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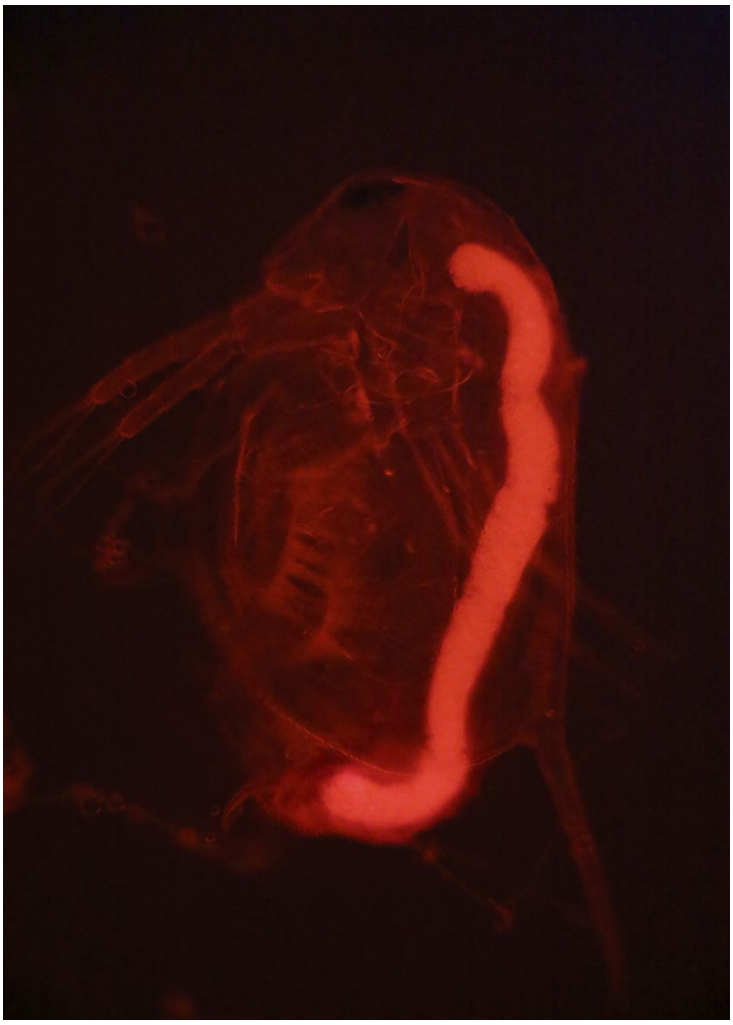
MIXiT: Towards quantifying impacts of microplastics on environmental and human health

Elena Gorokhova, Nazdaneh Yarahmadi, Hanna L Karlsson, Martin Ogonowski, Hoi Shing Lo, Sophia Reichelt



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MIXiT: Towards quantifying impacts of microplastics on environmental and human health

by Elena Gorokhova, Nazdaneh Yarahmadi, Hanna L Karlsson, Martin Ogonowski, Hoi Shing Lo and Sophia Reichelt

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Preface

The report *MIXiT: Towards quantifying impacts of microplastics on environmental and human health* presents the results of one of five funded projects within the call Microplastics from 2018. It is the result of a collaboration between the experts in environmental science (Stockholm University ACES and SLU), polymer chemistry (RISE), and biochemical toxicology (Karolinska Institute). The project focused on developing a framework for quantifying hazardous levels of microplastic and determining which properties of particles and materials should be considered.

The project has been funded with the Swedish Environmental Protection Agency's environmental research grant to support the Swedish Environmental Protection Agency's and the Swedish Agency for Marine and Water Management's knowledge needs.

This report is written by Elena Gorokhova, Nazdaneh Yarahmadi, Hanna L. Karlsson, Martin Ogonowski, Hoi Shing Lo, and Sophia Reichelt.

The authors are responsible for the content of the report.

Stockholm, March 2023

Marie Uhrwing
Department head
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Förord

Denna rapport med titeln: *MIXiT: Towards quantifying impacts of microplastics on environmental and human health* presenterar resultaten av ett av fem beviljade projekt inom utlysningen Mikroplaster från 2018. Rapporten är resultatet av ett samarbete mellan experterna inom miljövetenskap (Stockholms universitet ACES och SLU), polymerkemi (RISE) och biokemisk toxikologi (Karolinska Institutet). Projektet fokuserade på att ta fram ett ramverk för att kvantifiera farliga halter av mikroplast och bestämma vilka egenskaper hos partiklar och material som bör beaktas.

Projektet har finansierats med medel från Naturvårdsverkets miljöforskningsanslag till stöd för Naturvårdsverkets och Havs- och vattenmyndighetens kunskapsbehov.

Författarna av denna rapport är: Elena Gorokhova, Nazdaneh Yarahmadi, Hanna L. Karlsson, Martin Ogonowski, Hoi Shing Lo och Sophia Reichelt.

Författarna ansvarar för rapportens innehåll.

Stockholm, mars 2023

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Summary

The current environmental concern is that plastic debris, particularly microplastics, are ingested by aquatic biota, and microplastics transfer through the food web affects aquatic consumers and their ecological functions with potential effects on humans as the top consumers. However, the evidence for the biological effects of microplastics is scarce, and the mechanisms of these effects in aquatic biota and humans are largely unknown. Therefore, to assess the possible impacts of microplastic pollution on the environment and human health with scientific rigour, we need a sound methodology, which is currently missing. Thus, the primary motivation for the MIXiT project was to establish the principles for hazard assessment methods targeting microplastics as a case of solid waste type.

MIXiT project was focused on five main issues: (1) gathering global evidence for adverse effects of microplastics using meta-analysis of published data, (2) developing a test system for evaluating the effects of solid particles and the leachates, (3) preparation and standardisation of environmentally relevant microplastics for (eco)toxicological testing with considerations of particle morphology and physicochemical properties, (4) demonstrating approaches for microplastic effect studies using ecotoxicological test organisms and human cell lines with an emphasis on understanding the effect mechanisms and deriving effect concentrations suitable for regulatory work, and (5) modelling food-web transfer of microplastics using trophic guilds in the pelagic system of the Baltic Sea. The critical focus of the research is on designing appropriate animal and human tests and providing methodological recommendations on testing the hazardous properties of anthropogenic particles and their leachates.

The project outcome improved the methodology for deriving PNEC values via experimental testing and modelling and suggested test systems for deriving ecotoxicological (test species: algae, macrophytes, micro- and macro-crustaceans) and toxicological (human cell lines) threshold concentrations. In these test systems, specific solutions for exposure, controls, reference materials, and particle characterization were presented to allow obtaining datasets appropriate for PNEC derivation and applicable for chemical safety assessment under REACH.

The (eco)toxicity testing should employ aged material when testing microplastic effects because ageing drastically changes the chemical and physical properties of the material and its leaching components, with subsequent impact on the leachate toxicity. Moreover, appropriate methods should be used for artificial ageing when generating test materials.

The crucial aspects of experimental test design in microplastic effect studies in (eco)toxicology suggested by MIXiT are (1) using reference particles to disentangle particle effects induced by microplastics and appropriate controls for particle storage buffers; (2) controlling for the exposure concentrations encountered by the test cells/organisms, and particle size of the test suspension; (3) conducting tests evaluating the effects of both particulates and chemical exposure via leachates; and (4) deriving a two-component threshold with PNEC for microplastics conditional on the levels of the total suspended solids in the water, which makes it adaptable to different systems. Finally, regardless of the statistical significance of the experimental outcome, all test results must be reported to avoid publication bias.

In the hazard assessment, the thresholds based on the leachate toxicity should be combined with those derived from the particle concentrations. In our data, the leachate EC10 values were lower than those for the corresponding particulate material, suggesting that leachate testing can be sufficient for particles that cannot undergo translocation. Chemical analysis of the leachates should be conducted to identify the chemical substance behind the observed effects and set the hazard assessment into REACH perspective.

When setting and applying environmental thresholds for the mass-based microplastic concentrations in the water column, the suspended material's plastic and non-plastic components must be measured to relate the microplastic content to the total amount of the suspended matter. In our tests with *Daphnia*, the effect of microplastics was observed at total suspended solids exceeding 32 mg/L and microplastic percentage exceeding 2.4 %. A similar threshold for sediment-living biota (1.4 % of microplastic in sediment) was proposed by other researchers. Finally, the PNEC values based on the species sensitivity modelling can be used when more effect studies employing adequate design are available to include the representative concentration range and reference materials.

Sammanfattning

Det nuvarande miljöproblemet är ackumulationen av plastskräp, särskilt mikroplast (MP), i miljön. MP kan tas upp av vattenlevande organismer, och överföras genom näringskedjan vilket kan påverka ekosystemens funktioner och potentiellt ha effekter på människor som befinner sig högst upp i näringskedjan. Bevisen för de biologiska effekterna av MP är dock begränsade, och mekanismerna för dessa effekter i vattenlevande organismer och människor är i stort sett okända. För att kunna bedöma de potentiella effekterna av MP-föroreningar på både miljön och människors hälsa, saknas för närvarande robusta metoder, som skulle göra detta möjligt. Den primära målsättningen med MIXiT-projektet var att fastställa principerna för riskbedömningsmetoder som fokuserar på MP som en specifik typ av fast avfall.

MIXiT-projektet hade fem huvudsakliga syften som fokus: (1) att samla globala bevis för negativa effekter av MP genom metaanalys av publicerade data, (2) att utveckla ett testsystem för att utvärdera effekterna av fasta partiklar och lakvatten, (3) att utveckla metoder för att förbereda och standardisera miljörelevant MP med hänsyn till partikelmorfologi och fysikalisk-kemiska egenskaper för användning (eko)toxikologiska tester, (4) att demonstrera tillvägagångssätt för MP-effektstudier med användning av ekotoxikologiska testorganismer och mänskliga cellinjer, med fokus på att förstå effektmekanismerna och härleda effektkoncentrationer som är relevanta för regulatoriskt arbete, samt (5) att modellera överföring av MP genom näringskedjan med hjälp av trofiska nivåer i det pelagiska systemet i Östersjön. Det ultimata målet var att utforma grundläggande principer för ändamålsenliga tester av MP som är relevanta för djur och människor samt att tillhandahålla metodologiska rekommendationer för att undersöka de farliga egenskaperna hos antropogena partiklar och deras lakvatten.

Resultaten från projektet förbättrade metodiken för att härleda PNEC-värden (Predicted No Effect Concentrations) genom experimentell testning och modellering. Dessutom föreslog projektet testsystem för att härleda tröskelkoncentrationer för både ekotoxikologiska tester (inklusive alger, makrofyter, mikro- och makrokräftdjur) och toxikologiska tester (mänskliga cellinjer). Inom dessa testsystem presenterades specifika lösningar för exponering, kontroll, användning av referensmaterial samt karaktärisering av partiklar för att möjliggöra insamling av dataset som är lämpliga för att härleda PNEC-värden och som är tillämpliga för kemikaliesäkerhetsbedömning enligt REACH (Registration, Evaluation, Authorization and Restriction of Chemicals).

De viktiga aspekterna av experimentell testdesign inom (eko)toxikologi för MP-effektstudier, som föreslås av MIXiT, inkluderar följande: (1) användning av referenspartiklar för att särskilja effekterna som orsakas av MP från effekterna av andra partiklar, samt användning av lämpliga kontroller för partikellagring; (2) kontroll av exponeringskoncentrationer för testceller/organismer och partikelstorlek i testsuspensionen; (3) utförande av tester som utvärderar effekterna av både partiklar och kemikalier som exponeras genom lakvatten; och (4) härledning av en tvåkomponents-tröskel med PNEC för MP, baserat på nivåerna av totalt suspenderat material i vattnet, för att kunna anpassa det till olika ekosystem. Dessutom bör lämpliga metoder användas för att konstgjort åldra testmaterial vid behov.

Åldrat material bör användas i ekotoxicitetstestning för att undersöka MP-effekter, eftersom åldrandet avsevärt påverkar de kemiska och fysikaliska egenskaperna hos materialet och dess lakkomponenter, vilket i sin tur påverkar lakvattentoxiciteten. Slutligen bör alla testresultat rapporteras oavsett statistisk signifikans för att undvika publikationsbias.

I riskbedömningen kombineras tröskelvärden baserade på lakvattentoxicitet med de som härstammar från partikelkoncentrationer, och enligt våra data visade EC10-värdena för lakvatten lägre värden än motsvarande värden för partikelformiga material, vilket tyder på att lakvattentestning kan vara tillräcklig för partiklar som inte kan transporteras genom cellmembran. Det är också av vikt att genomföra kemisk analys av lakvatten för att identifiera de kemiska substanserna som ligger bakom de observerade effekterna samt för att kunna göra en farobedömning utifrån ett REACH-perspektiv.

Vid fastställande och tillämpning av miljögränser för massbaserade halter av mikroplaster i vatten är det viktigt att mäta både plast- och icke-plastkomponenterna (som t.ex. sedimentpartiklar) i det suspenderade materialet för att kunna relatera mikroplastinnehållet till den totala mängden suspenderat material. I våra tester med *Daphnia*-organismer observerades effekter av mikroplaster när det totala suspenderade materialet överskred 32 mg/L och andelen mikroplaster överskred 2,4 %. Liknande tröskelvärden har föreslagits av andra forskare för organismer som lever i sediment (1,4 % av mikroplasterna i sedimentet). Slutligen kan PNEC-värden baserade på artkänslighetsmodellering användas när fler effektstudier med adekvat design blir tillgängliga, för att täcka det representativa koncentrationsintervallet och inkludera referensmaterial.

1. Background

The global distribution and anticipated increase of environmental microplastic (MP) pollution are concerning, and assessing ecological and human health impacts due to MP exposure is a hot topic fuelled by these concerns. Today, MP is regarded as one of the emerging contaminants gaining the wide attention of environmental scientists, chemists, ecologists and toxicologists. Also, it is becoming increasingly evident that humans can be exposed to plastic particles via air, food and water (Akanyange et al., 2021). However, the risks posed to the environment and human health are still uncertain, and evidence is often weak. As a result, we often see ambiguous statements like *potentially harmful* or *could be hazardous*, often unsupported and misleading for policymaking. Much of this uncertainty is driven by severe limitations in the study methods for MP effects and reporting (Thiele and Hudson, 2021). The need for environmentally relevant approaches for MP effect studies prompted MIXiT.

