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1 Aquatic mesocosm strategies for the environmental fate and risk assessment of engineered
2 nanomaterials

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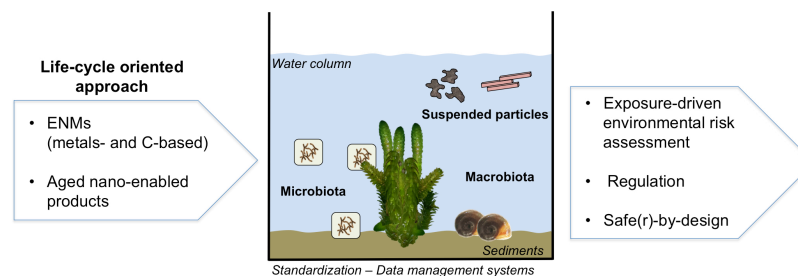
28 This review explores aquatic mesocosm outcomes in the study of engineered nanomaterial
29 fate and effects, across their life-cycle and in an environmental risk perspective.

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35 Table of Contents Art

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40 Abstract

41

42 In the last decade, mesocosms have emerged as a useful tool for the environmental study of
43 engineered nanomaterials (ENMs) as they can mimic relevant exposure scenario of
44 contamination. Herein, we analyzed the scientific outcomes of aquatic mesocosm
45 experiments, with regard to their designs, the ENMs tested, and the endpoints investigated.
46 Several mesocosms designs were consistently applied in the last decade to virtually mimic
47 various contamination scenarios with regard to ecosystem setting as well as ENMs class,
48 dose and dosing. Statistical analyses were carried out with the literature data to identify the
49 main parameters driving ENM distribution in the mesocosms and the potential risk posed to
50 benthic and planktonic communities as well as global ecosystem responses. These analyses
51 showed that at the end of the exposure, mesocosm size (water volume), experiment
52 duration and location indoor/outdoor had a major role in defining the ENMs/metal
53 partitioning. Moreover, a higher exposure of the benthic communities is often observed but
54 did not necessarily translate to a higher risk due to the lower hazard posed by transformed
55 ENMs in the sediments (e.g. aggregated, sulfidized). However, planktonic organisms were
56 generally exposed to lower concentrations of potentially more reactive and toxic ENM
57 species. Hence, mesocosms can be complementary tools to existing standard operational
58 procedures for regulatory purposes and environmental fate and risk assessment of ENMs. To
59 date, the research was markedly unbalanced toward the investigation of metal-based
60 compared to metalloids and carbon-based ENMs but also nano-enabled product. Future
61 studies are expected to fill this gap, with special regard to high production volume and

62 potentially hazardous ENMs. Finally, to take full advantage of mesocosms, future studies
63 must be carefully planned to incorporate interdisciplinary approaches and ensure that the
64 large data sets produced are fully exploited.

65

66

67 1. Introduction

68

69 Assessing the environmental impact and hazard of engineered nanomaterials (ENMs) is
70 challenging due to their specific physico-chemical properties compared to bulk and free-ion
71 species^{1,2}. In the last decade, an extensive amount of research has been carried out to
72 understand ENM fate and effects upon release in the environment. The majority of these
73 studies employed standardized experimental strategies, usually addressing single endpoints
74 in simplified systems (e.g. single-species ecotoxicity tests, one-parameter dependent
75 transformation), to provide straightforward information for regulatory purposes. However,
76 such approaches are often not representative of true environmental complexity and may not
77 reproduce mechanisms driving ENM behaviour and effects in the ecosystem. In recent years,
78 efforts have been made on the development and use of alternative testing strategies (ATS)
79 that enable the study of ENM environmental risk in more realistic frameworks, e.g. by
80 including the aging of ENMs in natural matrices as well as their interactions with natural
81 components during trophic or trans-generational transfers^{2,3,4}. Among the ATS, mesocosms
82 are considered invaluable tools, allowing for a more comprehensive simulation of key
83 ecological processes compared to other single standardized test or standard operational
84 procedure (SOP)^{2,5,6,7}.

85 Mesocosms can be defined as an enclosed and essentially self-sufficient experimental
86 environment and have been extensively used to investigate ecological processes and the
87 environmental fate and effects of xenobiotic chemicals^{8,9,10}. Mesocosms allow (I) self-
88 sustainability for the duration of the experiment, (II) feasibility of sampling and measurement
89 of multiple bio-physico-chemical endpoints and (III) the possibility to identify direct and
90 indirect effects of the tested compounds^{5,11,12}. Such strategies have already been
91 implemented to generate regulatory data in the evaluation of pesticide contaminations^{13,14}
92 and have recently been proposed for ENM environmental risk assessment (ERA). Regarding
93 ENM ERA, mesocosms enable to simultaneously investigate ENM behavior and effects using
94 an exposure scenario-driven approach^{5,15}. They provide exposure and hazard data that
95 account for ecological synergies but also interdependent transformation processes⁵. This is
96 particularly relevant, as the validity of conventional standardized tests is challenged by the

3

97 unique properties of ENMs and their dynamic evolution upon release in the ecosystem¹.
98 Exposure and hazard data obtained in ENMs dosed mesocosms can be gathered in
99 database and fed into fate models, hazard scoring schemes, and environmental risk
100 assessment models^{16,17}. They can also serve as input for high throughput experiments to
101 better elucidate the mechanisms involved².

102 While a definition of mesocosms has been debated with regard to the operational time and
103 size, terms such as microcosm and mesocosms have interchangeably been used in literature
104 for describing similar systems. A literature survey in 2012 focused on ENM studies in both
105 terrestrial and aquatic mesocosms, reporting only twelve instances of published works¹⁸.
106 However, during the last decade, the use of mesocosms in ENM fate and nanosafety studies
107 has consistently increased with more than 200 research items between 2008-2020 (source:
108 Web of Science; keywords nano*, mesocosms and microcosm) (Fig. 1). These works
109 investigated ENMs in either aquatic or terrestrial mesocosms and a part of these items have
110 already been reviewed with a focus on terrestrial environments¹⁹ and ENM toxicity⁴.

