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Inclusion of eco-corona formation and biotransformation in regulatory nanomaterial ecotoxicity and fate testing: review and insights from the MISTRA environmental nanosafety project

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Abstract

Background The eco-corona, consisting of environmental biomolecules formed around engineered nanomaterials (ENMs) when released to the environment, has gained increasing focus in the scientific literature and its role for ENM fate and toxicity is now widely acknowledged. The European chemicals legislation, REACH, entails reporting requirements when it comes to the transformation of nanoforms. Guidance provided by the European Chemicals Agency (ECHA) highlights eco-corona and biotransformation as relevant transformation processes. Still, no specific advice is given on how to test these processes. Based on the findings from the MISTRA Environmental Nanosafety project, we here map out methods to characterise ENM eco-corona and biotransformation and assess their effects. Furthermore, the regulatory relevance of the methods is evaluated.

Results We identified methods to assess both eco-coronas formed ex vivo (by interaction with natural organic matter-based solutions or solutions with animal secretes) and bio-coronas formed in vivo (via biotransformation, i.e., filtration of ENMs through living organisms). We recommend implementing these methods and methodological considerations in a future update of ECHA's guidance on ENM ecotoxicity and fate testing, both in the sections on transformation and aquatic pelagic toxicity. When exploring the characteristics and kinetics of eco-corona formation, various data are needed, including data on time-dependent interaction/adsorption/desorption between ENM and constituents in the medium (both with and without the addition of natural organic matter/biomolecules). It is, furthermore, proposed that environmental relevance is enhanced for hazard assessment of nanoforms in REACH. This can be done by incorporating eco-corona considerations in the persistency, bioaccumulative, and toxicity (PBT) assessment.

Conclusions We here propose to update ECHA 's guidance on ENM ecotoxicity testing and the PBT assessment required under REACH to include eco-corona considerations. If updated, this will aid in implementing information requirements on ENM transformation, increase the environmental relevance of ENM ecotoxicity tests, and reduce uncertainties in the extrapolation of ENM ecotoxicity data.

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Introduction

Engineered nanomaterials (ENMs) possess unique properties governed by a combination of their size, shape, surface chemistry, and phase composition, which enable their use in a wide range of applications [2]. The increasing consumption of ENMs in the European market [22] underpins the importance of ENM regulations to ensure safe and sustainable use. One of the main legislations relevant to ENMs in the European Union is the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulation [17]. From January 1st, 2020, a set of revised annexes to REACH came into force, making manufacturers, importers, and downstream users of nanoforms subject to a comprehensive set of nano-specific information requirements, including physico-chemical characterisation, environmental fate, and toxicity [9]. For instance, they have to report on the names or other identifiers of the nanoforms or sets of similar nanoforms of the substance as part of the substance identification. They also have to provide number-based particle number size distribution, surface functionalization or treatment, shape and aspect ratio, specific surface area by volume and/or by mass of these nanoforms. Specific toxicity-related test requirements have to be fulfilled by registrants for different nanoforms depending on their nature and likely route of exposure. For instance, an acute toxicity study has to be provided for the oral route or the inhalation route and at least one other route for substances manufactured or imported in 10 tonnes or more per year [23]. It is noteworthy that there are no nano-specific information requirements for ecotoxicity beyond that long-term testing should be considered early and some waivers should not be used for nanomaterials [9]. More recently, the European Commission updated the recommended definition of nanomaterials from 2011 [10, 23].

The European Chemicals Agency (ECHA) provides technical guidance to support manufacturers and importers of industrial chemicals meet the legal requirements of REACH [13]. The technical guidance constitutes essential support for the fulfilment of the legal requirements to ENMs, especially when it comes to assessing the risks of the materials, as standardised and suitable ENM risk assessment methods are still lacking in certain areas and/or hold challenges [49, 58]. ECHA recently updated their guidance document on physico-chemical properties and toxicological information requirements of ENMs [12]. However, the guidance document 'Appendix R7-1 for nanomaterials applicable to Chapter R7b Endpoint specific guidance' for ecotoxicological endpoints for ENMs has not been updated since 2017 [11]. The pending update of the nano-specific annex of ECHA Guidance R7b potentially leaves manufacturers and importers of ENMs unclear about how they can—or should—meet these requirements. Scientific research has, furthermore, advanced considerably since 2017 when it comes to ecotoxicological and fate testing of ENMs, e.g., for ecotoxicological algal testing (e.g., [3, 36, 60, 61]), ecotoxicity in sediment (e.g., [59, 66, 67]), trophic transfer (e.g., [16, 34, 40, 43, 44]), and species sensitivity distribution modelling (e.g., [62, 64]).

While advancements have been made in understanding exposure and effects in simplified test systems, much still remains to be discovered when introducing more complex chemical settings that to a higher degree resemble actual environmental exposure. One such example is the formation of eco-coronas, which is the result of the interaction between the ENM and natural biomolecules upon environmental entry, creating an adsorbed layer on most ENMs (partly or fully) [46]. The formation of such eco-coronas influences the chemical and physical properties of the ENMs, thereby potentially changing their stability, mobility, and toxic properties [4, 5, 15, 19, 24, 29, 31, 32, 38, 47, 68].

In ECHA's current guidance on ecotoxicity and fate testing [11], information on eco-corona formation and biotransformation is limited. Both are mentioned as important transformation processes that can influence the environmental behavior and fate of ENMs. However, no specific advice on how to test for eco-corona formation and biotransformation is provided. ECHA [11] describes that interactions between the test media and the test material should be considered as preparation before testing. It is further described that this includes interactions with dissolved organic matter (DOM) and NOM, and reference is made to the Organisation for Economic Co-operation and Development (OECD)'s guidance document on aquatic and sediment toxicological testing of nanomaterials [53]. In OECD [53], it is stated that DOM or NOM may be used as a stabilising agent when there is a "strong desire" to stabilise the test ENM or when "site-specific environmental relevance is sought". OECD [53] further describes that the effect of potential dissolution should be considered, as DOM may alter the bioavailability of released ions. In addition, tests both with and without DOM or NOM should be performed, and the most conservative hazard value of the

two tests should be applied. OECD [53] also states that suitable control tests should be conducted, such as tests with only DOM or NOM, and stresses the importance of characterising the DOM/NOM. In Table 6 in OECD [53], a test reporting checklist is presented. For 'test material stability in test media', reporting should include "agglomeration", "settling" (including, e.g., morphology changes), and "transformation/degradation". The latter includes "dissolution, change in dissolved ion concentration and/or MN diameter". Potential transformations must be assessed in the stock, test dispersions, and the test systems. Studies have, for example, clearly shown that solution sonication and further dilution of the stock solution considerably influence the ENM characteristics, dissolution, and administered ENM dose [31, 57]. Furthermore, potential effects on toxicity should be considered where relevant. Finally, coating/surface functionalization stability is listed as a reporting consideration. It is described that environmental realism should be integrated into the part of the ENM risk assessment that relates to exposure assessment, although environmental realism is beyond the scope of the guidance document, as stated in OECD [53].

