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**Nanomaterial safety for microbially-colonized hosts:  
Microbiota-mediated physisorption interactions and  
particle-specific toxicity**

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**Citation**

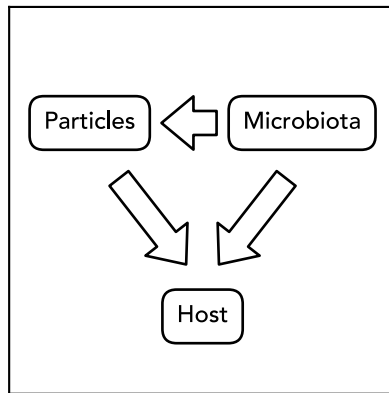
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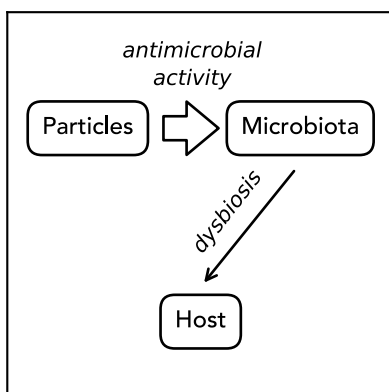


## CHAPTER 6

# General discussion

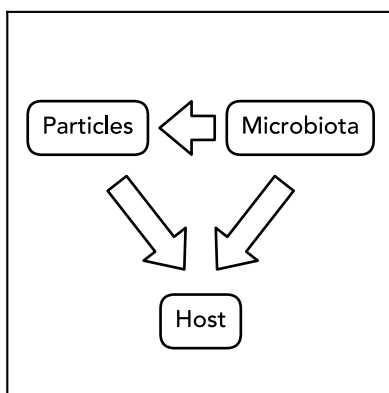
In the four years spanning the timeframe of this thesis (2018-2022), nanosafety research has made considerable progress by incorporating more realistic exposure scenarios in testing strategies, and by obtaining mechanistic insight into physiological responses to nanomaterials that can support predictive hazard assessment (e.g. Johnston et al. 2018; Kämpfer et al. 2020; Doak et al. 2022). As part of the progress made, the importance of microbiota-mediated toxicity, resulting from nanomaterial-induced changes in microbiota composition, has increasingly been demonstrated. In this thesis, we showed that an even wider array of interactions between hosts, microbiota and nanoparticles can influence nanomaterial toxicity. These interactions relate to key nanomaterials properties, including the large surface area of nanomaterials that is available for physisorption interactions, as well as particle-specific toxicity mechanisms. As described in this general discussion, this understanding of microbiota-dependent nanomaterial toxicity can further support the design of innovative tools and methods in support of regulatory decision-making. Ultimately, such a transition to microbiota-inclusive nanosafety testing can contribute to the evolving strategy of the European Union that is aimed to minimize the use of hazardous substances, including their (nano)forms, and where possible, detect and replace substances of concern early in the development pipeline, to continue to protect human and environmental health.

### 6.1 A new viewpoint on host-microbiota interactions in nanomaterial toxicity



Many experimental studies preceding the investigations presented in this thesis have shown that nanomaterials can disturb the composition and abundance of host-associated microbiota. When this results in dysbiosis, this can have detrimental consequences to hosts. It is hard to predict *if*, and under *what circumstances*, changes in microbiota composition affect host health. Due to functional redundancy between microbiota members, changes in microbiota composition do not necessary alter microbiota

functioning, for instance, in terms of metabolic activity, or immune system modulation. Moreover, it is still unclear *if*, and *at what pace*, host-associated microbiota can recover from nanomaterial-induced changes in composition. Nevertheless, the fact that many nanomaterials can alter the composition of microbiota, across diverse hosts, and following various exposure pathways, as summarized in **chapter 1**, is of concern to human, animal and environmental health.



