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

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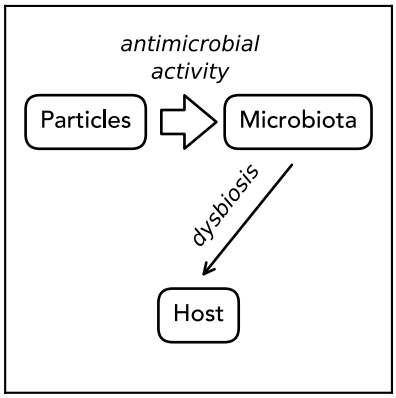
antimicrobial
activity
Particles
Microbiota
dysbiosis
Host

CHAPTER 1

General introduction

1.1 Where nano meets micro

Bacteria, archaea, fungi, slime molds and other amoeba, green microalgae, diatoms, oomycetes, dinoflagellates, ciliates, foraminifera, radiolarians, many other protists, bacteriophage and other viruses: a dazzling diversity and abundance of *microorganisms*[*] colonizes the environment. A large part of these microbes occurs 'free-living' in soil, water bodies, or air, where they aid, amongst others, in biogeochemical cycling, or the transformation of environmental pollutants (Madigan et al. 2011). Microbes can also colonize the tissue of plant and animal 'hosts', forming communities termed *microbiota*[*]. Whilst certain members of colonizing microbiota occasionally cause disease (Jochem and Stecher 2020), many members of host-associated microbiota interact beneficially with the host, supporting biophysiological homeostasis.

Substances that exert antimicrobial activity form one of the main threats to microbially-mediated biophysiological homeostasis. Many of the recently developed antimicrobial products consist of nanoparticles, which are either designed to combat infection or exhibit antimicrobial activity resulting from inherent physicochemical properties (Makabenta et al. 2021). Following the definition recommended by the European Commission (2011), *nanoparticles*[*] are particles that include at least one external dimension that is in between 1 to 100 nm, or alternatively, have a specific surface area exceeding 60 $m^2 \cdot cm^{-3}$. *Nanomaterials,*[*] in turn, are materials consisting for at least 50% of nanoparticles in terms of particle count. Owing to their innovative properties, nanomaterials enable technological advancement across many different sectors. For instance, nanotherapeutics enable the targeted, light-induced activation of cytotoxic agents for cancer treatment, thereby limiting unwanted side effects (Reeßing and Szymanski 2017); nanosensors can act as highly sensitive probes for the detection of environmental pollutants (Willner and Vikesland 2018); nanofertilizers and

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nanopesticides can facilitate the controlled release of agrochemicals (Hofmann et al. 2020); transparent nano-sized UV-filters are widely applied in sunscreens (Nasir et al. 2011); nanofabrics can dissipate heat in clothing (Iqbal et al. 2022); and as mentioned before, antimicrobial particles are incorporated in healthcare products such as bandages to combat infection.

The use of nanomaterials inevitably results in the release of nanoparticles into the environment. Along their transport through air and (waste)water, potentially attached to soil particles, food or microbes (Westmeier et al. 2018), a part of these nanoparticles will encounter plant or animal tissue. Depending on their exposure route, nanoparticles can reach different external tissues, including the epithelium of skin, leaves, and roots, as well as mucosa of gills, lungs, the mouth, and the gastrointestinal tract. Without any exception, these first-exposed tissues are colonized by microbiota. Hence, it is likely that nanomaterials and microbiota interact at the exposure interface, potentially shaping physiological responses of hosts to nanomaterials. The work presented in this PhD thesis was aimed to unravel via what mechanisms the interactions between hosts, microbiota and nanomaterials affect the safety of nanomaterials to microbially-colonized hosts.

1.2 Nanoscale properties affecting nanomaterial safety

The continued innovation in the field of nano-enabled products and materials offers a valuable opportunity to assess the safety and sustainability of these materials early in the development pipeline. This aligns closely with objectives of the European Union's '*Chemicals Strategy for Sustainability*' (2020), which, as part of the European Green Deal, aims to 'respond more rapidly and effectively to the challenges posed by hazardous chemicals' in the 'transition to chemicals that are safe and sustainable by design' (Doak et al. 2022). Of note, many of the first generation of engineered nanomaterials, including carbon and metal nanoparticles, nanotubes and nanowires, have already entered the market over 15 years ago (Science for Environmental Policy 2017). Currently, newly engineered nanomaterials mainly concern 'smart' nanocomposites and mixtures responding to external stimuli (Mech et al. 2022) and include exotic applications like self-propelled nanorobots (Novotný et al. 2020). Nevertheless, even for first generation nanomaterials, there is still a need for data and insight to be able to mechanistically predict the safety of nanomaterials to animals, humans and the environment, as based on characteristics of the nanoscale. Examples of nanoscale properties that can affect toxicity include the specific morphology (ranging from blunt and spherical shapes, to sharp needle-like shapes), increased reactivity, different or increased mobility, and altered optical, electronic and magnetic properties

(quantum effects) of nanomaterials (EFSA Scientific Committee, 2018). Two of such nanoscale properties affecting nanomaterial safety are highlighted in this thesis:

1. *A large surface area that is available for physisorption interactions.*
   A key intrinsic feature of all nanomaterials, is their large surface area resulting from the small size of nanoparticles. Biomolecules that are present in the surrounding of nanomaterials can adsorb onto this surface, forming several either tightly or loosely attached layers termed the *biocorona*[*] (Monopoli et al. 2012; Nasser et al. 2019). The main principles that govern these adsorption interactions, and the potential consequences thereof to nanomaterial safety, are increasingly well understood for proteins that interact with specific targets like receptors (Dawson and Yan 2021). Nevertheless, nanomaterials that are released into the environment, or taken up by a host, are exposed to many other biomolecules. Even in an ideal situation, where the biophysical conditions and biochemical composition of the current and previous surroundings of a nanomaterial have been fully characterized, it is extremely complicated to predict what biomolecules will associate with the nanomaterial surface, for how long, and in what orientation. Yet, this can affect how nanomaterials are recognized by (immune) cells of a host (Walczyk et al. 2010), and can influence the *colloidal stability*[*] of nanomaterials (Gebauer et al. 2012). The latter means that, depending on the chemical composition of the nanomaterial surface, particles will have a different tendency to remain dispersed, or to form agglomerates and aggregates. Considering the consequences of these effects on the immunotoxicity, biodistribution and reactivity of nanomaterials, the uncertainty in the biochemical composition of biocoronae forms one of the main challenges in predicting the effects of nanomaterials mechanistically.

2. *The release of toxic ions from metal nanomaterials in aqueous media.*
   Another challenge in detecting adverse effects for nanomaterials that are specific to the nanoform, is posed by the dissolution of soluble metal and metal oxide nanomaterials in aqueous media. Once dispersed, soluble nanomaterials will release metal ions into the exposure medium. These ions can be toxic at similar or even lower exposure concentrations than the corresponding nanoparticles (Zhai et al. 2016; Yang et al. 2017b; Brun et al. 2018; Sukhanova et al. 2018). Therefore, soluble nanomaterials in aqueous media represent mixtures which can exert toxic effects via their particles, ions, or the combination thereof. In order to determine if the nanoform of a substance contributes to its toxicity, it is necessary to differentiate between the toxicity of

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particles (*particle-specific** toxicity) and ions. Due to potential differences in the toxicity mechanisms of particles and ions, the toxicity of their mixture is generally modeled based on the accumulation of responses, rather than based the accumulative particle and ion concentrations (Zhai et al. 2016). Irrespectively, particles and ions often act in concert: ions that are released from particles may exert different toxic effects than ions originating from the corresponding bulk material. A clear example thereof is the 'Troyan horse effect', where nanoparticles first cross a biological barrier that is impermeable to ions, and subsequently release toxic ions from the particle core, or dissociate sorbed molecules from their biocorona, across this biological barrier (EFSA Scientific Committee, 2018).

The work presented in this thesis focuses on gaining mechanistic understanding of the effects of colonizing microbiota on physisorption interactions and particle-specific effects of nanomaterials. These investigations complement previous investigations, which specifically focused on the effects of microbial biotransformations on nanomaterial safety (section 1.3), and the risks of nanomaterial-induced *dysbiosis** (section 1.4). With regard to European nanosafety research, this thesis moreover directly contributes to the project PATROLS (www.patrols-h2020.eu): 'Physiologically-Anchored Tools for Realistic nanomaterial nanOmateriaL Safety testing'. As explained in detail by Doak et al. (2022), this project was aimed at delivering a suite of methods, tools and models that more accurately predict physiological responses to long-term or repeated exposure to low concentrations of nanomaterials. The microbiota-mediated effects that are studied in this thesis, are an example of the physiologically-relevant features and endpoints that were investigated within PATROLS to mimic more realistic exposure scenarios, in order to improve the predictive power of models and test systems.

1.3 Microbiota-mediated biotransformation of nanomaterials

To date, a handful of studies has shown how plant and animal microbiota can chemically transform nanomaterials (Avellan et al. 2018; Yin et al. 2019; Li et al. 2019b; Zheng et al. 2021; Li et al. 2022). As exemplified below, these microbiota-mediated transformations of nanomaterials can have important consequences on the fate and toxicity of nanomaterials.

Focusing on freshwater wetlands, Avellan et al. (2018) showed how microbiota-mediated transformations can affect the dissolution of nanomaterials. Specifically, microbiota associated with the freshwater macrophyte *Egeria densa* facilitated the dissolution of nano gold (nAu) via the formation of hydrogen cyanide. This is

remarkable, because the dissolution of nAu is thermodynamically unfavorable under realistic environmental conditions. Thus, microbiota can facilitate the oxidation of otherwise inert metal nanomaterials, changing the bioavailability and toxicity of these materials to biota.

Conversely, microbiota can also aid in the reduction of metal ions, thereby forming metallic nanoparticles. This activity has first been described for bacterial isolates that reduce silver ions (Ag^+) to form nano silver (nAg) (Lin et al. 2014). Interestingly, Yin et al. (2019) later confirmed that members of the human gut microbiota can also exert this activity, demonstrating the relevance of microbially-mediated nAg formation to human health. Because silver is generally considered to be less toxic in its particulate form, this activity may represent a detoxification mechanism against Ag^+.

Alternatively, intestinal microbiota can facilitate sulfidation reactions that transform ions that were shed from nAg into silver sulfide. Li et al. (2019, 2022) inferred this detoxification mechanism for microbiota in the intestines of the water flea *Daphnia magna*. The intestinal microbiota of *D. magna* acquired this protective function, as detected by a higher expression of genes that are involved in sulfate reduction, via changes in the microbiota composition over multiple generations of exposure to nAg. This illustrates how community-level differences in microbiota composition can introduce variability in the exposure dynamics of ingested nanomaterials, affecting the sensitivity of hosts to these materials.