111 Here, we reviewed papers focused on fate and impact of ENMs in aquatic mesocosms to
112 provide an overview of the relevant strategies, mesocosms designs, and their findings. An
113 extensive screening of these papers was carried out in order to select (I) experimental
114 mesocosm investigating ENMs and (II) studies focusing on the aquatic ecosystem. A final
115 selection of about fifty peer-reviewed papers was obtained excluding short-term
116 experiments (<1 week) with no acclimation time as well as single-endpoint and single-species
117 oriented studies, which have been already reviewed by Bour et al. (2015)⁴. A detailed list of
118 the selected literature and further information of the selection criteria are reported in the
119 Supporting Information (Table S1). A further aim of the present work is to relate the ENM
120 fate and nanotoxicity for aquatic organisms to the environmental risk in relevant
121 environmental scenarios. Statistical tools were employed to identify important parameters
122 determining the long-term partitioning of ENMs in aquatic environments. This will be used as
123 the basis to discuss ENMs exposure towards benthic and planktonic compartments as well as
124 ENMs hazard and systemic ecosystem responses to ENM contamination. Finally, the interest
125 of aquatic mesocosms in ENM lifecycle-oriented and ERA studies, as well as the current
126 limitations and knowledge gaps, will be discussed.

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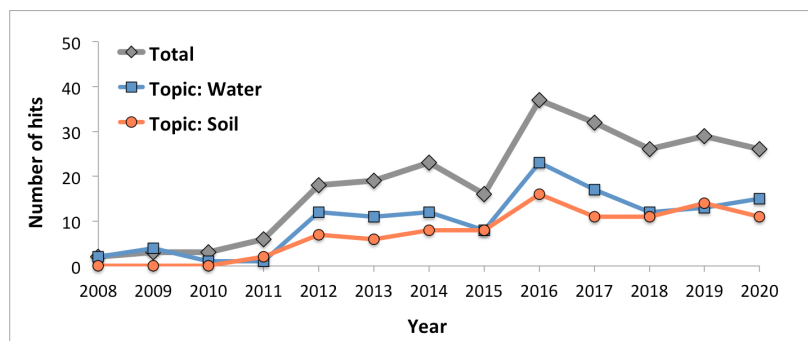


Figure 1. Web of Science outcome for nano* (topic, title) studies with topic of mesocosms and microcosms in the period 2008-2020 (grey, diamonds). In blue (squares) and orange (circles) the studies focused on water and soil compartments, respectively.

2. A large panel of ENM environmental exposure scenarios

2.1 Diverse aquatic mesocosm designs

Figure 2 provides an overview of the main designs found in the literature, where aquatic mesocosm strategies are categorized based on their location and the ecosystem mimicked. A detailed list of these works, with additional information regarding the ENM properties, experimental setups and endpoints addressed is provided in the Supporting Information (Table S1). In general, a major distinction in experimental approaches is achieved between indoor laboratory-scale facilities and outdoor systems operated in the natural environment (*ex situ* or *in situ*), which have been equally reported in literature. A further distinction is obtained along a salinity gradient, i.e. from freshwater (84% of the literature) to estuarine and seawater (16% of the literature) ecosystems (Fig. 2).

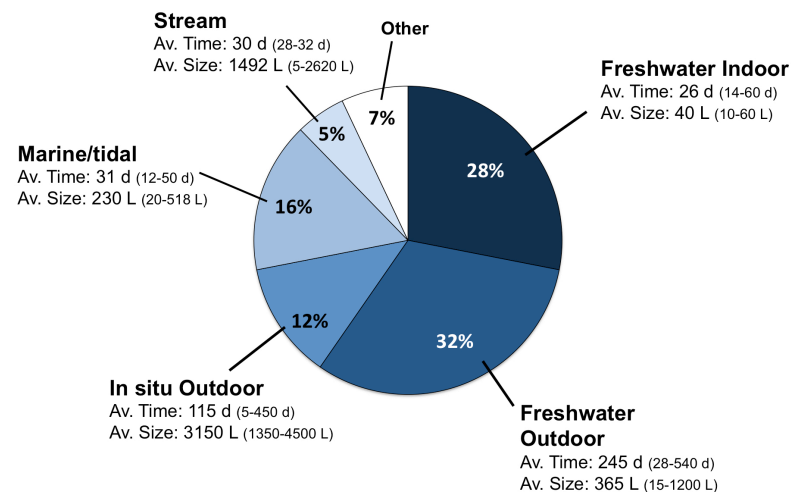


Figure 2. Mesocosm designs applied in the studies of ENM environmental fate with corresponding average experimental duration (Av. Time) and average water volume in the mesocosms (Av. Size). In parenthesis the range of minimum and maximum values applied for each design. "Other designs" refers to low-volume (<10 L) constructed wetland and sediment-oriented studies.

Freshwater indoor mesocosms have been implemented to mimic both lotic and lentic ecosystems. Relatively small aquaria (e.g. 10-15 L) were employed to study microbial communities²⁰ or specific trophic chains²¹, while larger modular set-ups (50-60 L tanks) were used to simulate complex ecological frameworks^{6,9,22,23,24}. These systems ensure the control of several parameters (e.g. pH, temperature, water volume, sediment depth) and allow high repeatability²⁵. Macro-organisms tested across these studies have included a variety of aquatic macrophytes and grazing animals as well as echinoderms, crustaceans, insects and amphibians larvae, and fishes. Authors used natural inoculums to colonize the mesocosms with primary producers sampled from the environment to be mimicked. Sediment matrices typically consisted of artificial sediments combined with sieved natural sediments. Similarly, aqueous phases consisted of commercial water with a pH and ionic strength close to the

170 actual environment spiked with water samples collected in the field²². This allowed
171 controlling the water chemistry and porous media properties (texture, density, metals
172 content) while providing natural inputs to the mesocosms. Indoor mesocosms have been
173 extensively used to study the fate of a wide range of ENMs. The exposure to the ENMs
174 usually took place over a period of several weeks (26 days on average) and was preceded by
175 an acclimation phase (approx. two weeks) for the equilibration of the (eco)system (e.g.
176 settling of suspended matter, pH/Eh stabilization, development of microbiotic communities).
177