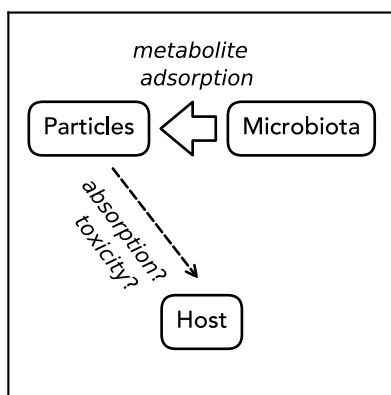
In this thesis, we took the opposite viewpoint on the role of host-associated microbiota in nanomaterial toxicity. We did so by examining how colonizing microbiota affect nanomaterial toxicity to the host. In principle, both the effects of microbiota on nanoparticle fate, bioavailability and biodistribution, as well as the effects of microbiota on the host's response to nanomaterials can alter nanomaterial toxicity to hosts. Differentiating between these effects is challenging, but it can be achieved by using

computational techniques (**chapter 2**), by focusing on specific life stages of host models (**chapter 3**), and by employing and combining different experimental strategies that are available for toxicological model organisms (**chapters 4,5**). This chapter elaborates on what we have learnt from this new viewpoint, specifically addressing particle-specific characteristics of nanomaterial toxicity. Firstly, interactions between microbes and particles that influence realistic nanomaterial exposure scenarios will be discussed ([section 6.2](#)), followed by the impacts of microbiota on particle-specific adverse effects of nanomaterials ([section 6.3](#)).

## 6.2 Accounting for microbiota in more realistic nanomaterial exposure scenarios

Microbiota-mediated physisorption interactions can affect the fate of nanoparticles and microbes. This section discusses how future tools for nanomaterial safety testing can account for these effects based on the findings of this thesis.

Nanoparticles can be purchased in a pristine form, free from endotoxins, as bare particles or with specific surface modifications. This pristine nature rapidly changes once nanoparticles enter the environment. In the environment, biomolecules adsorb onto the large surface area of nanoparticles, forming biocorona, consisting of tightly and loosely bound metabolite layers on the particle surface. Like any other chemical modification of the nanomaterial surface, including the application of surface coatings (e.g. consisting of polymers like polyethylene glycol or polyvinylpropylene) and functional groups (e.g. hydroxyl, amino, or phosphate groups), this can influence the colloidal stability (Gebauer et al. 2012; Panáček et al. 2018), circulation time (Li and Huang 2010) and biodistribution (Hussain et al. 1998; Thepphankulgarm et al. 2017) of nanomaterials in both in- and external environments. In **chapter 2**, we provide an overview of microbial biomolecules that can contribute to biocorona formation in the gastrointestinal tract. Many of these biomolecules fulfill essential roles in host-microbiota interactions, supporting amongst others the digestion of dietary fibers, energy supply to cells of the intestinal lining, signal transduction, and the control of inflammatory responses. For this reason, the potential interactions of these biomolecules with the nanomaterial surface should not be overlooked.

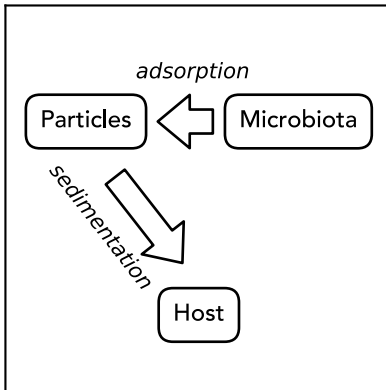


The results of **chapter 2** can be used to incorporate the effects of microbial metabolite physisorption into mechanistic pathways for nanomaterial safety assessment. To this end, the chapter ([section 2.2.6](#)) describes in detail how the adsorption affinity for microbial metabolites to metal and carbon nanomaterials can be predicted both quantitatively and qualitatively, as based on our results. Instead of repeating this rationale, we highlight three additional considerations for the relevance of this practice

to exposure scenarios for nanomaterials. Firstly, the models and simulations of **chapter 2** predict that it is particularly relevant to consider biocorona interactions for microbial metabolites from categories like lipids and bile acids. These categories comprise metabolites with hydrophobic sites that can interact with the hydrophobic regions of nanomaterials. This interaction type was inferred to contribute most to the overall adsorption affinity of microbial metabolites to nanomaterials. Secondly, our