1.1 Microplastic is everywhere, but does it pose a risk?

Despite the general perception of MP as hazardous waste, the evidence for biologically significant and ecologically meaningful effects concerning its environmental and human health impacts is limited, and no-effect results are also published (Ogonowski et al., 2018a; Yu et al., 2018), although to a lesser extent due to a publication bias in MP research (Reichelt and Gorokhova, 2020). Furthermore, the lack of systematic information on the particle properties of the test MP hampers read-across analysis for regulatory purposes. As a result, although the effect studies are numerous, no consistent picture emerges, and the effects have poor replicability even under virtually identical experimental conditions (Müller, 2021; Reichelt and Gorokhova, 2020). Moreover, the reports published to date rarely provide convincing evidence that MP effects (1) are attributed to the MP exposure *per se* and not the exposure to particulate material (Ogonowski et al., 2018a), (2) may occur at concentrations comparable to those found in sediments and surface waters (Lenz et al., 2016), and (3) propagate across biological organisation levels and cause adverse effects at the population level, which is the ultimate target for ecotoxicology. In the scientific literature, stating risks is often done without providing relevant evidence but referring to the presence of microplastics in the environment or their ingestion by organisms.

Weaknesses in experimental designs are often to blame for the adverse effects ascribed to MP (Adhikari et al., 2022). The significant artefacts of using (1) exposure conditions and concentrations that are orders of magnitude higher than environmentally plausible levels of MP (Lenz et al., 2016), (2) static exposure conditions for suspension-feeders and suspended microorganisms as the test species (Gorokhova et al., 2020; Reichelt and Gorokhova, 2020) resulting in erroneous dose estimates, (3) no reference materials to account for food dilution effects (Gerdes, 2021), and (4) failure to distinguish particle and chemical effects (Gerdes, 2021), are the major

drawbacks and sources for erroneous results in MP research. As a vast majority of the effect study reports suffer from at least some of these shortcomings and given the poor understanding of the effect mechanisms and lack of methodology for risk assessment, it is virtually impossible to provide recommendations for legislation and to support ecosystem management with threshold values representing environmentally acceptable levels of MP. As a result, there are no consistent science-based risk assessment programs for MP in either aquatic or terrestrial environments.

1.2 Plausible effect mechanisms

While the mechanisms and impacts of macroplastic litter on wildlife are apparent, we know relatively little about the MP effect mechanisms. Plausible adverse effects of MP on wildlife are commonly attributed to its persistence, ubiquitous ingestion by various animals, and capacity to act as a vector for the chemicals added during production or sorbed from the environment. Thus, MP impacts can occur via particle or chemical effects. In addition, as a unique microhabitat, microplastics can promote the formation of biofilm, with communities that differ from those on other substrates in the environment (Ogonowski et al., 2018b) and serve as carriers of antibiotic-resistant bacteria (Pham et al., 2021) and pathogens (Gorokhova et al., 2021), representing another plausible model of action for MP.

Separating particle and chemical effects of MP and assessing ecotoxicological responses from ecologically and environmentally relevant perspectives remains one of the most significant challenges in the field of MP ecotoxicology. The particle effects (de Ruijter et al., 2020), commonly related to physical damage and food dilution reducing nutritional uptake, are also typical for exposure to other solids, such as naturally occurring mineral particles. Furthermore, these natural particles can reach hazardous levels during resuspension events by dredging or heavy rainfalls (O'Connor et al., 2019). In this context, assessing MP impacts requires benchmarking their effects against other particle types.

1.2.1 Particle effects

The particle mode of action is related to the MP particle behaviour upon direct contact with cell/organism, such as irritation of the epithelium, crossing the cell membrane, translocation to body tissues, clogging feeding appendages and the gut, and food dilution. Effects, such as translocation, are partly size-dependent but can also be associated with other particle characteristics, e.g., specific shapes or material properties. For example, microplastic fibres induce higher mortality in water fleas than similarly-sized irregular fragments (Ziajahromi et al., 2018, 2017). Also, irregular pieces may have a longer gut residence time than spherical MP (Ogonowski et al., 2016), potentially impacting energy intake and chemical transfer.

Furthermore, particle exposure can alter animal feeding behaviour (McMahon and Rigler, 1965), with downstream food intake and assimilation effects. Also, the animals feeding non-selectively would experience food dilution effects when exposed to particle mixtures with high MP proportion relative to the food (Besseling et al., 2014). Besides shape and size, the physicochemical properties of MP, such as surface hydrophobicity and surface charge, are potential drivers of the particle effects (Lambert et al., 2017). For example, a positive surface charge increases

particle capture rate by zooplankton (Gerritsen and Porter, 1982), and amine-functionalised positively charged polystyrene particles appear more harmful than unfunctionalised particles (Bergami et al., 2016).

The uptake of MP by humans can occur through consuming terrestrial and aquatic food products, breathing air, and drinking water (Brachner et al., 2020). Recently, concerns have been raised that inhaled MP may induce lesions in the respiratory system, with effects being dependent on individual susceptibility and particle properties; moreover, this pathway is probably more hazardous than ingestion of MP (Kannan and Vimalkumar, 2021; Leslie et al., 2022).

It has been suggested that exposure to MP/NP can trigger toxicity pathways, with a prominent role in inflammation and oxidative stress in murine models. Once absorbed, MP/NP may act locally or access the bloodstream and, following the translocation process, reach various organs and tissues, including the gonads and placenta (Ferrante et al., 2022), opening new scenarios for toxicological evaluations (Leslie et al., 2022). Moreover, different mechanisms are strictly interconnected, and the induction of one pathway can trigger or sustain the others. However, as in the case of ecotoxicological effects, the chronic effect concentrations and underlying toxicological mechanisms by which MP may elicit effects are too poorly understood to make a science-based assessment of these risks (Kontrick, 2018).

1.2.2 MP as a vector for chemicals

The chemical impacts of MP are due to the release of toxic additives and modified transfer of chemical pollutants between the environment and the biota. Many plastic materials are excellent sorbents of hydrophobic organic contaminants (HOC). As the absorption rate increases with the surface-to-volume ratio, MP has higher sorption efficiency than macroplastics. Therefore, MP will accumulate higher levels of HOCs in a shorter time than macroplastic litter. With limited scientific recognition, HOC-carrying plastics were reported already during the 70s (Carpenter et al., 1972). The concept of microplastic acting as a "Trojan horse" that delivers pollutants to biota gained a foothold in the early phase of the MP research. What was first presented as a hypothesis (Cole et al., 2011) was quickly adopted as a theory and communicated to the research community and the general public.

Today, there is a general acceptance within the scientific community that the contaminant uptake via MP has a low probability (Koelmans et al., 2022). Moreover, the similarity between plastic and lipids implies that they have a similar capacity to accumulate contaminants when exposed to the same environmental concentrations. Modelling studies based on the thermodynamic relationships between plastic, HOC, and biota (Bakir et al., 2016; Koelmans, 2015) have helped set the course for this acceptance, while experimental studies are providing proof of concept (Gerdes et al., 2019b; Koelmans et al., 2017). In particular, HOC transfer from MP to biota has been demonstrated using experimental settings with $C_{Plastic}/C_{Lipid} >> 1$. However, this does not imply that MP will affect body burden and bioaccumulation *in situ*. This distinction between the principal possibility and environmental probability of transfer is fundamental from a hazard assessment perspective.

Whereas plastic leachate experiments usually lack environmental relevance and are of limited regulatory value (Delaeter et al., 2022), there are excellent examples of plastic litter effects on aquatic biota that are chemically driven and can occur at environmental concentrations. For example, the tire wear compound 6PPD-

quinone, a by-product of a standard tire manufacturing additive, is toxic to coho salmon at the concentrations present in road runoff (Tian et al., 2021). There are also good examples of unifying testing approaches to integrate leachate and particle effects in ecotoxicological testing (Beiras et al., 2012).

When designing effect studies, researchers predominantly use virgin plastic, i.e., new plastic material in the same state relative to its production, even though the ageing and fragmentation of large plastic items generate the most MP in the environment. However, ageing leads to diversification of the physical properties (McGivney et al., 2020) and surface chemistry (Lambert et al., 2017) compared to the original materials, with concomitant effects on the interactions with biological components in the test system. Furthermore, MP produced from fragmentation persisted in the environment for a long time, which has allowed the leaching of its additives to render particulate matter less toxic. However, at the same time, ageing can potentially increase the leachates' toxicity (Rummel et al., 2022), which was addressed by MIXiT.

2. MIXiT objectives

In MIXiT, we developed a framework for quantifying the hazardous properties of synthetic polymer microparticles and establishing environmentally acceptable MP levels in the water, sediment, and air. To move the field beyond the current state-of-the-art, we needed test methods that allow (1) delineating effects of different particulate materials (e.g., anthropogenic vs natural) in test systems, (2) estimating the critical levels of MP with varying physical and chemical properties in different environments to support risk assessment and regulatory work, and (3) high-throughput testing of solid polymer particles (to assess *particle effects*) and their leachates (to assess effects of *chemical exposure*), categorisation, and read-across for regulatory purposes.

The following objectives were put forward and achieved:

1. Development of appropriate test systems with environmentally relevant exposure scenarios, MP levels, and pathways;
2. Identification of morphological and physicochemical properties of MP that must be considered in their hazard assessment;
3. Integration of the bioassays for MP as particulates and their leachates to improve understanding of the effect mechanisms;
4. Establishing threshold values that correspond to NOEC levels for aquatic biota (exposure in pelagic and benthic habitats) and human cells (exposure via inhalation) using test MP with well-characterised properties and their leachates;
5. Generating recommendations on MP hazard assessment for the stakeholders.

3. Methods and main results

Various laboratory methods and techniques for conducting physicochemical, ecotoxicological, toxicological and microbiological evaluations were combined in different experimental studies. In addition, a meta-analysis of the published reports was applied to identify the global effects of MP across studies and species. Finally, we used dynamic modelling to estimate MP uptake in a simple food chain using relevant publications and assumptions.

To develop the methodology, which was the primary focus of the project, we **selected test materials** that were (1) representative of the most common polymer types in the environment, (2) size-fractionated to provide a biologically appropriate particle size for cell/organism interactions in (eco)toxicological testing, (3) used to produce leachates and thus enable testing of effects of particulate material and chemicals leaching under standard conditions, (4) containing a low but relevant number of additives to simplify data analysis and demonstration of the testing principles, and (5) subjected to ageing to represent environmentally relevant pathway to MP formation and understand the importance of using aged material in (eco) toxicological assessment.

3.1 Preparation and characterisation of test MP

Understanding and adequately simulating the most likely pathway for forming MP in the environment is crucial for producing relevant test material. Before plastic enters the aquatic environment as MP, it often has a degradation history. The degradation starts at manufacturing and continues during material usage, and its part of life as terrestrial and aquatic litter. To speed up natural degradation processes, *relevant accelerated ageing* is used that stimulates the same degradation mechanisms that affect plastic material properties during a product's service life (Jakubowicz, 2004; Vega et al., 2018). Since plastic materials consist of polymer/polymers and additives, it is essential to know the role of additives in the fragmentation and decomposition processes. The RISE team prepared test materials using accelerated thermo-oxidative ageing followed by mechanical grinding and physicochemical characterisation of test MP.

3.1.1 Source material and workflow

Low-density polyethene (LDPE) was chosen as a representative polymer as it is a ubiquitous environmental contaminant because of its wide use in packaging and short-life plastic products (Jadhav et al., 2021). Two different PE qualities provided by Borealis (Sweden), FT5236 (stabilised) and FT5230 (non-stabilised), were included. These granulated resins differed in the content of the stabilisers added to the resin. Secondary MP were prepared and used in the experiments (Figure 1).

The standard methods were used to characterise particle morphology, chemical composition and material structure, density, crystallinity, hydrophobicity, surface structure, and elemental composition (Woo et al., 2021). In particular, the presence of nano-sized particles in the leachates was assessed using dynamic light scattering, and the Fourier Transform Infrared (FTIR) imaging system was applied to determine the MP chemical composition and changes in the polymer structure. Differential Scanning Calorimetry (DSC) was applied to determine changes in crystallinity, density columns for polyolefin MP density, contact angle measuring system equipped with nano- and picolitre (DataPhysics Instruments) dosing systems to assess changes in hydrophobicity, image analysis for particle size and shape (e.g., aspect ratio or circularity), Size Exclusion Chromatography (SEC) to determine changes in molecule weight (Woo et al., 2021). All these methods are commonly used to characterize the ageing of polymers, including transformations of microplastics in the environment (Li et al., 2022), including embrittlement due to ageing (Garvey et al., 2020).

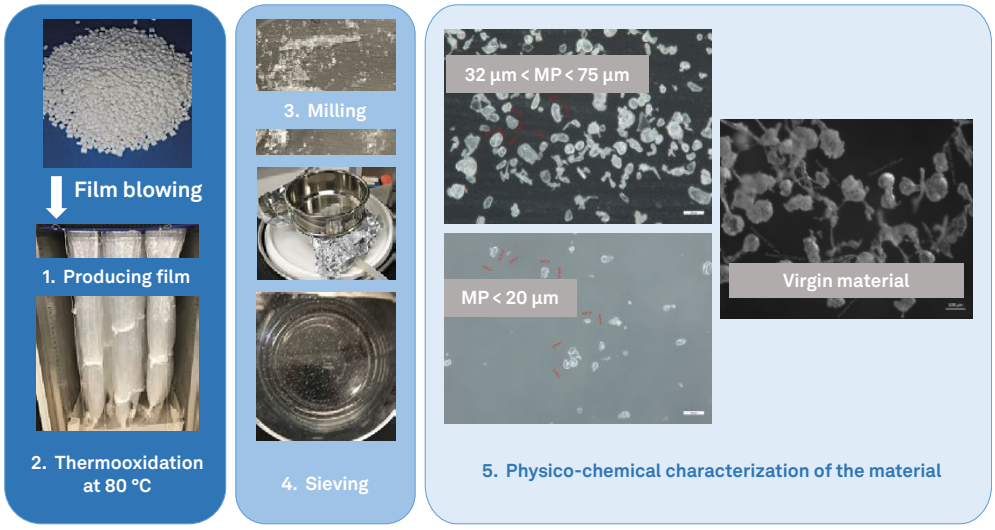

Figure 1. Workflow for preparation of test MP.

The resins (granulate form) were processed into thin films (approx. 20 µm thick) by film blowing to enable homogeneous degradation conditions. The film blowing was accomplished using a Brabander compact extruder, Brabander OHG, Duisburg, Germany, Screw diameter (D) of 19 mm and screw length of 25D with three individually controlled temperature zones. A screw with a compression ratio of 4:1 was used in all experiments. A Dynisco melt pressure transducer, model TPT463E-10M-6/18 Dynisco, Westwood, MA, USA, was positioned at the entrance of the die and connected to a Dynisco ER478 pressure indicator to measure the pressure loss over the die. The extruder was equipped with a conventional temperature-controlled film-blowing die having a diameter of 24 mm and a film-blowing tower, Brabander OHG, Duisburg, Germany, with a calendaring nip and take-off rolls.