178 **Freshwater outdoor mesocosms** achieve more realistic exposure scenarios at the expense of
179 less control and a lower feasibility compared to laboratory-scale set-ups. These experiments
180 account for seasonal changes and varied weather conditions (e.g. precipitation, drought).
181 They are prone to colonization by organisms from the surrounding environment, resulting in
182 an enhanced complexity but diminished control of parameters such as water volume,
183 temperature and overall biodiversity. Small 10-20 L mesocosms were occasionally employed
184 outdoors to monitor algae population response over several months after ENMs
185 contamination^{26,27,28}. The most consistently applied outdoor design made use of larger
186 facilities (365 L on average) optimized for longer exposure testing (245 d on average)^{6,9,29,30}.
187 While more challenging with regard to repeatability and the possibility for standardization,
188 these systems provide information about long-term contamination effects^{6,9,31} and ecosystem
189 response and recovery. For instance, freshwater, wetland ecosystems were simulated for
190 periods up to 1,5 years in rectangular-shaped containers (3.5 m³) lined with a uniform soil
191 layer (~20 cm) and a sloped-bed allowing humidity and redox gradients between
192 permanently and periodically flooded areas²⁹. The watertight enclosures were open at the
193 top to allow variation of the water volume due to rainfall, evaporation and transpiration.
194 Native plankton and benthic bacteria communities were brought with natural groundwater
195 and soil matrices. Macro-organisms tested include terrestrial and aquatic macrophytes,
196 molluscs, crustaceans, and fishes as well as unintentional insects.
197

198 **Stream freshwater mesocosms** allowed for the addition of ENMs to flowing water in order to
199 monitor their mobility and transformation during transport. Outdoor studies employed
200 hybrid flow-through/recirculating systems to model wastewater treatment plant and
201 constructed wetland effluents^{32,33}. Briefly, these consisted of two collecting pools connected
202 by 18 x 0.5 m sloped channels presenting riffle and glide sections. Engineered nanomaterial-
203 amended water in the top pool was gravity-fed to the bottom and then partially or totally

204 recirculated in order to maintain a stable volume and an essentially closed system. The
205 resulting mesocosms were colonized with insects, fishes, and snails and were tailored for
206 studying the effects of ENMs on the periphyton and algae populations. These systems
207 necessarily employ large water volumes and usually operate for short periods of time
208 (weeks), also to limit wastewater volume. Flow-through designs were also employed in an
209 indoor study, with small recirculating containers used for exposing snails and algae under
210 permanent water flowing conditions. Although less representative of whole stream
211 ecosystems, this approach presented several advantages such as the possibility to
212 accumulate large algae populations and to perform highly replicated experiments³⁴.
213

214 **In situ mesocosms** were obtained by contaminating an artificially bounded portion of a
215 natural lentic ecosystem. Furtado et al. applied such a strategy in two different studies where
216 round-shaped polypropylene sheeting, suspended from a floating ring and sealed to the
217 sediments, was directly placed in a sheltered bay of a littoral lake^{35,36}. These open-bottom
218 designs represent a straightforward approach for studying larger volumes (2400-3000 L) with
219 natural matrices. These studies were carried out over a relatively short-term of 30 d. Longer
220 exposure periods were achieved by Jovanovic et al., with a similar design where 1100 L
221 fiberglass tanks were attached to a platform anchored in the middle of a lake³⁷. Their
222 mesocosms were filled with sediments and filtered lake water followed by introduction of
223 zooplankton, macroinvertebrates, macrophytes and fishes before exposure to ENMs for
224 three months. Larger in situ mesocosms (> 5000 L) were also used for the evaluation of
225 community structures in contaminated ditches^{38,39,40}. These systems focused on sediments
226 and benthic community structure and recolonization of polluted areas following sediment
227 contamination by ENMs for periods of up to 15 months.
228

229 **Marine mesocosms** have been less implemented compared to freshwater ones. Some
230 pioneering works have been achieved in saltwater scenarios^{41,42,43}, which are to date the least
231 investigated compartments (approx. 15% of the reported literature). This can be ascribed to
232 the low predicted environmental concentrations (PEC) expected in marine environments due
233 to the ENM removal from the water phase at high salinities⁴⁴. In a few instances, indoor
234 facilities used small tanks (20 L) containing natural seawater and sediments for the study of
235 native bacteria and plankton communities^{42,43}. More recently, larger systems (>300 L) were
236 applied to study low-dose addition of ENMs to open water⁴⁵ or coastal ecosystems
237 previously colonized with molluscs and echinoderms⁴⁶. Tidal cycles in estuarine ecosystems

238 have also been mimicked. Buffet et al. investigated the response of benthic worms and filter-
239 feeders to ENM-contaminated intertidal sediments in outdoor facilities^{47,48}. In their study,
240 pre-contaminated sediments in ~5L containers were introduced in a top-tank and cyclically
241 received aerated natural seawater from a reservoir. Similar designs (with 2 reservoirs to mimic
242 the tide) have also been used indoors in more complex ecological setups including plants,
243 snails, clams, shrimp and fish^{41,49,50}.

244 245 2.2 ENM dosing scenarios and total doses 246

247 When selecting the ENM to be investigated in aquatic mesocosms, a broader attention has
248 been given to potentially hazardous materials, such as antimicrobial and (photo)catalytic Ag-,
249 Ti-, and Ce-ENMs (34%, 15%, and 16% of the aquatic mesocosms studies, respectively). Cu-,
250 Au- were also the subject of some studies, whereas little or no attention was paid to several
251 high production ENMs such as Zn-, Si-, Fe-, Al-ENMs and low production volume materials
252 such as quantum dots. For instance, Si-based ENMs were mostly neglected, despite SiO₂
253 ENMs being one of the most produced^{51,52}. Similarly, C-based ENMs (e.g. fullerenes,
254 nanotubes, graphene) were investigated to a lower extent (10% of the studies) compared to
255 metal-based ENMs. A graphene ENM was only recently included in a study despite its
256 increasing production and application rates⁵³. Carbon-based ENMs such as graphene and
257 CNTs can display enhanced toxicity when interacting with natural components^{54,55}, which
258 should be further characterized in realistic environmental settings. In general, the selection of
259 ENMs should be based on criteria identifying the potentially most impactful materials in
260 environmental contexts. For instance, Arvidsson et al., identified “global production volume”
261 and “short-term ecotoxicity” as relevant proxy measures for assessing ENMs environmental
262 impact⁵¹. They further provided a proof of concept assessment classifying several ENMs on a
263 low-to-high production vs toxicity basis. Similar criteria can be used to identify the most
264 relevant ENM to be investigated in future aquatic mesocosms.