results indicate that differences between experimental results for metal and carbon nanomaterials can result from microbiota-mediated biocorona effects. The predictions of the adsorption affinity for many metabolite categories differed between metal and carbon nanomaterials. Therefore, by affecting the extrinsic properties of metal and carbon nanomaterials differently, the adsorption of microbial metabolites to nanomaterials could contribute to different, microbiota-dependent responses of the host to these nanomaterials. Thirdly, in order to rationalize how microbiota-mediated biocorona formation can affect the responses of a host, advanced nanomaterials such as nanocarriers and biosensors can serve as examples. As noted before, vitamin B<sub>12</sub>, has for instance been employed to target pharmaceutical-loaded nanocarriers to specific cell types comprising transcobalamin (vitamin B<sub>12</sub>) receptors (Thepphankulngarm et al. 2017). Other nanomaterials have been designed for the detection and remediation of inorganic and organic environmental pollutant, like mercury (Chen et al. 2014a) and pesticides (Chen et al., 2016). Following similar principles, ingested nanomaterials may sequester enteric microbial metabolites, or may interact with specific cell types due to interactions between adsorbed metabolites and cell surface receptors.

Physisorption interactions can also influence the fate of nanoparticles and microbes in *in vitro* and *ex vivo* test systems for nanosafety testing. In these systems, nanoparticles are typically applied to the aqueous culture medium covering cells, tissues or organoids sitting on the bottom of a culture plate. This is similar to the acute toxicity test setup that is applied for the zebrafish larvae experiments in this thesis, where embryos or larvae sit on the bottom of a well plate, covered by a nanoparticle dispersion. In aqueous media, the exposure concentrations of nanomaterials are influenced by collisions between suspended particles, resulting in the continuous formation and breakup of aggregates in the water column. We observed such aggregates in all nanomaterial exposures that were performed for this thesis (**chapters 3,4,5**), and found that these aggregates increased in size, and settled to the bottom of exposure wells during the exposure time of the toxicity tests that were performed (1-2 days). Thus, vertical concentration gradients develop in many exposure setups for nanosafety testing, where the particle concentration at the bottom of the system increases gradually over time, until all particles have been deposited, or until a steady state with stably dispersed particles is reached. This has led to the development of dosimetry models for nanosafety testing (e.g. Hinderliter et al. 2010; DeLoid et al. 2016), that can be used to predict the delivered exposure concentrations as a function of time at the location of the test species or specimen.

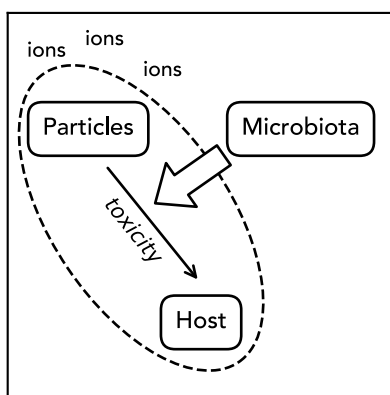


The results of **chapter 3** indicate that colliding nanoparticles do not only affect the exposure concentrations of nanomaterials, but can also influence microbial colonization dynamics. Briefly, focusing on nano-titanium dioxide ( $n\text{TiO}_2$ ), our results indicate that colliding particles and microbes can form heteroaggregates that facilitate the transfer of microbes to eggs, by way of the sedimentation of these aggregates onto the egg surface. Similarly, in both marine and freshwater ecosystems,

microbes have already been found to colonize the ‘plastisphere’, forming biofilms onto the surface of suspended microplastic particles (Kirstein et al. 2016; Arias-Andres et al. 2018). This contribution of this thesis to this insight is twofold. Firstly, we show that opportunistic pathogens do not only survive the antimicrobial activity of  $n\text{TiO}_2$ , but are also transferred to later (larval) life stages upon hatching (**chapter 3**). This underscores the concern that nanoparticles can facilitate the transfer of pathogenic bacteria to oviparous animals. Secondly, we show that these physisorption interactions between particles and microbes are also relevant to experimental test setups, which are a simplification of natural systems: even stagnant waters with little mixing induced by wind flow, tidal changes, and thermal convection are much more complex than our laboratory setup. This indicates that other laboratory setups that include microbial strains, such as advanced *in vitro* models for the intestine that mimic more realistic exposure scenarios by adding colonizing microbes (Kämpfer et al. 2020), should also account for the effects of nanoparticles on microbial colonization. For instance, temporarily separating the microbial colonization and nanoparticle exposure in experiments could minimize potential confounding effects of nanoparticle exposure on microbial colonization. Additionally, researchers can consider including non-toxic particles with similar physicochemical properties as the tested particle (e.g. surface functionalization, charge and specific surface area) as a ‘vehicle-control’ in experimental setups. Finally, it is recommended to track the abundance and (functional) composition of colonizing microbiota across the different experimental treatments over the exposure time to detect potential effects of the nanoparticle exposure on microbial colonization.

