Environmental impacts by fragments released from nano-enabled products: A multi-assay, multi-material exploration by "the SUN approach".

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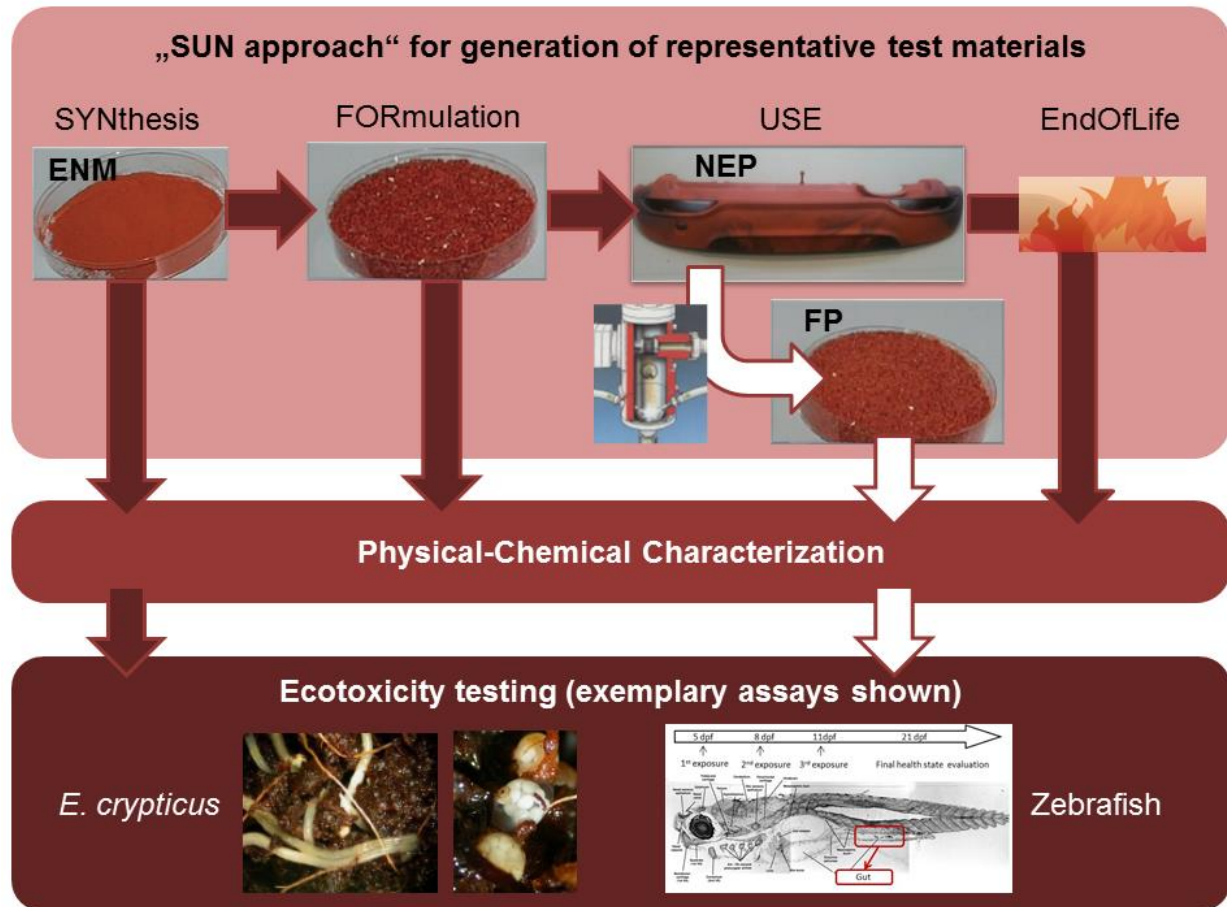38

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42   ABSTRACT

43   Nano-enabled products (NEPs) have numerous outdoor uses in construction, transportation or

44   consumer scenarios, and there is evidence that their fragments are released in the environment at

45   low rates. We hypothesized that the lower surface availability of NEPs fragment reduced their

46   environmental effects with respect to pristine nanomaterials. This hypothesis was explored by

47   testing fragments generated by intentional micronisation ("the SUN approach", Nowack et al,

48   ES&T **50** (2016) 2747). The NEPs were composed of four matrices (epoxy, polyolefin,

49   polyoxymethylene, cement) with up to 5% content of three nanomaterials (carbon nanotubes, iron

50   oxide, organic pigment). Regardless the type of nanomaterial and/or matrix used, it was observed

51   that nanomaterials were only partially exposed at the NEP fragment surface, indicating that mostly

52   the intrinsic and extrinsic properties of the matrix drove the NEP fragment toxicity.

53   Ecotoxicity in multiple assays was done covering relevant media from terrestrial to aquatic,

54   including sewage treatment plant (biological activity), soil worms (*Enchytraeus crypticus*), and

55   fish (zebrafish embryo and larvae, trout cell lines). We designed the studies to explore the possible

56   modulation of ecotoxicity by nanomaterial additives in plastics/polymer/cement, finding none. The

57   results support NEPs grouping by the *matrix* material regarding ecotoxicological effect during the

58   use phase. Further, control results on nanomaterial-free polymer fragments representing

59   microplastic had no significant adverse effects up to the highest concentration tested.

Introduction

Nanocomposites are a dominant class of nano-enabled products (NEPs), and especially those with a "durable" matrix, such as rubber, cements, plastics and coatings, are often in outdoor use.[1, 2] Processes such as wear, tear, manufacturing and shredding (mechanical processes) and photolysis, hydrolysis, aggressive use environments, thermal decomposition (chemical processes) contribute to the degradation of nanocomposites[3, 4] and induce releases into the environment at various time scales along the lifecycle.[5, 6] Pristine Engineered Nanomaterials (ENM) can induce adverse effects on environmental species in aquatic, sediment and soil compartments.[7] This is relevant for emission scenarios such as spills during the ENM synthesis phase of the lifecycle, but not for the NEP use phase. For NEPs made of chemically resilient nanocomposite materials, the only relevant case for NEP outdoor use, nanocomposite fragments of the NEPs are the dominant physical-chemical form of environmental releases during the use phase.[8-10] However, to the best of our knowledge no study has ever reported on environmental effects by NEP fragments. This is a serious gap in environmental risk assessment of nanomaterials. NEP fragments differ from pristine ENM in key physical-chemical properties such as composition, size, surface chemistry.[11] Their environmental release rates on the order of g/year[12] are too low to measure their properties and environmental effects directly for degrading NEPs, which in a controlled experiment requires exposure of various organisms up to high doses. To meet the gap, Nowack et al. proposed "the SUN approach": NEPs were micronized to generate fragmented products (FP) in kg quantities for characterization of properties and effects.[13] Our hypothesis for this study, based on limited reports on mammalian studies, is that FP will have lower environmental impacts compared to pristine ENMs due to the lower surface availability of ENM contained in FPs.