As is evident, some attention is given to ENM interaction with NOM in ECHA and OECD's guidance documents [11, 53], but there is a lack of specific methodological advice on how to test for these interactions. This is also highlighted in Nasser et al. [47] and Nasser and Lynch [46], which stress the need for updating international test protocols to include the influence of the eco-corona. In the scientific literature, methodological approaches have been proposed for, e.g., characterising small metabolite coronas in freshwater ecotoxicology with *Daphnia magna* and *Chlorella vulgaris* [21] and for facilitating eco-corona formation prior to ecotoxicity testing of *D. magna* [47].

The aim of this study has been to provide recommendations for the inclusion of eco-corona and biotransformation in ENM ecotoxicity and fate testing. The recommendations are targeted towards a future update of ECHA's guidance on ecotoxicity and fate testing [11] rather than the OECD GD, because the ECHA guidance provides specific advice directly to registrants on how to test for eco-corona formation and biotransformation. The recommendations are targeted at scientists and regulators working with ENMs and are based on findings from the MISTRA Environmental Nanosafety research project. In this project, a dedicated focus was directed towards eco-corona formation and its consequences for regulatory risk assessment, resulting in more than 60 scientific papers published since 2014 and listed at [41, 42]. These papers cover the topics of environmental transformation, fate, and effects of ENMs upon release into natural ecosystems, as well as proactive risk assessment, regulation, and safe handling of ENMs during end-of-life [42]. Out of the more than 60 papers, a little more than half are relevant to the topics of this paper. The forthcoming update of ECHA's guidance document on ecotoxicological and fate testing of ENMs [11] should consider this new knowledge pool. Here, we illustrate (by use of the MISTRA Environmental Nanosafety project as an example) how results from scientific projects could be used to provide recommendations on regulatory risk assessment. In the current study, technical advancements related to testing ENMs in complex settings are the focal point, i.e., considering eco-corona formation and biotransformation. Eco-corona is here defined as "*coronas formed and acquired by the ENM in the environment* via *interaction with macromolecules such as NOM or secreted biomolecules*". The definition is in line with Lynch et al. [35], which was one of the first studies to define and establish eco-corona as a term in the scientific literature. The corona formed via biotransformation is here termed biocorona and is also defined in line with Lynch et al. 35 as "*coronas formed by macromolecules produced* via *biological processes and acquired by the ENM as it interacts with living systems*". The recommendations provided in this study focus on lower trophic level organisms, i.e., algae and daphnia. These organisms are relevant as they make up the lower parts of the food chain and thus, potential effects occurring at this level can have consequences throughout the trophic chain. This focus also conforms with the efforts to reduce the use of vertebrate testing, and although cell lines enable high-throughput screening, their relation to whole organism effects is not fully established yet.

Methods

The ECHA guidance provides general advice on how to perform nanomaterials ecotoxicity and fate testing (Sect. 1.1), as well as specific advice for endpoints, such as aquatic pelagic toxicity (Sect. 1.2.1), toxicity for sediment organisms (Sect. 1.2.2), and degradation/biodegradation/transformation (Sect. 1.2.3) [11].

First, we analysed the more than 60 scientific articles that have been published in the MISTRA project that can be found at [41, 42] to thematically assess how they might be useful in a future update of the different sections of the ECHA guidance. This includes papers published during both phase I (2014–2018) and phase II (2018–2023) of the project. Second, we reviewed the papers identified as relevant for each section in the ECHA guidance with regard to key findings, strengths, and notable future research needs. Based on these analyses, we provide recommendations for additions to the future ECHA guidance document revision.

Results

Influence of eco-corona formation on ecotoxicity and environmental fate

One aspect that has been subject to several studies is ENM transformation, interaction with NOM, eco-corona formation, and adsorption of natural molecules. In ECHA's guidance on ENM ecotoxicity and fate testing (2017), it is described that no standard method is currently available when it comes to adsorption–desorption. Specifically, ECHA [11] refers to its guidance documents on physico-chemical properties and toxicological information requirements of ENMs, which since then have been updated [12]. According to ECHA [12] and Nielsen et al. [49], most of its advice on adsorption–desorption is still not relevant to most ENMs—in particular those with high dissolution or low dispersion stability. Similarly, no advice on how to assess corona formation is available [11, 12]. To address this, the following recommendations are provided.

Formation of eco-corona on ENMs can influence the ENM toxicity and behaviour and is, therefore, important to consider upon ENM ecotoxicity testing. In a meta-analysis of the scientific papers published up to 2020, Arvidsson et al. [1] found that EC_{50} and LC_{50} values obtained in short-term aquatic ecotoxicological tests without NOM addition generally could be used as conservative proxies for test results obtained with NOM addition for risk assessment purposes. This is supported by a review by Natarajan et al. [48] that looked into studies testing the effect of eco-corona on nanomaterial aquatic toxicity and found that NOM tends to decrease the toxic effect of nanomaterials. In long-term tests, Ekvall et al. [15] found that survival of *D. magna* exposed to tungsten carbide cobalt (WC–Co) NPs and cobalt (Co) NPs was higher when the NPs were mixed with eco-corona biomolecules (from *D. magna* feeding on algae) prior to the test. The toxic potency of the NPs was thereby reduced by the eco-corona, and the findings suggest that this reduction happened at the particle–organism interaction, as the eco-corona did not seem to influence nanoparticle (NP) uptake. The study indicates that at least under some circumstances, ENM toxicity is reduced upon environmental release. It was supported by the results obtained by Khort et al. [32], showing that pre-exposure of yttrium oxide (Y_2O_3) ENMs in eco-corona biomolecules increases survival probability of *D. magna* in the case of higher Y_2O_3 concentration (100 mg/L). Similarly, Khort et al. [31] also observed reduced toxicity of NPs under more natural conditions, i.e., presence of NOM and weathering. The study examined the effect of weathering (transformation/dissolution) and NOM adsorption (eco-corona formation) on the toxic potency [i.e., cytotoxicity, effect on membrane permeability, mitochondrial activity, lysosomal activity, and reactive oxygen species (ROS) generation] of metal and metal oxide NPs to piscine cell line RTGill-W1. In general, NOM reduced the intracellular ROS generation by 20–40% for all tested NPs. For tin (Sn) NPs, NOM reduced cytotoxicity due to stronger interaction with Sn NPs and thereby increased dissolution. This was, however, not seen for manganese (Mn) and nickel (Ni) NPs. The formation of an eco-corona (derived from excreted *D. magna* biomolecules) also showed reduced toxic effect of Y_2O_3 NPs when exposed to *D. magna* in a study by Kelpsiene et al. [29]. The decrease in toxicity was, however, only observed at the highest NP concentration (10 mg/L) and for NPs of sizes 30–45 ENM. For lower NP concentrations and other particle sizes, no effect of the eco-corona on toxicity was observed.