### 6.3 Effects of colonizing microbiota on nanoparticle toxicity

One of the key challenges in nanomaterial safety assessment, is to determine if the nanoform of a substance underlies its toxicity. If so, properties at the nanoscale, like the nanosized shapes, the high specific surface area and corresponding reactivity, or quantum effects of nanoparticles, contribute to nanomaterial toxicity (**chapter 1, section 1.2**). As part of this, it is important to differentiate between the effects of particles and shed ions of soluble nanomaterials. Since many of the nanomaterials that have been reported to interact with microbiota, can dissolve in aqueous media (see Fig. 1.1; Appendix Table S1), this also applies to investigations on the influence of microbiota on nanomaterial toxicity.



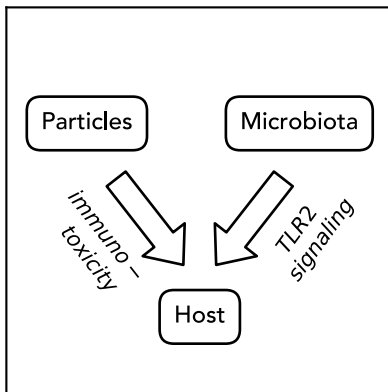
In **chapter 4**, we developed a protocol to quantify microbiota-dependent and particle-specific toxicity for soluble nanomaterials by means of the response-addition model (Bliss 1939). By testing the approach for the soluble nanomaterials nAg and nZnO, we could detect a marked protection of colonizing microbiota against the particle-specific toxicity of nAg in zebrafish larvae. Of note, the protocol can be adopted for any other test system or model organism that can be maintained under both

germ-free and microbially-colonized conditions. Amongst others, these include intestinal *in vitro* and *ex vivo* models (Pearce et al. 2018), algae, daphnids (Sison-Mangus et al. 2015; Callens et al. 2016; Manakul et al. 2017), fruit flies (Kietz et al. 2018), jewel wasps (Shropshire et al. 2016; Wang and Brucker 2022), mice (Kennedy et al. 2018) and rats (Qv et al. 2020). This diversity in available test systems and model organisms allows for interesting comparisons of microbiota-dependent, particle-specific nanomaterial toxicity between different hosts and host tissues.

In recent years, considerable progress has been made in identifying particle-specific toxicity mechanisms of nanomaterials. Briefly, while nanoparticles, their shed ions, and their associated reactive oxygen species (ROS) can damage cells both externally and internally in a multitude of ways, their cytotoxicity generally results in oxidative stress and inflammatory responses (Garcés et al. 2021). Understanding these toxicity mechanisms supports strategies that are aimed to predict the effects of nanomaterials under environmentally realistic conditions. Knowledge on particle-specific toxicity mechanisms can moreover be employed to integrate human and environmental nanomaterial safety testing. Additionally, it can be implemented in the 'safe- and sustainable-by-design' process, promoted by the European Commission (2020) with the



aim to support the development of new nanomaterials that provide functions or services with minimized harmful impacts to human health and the environment (Mech et al. 2022).



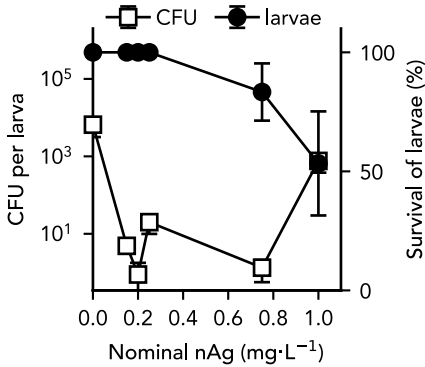
Focusing on the specific case of nAg toxicity in zebrafish larvae, the results of **chapter 5** demonstrate the importance to consider host-microbiota interactions in strategies that apply particle-specific toxicity mechanisms for the design and risk assessment of nanomaterials. These results reveal that, in addition to the effects of microbe-particle interactions on the fate, bioavailability and biodistribution of nanomaterials ([section 6.2](#)), interactions between microbiota and the host influence the

