Report on (feedback on) initial DEB-based AOP for chronic ecotoxicity to ENMs (based on workshop)

DELIVERABLE 6.4_v1

Document History

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Abstract

This deliverable is presents major work and efforts in WP6 of RiskGONE to develop an adverse outcome pathway (AOP) for the chronic (reproductive) toxicity of nanomaterials (NMs) to daphnia. Daphnia species are one of the most important model organisms utilised for standardised toxicity testing. Their importance arises because of their role as a keystone species in the food chain and their sensitivity to pollutants which makes them an excellent indicator species. Another advantage of daphnids for ecotoxicity testing is their asexual (parthenogenic) reproduction mode under normal conditions. Under stressed conditions (e.g., crowding, lack of food, pollutants), they switch to production of males leading to sexual reproduction and formation of resting eggs or ephippia, that can survive the harsh conditions and hatch when conditions improve.

While there is a growing body of chronic toxicity studies utilising daphnids and various NMs, only a small sub-set of those papers have the necessary mechanistic insights and/or omics analysis to allow determination of so-called key events (KEs) and their linkage into an AOP. Mapping these papers to the existing AOP for (endocrine disruption based) impairment of reproduction led to the suggestion of particle accumulation in the gut as the molecular initiating event. Accumulation of NMs in the guts of daphnids and git blockage thus appears to be an important mechanism of toxicity, leading to reduced calorific uptake and thus reduced growth and reproduction. Food scarcity is similarly linked to reduced calorific intake, and thus to reduced growth and development that may lead to delayed sexual maturity and thus impacts on reproduction. Thus, we have also leveraged the large amount of data on food scarcity on daphnia reproduction to strengthen the weight of evidence for the gut-blockage induced AOP.

A well-established model for analysing the energy trade-offs between growth and reproduction and the impacts of stresses on these trade-offs is the Dynamic Energy Budget (DEB) model. This model describes the rates at which an organism assimilates and utilises energy for maintenance, growth and reproduction, as a function of the state of the organism and of its environment. Thus, we have utilised the DEB model to develop a first AOP for NMs-induced reproductive impairment and induction of males in daphnids. We are building the evidence basis, having identified the KEs and their relationships, determined the strength of evidence for each KE and will shortly be sharing the proposed AOP via the AOP-Wiki. To date, there are very limited AOPs for daphnia, and thus we are also working to build a community of researchers working on all aspects of daphnia AOPs via the newly established Daphnia Portal on WikiPathways.

A series of presentations and discussion workshops with experts in daphnia biology and NMs have been held over the Autumn 2021 to further build the consensus on the AOP and based on these discussions some further refinements are underway. Complete documentation and demonstration of the weight of evidence (WoE) for the AOP and the underpinning DEB model is also underway and will be reported in RiskGONE Deliverable Report D6.5.

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List of Abbreviations

AmP – Add my Pet

AOP - Adverse Outcome Pathways

D – Deliverable

DEB – Dynamic Energy Budget

ENM - Engineered nanomaterial

ERA – Environmental Risk Assessment

FAIR - Findable, Accessible, Interoperable and Reusable

FV - Fenvalerate

GUTS - General Unified Threshold of Survival

IATA - Integrated Approaches to Testing and Assessment

KE – Key Event

M – Month of the project timeline

MIE - Molecular Initiating Event

NEC – no-effect concentration

NCS – Nano Safety Cluster

NOM – natural organic matter

PCA – Principal Component Analysis

PE - polyethylene

RG – Risk Governance

RGC - Risk Governance Council

RGF – Risk Governance Framework

RR – Round Robin

SDS – Sodium Dodecyl Sulphate

SOP - Standard Operating Procedure

TEM – Transmission Electron Microscopy

TKTD - Toxicokinetic-Toxicodynamic

TG – Test Guidelines

WoE - weight of evidence

WP – Work Package

1. Introduction

Adverse outcome pathways (AOPs) are important instruments to expand the use of mechanistic toxicological data and facilitate development and implementation of Integrated Approaches to Testing and Assessment (IATA) (OECD, 2017). Application of the AOP concept has potential in the long term to transform the practice of (eco)toxicity testing, reducing the reliance on fish and other sentient species, thus providing tangible 3R's (Replacement, Reduction and Refinement of Animal Testing) benefits, for example, by increasing confidence in cross-species extrapolations and read-across approaches, as well as perpetuating the development of new non-animal methods for use in safety assessment. A recent review of areas with scope for AOP development for environmental risk assessment (ERA) highlighted the need for development of chronic toxicity AOPs and AOPs for multigenerational effects of chemicals. Both areas are of particular interest to the RiskGONE WP6 partners, and for which we know we have available in-house data and expertise to build the AOPs on.

At the time of writing the RiskGONE proposal (June 2018) there were no ecotoxicity AOPs in the AOPWiki and a search of numerous terms (Daphnia, bacteria etc.) produced no results. There were only some results for energetics, reactive oxygen species (ROS) and chronic cytotoxicity. An updated search at the start of this task in February 2020 identified some progress towards daphnia-related AOPs with a focus on endocrine disruption and oxidative stress. Two daphnia-related AOPs are currently under development in the AOPWiki: AOP 201 "Juvenile hormone receptor agonism leading to male offspring induction associated population decline" and AOP216 "Excessive reactive oxygen species production leading to population decline via follicular atresia". Building on these, and the extensive datasets generated by UoB on chronic and multi-generational toxicity of nanomaterials (NMs) to *Daphnia magna*, we decided to focus initially on development of an AOP for NMs-induced impairment of daphnia reproduction.

Within RiskGONE, an extensive literature search based on chronic daphnia exposure to NMs was performed to ascertain the potential application of chemical AOPs for NMs and to identify some testable molecular initiating events (MIEs) and key events (KEs) that may be adaptable for testing of NMs effects in daphnia. In parallel, a top-down approach was applied by searching the AOP-Wiki for daphnia-relevant adverse outcomes (AOs). Although this seacrh was fairly limited, it provided a starting point for AOP developmental approach and identified the specific genes involved in juvenile hormone receptor induction leading to male induction (AOP 201) and excessive oxidative stress leading to oocyte apoptosis associated reproduction decline (AOP 216). This dual approach enabled a multi-pathway AOP, from growth inhibition leading to delayed reproduction and decreased fertility, through induction of males and reduced mating success, all converging in reduced reproductive success and population decline, to be developed. Additional eperimental evidence for the MIEs and KEs is being generated to increase the confidence in the NMs-related AOP and the set of interlinked pathways related to reproductive impairment in daphnia. The AOP starts from an MIE of gut overload leading to KEs of reduced calorific consumption, reduced growth including reduced caparapce shedding, induction of males, and reduced mating success. The identified key events and the suggested MIE are currently being evaluated on the basis of the weight of evidence (WoE) supporting them and the resulting confidence in them, following the guidance described in the OECD "Users' Handbook supplement to the Guidance Document for AOP".

Since reproduction is a highly energy intensive process, modeling of toxicity data can also give insights into how individual toxicity could affect the ecosystem dynamics as a whole. The Dynamic Energy Budget (DEB) model is a type of toxicokinetics-toxicodynamics (TKTD) model that utilises common end points such as growth and reproduction to access how, under various exposure conditions, daphnia

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can divert their energy expenditure for either maintainence, growth or reproduction. Thus, DEB modelling is being used to provide an additional line of evidence to support the transition to reduced growth and reduced reproduction in the AOP model.

2. Background information

2.1 Daphnia as a model organism for toxicity testing

The importance of the cladoceran Daphnia as a model organism for ecotoxicity testing has been well-established since the 1980s when it was standardised for regulatory testing of chemicals, due to its keystone status in the foodchain and sensitivity to pollutants which make it an essential indicator species. The mapping of the genome of its members *D. pulex* in 2012 and *D. magna* in 2017 further consolidated its utility for ecotoxicity testing by demonstrating the responsiveness of its genome to environmental stressors. Its short lifecycle and parthenogenetic reproduction make it hugely useful for assessment of development toxicity and adaption to stress.

These freshwater crustaceans are filter feeders, and their filtrating apparatus helps to uptake small food particles from sediments and water (Geller and Müller 1981). Via the dietary and waterborne routes, daphnids ingest particulates with sizes up to 50 µm through mechanical sieving mechanisms (Gophen and Geller 1984). The water flow produced by their back legs, and the swimming and stirring movement of the daphnids help to disperse the particulate matter in the water environment and favour its uptake. The filtration and ingestion processes rely on the food concentration (Ebert 2005). Daphnia feed mainly on phytoplankton, like green algae (high quality food, as *Scenedesmus* sp.), bacteria and organic detritus (low quality food). Digestion and assimilation occur in the daphnids' midgut, while peristalsis movements mix the food and increase the efficiency of these processes. A great number of microvilli covers the intestinal lumen, augmenting the gut surface contact with particulate food, contributing to their absorption. Therefore, *Daphnia* sp. are the likely entry for contaminants and NMs playing a vital role for energy transfer in the food chain as a primary consumer (Schwarzenberger and Fink 2018).

Under favourable environmental conditions (e.g., within the optimal range of parameters shown in Table 1), Daphnia reproduce parthenogenetically (Figure 1).

Table 1: Recommended conditions for optimal culture growth of Daphnia

Factor	Optimal Range
pH	7.0 - 8.6
Temperature	20 – 25 °C
Dissolved oxygen	> 6 mg/L
Water Hardness	160 – 180 mg $CaCO_3$/L
Light/ dark cycle	16 light/ 8 dark

Source: http://ei.cornell.edu/toxicology/bioassays/daphnia/culture.html

Parthenogenesis is a type of asexual reproduction in which the offspring develops from unfertilized eggs. Female Daphnia produce more female Daphnia without requiring a male to fertilize the eggs. Eggs are produced in the ovaries, and released via the oviduct into the brood chamber where they continue to develop, eventually hatching (within 1 to 3 days) into young neonates which are genetically virtually identical to the parent. The young neonates are then released to the outside at the next moult or shedding of the exoskeleton called a carapace. Usually, one clutch of eggs is produced for each adult instar (a phase between two periods of moulting in the development of an insect larva) with between 2 and 100 eggs per clutch. The newly hatched daphnids must moult, i.e., shed their outer carapace, several times before they are fully grown into an adult, usually after about two weeks. Each moult represents the transition from one instar to the next. Thus, the young are small copies of the adult; there are no true nymphal stages. Fully mature females are able to produce a new brood of young about every ten days under ideal conditions. The reproduction process continues while the environmental conditions continue to support their growth.

Figure 1: **Left:** The reproductive cycle of daphnia: under normal conditions daphnia reproduce asexually producing clonally identical offspring (so-called parthenogenetic reproduction). However, under stressed conditions males are produced and males and females mate to produce diapausing eggs that survive harsh conditions and hatch later when conditions improve. **Right:** Light microscopy images of parthenogenic reproduction with normal eggs versus sexual reproduction whereby females produce diploid resting eggs of ephippia, that are released, and can hatch at a later point regenerating the females.

Under harsh environmental conditions, or in response to some pollutants including some NMs, production of new female generations ceases and parthenogenic males are produced instead (see Figure 1). However, even in harsh environmental conditions, males may make up considerably less than half the population. Males tend to be much smaller in size than females and are also distinguished by larger antennules. The males then fertilise the eggs forming ephippia or resting eggs, which can survive in the sediment for long periods of time (years) and then hatch when conditions improve, allowing the parthenogenetic reproductive cycle to begin again.