Finally, the work of Zheng et al. (2021) shows how bacterial cell membranes can facilitate the biotransformation of rare-earth metal nanomaterials. Although mineable sources for these metals are uncommon, rare-earth metals are abundant in the Earth crust (Campbell 2014). Moreover, the nanoform of rare earth metals can be applied in many green and high-tech products, including supercapacitors, batteries, sensors and solar cells (Huang and Zhu 2019). Focusing on a representative rare-earth oxide metal nanoparticle, nano lanthanum oxide (nLa_2O_3), Zheng et al. (2021) showed that interactions of nLa_2O_3 with the external membrane of bacteria results in the formation of nano lanthanum phosphate. The reaction disrupted the cell membrane of exposed bacteria via the dephosphorylation of phospholipids. This specifically occurred on the (outer) cell membrane of Gram-negative bacteria, which, as opposed to the cell membrane of Gram-positive bacteria, is not covered by an external peptidoglycan layer. This layer of peptidoglycan can thus protect Gram-positive bacteria against detrimental interactions between nanoparticles and cell membranes. As a consequence, exposure to nLa_2O_3 resulted in a reduced relative abundance of Gram-negative bacteria in bronchoalveolar microbiota of exposed mice. In this way, the microbiota-mediated biotransformation of nanomaterials can contribute to nanomaterials-induced *dysbiosis*,* as further described in section 1.4.

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1.4 Nanomaterial-induced dysbiosis

In striking contrast to the limited number of studies reporting effects of microbiota on the chemical biotransformation of nanomaterials (section 1.3), a rapidly increasing number of studies investigated the potential of nanomaterials to induce or cure *dysbiosis*[*] (Fig. 1.1a; Appendix Table S1).

Dysbiosis is a state characterized by an altered abundance of *beneficial microbiota*[*] members, which is often accompanied by increased inter-host variation in microbiota composition (Duperron et al. 2020). It is mostly studied and described for bacterial members of microbiota, whilst other members, like fungi, archaea, protists, microalgae, and viruses, also contribute to host health. Beneficial host-microbiota interactions contribute to the digestion of food and the uptake of nutrients, affect neurobehavioral development, support the defense against pathogens, and prevent oversensitive immune responses. Disturbance of these intricate interactions between hosts and microbiota has been linked to diverse pathologies, including inflammatory bowel disease, obesity, diabetes, kidney disease, cardiovascular disease, autism spectrum disorders and cancer (DeGruttola et al. 2016; Wilkins et al. 2019).

Roughly, the existing research on nanomaterial-induced dysbiosis can be divided into four groups (Fig. 1.1a), which either focus on the safety of nanomaterials to *human and animal health*[*] or *environmental health*[*], including the health of crop species, or focus on the efficacy of fertilizers or nanotherapeutics exerting microbiota-dependent effects. With regard to nanosafety testing, the majority of these investigations focuses on nanoparticles that exhibit antimicrobial activity, like nAg, nano zinc oxide (nZnO) and nano titanium dioxide ($nTiO_2$). These particles, and in particular nAg and $nTiO_2$, are also the most commonly applied particles in industrial consumer products (Eduok and Coulon 2017). Many of the remaining studies focus on nano copper oxide, nano selenium and nano cerium oxide (nanofertilizers), or functionalized carbon, iron and iron oxide nanoparticles (nanosupplements and -therapeutics). Collectively, this body of work demonstrates that nanomaterials can cause shifts in the composition of gut, mouth, lung and rhizosphere microbiota, across a great variety of hosts, including fish, aquatic and terrestrial invertebrates, plants and trees, mice, rats, chicken, pigs, and humans (Fig. 1.1b-d). The exposure routes, exposure duration and exposure concentration applied in these experiments are summarized in Fig. 1.2, and are included in Table S1 of the appendix.

Nanomaterial-induced changes in microbiota composition often coincide with physiological changes in the host. These changes include beneficial effects on host health, such as improved crop growth (Shcherbakova et al. 2017; Dai et al. 2020; Wang et al. 2022), the prevention of diarrhoea among cattle (Xia et al. 2017), and the inhibition of lesions associated with dental caries (Naha et al. 2019; Ostadhossein et al.

2021). However, nanomaterials have also been found to increase the susceptibility of hosts to pathologies that have been related to dysbiosis, such as colitis (Mu et al. 2019) and obesity (Kurtz et al. 2020; Zhu et al. 2021). While these physiological effects might result from changes in microbiota composition, it is often hard to differentiate between the direct effects of nanomaterials on host health, and cascading effects, resulting from nanomaterial-cured or -induced dysbiosis.

Several recent studies addressed the causality between nanoparticle-induced pathologies and changes in microbiota composition using probiotics and fecal microbiota transplants (Li et al. 2019a; Ju et al. 2020; Zhao et al. 2020, 2021). The administration of probiotic *Lactobacillus rhamnosus* GG bacteria, for instance, ameliorated $nTiO_2$-induced intestinal inflammation in juvenile rats (Zhao et al. 2020), and decreased $nTiO_2$-enhanced susceptibility to diet-induced metabolism syndrome in mice (Zhao et al. 2021). Similarly, a probiotic cocktail comprising 11 Lactobacillus strains and 5 Bacteroidetes strains could rescue mice from nano silicon dioxide ($nSiO_2$)-induced lung inflammatory injury (Ju et al. 2020). By contrast, in each of these cases, the inflammatory injury resulting from nanoparticle exposure could also be induced by the administration of fecal microbiota from nanoparticle-exposed individuals.