82   Here we deliver an exploration of ecological effects of multiple NEP-FPs by multiple ecotoxicity

83   assays, supported by an extensive physical-chemical characterization of the properties of the FPs

84   generated by "the SUN approach" (**Figure 1**). Specifically, we compare different ENM in the same

85   matrix, and additionally explore one ENM in different matrices. We selected a balanced choice of

86   NEPs: this includes highly studied nanomaterials, of which we chose a family of NEPs with

87   multiple walled carbon nanotubes (CNTs) in polyolefins for conductive functionality / in cement

88   for electromagnetic shielding functionality / in epoxy for lightweight functionality in automotive

89   / construction / airplane applications, respectively.[14] Additionally, we selected a family of NEP

90   made from less well studied ENM from commercially well-established particulate materials,

91   which are now identified as nanomaterials in regulatory terms, specifically transparent halogen-

92   organic and inorganic pigments for automotive parts and coatings.[2] These constitute the large

93   volume reports in the French nanomaterial registry with above 100 ton/y nanoform production or

94   import in France, far above CNT.[15] Due to the history of widespread use of nanoforms of these

95   materials in coating, ink, plastic components of consumer products, a historical and ongoing

96   dispersive emission of NEP fragments into the environment has to be anticipated. We previously

97   verified that the ENMs are present in and on the FPs, and that even after extensive (365 MJ/m²)

98   UV radiation less than 6 ppb of the ENM content migrates or leaches from the weathered FPs

99   (WFPs),[16] such that testing of environmental effects can be approximated by testing FP only. This

100   reasoning is specific to environmentally persistent matrices such as polyolefins or cements, as

101   tested here. Our study is the first to enable their environmental risk assessment with full

102   consideration of the nanostructures induced by the ENM content.

103

Figure 1. How can we assess environmental impacts from nano-enabled products? Representative test materials across all lifecycle phases are needed: The pristine ENM powder (here an organic pigment), represents spills during nanomaterial synthesis (SYN), and the pigment-in-polymer masterbatch granules represent spills during formulation (FOR). But the nano-enabled product (NEP), a 1.4m-wide car bumper in one of our case studies, releases fragments only at very slow rates.[12] Specifically to the "SUN approach", highlighted by white arrows, mechanical micronisation generates fragmented products (FP) to represent the real-world NEP releases with greatly increased surface and surface-accessible ENM.[13] Photographs illustrate test materials in 10 cm petri dishes (no ashes remain after incineration in the specific case). Materials representing all lifecycle phases were compared regarding their physical-chemical properties. The most relevant

115   materials were compared regarding their ecotoxicity by multiple assays, of which the lower panels

116   show: Enchytraeidss (soil): *Enchytraeus crypticus.* in culture soil and a detail of the cocoons with

117   eggs inside, and the Zebrafish (aquatic): *Danio rerio* development assay using water-

118   accommodated fractions. We assessed six NEPs and their ENM-free control matrices in a

119   systematic variation of ENMs and of matrices (see Figure 3).

120

121   **Materials and Methods**

122   *ENM, synthesis phase and NEP, formulation phase:* The test panel consisted of three ENM and

123   four matrices. The same nanomaterials have been used previously for extensive human toxicity

124   testing.[17, 18] "CNT" designates multi-wall CNT, CAS 308068-56-6, and is the identical grade as

125   the batch distributed as NM400 in the OECD sponsorship program. "OrgPig" designates di-keto-

126   pyrrolo-pyrrole, CAS 84632-65-5. "Fe_2O_3" designates hematite, CAS 1309-37-1 (**Table SI_1**).

127   All ENM were formulated into polyolefins (high density polyethylene, HDPE, and polypropylene,

128   PP). Additionally to PP, the CNT was integrated in three complementary matrices:

129   Polyoxymethylene ("POM") nanocomposite identical to the FP used in a human tox study[19],

130   cement ("cement") nanocomposite identical to the FP used in a human tox study[19], epoxy

131   ("epoxy") nanocomposite identical to those applied in the NanoRelease interlab testing (**Table**

132   **SI_2**).[20] Comparative testing of environmental weathering releases was performed on the identical

133   nanocomposites, finding release rates that increase across five orders of magnitude in the order

134   polyolefin – POM – epoxy – cement.[21]

135   *NEP fragmentation, use phase:* We implemented "the SUN approach" (**Figure 1**) and performed

136   cryo-milling for all NEPs as listed in **Table SI_2**. In short, the OrgPig_PP, Fe2O3_PE,

137   CNT_epoxy, CNT_PP nanocomposite materials and the PP, PE, epoxy nano-free controls were

138   frozen to cryogenic temperatures (at -193°C, liquid N_2) in order to maximize brittleness.[13]

139   Additionally, in order to remain comparable to earlier human tox investigations on sanding dusts,

140   limited tests were performed on CNT_POM and CNT_cement fragments and their POM, cement

141   control fragments. These were generated by sanding (1.8 m/s, grit 80, 10 N) of the same NEP batch

142   that was previously investigated by rat in vivo studies.[19] See SI for fragmentation details.

143   *FP size analysis*: Size distributions have been previously reported.[13] and are included by the

144   median diameters here. In short, FP were dispersed in a concentration of 1 g/l by sonication in

145   water containing 0.5 g/l SDS (Sodiumdodecylsulfate), and were characterized by laser diffraction

146   (Malvern Mastersizer 3000).

147   *FP composition analysis:* The content of ENM in several FP was not known previously, and was

148   investigated here by ICP-MS for the inorganic ENM and by combustion for the organic pigment.

149   In the case of CNT, the detection was based on the content of Al and Co in the used CNT which

150   remains from their production process. The Al and Co content is known from earlier release

151   investigations on the same batch of CNT_epoxy.[20]

152   *FP surface chemistry analysis (X-ray photoelectron spectroscopy, XPS + hydrophobicity):*

153   Surface composition was investigated by XPS to determine element concentrations and content of

154   ENM at the fragments' surface. Phi XPS 5500 with 300 W monochromatic Al-K alpha radiation,

155   pass energy for surveys 117 eV. The XPS penetration depth of 10 nm is around three orders of

156   magnitude smaller than the FP diameters. Identical analysis was performed on pristine ENM. To

157   evaluate the hydrophobicity, contact angle measurements were performed by placing a sessile drop

158 of water onto a round pressed plate of ENM or FP respectively with a diameter of 3 cm. Triplicates

159 had standard deviations between 2° and 6°.

160 *FP "biologically accessible" fraction of ENM:* Biologically accessible ENM are defined as those

161 that are in contact to the surrounding liquids, and are considered as the fraction that can potentially

162 elicit an ENM-specific effect. FP were dispersed in 1% HCl and shaken for up to 22h. At each

163 sampling, the suspension was left to settle for 10 minutes, then sampling an aliquot for ICP-MS to

164 determine Al, Co, and Fe concentrations. The HCl concentration chosen is enough to keep traces

165 of these elements in a truly dissolved state, while it is not expected to significantly corrode the

166 organic matrix. The method was adapted from Schlagenhauf et al.[22]

167 *FP "dispersability" in liquid media:* FP were dispersed by testing both bath sonication (30 min,

168 power 35W) and probe sonication (15 min, power 100W) into different media, specifically:

169 distilled water (DW) + sodium dodecyl sulfate (SDS) at 20 mM; DW + Suwannee River Humic

170 acid (SRHA) at 0.2% w/w; Artificial freshwater (AFW, adhered to OECD 203 protocol[23]) + SRHA

171 at 0.2% w/w. As expected from polyolefin density, flotation of FPs on the water surface was

172 observed. The results were confirmed within the concentrations range from 10 mg/l to 10 g/l by

173 means of Dynamic Light Scattering (DLS, multi-angle Nicomp ZLS Z3000 Particle Sizing

174 System), Centrifugal Separation Analysis (CSA, LUMiSizer 651, L.U.M. GmbH) and number-

175 based Transient Resistive Pulse Sensing (TRPS, izon qnano).