D. magna are a well established test organism in toxicity studies and have a range of chronic toxicity end points, such as growth (eye-tail length), reproduction (total offspring and time to first brood), induction of males and resting egg production, which are are all well established end points. These can be further complemented with sublethal markers such as lipid deposits, morphological defects, delays in moulting and changes to kairomone signalling. With increasingly complex experimental designs, such as chronic, pulsed and multigeneration toxicity studies, there is a range of data that can be used to further the understanding of MIE) and how this can lead to AOPs for *Daphnia* toxicity.

2.1.1 Reproductive test (OECD 211)

Daphnia are a well-established and widely used model organism for freshwater toxicity testing due to their keystone status in the environment, rapid parthenogenetic reproductive cycle and sensitivity to a range of xenobiotics which they are exposed to as a result of their filter feeding behaviour. A broad set of behavioural and morphological changes can be observed in *Daphnia* when exposed to environmental stimuli, which forms the foundation of defined and standardised protocols for chemical toxicity testing, such as the OECD Test Guideline (TG) 202 (Acute toxicity) and TG 211 (Reproduction) tests and the EPA testing methods (Biesinger, OECD 2004, OECD 2012, Maxwell, Schnitzler et al. 2014).

Endpoints used for toxicity testing with *Daphnia* encompass acute responses, such as death (measured as immobilisation in the OECD TG 202), to changes in life history traits during the chronic test (OECD TG 211) including both reproductive changes, such as an increase or decrease in the number of neonates per adult daphnid or a delay between broods, in addition to delays or reductions in growth. Further to the standard test endpoints, phenotypic changes can be observed, such as additional spines on the helmet, variability in lipid deposits and behavioural changes, such as swimming activity (Karatzas, Melagraki et al., Colbourne, Pfrender et al. 2011, Chevalier, Harscoët et al. 2015, Tkaczyk, Bownik et al. 2021).

The effect of pollutants on life history traits, such as survival, growth and reproduction, provides sensitive information for ecological stress and chemical toxicity (Besseling et al., 2014). Studies have criticised the use of effective concentrations (EC_{20}) obtained from acute toxicity testing (where the organisms are not fed) since the EC can significantly change in chronic studies in the presence of food and natural organic matter (NOM) (Allen et al., 2010, Blinova et al., 2013). Thus, extrapolating from acute to chronic concentrations is challenging, especially in the case of multi-generational studies where impacts in the parent (F0) generation can be passed onto offspring. The enhanced uptake of xenobiotics (including nanomaterials) via food sources may explain the high population mortality in the F0 (parent) generations under chronic exposure conditions compared to the lower uptake directly from the water phase, leading to interrupted metabolic pathways, cellular signalling, and enzyme function (Kim et al., 2014). Zhu (2010) also reported acute studies, in which daphnids exposed to 50 and 100 mg L^{-1} uncoated TiO_2 NMs (21 nm) had between 10 and 20% immobilization with no significant mortality. However, in chronic exposures (3-21 days) to 0.1 mg L^{-1} uncoated TiO_2 NMs, the mortality increased significantly ranging from 13-100% in strong agreement with our studies with pristine TiO_2 NMs (Ellis et al., 2021). However, the same particles aged for 6-months in salt-only or NOM-containing media displayed reduced toxicity (Ellis et al., 2021).

The standard OECD reproductive test for *Daphnia magna* is over a single generation, and measures the time to the 1st and subsequent broods (noting any delays) and the number of offspring per brood (which is then divided by the number of adults to get the average offspring per adult), as shown

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schematically in Figure 2. The test continues until the organisms have had 5 broods, or in cases where the adults died from the exposure it terminates when there are no further adults or no further offspring. Additional aspects can be also noted, such as numbers of aborted eggs (eggs that didn't hatch) and the presence of male offspring, which is a clear sign of pollution-related stress induced by exposure to the NMs, as well as observing any morphological defects (e.g., loss of tail length, increased lipid deposits, change in body shape, delays in moulting etc). Indeed, using the images from the multigenerational studies, we were able to develop a machine learning model for prediction of NMs' toxicity to daphnids across generations (Karatzas et al., 2020). It should be noted that for the multi-generational approach that we have been developing at UoB, the parent (F0) generation is exposed and the offspring are removed within 24 hours of birth – the offspring are then split into two groups, one of which continues to be exposed to the same NM in the same medium for the subsequent generations, while the other half are placed into medium only and are called the recovery group (see Figure 3). This parallel approach allows us to look at how the daphnids potentially adapt to the presence of NMs (continuously exposed), and whether there are any epigenetic effects passed onto the offspring from the parental exposures. We note also that the subsequent generations are typically produced using the 3rd broods from the F0 (parent) generation, as there are considered the most genetically stable.
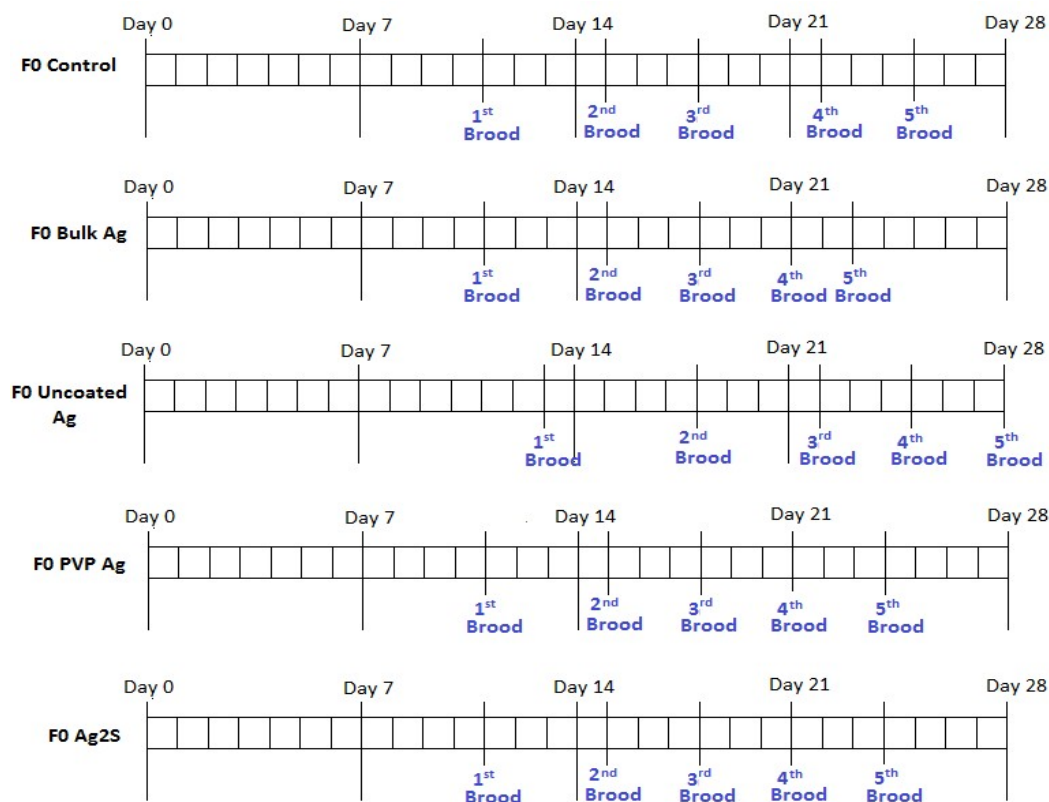

Figure 2: Schematic illustration of the standard OECD TG 211 reproductive assay showing the F0 (parent) exposure and the timings of the various broods. The controls are the *Daphnia* in pure medium with no NMs, while the subsequent rows illustrate the impact of various silver (Ag) NMs on the timings or the first and subsequent broods (offspring).
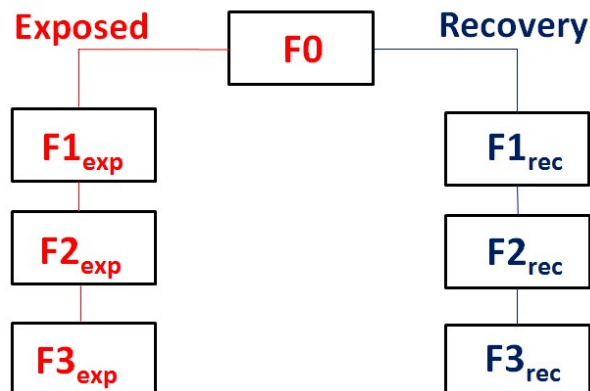
Figure 3: Multigenerational design showing the recovery and exposed generations after the F0 parental exposure. Note, the F1rec generations are born into exposure and then removed (within 24 hours post birth) to assess the recovery in the following generations.

As the medium composition affects NM physicochemical properties (Tejamaya et al., 2012, Römer et al., 2011) and their toxicity (Loureiro et al., 2011), it is essential to consider more environmentally relevant media for NMs ecotoxicology studies (Prasad et al., 2013, Shen et al., 2015) as well as assessment of appropriately transformed NMs under relevant exposure conditions. Hammes et al. (2013) classified European water types (Class I-VI) based on their chemical properties (pH, ionic strength, composition and NOM content for prediction of the stability of NMs. Application of these water classes for eco-toxicology assessments would provide more representative conditions and allow comparison of differently stabilized and transformed NM variants based on core speciation, acquisition of a NOM corona and other environmental transformations. Environmental exposure assessment should consider older and/or transformed NMs in addition to the pristine (often highly reactive) forms. In the absence of realistic media and environmental ageing, the data is neither predictive or reflective of real NM exposure scenarios nor appropriate for risk assessments (Lowry et al., 2012). The toxicity of a set of Ag and TiO_2 NMs was determined in a salt only medium (HH Combo), optimised for *Daphnia magna*, and two representative waters as defined by Hammes et al. (2013), namely the Class I and Class V waters with different amounts of NOM. Using these representative natural waters allows exploration of the role of formation of a biomolecule corona around the NMs, which can reduce their "stickiness" which may reduce their toxicity.

The compositions of the water used in the bulk of the data supporting the development of the AOP and the DEB-model are presented in Table 2 below. We have demonstrated that the current use of pristine NMs in simple synthetic media significantly overestimates the NM hazards and risks, as this type of medium does not account for the corona formation on the nano-surface under real environmental conditions and the various possible transformations of NMs including agglomeration or dissolution (Ellis et al., 2020a).

Table 2: Compositions of the optimised medium for culturing of *Daphnia*, the High Hardness Combo (HH Combo) medium, and the representative test waters with various amounts of NOM, and representative of northern Europe / Scandinavia (Class I) and UK and Spain/Portugal (Class V).

Class I river water		Class V river water		HH combo medium	
Compound	Stock mg L^{-1}	Compound	Stock mg L^{-1}	Compound	Stock (g L^{-1})
Calcium Sulfate Dihydrate	1.722	Calcium Sulfate Dihydrate	155.02	Calcium Chloride, Dihydrate	110.28
Calcium carbonate	2.002	Calcium carbonate	8.007	Magnesium sulphate heptahydrate	113.5
		Magnesium carbonate	45.517	Potassium phosphate dibasic	1.742
	µL L^{-1}		µL L^{-1}	Sodium nitrate	17
Calcium Nitrate Tetrahydrate*	5	Calcium Nitrate Tetrahydrate*	165	Sodium metasilicate nonahydrate	28.42
Sodium bicarbonate*	13.5	Sodium bicarbonate*	696.6	Boric acid	24
Calcium chloride*	7.5	Calcium chloride*	363.5	Potassium chloride	5.96
Potassium bicarbonate *	11.6	Potassium bicarbonate *	85.9	Sodium Bicarbonate	63
Magnesium nitrate Hexahydrate	48.9				
	mL L^{-1}		mL L^{-1}		
NOM (1g/L)	1.84	NOM (1g/L)	4.6	Not applicable	Not applicable
pH	7.3-7.8	pH	7.6-7.8	pH	7.6-7.8

*Stock solutions of 1 mol L^{-1}. NOM = Natural organic matter.