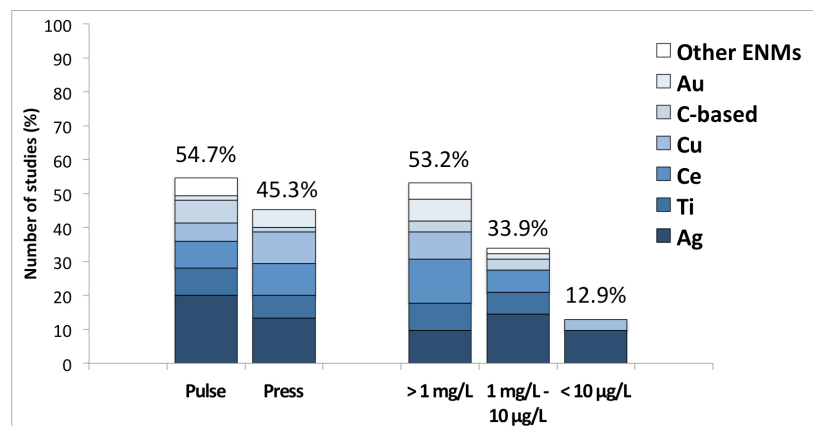
265 Whatever the ENMs, the absolute dose injected can strongly affect both their exposure and
266 hazards of the mimicked ecosystem, as many physico-chemical processes (aggregation,
267 dissolution, adsorption) are kinetically controlled by the concentration^{31,56}. With a few
268 exceptions, contamination of the mesocosms at low mg.L⁻¹ levels has emerged as the best
269 compromise between environmentally relevant concentrations and qualitative and
270 quantitative analyses in the complex matrices investigated. To date, half of the mesocosms
271 experiments used mg.L⁻¹ contamination levels, whereas less than 20% were carried out at the

272 more environmentally relevant low µg.L⁻¹ levels (Fig. 3). Although lower than the
273 concentrations typically applied in *in vitro* ecotoxicity studies, these ranges are higher than
274 the PECs, mostly expected in the range of ng.L⁻¹ and µg.Kg⁻¹ in water and sediments,
275 respectively⁴⁴. A well-known challenge in mesocosm studies performed at environmentally
276 relevant concentrations is the analytical feasibility of detecting and quantifying low
277 concentrations in matrices with high natural backgrounds (e.g. detecting Ti-ENMs within
278 sediment naturally rich in endogenous Ti). Recent studies confronted these issues by
279 applying powerful analytical methodologies using spICP-MS (for metal-based ENMs)^{35,45,57} or
280 optimized HPLC-MS (for carbon-based ENMs) able to detect pg.L⁻¹ levels⁴⁶. The use of
281 isotopically-labeled ENMs shows a great potential for detecting and discerning ENMs apart
282 from the natural background at realistic contamination concentrations and can be applied to
283 a wide range of metal, metalloid or carbon-based ENMs. For instance, similar techniques
284 enabled the detection of ¹⁰⁹Ag-enriched Ag-ENMs⁵⁸ or multi-isotopically labelled
285 ¹¹¹Cd/⁷⁷Se/⁶⁸ZnS QDs^{59,60} at concentrations as low as ng.L⁻¹ in complex matrices. Furthermore,
286 understanding the isotopic fingerprint of ENMs can help distinguish natural from
287 manufactured ENMs, and even the synthetic methods and manufacturers of engineered
288 ENMs⁶¹. Such methodologies have surprisingly received scarce attention in mesocosm
289 investigations (e.g. ¹⁴¹Ce-enriched Ce-ENMs⁶²) and may represent a straightforward solution
290 for overcoming the current dosing limitations in mesocosm studies.

291 More than addressing the absolute doses, several studies assessed the role of the dosing
292 scenario in the global response of the ecosystems to ENM contamination^{6,9,22,31,33}. Two main
293 mesocosm dosing procedures were published *i.e.* (I) “pulse” treatments that mimic single
294 contamination events and (II) “press” treatments describing continuous addition of low
295 doses of ENMs over the duration of the experiment. These are also referred to as acute or
296 chronic exposures, respectively. Although equally reported in literature (Fig. 3), in recent
297 years press contamination was increasingly applied in order to mimic the chronic exposures
298 expected in aquatic ecosystems^{31,63}. The mesocosms dosing influenced both the ENMs fate
299 and effects, affecting partitioning between the environmental compartments and
300 bioaccumulation and biomagnification in organisms^{31,64}. Using multivariate analysis, Nassar et
301 al. recently highlighted that the dosing scenario constituted the main parameter to consider
302 when investigating the environmental risk for a given ecosystem exposed to ENMs with
303 different sizes and coatings⁶⁵.

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309 Figure 3. The dosing procedure (left) and ranges of concentrations (dose, right) applied for
310 aquatic mesocosm contamination. The dose concentration ranges refer to the total ENMs
311 input in the mesocosms at the end of the experiment.
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2.3 ENM partitioning within ecological compartments

315 More than the absolute dose, the environmental risk posed by ENMs stems from their
316 accumulation and speciation within ecological compartments. ENM partitioning in
317 mesocosms is driven by bio-physico-chemical transformation processes leading to the
318 distribution of large aggregates in the sediments and more well-dispersed ENMs or
319 dissolved metals in the water column. Espinasse et al., reported short aqueous residence
320 times in a wetland mesocosm for a range of ENMs ($\text{Ag} > \text{TiO}_2 > \text{SWCNT} > \text{CeO}_2$ ENMs) with
321 half-lives in the water ranged from 36 h for CeO_2 -NPs to 10 d for Ag -NPs⁶⁶. Such an
322 environmental partitioning results from dynamic (bio)transformations in the ecosystem
323 regulated by environmental factors such as the water chemistry, the ENM properties, the
324 exposure scenario and the mesocosm design.

325 To investigate the contribution of each of these parameters on the ENM partitioning,
326 multivariate analyses were performed on data extracted from the papers reviewed herein.
327 Among the fifty peer-reviewed papers initially screened, 31 that comprehensively reported
328 the concentrations of metals detected in the environmental compartments as well as data
329 about both the ENMs used (e.g. composition, size, coating) and experimental parameters
330 applied (e.g. dose and dosing, mesocosm location, duration and total water volume of
331 mesocosm) were selected (Supporting Information, table S2). It must be noted that the
332 analysis did not include environmental factors that showed low variability between the
333 studies (e.g. pH, 8.2 ± 0.6 in average) or large daily or seasonal changes during the
334 experiments (e.g. pH, T, dissolved O_2). Parameters that were not comprehensively
335 investigated/reported across the studies under exam (e.g. organic and total suspended
336 matter) were not included in this analysis. ENM partitioning was expressed as the percentage
337 of injected metals found in the water column at the end of the experiments (107 d on
338 average), which is an indicator of ENMs environmental distribution often reported in
339 literature.