sensitivity of the host to adverse effects of nanomaterials. In part, these host-microbiota interactions were mediated via toll-like receptors (TLRs), and concerned a pro-inflammatory cytokine response of the innate immune system against nanomaterials. The evolutionary conservation of these targets suggests that the results from this single case may apply to a wider range of organisms and nanomaterials. In the following paragraphs, we discuss this with regard to strategies that are aimed to 1) extrapolate findings from acute to chronic and repeated exposures ([section 6.3.1](#)); 2) employ grouping and read-across of nanomaterials for risk assessment ([section 6.3.2](#)); and 3) use conserved molecular targets to predict nanomaterial toxicity across a wider range of species ([section 6.3.3](#)). In support of this discussion, Fig. 6.1 to Fig. 6.3 provide several additional research findings which have not been presented in the previous chapters of this thesis.

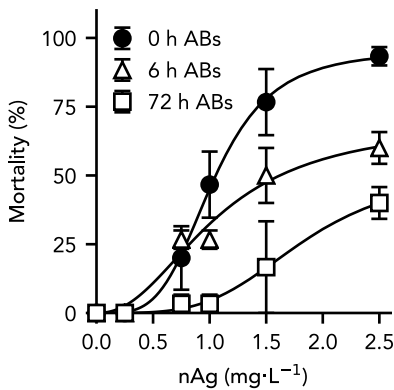
### *6.3.1 Extrapolating microbiota-dependent nanoparticle toxicity from acute to chronic or repeated exposures*

The loss of microbiota at sublethal exposure concentrations (Fig. 6.1) resembles the germ-free condition tested in **chapter 4** and **chapter 5**. We found that this microbiota-deficiency substantially increases the sensitivity of zebrafish larvae to nAg. This could imply that the loss of protective microbiota can sensitize the host to the immunotoxic effects of nanomaterials over longer term or repeated exposures. Similarly, co-exposure to (other) antimicrobial agents might intensify the immunotoxic effects of nanomaterials. This is a particularly relevant consideration in view of the widespread use and release of antibiotics in the environment (Larsson and Flach 2021; Wilkinson et

Considerations for acute to long-term exposure extrapolations: <sup>a)</sup>

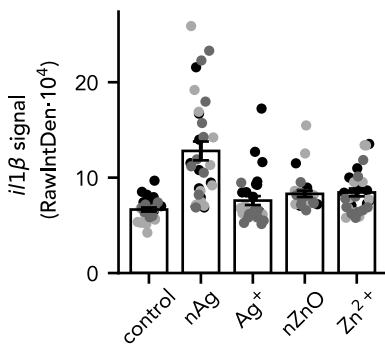


**Figure 6.1:** Antimicrobial effects of nAg at sublethal exposure concentrations may sensitize zebrafish larvae over longer term exposures. Colony-forming unit (CFU) count (white squares, left axis) and zebrafish larvae survival (black circles, right axis) are shown for two-day exposure (3-5 dpf) to nominal nAg concentrations ranging from 0.1 to 1.0 mg Ag·L<sup>-1</sup>.



**Figure 6.2:** In contrast to germ-free conditions, antibiotics reduce the sensitivity of zebrafish larvae to nAg. The figure shows zebrafish larvae mortality for two-day exposure (3-5 dpf) to nAg following a pre-exposure of 0 h (black circles), 6 h (white triangles) and 72 h (white squares) to an antibiotic-antifungal cocktail comprising Ampicillin (100 µg·mL<sup>-1</sup>), Kanamycin (5 µg·mL<sup>-1</sup>) and Amphotericin B (250 ng·mL<sup>-1</sup>). Nominal exposure concentrations of nAg are shown.

Considerations for read-across: <sup>a)</sup>



**Figure 6.3:** Particle-specific *il1β* expression could serve as a potential biomarker for immunotoxic effects that are influenced by colonizing microbiota. Bars present the *il1β* signal, as determined following the procedure described in chapter 5, following two-day exposure (3-5 dpf) to sublethal (nominal) exposure concentrations of nAg (0.25 mg Ag·L<sup>-1</sup>) and nZnO (2.5 mg ZnO·L<sup>-1</sup>), to corresponding shed-ion concentrations of Ag<sup>+</sup> (0.05 mg Ag·L<sup>-1</sup>) and Zn<sup>2+</sup> (1.1 mg Zn·L<sup>-1</sup>), or to no particles or ions (control). Both the protective effect of microbiota (chapter 4) and enhanced *il1β* signal were only detected for nAg.