2.2 Dynamic Energy Budget modelling

The DEB theory modelling is a branch of TKTD modelling that can be used to explore the variation of energy allocation in organisms during their lifetime under changing environmental conditions. TKTD modelling allows for comparison for mechanism-based effects between toxicants, test species and development stage, test endpoints and environmental conditions and can simulate the effects over a range of time points (Jager et al. 2011). DEB theory has a wide range of potential applications, from fisheries management for estimating quotas based on changing environmental parameters to wastewater treatment work applications for calculating effective microbial action. DEB theory can also be applied in ecotoxicology, forming DEBtox models (https://www.debtox.info/index.html) to explore the change in life history traits due to exposure to toxicants. DEBtox models are formed of two parts: (i) the physiological element which is underpinned in DEB theory and describes the energy flow and allocation within the test species and (ii) the TKTD aspect which covers the uptake and effect of the toxicant on the test organisms.

TKTD models such as the General Unified Threshold of Survival (GUTS) models, which predict the survival rate of organisms when acutely expose to a chemical, are considered ready for use for risk assessments by the European Food Safety Authority (EFSA Panel on Plant Protection Products and their Residues (PPR), 2018). At the time that the GUTS model was approved by EFSA, it was reported that there was not enough data on DEBtox model outputs to classify DEBtox as ready for use in risk assessments, however it was noted that there was great potential for use of DEBtox in the future when the state of the science was adequately expanded. To date, the collaborative compilation of an online, readily accessible database for DEB modelling with different test organism is underway and called 'Add my Pet' or AmP, with over 2864 entries of a combination of species and toxicity studies at varying stages of completeness as of March 2021 (AmP 2021). *Daphnia magna* currently score 6/10 for

completeness within the database, meaning there is data available for modelling with reproduction, feeding, growth and respiration as a function of length or weight for the organism for at least one food level (Kooijman and Gergs 2019). The development of DEBtox modelling for ERA and monitoring of potential effects will be beneficial as the GUTS model is specifically designed for absolute end points such as death or immobilization and cannot be applied to continuous variations in response such as growth or reproduction. Therefore, DEBtox would be a useful tool in utilising these chronic testing datasets to understand the variations in toxicity response. Moreover, DEBtox models can be used to predict survival rate under untested exposure conditions and to explore the effects on growth and reproduction of toxicants over time.

2.2.1 Model theory

The DEBtox model works on the assumption that energy is allocated to one of the four main avenues: growth, maintenance, maturity and reproduction. The Kappa allocation (κ) is one of the fundamental parameters of the model, and it is designed to ensure that the initial energy constraints, i.e., the somatic maintenance of the organism, is considered when allocating energy from the reserve. A set fraction, κ, from the mobilised reserve will be allocated to somatic maintenance before the remaining $1-\kappa$ is allocated for maturity, maturity maintenance and reproduction. Within the κ allocation, somatic maintenance is the priority accounting for aspects such as maintaining structure i.e., synthesising proteins, maintaining concentration gradients, running channels such as potassium or sodium channels against the gradient, osmotic maintenance and movement costs. After meeting the somatic requirements of the organisms, the remaining mobilised κ fraction can then be utilised in growth. For example, *Daphnia* are isomorphs, with a κ allocation of approximately 0.8 on average and can be modelled using a standard DEB model framework.

Somatic maintenance is relative to the structure and therefore size of the organism (surface area and volume specific) and these costs are assumed constant within the DEB model once internal homeostasis is established. In addition, by decreasing the fluctuations in food it allows a higher level of homeostasis to be established between the different energy fluxes in the organism. These energy fluxes are shown schematically in Figure 4.
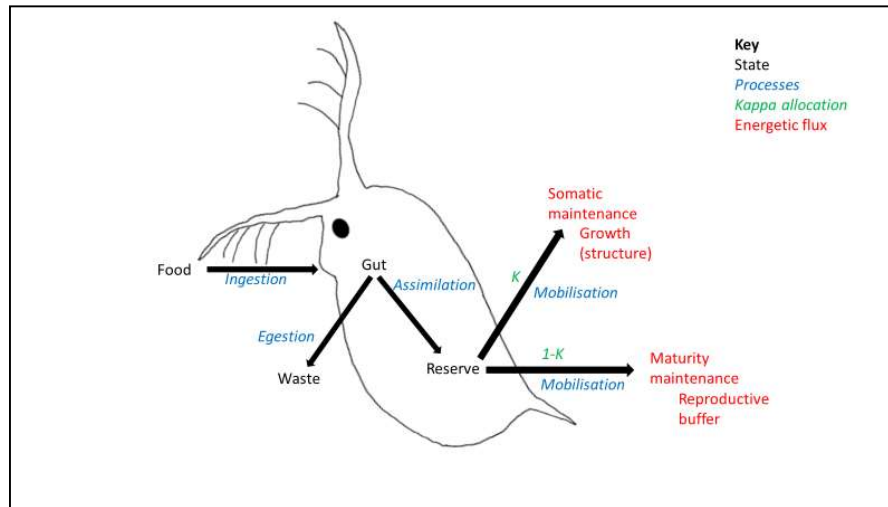
Figure 4: Scheme for the energy flow in a *Daphnia* (isomorph) standard DEB model. κ (kappa) is the energy allocated to somatic maintenance and growth and represents a fixed fraction of energy mobilized from the reserve. $1 - \kappa$ is the remainder of mobilised energy and is allocated to either maturation (juveniles) or reproduction (adults). Mature animals sink energy into the reproductive buffer until it is emptied during reproductive events (i.e., gamete release).

2.2.2 Daphnia response to food shortages and/or overcrowding

As previously stated, the DEBtox model follows the κ allocation rule, however, a switch in energy allocation in favour of somatic maintenance (an increase in k) can be used to explain a change in life history traits in organisms by increasing the mobilised reserve available for somatic maintenance and therefore growth, whilst leading to a decrease in the available fraction for maturation and reproduction (1-k) which can be observed in the change of life history end points in chronic tests to favour growth over reproduction.

Previous work with NMs has shown that the uptake and impacts of the NMs are also affected by the presence of food, both in terms of the amount assimilated and the amount retained in the gut over extended times. Particles can fit in between the microvilli and thus get "stuck" as a result of the peristaltic pressure of the filter-feeding nature of daphnids (see for example Nasser and Lynch, 2019).
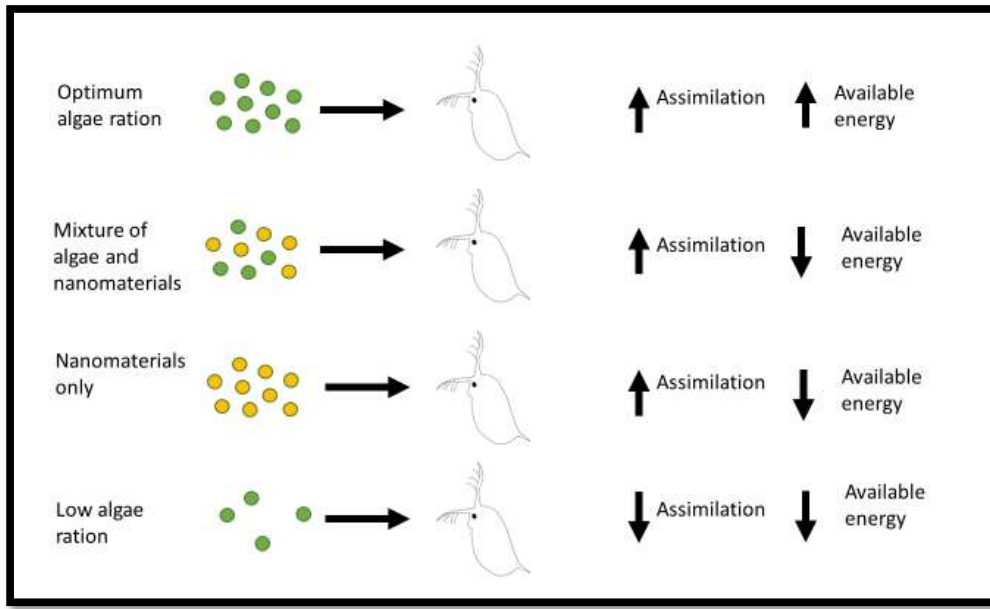
Figure 5: Schematic illustration of a study design to explore the impact of food and/or nanoparticle availability on *Daphnia* processes such as assimilation and the resulting energy availability. Co-exposure of a mixture of algae and nanoparticles leads to enhanced uptake of the nanoparticles, but also to greater clearance as less of the NMs get trapped in the microvilli (Nasser et al., 2020).

At high population densities, organisms can be impacted by a combination of over exploitation and rapid depletion of available resources, such as food, in addition to negative interference between individuals often referred to as crowding. Crowding and overcrowding can impact fitness by direct interference between individuals or through chemicals released by the organism (such as olfactory cues) which can lead to reduction in growth, reduction in offspring fitness and increase mortality (Gergs, Preuss, and Palmqvist 2014).

A study using *Daphnia* as a model to explore the assumptions of uptake and allocation of energy in an individual tolerance based DEB model for resource shortage and crowding demonstrated that the combination of resource-related and crowding-related hypotheses allowed for accurate model predictions of daphnid population dynamics (Gergs, Preuss, and Palmqvist 2014). Through these models, it was demonstrated that there are a range of mechanisms that can differ based on the population density (crowding) levels which contribute to the negative feedback between the population density and the individual life history of daphnids. The impact of crowding during *ad libitum* food supply has previously been show to lead to the daphnids producing fewer but larger neonates with higher levels of individual fitness (i.e., higher lipid concentrations with a greater starvation tolerance) when compared to non-crowded control populations (Cleuvers, Goser, and Ratte 1997). This life-strategy shift from the F_0 generation could be a preventive strategy to produce more resilient and fewer offspring to increase the F_1 likelihood of survival with increasingly depleted resources in a fluctuating environment.

2.2.3 *Daphnia* response to toxicants

Response to toxicants can be very specific depending on the nature of the exposure. Physical toxicants such as NMs or micro/nanoplastics could lead to physical impedance in the daphnids guts compared to a chemical based exposure, leading to a shortfall in assimilation of suitable food and a decrease in the reserve, similar to that experience during food shortages. However, in addition to the shortfall in the reserve, the daphnids will also have the energy burden for the removal of the physical toxicant from their guts which could lead to a mechanistic toxicity response that exasperates the depletion of the energy reserve further than a limitation to available food.

A recent study explored the hypothesis that if food level only affected accumulation kinetics and resource allocation, then the intrinsic sensitivity to a specific toxicant should be the same for all food regimes (Pieters et al., 2006). Thus, the study examined results of two recently conducted life-cycle studies on the combined effects of food level and a pulsed exposure to the pyrethroid insecticide fenvalerate (FV) on *D. magna*. The model described the effects of the time-varying exposure well, and indicated that when the animals did not die from exposure to FV, full reversibility of toxic effects was possible, allowing a complete recovery. Results revealed furthermore that the data from both studies could be described by the same no-effect concentration (NECs) for survival and assimilation, killing rate and tolerance concentrations and therefore concluded that food-dependent FV toxicity can be explained by altered TK and resource allocation, but not by changes in the intrinsic sensitivity of the daphnids. This study implies that the effect of pesticide application in the field depends on the trophic state of the receiving water body, but also that full recovery of survivors is possible after FV application.