The produced films were subjected to accelerated thermo-oxidative ageing in force-ventilated ovens at 80 °C. The films were cut into rectangular sheets of suitable size for hanging freely in the ovens to ensure that all sheets were in contact with fresh air throughout the duration of the ageing. The films were frequently checked for signs of embrittlement to ensure that they were sufficiently degraded before removal from

the ovens, and the time required for sufficient ageing and embrittlement, varied due to the different content of the stabilisers in the resins. The film containing low amounts of stabilisers (hereafter referred to as non-stabilised LDPE) was subjected to ageing for six months, while the film containing higher quantities of the stabiliser (stabilised LDPE) required 12 months to show signs of ageing.

Moreover, when the stabilised LDPE was examined, a mosaic colouration in the film was observed. While the colouration of the aged film was similar to the unaged in some parts, in which a great deal of ductility was retained, other parts of the film were discoloured (yellowish appearance) and significantly more brittle than the unaged film. This indicates that the film degradation was uneven. We, therefore, decided to handle these different parts (non-coloured parts and those showing noticeable discolouration) of the aged film as two different materials throughout the project. Five test materials were prepared using the LDPE films, and two raw materials (granulate form) were used in the project (Table 1).

Table 1. List of the materials for preparing the test MP by the relevant accelerated ageing.

Abbreviation	Description of the test material
PE-US/UA-g	LDPE, **non-stabilised** and **unaged** in granular form (raw material)
PE-S/UA-g	LDPE, **stabilised** and **unaged** in granular form (raw material)
PE-US/U-f	LDPE, **non-stabilised** and **unaged** in film form
PE-US/A-f	LDPE, **non-stabilised** and **aged** (6 months at 80 °C) in film form
PE-S/UA-f	LDPE, **stabilised** and **unaged** in film form
PE-S/A-f	LDPE, **stabilised** and **aged** (12 months at 80 °C) in film form (not discoloured)
PE-S/A-f-col	LDPE, **stabilised** and **aged** (12 months at 80 °C) in film form, **discoloured** (yellow)

Characterisation of the LDPE films. The different film materials (stabilised, unaged and aged, and non-stabilised unaged and aged) were characterised to provide information regarding the physical and chemical properties and how these changed during the ageing. When exposed to oxidative environments, polyethene materials may be chemically and physically altered by the degradation causing changes in physical (e.g., density, brittleness, wettability) and chemical (functional groups, molecular weight, content of low mass substances) properties. These changes may affect responses in the effect studies but also how the MPs interact with water, air, biofilms, etc., affecting how they may be transported in the environment.

- **Density** measurements were performed using a density column (ISO 1183-2) with control beads covering the density range ρ = 0.8899–0.9798 g/ml. Pieces of each film were melted into more beadlike geometries, more suitable for measurements in the density column. One potential drawback of performing the re-melting of the films is that the crystallinity may be altered, affecting the measured density.

  The results of the density measurements indicated that the samples, although re-melted, were slightly too small and irregular in shape to obtain reliable measurements as variations were found within each sample (Table A1.2) due to, e.g., irregular shapes that may enclose air bubbles affecting the estimated density values. The obtained values were, however, lower than expected because LDPE degradation often leads to an increase in crystallinity which, in turn, should increase the density of the material.

Table 2. Measured density values ([g/cm^3], n = 4) of re-melted beads from the test films.

Test material	Average density	Value 1	Value 2	Value 2	Value 4
PE-US/UA-f	0.912	0.9168	0.9196	0.9096	0.9025
PE-US/A-f	< 0.8899*	< 0.8899*	< 0.8899*	< 0.8899*	< 0.8899*
PE-S/UA-f	0.903	0.9050	0.9169	0.8914	0.8986
PE-S/A-f	0.899	0.9040	0.9011	0.8995	0.8923

* Outside the control beads range.

- **Fourier Transform Infrared Spectroscopy (FT-IR)** is a technique suitable for identifying a polymeric material and monitoring changes in the chemical structure (e.g., functional groups) as different chemical bonds absorb energy at specific wave numbers. By analysing the test films we could monitor changes in their chemical structure under the progression of ageing and provide information on the presence of different functional groups (e.g. carbonyl groups) formed by the oxidation of the materials.

  We used a Spectrum One FT-IR spectrometer (PerkinElmer, Waltham, MA, USA) equipped with a universal ATR unit to collect at least three spectra of 16 scans from each of the three pieces of film for each of the five materials, rendering a total of nine spectra for each material. The collected spectra were evaluated using OMNIC software.

  The FT-IR spectra from the unaged films (Figure A1.1) presented no indication of thermo-oxidative degradation of the materials, as no noticeable peaks were found in the carbonyl region (approx. 1600–1800 cm^{-1}). Furthermore, the aged films (PE-US/A-f and PE-S/A-f-col) presented a high absorbance in the carbonyl region, while the stabilised and aged film without discolouration (PE-S/A-f) only showed minor absorbance in this region.

  The absorbance in the carbonyl region (Figure 2) consisted of multiple peaks assigned to different carbonyl compounds based on previous studies (Gulmine et al., 2003). The highest absorbance was seen at 1714 cm^{-1}, interpreted as ketones, followed by absorbance at 1733 cm^{-1}, interpreted as esters and/or aldehydes and 1780 cm^{-1}, interpreted as γ-lactones. Furthermore, there were indications of a minor shoulder at 1700 cm^{-1} that could be construed as carboxylic acids.

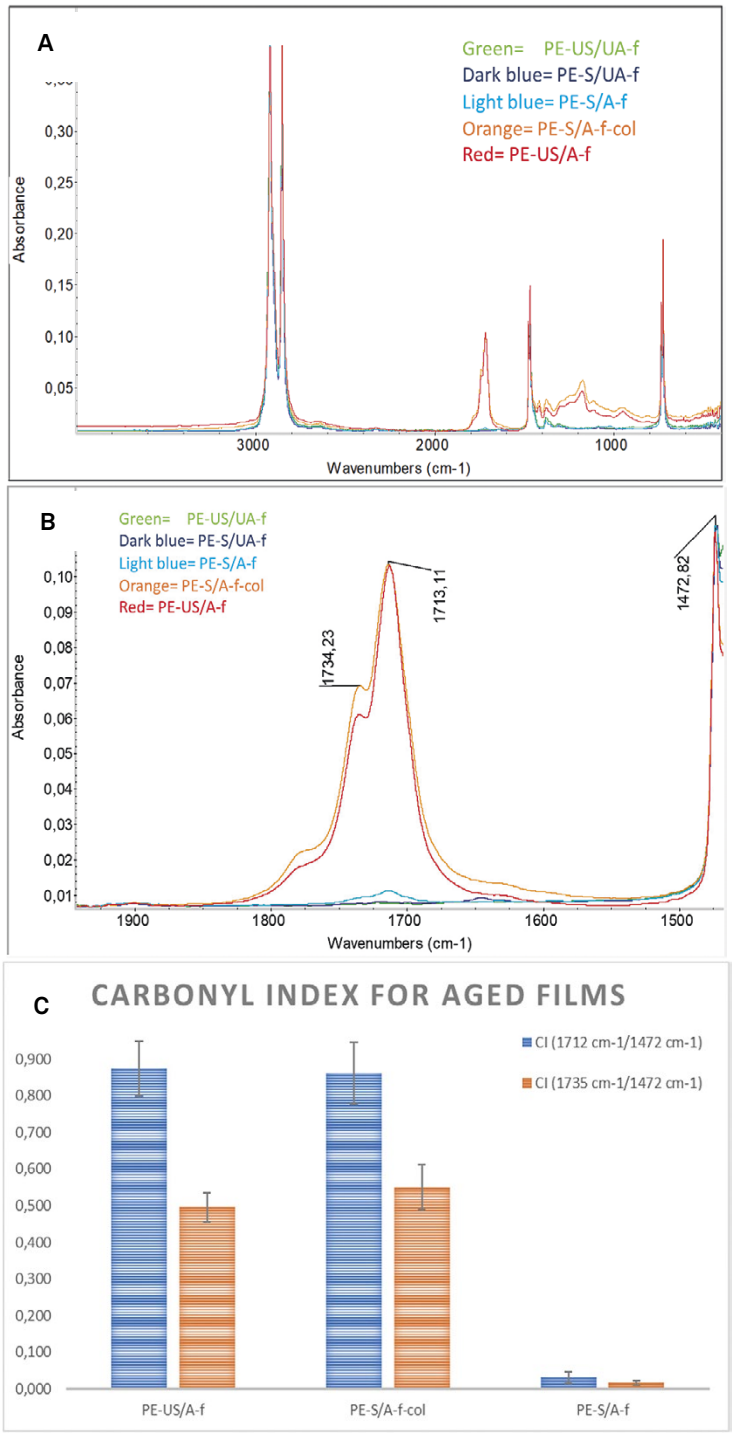

Figure 2. FTIR results for the representative FTIR-spectra (A) covering the entire scan range and showing a significant difference in absorbance in the carbonyl region and below 1300 cm^{-1} for the PE-US/A-f and PE-S/A-f-col samples as compared to the other materials; (B) focusing on the carbonyl region and showing a significant absorbance in the carbonyl region for the PE-US/A-f and PE-S/A-f-col films, minor absorbances for the PE-S/A-f sample and no noticeable absorbance for the unaged samples, and (C) calculated carbonyl index for these materials and regions.

The carbonyl index was calculated from the different spectra using the peak intensities ratios of the carbonyl peaks wavenumbers 1714 cm^{-1} and 1734 cm^{-1} and the reference peak at 1472 cm^{-1}. In addition to the significant absorbance in the carbonyl region, samples PE-US/A-f and PE-S/A-f-col also presented overlapping absorbance bands below 1300 cm^{-1} which could be from C-O-C-groups (1300–1000 cm^{-1}) and unsaturated C = C-bonds (1000–650 cm^{-1}).

- **Differential Scanning Calorimetry (DSC)** was used to measure crystallinity and oxidation onset temperature as a proxy for the stabilisation degree in the polymers. The measurements were performed with a DSC instrument (Mettler Toledo GmbH, Greifensee, Switzerland) equipped with a gas controller and a sample robot. The DSC's temperature and heat flow accuracy were calibrated using pure indium and zinc as reference materials.
  - *Crystallinity.* The film samples were obtained by punching out a stack of several layers of film to acquire sufficient sample mass. Then, thin discs were cut from the granular material using a scalpel. The samples were subjected to a temperature regime based on ISO 11357-3, i.e., two heating cycles ranging from 30 °C (5 °C for the second heating cycle) to 160 °C for calculating the inherent crystallinity (first heating) of the films and granulates and the crystallinity of the materials after that the thermal history have been erased (second heating). All samples were analysed in triplicates in a nitrogen atmosphere (50 ml/min N_2) using a heating rate of 20 °C/min. The crystallinity was calculated as the ratio between the recorded enthalpy of fusion (ΔH_m) for each melting peak and a reference value (ΔH_{100} = 293 J/g) for a 100 % crystalline LDPE material. A significant increase in crystallinity of the non-stabilised aged film compared to the unaged films was found.

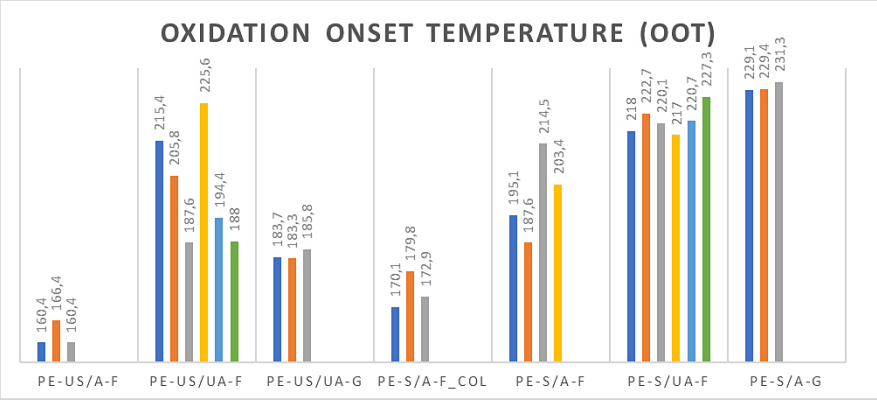

Figure 3. Variability of individual OOT measurements (n = 3 to 6) using an onset threshold value of 0.2 W/g. The material is indicated on the x-axis, and the OOT value is shown on the top of each bar. See Table 1 for sample coding.

  - *Oxidation onset temperature (OOT).* A sample consisted of either a circular film monolayer or a thin slice from the centre of the granulates, with three to six replicates analysed for each material. The applied temperature regime based on ISO 11357-6 was a start temperature of 30 °C and a heating rate of 20 °C/min in oxygen (O_2, 50 ml/min). Each experiment was automatically terminated when the exothermal signal (from oxidation) reached 10 W. The OOT were

subsequently evaluated using an onset threshold value of 0.2 W/g. The degree of stabilisation was highest for the granular material (PE-S/UA-g), followed by the unaged film (PE-S/UA-f), exhibiting a minor reduction, probably due to the additional melt processing step. For the stabilised PE, the aged sample without discolouration (PE-S/A-f) showed a lower OOT and more considerable inter-replicate variation, and the aged and discoloured film (PE-S/A-f-col) presented the lowest OOT. The non-stabilised PE did not show the same trend as the stabilised PE, as the unaged film had a high variance in the OOT values, often exceeding the value presented for the granular raw material. The non-stabilised aged material, however, showed the lowest OOT of all samples, as expected (Jakubowicz, 2004; Vega et al., 2018).

- **Gel permeation chromatography (GPC)** was used to evaluate the effects of ageing on the molecular weight (Mw) of the PE materials. The molecular weight was high for the unaged samples and the PE-S/A-f, while it was about 90 % lower for the PE-US/A-f (Figure 4), indicating that the thermo-oxidative degradation caused a significant chain-scission of the polymers.

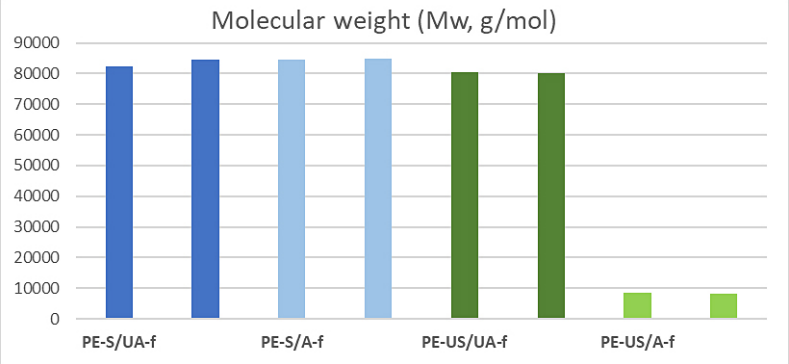

Figure 4. Molecular weight (Mw) measured by conventional high-temperature GPC. No measurements were obtained for PE-S/A-f-col samples.

- **Contact angle (dynamic)**, Θ (theta), is a quantitative measure of the wetting of a solid by a liquid. The contact angle is geometrically defined as the angle formed by a liquid, in our case, water, at the three-phase boundary where a liquid interacts with a surface. Measuring contact angle informs about the material hydrophobicity (Piao et al., 2010).