340 PERMANOVA analysis (Supporting Information, Table S3) showed that at the end of the
341 exposure mesocosm size (water volume), experiment duration and location had a major role
342 in defining the metal partitioning ($p\text{-value} < 0.05$). In Canonical Correlation Analysis (Fig. 4), a
343 strong linear correlation was identified between the mesocosm water volume and the
344 percentage of metals in the water ($R^2 = 0.163$, $p\text{-value} \leq 0.001$). This is attributed to a higher
345 water height in larger mesocosms, resulting in longer settling time and metal persistency in
346 water. Conversely, the experiment duration was inversely correlated to the percentage of

347 metals in water ($R^2 = 0.049$, p -value = 0.023), which is consistent with the kinetics of ENMs
348 settling onto surficial sediments.

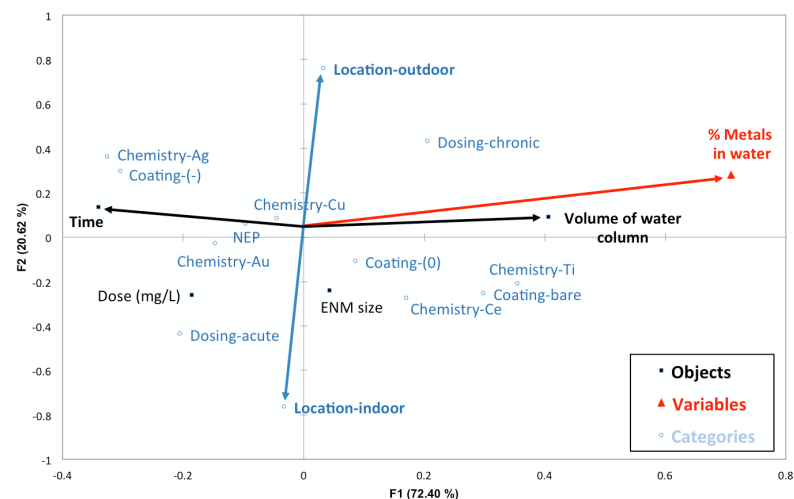
349 The mesocosm location (i.e. indoor or outdoor) had a significant impact on the system
350 variance (PERMANOVA, $R^2 = 0.064$, p -value ≤ 0.005) but in Canonical Correlation Analysis
351 this parameter was poorly correlated with the percentage of metals in the water. It is
352 noteworthy that the long-term accumulation of the metals/ENMs in the sediments was
353 observed independently of the location, suggesting that both indoor and outdoor designs
354 were consistent in reproducing the behaviour of metal ENMs in aquatic ecosystems on the
355 long-term.

356 It must be noted that, since the data used for this statistical analysis referred to the end of
357 the experiments (107 d on average), the results presented here may underestimate the role
358 that parameters played over the short term. For instance, the intrinsic ENM physico-chemical
359 properties (size, chemistry, coating), the water chemistry (freshwater vs seawater), and the
360 dosing scenario had no major role in defining the metal partitioning at the end of the
361 experiment (p -value >0.05). For the dosing scenario, the Canonical Correlation Analysis
362 suggests that press dosing (chronic) was directly correlated with the percentage of metals in
363 the water, whereas pulse dosing (acute) was inversely correlated (Fig. 4). This was consistent
364 with the observations reported in literature, i.e. that for a given ENM dose pulse additions
365 caused a short-term higher aqueous concentration but also rapid settling of the ENMs onto
366 the sediments, whereas press treatments lead to a higher persistence of ENMs in the water
367 column on the longer term. For instance, Colman et al. showed that, in wetland mesocosms,
368 water [Ag] in chronic exposures matched those of the acute treatment after three weeks, and
369 were ~200-fold higher after 7 months at equal total doses³¹. In pond mesocosms, Ce-ENMs
370 sedimentation rates were not significantly different following pulse or press dosing, but
371 chronic addition resulted in a higher final Ce concentration in water column after 1 month²².
372 Comparable behaviors were also observed in stream³² or saltwater ecosystems^{41,43,49}.
373 Nonetheless, a limitation in the statistical analysis was due to the lack of data available about
374 important parameters driving ENM fate. For instance, while conductivity was often reported
375 as an estimate of mesocosm water ionic strength, the ionic species in solution were seldom
376 characterized. Similarly, while macrophytes and associated biofilm have been described as a
377 main factor for ENM fate^{e.g.67,68}, estimates of the biomass and surface area are missing.
378 Statistical analysis such as the one presented here will benefit from more comprehensive
379 datasets including such data and future mesocosms studies are expected to fill these gaps.

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384 Fig. 4. Canonical Correlation Analysis showing the relationships between qualitative (blue)
385 and quantitative (black) parameters and the amount of metals in the water column (%),
386 at the end of the experiments. The arrows identify the significant parameters (p -value <0.05 in
387 PERMANOVA) and the angle between the arrows indicates the degree of linear correlation
388 between them.

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391 3. Environmental risk for aquatic ecological niches

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394 3.1 The benthic compartment: high exposure to less reactive ENMs

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396 A predominant accumulation of the ENMs and metals onto the sediment was transversally
397 reported across the studies, driven by homo- and/or hetero-aggregation with natural