Likewise, fecal microbiota transplants could reproduce the protective effects of nanomaterials against dysbiosis-related pathologies in two studies on functionalized metal nanoparticles (Deng et al. 2021; Sharma et al. 2022). The first of these studies concerns selenium@albumin complex nanoparticles (nSe@albumin) which offer protection against chemotherapy-associated intestinal mucositis, a common side effect in cancer treatment (Deng et al. 2021). Fecal microbiota transplants from mice exposed to these particles exerted similar protective effects against cisplatin-induced mucositis as nSe@albumin exposure. The second study focused on the protective effect of functionalized gold nanoparticles (nAu), capped with *Cinnamomum verum*-derived bioactives. These particles reduced the susceptibility of mice to high-fat diet induced obesity (Sharma et al. 2022). Similarly, the administration of fecal microbiota obtained from nAu-exposed mice reduced weight gain in healthy mice.

The examples of nanomaterial-induced and -cured pathologies, resulting from nanomaterial-induced changes of microbiota composition, show the importance to consider potential cascading effects due to nanomaterial-induced changes in host-associated microbiota in nanosafety testing.

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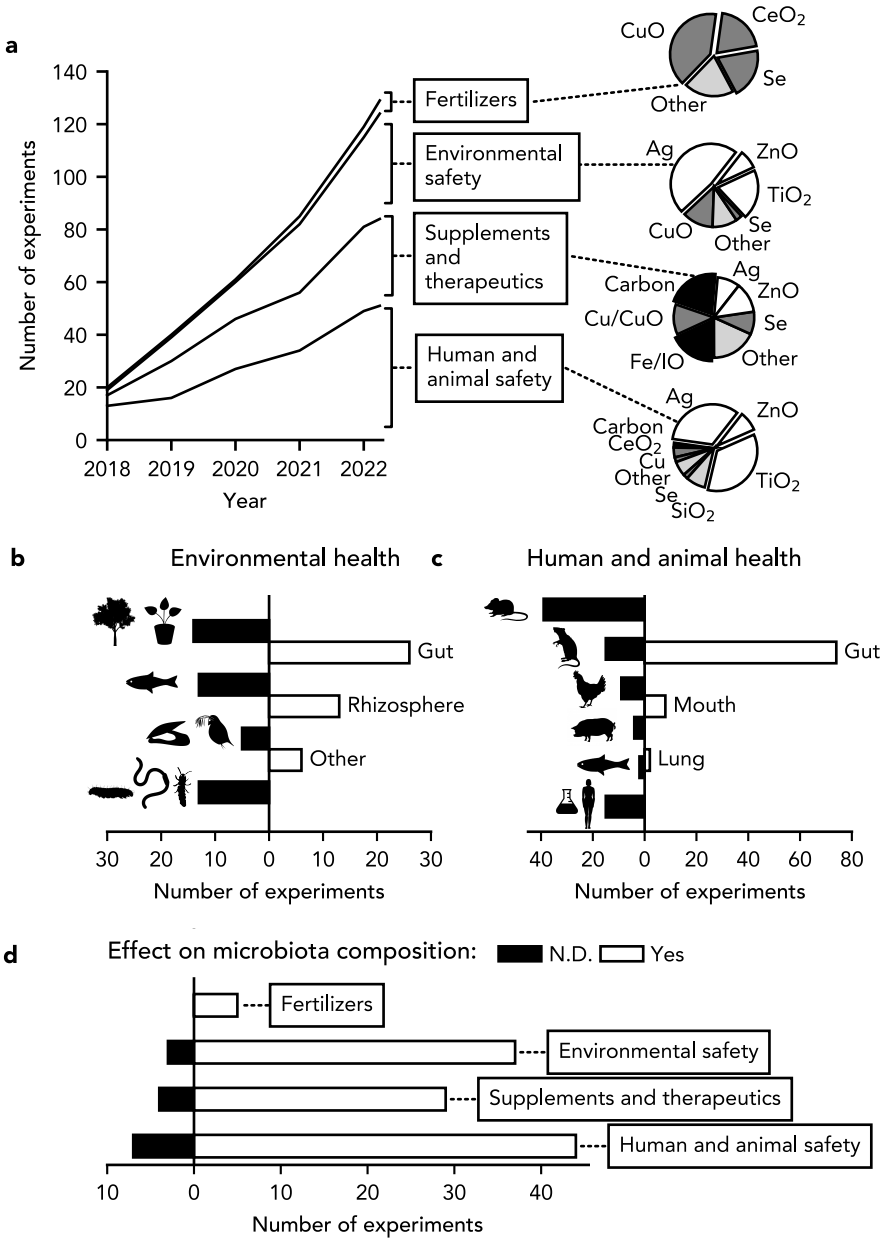

Figure 1.1: *Research investigating the effects of nanomaterials on the composition of host-associated microbiota.* [a] **a**) Accumulative number of studies over time, focused on environmental health ('fertilizers' and 'environmental safety testing') or human and animal health ('supplements and therapeutics' and 'human and animal safety testing'). Pie charts indicate what nanoparticles have been investigated in the concerning studies. Wedge color indicates if particles have mainly …

[a] Literature was retrieved from the Web of Science Core Collection database, accessed on 27 March 2022 through Leiden University's library, using the search string '(nanomaterial* OR nanoparticle*)' for the title, and the search string '(microbiome OR microbiota)' for the abstract of articles.