<sup>a)</sup> The corresponding data is available via Zenodo (DOI: 10.5281/zenodo.7066692).

al. 2022). However, predicting the effects of such mixed exposures is complicated by potential direct and microbiota-independent effects of antibiotics on host cells (Yang et al. 2017a). In agreement with the immuno-suppressive effects of broad-spectrum antibiotics (Oehlers et al. 2011), we found that pre-treatment to an antibiotic-antifungal cocktail, comprising Ampicillin, Kanamycin and Amphotericin B, reduced the sensitivity of zebrafish larvae to nAg toxicity (Fig. 6.2). This illustrates that common environmental pollutants, exerting microbiota-independent effects, should not be overlooked when assessing the consequences of the potential loss of protective microbiota on microbiota-dependent nanomaterial toxicity over longer-term and repeated exposures. These include, and are not limited to: endocrine-disrupting pollutants, carcinogenic, mutagenic and reprotoxic (CMR) pollutants and neurotoxic pollutants. Nevertheless, many of these pollutants, such as (nitrated) polyaromatic hydrocarbons (PAHs), nitrotoluenes, polychlorobiphenyls (PCBs), metals (like mercury), and azo dyes, can undergo microbiota-mediated transformations that affect their toxicity (Claus et al. 2016). Therefore, in many cases, both microbiota-dependent and microbiota-independent pathways should be taken into account when unravelling toxicity mechanisms for environmental pollutants.

### *6.3.2 Read-across for microbiota-dependent nanoparticle toxicity*

Although we specifically dissect a microbiota-dependent toxicity mechanism for nAg (**chapter 5**), the observed protective effect of colonizing microbiota against nanomaterial toxicity could apply to other nanomaterials that elicit a comparable pro-inflammatory innate immune response. To date, a very diverse set of nanomaterials, including metal nanomaterials, carbon nanotubes and fullerenes, have been found to induce pro-inflammatory innate immune responses (Cronin et al. 2020). These materials interact with diverse cellular and non-cellular components of the innate immune system, responding to foreign structures, nanoparticle-induced oxidative stress and nanoparticle-induced cell damage (Engin and Hayes 2018). Whether a nanomaterial causes an innate immune response, and if so, what components of the innate immune system are involved in this response, depends on many factors, including the physicochemical properties of nanomaterials, such as core composition, surface modification, size, shape, and acquired biocorona, as well as on nanomaterial fate and biodistribution (Engin and Hayes 2018). This complicates the use of immunotoxic endpoints for read-across. In fact, in **chapter 4**, we show that colonizing microbiota protect zebrafish larvae against immunotoxic nAg, but do not offer protection against nano-zinc oxide (nZnO), while nZnO is also known to exert immunotoxic adverse effects. Nevertheless, in contrast to the results for nAg, presented in **chapter 5**, nZnO did not elicit a pro-inflammatory cytokine response as observed

using an *il1 $\beta$*  reporter line (Fig. 6.3). Although we cannot differentiate between microbiota-dependent and microbiota-independent nanomaterial immunotoxicity based on a single comparison, this could be a first indication that enhanced *il1 $\beta$*  expression might serve as biomarker to detect microbiota-dependent nanomaterial immunotoxicity. Of note, in a review on the use of cytokines as biomarkers for nanoparticle immunotoxicity, Elsabahy and Wooley (2013) discuss that both nAg and double-walled carbon nanotubes have been found to trigger the release of IL1 $\beta$  in human monocytes. Ultimately, such comparisons, further linking material properties to toxicity pathways, may facilitate read-across once immunotoxicity mechanisms have been dissected for a larger set of nanomaterials, organisms and cell types.