  The thin films were attached to glass plates with double-sided adhesive tape and flattened by pressing them with a clean (rinsed with ethanol and water) glass slide for a few minutes. Then, the contact angle of the different films was measured using a DataPhysics OCA40 micro (DataPhysics GmbH, Germany) instrument by applying water droplets (5 µL) using a needle with OD 0.31 mm and a pump speed of 0.5 µL/s. First, the static contact angle was measured 10 s after the droplet had been added to the surface. After this, the needle was inserted in the droplet (5–9 droplets for each film), which allowed for altering (increasing/decreasing) the droplet volume to measure the advancing and receding contact angles.

  The contact angle in PE-US/A-f and PE-S/A-f-col were low, especially for the receding droplet, which indicates that these surfaces have a higher affinity for

water, probably due to the presence of polar groups (i.e. carbonyl groups) on the film surfaces as a result of the thermo-oxidative PE degradation, which is in line with the FTIR-measurements. The non-stabilised aged film had the highest contact angle and surface polarity (Table 3).

Table 3. Summary of results for the contact angle (°) measurements (mean ± SD) in the test materials. The contact angle is calculated using tangent for droplets (static), advancing and receding contact angles with a needle in the droplets. See Table 1 for the abbreviations of the test materials.

Test material	Contact angle drop	Contact angle advancing	Contact angel receding
PE-US/UA-f	97 ± 2	107 ± 2	89 ± 1
PE-US/A-f	84 ± 2	90 ± 3	66 ± 3
PE-S/UA-f	94 ± 3	98 ± 2	88 ± 2
PE-S/A-f	100 ± 2	105 ± 1	90 ± 2
PE-S/A-f-col	84 ± 2	91 ± 3	66 ± 3

- **The efficiency of the size fractionation of the test particles** was determined using size and shape characterisation for the particles from initial grinding trials and unaged raw material (PE-S/UA-g). The particles were characterised by laser diffraction (Mastersizer 3000, Malvern Instruments) in a solution of isopropanol (IPA) with and without an added surfactant (Lutensol AO8). The average particle size of PE-S/UA-g (sieved with 80 µm) was about 160 µm, with less than 1 % of the particles being < 20 µm. Moreover, the particles had very irregular shapes, where semi-spherical beads carried long, thin plastic tails or multiple beads interlinked by the thin tails (Figure 5). Therefore, with its native ductility, the unaged plastic was not sufficiently brittle to produce MPs in the required size range (< 20 um) using the applied grinding procedure.

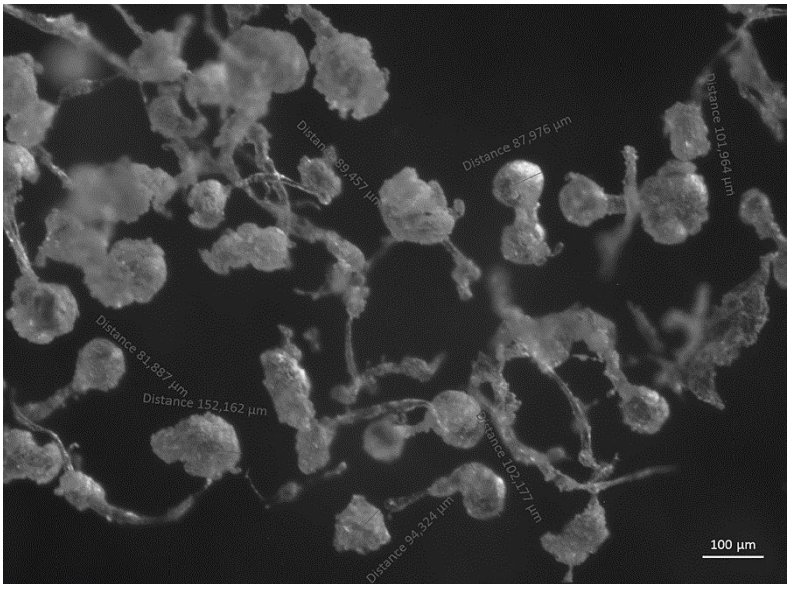

Figure 5. Optical microphotograph showing the irregular shapes of the MPs produced from unaged stabilised LDPE.

- **Particle size distribution analysis** was conducted by the laser diffraction method, also known as laser diffraction spectroscopy, which utilises diffraction patterns of a laser beam passed through any object ranging from nanometers to millimetres in size to measure the geometrical dimensions of a particle. The sample has been analysed with light scattering/diffraction using Malvern Mastersizer 3000 with the measuring cell Hydro SM cell, which holds 120 mm of liquid and the range of 0.020–3 500 µm. The PE-S/A-f (stabilised aged LDPE; < 20 µm, 8 ml) was diluted to 16 ml with 95 % ethanol, sedimented, and left to mix for 60 min in a Heidolph reax 2 (speed 5). Then, the mini shaker was run for 10 sec before withdrawing 5 g of sample, diluting it with 110 ml of 95 % ethanol in the instrument and taking measurements at a stirring speed of 2 000 rpm. No submicron (< 1 µm) particles were observed for MP in the < 20 µm fraction (Figure 6).

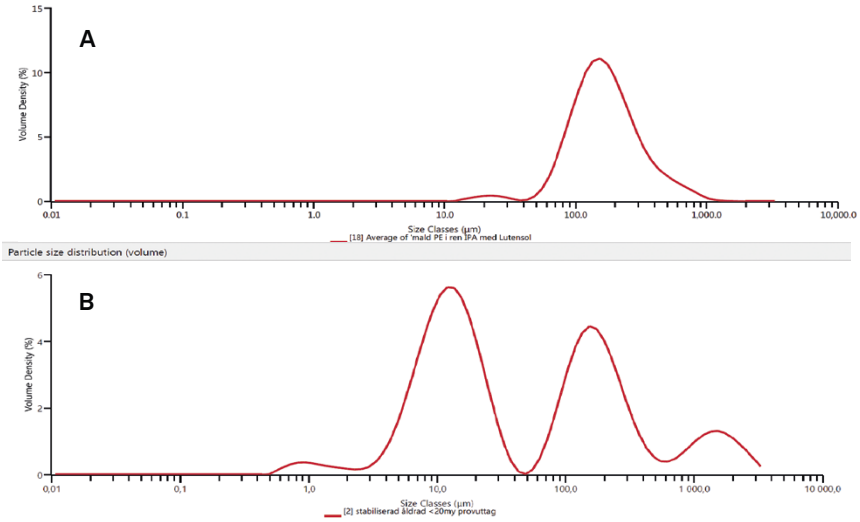

Figure 6. The particle size distribution for MP was sieved with (A) 80 µm and (B) 20 µm mesh.

3.2 Designing a test system for ecotoxicological assessment of MP effects

To answer whether MP poses a risk to the environment, we need to develop experimental designs that identify environmentally relevant effects that help understand particle and chemical effects and whether there is a potential for these to occur *in situ*. For example, particle exposures generate behavioural adaptations, such as avoidance and altered feeding in filter-feeders that are common test organisms in MP effect testing. In addition, because MP is just a fraction of suspended solids in the water column or sediment, their effects should be evaluated in mixtures with other solids.

We proposed a novel method, the *MP ratio test*, for testing MP effects in mixtures with reference particles via analysis of dose-response data for particle combinations with increasing MP proportions (Gerdes et al., 2019a). The MP load in the system can vary, mimicking environmentally relevant levels, while the suspended solid

(TSS, i.e., the sum of MP and reference particles) concentration is constant across the treatments with different MP levels. This ecologically and environmentally relevant exposure scenario allows analysis of the particle effects reflecting the real-life exposure scenario. The effect concentrations (Figure 7, Step 2) can be expressed as two threshold values (Step 3) describing (1) tolerable level of TSS (NOEC for TSS; No Effect Concentration) and (2) safe contribution of MP (%MP) conditional on the tolerable TSS level. The critical threshold for %MP present in the mixture and exerting no effect in the test system was termed NOE%MP (No Effect Percentage of Microplastics).

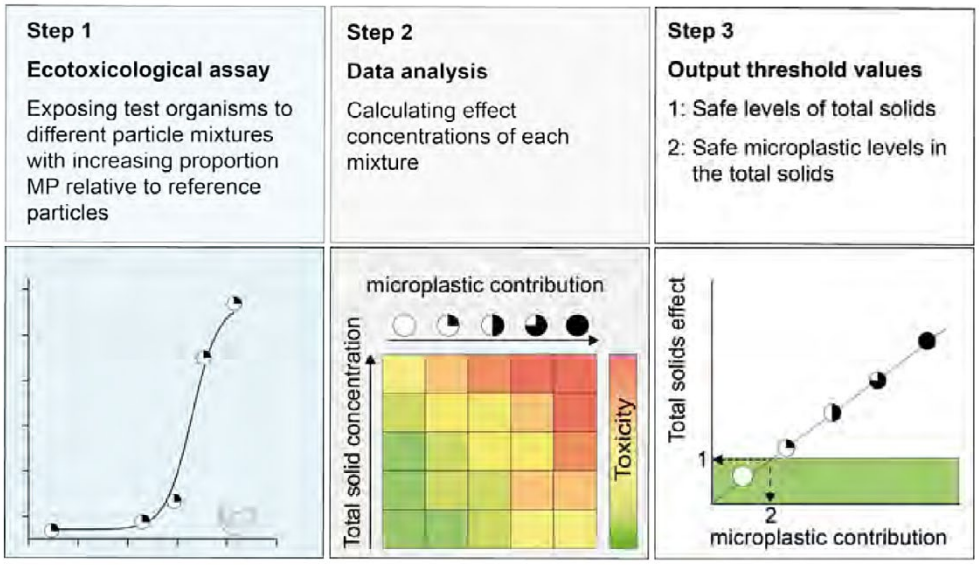

Figure 7. The concept of the MP ratio test. *Step 1*: Dose-response effect assessment, e.g., the *Daphnia* sp. Acute Immobilisation Test, across mixtures with an increasing ratio between MP and reference particles. *Step 2*: Data analysis to acquire effect concentrations, e.g., EC10, in the mixtures. *Step 3*: Modelling the relationship between the effect concentration of each mixture and microplastic contribution in this mixture to derive two thresholds (1) tolerable levels of SS (i.e., NOEC for suspended solids) and (2) No-observed-effect concentrations for MP levels in the mixture with the non-harmful SS value (NOE%MP, mass-based).

Acute Immobilisation Test for *Daphnia* with kaolin as reference material and PET as MP was used to derive hazard thresholds for the MP contribution to the suspended matter conditional on the TSS in the water column. No measurable effects were observed at the suspended solid concentrations of up to 32 mg L^{-1}. However, the mixture became more hazardous when the PET proportion exceeded 2.4 %. These values of total suspended solids are not unusual, although the amount of MP is around three orders of magnitude higher than the reported levels in aquatic environments (Dibke et al., 2021).

The hazard level for MP contribution to TSS that we found for daphnids is close to the EC10 of 1.1 % MP per sediment dry weight reported for a benthic macroinvertebrate (Redondo-Hasselerharm et al., 2018). Together, these findings support the possibility of benchmarking MP effects against the lower 95 %-confidence bound of the reference material and using the MP corresponding contribution to the mixture as the environmentally safe MP level. Thus, the method allows estimating MP threshold levels in water and sediments that can be used for hazard assessment.

The NOE%MP value for *Daphnia* is a percentage of microplastic in the mixed solids in the water column that does not cause measurable effects; this value can be used as a safe level threshold for MP in surface waters.

3.3 Ecotoxicological assays

3.3.1 Testing MP and leachate effects in macrophytes

Macrophytes are the major primary producers in coastal environments and accumulation hotspots for MP because they have a high capacity to retain plastic litter (Karalija et al., 2022). This litter slowly breaks down in the macrophyte beds, producing microplastics and leachates containing additives, their breakdown products and releasing chemicals sorbed during exposure to contaminants in the environment. Therefore, macrophytes are relevant organisms for assessing plastic leachate effects.

We tested leachate-induced response in red macroalga *Ceramium tenuicorne*, one of the Baltic region's most relevant standardised macroalgal test species (Eklund, 2005). The test MP with and without stabilising additive Irganox 1076 (primary phenolic antioxidant) and subjected and not subjected to the accelerated thermo-oxidative ageing (Table 1) were used to generate leachates according to the standard procedure (CEN - EN 12457-2, 2002). The algae were exposed to MP leachates for seven days at a geometric concentration range corresponding to 0.1–100 g L^{-1} of plastic material. As endpoints, we measured ecophysiological responses related to photosynthesis, pigment concentrations, and antioxidative status of the algae; in the context of sublethal effects in plants, these responses were suggested to be the most sensitive.

All tested plastic leachates induced sub-lethal dose-dependent responses for all endpoints (Figure 8), with a reduction of pigments and increased total antioxidant capacity (water-soluble antioxidants, a reference for antioxidant effectiveness) at increasing leachate concentrations. These responses are consistent with oxidative stress and the inadequate capacity of the plants to counteract ROS production. The EC10 values were 0.13–2.06 g L^{-1} among the test materials; these EC10 values are sufficiently low to fall in the range of the plastic litter concentrations observed in the hot spots of plastic litter pollution in the environment.

As hypothesised, there were tendencies that the effects depended on whether the plastics were aged and contained additives. However, there were also differences in the endpoint sensitivity towards plastic materials. For example, the most toxic leachate was PE-Irg-aged, with the lowest EC10s for the antioxidant capacity and chlorophyll-*a* among the tested plastics, but the carotenoid concentration was the least affected compared to other plastics. In contrast, PE-aged leachate affected total carotenoid concentrations the most while also strongly affecting antioxidant capacity and chlorophyll-*a*. The other two plastics, however, did not show consistent responses among the endpoints. Thus, ageing was the most influential factor for leachate toxicity.