176 ***Ecotoxicological testing of fragmented material***

177 Testing aimed to cover from aquatic to terrestrial exposure, using a suite of standard organisms

178 and associated endpoints as these are among the current mandatory framework. As shown in Figure

179 1, each phase of the lifecycle may result in environmental emission by spills during SYN and FOR,

180 or by slow micronisation and degradation during USE or finally by landfilling of remaining solids

181   after incineration (polymers) or after destruction (cements), impacting sewage treatment plants,

182   soils and other compartments.

183   *Sewage sludge treatment plant function*

184   Effects on the biological function of a sewage treatment plant (STP) were investigated based on

185   the OECD Test Guideline 303A (OECD, 2001). A lab-scale STP (behrotest® Laborkläranlage

186   KLD 4N, Germany) with a denitrification and nitrification reactor and a secondary clarifier was

187   used. The pristine Fe_2O_3 and the fragmented $Fe_2O_3\_PE$ were added continuously over 10 days with

188   the synthetic sewage into the denitrification reactor of the model STP. Effects on the biological

189   function of the STP were assessed by measurements of the elimination rate of dissolved organic

190   carbon and the determination of nitrate, nitrite and ammonia in the effluent of the sewage treatment

191   plant, representing the denitrification and nitrification processes. Treatments included from 0.04

192   mg/L (environmental relevant) to 1.0 mg/L (worst case scenario).

193   *Microbial function - Short and long-term effects on the soil microflora*

194   The nitrite content was determined using the short-term potential ammonium oxidation test as

195   recommended by ISO Guideline 15685 (2012). The effect on ammonia oxidizing bacteria was

196   determined 24 h and 28 days after application of the nanomaterials (10 and 1000 mg/kg dry matter

197   soil) into reference soil.

198   *Long term effects via sewage sludge spread into agricultural soil*

199   After 10 days continuous addition of the nanomaterial into the STP, the sludge was dewatered and

200   added to the reference soil in accordance with the German sewage sludge ordinance, which states

201   5 tons per hectare over 3 years can be spread on agricultural areas. 1.67 g of dry matter sludge

202   were introduced into the soil, under the assumption of a soil depth of 20 cm and soil density of 1.5

203   g/m^3, corresponding to test concentrations of 0.4 and 10 mg/kg dry matter soil. The soil was

204 incubated at 20°C in an incubation chamber in the dark. The long term effect of the nanomaterials

205 added via sewage sludge on the ammonia oxidizing bacteria was investigated after 30, 60, 100 and

206 140 days of incubation.

207 *Enchytraeid Reproduction Test (ERT):*

208 Cultures of test species *E. crypticus* (Oligochaeta: Enchytraeidae, **Figure 1**) were kept in agar

209 plates for several years.[24] The standard LUFA 2.2 natural soil (Speyer, Germany) was used.

210 Spiking followed the recommendations for nanomaterials[25, 26] The standard guideline (ISO 2004;

211 OECD 2004) was followed, but instead of using adults, 10 synchronized age (17-18 days)

212 organisms were used. Exposure concentrations ranged from 0-3200 mg/kg soil dry weight for both

213 the ENM and the FP, i.e. for the FP exposure there was a corresponding lower % of ENM. Test

214 ran at 20 ºC and 16:8h photoperiod during a period of 28 days. Endpoints assessed included

215 survival (number of adults) and reproduction (number of juveniles).

216 *Hatching, survival and growth test on zebrafish embryos and larvae*

217 Zebrafish embryos and larvae were used to assess the hazard potential in a model organism

218 frequently used in nanomaterial safety studies (**Figure 1**). Motivated by FP hydrophobicity and

219 lack of dispersability, sample preparation for aquatic compartment testing (see details in SI)

220 targeted the water-accommodated fraction as recommended by an OECD workshop.[27] Healthy

221 embryos at the same developmental stage (2 hour post fertilization, hpf) were selected and placed

222 in 96-well plates with one embryo per well.[28, 29] One hundred microliters of CNT, or FP of CNT-

223 cement and CNT-POM was added to the wells at 4 hpf. To achieve robust statistical calculation,

224 five replicate trials were carried out, each using 12 embryos. Observations of adverse biological

225 outcomes, including hatching interference, phenotypic abnormalities and mortality (necrosis of the

226 embryos), were carried out every 24 h for five consecutive days.

227 Using our previously developed pulse exposure procedure,[30] we further investigated the hazard

228 potential of the CNT and FP from CNT-nanocomposite in zebrafish larvae. As shown in **Figure**

229 **SI_5**, the pulse exposure was conducted using groups of 30 growth-synchronized larvae at 5, 8

230 and 11 days post fertilization (dpf). The exposure was performed in Petri dishes for 6 h each time

231 by immersing the larvae in 3 mL of the suspension of CNT or FP. The larvae were maintained in

232 standard aquarium tanks for further observation of development and survival until 21 dpf. The

233 overall health status of the larvae was assessed at 14 dpf based on gross morphology, body length

234 and weight, and number of calcified vertebrae. To assess morphology, larvae were anesthetized in

235 0.02% tricaine and embedded in low-melt agarose gel. This allowed positioning to obtain lateral

236 views to assess morphology, body length, and fin structure.

237 *Cytotoxicity on fish cell lines*

238 Two fish derived cell lines were used: RTL-W1[31] and CLC.[32, 33] For details see SI. Cells were

239 exposed for 24 h to appropriate concentrations of the original NM or of the FP (0.78 to 100 µg/mL)

240 using 96 well plates. Two independent experiments were carried out, with treatments applied in

241 triplicate in each experiment, Fresh suspensions were prepared for each experiment in milliQ water

242 at 10 mg/ml by sonication for 20 min in an ice-water bath using a probe sonicator (Vibra cell

243 VCX130, Sonics & Materials Inc., Newtown, CT, USA) (2 mm microtip, 80% of amplitude

244 continuous mode). 75 µl were then mixed with 25 µl of bovine serum albumin (BSA, 80 mg/l)

245 solution, added to 7.4 ml of culture medium, and sonicated for 10 min in a bath sonicator (S 40 H

246 Elmasonic, Elma, Germany). Cells were exposed to ½ serial dilutions of this suspension in culture

247 medium. Cytotoxicity was measured by three different assays.[34, 35]

248

Results & Discussion

Enabled by the "SUN approach" (**Figure 1**), we followed nanomaterials along their lifecycle, both by physical-chemical and by ecological methods. The pristine ENM, representing the SYN (as SYNthesized) phase, and the fragmented product, representing releases during the USE (in USE) phase, were characterized in terms of size, composition, and surface properties **(Figure 2** and **Table SI_3**). Additionally, selected properties were available from our previous publications or from gap-filling original data for the FOR (FORmulation) and EOL (End Of Life) phases. **Figure 3** thus constitutes a "lifelog" of nanomaterials from cradle to grave.