While such approaches have not yet been applied to NMs, proof of principle exists providing a sound basis on which to explore the implications of NM assimilation on resource allocation and energy availability in *D. magna*.

2.2.4 Limitations of DEB modelling with daphnids

The moult cycle of *D. magna* is not widely considered in the DEBtox modelling design, and this is something that could improve the accuracy of the model, as previously shown in crabs with a similar moult cycle. This could be useful across all crustacean groups with similar moulting cycles that are used for modelling different parameters and will account for the slight jump in the size of the organisms based on the moulting cycle.

The variability of energy budgets due to deficits in available energy due decreases in assimilated energy as a result of ingestion of NM can be supported by DEBtox theory to model the population level impacts of the individual AOP effects.

"By modelling these outputs using the DEB framework, we can further understand the relationship between the growth and reproductive success of each population. When combined with the increased understanding of MIEs and KEs for the AOPs, we can further evidence the KEs, and understand how these markers could be used to monitor sublethal toxicity. The experimental data will also support an updating of ecotoxicity standard operating procedures and the Test Guidance documents. Collectively, the experimental data, the DEB modelling and the AOP for D. magna reproduction will provide a greater understanding of NMs impacts for not only the individuals and the immediate population, but also the ecosystem as a whole. This could significantly support the environmental risk assessment and monitoring efforts for NMs." – SETAC presentation abstract (Katie Reilly & Iseult Lynch)

Further developments within this field would be benefited by publication of raw datasets for both chronic and acute studies as opposed to only including the summary statistics such as LC_{50} values or key effect concentrations as this will allow for model development and validation using the time series data which cannot be done using the summary statistics only (Jager et al. 2011).

2.3 Development of Adverse Outcome Pathways

The AOPs are emerging as a useful tool to capture the complex mechanistic basis of toxicity in linear modules (or networks) of causally linked biological events spanning multiple levels of biological organization from molecular, cellular, tissue and organ levels to individuals and whole populations (Ankley et al. 2010). It is clear that the degree of mechanistic insight is significantly higher at molecular and cellular levels than at whole organism level as shown in Figure 6. Thus, AOPs are an important means to bridge apical endpoints coming from OECD standard tests with mechanistic insights arising from toxicogenomics studies, for example. AOPs facilitate a systematic curation, organization and application of mechanistic information (OECD 2016), by connecting an initial trigger of toxicity at a molecular level (molecular interactions with stressors, termed MIEs) to an apparent toxicity or AO associated with chemical exposure (Halappanavar, 2021). AOPs are 'chemical agnostic' (i.e., AOPs can be triggered by various stressors) and describe dynamic processes of toxicity. The individual components of the AOP include an MIE, a series of KEs, which are measurable biological changes that occur between a MIE and its eventual AO, linked by the key event relationships (KERs; Halappanavar et al., 2019).

Within RiskGONE WP6, there is a strong focus on *D. magna*, and a major effort underway to revise and update the OECD TG for chronic toxicity in daphnids (OECD TG 211) looking at reproductive decline induced by exposure to NMs, and extending it to include multi-generations based on our observations that impacts in subsequent generations may be more severe than in the parent generation (see also section 2.1.1), as documented in recent papers from our team assessing the impacts of Ag and TiO_2 NMs on daphnids (e.g., Ellis 2020a, Ellis 2020b, Ellis 2021). We thus proposed to map the existing data from chronic multi-generational tests, which were supplanted with toxicogenomics data and other molecular level end-points, onto the AOP framework and to develop a putative AOP for NMs-induced indication of males, reproductive failure and population decline, as shown schematically in Figure 7.
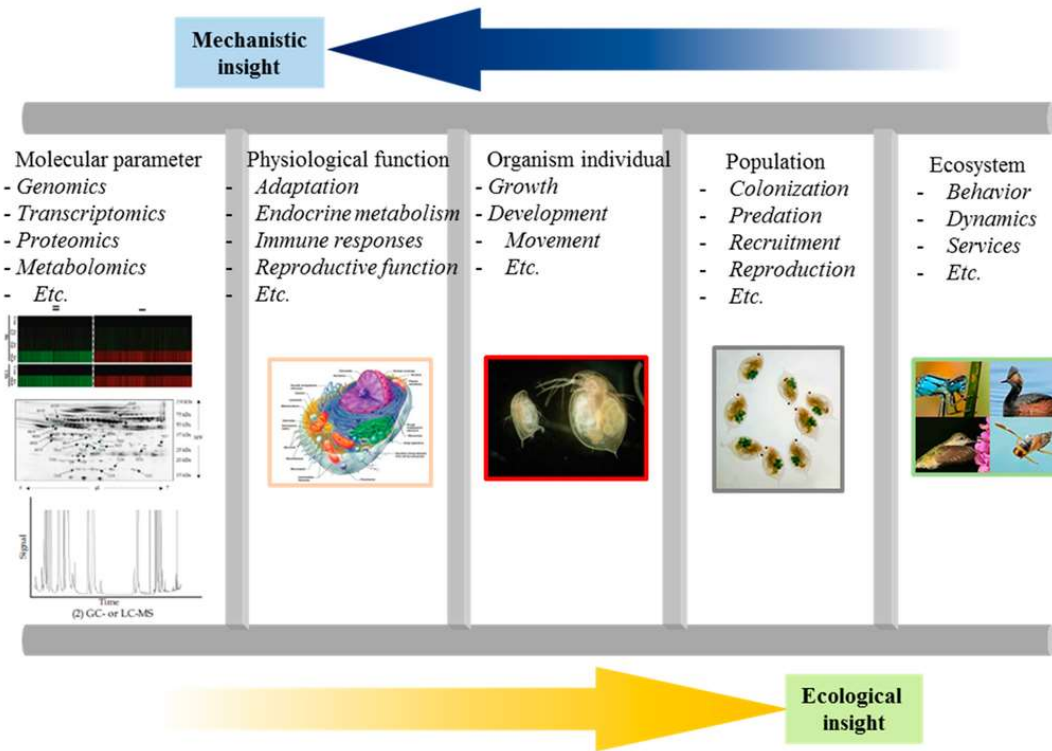
Figure 6. A multilevel framework for ecotoxicogenomic studies at multiple levels ranging from molecular, physiological, organismal, and population in ecosystem. From Kim et al., 2015.
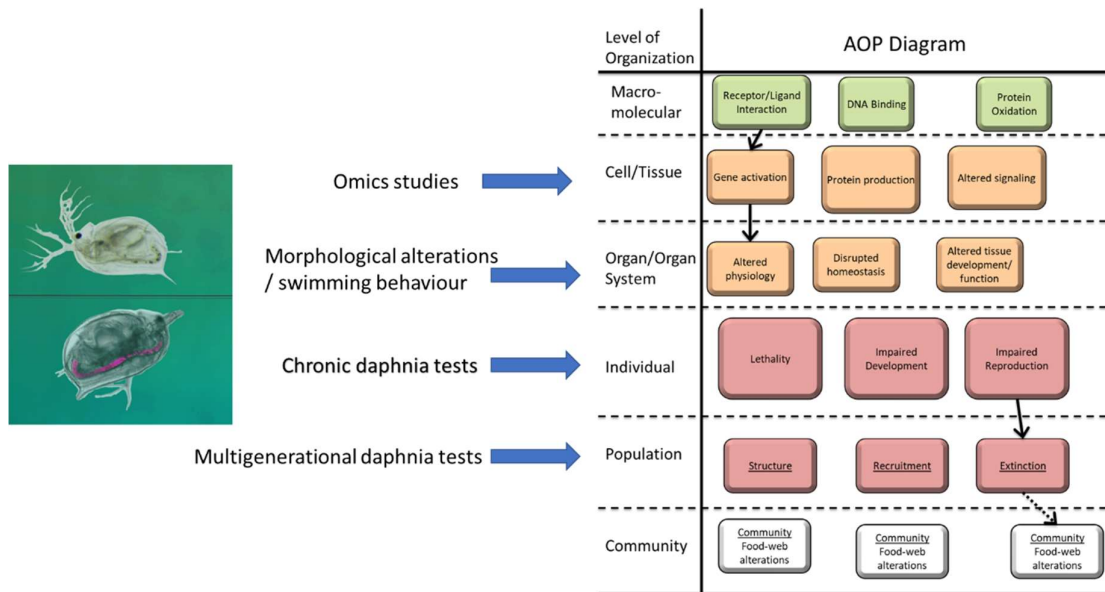

Figure 7. Schematic illustration of the mapping of experimental endpoints and data to a generic AOP structure which indicates the types of aspects to consider and map.

3. Methodology

3.1 Alignment with and learning from WP5 activity

The efforts of WP6 are intentionally aligned with those of WP5, which is a much larger WP with more partners, and since AOPs are more established for human-related AOs. Building on the approach developed in WP5, a combined bottom—up and top-down literature review strategy was explored, coupled with an exploration of existing AOPs for daphnids where these exist. For example, WP5 took the approach of using existing AOPs to generate simple and testable strategies to predict if a given NM has the potential to induce a specific MIE leading to an AO through a series of KEs. Firstly, the WP5 partners identified potential MIEs or initial KEs reported for NMs in the literature. Then, the searched the identified MIE or initial KEs as keywords in the AOP-Wiki to find associated AOPs. Finally, using two case studies, the WP5 partners demonstrated how *in vitro* strategies can be used to test the identified MIE/KE, and published the approach in Murugadoss et al., 2021.

3.2 Literature Analysis – bottom-up and top-down

A review of the Web of Science database using the search terms "Nanomaterial AND daphnia AND chronic" or "Nanomaterial AND daphnia AND multigeneration" was performed (as shown in Figure 8). This was followed by an initial analysis of the relevance of the identified papers, while non-relevant papers were removed. The remaining 64 papers were distributed among the partners for the extraction and analysis of the data. Templates were developed to capture: i) the NMs' characteristics and the exposure characteristics; ii) the endpoints studied and the outcomes of the exposures, and were supplemented with iii) the ToxR and GuideNano tools for scoring of the data quality. The data are being used to provide "WoE" analysis for each of the end-points and to map the available data into the categories of the AOPs – i.e., macromolecular, cellular, organ/system, individual, population and community.

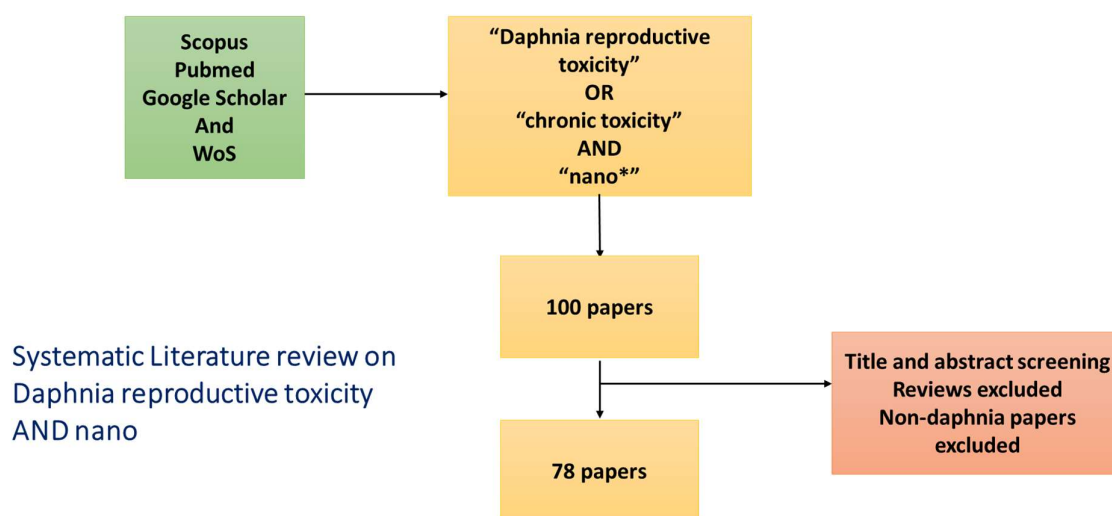**Figure 8:** Bottom-up literature review of available literature on chronic (reproductive) toxicity of NMs to daphnids. The acquired papers were curated for all NMs physico-chemical information and for all biological end-points, including life stage information, such as time to first and subsequent broods, number of offspring per brood and per daphnid, as well as assessment of any morphological effects and other aspects of relevance.