Another study to find mixed/limited effects of eco-corona was conducted by Mei et al. [40]. The authors tested the influence of eco-corona, in the form of excreted biomolecules from *D. magna*, on Co NPs on uptake/transfer in an aquatic food web consisting of algae (*Scenedesmus sp.*), zooplankton (*D. magna*), and fish (*Carassius carassius*). Low or no interaction, in terms of heteroagglomeration, was observed between the algae and biomolecules, and this likely explains the lack of an impact the biomolecules had on the Co uptake and transfer throughout the food web [40]. Low interaction between NOM and NPs was also observed in Hedberg et al. [24] that investigated interactions between WC NPs and humic as well as hydroxybenzoic acid. The low interaction resulted in considerable agglomeration and sedimentation of the WC NPs. Hedberg et al. [24] also studied the interaction of the WC NPs with *D. magna* and found that WC was taken up in the organisms, despite the sedimentation. The WC uptake caused no acute toxicity to *D. magna*.

Based on this, it is recommended to test the aquatic toxicity on weathered/transformed ENMs and in media that to a higher degree represent natural waters. Specifically, this means with the presence of natural biomolecules, such as done in the experiments performed by Ekvall et al. [16]. In the following, methodological approaches on how to test the influence and characteristics of eco-corona formation are provided.

Methods for eco-corona preparation/weathering

To prepare the NOM-containing test solution, Suwannee River NOM (obtained from the International Humic Substances Society, USA, presented as a heterogeneous mixture of various molecules, predominantly fulvic and humic acids) should be introduced into artificial freshwater to achieve a NOM concentration of 10 mg/L (Fig. 1A). The solution should be magnetically stirred for 2 h to

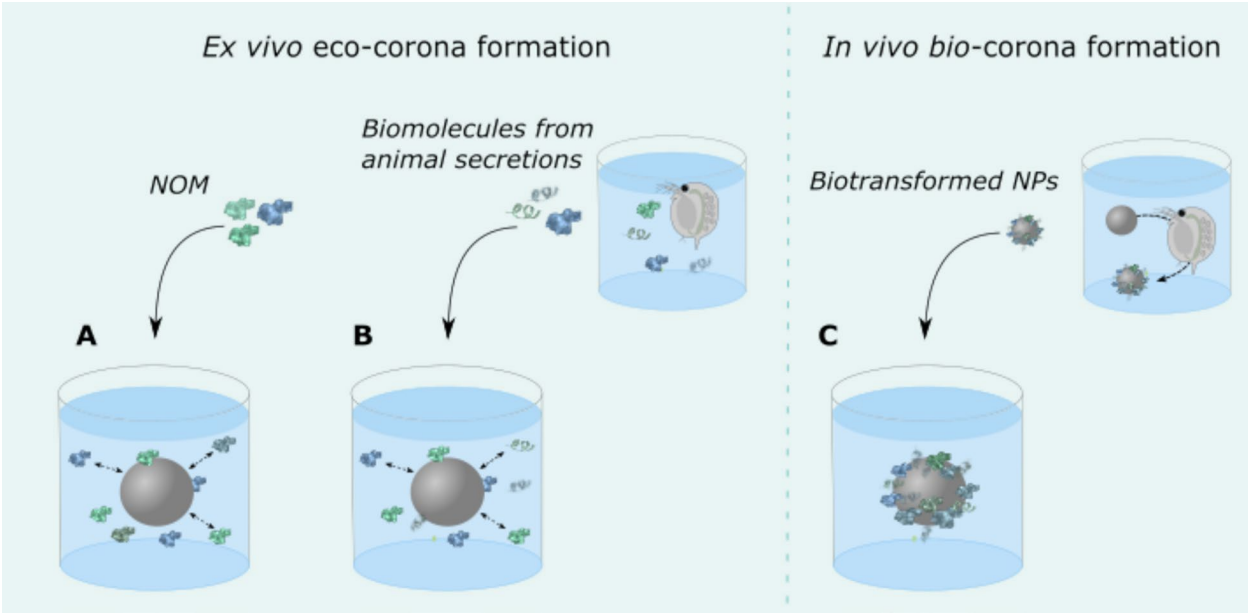

Fig. 1 Examples of different coronas obtained in experimental test systems. Ex vivo eco-corona formation involves adding nanoparticles (NPs) to aquatic media containing natural organic matter (NOM) (**A**) or to solutions with biomolecule secretions from organisms (**B**). In vivo bio-corona formation includes biotransformed NPs (e.g., NPs ingested and egested by living organisms such as *Daphnia magna*) (**C**). Modified from Nielsen [50] and inspired by Wheeler et al. [68]

ensure homogeneity. Subsequently, the pH of the solution needs to be adjusted to 6.2 using nitric acid (HNO_3) or sodium hydroxide (NaOH). The prepared solution should be stored in a refrigerator at approximately 4 °C. Before use, the solution needs to undergo additional magnetic stirring for 30 min, and the pH should be adjusted if necessary [5, 24, 30–32, 57].

Excreted biomolecules from *Daphnia magna* feeding on green algae (Fig. 1B) can be collected and used to prepare eco-corona biomolecule solutions. Following the procedure in Ekvall et al. [15], *D. magna* should feed on algae for 2 weeks, after which *D. magna* and remaining algae and debris products are filtered out of the solution. The biomolecules left in the solution can then be bound using a strong anionic exchanger. The predominant components in the eco-corona biomolecules were lactate ($CH_3CH(OH)COOH$) and unidentified metabolites of fatty acids, characterized by methyl ($–CH_3$) groups linked to methine (=CH–), hydroxyl (–OH), or nitrogen, along with methylene ($–CH_2$) groups of an unknown nature [15]. To enable comparison to proposed OECD Test Guidelines for NP dispersion stability, the equivalent total organic carbon (TOC) concentration should be 10 mg/L [43, 51]. The resulting solution should undergo magnetic stirring for 2 h to ensure homogeneity. Subsequently, the pH is adjusted to 6.2 using HNO_3 or NaOH. In general, the prepared solution should be used promptly to minimise the risk of biomolecule degradation. Alternatively, it can be stored in a refrigerator at around 4 °C. In the latter case, before use, the solution should undergo an additional 30 min of magnetic stirring, and the pH needs to be readjusted if necessary to maintain consistency and reliability in experimental conditions [5, 32].