Our specific understanding of lifecycle phases differentiates:
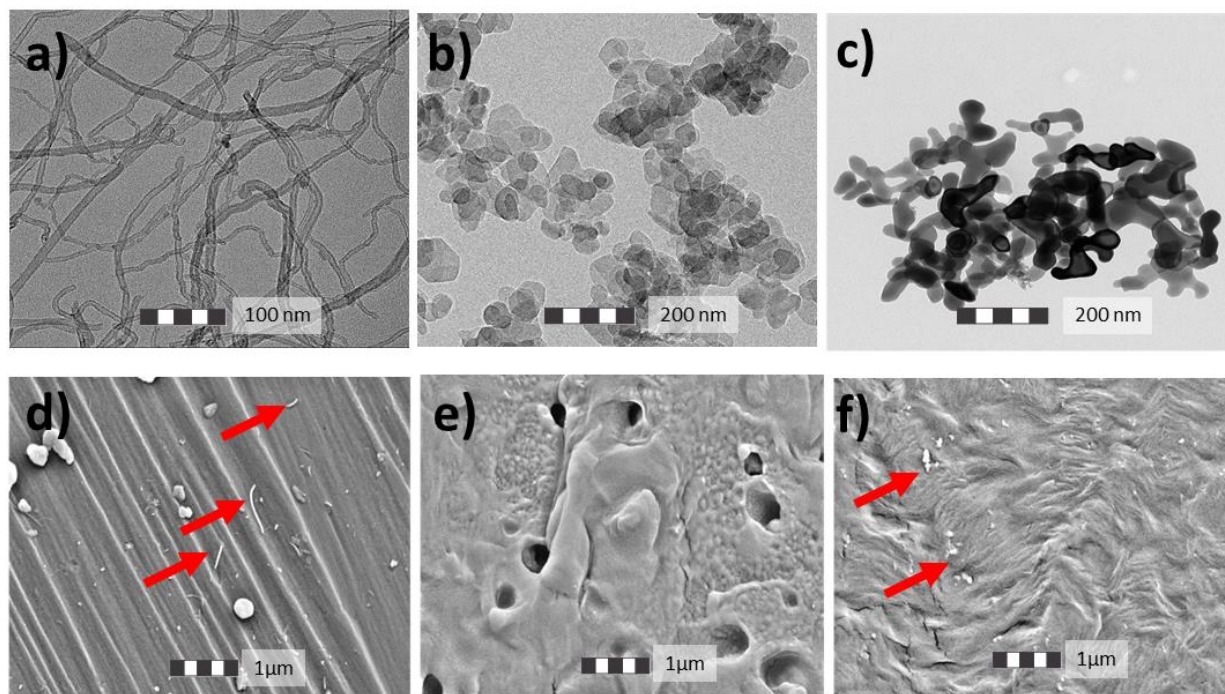
- SYN = synthesis of the pristine ENM, typically as powder. Industrial process by chemical operations. Risk assessment prioritizes occupational human aerosol exposure and environmental emissions by spills and waste streams.

- FOR = formulation of the ENM as an additive in a product matrix. Industrial manufacturing process that produces a nano-enabled product (NEP). Our case studies used ENM masterbatch (for thermoplastic polymers PP, PE, POM), or ENM suspensions in liquid precursors (epoxy polymer and cement).

- USE = consumer use of the NEP. Risk assessment needs to consider various product-specific scenarios with durations between days and decades. For our specific case studies, environmental emissions of FP by weathering, wear and tear during outdoor use take priority.

269   • EOL = end of life of the NEP by recycling or disposal by incineration or landfill. For our

270   specific case studies Table 3 reports the results measured for incineration of epoxy[36] and

271   PP[37, 38] or landfill of cement FP.

272   Previously we reported the FP size as a basic descriptor.[13] Here we go further to characterize the

273   composition and surface chemistry by several descriptors that are most relevant for nanoscale- or

274   micro-particles. The actual ENM content in FP (USE phase) was found to be minimally above the

275   ENM content of the FOR phase, resulting in a small kink in the "nanomaterial lifelog" between

276   FOR and USE phase (**Figure 3**, panel "composition"). The increase in ENM content correlates

277   roughly with the brittleness of the matrix, as it is least with PP and PE, higher with epoxy and

278   cement. Transmission Electron Microscopy (TEM) scans of iron oxide pigments extracted from

279    $Fe_2O_3\_PE$ fragments showed no significant change of nanomaterial structure due to the

280   fragmentation process (ultramicrotomy data not shown). As the changes of composition are

281   minimal between FOR phase and USE phase (**Figure 3** and **Table SI_3**), whereas only the USE

282   phase potentially releases fragmented product by diffuse emissions into the environment, we

283   focused testing on the SYN and USE phases. Sizes after sieving were above the inhalable range

284   for all cryo-milled FP (**Figure 3**, panel "size"), in accord between laser diffraction, suspension-

285   based analytics and microscopy. [13] The amounts of the sieved fragments were below 0.5% of

286   cryomilling output, and were provided for ecotoxicological testing. Additionally, FPs produced by

287   sanding CNT_POM_FP were consistent in size with the other FP generated by cryo-milling, as

288   the CNT_POM_FP had < 1 % passed through a 2.7μm filter, and < 60 ppm were below 0.1 μm

289   (both w/ and w/o CNT); 3 % of the CNT_cement_FP passed through a 2.7 μm filter, and 100 ppm

290   were below 0.1 μm.[19] High resolution images reveal protrusions of the inorganic Fe_2O_3 pigment

291   and CNTs at the surface of corresponding fragments (red arrows in **Figure 2**), whereas the lower

292 concentration and lower electron density of OrgPig caution against an interpretation of absence of

293 protrusions from SEM results.

294

295 **Figure 2** SEM scans to characterize surface structures of a)-c) ENM (representative for lifecycle

296 SYN phase) and d)-f) fragments of NEP containing the same ENM (FP, representative for lifecycle

297 USE phase). a) CNT; b) halogen-organic diketopyrrolopyrrole pigment red 254 (OrgPig); c) Fe_2O_3

298 pigment red 101; d) FP of nanocomposite CNT_epoxy; e) FP of nanocomposite OrgPig_PP; f) FP

299 of nanocomposite Fe_2O_3_PE. Red arrows highlight protrusions of ENM on FP surfaces. See

300 **Figure SI_1** for pure matrix control fragments and whole-particle scans.