398 colloidse.g.^{69,70} and interaction with various aquatic organisms⁶⁶. Thus, benthic organisms are
399 predominantly exposed to aggregated ENMs, accumulated at the top sediment layer (0-1
400 cm)^{23,71,72}. Once in the sediments, ENMs can be remobilized via bioturbation, feeding, and
401 entered the food web. Studies highlighted ENMs biomagnification and ENMs trophic
402 transfers, e.g. from biofilm to insect larvae, molluscs and fish^{24,41,64}.
403 The risk posed to the benthic communities did not only depend on the relatively more
404 important doses in sediments but also on the metals speciation. Studies described
405 (bio)transformation processes are relevant in such dynamic redox environment and after
406 interaction with benthic biota. Oxidation/sulfidation is a major transformation pathway for
407 ENMs such as Ag, Zn and Cu in sub-oxic and anoxic environments^{29,56}. Lowry et al., showed
408 that sulfidation of Ag-ENMs in freshwater sediment and organisms involves the oxidation of
409 Ag(0) to Ag(I) and subsequent formation of Ag₂S and sulfhydryl compounds²⁹. The
410 transformed Ag-ENMs remained bioavailable to benthic species^{29,31,66}. However, the
411 diminished solubility and reactivity of these by-products translated to slower metal
412 dissolution, lower uptake and weaker damage on aquatic plants, benthic microbial
413 community and (in)vertebrate compared to pristine ENMs^{73,74,75}. In the case of Cu-ENMs,
414 sulfidation could even enhance mobility and dissolution, contributing to a faster
415 transformation of the pristine ENMs via Cu-S- and Cu-O- binding with organic matter⁶⁷.
416 Grazing benthic macroorganisms such as snails and filter feeders have been shown to
417 accumulated Ag-, Ce-, Cu- and C-ENMs^{23,46,48,76} and had the highest concentration factor in
418 complex food webs exposed to Au-ENMs⁴¹. In estuarine ecosystems, evidence of DNA
419 damage and oxidative stress in clams exposed to Cu- and Ag-ENMs is attributed to the
420 uptake of aggregated ENMs containing redox active metals⁴⁸. In freshwater ecosystems,
421 ENM contamination led to behavioural alterations of bivalve filtering capacity (Ce-ENM⁷⁶)
422 and molluscs excretion rates (Au- and Cu-ENMs⁷⁷). Ce-ENMs also induced oxidative stress to
423 snails and genotoxic effects in amphibian larvae^{24,78}, linked to the uptake of aggregated
424 ENMs and the subsequent bioreduction of Ce(IV) in Ce(III)^{70,76}. In these studies, citrate coated
425 Ce-ENMs were less aggregated and more bioavailable⁷⁶, but caused lower oxidative stress,
426 likely because of the antioxidant properties of the organic ligands²³.
427 Macrophytes and benthic algae were found to be an important target for Ce-³², Ti-^{33,34}, Ag-³¹
428 and Cu-ENMs^{67,79}. Aquatic plants present a high surface area and could sequester a
429 significant fraction of ENMs in the long term, mostly due to adsorption and uptake onto
430 plants roots, tissues and associated microbiome^{67,68,80}. These processes influence ENMs
431 cycling by immobilizing the ENMs and reintroducing them in the food web^{29,66}. However,

432 most studies did not identify the main interaction mechanisms between ENMs and plant
433 tissues. Negative effects on periphyton endpoints (e.g. algal density, viability, diversity,
434 chlorophyll production, biomass growth) were also observed at relatively high doses (> 1
435 mg/L) of Ti-^{27,33}, Al-²⁶, and Fe-ENMs²⁸ in lotic and lentic ecosystems.
436 Biofilms associated with sediment and benthic organisms were also often identified as an
437 important sink^{e.g.41,81} and can contribute to the immobilization of the ENMs as well as their
438 biotransformation^{68,82} and entry in the food web⁵⁰. However, the impact of ENMs on benthic
439 microbiota appeared to be limited, even in the case of the expectedly toxic Ag- and Cu-
440 ENMs. Several studies found negligible effects on prokaryote abundance and diversity in
441 saltwater⁴³ and freshwater sediments⁸³. On a longer term, the stability of microbial
442 community function and structure was associated with a developed tolerance to metals in
443 constructed wetlands⁸¹. Interestingly, other studies highlighted how resilience depends on
444 population density and how specific taxa were more sensitive to contamination⁸⁴.

445 446 447 3.2 The planktonic compartment: low exposure to more reactive species 448

449 Because ENMs primarily partition onto the sediments, most of the research focused on the
450 fate and effects in the benthic compartments and data available on planktonic communities
451 are limited. However, despite the presence of low ENM concentrations in the water column
452 over time, exposure of planktonic biota involved potentially more bioavailable, reactive and
453 consequently potentially toxic ENMs and metals.
454 Several works investigated the role of dissolution as a transformation pathway that might
455 release toxic by-products as free-metal ions. Avellan et al., recently showed the
456 (bio)dissolution of Au-ENMs by bacteria within biofilms growing on macrophytes and the
457 metals complexation in stable cyanide, hydroxyl or thiolic derivatives^{68,82}. A rapid dissolution
458 was also observed in freshwater for Cu nano-pesticides⁸⁵ and Al-ENMs²⁶ and it can be
459 expected for ENMs such as ZnO, whose toxicity has been related to ions rather than
460 nanoparticulate forms⁵⁶. In other studies, quantitative assessment of dissolution was difficult
461 to estimate due to re-complexation of the resulting ions with natural suspended matter.
462 Furtado et al., found relatively low dissolution rates of Ag-ENMs in freshwater lake
463 mesocosms at low ionic strength and high dissolved organic carbon (DOC), and concluded
464 that free metals such as Ag⁺ ions were removed by complexation with natural organic matter
465 (NOM)³⁵. Hence, while ENMs were observed to decrease in size, they may have formed

466 larger hetero-aggregates rich in metals. Other studies showed that plant derived DOC
467 stabilized the Ag- and Ce-ENMs in suspension and that the adsorption of organic ligands
468 blocked the oxidation sites and prevented dissolution^{86,87}.
469 Furthermore, decreasing the ENM size, due to the progressive desorption of metals, can
470 dramatically change their behavior, leading to enhanced mobility, reactivity, and toxicity. In
471 wetland mesocosms, smaller Ce-ENMs interacted more with suspended solids and were
472 significantly more bioavailable to (micro)organisms⁸⁸. Similarly, in a marine ecosystem, the
473 negative effects of Ag-ENMs to plankton communities were associated with diminishing sizes
474 and dissolved silver ions in solution⁴⁵. Tsiola et al., showed that Ag-ENMs and the resulting
475 dissolved Ag ions significantly affected seawater planktonic microbial communities⁴⁵. In
476 particular, they observed noxious effects on specific species and highlighted the potential
477 damage to photosynthetic activity of cyanobacteria. In freshwater, Ag-ENMs affected the
478 abundance and had a hormesis effect on the planktonic microbial communities⁸⁹. In the
479 water column, a bioaccumulation of Ag-ENMs was found in dragonfly larvae²⁹, whereas the
480 toxicity of Ce-ENMs was observed in amphibian larvae⁷⁸. A significant bioaccumulation of
481 Ag-ENMs and Ti-ENMs was also observed in higher organisms such as fish species^{21,29}.