### 6.3.3 Cross-host extrapolation for microbiota-dependent nanoparticle toxicity

Most of the experimental work in toxicology employs the benefits of a selection of test systems and model organisms that are easy to handle, have been well characterized in terms of their genomes and physiology, and ideally, reduce, refine and replace the use of (vertebrate) animal models. Based on these criteria, we adopted zebrafish larvae as an ideal model organism for the experimental work performed for **chapters 3-5**. When the results from these investigations are used to inform human and environmental effect assessment, this requires the extrapolation of results to different organisms, which is known as cross-species extrapolation. In view of the additional challenge in microbiota research, to account for differences in microbiota composition that exist between different host species, and even between different individuals of the same species, we refer this challenge as ‘cross-host extrapolation’ in the remainder. Fortunately, despite these differences in microbiota composition between hosts, the functions that are performed by microbiota of different hosts are generally well conserved (Rawls et al. 2006; Gaulke et al. 2020). This also applies to the protective effect of colonizing microbiota against nAg toxicity (**chapters 4-5**), which was consistently observed despite differences in the microbial taxa between larvae of different parental lines, as detected using 16S rRNA profiling (Table S2). Overall, this encourages the use of functional endpoints to assess microbiota-related toxicity outcomes across hosts.

Mechanistic insight into the pathobiological response of hosts to environmental stressors can facilitate cross-host extrapolation, using adverse outcome pathways (AOPs) as a framework in combination with toxicokinetic and toxicodynamic traits (Spurgeon et al. 2020). When responses are governed via specific receptors, the conservation of these targets across different species can be used as a first criterium to identify species for which similar responses can be expected. This rationale has been incorporated in tools and databases that have been designed to support cross-species

extrapolation, such as SeqAPASS (LaLone et al. 2016; <https://seqapass.epa.gov/seqapass/>) and ECOdrug (Verbruggen et al. 2017; <http://www.ecodrug.org>). For nanomaterials, which typically act via multiple molecular targets, such analyses are less straightforward. Nonetheless, we found that the influence of microbiota on nanoparticle toxicity is mediated via TLR2 (**chapter 5**). The recognition of commensal microbiota by receptors of the TLR family is highly conserved among vertebrate animals (Dierking and Pita 2020), and, despite of its absence in fruit flies, it has even been identified in basal metazoans like *Hydra* (Franzenburg et al. 2012). Clearly, there are many ‘candidate hosts’ which may benefit from the protective effect of microbiota against nanomaterial toxicity. Further research, comparing the different organs, tissues and cell types that are being exposed to nanomaterials, and investigating the conservancy of signaling pathways downstream of TLR2, such as those involved in innate immune responses and tissue regeneration, can help to elucidate the applicability of this cross-host extrapolation.

#### 6.4 Conclusions and recommendations

Following up on the recent call for ‘microbiome-aware ecotoxicology’ (Duperron et al. 2020), this thesis illustrates, in a case-by-case manner, the specific relevance of host-associated microbiota in the field of nanotoxicology. In accord with basic principles of microbial ecotoxicology, which have been anchored in its definition (p. 31; Ghiglione et al. 2016), our investigations reveal that microbiota can also influence nanoparticle fate in realistic exposure scenarios for nanomaterials. In addition to the impacts of nanomaterials on the integrity of host-associated microbial communities, which have specifically been investigated by the time of writing, our work moreover demonstrates that interactions between microbiota and specific targets of the host can shape the host’s sensitivity to particle-specific nanomaterial toxicity. The evolutionary conservation of these targets supports the interesting hypothesis that these important interactions apply to a wider range of hosts and nanomaterials.

In a field that already faces great diversity in the intrinsic and extrinsic properties that influence nanomaterial toxicity, including host-microbiota and microbiota-particle interactions in models and test systems could add undesirable complexity to tools and methods for nanosafety testing. At the same time, current developments in nanosafety assessment strive for more realistic exposure characterization, and improved pathophysiological relevance of models and test systems. In view of this, I envision that the findings presented in this thesis could be used as a guideline to rationalize under what specific conditions, and for which kind of nanomaterials, host-microbiota and microbiota-particle interactions should be considered *a priori* in the selection,

development and application of models and tools for nanosafety testing. These results could moreover be inquired to recognize the potential influence of microbiota on test outcomes and environmental monitoring results *a posteriori*. This merits including functional endpoints and biomarkers as (proxy) measures for microbiota integrity, thereby enabling the extrapolation of results from acute to chronic or repeated exposure regimes, between different kinds of nanomaterials, as well as between different hosts. Altogether, this could aid in the safe-and sustainable-design of nanomaterials, and could support more realistic, physiologically relevant nanosafety assessment, advancing towards ‘microbiota-inclusive nanotoxicology’.