… been investigated in relation to nanosafety (white wedges), nanosupplements and -therapeutics (black wedges), nanofertilizers (dark gray wedges) or none of the above (light gray wedges). **b**)-**c**) Investigated host species (left, black bars) and microbiota type (right, white bars). Host symbols (top-down) depict, for environmental health: plants and trees, fish, aquatic invertebrates (daphnids, flat worms, mussels, and fly larvae) and terrestrial invertebrates (collembolans, earth worms and silk worms); and for human and environmental health: mouse, rat, chicken, pig, fish and human (*in vitro* and model gut systems). **d**) Number of experiments that observed effects (white bars), or no effects (black bars) on microbiota composition. Details of the studies are included in the appendix (Table S1). *Abbreviations*: Ag, silver; CeO_2, cerium dioxide; Cu/CuO, copper/copper oxide; Fe/IO, iron/iron oxide; N.D., not detected; Se, selenium; SiO_2, silicon dioxide; ZnO, zinc oxide.

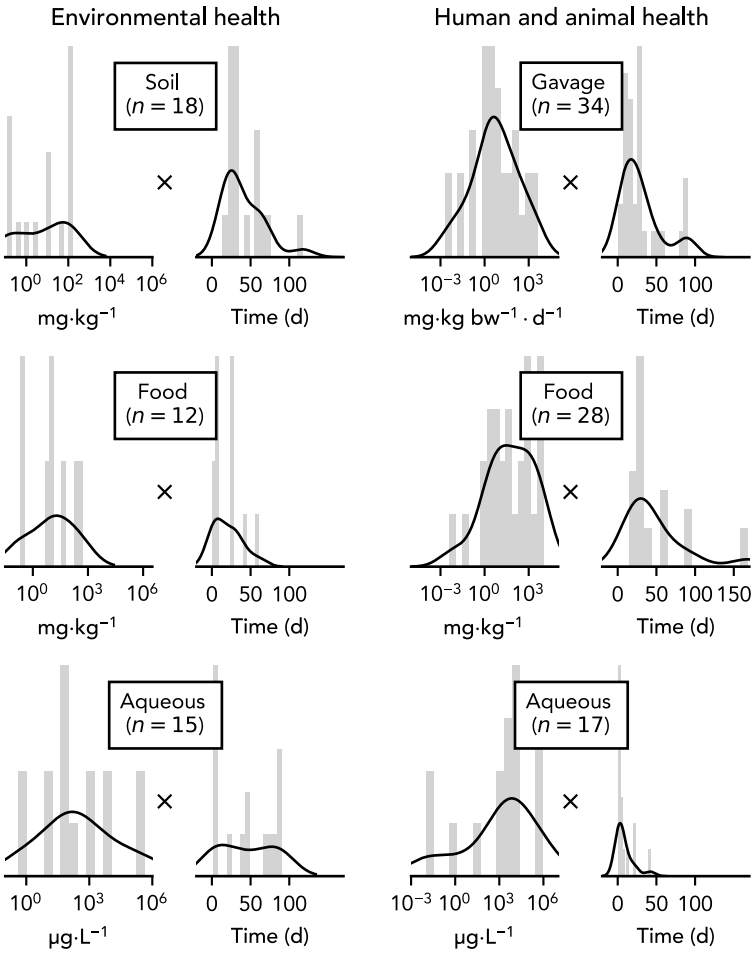

Figure 1.2: *Distribution of the exposure concentration and exposure time applied to investigate potential impacts of nanomaterials on host-associated microbiota.* Density curves and histograms depict distributions for studies concerning environmental health ('nanofertilizers' and 'environmental safety'; left column), and animal and human health ('supplements and therapeutic' and 'human and animal safety'; right column). Rows separate studies applying different exposure routes. Other experimental details are included in Fig. 1.1 and appendix Table S1.

1.5 Research question and aims of this thesis

Given the main focus of microbiota-related nanosafety testing on the chemical biotransformation of nanomaterials (section 1.3) and dysbiosis-related pathologies (section 1.4), the effects of colonizing microbiota on physisorption interactions and the particle-specific toxicity of nanomaterials remain largely unexplored. These topics are investigated in this thesis, by addressing the following research question using a combination of computational methods (section 1.6) and zebrafish larvae experiments (section 1.7):

> *'What mechanisms govern the physisorption-driven and particle-specific effects of colonizing microbiota on nanomaterial toxicity to microbially-colonized hosts?'*

Firstly, **chapter 2** and **chapter 3** of this thesis aim to gain mechanistic understanding of the effects of microbiota-mediated physisorption interactions on nanomaterial safety. To this end, **chapter 2** investigates what metabolites with a microbial origin can be predicted to adsorb onto ingested nanomaterials in the gastrointestinal lumen. In this way, this chapter initiates research on the potential 'microbial fingerprint' of the nanomaterial biocorona. Zooming out to the scale of microbes, **chapter 3** subsequently focuses on the potential of nanoparticles to adsorb onto colonizing microbes. As shown for the physisorption of nanomaterials to pollen, fungal spores (Westmeier et al. 2018) and orally-administered bacteria (Akin et al. 2007; Chen et al. 2021), this can have consequences to the transfer of nanoparticles through ecosystems, and through a host its body.