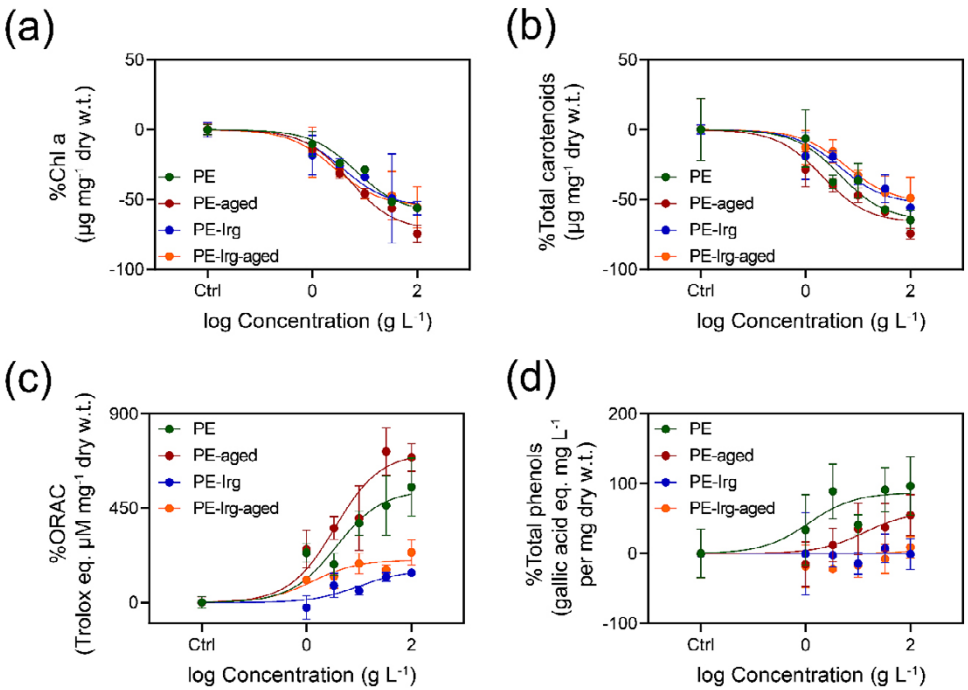

Figure 8. Dose-response curves of sub-lethal endpoints including (a) chlorophyll *a*, (b) total carotenoids, (c) antioxidant capacity measured by ORAC assay, (d) total phenol, as the functions of leachate concentrations (0.1–100 g L^{-1}) extracted from four plastic materials. All values are relative to controls (mean ± SD, n = 3).

3.3.2 Behavioural responses to MP exposure

Coastal, soft-bottom ecosystems are particularly susceptible to MP pollution because they are deposition areas for solid and soluble contaminants. Adding MP or any other inert particles to the sediment may dilute its nutritional content and modify structural integrity, leading to adverse effects on sediment-living biota. Therefore, benthic species should be included in test batteries for MP effect assessment.

Behavioural endpoints are sensitive but underutilised indicators of habitat quality linked to physiological responses, such as metabolism, which is essential for understanding the mechanisms behind the MP effects. For example, alterations in the swimming behaviour of *Daphnia magna* exposed to MP were observed, indicating that downstream responses in food acquisition and growth can be related to inefficient time and energy budgets (Gorokhova et al., 2018). Therefore, we studied the behaviour of the benthic deposit-feeding amphipod *Corophium volutator* exposed to sediments enriched in MP. We also measured the Electron Transport System activity (ETS) as a proxy for respiration in the animals facing varying sediment quality (Gerdes, 2021). The natural sediment was manipulated by dilution with two common plastic materials (polystyrene PS and polyethene terephthalate PET, aged and virgin) and kaolin clay as reference material at 1 and 10 % sediment dry weight.

We did not find any significant effects of either material type or ageing status on behavioural traits (Figure 9). However, a significant increase in erratic non-feeding movements was observed in the 10 % mixture treatments regardless of whether PS, PET or clay was added to the sediment. These non-feeding movements suggested a general stress response to the altered habitat quality. There was also a consistent

preference for the natural sediment, as evidenced by a higher burrowing frequency than mixed sediments. Even though the addition of the inert microparticles diluted the organic carbon content of the sediment, and amphipod movement increased nearly 4-fold, their feeding activity was not altered in any detectable way by the exposure. The ETS activity was positively correlated with the increased physical activity due to the non-feeding movements but not food acquisition behaviour.

In conclusion, the effects of MP on the behaviour and metabolic state of deposit-feeding amphipods are similar to other nutritionally inert particles. The adverse effects are more of a reaction to a general disturbance of the sediment structure than material specific. Compared to the published data on MP effect concentrations in benthic organisms, behavioural endpoints are sensitive and informative when studying animal responses to alterations in sediment quality; thus, they may provide complementary information for developing sediment quality criteria.

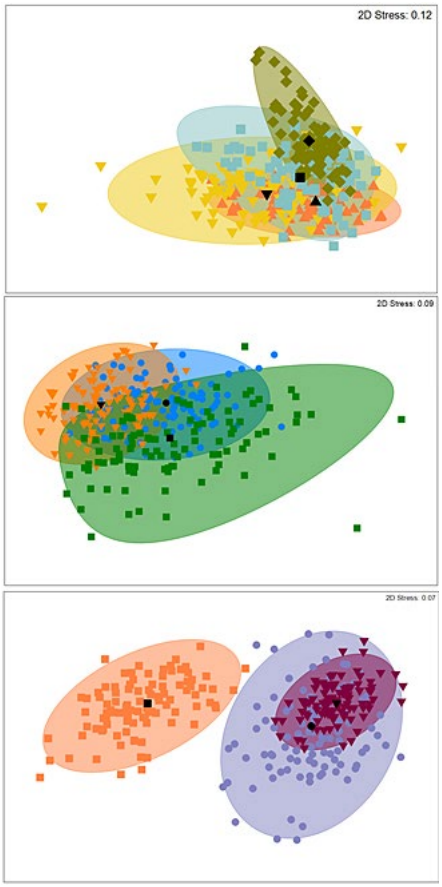

Figure 9. Bootstrapped non-metric multi-dimensional scaling (nMDS) of the behavioural responses in the amphipod *Corophium volutator*. The response consisted of several metrics (feeding and non-feeding movements, burrowing behaviours, etc.) measured in the amphipods exposed to sediments mixed with either PS, PET or clay in different proportions (none, 1 % and 10 % of the added plastic material). Black symbols represent the group centroids across, and shaded areas show 95 % confidence regions. The clustering of the samples indicates that behavioural responses of the individual amphipods are: (A) similar between the aged (green: PS, blue: PET) and virgin (yellow: PS, pink: PET) plastics; (B) similar among the test and reference materials (orange: PS, blue: PET and green: clay); and (C) different between the exposure levels when all data for the test and reference materials are pooled (addition levels: 10 % indicated in orange, 1 % – dark purple, and no addition – light purple); observed the difference between the treatments with low (0–1 %) and high (10 %) levels of the added materials.

3.3.3 MP as a carrier of pathogens in ecotoxicological assay

The surface of plastic materials, including MP used in testing ecotoxicological and toxicological effects in MIXiT (Ogonowski et al., 2018b), harbours unique bacterial assemblages. Moreover, due to ageing and promoting the propagation of some microbial organisms, plastic surfaces undergo various physicochemical transformations, changing particle capacity to sorb chemicals and aggregate with other particulates (McGivney et al., 2020). Consequently, concerns have been raised that ingested microplastic may affect the consumer gut microbiota and spread pathogens in animal populations (Rogers et al., 2020).

We hypothesized that in an ecotoxicity assay with a mixture of polystyrene (PS) and clay: (1) the microbiome of the test animals inoculates the system with bacteria (Gorokhova et al., 2021); (2) the relative contribution of PS and the total amount of suspended solids (SS) select for specific bacterial communities (Gorokhova et al., 2021); and (3) particle aggregation is affected by biofilm presence (Motiei et al., 2021) and composition (Gorokhova et al., 2021), with concomitant effects on the animal survival. These hypotheses were tested by exposing *Daphnia magna* to mixtures of PS and clay at different concentrations of SS (10, 100, and 1000 mg/L) and varying microplastic contributions (%PS; 0–80 %). After the exposure, we recorded animal survival, examined the biofilm communities by 16S rRNA gene sequencing and analysed particle size distribution.

The biofilm communities diverged from the *Daphnia* microbiota used to inoculate the system, with an overrepresentation of predatory, rare, and potentially pathogenic taxa in the biofilms. Moreover, the bacterial diversity was stimulated by %PS and decreased by predatory bacteria. Daphnid survival in the experiment was affected by particle aggregation and biofilm composition, namely Bdellovibrionaceae relative abundance (Figure 10). The high death rate was associated with a smaller aggregate size and high Bdellovibrionaceae contribution to the biofilm communities. The adverse effects of suspended solids on the test animals were mediated by the aggregate size (high SS → smaller particles → higher mortality) and relative abundance of Bdellovibrionaceae (high SS → more predatory bacteria → higher mortality). The effect of polystyrene was mediated by its negative impact on Bdellovibrionaceae, thus, ameliorating the adverse effects of suspended solids (Figure 10).

In conclusion, particle aggregate size and biofilm composition were the primary drivers of animal survival, with a higher death rate associated with exposure to smaller particles and predatory bacteria. These findings imply that the ecotoxicological testing for solid waste materials, including MP, should consider the biofilm formation, particle size distribution, and ecological interactions in the biofilm. All these factors are needed to interpret particle aggregation in the exposure system and its concomitant effects on potentially harmful microorganisms and test animals.

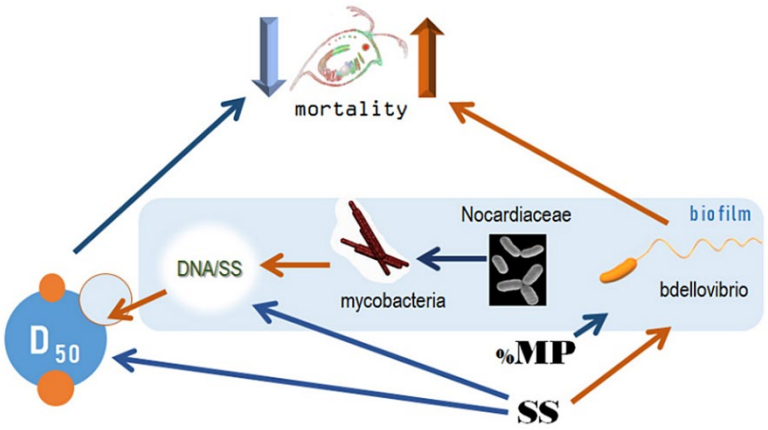

Figure 10. The pathways for the causal relations behind the variability in the *Daphnia* mortality under exposure to suspended solids (polystyrene and clay). Orange and blue arrows depict positive and negative relationships, respectively. SS: suspended solids (mg/L); percent MP: proportion of polystyrene microplastics in the exposure mixture; D50: median aggregate size (µm); DNA/SS: relative contribution of the biofilm to the particle mixture (µm/mg).

3.4 Toxicological assays

The toxicological assays aimed to (1) understand MP cytotoxicity in human cells and (2) explore the effects of MP-leachates. Inhalation was considered the primary exposure route. Therefore, the test system for human exposure was based on macrophages and lung epithelial cells. In general, any particle in the human body will be engulfed by the professional phagocyte cells, such as macrophages. The monocyte cell line THP-1 was differentiated into macrophages (using PMA) and the cytotoxicity and inflammatory effects of MP were tested. Macrophages are found in many different organs, where they engulf foreign objects, including particles, and thus, this cell model is also relevant for non-lung exposures. To address inhalation perspective, selected MP were evaluated for the DNA damage effects using human bronchial epithelial cells (HBEC).

The particles/leachates effects were addressed in the following studies (Figure 11):

- Investigation of cytotoxic and oxidative stress potential of four primary PS particles (different sizes and surface modifications) and three secondary MPs (PS, PET, PE) using three model systems (lung epithelial cells, macrophages, and a co-culture).
- Study of the cytotoxic effects of the test MPs (LDPE; i.e., stabilised/aged, stabilised/unaged, non-stabilised/aged, non-stabilised/unaged) in human-derived macrophages.
- Evaluation of the cytotoxicity of MP leachates (in medium and saliva + gastric juice) generated from the test LDPE materials (stabilised/aged, stabilised/unaged, non-stabilised/aged, non-stabilised/unaged).

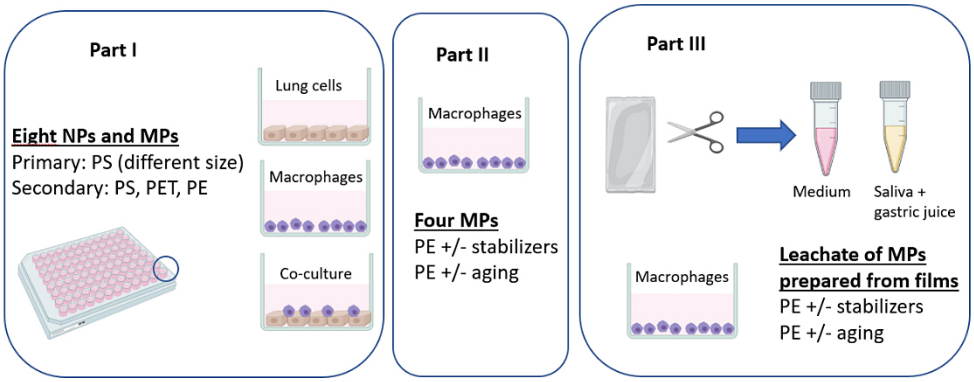

Figure 11. Overview of the toxicological studies.

3.4.1 Cytotoxic and oxidative stress potential of primary PS particles

We tested cytotoxicity and oxidative stress in three cell models exposed to eight NPs and MPs (Table 4). The particles were suspended in a solution consisting of distilled water, anionic surfactant (0.02 %) and/or sodium azide (< 0.09 %), except the NH_2-PS. For testing vehicle toxicity, the particles were removed using centrifugal filter tubes.

- **SEM analysis** was used to confirm the size and shape of the different NP/MP. The samples were prepared for imaging with a Leica CPD030 critical point dryer and a Qourum Q150T ES sputter coater. All particles except 50–55 nm particles were mounted on a polyethersulfone membrane; the 50–55 nm particles were mounted on an EM grid. Imaging was done by Zeiss Gemini Ultra 55 scanning electron microscope (SEM) equipped with an on-axis SE detector (InLens SE) or Everhart-Thornley SE detector (SE2). The images confirmed the round shape of the primary PS particles and showed an irregular morphology and more varying size of the secondary MP (PS, PET and PE; Figure 12).

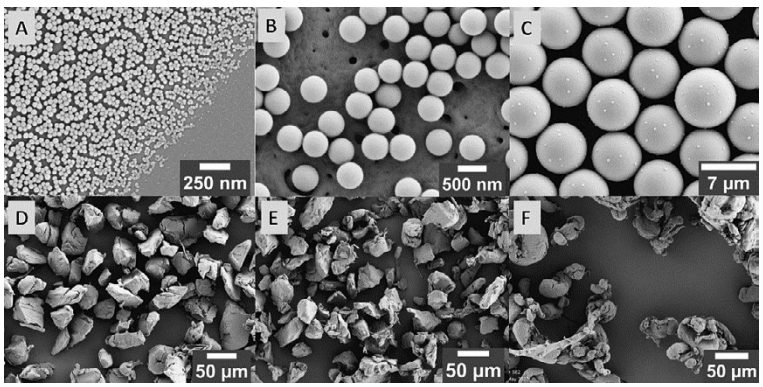

Figure 12. SEM-images of primary PS particles: (A) 50 nm, (B) 500 nm, and (C) 7 µm; and secondary particles: (D) PS-UV treated, (E) PET-UV treated, and (F) thermo-oxidized LDPE.