301

302 "Protrusions"[22, 39] of ENM on the surface of fragments were investigated by multiple approaches:

303 surface chemistry (XPS, elements and C(1s) line shift identification), hydrophobicity (sessile water

304 drop contact angle), morphology Scanning Electron Microscopy (SEM). As hypothesized in the

305   introduction, the surface of all investigated FP materials is mainly composed of carbon (**Table**

306   **SI_3** and **Figure 3**, panel "surface chemistry"). In case of the epoxy-based materials, further

307   elements such as carbon and nitrogen are present in higher contents as well. The XPS

308   quantification of different species by C(1s) line shift (**Figure SI_2**) analysis finds a *surface* content

309   of CNT in CNT_PP_FP of 4.3%, which is slightly increased above the *bulk* content determined by

310   ICP-MS of 2.7%. Also the Al and Co acid leaching analysis showed that the CNT are accessible

311   to the liquid phase on the surface of the CNT_PP fragments, as these released Co and Al (**Figure**

312   **SI_3**). The biologically accessible concentration of Al was deduced to be 27.0 $\mu g/m^2$ on FP with

313   CNT and 7.6 $\mu g/m^2$ without; the respective values for Co were 2.3 $\mu g/m^2$ and 0.1 $\mu g/m^2$ (**Table**

314   **SI_4** and calculation there). Correcting for the control background, and using the diameter, density

315   and metal content in this specific CNT grade, we deduce from the acid leaching that 6.2 % of the

316   surface area provide contact to CNT. Considering the approximations made, this value is in

317   excellent accord with the XPS result and predicts that protrusions of ENM modulate the surface

318   chemistry of FP, but do not dominate it. For CNT in epoxy and for Fe_2O_3 in PP however, leaching

319   results are convoluted by contaminations of the matrix with the target analytes (Al, Fe) (results in

320   the SI). Further work will aim at determining whether these metals are embedded in the NEP or if

321   the NEP fragmentation process contaminates the FP. The same methods of XPS line shift, tracer

322   ion release and TEM analysis, found significant protrusions on brittle CNT_epoxy[22] or

323   CNT_cement,[39] but low prevalence in tough materials such as CNT_POM or CNT_PP.[39, 40] This

324   is in accord with our results.

325   For organic pigment, XPS line shape analysis identifies an enrichment at the FP surface with up

326   to 9% on the FP surface compared to 0.21% within the FP bulk (**Figure SI_2**), but may be less

327   reliable because only CNTs emit C(1s) photoelectrons at a distinct energy shift, whereas the

328 organic pigment signal overlaps with the PP matrix signal. The water contact angle was considered

329 as simple measure of surface-mediated (extrinsic) interactions. The contact angle results highlight

330 that the FP surface hydrophobicity is clearly dominated by the matrix, not by the ENM (**Figure 3**

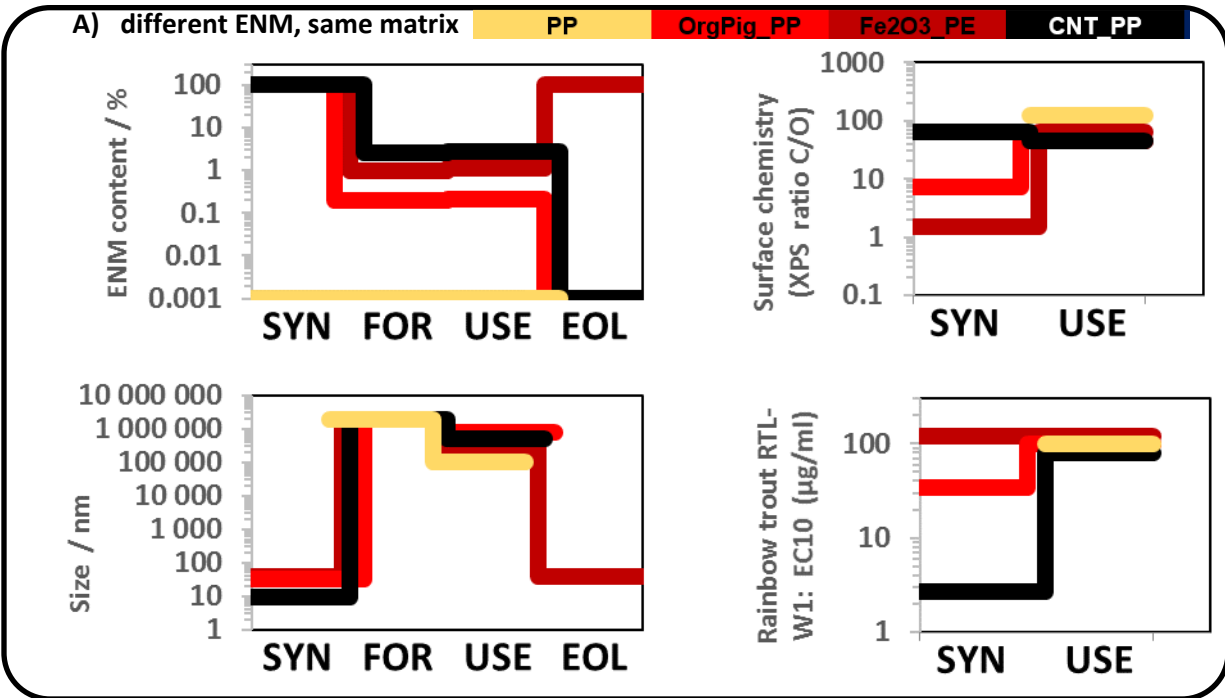331 panel "surface chemistry" and **Figure SI_7).**

332 Surface chemistry controls nano-bio-interactions, but also impacts fate and transport. In

333 biologically irrelevant media such as a 1:1 acetone:water mixture, the FP can be solubilized and

334 dispersed, but in environmentally relevant media such as artificial fresh water with or without

335 natural organic matter the FP remain agglomerated and tend to float (especially the polyolefins PE

336 and PP). In accordance to the results from laser diffraction reported earlier,[13] no significant fraction

337 below 1μm diameter was detected by measurements with the nanospecific methods of DLS and

338 CSA, using either SDS or SRHA as dispersants. By the nanospecific counting method of TRPS,

339 all FP showed the presence of fragments in a size range between 200 and 800 nm, but the number-

340 based concentrations ranged between 10^6 and 10^7 #/ml for 10g/l FP suspensions (**Table SI_5**).