In both scenarios, NP powders should be dispersed in ultrapure water through tip sonication in a constant mode with 20% amplitude (tip diameter is 6 mm) in acid-cleaned glass vials to create stock solutions of NPs at a concentration of 1 g/L before the addition of the eco-corona. Following this, the stock solution should be further dispersed into test solutions using Vortex to achieve the desired NP concentration. The sonication duration, either set at 3 min [24],Kelpsiene et al. [29], 5 min [5, 30, 31], or determined specifically for the ENM of interest [32]—in this case also 5 min, to ensure optimal dispersion (and lowest amount of ENM dissolution and surface changes) within the shortest possible time [57].

Methods for eco-corona characterization

To investigate and characterise the kinetics and extent of adsorption of biomolecules onto the NPs creating the eco-corona, several studies applied attenuated total reflection–Fourier transform infrared spectroscopy (ATR–FTIR) with a platinum ATR–IR accessory for in situ investigations (e.g., [5, 15, 31, 32, 39, 40]). The accessory is composed of a diamond crystal with a 45° angle of incidence for the IR beam onto which a layer

of the NPs of interest was created [5, 31, 32, 40]. The method has been applied to, e.g., Co, Sn, Ni, Mn NPs and several metal oxides [5, 31, 32], Cu, Al, Mn, and SiO_2 [57] as well as WC–Co NPs [15]. In Khort et al. [31], adsorption was studied in freshwater with and without NOM. Background spectra were measured in ultrapure water and subsequently solutions of freshwater (with and without NOM) were added. Spectra were then collected every 10th–20th minutes up to 6 h. Finally, the NP layer was rinsed with ultrapure water to investigate the strength of the adsorption. In the study conducted by Khort et al. [32], the dynamic change in peak areas of the ATP–FTIR spectra, acquired at 20-min intervals, was conducted to quantify the dynamic change in the adsorption of carboxylates, carbonates, and sulphates on the surface of Y_2O_3 ENMs, exposed to either freshwater only or freshwater containing eco-corona biomolecule solutions. The data were used to calculate the kinetics of the adsorption of functional groups of the eco-corona.

Khort et al. [31] found that in solutions with NOM present, NOM adsorbed to most of the NPs, resulting in smaller negatively charged colloids compared to treatments without NOM. A somewhat related study, Mei et al. [39], looked at interactions between Co NPs and biomolecules of different structures and properties in phosphate-buffered saline—simulating physiological conditions. The study aimed to explore the adsorption of different biomolecules, whether bio-corona formation affects Co NPs stability and mobility as well as prevents Co NPs agglomeration, and finally, the effect of bio-corona formation and biomolecule characteristics on NP dissolution. Adsorption of the different biomolecules to Co NPs differed depending on surface characteristics and NP dissolution. Low or no adsorption of amino acids was observed, while larger biomolecules (e.g., polyglutamic acid and polylysine) adsorbed onto the Co NPs in parallel with the adsorption of phosphate in the bio-corona. The study recommended taking the presence of phosphate and biomolecule content of test media into account in ENM ecotoxicity testing, since they both influence the surface and particle characteristics, as well as dissolution patterns, and largely depend on both ENM and biomolecule type and characteristics. The study by [39] used ATR–FTIR for in situ studies of biomolecule adsorption to a Co NP layer (deposited on an ATR crystal). The Joint Expert Speciation System (JESS) was applied to perform solution speciation estimations to gain knowledge on the complexation of Co ions and amino acids and phosphate. X-ray photoelectron spectroscopy (XPS) was used to study the composition of the outermost surface of the NPs. Another study to apply ATR–FTIR to measure ENM surface adsorption is Pradhan et al. [57]. Here, adsorption of humic acid and dihydroxy benzoic acid (DHBA), representing a degradation product of HA, onto Cu, Al, Mn, and SiO_2 NPs in synthetic freshwater was studied. The study investigated the stability, mobility, and dissolution of the NPs. Finally, Hedberg et al. [24] also applied ATR–FTIR to investigate biomolecule (in the form of humic acid or DHBA) adsorption to WC NPs. Low or no adsorption was observed, which could hinder high agglomeration and sedimentation of the NPs (due to density and strong van der Waals forces).

Another approach to investigate ENM adsorption processes is predictions using molecular dynamic (MD) simulations, which, e.g., were applied by Kolman and Abbas [33]. Here, adsorption of four derivatives of benzoic acid (common degradation products of organic matter) onto charged silica surfaces was studied. The authors developed a MD silica model and explored aggregation (with clustering simulations), interaction between single molecules and silica surfaces (pulling simulations), and adsorption of multiple molecules onto silica surfaces (adsorption simulations). By use of the simulations, it was explored how the molecular structure of model molecules, pH (ranges: basic, neutral, and acidic), and ion concentration influence adsorption.

When investigating ENM adsorption upon exposure to aquatic environments, information on changes in physico-chemical characteristics such as hydrophobicity, mobility, stability, dissolution, and surface composition can be determined in situ. Hydrophobicity can be measured by dye staining [7, 49]]; changes in stability in terms of particle size distribution and extent of sedimentation can be assessed using different light scattering methods, such as photon cross-correlation spectroscopy (PCCS) (e.g., [57, 70]), laser diffraction spectroscopy (LDS) (e.g., [55]), dynamic light scattering (DLS) [65], nanoparticle tracking analysis (NTA) (e.g., [5, 32, 37]]), and differences in surface charge by means of zeta potential measurements (e.g., [31, 65, 71]]); dissolution using single particle inductively coupled plasma–mass spectrometry (ICP–MS) (e.g., [6]) or atomic absorption spectroscopy (AAS) (e.g., [31]) and ATR/FTIR (e.g., [32, 45]).

An important general aspect to consider when studying ENM surface transformations (such as adsorption and eco/bio-corona formation) is that these transformations are particle surface- and environment-specific [5, 31]. For ENM transformation testing, the composition of the ENM surface and the expected environmental fate prior to testing must be considered. In addition, surface transformation is time- and exposure-dependent [5], stressing the importance of adjusting these parameters to reflect the expected exposure scenario. The presence of NOM also seems to affect other transformation processes of ENMs (if NOM adsorbs to the particles). Chang

et al. [5] found that the presence of both NOM and eco-corona biomolecules reduced NP agglomeration and may thereby enhance mobility. Khort et al. [30, 31] similarly found that NOM, as well as eco-corona biomolecules adsorption to NPs "can provide some colloidal stability".

