang, X. Zhang, Y. Chang, Y. Chen, *Nanotechnol.* 20 (2009) 195103.  
746 <https://doi.org/10.1088/0957-4484/20/19/195103>
- 747 [66] S. Kashiwada, *Environ Health Perspect.* 114 (2006) 1697-1702.  
748 <https://doi.org/10.1289/ehp.9209>
- 749 [67] J. Duan, Y. Yu, H. Shi, L. Tian, C. Guo, P. Huang, X. Zhou, S. Peng, Z. Sun, *PLoS ONE*  
750 18 (2013) e74606. <https://doi.org/10.1371/journal.pone.0074606>
- 751 [68] K.J. Ong, X. Zhao, M.E. Thistle, T.J. McCormack, R.J. Clark, G. Ma, Y. Martinez-Rubi,  
752 B. Simard, J.S.C. Loo, J.G.C. Veinot, G.G. Goss, *Nanotoxicol.* 8 (2014) 295-304.  
753 <https://doi.org/10.3109/17435390.2013.778345>
- 754 [69] K.-T. Kim, R.L. Tanguay, *Environ. Health Toxicol.* 29 (2014) e2014021.  
755 <https://doi.org/10.5620/ehp.e2014021>