341 This converts to a low ppm level mass content of fragments with diameters below 1μm.

342 In a very condensed presentation of size, surface chemistry and composition, **Figure 3** summarizes

343 the dramatic changes of physical-chemical properties along the NEP lifecycle.
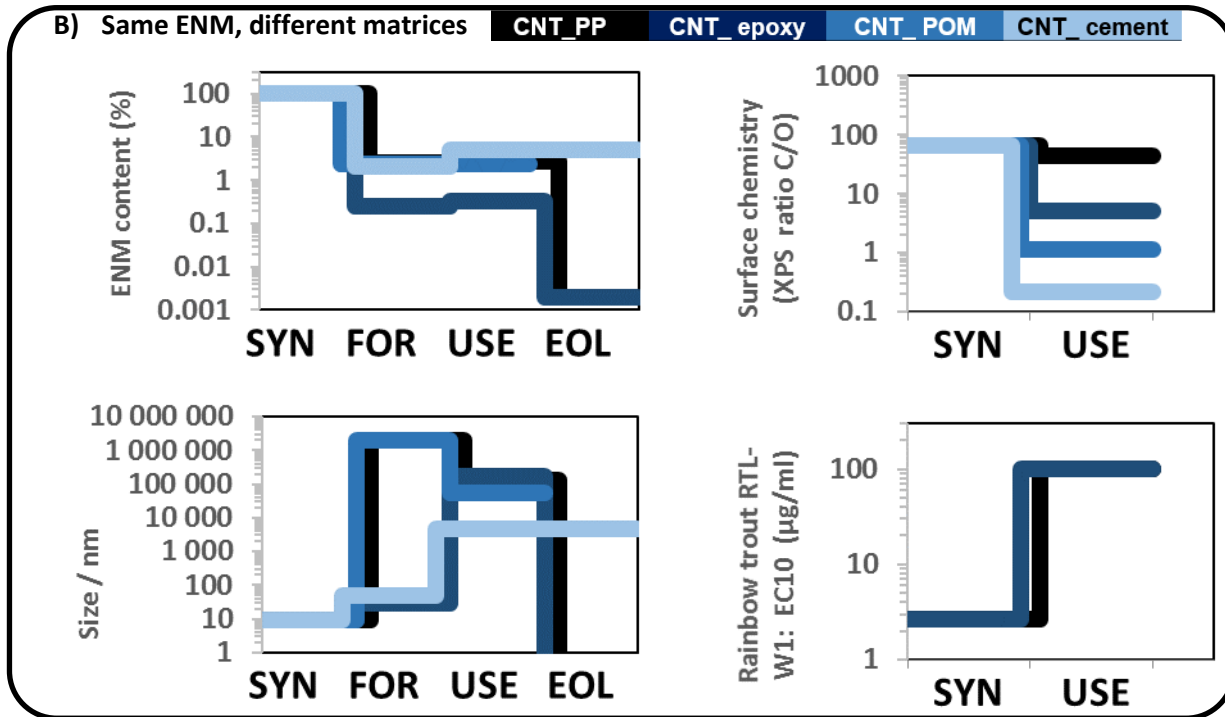
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350 **Figure 3** Tracking nano-enabled products along their lifecycle stages SYNthesis, FORmulation,

351 USE, End-Of-Life: A) different ENM, same matrix; B) same ENM, different matrices. Panels

352 summarize the key descriptors of Size (median diameter), Composition (ENM content), Surface

353 Chemistry (C/O ratio) and Ecotoxicity (represented by our most sensitive assay, rainbow trout

354 RTL-W1). Ecotoxicity EC10 values are lower limits for the USE phase. Color code of NEPs is

355 indicated above the figure and matches Table 1. See beginning of results section for the scenario

356 assumptions on SYN-FOR-USE-EOL phases.
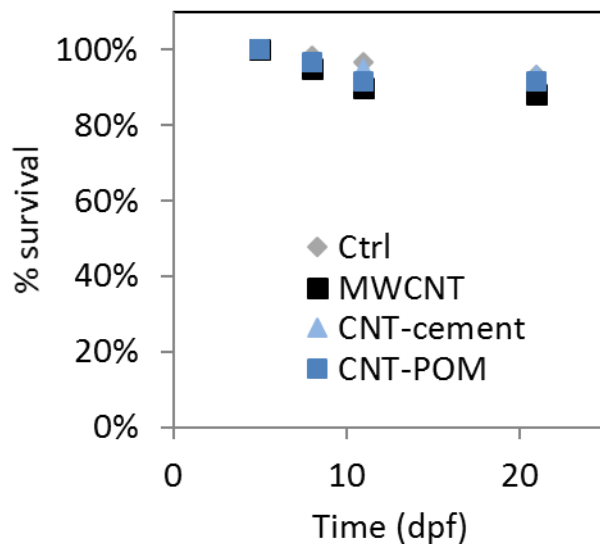
357

358

359 *Ecotoxicity effect characterisation*

360 No hatching or survival rate of zebrafish *embryos* were affected by CNT or the FP (**Figure SI_5**),

361 likely due to low heavy metal content from the CNT and little to no penetration through the chorion

362 of the embryos. With the pore size of the chorion being at the range of 200~500 nm, it was

363 anticipated that there was little to none penetration of CNT and FP into the chorionic space. As

364 shown in **Figure 4**, both CNT and CNT-composite showed little to no toxicity as reflected by the

365 survival and growth of zebrafish *larvae* subject to aqueous exposure. Even though in the zebrafish

366 assay CNT were protruding and entirely biologically accessible after shaking in solution or

367 sonication from CNT-cement_FP (**Figure SI_4**), whereas there was no protrusion from CNT-

368 POM_FP, the composites showed no adverse effects on zebrafish embryo hatching as well as

369 survival and growth of zebrafish larvae. These results show that CNT nanocomposites do not

370 appear to have negative impact on zebrafish. Zebrafish embryo hatching was previously

371 demonstrated to be affected by heavy metal ions, such as Zn^{2+}, Cu^{2+}, Ni^{2+} and Cr^{3+}.[28] The lack of

372 hatching interference observed here suggests that the heavy metal leaching, observed only in HCl

373 to reach ppb levels during 22h (**Figure SI_3**), remained low during the test but may contribute to

374 long-term effects in the environment. One may differentiate that only free ENM are biologically
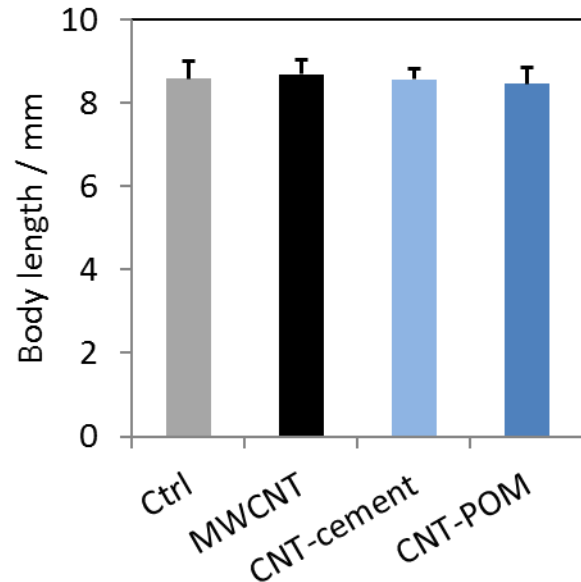
375   *available* in the sense that they can be taken up, whereas ENM protrusion on a larger fragment are

376   only *accessible* in the sense that they interact with e.g. the chorion or cell membrane.

377

378   **A)**

379

380   **Figure 4**. Aquatic compartment screening on zebrafish *larvae*: Both A) survival (standard

381   deviation of N=30 replicates were determined for each time point and were below 5% for all) and

382   B) body length at day 14 showed no statistically significant differences between the control and

383   exposure of CNT and FP. Pulse exposure was performed on 5, 8 and 11 dpf (day post fertilization)

384   (see scheme in **Figure 1**). See **Figure SI_5** for zebrafish *embryo* development and growth results.