Table 4. Physiochemical properties of MP tested in human cells.

Particle type	Shape	Polymer/ source	Post-treatment/ Surface groups	Size	Type
Primary	Spherical	PS Magsphere Inc.	Non-functionalized	~ 50 nm	NP
				~ 520 nm	NP
				~ 7 µm	MP
			Amine group (NH_2-)	~ 55 nm	NP
Secondary	Irregular	PS Goodfellow	Produced by cryo-milling 3–5 mm pellets + UV radiation	~ 50 µm	MP
		PET Goodfellow	Produced by cryo-milling 3–5 mm pellets + UV radiation	~ 50 µm	
		LDPE Borealis	Film with and without additives; particles produced by cryo-milling alone or cryo-milling + thermo-oxidation 80 °C	~ 40 µm	

- **Toxicity testing** was conducted using human bronchial epithelial cells (HBEC3-kt, from ATTC), THP-1 human monocytes (THP-1, from ATTC) differentiated into macrophages (called dTHP-1), and a co-culture of these prepared by harvesting freshly differentiated macrophages and adding these on top of HBEC cells at a ratio of 10:1 between HBEC and dTHP-1 cells. Cytotoxicity was tested using Alamar blue assay, and oxidative stress was measured using the DCFH-DA assay. The "cell dose" of the fluorescent particles was determined following incubation for 3 h (six wells). Then three of the six wells were washed, and fluorescence was measured at 485/590 nm excitation/emission. The cellular uptake/interaction was quantified as the mean fluorescent signal from washed wells as a percentage of the mean fluorescent signal from the full dose wells. The results showed that the actual cell dose was around 5–10 % for all concentrations and independent of the particle size.
- Moreover, a **cytotoxic effect of the vehicle** (anionic surfactant, 0.02 %, and sodium azide, < 0.09 %; Figure 13) was observed for HBEC cells but not THP-1 cells. This is likely because HBEC cells are cultured under serum-free conditions (with some proteins added), whereas the THP-1 cells are cultured in 10 % FBS. As expected, the aminated particles exhibited significant toxicity in both cell types. However, no cytotoxicity and oxidative stress were observed for the secondary MPs (Figure 14), suggesting no cell uptake (probably due to the relatively large particle size) and no measurable membrane damage.

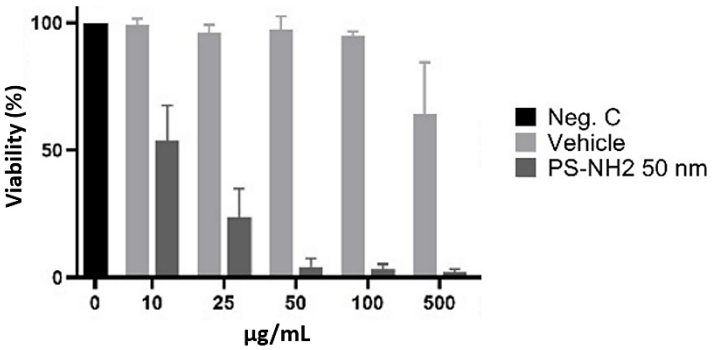

Figure 13. Cell viability following a 48 h exposure of HBEC cells to aminated PS particles (dark grey) and vehicle (light grey).

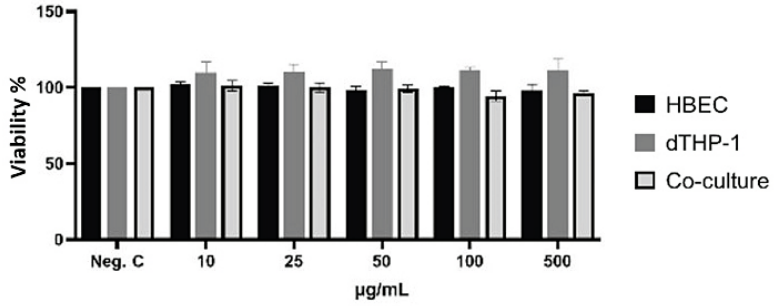

Figure 14. No decrease in cell viability was observed in three different cell models exposed to the secondary MP (PS) for 48 h. Cobalt nanoparticles (25 µg/mL) were used as a positive control showing decreased viability.

In conclusion, the secondary MP tested were non-toxic at the experimental conditions. Taken together, these results highlight that the possible toxicity of the vehicle needs to be considered for particles supplied in suspension. Furthermore, the *cell dose*, i.e., particles coming in contact with the cells, is likely to be only a few percent of the nominal dose.

3.4.2 Cytotoxic effects of the MP with four additive/ageing combinations

The cytotoxicity of the LDPE particles produced by RISE (Table 1) was tested using:

- stabilised/aged (S/A)
- stabilised/unaged (S/UA)
- non-stabilised/aged (NS/A)
- non-stabilised/unaged (NS/UA)

We exposed macrophages (differentiated THP-1 cells) to these particles and tested the viability as previously described. Increased viability for the stabilised/aged MP was observed, but no adverse effects (Figure 15). The reason for the increased viability for only the PE-S/A is somewhat unclear but may be related to an increased cell dose of the aged (compared to non-aged) because these particles may be more brittle, in combination with an effect of the stabiliser (Irganox 1076), which is a potent antioxidant. Thus, the results suggest that PE microplastics have no cytotoxic effects at the doses tested, but a stimulatory effect on cell viability was induced.

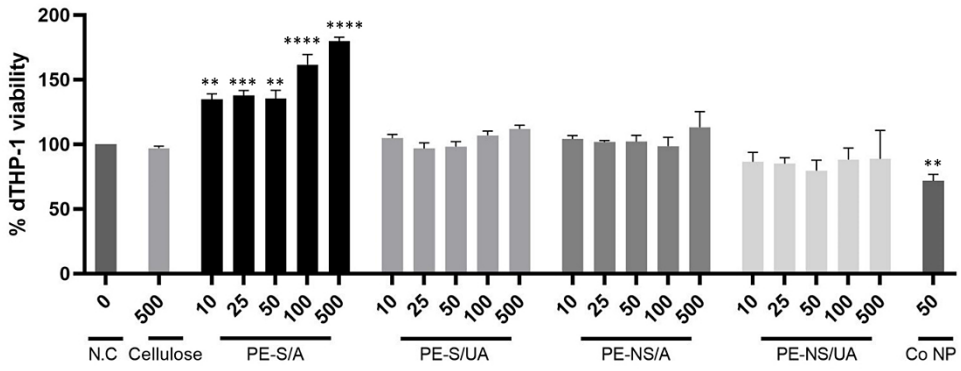

Figure 15. Viability of macrophages following exposure to LDPE microplastics; stabilised/aged (S/A), stabilised/unaged (S/UA), non-stabilised/aged (NS/A), non-stabilised/unaged (NS/UA).

3.4.3 Toxicity of MP leachates

Testing of toxicity of the leachates was conducted using the same materials as in Section 3.4.2 (Figure 15). The leaching was performed in cell medium and saliva/ gastric juice (Table 5). Particles were weighed and leached in cell medium (RPMI Medium 1640, with 10 % FBS, 1 % PEST, 1& L-glutamine) at 1 mg/mL concentration in darkened glass vials. Vials were incubated at 37 °C 180 rpm shaking for 24 hours. Leaching was also performed in artificial saliva (5 min, 37 °C) followed by artificial gastric juice (2 h, 37 °C) under shaking.

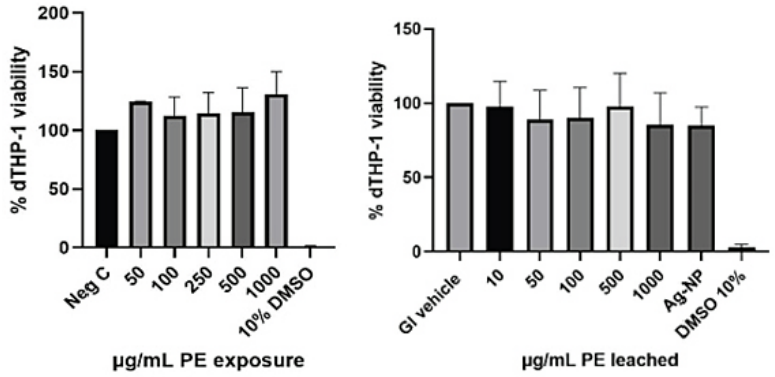

Figure 16. Viability of macrophages following exposure to medium leachate (left) or leachates from artificial saliva and gastric juice (right) for the stabilised/aged MPs.

None of the leachates caused significant adverse effects on macrophage cell viability (Figure 16), although the standard deviation was sometimes high. Therefore, leachates from the materials tested do not cause any acute cytotoxic effects; however, more subtle stimulating effects were found and should be further explored.

Table 5. Composition of artificial saliva and gastric juice used to prepare leaching media for testing effects of LDPE leachates.

Saliva		Gastric juice	
	g/l		g/l
KCl	0.896	NaCl	2.752
KSCN	0.2	$NaH_2PO_4 \cdot H_2O$	0.306
NaH2PO4.H2O	1.021	KCl	0.824
Na2SO4	0.57	$CaCl_2$	0.302
NaCl	0.298	NH_4Cl	0.306
NaHCO3	1.694	37 % HCl	
urea	0.2	glucose	0.65
		glucuronic acid	0.02
amylase	0.29	urea	0.085
uric acid	0.015	glucosaminehydrochloride	0.33
mucin	0.025		
Milli Q Water		BSA	1
		pepsin	2.5
		mucin	3
		milli-Q water	

3.5 Meta-analysis

Two meta-analysis studies were conducted to evaluate MP effects in (1) standardised ecotoxicity testing using a standard algal growth inhibition test with minimum variability across the studies that all followed OECD guidelines and (2) a broad range of studies reporting effects across different levels of biological organisation in a variety of species and endpoints.

3.5.1 Meta-analysis of MP effects in standard tests on growth inhibition in microalgae

Standardised ecotoxicological testing of MP effects should include primary producers at the base of the food web because they are at risk of being exposed to both plastic litter and its leachates. Furthermore, negative effects on primary producers may translate into adverse effects on consumers. In addition, the interaction with bacterio-and microplankton can alter biochemical cycling and impact the fate of polymers. Primary producers, such as microalgae, have shown both stimulation and impairment of growth measured using cell counts and autofluorescence, which are the two standard endpoints in the algal growth inhibition assay used for toxicological testing following OECD guidelines. Prompted by these conflicting outcomes and to identify drivers of adverse outcomes, a meta-analysis was carried out using 20 studies published over the last ten years, with five polymers in size range from 0.04 to 3 000 µm and 16 algal microalgal species. Three separate random effect models were set up to investigate MP concentration effects. The third model included the most substantial effect size observed across the studies regardless of the MP concentration. Test species, particle concentration, size and shape were recorded to identify significant predictors.

None of the three models showed a significant negative growth effect in algae exposed to micro-and nanoplastics (*Full-range model;* Figure 17). All models showed a strong heterogeneity between the outcomes. Polymer density had a significant moderating effect with a greater risk of growth impairment at lower densities. However, neither micro- and nanoplastic concentration nor material type or shape contributed to the variation of study outcomes.

Additionally, we observed a significant publication bias towards small studies reporting negative results (Figure 17), implying that a study reporting inhibitory effects is more likely to be published, even though the results would be less reliable and the sample size is small. Given that plastic litter has become a hot topic in the current environmental research fueled by keen public interest, discovering indications of publication bias was not particularly surprising. However, the magnitude of the bias was not sufficiently large to invalidate the meta-analysis outcome.

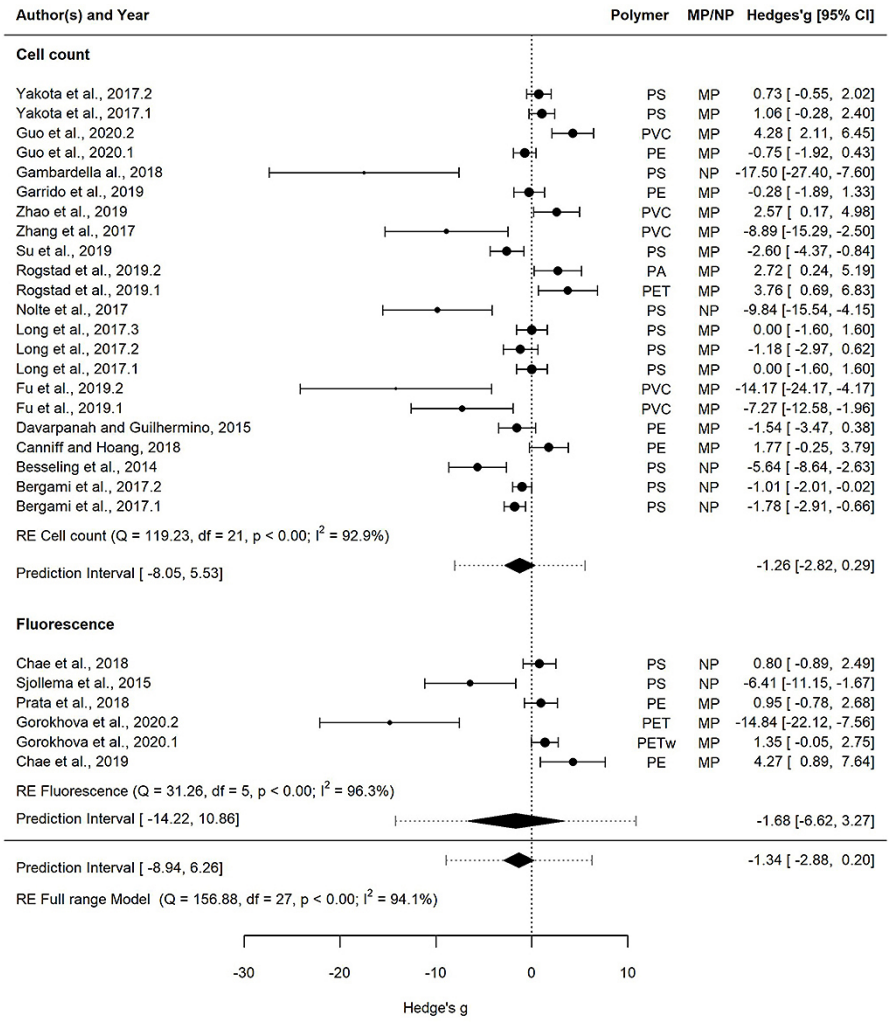

Figure 17. Forest plot of the full-range model with the effect size expressed as Hedge's g and associated 95 % confidence intervals. The symbol size is proportional to the weight assigned to the study, and the overall pooled effect is depicted as a diamond with the prediction interval. The estimates are separated based on different endpoints (cell count and fluorescence). Multiple entries represent studies that used several test species and/or different polymers.