482

483 3.3 Global ecosystem response

484

485 Mesocosm studies provide a global view of the ecosystem responses including toxicity
486 arising from indirect interactions with ENMs as well as shifts in complex ecological processes
487 and ecosystem functioning^{30,78}. For instance, the noxious effects of Ce-ENMs toward
488 secondary consumers in pond mesocosms was not observed in parallel experiments with
489 direct or trophic chain exposures⁷⁸. The authors attributed the toxicity to changes in the
490 microbiota community, affecting the nitrogen cycle and litter decomposition. Thus, the
491 ecosystem equilibria may be altered following disturbances to key ecological processes
492 related to biota respiration or nutrient production and consumption. Toxicity to detritivores
493 such as chironomid larvae²⁴, combined with shifts in the structure of fungal and bacteria
494 communities^{90,91,92}, have also been shown to alter organic matter decomposition and the
495 availability of nutrients.

496 A decrease in chlorophyll production and algal biomass as well as variations of abiotic
497 parameters such as redox potential and dissolved oxygen were reported after exposure to
498 Fe-^{20,28}, Al-²⁶ and Ti-ENMs²⁷. Further studies suggested a limited impact on the long-term,
499 but effects of Ag- and Cu-ENMs on photosynthesis and methane metabolism⁸³.

17

500 In a few instances, the authors reported major ecosystem disruption and were able to
501 characterize a complex cascade of effects following contamination. A strong eutrophication
502 was observed in wetland mesocosms due to a synergistic effect of either Au- or Cu-ENMs
503 with nutrient enrichment, which led to an extended algal bloom (>50 d) in comparison with
504 nutrient only treatments⁹³. In this case, an increase in water turbidity eventually affected plant
505 growth and caused up to 92% reduction of the ecosystem primary production. Colman et al.,
506 related the toxicity of Ag-ENMs to macrophytes and phytoplankton with a general disruption
507 of the ecosystem resulting in increased methanogenesis³⁰. In this study, an initial damage to
508 autotrophs led to the release of DOC and an increase of heterotrophic prokaryote
509 abundance and respiration. In turn, the consumption of dissolved O₂ and diminished
510 photosynthetic activity resulted in hypoxia, which combined with elevated CO₂ and
511 decreased methane consumption led to a 40-fold increase of CH₄ in the wetland. Although
512 such processes were observed after single pulse additions at relatively high doses of Ag-
513 ENMs, a similar response of a lower magnitude was also identified in further experiments at
514 lower concentrations³¹.

515 Other studies reported minor or no changes in the ecosystem functioning, but identified
516 negative effects on specific ecological niches. In lake mesocosms, amendment of Ti-ENMs
517 led to a significant decrease of soluble reactive phosphorus and although a wide range of
518 organisms (phytoplankton, plants and fishes) was unaffected³⁷. The authors reported a
519 significant decrease in the biomass of Rotifer species, which typically display high
520 reproductive rates and tolerance to environmental changes. Similarly, in two different
521 studies, the exposure to Ag-ENMs had no major consequences on most organisms under
522 investigation but affected benthic nitrifying bacteria, decreasing nitrification rates in the
523 ecosystem and altering the nitrogen cycling^{72,84}. Recently, Li et al., further investigated these
524 processes, with a focus on the synergies between ENM and endogenous metal species⁹⁴.

525

526 4. Environmental perspectives and current knowledge gaps

527

528 4.1 Need for life-cycle oriented approaches

529

530 ENMs release in the environment can occur at any stage of the value chain, i.e. from
531 synthesis to application/use and eventual disposal (Figure 5). The environmental research so
532 far has mainly focused on pristine ENMs (85% of mesocosm studies considered herein) rather
533 than nano-enabled products (NEPs). However, given the strict control at production and
534 processing sites, incidental discharge into the environment may be more relevant during the

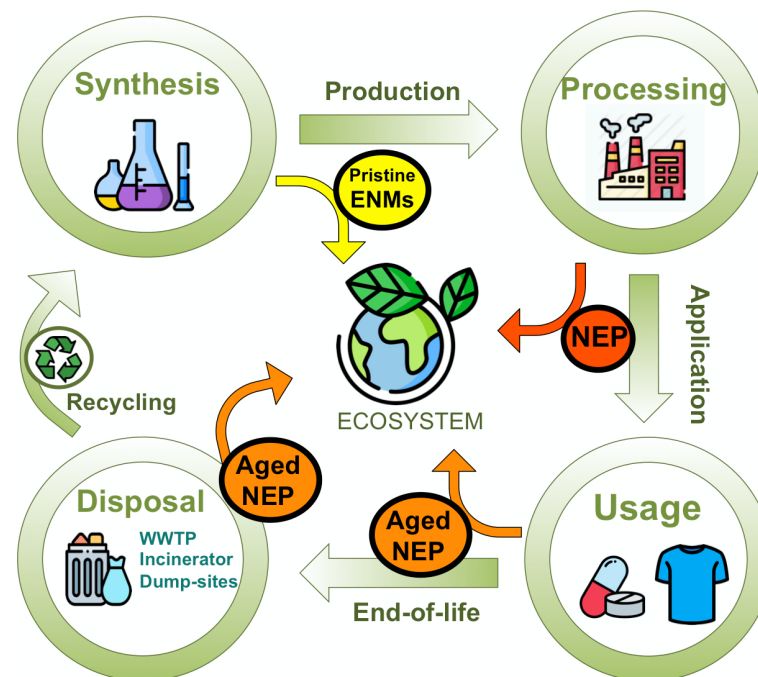
18

535 use and end-of-life phases. Environmental research on aged ENM by-products and NEPs is
536 still very limited, especially in the case of mesocosms studies (11% and 4% of the studies
537 considered herein, respectively).