In parallel, a search of the AOP Wiki for daphnia-related AOPs was performed. There are very limited AOPs for ecotoxicity to date, with just two of relevance to *D. magna* reproduction, including AOP 201 "Juvenile hormone receptor agonism leading to male offspring induction associated population decline" and AOP 216 "Excessive reactive oxygen species production leading to population decline via follicular atresia" (ROS production leading to reproduction decline). A targeted or top-down literature analysis was performed based on the key events from AOP201 as shown in Figure 9.

Figure 9: Bottom-up literature review based on key events from AOP201 and NMs.

Using the methodology outlined in the OECD Nano-AOP working group, the identified biological events associated with chronic and/or multi-generational toxicity of NMs to daphnids were evaluated to identify potential KEs which were assessed for the three main criteria: plausibility, measurability, and regulatory relevance. The KEs were also assessed for cross-AOP application.

3.3 DEB modelling approach

Within the DEB research community, models have been developed in MATLAB and R. Due to the open source nature of R, several packages can be used as a starting point for DEB modelling with daphnids. SimecolModels and NicheMapR are both often referred to within the GitHub for DEB model development, and thus allowed us to start from a well-developed model already, although which had not been applied previously to nanomaterials. Example of SimecolModels DEB outputs.

DEB modelling packages in R utilise the following parameters as summarised in Table 3, with some of the key constants shown in Table 4 and an example of the output shown in Figure 10.

Table 3: Technical application and notation used in the model development

Notation	Parameter/ equation
k	kappa allocation
k_x	Assimilation efficiency
k_R	Efficiency of reproduction
k_J	Efficiency of maturation*
$\dot{v}$	Energy conductance
$\{\dot{p}_{Am}\}$	Surface area maximum
$\{\dot{p}_m\}$	Volume specific maintenance costs
$\{\dot{p}_T\}$	Surface specific maintenance costs
$[E_G]$	Specific cost of growth
E_H^b	Maturity at birth
E_H^p	Maturity at puberty (maximum maturity)
$\dot{r}_B$	Von Bertanffly Growth constant

Table 4: Values of key parameters used in the DEB modelling to support the AOP development for reproductive toxicity to daphnia.

Key Parameters	Value
Arrhenius temperature	6400K
Standard kappa allocation	0.8

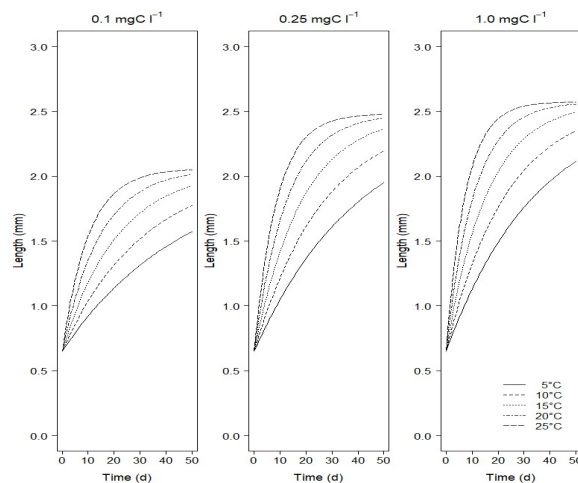**Figure 10**. An example DEB model output available within the SimecolModel package, scaling the *D. magna* growth for both food and variations in temperature over time. This model has many of the parameters for daphnids built into the package. As such, we started from this model and began fitting our data and to consider the impacts of nanomaterials and their accumulation in the daphnids gut.

3.3.1   Methodology: exposure to analysis

***Daphnia* chronic exposures**

Daphnia chronic toxicity response can be established using total reproduction and growth over a 21-day testing duration. The chronic toxicity exposures are based on the OECD TG 211 *daphnid* chronic reproduction test (OECD 2012). Observations are made over the test durations for time to first brood, time between broods, total neonates per brood (and over the whole period) and growth over time, often measured from the eye to the tail spine. These observations allow for sublethal toxicity to be observed in the daphnids and the impact of the toxicant on the reproductive health of the daphnids to be determined.

Resources and reagents

- Aerated medium or borehole water
- Glass pipette
- Light box
- Test vessels (50mL glass vials) and racks
- *Chlorella vulgaris* algal feed (refrigerated to 4°C)
- Nikon stereomicroscope with camera (or microscope with a camera fitting to enable images to be taken)
- Access to image analysis software (such as Image J- which is open access ImageJ (nih.gov))

Protocol

1) Neonates were filtered from the running cultures and pooled from the different culturing jars. NB. Daphnid exposures are typically done with broods 3-6 from the running cultures. Cultures are maintained in the same medium that the exposure will be conducted in to remove any confounding factors associated with the change in medium.
2) Neonates were then allocated to a test vessel from the pooled stock to ensure that there is no bias associated to the different culture jars that could compound the results.
3) Daphnids were maintained individually in the 50mL test vessels, with typically 12 replicates per treatment.
4) The toxicant was then added to the test vessel in the nominal concentration outlined in the study (25 mg/L 1-5µm polyethylene beads).
5) The labelled test vessels were then pooled and stored within the CT laboratory for the duration of the test.
6) For observation of results, test vessels were randomly selected, and results recorded (Table 6). Typically, daphnids were imaged on Day 0, 7, 14 and 21 during chronic tests and neonates were counted daily.
7) For imaging, daphnids were removed from the test vessel and placed on a glass slide, excess medium was removed to limit the daphnia movement to enable a clearer image to be taken. The slide was transferred to the microscope stage, focus adjusted, and image taken as quickly as possible to reduce the stress to the daphnia. Once imaged, the daphnid was returned to the test vessel. Light intensity and magnification were recorded on the respective observation sheets at the time of imaging. A scale bar was included with each photo to enable subsequent growth measurements.
8) Neonates were removed from the test vessel at time of observation. Care was taken to ensure minimal medium was removed.
9) Testing medium was replenished three times per week as outlines in steps 3 and 4 above.

10) Images were measured for total growth (centre of the eye to base of the tail spine) and total length of tail to allow for total growth to be calculated using Image J software (Figure 11).
11) At the end of the 21-day testing period daphnia would be discarded or could be retained for subsequent lipid analysis.

Table 5. Daphnid observation data capture sheet for chronic toxicity studies

Date	Day of Culture	Culture/Jar						Offspring						Food	Media Change	Observations/Comments
		1	2	3	4	5	6	1	2	3	4	5	6			

In addition, measurements can be taken for growth over time using image analysis software, such as Image J. This allows the growth of the daphnids to be measured by using the captured microscope images from the study. Typically, measurements are taken from the centre of the eye to the base of the tail spine (Figure 11) and then from the base to the tip of the tail for tail length. Calibration is based on the scale bar for the respective images.

Figure 11. Example of image analysis in Image J measuring the growth of the daphnid. Red line depicts the scale bar and yellow line is the length being measured.

Analysis for DEB models

The observations were compiled into an excel sheet and saved as a csv to enable the data to be read directly into R, any missing observations were replaced with NA (example in Table 6 below). This included compiling the neonate numbers from the lab data sheets, time to different broods, and the measurements taken in Image J for the daphnids over time.

Table 6. Example of compilation of data, formatted to read into R.