Testing nanomaterial bio-corona formation during biotransformation

A bio-corona may form in vivo as a result of biotransformation after uptake of an ENM in an organism (Fig. 1C). Kelpsiene et al. [28] investigated the biotransformation of polystyrene NPs, more specifically, the uptake and transfer of the NPs through the digestive tract of *D. magna*. The study compared small aminated PS NPs, which are known to be acutely toxic (in sizes around 50 nm), and larger (200 nm) aminated or small and larger carboxylated PS NPs that are non-toxic in acute testing. When passing through *D. magna*, the small toxic aminated PS NPs bound smaller, more acidic, and a higher amount of proteins when compared to non-toxic carboxylated PS NPs. In addition, the study showed that besides proteins, other substances bound to the NPs, e.g., triglyceride lipids.

Mattsson et al. [38] investigated gold (Au) NPs filtrated through *D. magna* and compared their aggregation and protein adsorption to Au particles incubated with excreted proteins from *D. magna* and to particles in media only. Filtrated and protein-incubated Au NPs were shown to exhibit less aggregation than particles in media only. This shows that biological interaction with NPs may change their aggregation state and behavior during an on-going experiment [38]. Frankel et al. [19] also observed a change in aggregation when protein-induced PS NP aggregates were filtrated by *D. magna* and attributed the aggregate size decrease to protein digestion in the zooplankton intestine. Interestingly, the filtrated Au NPs bound fewer unique proteins than Au NPs incubated with secreted proteins, with only a few overlapping proteins between the two conditions, indicating that the bio-corona may change on a short temporal scale [19]. Furthermore, particles undergoing preconditioning, i.e., only incubation of excreted biomolecules, may not properly represent natural conditions.

Kelpsiene et al. [29] found that upon exposure to *D. magna*, the bio-corona on Y_2O_3 NPs was mainly composed of proteins, such as Cu–Zn superoxide dismutase, apolipophorins, and vitellogenin-1, irrespective of the NP size. However, larger Y_2O_3 NPs (30–45 nm) showed a higher number of associated proteins in the bio-corona. The process of biotransformation was shown to influence the toxicity of the NPs, with prefiltration by *D. magna* leading to a reduced dissolution of yttrium and an increased uptake of both dissolved and non-dissolved Y_2O_3, which was found to be size-dependent. Notably, the biotransformation process, through the adsorption of natural biomolecules to the NP surface, altered the toxicological outcomes, as prefiltration of certain sized NPs (30–45 nm) decreased the toxic effects on *D. magna*. The study underscores the complex interplay between the physicochemical characteristics of NPs and the biological environment, which can lead to a varied toxicological response in aquatic organisms.

In the study by Mei et al. [40], also described in Sect. "Influence of eco-corona formation on ecotoxicity and environmental fate", the influence of eco-corona formation (composed of organic matter excreted by *D. magna*) on trophic transfer was, furthermore, explored for Co NPs in a food web consisting of algae (*Scenedesmus sp.*), zooplankton (*D. magna*), and fish (*Carassius carassius*). Algae were pre-exposed to Co NPs (with and without excreted biomolecules from *D. magna*) and then fed to *D. magna*. As mentioned, excreted biomolecules did not change Co NP uptake in *D. magna* when feeding on the algae—likely because of low/no heteroagglomeration between the algae and Co NPs. Accordingly, the presence of eco-corona formation from biomolecules did not affect the uptake of Co in the fish that was subsequently fed with the daphnia [40]. However, the Co NPs were transferred through the trophic levels in the food chain, and during this, they gained different properties. Mei et al. [40], therefore, recommend that risk assessment of ENMs is conducted on weathered and transformed particles instead of pristine ones. To measure the kinetics of adsorption of algae and excreted biomolecules from *D. magna* onto Co NPs, they conducted in situ measurements by means of ATR–FTIR using a Bruker Tensor 37 FTIR spectrometer with a platinum ATR–IR accessory comprised of a diamond crystal with an angle of incidence of 45° for the IR beam. An MCT (mercury cadmium telluride) detector with a ZnSe window with a working wavenumber range around 650 cm^{-1} was applied to improve the sensitivity in adsorption measurements on Co NP films. Furthermore, the study explored total concentrations of Co derived from heteroagglomeration with Co NPs and algae inside *D. magn*a and *C. carassius*, as well as dissolved Co NPs in simulated fish gut solutions and stomach. For these measurements, graphite furnace–atomic absorption spectroscopy (GF–AAS) was applied. Finally, Mei et al. [40] assessed the apparent surface charge/zeta potential of the Co NPs in different solutions, i.e., tap water, algae solution, and algae solution with excreted biomolecules, to achieve information on particle stability in solution.

Discussion

Update of ECHA's guidance regarding transformation

Tables 1 and 2 list scientific approaches to characterise and test the influence of ENM eco-corona formation and biotransformation. We believe that the methods can provide helpful support for registrants of nanoforms under REACH [9] if taking into consideration in the technical guidance provided by ECHA on ENM ecotoxicity and fate [11]. As mentioned in the introduction, ECHA [11] highlights eco-corona formation and biotransformation as important transformation processes under section '1.2.3.3 Transformation', but contains no specific instructions on how to assess them. Table 1 provides methods for testing the formation of ENM eco-corona.

When investigating eco-corona formation, information should be obtained regarding, first of all, kinetics, i.e., time-dependent interaction/adsorption/desorption between medium constituents and the NPs (both with and without the presence of NOM/biomolecules). These processes can greatly influence NP dissolution and aggregation/agglomeration [25] and it is, therefore, also important to obtain information on time-resolved dissolution rates, both with and without NOM/biomolecules, and time-dependent stability/sedimentation, both with and without NOM/biomolecules (see Table 1). To gain information on dissolution and dispersion stability, guidance documents (such as OECD's test guideline 318 on dispersion stability of ENMs in simulated environmental media [51] and OECD's guidance document for the testing of dissolution and dispersion stability of ENMs and the use of data for further environmental testing and assessment strategies [52] can be of help. For the methods presented in Table 1, testing environmentally relevant concentrations may be challenging if particle concentrations are lower than the detection limits. Furthermore, especially for metal and plastic NPs, environmentally relevant aquatic concentrations are generally unknown [32].