Secondly, **chapter 4** and **chapter 5** focus on the effects of colonizing microbiota on particle-specific toxicity mechanisms for nanomaterials. To do so, **chapter 4** of this thesis aims to determine if different metal nanomaterials exert particle-specific toxicity in a (partly) microbiota-dependent manner. Subsequently, **chapter 5** aims to unravel if and to what extent signaling pathways for the recognition of microbiota are involved in this particle-specific toxicity.

1.6 Chemoinformatic approaches to study microbiota-mediated physisorption

Computational methods provide a means to study biophysical interactions between microbiota and nanomaterials prior to and in support of more costly laboratory-based experiments. In this thesis, we combine two different chemoinformatic approaches, namely, quantitative structure-activity relationship (QSAR) models and molecular dynamics (MD) simulations. As described below, these techniques differ fundamentally in the way how the chemical properties or biological activity of substances are predicted.

For this reason, these methods can provide complementary insight into the physicochemical properties, biological activity and molecular interactions of substances.

The methodology of QSAR models originates from the work of Corwin Hansch and Toshio Fujita in the early 1960s. They showed how the median toxic concentration of substances can be predicted based on a hydrophobicity term, an electronic term and a steric term (Hansch and Fujita 1964; Gramatica 2008). Each of these explanatory properties can be derived from the chemical structure of the substances. As such, Hansch and Fujita showed that relationships exist between the information that is included in the chemical structure of substances, and their biological activity. Similarly, chemical and physical properties can be predicted based on the chemical structure of substances. In order to derive structure-activity relationships, QSAR models require 'training sets' with substances for which the physical, chemical or biological properties of interest have previously been derived, either experimentally, or using modeling approaches. First, molecular descriptors are computed based on the chemical structure of these substances, using chemoinformatic tools. An overview of available descriptors is given by Grisoni et al. (2018). Next, these descriptors are linked to the physical, chemical or biological property of interest. This can be done using diverse models, ranging from traditional multiple linear regression, to machine learning algorithms like decision trees and artificial neural networks (Gini 2018). Finally, the structure-activity relationships, as given by the models, can be applied to a 'test set', comprising substances that are similar to the training set with respect to the structural features included in the models. Across many different fields, ranging from toxicology to drug discovery, this approach nowadays enables the quantitative prediction of biological, chemical or physical properties of substances based on their chemical structure.

The MD methodology finds its origin in the early 1950s, when Berni Alder and Tom Wainwright had the opportunity to use the spare capacity of a powerful (IBM-704) computer at Lawrence Livermore Laboratories to perform pioneering work on molecular simulations (Battimelli and Ciccotti 2018). In this work, Alder and Wainwright applied physical laws to obtain insight into the motion of hard spheres in a liquid-solid phase transition (Alder and Wainwright 1957). Later on, Martin Karplus and colleagues showed that classical mechanics could also be used to describe the motion of hydrogen atoms in the exchange reaction of H with H_2 (Karplus et al. 1965). This important finding – which contributed to the chemistry Nobel Price in 2013, jointly awarded to Martin Karplus, Michael Levitt and Arieh Warshel – led to the application of physical laws to describe the trajectories of heavier atoms, like carbon, nitrogen and oxygen. Thereby, it created opportunities to obtain molecular information from MD simulations in the study of biological phenomena and in drug design (McCammon et al. 1977; Cheng and Ivanov 2012; Lindahl 2015). Nowadays, the field of

MD simulations includes diverse specializations. We refer the interested reader to in-depth literature on such specializations, enabling, for instance, the simulation of the formation and breakup of chemical bonds (Senftle et al. 2016; Behler 2017), or the simulation at coarser spatial and temporal scales (Barnoud and Monticelli 2015; Vassaux et al. 2020).

1.7 Zebrafish larvae as a model host

Zebrafish larvae are widely adopted as a model organism for research in the fields of developmental biology, ecotoxicology, pharmacology, oncology and immunology. Advantages of zebrafish larvae that are employed within these research areas include their ease of handling, short generation time, high fecundity, *ex vivo* development, transparent eggs and larvae, and many (>70%) human ortholog genes (Howe et al. 2017). Additionally, there are many valuable resources available for zebrafish research, including a rich collection of scientific literature and protocols (https://zfin.org; 'ZFIN Protocol Wiki'), standardized toxicity tests (OECD Test No. 236 'Fish Embryo Acute Toxicity Test'), and transgenic reporter lines and mutants. The ease to derive germ-free zebrafish larvae, available procedures that allow for the (re)colonization of larvae by microbes of interest (Pham et al. 2008), and the possibility to track live microbes *in vivo* using imaging techniques (Stephens et al. 2015; Wiles et al. 2016; Koch et al. 2018), moreover support the use of zebrafish larvae in microbiota research.

Several developmental transitions mark important colonization phases for early life stages of *teleost fish** (Llewellyn et al. 2014) like zebrafish (Fig. 1.3). The first microbes that colonize their tissues, occur on the outer membrane of zebrafish eggs (the *chorion**). Presumably, part of these early colonizers are transferred vertically, that is from parental fish, along the passage of eggs through the oviduct. Additional microbes that colonize the eggs are horizontally acquired from the environment of the eggs. Around two days following external fertilization, zebrafish *larvae** hatch from their eggs. Since it is assumed that chorion microbes cannot pass the chorion membrane, this time of hatching most likely marks the onset of the colonization of larvae. Microbes first colonize the external tissues of larvae, such as skin and gill mucosa. Later on, when larvae open their mouth (~3 days post-fertilization (dpf)) and start feeding independently (~5 dpf), microbes colonize their gastrointestinal tracts (Llewellyn et al. 2014). The core composition of zebrafish microbiota, which varies across development, and is taxonomically distinct, but functionally conserved in comparison to mammalian microbiota, has been studied extensively by Roeselers et al. (2011), Stephens et al. (2016) and Gaulke et al. (2020). Of note, from 6 dpf onwards, the use of zebrafish larvae for animal experimentation is restricted by European legislation (EU Animal Protection