385

386

387   Results of the toxicity of the original material and of the FP (SYN and USE phases, respectively)

388   on RTL-W1 cells, represented as the most sensitive assay, are summarized by **Figure 3** and **Figure**

389   **SI_6**. In both fish cell lines, RTL-W1 and CLC, the original NM (SYN phase) caused decreased

390   cell viability. The lowest EC50 was detected in RTL-W1 cells by means of the 5-

391   carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM) assay, 23.56±5.0 µg/ml. The

392   toxicity in the CLC cells was below EC50 hence this was not calculated. The exposure of cells to

393   the FP (USE phase) did not cause any toxicity, even at the maximal concentration used, 100 µg

394   FP/ml. However, 100 µg FP/ml contains nanomaterials at <5 µg/ml. In addition to this low

395   concentration of NM, the low density of FPs causes their flotation and hence the contact with the

396   cells was minimal. Other alternatives, as cells in suspension instead of attached to the bottom of

397   the plates or an inverted culture should be tested in future to enhance the sensitivity of the assay,

398   as demonstrated for PP-based incineration-FPs.[41, 42]

399   On ammonia oxidizing bacteria, the reference products and the pristine nanomaterials Fe_2O_3 Red

400   101, Organic Pigment Red 254 and CNT showed no effects at test initiation and after 28 days. A

401   comparable result was found for the fragmented products Fe_2O_3_PE, OrgPig_PP, and CNT_epoxy,

402   although for Fe_2O_3_PE and CNT_epoxy at 1000 mg/kg dry matter soil inhibitory effects of 23%

403   and 38%, respectively, were determined at test start. However, this effect could not be confirmed

404   after 28 days of incubation. The results are presented in **Table SI_4**. Thus, after 28 days, EC50

405   values were above 1000 mg/kg dry matter soil for all products tested. No impact occurred on the

406   biological function/microbial activity of the sewage sludge neither on the elimination of dissolved

407   organic carbon nor on the denitrification and nitrification processes. There were no effects due to

408   the pristine and fragmented products observed on the activity of the ammonia oxidizing bacteria

409   over 140 days after application of the nanomaterials via sewage sludge into soil.

410   Results for *E. crypticus* showed no effects on survival for any of the FP (**Figure 3, Figures SI_7**

411   and **SI_8**). Effects in terms of reproduction showed increased sensitivity, with the pure NM (SYN

412   phase) having different EC10, specifically OrgPig had a measurable effect (EC10=2009mg/kg),

413   but not Fe_2O_3 or CNT. All FP, both NM-containing FP and the pure polymer FP (pure PE, pure

epoxy, pure PP) caused no effect in the tested range (0-3200mg/kg). Such results are not unexpected given the low degradability of the tested matrix or the low fraction of NM embedded in the matrix. The added characterization of the NEP further confirms the hypothesis. Of note, for OrgPig_FP, a 10% reduction in reproduction occurs for 800 mg/kg although no effects occurred at higher concentrations. A non-monotone dose response has been reported before for Ag[26] and Ni[43]. This could be related to the potentially reduced agglomeration at lower concentrations hence higher exposure and effects. However, we do not have evidence to support this hypothesis for the FP with or without embedded ENMs.

Figures 3 and **Table 1** summarize the results of our explorations: At the ENM concentrations tested, the NEP matrix properties dominated the intrinsic and extrinsic FP properties, including their ecotoxicity in a wide range of assays covering sludge, soil, and fish. Two orthogonal comparisons help to identify the mechanisms:

a. The surface chemistry, hydrophobicity and the size of fragments assimilate in the USE phase of different ENM in the same matrix. (**Figure 3a**, **Figure SI_7**)

b. Conversely, for one type of ENM in NEPs with different matrices, the surface chemistry and size of USE phase fragments diversifies. (**Figure 3b**)

432   **Table 1:** Effects of USE phase Fragmented Product (FP) tested in various ecotoxicity assays.[1] No

433   FP elicits a significant effect. NT: Not Tested.

	PP	OrgPig_ PP	Fe_2O_3_ PE	CNT_ PP	CNT_ epoxy	CNT_ POM	CNT_ cement
RTL-W1 EC10	> 100 µg/mL	> 100 µg/mL	> 100 µg/mL	> 100 µg/mL	> 100 µg/mL	NT	NT
CLC EC10	> 100 µg/mL	> 100 µg/mL	> 100 µg/mL	> 100 µg/mL	> 100 µg/mL	NT	NT
Soil microflora EC10	> 1000 mg/kg	> 1000 mg/kg	> 1000 mg/kg	NT	> 1000 mg/kg	NT	NT
STP biology EC10	NT	NT	> 6000 mg/kg	NT	NT	NT	NT
E. crypticus EC10 repro.	> 3200 mg/kg	>3200 mg/kg	> 3200 mg/kg	> 3200 mg/kg	> 3200 mg/kg	NT	NT
ZF embryo NOAEL	NT	NT	NT	NT	NT	> 5 mg/ embryo	> 5 mg/ embryo
ZF larvae NOAEL	NT	NT	NT	NT	NT	> 50 mg/mL	> 50 mg/ mL

434

435

[1] RTL-W1, fibroblast from biliary ducts of rainbow trout. CLC, macrophage cell line from carp,. *Enchytraeus crypticus* survival is not affected, results reported are EC10 of reproduction. Soil microflora, short-term potential ammonium oxidation test based on ISO Guideline 15685. STP biology, biological function of a sewage treatment plant (STP) based on OECD Guideline 303A. ZF embryo, zebrafish embryos exposed to the water-accomodated fraction of the FP mass indicated, assessing hatching percentage and survival rate vs. control embryos. ZF larvae, zebrafish larvae exposed repeatedly to the water-accomodated fraction of the FP concentration indicated, assessing body length and survival rate vs. control larvae.

436 Both categories of lifecycle-induced ENM transformation, assimilation of properties and

437 diversification of properties by product formulation and fragmentation, have been predicted earlier

438 by Mitrano et al.[10] Here we studied specific examples with polymeric and cementitious matrices

439 with organic, carbonaceous and inorganic ENM, and explore their implications for environmental

440 hazards. In both transformation categories, we find that the physical-chemical properties of the

441 bulk matrix predominantly determine the physical-chemical properties of the fragments released

442 during USE, with little modulation by the ENM that was embedded in the NEP. With the specific

443 ENM and matrices, the ecotoxicity of fragments released during the lifecycle of NEPs is not

444 affected by the ENM.