Table 2 provides methods for testing bio-coronas formed in vivo via biotransformation, and similarly, it is indicated which methods are relevant for Sect. 1.2.3.3 on transformation. As can be seen, the methods require somewhat more complex test setups that have so far not been applied as broadly. However, the identification of proteins on NPs after filtration can, as seen above, give interesting information on how the bio-corona can be formed inside living organisms and influence the behavior, e.g., by reducing aggregation [38]. For application and further development of such methodological approaches, some experimental challenges must be considered, which to some extent make evaluation and extrapolation of the toxicity results more difficult. In practice, the number of NPs in an experiment must be high enough to detect and identify the proteins. This means that the concentration of particles in the published studies is magnitudes higher than what is desired in toxicity tests aimed at simulating environmentally relevant exposures. High concentrations may cause other problems, such as fast mortality (if NPs are acutely toxic, as in the case of small aminated PS NPs) or a mechanical effect on the *D. magna*, potentially resulting in debris falling off from the animals—a phenomenon that will ruin the protein analyses.

Update of ECHA's guidance regarding aquatic pelagic toxicity

In the chemical safety assessment required by REACH for nanoforms registered in quantities of more than 10 tonnes, ENM transformation should be characterized as part of the exposure assessment. Furthermore, the OECD's guidance document on aquatic and sediment toxicological testing of nanomaterials [53] describes that environmental realism should be incorporated in the exposure assessment step of a risk assessment, as described in the introduction. These are important requirements and advice on exposure when it comes to promoting environmental relevance in regulatory risk assessment. We propose that emphasis is also put on incorporating environmental relevance in the element of risk assessment that relates to hazard assessment. This is also urged by Nasser et al. [47], which propose updating international protocols to include biomolecules as part of ecotoxicity testing, since ecotoxicity tests without biomolecules can produce misleading results. Nasser et al. [47] point to different mechanisms by which eco-coronas can influence NM ecotoxicity. For example, eco-coronas can lead to both decreased and increased NM agglomeration, and in cases where agglomeration increases, organisms like *D. magna* can find the particles more attractive as food, especially combined with the organic coating of the particles. Increased NM uptake can lead to increased toxicity, dependent on biomolecule concentration in the medium and duration of the interaction between NM and biomolecules [47]. While we highly support the inclusion of eco-corona formation aspects in international protocols (e.g., OECD) on ENM ecotoxicity testing, we advocate that guidance is supplied in the meantime to help registrants of nanoforms increase the environmental relevance of their ecotoxicity tests, although eco-corona formation is not part of the information requirements for ecotoxicity in REACH. Methodological approaches for testing the influence of eco-corona (Table 1) and biotransformation (Table 2) on ENM toxicity are, therefore, proposed here with relevance for section '1.2.1 Aquatic pelagic toxicity' in ECHA [11].

Table 1 Methods identified to prepare ENM eco-corona/weathering, to characterise eco-corona and to assess eco-corona effect

Aim	Method	NP type	References
Preparation of NOM-containing solutions with Suwannee River NOM	10 mg NOM/L artificial freshwater, 2 h magnetically stirring, pH to 6.2. 4°C Storage. Before use, 30 min of magnetically stirring and adjust pH again	WC, Co, Ni, Co–Ni, Mn, Sb, ZnO, NiO, Sb_2O_3, Y_2O_3, Co_3O_4, Cu, Al, SiO_2	[5, 24, 31–33, 57]
Preparation of eco-corona-based solutions, using excreted biomolecules from *Daphnia magna* feeding on green algae	Biomolecules extracted from *D. magna* and green algae solution. Dilute to 10 mg biomolecules/L artificial freshwater. 2 h magnetically stirring, pH to 6.2. Store at 4°C. Before use, magnetically stir for 30 min and adjust pH	WC–Co, Co NPs	[5, 15, 32]
Adsorption of algae and excreted biomolecules from crustacean *D. magna* onto NPs	ATR–FTIR by use of a Bruker tensor 37 FTIR spectrometer with a Platinum ATR–IR accessory (ATR–IR accessory comprised of a diamond crystal with an angle of incidence of 45 for the IR beam) Creation of film of NPs on the ATR crystal by particle dispersion of 25 mg NPs in 6 mL ethanol using tip sonication. Transfer of300–350 µL sonicated solution to ATR crystal and leave NP film to dry in ambient air (2 h)	Co, WC–Co, Sn, Ni, Mn, Cu, Al, SiO_2, Y_2O_3, NPs	[5, 15, 31, 32, 39, 40, 57]
Adsorption of biomolecule in the form of humic acid or dihydroxybenzoic acid (DHBA) to NPs	ATR–FTIR by use of a Bruker tensor 37 FTIR spectrometer with a Platinum ATR–IR accessory NP film is created as described on row above (row 3)	WC NPs	[24]
Adsorption of algae and excreted biomolecules from crustacean *D. magna* onto Co NP films	ATR–FTIR by use of a Bruker tensor 37 FTIR spectrometer with a Platinum ATR–IR accessory. A MCT (mercury cadmium telluride) detector with a ZnSe window with working wavenumber range around 650 cm-1 NP film is created as described on row 3	Co NPs	[40]
Complexation of Co ions from Co NPs and amino acids and phosphate/Complexation of free tungsten (W) with simulated surface water and dihydroxybenzoic Acid (DHBA)	Solution speciation estimations by use of Joint expert speciation system (JESS)	Co NPs/WC NPs	[24, 39]
Composition of the outermost surface of the NPs	X-ray photoelectron spectroscopy (XPS)	Co NPs	[39]
Interaction between organic molecules and solid surfaces	Silica Molecular Dynamics model developed to explore aggregation (with clustering simulations), interaction between single molecules and silica surfaces (pulling simulations) and adsorption of multiple molecules onto silica surfaces (adsorption simulations) (to compare/supplement experimental data)	Charged silica surface (not NP)	[33]
ENM hydrophobicity in aquatic environments	Dye staining	-	[7, 49]
Uptake of Co derived from heteroagglomeration with Co NPs and algae inside *D. magna* and *C. carassius*	Graphite furnace atomic absorption spectroscopy (GF-AAS)	Co NPs	[40]

Table 1 (continued)