Medium	Daphnia	body1	tail 1	body2	tail2	body3	tail 3	body4	tail4	body5	tail5	body6	tail6	body7	tail7	body8	tail8	body9	tail9	body10	tail10	body11	tail11	body12	tail12	body13	tail13	body14	tail14	body15	tail15	body16	tail16	body17	tail17	body18	tail18	body19	tail19	body20	tail20	body21	tail21	No_1broo	Time_1bro	No_2broo	Time_2bro	No_3brood	Time_3bro	No_4brood	Time_4bro	No_5brood	Time_5bro	Total_neo	growth_bo
HH_PE	PE1	1010.5	353.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	NA
HH_PE	PE2	965.8	324.8	1158.1	286.1	NA	NA	1003.9	132.5	1163.1	253.2	2698.8	325.4	2685.8	291.2	2724.9	281.8	3224.9	335.4	3181.8	381.6	3303.8	365.1	2877.9	319.4	2872.7	349.6	2878.5	318.6	NA	NA	3555.1	433.7	3563.1	475.4	3783.9	402.2	3688.6	455.3	3731.3	399.9	3980.6	457.7	17	14	12	16	16	20	NA	NA	NA	NA	45	3014.8
HH_PE	PE3	980.9	357.9	978.8	339.2	NA	NA	830.8	212.5	NA	NA	2354.7	536.7	2963.6	540.1	2933.6	589.2	2906.7	539.5	3485.5	616.3	3552.7	611.7	2936.9	528.6	3009.1	487.3	3007.3	463.9	NA	NA	3650.9	556.4	3758.1	572.6	3673.3	534.6	3931.1	564.5	3905.5	546.4	4220.4	490.9	15	12	11	16	24	19	NA	NA	NA	NA	50	3239.5
HH_PE	PE4	943.9	362.4	936.4	353.7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	NA
HH_PE	PE5	1564.1	419.4	1532.7	368.3	NA	NA	973.4	219.3	1260.7	229.2	25.1	481.6	3113.1	528.6	3161.2	579.2	3132.6	501.2	3593.4	572.6	3609.1	570.6	2985.6	447.7	3081.9	454.2	3034.6	433.9	NA	NA	3576.1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	18	12	12	14	NA	NA	NA	NA	NA	NA	30	2012
HH_PE	PE6	1227.5	395.1	1191.8	356.5	NA	NA	887.1	173.1	871.5	170.5	2355.1	402.5	2365.7	417.4	2938.6	449.1	2931.2	472.7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	NA
HH_PE	PE7	1189.1	371.1	1172.3	376.4	NA	NA	936.3	202.7	951.1	228.4	2312.6	593.4	2365.7	551.9	2344.1	551.5	2836.7	595.6	3593.4	572.6	3331.4	571.2	2791.9	442.5	2729.1	497.7	3058.3	448.8	NA	NA	3917.8	517.1	3959.5	514.1	3994.5	555.3	3949.9	458.1	4299.3	463.7	4230.1	465.5	20	13	19	16	21	21	NA	NA	NA	NA	60	3041
HH_PE	PE8	1044.5	315.7	1031.5	280.3	NA	NA	805.3	152.9	NA	NA	2136.5	345.2	2112.8	292.9	2776.4	412.9	2803.8	491.9	3209.9	474.5	3202.1	448.4	2668.8	377.1	2743.1	375.3	2732.2	399.8	NA	NA	2868.4	487.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	1823.9
HH_PE	PE9	1064.6	355.3	1071.3	332.9	NA	NA	817.7	172.9	1123.9	211.8	2243.4	440.6	2258.6	447.4	2789.2	435.1	2778.7	382.6	326.1	538.4	3267.3	517.4	2717.8	373.8	2918.4	453.4	2920.9	461.7	NA	NA	3680.9	506.3	3680.6	487.6	3709.4	507.9	3972.9	444.9	4008.2	490.2	3910.1	432.6	17	11	17	15	22	19	NA	NA	NA	NA	56	2845.5
HH_PE	PE10	1185.6	374.3	1194.7	339.6	NA	NA	883.1	165.2	1814.5	332.2	2388.3	430.1	2399.5	395.2	2944.3	376.3	2976.4	421.2	3238.2	431.8	3172	422.6	2667.1	340.6	2659.6	352.9	2556.7	333.5	NA	NA	3241.4	420.2	3628.6	545.3	3590.4	520.2	3518.4	483.4	4027.7	488.7	4098.6	516.2	21	12	17	15	20	19	NA	NA	NA	NA	58	2913
HH_PE	PE11	1128.9	323.9	1108.8	315.4	NA	NA	926.9	191.9	1949.6	411.5	1862.3	414.6	2549.1	450.6	2994.8	558.2	3100.7	505.4	3093.5	588.8	3207.6	502.5	2615.5	414.6	2587.1	417.5	2925.1	471.4	NA	NA	3430.3	531.9	NA	NA	3907.8	621.5	3919.7	574.8	4147.5	614.3	4175.2	572.6	12	13	7	15	18	16	20	20	NA	NA	57	3046.3
HH_PE	PE12	1180.5	373.9	1132.8	379.3	NA	NA	914.8	187.6	1874.3	374.5	2469.3	518.2	2483.8	466.7	2984.1	523.4	3020.4	538.1	3422.1	590.8	3405.4	596.4	2769.8	521.2	3156.5	514.1	3131.5	509.1	NA	NA	3682.8	599.7	3955.1	641.4	NA	NA	NA	NA	NA	NA	NA	NA	20	12	16	15	NA	NA	NA	NA	NA	NA	36	NA
HH	HH1	1358.2	416.1	1331.5	422.4	1576.1	388.2	NA	NA	NA	NA	2717.3	504.7	2682.5	550.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	NA
HH	HH2	1187.8	367.8	1162.7	389.6	1412.5	362.3	NA	NA	NA	NA	1901.2	395.9	1969.2	385.8	2018.7	368.9	2007.6	397.2	1933.1	389.6	2082.8	394.1	1821.6	290.5	1788.4	315.6	1815.8	261.8	1722.7	261.7	NA	NA	1840.4	344.7	2295.1	298.4	2349.3	223	2320.8	241.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	1133
HH	HH3	1292.8	428.6	1219.9	398.7	1463.4	401.1	NA	NA	NA	NA	2620.1	518.4	2620.3	504.1	3290.8	533.8	3322.9	513.3	3284.8	583.6	3933.3	597.7	3210.1	467.8	3237.8	504.9	3550.5	476.1	3528.8	490.1	NA	NA	3705.6	484.9	4543.9	524.7	4487.5	578.1	4737.5	558.6	NA	NA	13	11	31	13	34	16	29	19	NA	NA	107	3444.7
HH	HH4	931.6	311.2	1068.7	315.6	1339.3	340.4	NA	NA	NA	NA	2372.2	466.8	2369.1	404.2	2905.1	453.1	2845.9	487.9	3496.6	510.4	3449.6	504.1	2852.8	405.2	3173.9	377.2	3114.7	448.7	3131.6	383.6	NA	NA	3326.5	403.2	4071.9	541.3	4243.9	389.7	4208.3	364.4	NA	NA	23	12	26	16	15	18	29	20	NA	NA	93	3276.7
HH	HH5	1066.2	411.6	1271.6	383.3	1623.4	403.6	NA	NA	NA	NA	2766.5	518.8	2731.6	533.5	2697.2	485.3	3408.6	571.7	3414.4	578.8	3874.9	605.9	3262.4	505.4	3262.3	487.1	3228.4	484.5	3518.1	504.3	NA	NA	3483.1	466.7	4340.1	552.9	4390.1	554.3	4357.5	542.9	NA	NA	12	10	34	14	26	16	31	20	NA	NA	103	3291.3
HH	HH6	1179.1	393.4	1156.7	370.9	1477.5	359.6	NA	NA	NA	NA	1824.7	339.7	1975.1	409.6	1983.5	391.6	1987.6	406.7	2142.8	367.9	2071.8	356.9	1688.5	340.7	1811.6	289.5	1796.2	334.9	1834.9	330.7	NA	NA	3331.5	483.9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0	2152.4
HH	HH7	1035.5	380.4	1198.9	343.3	1617.4	363.6	NA	NA	NA	NA	2797.1	524.4	2764.9	529.5	2756.9	525	3415.3	549.6	3412.8	559.3	3745.3	596.4	3127.6	470.1	3151.3	457.7	3077.3	469.3	3325.9	472.1	NA	NA	3377.1	479.8	4241.6	545.2	4223.4	539.7	4162.9	519.8	NA	NA	17	10	34	14	30	16	33	20	NA	NA	114	3127.4
HH	HH8	1046.4	402.2	1249.4	362.9	1589.1	398.3	NA	NA	NA	NA	2686.7	539.9	2631.9	560.9	2616.4	459.6	3286	572.6	3349.1	538.6	3816	539	3185.5	465.5	3213.2	457.3	3329.9	473.9	3406.1	448.7	NA	NA	3507.9	402.1	4319.2	494.8	4290.1	526.6	4296.8	498.8	NA	NA	29	14	31	16	24	20	NA	NA	NA	NA	84	3250.4
HH	HH9	1318.4	416.6	1267.1	421.2	1564.7	408.9	NA	NA	NA	NA	2656.7	482.1	2641.8	594.6	2691.6	517.9	3243.8	585.2	3249.4	617.6	3708.4	607.2	3140.3	540.9	3044.9	549.5	3398.4	533.7	3383.2	504.2	NA	NA	3345.1	560.5	4167.4	416.3	4240.5	422.6	4239.2	438.3	NA	NA	9	10	29	13	30	16	32	20	NA	NA	100	2920.8
HH	HH10	1066.3	402.8	1163.7	344.3	1222.2	369.9	NA	NA	NA	NA	1904.3	380.9	2557.8	482.8	2620.1	489.6	3218.8	566.2	3214.8	536.4	3801.7	594.9	3188.1	480.1	3148.6	499.1	3460.5	NA	3467.3	520.4	NA	NA	3631.9	463.9	4286.6	557.3	4271.6	572.5	4351.5	496.9	NA	NA	30	13	34	16	24	19	NA	NA	NA	NA	88	3285.2
HH	HH11	1064.3	360.6	1342.4	371.6	1315.1	378.9	NA	NA	NA	NA	2395.6	549.2	2925.7	597.4	2980.7	648.9	3532.8	680.1	2616.8	635.5	3580.2	690.9	3315.5	560.5	3346.4	552.5	3353.6	549.7	3560.7	574.9	NA	NA	3590.3	563.1	4311.1	642.4	4295.2	645.1	4333.7	634.5	NA	NA	20	11	32	14	29	17	33	20	NA	NA	114	3269.4
HH	HH12	980.2	378.1	1274.5	359.2	1231.3	353.4	NA	NA	NA	NA	2197.2	432.1	2834.4	508.7	2762.3	512.8	3425.9	618.9	3379.1	605.1	3397.8	587.1	3191.1	519.1	3218.1	523.5	3131.8	3131.8	3503.1	459.8	NA	NA	3480.7	475.2	4246.5	633.9	4362.5	559.1	4296.7	551.1	NA	NA	19	11	19	14	22	17	27	20	NA	NA	87	3316.5

For the initial trial of the DEB model and adaptation of the packages available in R currently, the observations for each exposure group (n=12) were averaged for each observation time point, leading to a total of 21 measurements of growth for the exposure and control group respectively and used going forwards within the model. This was used in combination with the parameters outlined on AmP to modify the R script available from R for Science – musselDEB.

4. Results and Discussion

4.1 Initial AOP for NMs-induced impairment of reproduction

Only three papers were identified from the top-down search based on AOP201, as shown in Table 8. However, these papers provided important insights into the likely MIE - namely that physical blockage of the gut occurs as a result of accumulation of NMs in the gut. This is consistent with the Transmission Electron microscopy (TEM) imaging from exposures of daphnia to TiO_2 NM, performed in both salt only and NOM containing medium, as well as on pristine (freshly dispersed) versus environmentally aged (in the media for 6 months prior to exposure) NM, as shown by the representative images in Figure 12.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 814425.
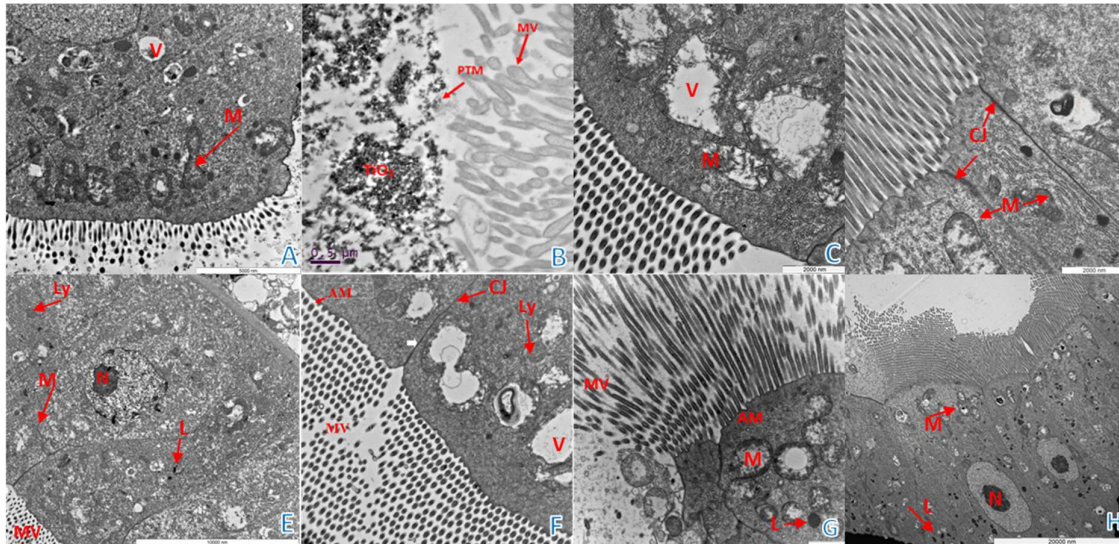
Figure 12: TEM images of daphnid gut cells: **A)** control daphnid gut, **B)** pristine uncoated TiO_2 NMs in culturing medium (evidence of lipofuscin and autophagy vacuoles), **C)** TiO_2 uncoated NMs aged in culturing medium, **D)** uncoated TiO_2 NMs aged in artificial river water, **E)** control daphnid gut, **F)** TiO_2 PVP NMs aged in culturing media, **G)** pristine PVP TiO_2 NMs in artificial river water, and **H)** aged PVP TiO_2 NMs in artificial river water. **KEY:** mitochondria (M), cell junctions (CJ), nucleus (N), apical membrane (AM), microvilli (MV), peritrophic membrane (PTM), vacuole (V), lysosome (L) and secondary lysosomes (ly). From Ellis et al. 2021.