445 However, the validity of each individual ecotoxicity assay –that were not developed for FP testing–

446 is challenged by one or other FP property. For some materials (low density of polyolefins)

447 floatation limits the delivered dose; this is, however, no issue for FP testing of epoxy, POM and

448 cement, which are denser than water. Further, the size range of the tested fragments is larger than

449 generally adequate for the tests and species. To reduce this uncertainty, we sieved away 99.5% of

450 the larger particles so that exposure was targeted to smallest sizes. And yet, for organisms like *E.*

451 *crypticus*, the FP sample contains fragments that can be larger than the organisms themselves. A

452 detailed discussion can be found in a recent opinion paper.[44] Enchytraeids and other oligochaetes

453 are exposed via soil ingestion and skin adsorption, hence in this case risk is reduced or absent via

454 exposure. Also the absence of adverse effects on the microbial soil compartment are at least

455 partially due to the reduction of exposure, because the thermoplastic matrices have low

456 degradation and diffusion.[9, 45] No effects on soil microflora were found for relatively long periods

457 up to 140 days, but also a high bacterial resilience may contribute to this absence of effects.

458 Follow-up options include long term aging of FPs before testing or much longer term biota

459   exposure. Additional species can be added, e.g. filter feeding marine organisms may be susceptible

460   both to direct effects of microplastic particles[46] or to vector effects of adsorbed toxicants.[47] One

461   may speculate that toxicant affinity is modulated by the surface chemistry, even if we found surface

462   chemistry to be dominated by the matrix, not by the ENM.

463   There is no directly comparable study on nanocomposites. One paper reported an unconventional

464   ecotoxicity assay by exposing fruit flies to an environmentally aged CNT-composite, finding no

465   specific effects.[48] Seven papers assessed the toxicity for humans by sanding fragments released

466   from NEPs, and focused on inhalation exposure[19, 22, 49-53] and oral exposure[54]. For paints and

467   plastics NEPs with silica, titania and CNT additives, the studies report no additional toxicity by

468   the ENM in the primary target organ, [19, 22, 49-54], only one study found secondary effects in the

469   liver[53]. Models for environmental risk assessment currently assume the fate and effects of NEP to

470   be determined by ENM properties.[6] This assumption needs to be revisited in light of the present

471   finding of NEP environmental effects determined by *matrix* properties, and of their significant

472   differences in key physical-chemical properties such as composition, size, surface chemistry.[11] We

473   designed the studies to explore the possible modulation of ecotoxicity by nanomaterial additives

474   in plastics/polymer/cement. We found no such modulation despite a systematic variation of (Fig.

475   3A) different nanomaterials and (Fig. 3B) different matrices and (Table 1) test organisms

476   representing different environmental compartments. In a separate paper, the Predicted

477   Environmental Concentrations (PEC) based on material-flow exposure models for nanomaterials

478   and Predicted No Effect Concentrations (PNEC) were estimated with species sensitivity

479   distribution models. PEC/PNEC risk ratios were obtained for the case studies, species and

480   environmental compartments described above and indicate no risks based on the given data.[55]

481   With regard to our primary motivation by nanomaterial risk assessment, the present results lend

482   support to grouping of ecotoxicity during the USE phase: the group would be defined by the *matrix*

483   material, potentially limited to a certain maximum ENM content that is relevant for ENM

484   application as functional additive (typically below 5%). The ecotoxicity of fragments emitted

485   during the USE phase would be read across from the pure matrix material. The grouping by matrix

486   would probably fail for easily hydrolysible matrices,[8] which would release free ENM and were not

487   tested here, and for NEPs using dissolving ENM such as Ag, CdTe, which were tested and

488   reviewed elsewhere.[9, 56-58] Further, secondary fragmentation and release of free ENM may occur

489   during transport to the final sink in soils or sediments over time scales longer than the standard

490   test duration for organisms. However, secondary ENM release by weathering and migration was

491   independently tested on the identical NEP, and found strong containment by the polyolefin

492   matrix.[16] Another experimental study on tire wear found up to 0.045% releases of free ENM for

493   secondary UV/hydrolysis degradation, as this only affects a sub-µm-thin surface layer of many-

494   µm-diameter FP particles.[59] Further, any secondary fragmentation by UV degradation is again

495   mostly determined by the matrix, with benchmarks of labile (epoxy), intermediate (Polyamide,

496   POM) and resilient (PE, PP, cement) matrices.[21] Thus, grouping by matrix can be applied to a wide

497   range of matrices and ENM that is delimited by the intended persistence during use.

498   As a secondary interpretation, one may identify the FP material as microplastics. The size range

499   and composition match the definition of e.g. the Danish EPA.[60] Plastics, including commodity

500   plastics with widespread use, are routinely colored with pigments such as Fe_2O_3, whereas the

501   Organic Pigment Red 254 is rather a high-performance pigment class, and CNTs are currently

502   lower volume material.[15] Our results on PP, POM, epoxy and PE, with and without ENM, can be

503   interpreted as one of the first studies on the effects of microplastics on organisms in soils, fish, and

504 on the sewage treatment plant biological activity. We found no significant effects up to the highest

505 concentration tested. Of note, considerations of physical-chemical similarity as proposed by Hüffer

506 et al.[11] would position our mechanically shredded plastics with hydrophobic, irregular surfaces

507 and low aquatic dispersability as more similar to real world secondary microplastics of polyolefin

508 (packaging), polyamide (fishery nets)[61] or rubber (tire wear)[60] than charge-stabilized polystyrene

509 beads that are simply more convenient in (eco)toxicity assays.[62] "The SUN approach" thus

510 contributes to environmental hazard screening of both nano- and micro-structures.

511

512

516

517   **Supporting Information**: additional SEM scans of shape and surface structure of FPs;

518 composition and preparation of nanomaterials and NEPs; micronisation of NEPs to FPs; physical-

519 chemical properties of FPs in tabular format expanding the graphical representation in Figure 3;

520 FP surface chemistry analysis by XPS line shape and acid leaching; FP dispersability in ecological

521 media; preparation of FP water-accomodated fractions for zebrafish testing; zebrafish larvae

522 results; soil microorganism results; cytotoxicity results; *E. crypticus* results.

523

524

525 **Glossary / List of abbreviations**

526 AFW: Artificial freshwater

527 BSA: Bovine serum albumin

528 CFDA-MS: 5-carboxyfluorescein diacetate acetoxymethyl ester

529 CNTs: Carbon nanotubes

530 CSA: Centrifugal separation analysis

531 DLS: Dynamic light scattering

532 dpf: days post fertilization

533 DW: Distilled water

534 EOL: End of life

535 ENM: Pristine engineered nanomaterials

536 ERT: Enchytraeid reproduction test

537 FOR: Formulation

538 FP: Fragmented products

539 HDPE: High density polyethylene

540 hpf: Hour post fertilization

541 NEPs: Nano-enabled products

542 OrgPig: Di-keto-pyrrolo-pyrrole

543 PE: polyethylene

544 PEC: Predicted environmental concentrations

545 PNEC: Predicted no effect concentrations

546 POM: Polyoxymethylene

547 PP: Polypropylene

548 SDS: Sodiumdodecylsulfate

549 SEM: Scanning Electron Microscopy

550 SRHA: Suwannee river humic acid

551 STP: Sewage treatment plant

552   SYN: Synthesis

553   TEM: Transmission Electron Microscopy

554   TRPS: Transient resistive pulse sensing

555   WFPs: Weathered fragmented products

556   XPS: X-ray photoelectron spectroscopy

557

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