Aim	Method	NP type	References
Changes in toxicity of NPs towards *D. magna*	Evaluation of survival of *D. magna* exposed to Y_2O_3 NPs in different test solutions	Y_2O_3 NPs	[29, 32]
Changes in particle size distribution of Co NPs in tap water with algae solution with and without the presence of excreted biomolecules	Dynamic light scattering spectroscopy (DLS) by means of photon cross-correlation spectroscopy (PCCS)	Co NPs	[40]
Changes in surface charge of Co NPs in tap water, algae solution and solution with excreted biomolecules	Measurement of zeta potential by a Zetasizer Nano ZS instrument using the Smoluchowski Method	Co NPs	[40]
Changes in surface charge of NPs in freshwater with eco-corona solution	Measurement of zeta potential by a Zetasizer Nano ZS instrument using the Smoluchowski Method	Co NPs, Ni NPs, NiO, Co_3O_4 NPs	[5, 32]
Changes in surface charge of NPs in freshwater with eco-corona solution	Measurement of zeta potential by a Zetasizer Nano ZS instrument using the Smoluchowski Method	Y_2O_3 NPs	[32]
Changes in colloidal stability of NPs in freshwater with eco-corona solution	Measurements of particles concentration, median size and size distribution by Nanoparticle Tracking Analysis	Co NPs, Ni NPs, NiO, Co_3O_4 NPs	[5]
Changes in colloidal stability of NPs in freshwater with eco-corona solution	Measurements of particles concentration, median size and size distribution by Nanoparticle Tracking Analysis (NTA)	Y_2O_3 NPs	[32]
Changes in dissolution of NPs and NPs in freshwater with eco-corona solution (biodissolution)	Graphite furnace–atomic absorption spectroscopy (GF–AAS)	Co, Co_3O_4 NPs	[5]
Changes in dissolution of NPs in freshwater with eco-corona solution (biodissolution)	Inductively coupled plasma–mass spectrometry (ICP–MS, Perkin Elmer 350D, USA)	Y_2O_3 NPs	[29, 32]
Changes in particle size distribution of NPs in tap water	Differential centrifugal sedimentation (DCS), Transmission electron microscopy (TEM)	Y_2O_3 NPs	[29]

Al: aluminum; ATR–FTIR: attenuated total reflection–Fourier transform infrared spectroscopy; Co: cobalt; Co_3O_4: cobalt(II,III) oxide; Cu: copper; Mn: manganese; Ni: nickel; NiO: nickel oxide; NOM: natural organic matter; NP: nanoparticle; Sb: antimony; Sb_2O_3: antimony trioxide; SiO_2: silicon dioxide; Sn: tin; WC–Co: tungsten carbide cobalt; Y_2O_3: yttrium oxide; ZnO: zinc oxide

Table 2 Methods identified in the MISTRA Environmental Nanosafety project to characterize and test influence of bio-corona formed in vivo via biotransformation on ecotoxicity of ENMs

Aim	Method (test setup and transformation assessment)	NP type	Reference(s)
Bio-corona formed via biotransformation (in vivo)			
Biotransformation in terms of bio-coronate formation upon uptake through *D. magna*	Filtration of NPs in *D. magna*, after optimizing the protein binding by particle concentration and filtration time to reduce *D. magna* death Characterization of proteins on NPs after desorption using SDS, separation by gel electrophoresis, followed by identification of proteins by mass spectrometry	Aminated and carboxylated PS NPs in different sizes	[28]
Effect of bio-corona obtained via filtrated NPs by *D. magna* (i.e., biotransformation)	Filtration of NPs in *D. magna*, evaluation of survival of *D. magna* Measurement using ICP–MS, DSC, TEM, SDS–polyacrylamide gel electrophoresis, followed by Liquid chromatography–mass spectrometry	Y_2O_3 NPs	[29]
Transformation during transfer in aquatic food web (i.e., algae (*Scendesmus sp.),* crustacean (*D. magna*) and fish (*Carassius carassius*))	Algae pre-exposed to Co NPs (with/without excreted biomolecules from *D. magna*), fed to *D. magna.* Fish subsequently fed with the daphnia. Algae/biomolecule onto NPs, measured by ATR–FTIR. I*n situ* adsorption experiments on NP films, determined using a MCT (mercury cadmium telluride) detector with a ZnSe window. Graphite furnace–atomic absorption spectroscopy (GF–AAS) to explore Co concentrations from heteroagglomeration with Co NPs and algae inside *D. magn*a and *C. carassius*, and dissolved Co NPs in simulated fish gut solutions and stomach	Co NPs	[40]
Transformation of NPs through filtration by *D. magna*	Filtration of Au NP in *D. magna*. Effect on aggregation evaluated by dynamic light scattering (DLS), differential sedimentation centrifugation (DSC), and UV-absorption. Adsorbed proteins desorbed by Sodium dodecyl sulfate (SDS), separated by gel electrophoresis and identified by mass spectrometry	Au NPs	[38]
Transformation of protein-induced aggregates by filtration by *D. magna*	Size controlled aggregates of immunoglobulin G and 50 ENM aminated PS NPs, by optimising the ratio between proteins and particles. Size before and after filtration by *D. magna* evaluated by dynamic light scattering (DLS). Effect of filtration compared to PS NPs of the same size as the aggregates	Aminated polystyrene (PS) NPs of different size	[19]

Au: gold; DSC: differential sedimentation centrifugation; NP: nanoparticle; Co: cobalt; PS: polystyrene; SDS: sodium dodecyl sulfate; Y_2O_3: yttrium oxide

Inclusion of eco-corona considerations in hazard assessment

We recommend enhanced environmental relevance in the hazard assessment of nanoforms registered in REACH. The methods proposed can be used to help conduct more relevant persistency, toxicity, and bioaccumulative (PBT) assessment (a requirement in REACH as part of the hazard assessment). Persistency (P) of ENMs is here understood as the persistency of the nanoform. Dissolution can lead to ENMs ceasing to be in the nanoform and is, therefore, a relevant fate process to consider here. Biodissolution testing is, therefore, recommended to assess persistency in more environmentally relevant conditions, i.e., environmental media containing NOM or other biomolecules (illustrated in, e.g., [5, 18, 29, 32]). ENMs can also cease to be in the "nanoform" via aggregation/agglomeration processes (although not according to the current definition of ENMs adopted by the European Commission) and testing these processes in the presence of biomolecules is, therefore, also relevant. Such studies have been performed by, among others, Gallego-Urrea et al. [20] with fulvic acid, Cumberland and Lead [8] with fulvic, humic, and peat humic acid, Peng et al. [56] with humic acid, citric acid, and l-cysteine, and Mattsson et al. [38] with *D. magna* conditioned test medium and NPs ingested and egested by *D. magna*. It is also recommended to include eco-corona considerations in the toxicity (T) testing of ENMs. This could be achieved by conducting *D. magna* toxicity tests (a basis test in environmental chemical regulation) in environmentally relevant aquatic media, such as media containing excreted biomolecules from *D.* magna (as illustrated by, e.g., [27, 30]). Isolation of eco-corona biomolecules from *D. magna* secretes was achieved by Grintzalis et al. [21] on

amino-functionalised PS NPs by use of a semi-quantitative technique involving untargeted mass spectrometry metabolomics and organic solvent extraction. The technique was able to reproducibly identify eco-corona molecules. Nasser et al. [47] support adapting *D. magna* toxicity testing with eco-corona aspects and highlight *D. magna* as an essential test organism. More specifically, the authors recommend preconditioning ENMs in biomolecule-containing medium before exposure to *D. magna*. Finally, we here propose that bioaccumulation (B) can also be tested under the proposed conditions (e.g., presence of NOM and/or other biomolecules). *D. magna* is proposed as the main test organism due to its strong interaction with particles and placement in the aquatic food web, acting as a potential "point of entry" for hazardous substances into the food web [54]. For the implementation of this adjusted PBT assessment, methods to assess P, B, and T with eco-corona considerations must be further validated and/or developed.