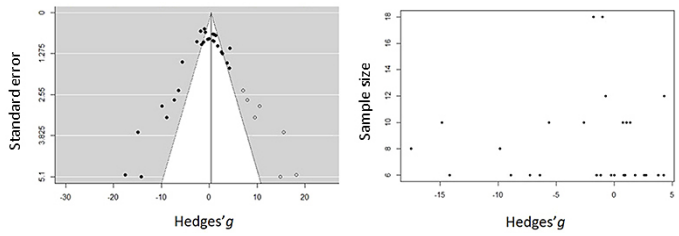

Figure 18. The funnel plot for the estimated probability of publication bias for studies used in the meta-analysis (left panel) and the relation between sample size and effect size (right panel). Each dot represents a study; the hollow symbols are the studies filled by the trim and fill procedure, which would balance the asymmetry in the funnel. The white area depicts the significance between 0.05 and 1. The grey area is between 0 and 0.05.

3.5.2 Meta-analysis of MP effects across different levels of biological organisation

In aquatic environments, MP contribute very little to the total amount of suspended particulates. To understand whether MP effects are fundamentally different from other natural particles, we performed a hazard assessment comparing the effects of MP and other suspended solids (SS) using data from the scientific literature (Ogonowski et al., 2022). The published data for various organisms (VKM, 2019) were complemented with effect studies on SS reviewed earlier (Gordon and Palmer, 2015; Ogonowski et al., 2018a). The exposure and effect datasets were used to extract effect concentrations as the lowest observed effect concentrations (LOEC), effect concentrations derived from dose-response relationships (EC10, EC20, EC50, LD50), and no-effect concentrations defined as the highest observed-no-effect concentration (HONEC). The primary data in the form of varying dose descriptors other than the no-observed effect concentrations (NOECs) were converted to estimated NOECs using a conversion factor specific to each descriptor (Adam et al. 2019).

The compiled dataset was restricted to studies in which aquatic organisms were directly exposed to MP or SS added to the medium. Thus, studies in which the particles were incorporated into food were excluded. Furthermore, data on fibrous particles were omitted since this particle shape was exclusive to MP. Studies employing particles < 0.98 µm (clay-sized particles) were also removed since nano-sized particles' mode of action may differ due to their capacity to pass biological barriers and cell membranes. As a result, the reported PNEC values for nanoplastics are lower than for microplastics, e.g., preliminary safe standards of 0.33 µg/L and 1.1 µg/L for microplastics and nanoplastics, respectively, have been proposed (Besseling et al., 2019).

Since the primary mode of action of microparticles > 1 µm is assumed to be food dilution (de Ruijter et al., 2020), we further restricted the data to those with ingestion as the main route of exposure, which retained only consumers (feeding stages) in the final dataset. We considered individual- and population-level endpoints (Galloway et al., 2017), i.e., growth, mortality and reproduction. The data subset used for analysis consisted of 43 studies (MP: 28 and SS: 16) and 200 biological endpoints (MP: 123 and SS: 77). Mass- and particle-based concentrations were converted to volumetric units (Koelmans et al., 2020).

We used two different models and approaches to assess the effects:

- *A novel two-step approach to probabilistically model Species Sensitivity Distributions (SSDs)* allowed the assessment of the average hazard over diverse species. However, the original method was developed for soluble toxicants and did not account for the heterogeneity in particle characteristics that may affect the response. We, therefore, used multiple regression to standardise the exposure parameters and particle characteristics and then ran the SSD analysis, allowing for a comparison of the effects.
- *A Bayesian mixed model* allowed standardisation of the exposure parameters, such as exposure time, particle shape and size, to be comparable across the particle types.

The two methods converged at the same conclusion indicating that an average MP is approximately ten-fold more harmful than a natural SS particle (Figures 19 and 20). However, the uncertainties around these estimates were substantial, with considerable overlap in credible intervals. We also found a weak indication that organisms are more sensitive to smaller particles – particularly those in the clay and silt size fractions, which is in line with MP reports (Ziajahromi et al., 2018). Also, organisms inhabiting clear, oligotrophic waters appear more sensitive than those adapted to turbid environments; hence, adaptations among species to cope with non-palatable particles are expected.

Although we found some support for MP being more potent to cause effects than natural microparticles, there are limitations to this and other meta-analyses investigating MP effects (Doyle et al. 2022). For example, concentration-dependent dose metrics like the NOEC and LOEC are problematic because MP studies tend to employ lower test concentrations than SS studies. Therefore, the MP datasets are prone to generate lower effect concentrations than SS datasets. Moreover, MP studies often use virgin plastics containing additives, whereas environmental MP are the products of ageing and fragmentation of plastic litter, and chemicals present in the virgin material leach out during these processes. Also, MPs used in experimental studies are stored as suspensions with preservatives which can induce toxic effects on their own if carried over to the test vessels.

We conclude that MP in the 1–1 000 µm range are more likely to affect aquatic organisms than natural suspended solids; however, further studies are warranted due to the high uncertainty of our estimates. Also, more experimental data are needed for MP to establish whether this pattern holds for aged particles.

Nevertheless, these results align with previous effect assessments and meta-analyses on the subject (Ogonowski et al., 2018a). We would, however, like to emphasise that hazard assessment for particulate materials based on literature data can be difficult to interpret unless test designs are standardised and stable exposure conditions are maintained (Gerdes et al., 2019a; Gorokhova et al., 2021, 2020; Motiei et al., 2021), adequate reference particles are used, and dose-dependent point-estimates are derived. The MP-ratio test developed for planktonic organisms in this project (Gerdes et al., 2019a) and a similar test for deposit feeders (Redondo-Hasselerharm et al., 2018) are excellent examples of the test systems that could improve hazard assessments for particulate materials and further our understanding of the mode of action for solid waste particles, including MP.

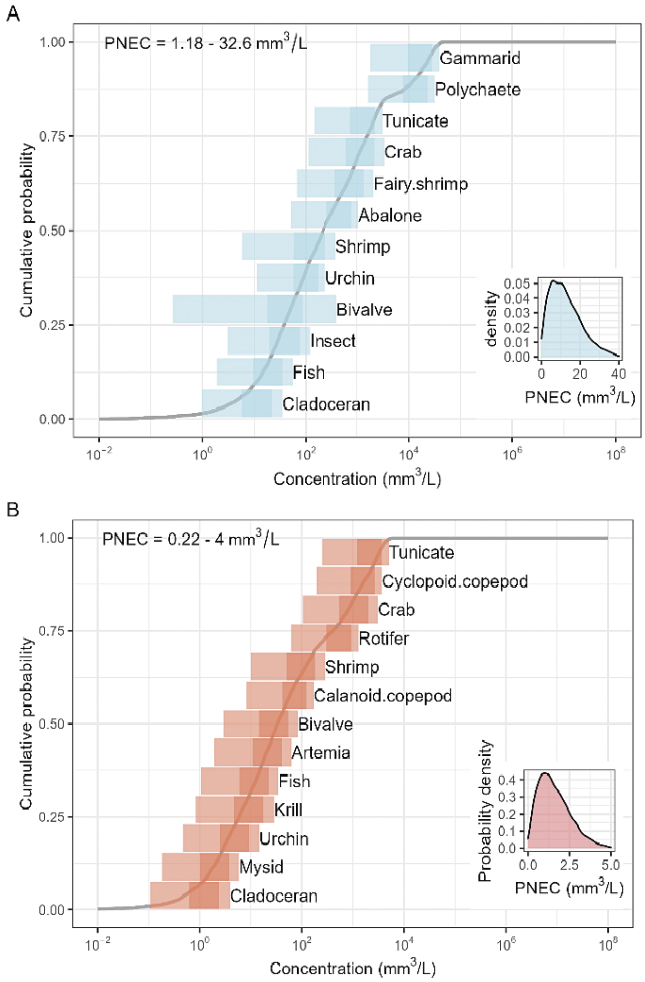

Figure 19. Probabilistic species sensitivity distributions for volume-based effect data corrected for inter-study differences in the particle characteristics and exposure conditions for (A) suspended solids (sediments), estimated median PNEC = 11.1 mm³/L, and (B) microplastics, median PNEC = 1.4 mm³/L. The dark-shaded horizontal bars represent the 25–75th percentile range, and light-shaded areas are the 5–95th percentile range.

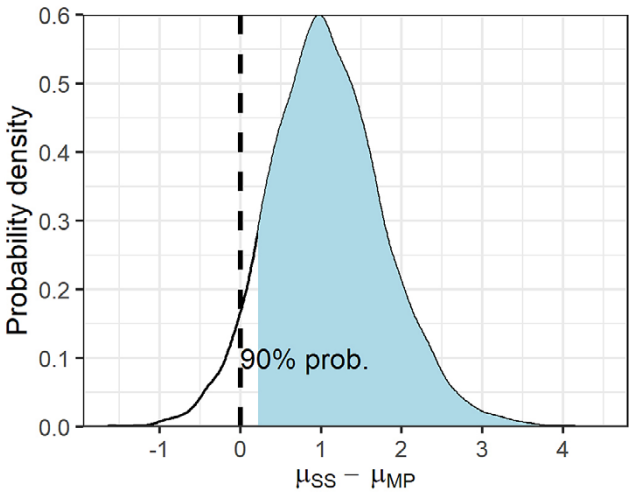

Figure 20. The marginal mean difference in the posterior probabilities between suspended solids (SS) and microplastic (MP) groups in the Bayesian mixed model. The shaded area shows a 90 % probability (one-sided test) that MP have a lower estimated No-Effect-Concentration than SS.

3.6 Modelling body burden of microplastic in a simple food web

Microplastic hazard assessment in the marine environment requires quantification and understanding of MP transfer in the food web. However, gathering quantitative data on MP body burden in biota is difficult due to the analytical challenges with material identification and unsettled sampling methodologies. Therefore, a modelling approach is helpful because it can help predict body burden using data on microplastic occurrence in the environment and knowledge of the food web topography (Alava, 2020).

A mass balance model was applied to predict microplastic body burden in the Baltic herring; the model is relatively simple and based on the microplastic abundance in the water and physiological rates of the food processing by the fish (Ogonowski et al., 2019). To add food-web complexity, a trophic guild structure *zooplankton – invertebrate zooplanktivore – herring* was incorporated in this model; this was implemented in the BSc Thesis project (Månsson, 2020). This trophic guild is typical for the Baltic pelagic food webs and exists in any aquatic system, where small zooplanktivorous fish and large predatory invertebrates (such as mysids, jellies, chaetognaths, etc.) share the same food source (zooplankton).

In this model (Figure 21), MP can enter aquatic consumers both directly (by ingestion of MP mistaken for food particles) and indirectly (through secondary consumption). Regardless of the ingestion route, possible negative effects on digestive functions, food intake and growth can occur. Using the revised model, we (1) evaluated how the mixed diet (zooplankton and mysids) affected the total MP body burden in the fish and (2) calculated the relative contribution to the total MP burden in herring via secondary consumption.

A dynamic model predicting MP occurrence in Baltic herring in a trophic guild was developed and compared to the earlier model that simulated the direct uptake of MP from the water by herring of 20 cm. In the revised model, the MP uptake involves two pathways: filtration associated with feeding on zooplankton (direct intake) and predation on mysids feeding on zooplankton in the waters contaminated with MP (indirect intake).

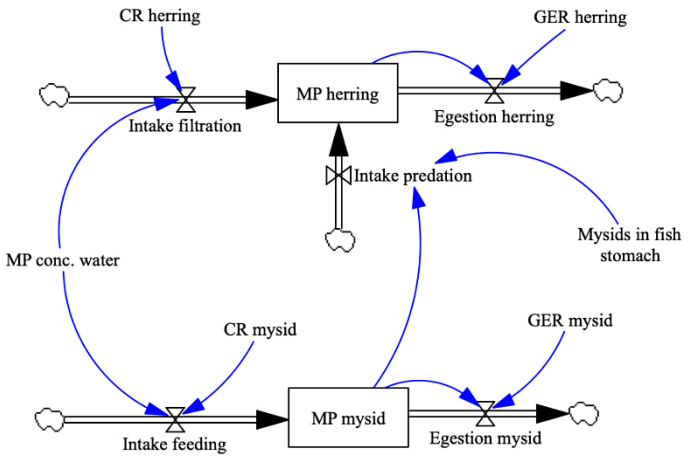

Figure 21. The model (Vensim PLE®) predicts MP burden in Baltic herring via direct ingestion of suspended MP and secondary consumption via predation on mysids.

The predicted MP burden in Baltic herring in the trophic guild was 2.7 MP ind.$^{-1}$, which is in the range of the values reported from the field observations (0–20 MP ind.$^{-1}$) and 30 % lower compared to the model predictions for the fish with exclusively zooplanktivorous diet. Thus, for particles 1–5 mm, predation on mysids contributes negligibly to the ingestion of MP, and direct ingestion of MP is the main source of MP intake by herring.

The modelled MP abundance in mysids was low but reasonable, considering that their ingestion of a 1-mm MP ought to be extremely low at the ambient MP concentrations, which, in turn, leads to the negligible contribution to MP body burden in herring. As a result, the mysids "dilute" the MP body burden originating from the direct uptake. When modelling transfer of MP of this size (1–5 mm), this would also be true for other invertebrate zooplanktivores in various trophic guilds because they have a lower filtration efficiency than fish and thus a diluting effect on the MP body burden. However, these invertebrates, including mysids, commonly ingest smaller MP particles (< 50 µm). Hence, to further develop the modelling approach for analysis of the food web transfer of MP, determination of the MP contamination and safe levels of MP in the environment, we need to focus on the smaller MP commonly found in invertebrates.

4. Discussion

Regulatory concerns call for defining specific risks of MP exposure and thresholds applicable in the impact assessment and derivation of environmental quality standards. During the last decade, much progress in MP identification and abundance analysis has been achieved due to massive funding support and research efforts worldwide. In contrast, the MP risk assessment remains problematic; the current approaches are based primarily on the MP occurrence in the environment and the chemical composition of virgin materials (Adam et al., 2021; Yuan et al., 2022). Furthermore, no risk assessment framework considers the multidimensionality of microplastic particles against the background of numerous natural particles, although modelling approaches have been suggested to address this issue (Koelmans et al., 2022).

Several authors have recently proposed tentative 'effect thresholds' for microplastics for the marine environment (Adam et al., 2019; Besseling et al., 2019; Ding et al., 2022; Koelmans et al., 2020; Kong and Koelmans, 2019; Redondo-Hasselerharm et al., 2018). Whereas derivation methods of these values vary, they all indicate that no immediate risk can be expected given the available data. However, some estimates suggest that microplastic concentrations in near-shore surface waters at 'hot-spot' locations could threaten the most sensitive species (Besseling et al., 2019). However, there is also a consensus that current information is insufficient to derive robust predicted no-effect concentrations (PNECs) for microplastics that could be used to justify a conclusion that risks are adequately controlled, either based on current exposures in the environment or exposures that are forecast to occur in the future. Also, the data used to derive these estimations are not fully admissible according to REACH criteria.