538 Recent studies have stressed the need for life-cycle oriented approaches considering realistic
539 exposure scenarios and the actual state of ENMs entering the environment^{6,95,96} and their
540 further aging in natural matrices^{3,97}. Aquatic mesocosms represent an invaluable tool for life-
541 cycle oriented studies, as they implicitly account for the bio-physico-chemical aging of these
542 NEPs and aged ENMs and can highlight differences in environmental fate and impacts over
543 long periods. For instance, an estuarine mesocosm experiment focusing on consumer
544 products containing Ag-ENMs, found a gradual release of up to 99% of the Ag ions over 60
545 days⁴⁹. The Ag released by three different NEPs displayed higher persistence in the water
546 column compared to ionic Ag and pristine Ag-ENMs, and enhanced bioconcentration in
547 biofilm, snails and clams. On the contrary, Auffan et al., reported diminished aqueous
548 persistence of Cu-ENMs associated to fragmented paint NEPs⁷⁸. In their study, only 1% of
549 the Cu remained in the water column after 28 days, compared to 10% of the pristine ENMs.
550 In addition, the paint matrix prevented (bio)transformation of the ENMs, with regard to
551 dissolution and complexation. More recently, Chatel et al., reported an enhanced
552 bioaccumulation of pristine Ti-ENMs compared to cement-embedded counterparts in
553 freshwater snails⁹⁹.

554 These studies highlighted a significant difference between pristine and matrix-embedded
555 ENMs, suggesting that most results gathered to date should be considered with caution, as
556 they may not be representative of the actual behavior of ENMs entering the ecosystem. The
557 characterization of pristine ENMs is still fundamental for risk assessment and management,
558 and is the basis for understanding transformed ENM properties⁵⁶. However, future research
559 should focus on NEPs especially in the context of safe(r)-by-design products¹⁰⁰. In particular,
560 priority should be given to reproducing aged NEPs from realistic sources and to the
561 application of relevant dosing for better mimicking real contamination scenarios. In this
562 framework, protocols/strategies have been developed for the production of suitable
563 materials, i.e. fragmented NEP and relative ENMs-free products to be used as controls^{95,96}.
564 Better use of these protocols should overcome current limitations in the understanding of
565 NEPs environmental release, exposure, risk, and life cycle assessment^{101,102}.

566
567



568
569 Figure 5. The potential release of pristine engineered nanomaterials (ENMs), nano-enabled
570 products (NEPs), and aged NEPs along ENM value chain.

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573

4.2 Toward the standardization of mesocosm experiments in nanosafety

574 Although the popularity of mesocosms and similar ATS is arising, especially for ecotoxicity
575 purposes^{2,4}, such methodologies are surprisingly under-utilized for ENM environmental risk
576 assessment. Main limitations identified are the lack of standardized protocols and the lack of
577 comprehensive data sets applicable to all potential contamination scenarios. However,
578 depending on their application, ENM contamination scenarios will likely concern specific
579 environmental compartments, whose assessment should be grouped and prioritized. For
580 instance, Ti-ENMs released from sunscreen consumer products accumulate in sediments in
581 near-shore freshwater and seawater environments and likely affect offshore environments to

582 a lower extent^{103,104,105}. Similarly, WWTP and constructed wetland effluents as well as river
583 sediments downstream of production sites were identified as hotspots of ENMs
584 contamination^{106,107}. In this context, mesocosms should be seen as tools for regional-scale
585 evaluations of such impacted areas, i.e. providing information about ENM fate and effects
586 under the most relevant conditions. While the current lack of standardization of mesocosms
587 translates to a lack of regulatory relevance, the same argument may be valid contrariwise, as
588 the lack of regulatory relevance currently hampers mesocosms use. Standardization may be
589 considered challenging, due to the overall complexity of mesocosms compared to other
590 more classic approaches. However, it must be noted that specific designs, e.g. for mimicking
591 pond and wetland environments, have been consistently applied in the last decade with
592 essentially identical procedures. Similarly, dual-tanks systems for mimicking the tidal cycle
593 have been applied and optimized to reproduce estuarine environments. These designs have
594 allowed for the study and comparison of several ENM classes in potentially affected
595 environmental compartments, and provide set up examples for future research
596 investigations. In general, no mesocosms design is *a priori* better than another, since the
597 design should be tailored to mimic the exposure scenario of interest. Nonetheless, the
598 design must be comprehensive of the ecosystem setting and complexity, for instance with
599 regard to micro-biota and organisms, which should be representative of both the planktonic
600 and benthic compartments. Biota populations and mesocosm sizes should also be
601 harmonized, in order to avoid stress or behavioural changes e.g. due to population densities.
602 According with the studies reviewed here, experimental periods of several weeks or months
603 are recommended, in order to allow mid- to long-term observations of ENMs fate,
604 ecosystem response and trajectory. Furthermore, an acclimation period is also
605 recommended prior contamination to better mimic the equilibrated ecosystem. Finally,
606 mesocosms are self-sufficient environments and once operating should not be extensively
607 manipulated except for sampling, e.g. with the addition of organisms, food sources or
608 nutrients. Future studies will need to further identify and characterize the most important
609 parameters influencing ENM exposure and hazard, which will in turn define the designs
610 applied to future experiments.

611
612
613 5. Conclusion
614

615 Mesocosms are increasingly being used for the study of ENM environmental fate and effects,
616 and were reviewed here with a focus on aquatic ecosystems. When compared to most SOP,
617 the advantage of mesocosms is the possibility to mimic realistic contamination scenarios,
618 comprehensive of natural complexity and synergies between contaminants and natural
619 components. Addressing multiple endpoints during dynamic ecological processes allows a
620 realistic, kinetic-oriented investigation of ENM exposure and hazards of target ecological
621 niches. Furthermore, mesocosms incorporate experimental periods of months to years, thus
622 allowing the long-term characterization of ENM contamination and ecosystem response and
623 recovery.

624 Different mesocosms designs were consistent in showing that ENMs entering the aquatic
625 environment will be predominantly removed from the water column and accumulate onto
626 the sediments. Statistical analysis suggests that such an accumulation will occur in the long-
627 term, independent of ENM physico-chemical properties. As a result, the potential risk for
628 aquatic benthos is defined by a higher accumulation of less reactive species (e.g. homo- /
629 hetero-aggregated, sulfidised). On the contrary, the risk for planktonic species will result
630 from a lower concentration of potentially more reactive ENM/metals.

631 Future studies will need to address current knowledge gaps, with special regard to the
632 identification of the most environmentally relevant ENMs/NEPs and their realistic dosing. In
633 addition, the scientific outcome to date had a stronger focus on the characterization of the
634 ENM fate than ecological effects and ecosystem responses, which are crucial for risk
635 assessment. Large and heterogeneous datasets are produced within mesocosms that should
636 be fully exploited. In this context, researchers should carefully define analytical and operative
637 strategies in advance, in order to generate findable, accessible, interoperable and reusable
638 data for the nanosafety community and stakeholders.

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