The above-mentioned recommendations will not influence the methodology for predicted no-effect concentration (PNEC) determination by the assessment factor (AF) approach. Compared to PNEC obtained by OECD TGs for base-set organisms (fish, crustaceans, algae) with no consideration of eco-corona, the actual values derived may, of course, be different, but the method of deriving PNEC (lowest effect concentration divided by an AF) will be the same as today and with the same values for AFs (i.e., 1–1000 for freshwater assessments). However, for ENMs, a much stronger emphasis should be put on the use of species sensitivity distribution (SSD) for PNEC derivation [62]. In brief, PNECs derived by SSD approaches are based on an estimation of the 5% hazard concentration (HC5), which is divided by an AF. Probabilistic and deterministic approaches to this have emerged recently and can be applied to data-poor and data-rich ENMs, respectively, as described and tested by Wigger et al. [69] and Sørensen et al. [62]. Since testing according to our recommendations will lead to results of higher environmental relevance by taking eco-corona influences into account, these data should be used to estimate HC5 by SSD. In this case, it might be argued that a lower (or sometimes even no) additional AF needs to be applied to derive the PNEC due to the higher environmental relevance of the underlying test results.

Final remarks on increasing environmental relevance of nanomaterial ecotoxicity and fate

Besides the specific recommendations mentioned in 4.1–4.3, some overall recommendations to increase the environmental relevance when conducting ENM hazard assessment can be derived. First of all, it is recommended to conduct ecotoxicity tests on weathered/transformed ENMs (instead of pristine/"as manufactured" particles) in media that to a higher degree represent natural environments (e.g., [15, 16, 40, 63]). Currently, the effect of pristine nanoparticles does not reflect the effect levels observed for testing transformed nanoparticles, and the method of assessment factors markedly exacerbates this trend due to inherent differences in assessing a dissolved chemical and a physical entity. Consequently, increasing the environmental realism by testing weathered/transformed material makes a better representation of the interactions that can be expected in the environment, thus bridging the gap that has to be covered by assessment factors during the extrapolation to the environment. Furthermore, it should be clarified that for ENM surface transformation testing, the composition and characteristics of the ENM surface and of the expected environmental fate prior to testing must be considered, because these transformations are clearly material-, surface- and environment-specific. In addition, it should be understood that ENM surface transformation is time- and exposure-dependent, and these parameters must, therefore, be adjusted to reflect the expected exposure scenario to the possible extent when testing the ENMs [5]. Another aspect is to consider various and more natural exposure scenarios when conducting hazard testing of ENMs. This can be done by resuspending the ENMs to mimic natural wave and current actions, as done in Ekvall et al. [14]. Ekvall et al. [14] observed stronger effects on *D. magna* when resuspending test organism food (algae) and test material (WC NPs) during exposure. And finally, it is recommended to conduct long-term studies with environmentally relevant concentrations testing to better predict effects on natural aquatic environments [27]. It should be noted that studies at the laboratory scale are, and have been, important for our understanding of the fate and effects of NPs and will still serve as the primary tool for implementation at a regulatory level. However, continuous efforts to increase and understand the environmental interaction and transformation are necessary to adequately extrapolate laboratory findings to the environment and ultimately assess the environmental risk of nanomaterials.

Conclusions

Addressing the formation of corona is key when it comes to future updates of ECHA's guidance on ENM ecotoxicity and fate testing. Through our analysis, we find that it is of high importance to investigate the kinetics of eco-corona formation and particle transformation to understand the time-dependent interaction/adsorption/desorption between the constituents of the medium and the ENMs (with and without the presence of biomolecules), as well as data on surface composition, as well

as time-resolved dissolution rates and time-dependent particle stability/sedimentation (with and without the presence of biomolecules). We, furthermore, propose to enhance the environmental relevance of the hazard assessment of nanoforms, required under REACH, by including eco-corona considerations into the PBT assessment. An enhanced environmental relevance in the PBT assessment will arguably lead to the lowering of the uncertainties linked to the extrapolation of ecotoxicity data, which is particularly challenging for ENMs. Finally, we recommend expanding the temporal and spatial scales to bridge the extrapolatory gap between short-term laboratory and environmentally relevant exposures to adequately assess the fate and effects of NPs and allow for a more robust risk assessment of nanoparticles.

Abbreviations

AAS	Atomic absorption spectroscopy
AF	Assessment factor
Al	Aluminum
ATR–FTIR	Attenuated total reflection–Fourier transform infrared spectroscopy
Au	Gold
Co	Cobalt
Co_3O_4	Cobalt(II,III) oxide
Cu	Copper
DHBA	Dihydroxybenzoic acid
DOM	Dissolved organic matter
DSC	Differential sedimentation centrifugation
ECHA	European Chemicals Agency
ENM	Engineered nanomaterials
GF–AAS	Graphite furnace–atomic absorption spectroscopy
HC5	5% Hazard concentration
HNO_3	Nitric acid
Hydroxyl	–OH
ICP–MS	Inductively coupled plasma–mass spectrometry
JESS	Joint expert speciation system
Lactate	$(CH_3CH(OH)COOH)$
LDS	Laser diffraction spectroscopy
MCT	Mercury cadmium telluride
Mn	Manganese
MD	Molecular dynamic
NaOH	Sodium hydroxide
Ni	Nickel
NOM	Natural organic matter
NP	Nanoparticle
NTA	Nanoparticle tracking analysis
OECD	Organisation for Economic Co-operation and Development
PBT	Persistency, bioaccumulative and toxicity
PCCS	Photon cross-correlation spectroscopy
PNEC	Predicted no-effect concentration
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
ROS	Reactive oxygen species
Sb	Antimony
Sb_2O_3	Antimony trioxide
SDS	Sodium dodecyl sulfate
SiO_2	Silicon dioxide
Sn	Tin
SSD	Species sensitivity distribution
TEM	Transmission electron microscopy
TOC	Total organic carbon
WC–Co	Tungsten carbide cobalt
XPS	X-ray photoelectron spectroscopy
Y_2O_3	Yttrium oxide
ZnO	Zinc oxide

Supplementary Information

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Additional file1 (DOCX 20 kb)

Additional file2 (DOCX 18 kb)

Additional file3 (DOCX 96 kb)

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Availability of data and materials
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Declarations

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