Based on the mapping in Table 7 of the impacts of NMs on each of the KEs in AOP201, a first iteration of the network of pathways leading to decreased reproduction was developed. Based on the available data, we propose 5 interlinked AOs, including decreased growth and decreased fertility, induction of males, reducing mating success (under stressed conditions where sexual reproduction is needed), all converging in decreased reproductive success, as shown schematically in Figure 13. A total of 22 KEs are currently identified, although these may evolve and their plausibility and regulatory relevance (as per the OECD guidance for development of AOPs) as we work through the full documentation of the WoE for each. All are triggered by the MIE of physical blockage of the gut, leading to decreased calorific intake. Applying the DEB model, which is a key method to assess the energetic trade-offs between growth and reproduction, is a logical extension to provide further mechanistic insights.

Table 7: Mapping of the literature review results on NMs impacts on key events from AOP201 indicating physical blockage of the gut as the MIE.

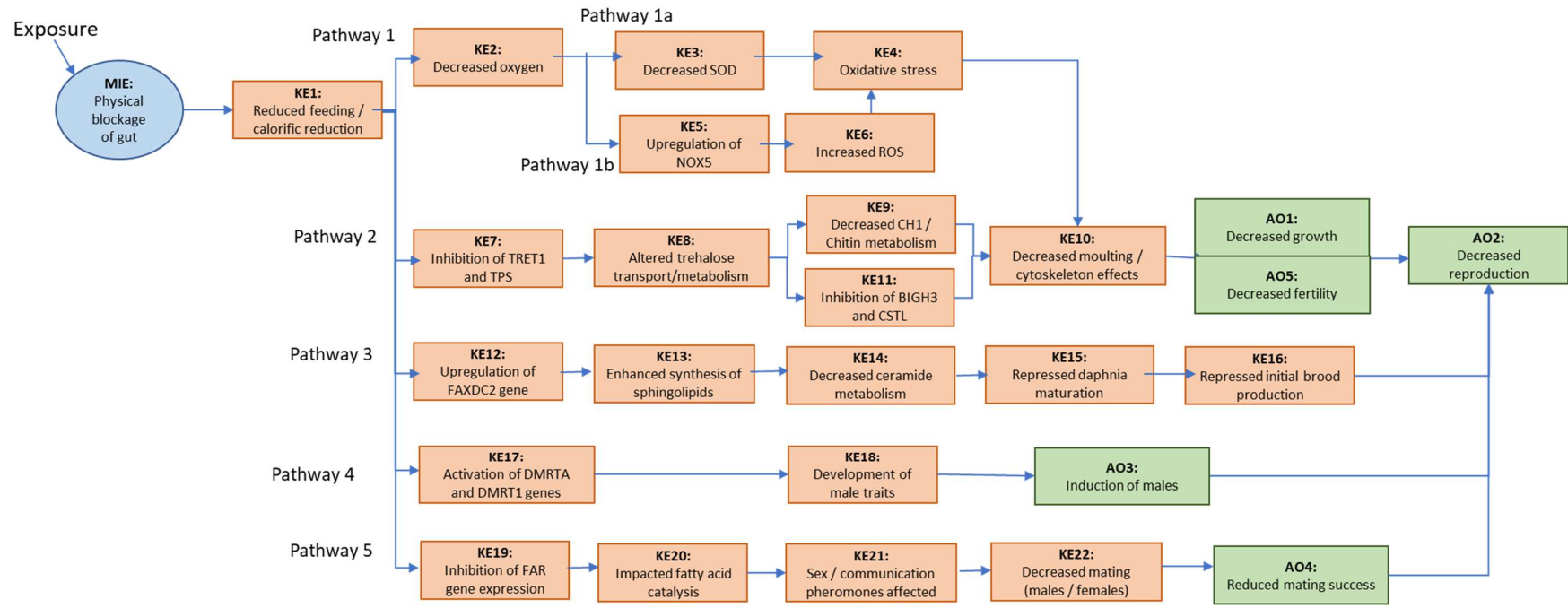| Test protocol	Toxicity result EC50	Molecular initiating event	key event 1 organelle response	key event 2 Cellular response	key event 2 Cellular response	key event 2 Cellular response	key event 3 Tissue response	key event 4 organ response	Adverse outcome Organism level	Adverse outcome population level	Other results
OECD 211	0.913 g/L								affected reproduction	reduced the number of female offspring, no effect on male offspring	
OECD 211	n.a.	physical damage	decrease cellular and tissue oxygene level and energy intake	oxidative stress			aborted eggs		affected reproduction, no effect on growth and mortality		
	n.a.								low mortality, significant growth reduction, affected reporduction	reduction in number of broods and total offspring	
	n.a.		reduction in food and energy intake	chemical and oxidative stress	impaired detoxification processes		endocrine toxicity	neurotoxicity	affected reproduction		
	n.a.										
	n.a.								reduced fertility, developmental effects		
OECD 211		blockage in digestive tract	decreased feeding and energy intake	inhibition of TRET1 and TPS gene expression	affected trehalose transport and metabolism	inhibition of expression of CHI gene isoforms (Chitin metabolism inhibition)	interference with exoskeleton and molting	decreased fecundity	decreased growth an reproduction	decreased reproduction	transcriptome expression (NR, Swiss-Pro, Pfam, COG, GO, KEGG)
					affected trehalose transport and metabolism	inhibition of BIGH3 and CSTL expression	decreased tissue growth and molting		decreased growth		
					inhibition of FAR gene expression	affected catalysis of the activated fatty acids reduciton to fatty alcohols	affected sex or communication pheromones		decreased mating success between male and female		
					upregulation of FAXDC2 gene	enhanced synthesis of sphingolipids	decrease in ceramide level		decreased reproduction		
					upregulation of NOX5 gene	increased ROS production					
					activation of DMRTA and DMRT1 genes				affected development of male traits	reproductive modes shifting	

Figure 13. First effort to map the AOP network for decreased reproduction in *Daphnia magna*, based on experimental data generated utilizing the OECD 211 test supplemented with additional mechanistic end-points. Five interlinked Adverse Outcomes and 22 key events are identified, arising from a MIE related to particle accumulation in the daphnid gut and leading to reducing feeding and calorific intake.

4.2 DEB modelling – initial results

Our first DEB modelling was performed on a dataset generated by UoB on the impacts of polyethylene (PE) particles alone and in combination with chemical toxicants (either diclofenac or sodium dodecyl sulfate, SDS) as part of the PhD work of Katie Reilly. This dataset provides a clear way to isolate effects from the chemicals versus the particles and their combined effects. As per the previously mentioned study which demonstrated the use of DEB modelling to probe whether exposure to pesticides alters the intrinsic sensitivity of the daphnids or just alters the toxicokinetics and resource allocation, the goal of the current work is to explore the same question with NMs and assessing their impacts on calorific uptake and resource allocation.

A principal components analysis (PCA) was used to visualise the initial variation between the growth (y) and reproduction (x) of daphnids exposed to different combinations of PE particles or chemical toxicants (either diclofenac or SDS) in HH COMBO medium over a 21-day test period, performed in accordance with the OECD TG 211 chronic daphnia test. The PCA plot, shown in Figure 14, indicates that the highest variability was in the number of neonates produced for most exposure group. However, there was more variability in growth for that population for the combined exposure of diclofenac and PE beads. This can be further investigated by DEB modelling, as shown in outline below.
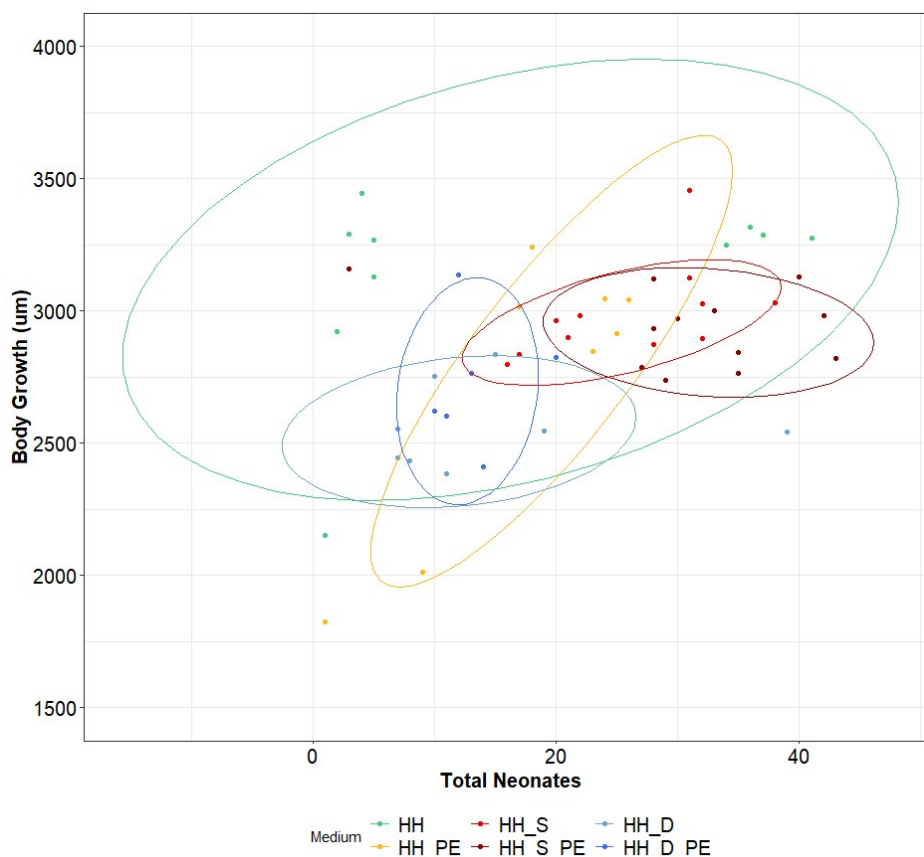

Figure 14. PCA plot of the reproductive output (total number of neonates) versus growth (shown as body length on the y-axis) for *D. magna* exposed in accordance with the OECD 211 reproductive test in salt-only medium (HH Combo) to PE alone or in combination with co-pollutants diclofenac or SDS.

As part of the DEB modelling, the input parameters were modified as follows to account for the exposure conditions, number of daphnids, number of offspring etc.

Parameter values:

param <- c(X = 75,   # scaled functional response (food/(food+ks) concentration, mol C/m^3

      X_K = 35,   # half-saturation constant for uptake, mol C/m^3

      Temp = 20,   # temperature, degrees C

      T_A = 6749, # Arrhenius temperature, degrees C

      T_0 = 20,   # reference temperature, degrees C

      p_Am = 80,   # max. surface area spec. assimilation rate, J/(d*cm^2)

      p_M = 18,   # volume specific somatic maintenance costs, J/(cm^3*d)

      ec = 0.02,   # energy conductance, cm/d

      E_G = 1200, # volume specific cost of structural volume, J/cm^3

      kap = 0.8,   # fraction of mobilized reserves allocated to soma, -

      del_M = 0.3) # shape coefficient.