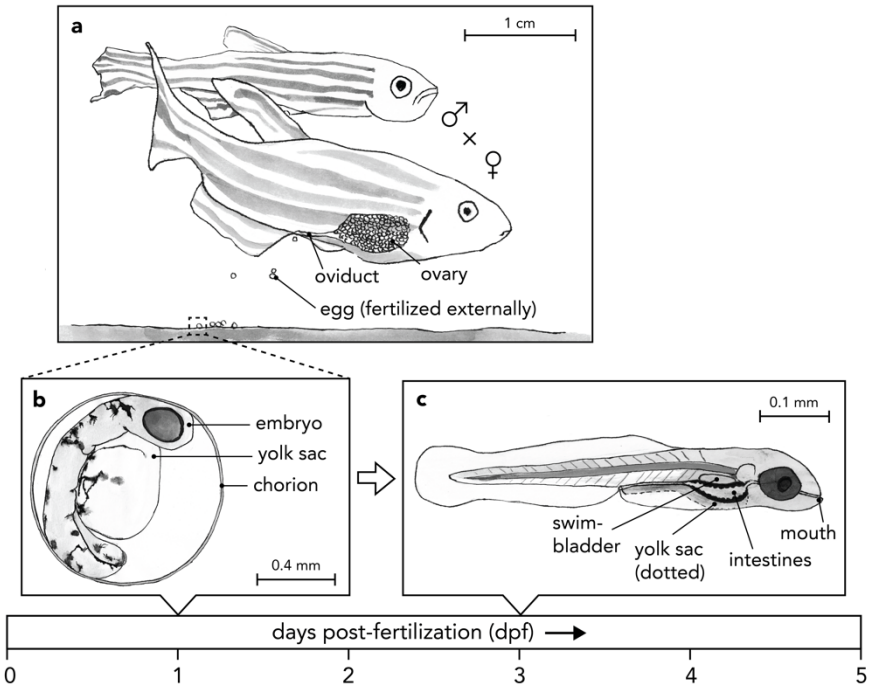

Figure 1.3: *Early zebrafish development with relevant structures for microbial colonization.* **a**) Mating zebrafish females release eggs from their ovaries via the oviduct into the water column. Maternal microbes that stick onto the chorion membrane of released eggs may be transferred to the offspring (vertical colonization). **b**) Following external fertilization, zebrafish embryos develop *ex vivo* inside of the protective chorion of eggs. Microbes from the water column further colonize the chorion (horizontal colonization). It is assumed that microbes cannot pass the chorion membrane. **c**) Around 2 days post-fertilization (dpf), larvae hatch from zebrafish eggs. Microbes from the chorion and water column can colonize the skin and gill tissues of larvae. Once larvae open their mouth at 3 dpf, microbes start to colonize intestinal epithelia. Around 5 dpf, larvae do no longer feed on the yolk sac, and start feeding independently. This facilitates the further development of the intestinal microbiota.

Directive 2010/63/EU), in support of the 3R's aim to Reduce, Refine and Replace animal experimentation. This means that zebrafish larvae are generally studied in the absence of diet-induced variation in microbiota composition.

In addition to the above milestones for microbial colonization, distinct phases in the maturation of the zebrafish immune system can influence the toxicity of nanomaterials. Primitive macrophages and neutrophils are formed in zebrafish embryos during the first day of development, around 15 and 18 hours post-fertilization (hpf), respectively (Herbomel et al. 1999). Neutrophils become mature from 24 to 48 hpf (Bennett et al. 2001; Lietschke et al. 2001). In the meantime, around 26 hpf, the blood circulation starts. Maturation of the adaptive immune system takes considerably longer

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(up to 4-6 weeks) (Lam et al. 2004), offering the opportunity to study innate immune responses in isolation of the adaptive immune system.

In this thesis, we employ the advantages of zebrafish larvae for (eco)toxicological investigations and microbiota research. **Chapter 3** focuses on zebrafish colonizing microbiota at the earliest stage of colonization, on the chorion membrane. **Chapter 4** and **chapter 5** focus on later stages including early colonization of the intestinal mucosa (3-5 dpf-old larvae). Larvae of this life stage are also used for studying physiological responses to nanoparticles in the presence of a functional innate immune system. In **chapter 3** and **chapter 5**, the transparency of zebrafish embryos and larvae allows for the localization of nanoparticles, microbes and physiological responses in and on embryos and larvae. In **chapter 4**, available protocols for the derivation of germ-free zebrafish larvae are optimized for nanosafety testing, thereby providing a method to detect microbiota-dependent and particle-specific nanomaterial toxicity. Finally, in **chapter 5** this method is applied in combination with several available zebrafish mutant lines, to test the contribution of specific elements of signaling pathways to microbiota-dependent nanomaterial toxicity.

1.8 Outline of this thesis

This thesis consists of six chapters. Each of the four research chapters, following on this general introduction of **chapter 1**, investigates the interactions between hosts, microbiota and nanoparticles from a different viewpoint. The final chapter discusses the implications of the obtained knowledge to human and environmental nanomaterial safety testing, focusing on different actors and common strategies in the field of nanotoxicology.