The parameters also included a data-frame of daphnid growth response over 21 days based on the experimentally determined mean sizes measured using ImageJ analysis:

dat <- data.frame(day = c(1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20,21),

      length = c(1123.825, 1137.2, 1137.2, 897.93, 1376.0875, 2084.61, 2529.77,

              2859.12, 2971.21, 3015.988889, 3339.044444, 2781.255556,

              2861.944444, 2916.122222, 2916.122222, 511.522222,

              3757.5, 3776.55, 3830.1, 4019.916667, 4102.5))

This set of input data leads to an output plot as shown in Figure 15, which presents the observed data plotted along the y axis, and the 'estimated model output' along the x-axis.
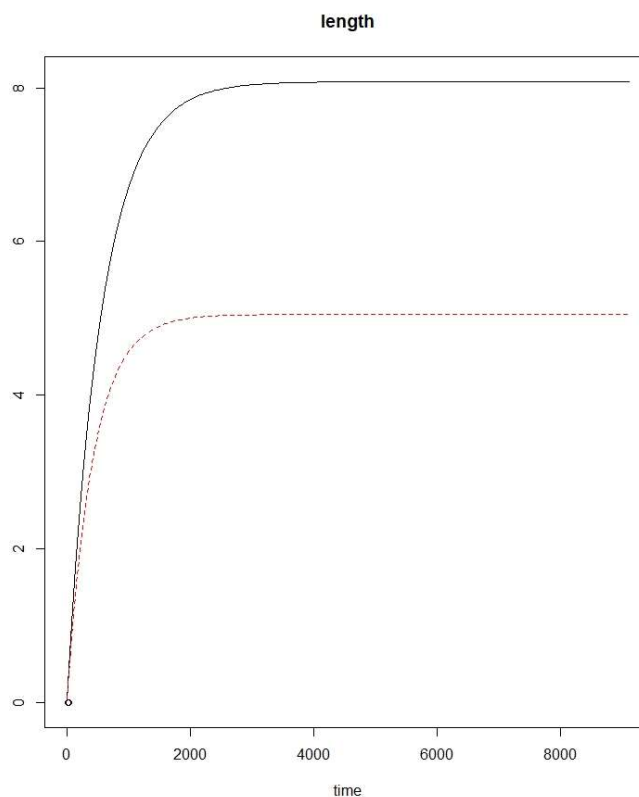
Figure 15: Initial output from our DEB-model for chronic exposure of daphnids to NMs, which will feed into the weight of evidence for the AOP for decreased reproductive success of daphnids.

4.3 Integrating additional mechanistic evidence

4.3.1 Impacts of nanomaterials on gut interactions and gut chemistry

The gut luminal chemistry is of particular interest for comprehending the fate and effects of NMs on the organism after the uptake. The pH (6.8-7.2), as well as the presence, concentration and types of ions (e.g. Na^+, Ca^{2+} and Mg^{2+}), NOM, cuticle, and redox chemistry are factors that interfere with the absorption of particulates in the gut and acting as a barrier in NMs exposure (van der Zande et al., 2020). According to their location in the intestine, NMs acquire an unique eco-corona profile (Chetwynd and Lynch 2020, Cui et al. 2020). Because of their nature, NMs are dispersed in the luminal liquid, rather than dissolved, and a wide diversity of macromolecules (solid-phase food, exudates, digestive enzymes, and proteins) are present in the intestine or obtained from water and might be considered as colloidal system components (van der Zande et al., 2020). The composition of the digestive tract and the interaction forces involved with the gut lumen matrix determine NMs' bioavailability, potentiating or mitigating its toxicity to the daphnids (Cui et al. 2020). Consequently, the physicochemical characteristics of NMs and natural biological constituents must be studied regarding colloid chemistry, including non-DLVO and DLVO forces (Christenson 1984), to determine their colloidal behaviour and impacts on daphnids' physiology.

Ongoing mining of the literature on the impact of food shortage on *D. magna* reproduction is currently being undertaken to provide further evidence for the KEs identified in AOP201 and in our putative NMs-induced calorific reduction leading to impaired reproduction.

4.3.2 *Daphnia* genome and key pathways in NMs toxicity

Investigations of how NMs perturb biological processes and the level of uncertainty at predicting dose response relationships and AOs is low regarding how NMs cause harm to human health, mainly due to the issues around how NMs are regulated under chemical frameworks (Hansen 2017). In addition, we must understand the exposure limits of humans and the permissible levels of NM pollution in the environment. Due to the shared mammalian biology, information safeguarding human health relies on the toxicological information from mice and rat *in vivo* studies (Festing and Wilkinson 2007, Franco 2013), as well as fish and algae, which have been traditionally used to set regulatory limits to protect human and environmental health (Brunner et al. 2009, Kaltenhäuser et al. 2017).

The principles of the 3R's were developed over 50 years ago providing a framework for performing more humane animal research (https://nc3rs.org.uk, Burden et al. 2017, Sneddon et al. 2017). Advancement of animal genome research over the last 20 years have led to the significant understanding of the relationship between simple organisms and their changing environment (van Straalen and Feder 2012). Moreover, the advancement in evolutionary developmental biology and ecological functional genomics, has identified a possibility to reduce the use of animal experimentation by the use of simple model organisms. This technology (originated from sequencing technologies) is used to identify genetic variation under natural selection (Feder and Mitchell-Olds 2003, Leinonen et al. 2013, Grummer 2019) in model test organisms, which are accessible to both laboratory and field studies along defined environmental gradients (Spanier et al. 2017). Furthermore, comparative studies into the evolution and conservation of genes and genomes, provides significant information on genetic diversity and similarities among major groups of organisms from simple organisms to larger invertebrates (Thomson et al. 2009).

Being able to identify the genomic expression profile enables us to identify how genes are regulated under different ecological conditions and identify how these expressions are linked to phenotypic change (Rozenberg et al. 2015, Hales et al. 2017). It is well understood that phenotypical variation is substantially due to gene and environment interactions that were essentially shaped by evolution, or by environmental stress that predictably disrupts the normal functioning of genes (Hodgins-Davis and Townsend 2009, Moyerbrailean et al. 2016, Alexander-Dann et al. 2018).

The expression profile (when compared to a nano treated control) all genes that are expressed in response to a particular initiating event, is called "molecular phenotype". The transcription of the "molecular phenotype" is based on the evolutionary history of populations (Ravindran et al. 2019). Hence, the transcriptional responses of *Daphnia* exposed to environmentally available NMs are a rich source of both phenotypic and genotypic information about the mechanisms of adaptation. This method aligns human- and eco-toxicology towards a more general understanding of how exposure to NMs disrupts biological processes that otherwise ensure animal (including human) health. Evidence is growing on the feasibility of classifying the effect of NMs on human, based on gene expression monitoring using distantly related environmentally relevant model organisms such as *Daphnia*.

We are also supplementing the WoE utilizing literature on resource shortages, and utilizing gene expression data from our Ag and TiO_2 NMs exposure studies (currently being analysed via the NanoSolveIT toxicogenomics preprocessing and processing pipeline).

5. Dissemination and feedback to date

A workshop to gain feedback on the proposed model was initially foreseen for M24 of the project, in order to present the initial findings and proposed network model, for feedback, integration of existing data and refinement of the model. This has not yet been organized, although the model has been presented at multiple conferences, such as the NanoSafe conference in 2020, and workshops during 2021 to gain support and feedback on it.

Additional feedback will be sought via the AOP-Wiki whereby the suggested AOP will be documented in the wiki and feedback on the draft will be collected, and it can be further refined and its domain of applicability tested and refined. Initial work to draft the entry for the AOP-Wiki is underway and Deliverable report D6.5 will provide the full update and a link to the putative AOP.

A further route to gather feedback will be the extension of AOP-Wiki to include also an AOP portal for *Daphnia* (see section 5.1 below) and to encourage establishment of a community of researchers working on elucidating AOPs for daphnids. This is described in further detail below.

5.1 Establishment of the *Daphnia* AOP portal

As part of RiskGONE's efforts to establish the AOP for NMs-induced reproductive decline, and in response to the significant lack of daphnia-related information in the AOP-WEiki, we decided, in collaboration with the NanoSolveIT and NanoCommons projects, to set up a *Daphnia* portal in the WikiPAthways space as shown in Figure 16 (http://daphnia.wikipathways.org/).
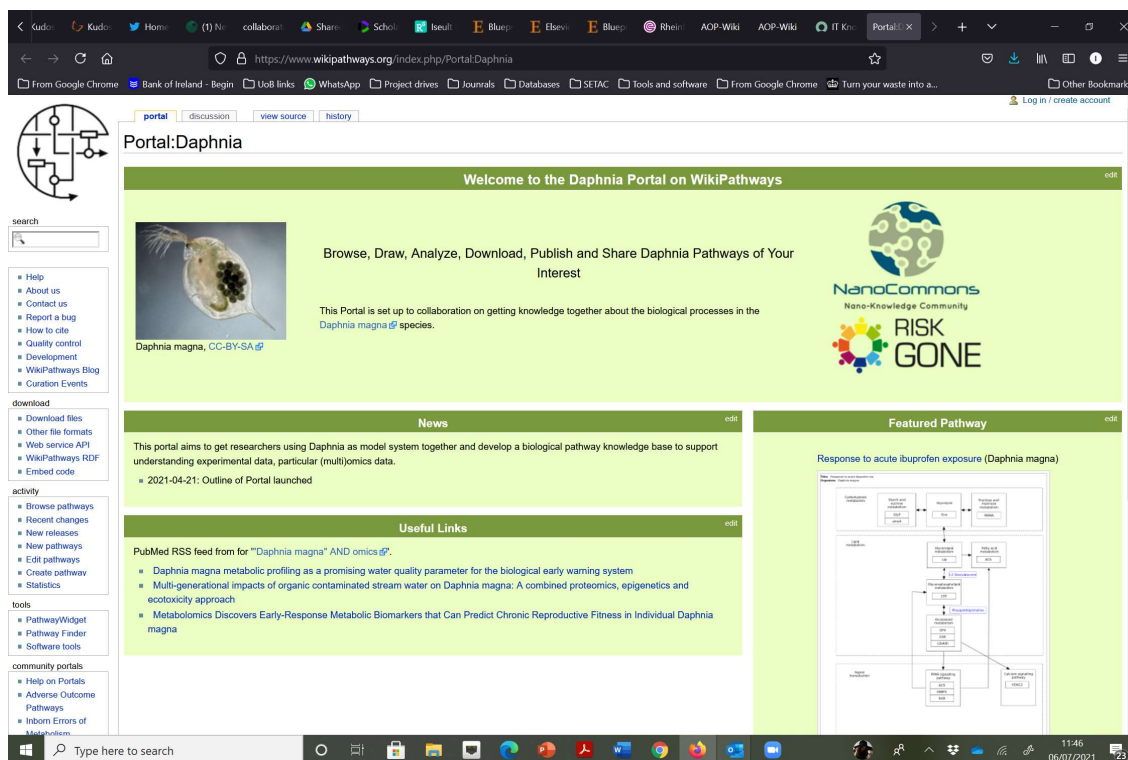
Figure 16: Screenshot of the newly established daphnia portal in WikiPathways. Next steps involve adding our putative pathways for additional commenting by the wider community.

A related task that we want to continue with is *Daphnia* ID mappings. An ID mapping database in BridgeDb format would allow WikiPathways to link to their database, as well as open up ways to do pathway analysis. Some efforts towards this already exist, such as the FleaBase: http://wfleabase.org/maps/ which will be built upon and furthered with tools to link to NMs. Similarly, there is the *Daphnia* Stressors database, which uses BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) annotation for genes up or down regulated by stressors, and has a small number of NMs as stressors: http://www.daphnia-stressordb.uni-hamburg.de/dsdbstart.php.

6. Conclusions and next steps

This deliverable report presented the current state of development of the RiskGONE DEB model-based AOP for NMs-induced reduction in reproduction of *D. magna*. Based on the existing AOP201 on impaired reproduction, we integrated existing evidence on NMs' accumulation in the gut, which leads to the daphnids feeling full and grazing less, leading to reduced calorific intake, reduced growth and thus reduced reproduction. The reduced calorific intake leads to reduced energy for allocation to growth and reproduction. Feedback from the wider community, of AOP experts and daphnia biologists is currently being sought to further strengthen the evidence basis for the model and the overall AOP. Deliverable D6.5 will present the final version of the AOP and the changes resulting form these consultations and discussions.

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