Chapter 2 investigates the potential of biomolecules that originate from intestinal microbiota to adsorb to different carbon and metal nanomaterials. First, a concise overview of microbial metabolites that are available for these physisorption interactions is generated. Subsequently, the adsorption affinity for these metabolites is predicted statistically, using QSAR models, and computationally, using MD simulations. Finally, key interaction types for these physisorption interactions, derived using both methods, are compared and discussed in relation to the biological functions of the concerning metabolites.

Chapter 3 focuses on adsorption interactions between $nTiO_2$ and microbes that occur on the chorion of zebrafish eggs. The abundance of sorbed particles and microbes on the chorion is examined in relation to the potential of particles to reach internal (embryonic) structures. Different imaging techniques are combined, including two-photon microscopy, confocal microscopy and particle-induced X-ray emission analysis.

The effects of $nTiO_2$ on colonizing microbiota of zebrafish eggs, and potential cascading effects on microbiota of hatched larvae, are assessed in terms of microbiota abundance and functional composition.

Chapter 4 optimizes protocols for the derivation of germ-free zebrafish larvae for nanosafety testing, to test whether nAg and nZnO exert microbiota-dependent, particle-specific toxicity. The response addition model is applied to quantify the particle-specific contributions to toxicity. Additionally, the antimicrobial activity of nAg and nZnO is quantified *in situ*, and nAg-resistant microbes are isolated and identified from exposed larvae.

Chapter 5 combines the germ-free methods of **chapter 4**, in combination with available mutant lines, to explore how components of the Toll-like receptor signaling pathway, involved in the recognition of commensal microbiota, affect nAg toxicity. To gain further mechanistic understanding of these identified effects of colonizing microbiota on nAg toxicity, the accumulation of silver in larvae and in intestinal tissue is quantified over the exposure time, and related pro-inflammatory responses are localized using a transgenic zebrafish line.

Chapter 6 concludes with a general discussion on relevant interactions between particles, colonizing microbiota and hosts in nanosafety testing, as studied in **chapter 2-5**. This final chapter elaborates on the observed influence of colonizing microbiota on particle-specific exposure scenarios, and the microbiota-dependent sensitivity of hosts to nanomaterials. The obtained mechanistic insight is discussed in relation to overarching objectives in the field of (nano)safety testing, including the prediction of chronic effects, the application of read-across, and the use of conserved pathways for cross-species extrapolation. The chapter concludes with recommendations for a transition to rapid and effective microbiota-inclusive nanosafety testing.

Definitions

Beneficial microbiota

A community of microbes that colonizes a specific habitat, including host tissue, with a positive influence on environmental, human or animal health (see below).

Biocorona (plural *biocoronae*)

The collection of biomolecules that forms both tightly and loosely attached layers onto the surface of nanomaterials. Biocoronae can be termed *ecocoronae* when they are formed by biomolecules from the external environment of organisms.

Chorion

Outer protective envelope surrounding the developing embryo in eggs of reptiles, birds, mammals, cephalopods (squids, octopuses, cuttlefish, etc.), pterygotan (i.e. winged or secondarily wingless) insects and teleost fish (see below).

Colloidal stability

The extent to which particles in aqueous media remain dispersed. The higher the colloidal stability of particles, the more these particles will remain dispersed, and the lower the tendency of these particles to form agglomerates and aggregates.

Embryo

The earliest life stage of plants animals, which, in case of oviparous animals, develop inside of the protective envelopes of an egg from the moment of fertilization up to the time of hatching.

Dysbiosis

A state where microbiota do no longer have a positive influence on environmental, human or animal health (see below). In comparison to beneficial microbiota, dysbiosis is characterized by the altered abundance of beneficial microbes and/or by increased variation in microbiota composition between different individuals of the same host.

Environmental health

Within the context of this thesis: the productivity, sustainability and biodiversity of ecosystems and agricultural land, as studied in relation to the intentional or coincidental release of nanofertilizers and other nanomaterials into the environment.

Human and animal health

Within the context of this thesis: the maintenance of biophysical homeostasis and the absence of disease in animals including humans, as studied in relation to the application or coincidental exposure to nanopharmaceuticals, nanosupplements, or other nanomaterials.

Larva (plural *larvae*)

An early life stage of animals prior to metamorphosis into the adult life stage. For oviparous animals, the time of hatching marks the onset of the larval life stage.

Microbial ecotoxicology

According to Ghiglione et al. (2016): 'a branch of science that studies both

(i) the ecological impacts of chemical (synthetic or natural origin) or biological (toxic species) pollution at the microbial scale and the various functions that they ensure in the ecosystems and

(ii) the role of microbial communities in the ecodynamic of the pollutants (source, transfer, degradation, transformation).'

Microorganism (or shortly, *microbe*)

An organism that is invisible to the naked eye.

Nanomaterial

A material consisting for at least 50% of its particle count out of nanoparticles (see below).

Nanoparticle

Any particle with at least one dimension within the range of 1 to 100 nm, and any particle with a specific-surface area exceeding 60 $m^2 \cdot cm^{-3}$.

Particle-specific

Describing properties, phenomena and effects that result from the presence of particles of a certain material, rather than from the release of ions from this material.

Teleost fish

Evolutionary lineage comprising diverse ray-finned fish, including zebrafish, that can extend their jaw outwards from the mouth owing to their movable premaxilla.