Our findings contribute to improving the methodology for deriving PNEC values, both in experimental testing and SSD modelling. We also suggested improvements in the test systems for deriving ecotoxicological (test species: algae, macrophytes, micro- and macro-crustaceans) and toxicological (human cell lines) threshold concentrations. Addressing these crucial methodological issues of exposure, controls, reference materials, and particle characterization would allow obtaining datasets appropriate for PNEC derivation for chemical safety assessment under REACH.

4.1 Test systems and their relevance for regulatory applications

Our methodological contributions to the field of MP effect testing systems are (1) a new design for an assay testing MP in suspension suitable for filter-feeders, and unicellular algae, (2) improved an existing assay testing leachates of solid waste materials (including MP) by using sub-organismal effects in a standard toxicity test species macrophyte *Ceramium*, and (3) behavioural assay for testing MP effects in sediment. The heterogeneity in the experimental settings used by different laboratories for MP effect studies limits the comparability and reliability of the reported effects and effect concentrations that can be used for hazard assessment. Therefore,

our exposure design with stable exposure over time, reliable information about the exposure levels, and more sensitive endpoints (e.g., antioxidative capacity, pigment and energy-processing markers, behavioural reactions) to detect subtle changes is a significant step forward in standardizing experimental protocols. Furthermore, using reference particles to disentangle particle effects from those induced by chemical exposure via leachates will provide environmentally relevant effect estimates. Finally, the two-component threshold with PNEC for MP is conditional on the levels of the total suspended solids in the water, which makes it adaptable to different systems.

We identified several methodological issues that should be considered in MP effect testing:

- Commercial NP/MP provided in suspensions may contain substances that are toxic to the cells (e.g., surfactants or biocides, such as sodium azide). Therefore, control incubations should include particle-free storage buffers in appropriate concentrations.
- Controls should include particle-free controls (growth media only) and particle controls using reference material, i.e., either natural particles or benchmark plastic particles with similar particle size distribution as the test MP (Reichelt and Gorokhova, 2020). The reference material is needed to delineate the effects of MP and those of any other particulates in the water (Gerdes et al., 2019a).
- In static tests with cell culture adhered to the bottom, the *cell dose* (i.e., particles coming in direct contact with the cells in the exposure vessel/plate) can be as low as 5 % of the nominal dose. Therefore, *de facto* exposure concentrations must be considered when analysing dose-response relationships. Also, information on the specific gravity of the material tested and particle aggregation in the media should be used to derive the actual exposure levels.
- When testing particle effects on filtrators in ecotoxicological settings, care should be taken to prevent the test particles from settling in the exposure vessels and thus maintain constant exposure. These tests should be conducted using rotating plankton wheels. In line with that, other studies also suggest that stirring is not sufficient to prevent particles from setting (Salaberria et al., 2020).
- In bioassays with sediments, any manipulations with natural sediment related to adding solid ingredients, such as mineral clay or plastics, can induce adverse behavioural effects in sediment-living animals due to changes in the substrate texture, grain size distribution and organic content, with concomitant effects on growth and metabolism of the test organisms.
- Well-characterised test materials must be used in terms of the polymer and particle size distribution because, due to the particle aggregation in the incubation systems, the actual size of the aggregates containing MP is different from their nominal size in the stock suspension. The aggregate size and topology are the main drivers of the effects on planktonic species, as shown for algae (Gorokhova et al., 2020) and *Daphnia* (Motiei et al., 2021).
- Regardless of the statistical significance of the outcome, all positive and negative test results must be reported to avoid publication bias (Reichelt and Gorokhova, 2020). In addition, the primary data must be made available for the scientific community to use in meta-analysis and data synthesis for hazard assessment.

4.2 MP and leachate effects across materials and test systems

Non-functionalized polystyrene nanospheres were cytotoxic at the highest dose tested (500 µg/mL), which greatly exceeds the PNEC values suggested for nanoplastics, 1.14 µg/L (Besseling et al., 2019). Similar exposure levels for small-sized MP (< 10 µm) were reported as inhibitory for the growth of unicellular algae when administered as a suspension in static tests (Reichelt and Gorokhova, 2020).

The secondary MP (PS, PET and PE, < 50 µm) that were first milled and then aged using UV-light were not non-cytotoxic in all three cell models at all doses tested (up to 500 µg/mL), most probably due to the large particle size. In algae (Gorokhova et al., 2020) and *Daphnia* (Gerdes et al., 2019a; Motiei et al., 2021), exposure to these MP caused growth inhibition and mortality, respectively, but at the concentrations of total suspended solids exceeding 32 mg/L and containing 2.4 % PET, translating into PNEC of 0.8 mg/L PET in highly turbid water. These levels greatly exceed PNEC values for MP [0.33 µg/L (Besseling et al., 2019) and 0.04 µg/L (Adam et al., 2019)] but are below the values based on our SSD modelling (Section 3.5.2). In the sediment-based assay with amphipods (Section 3.3.2), effects of particle addition to the sediment were found when any 'foreign' (either PET/PS or clay) particles replaced 10 % of the natural sediment, with no effect of material type on the behavioural and physiological responses of the animals. Although the PSD of the added materials differed between MP (< 50 µm) and clay (< 20 µm), the overall grain size distribution of the experimental sediments was very similar and explained the observed responses (Gerdes, 2021).

The leachates from the aged LDPE contained Tris(1,3-dichloroisopropyl) phosphate (TDCPP), a chlorinated organophosphate. Organophosphate chemicals are potent pro-oxidative agents that have a wide variety of applications and are used as flame retardants, pesticides, plasticizers, and nerve gases (Pearson and Patel, 2016). Moreover, the aged LDPE contained up to 10-fold higher TDCPP levels than non-aged material (NSNA & SNA: < 0.04 mg/L and NSA & SA: 0.12–0.55 mg/L; GC-MS characterization of leachate conducted by RISE). Accordingly, testing ageing and additive effects on the leachate toxicity provided evidence that ageing is a primary driver of the oxidative stress caused by the leachate. Whereas no effect was observed in the viability of macrophages, and there were no apparent changes following exposure to leachates in medium or artificial saliva and gastric juice (Figure 15), the effects in algae were strong (Figure 8).

In *Ceramium*, the decrease in pigments (chlorophyll-*a* and carotenoids) and a simultaneous increase in total antioxidant capacity (ORAC) indicate that the first line of the antioxidative defence (e.g., the pigments) fails, and other low-molecular water-soluble compounds are compensating for the decline in the plant pigments; this is also the mechanism suggested for plants (Pérez-Gálvez et al., 2020). However, the EC10 values for all subcellular responses were above the relevant PNEC values. Notably, the materials containing additive Irganox 1076 appear to have lower effects on the prooxidative processes in *Ceramium*, probably because Irganox 1076 is a potent antioxidant. Therefore, the chemical nature of the additives must be considered when interpreting the effects of MP and their leachates.

Similar to the sediment-based assay, the primary drivers of the effects observed in algae and daphnids were not the MP *per se* but the size of the aggregates formed

during the exposure and their behaviour (Gorokhova et al., 2021, 2020; Motiei et al., 2021), and biofilms with diverse bacterial communities inhabiting these aggregates (Gorokhova et al., 2021). Also, the aggregation is a function of particle properties, e.g., z-potential, density, crystallinity and particle size; therefore, analysis of these features provides essential information on the effect drivers (Section 3.1).

4.3 Test materials and their physicochemical properties

Our results showed that ageing could drastically change microplastics' chemical and physical properties (Table 3) and the leaching components, with subsequent effects on the leachate toxicity. These findings demonstrate that virgin MP should not be used in impact studies and models deriving PNEC values. Furthermore, as MPs are usually exposed to many physicochemical factors rather than individual, single impacts, the combined effects on the molecular composition of the material and its behaviour in exposure systems are of great importance. Our findings also suggest that an essential step towards improving the current practice of MP effect testing is always including particle and leachate testing in the assessment battery. Such a measure is likely to enhance the understanding of MP impacts and the mode of action for specific materials resulting in more robust data. The potential bio-accumulation properties and hazards of microplastics that are thought to be formed during the degradation are poorly understood, which prevents an assessment of the risks posed by relevant breakdown/transformation products of microplastics in the environment.

4.4 Modelling approaches for deriving threshold values and identification of relevant test species and endpoints

Our meta-analysis using SSD models suggests that MP may be slightly more likely to cause effects than mineral solids, particularly to organisms in clear waters, such as alpine lakes and tropical seas. Although high uncertainties surround the results, the apparent difference in toxicity is partly due to systematic differences in experimental designs that cannot be accounted for statistically. Also, the PNEC values obtained by these models are much higher than those suggested using similar approaches in earlier studies. Given this conflicting information, we need more data from comparative experiments with plastic and non-plastic particles in mixtures, where the effects of associated chemicals are accounted for, and dose-dependent point estimates are used to assess the MP impacts in the field relative to those of SS. Moreover, the adverse effects of the observed responses on the organism's physiology, growth and reproduction must be established before using these observations for deriving PNEC values.

Several issues complicate microplastic risk assessment in the marine environment, as shown by modelling MP transfer from plankton to herring (Figure 20). First, predictive modelling is dependent on relevant and up-to-date observational data. However, gathering quantitative field data is challenging due to the small size

of plastic particles, analytical challenges with material identification, unsettled sampling methodologies, and spatial and temporal variabilities. Nevertheless, the model demonstrated that microplastic is not expected to accumulate in the gastrointestinal tracts of higher-level biota based on zooplankton-mysid-herring interactions. To better inform models such as this and therefore improve their accuracy, it is important to gain a better understanding of microplastic retention times in biota and the interaction between microplastics and resources utilised by benthic macroinvertebrates and fish, such as plant material and allochthonous detritus (O'Connor et al., 2022).

5. Project outcome

5.1 Peer-reviewed published papers and reports

Gerdes, Z., Hermann, M., Ogonowski, M., Gorokhova, E., 2019. A novel method for assessing microplastic effect in suspension through mixing test and reference materials. Scientific Reports 9. https://doi.org/10.1038/s41598-019-47160-1

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VKM, Janneche Utne Skåre, Jan Alexander, Marte Haave, Ignacy Jakubowicz, Helle Katrine Knutsen, Amy Lusher, Martin Ogonowski, Kirsten Eline Rakkestad, Ida Skaar, Line Emilie Tvedt Sverdrup, Martin Wagner; Angelika Agdestein, Johanna Bodin, Edel Elvevoll, Gro-Ingunn Hemre, Dag Olav Hessen, Merete Hofshagen, Trine Husøy, Åshild Krogdahl, Asbjørn Magne Nilsen, Trond Rafoss, Taran Skjerdal, Inger-Lise Steffensen, Tor A Strand, Vigdis Vandvik, Yngvild Wasteson (2019). Microplastics; occurrence, levels and implications for environment and human health related to food. Scientific opinion of the Scientific Steering Committee of the Norwegian Scientific Committee for Food and Environment. VKM report 2019:16, ISBN: 978-82-8259-332-8, ISSN: 2535-4019. Norwegian Scientific Committee for Food and Environment (VKM), Oslo, Norway.

Gorokhova, E., Ek, K., and Reichelt, S. (2020). Algal Growth at Environmentally Relevant Concentrations of Suspended Solids: Implications for Microplastic Hazard Assessment. Front. Environ. Sci. 8. https://doi:10.3389/fenvs.2020.551075

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Rogers, K. L., J. A. Carreres-Calabuig, E. Gorokhova, and N. R. Posth. (2020) Micro-by-micro interactions: How microorganisms influence the fate of marine microplastics. Limnology and Oceanography Letters 5: 18–36. https://doi:10.1002/lol2.10136. **Top cited paper in L&O Lett in 2020**.

Reichelt, S., Gorokhova, E., 2020. Micro- and Nanoplastic Exposure Effects in Microalgae: A Meta-Analysis of Standard Growth Inhibition Tests. Front. Environ. Sci. 8. https://doi.org/10.3389/fenvs.2020.00131

Gerdes, Z., Ogonowski, M., Nybom, I., Ek, C., Adolfsson-Erici, M., Barth, A., Gorokhova E., 2019. Microplastic-mediated transport of PCBs? A depuration study with *Daphnia magna*. PLoS ONE 14, e0205378. https://doi.org/10.1371/journal.pone.0205378

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Motiei, A., Ogonowski, M., Reichelt, S., Gorokhova, E., 2021. Ecotoxicological assessment of suspended solids: The importance of biofilm and particle aggregation. Environmental Pollution 280, 116888. https://doi.org/10.1016/j.envpol.2021.116888

5.2 Preprints

Gerdes Z., Ogonowski M., Krång A.-S., Gorokhova E. 2022. Insufficient evidence for behavioural and metabolic effects in a sediment-dwelling amphipod exposed to microplastics. *bioRxiv*.

Ogonowski M., Wagner M., Rogell B., Haave M. and Lusher A. 2022. What is particular about microplastics? A meta-analysis of the toxicity of microplastics and suspended sediments. *bioRxiv*.

5.3 Manuscripts under preparation

Montano Montes A, Tagaras N, Vallabani S, and Karlsson HL. Low cytotoxicity of nano- and microplastics in human lung cells and co-cultures.

Lo, Hoi Shing, Montano Montes A., Yarahmadi, N., Jakubowicz I., Karlsson HL, and Gorokhova, E. The effect on viability of PE microplastics and leachates: role of aging and stabilisers.

Lo, Hoi Shing, Yarahmadi, N, Jakubowicz I., Gorokhova, E. Plastic leachates stimulate antioxidant activity in red alga *Ceramium tenuicorne*.

Reichelt S, El-Shehawy R, Gorokhova E. Bacterial biofilms in ecotoxicity testing with solid particulates: implications for microplastic effect studies.

5.4 PhD Thesis

Gerdes, Z., 2021. Exploring the ecotoxicity of microparticle debris. PhD Thesis, Stockholm University, Stockholm, Sweden.

Motiei, A., 2021. Microbiome Of Ecotoxicity Assays. Stockholm University, Faculty of Science, Department of Environmental Science, Stockholm, Sweden.

Reichelt, S.(planned defense 2023). Micro-by-micro interactions: the role of microorganisms in fate and impact of microplastics.

Montano Montes A (planned defense: 2024). Health effects of nano- and microplastic particles – studies using advanced cell models.

5.5 MSc and BSc Theses

Tagaras, N. 2021. Evaluation of pulmonary toxicity of micro- and nanoplastics using mono- and co-culture models. Master thesis, Karolinska Institutet, Stockholm, Sweden.

Österblad, M. 2021. Emissions from road markings : Toxicity tests of leachates from road marking products on the microalgae *Raphidocelis subcapitata*. Master Thesis; Stockholm University, Faculty of Science, Department of Environmental Science, Stockholm, Sweden.

Månsson, A. 2020. Modeling body burden of microplastic in a simple food web. Bachelor Thesis, Stockholm University, Stockholm, Sweden.

Helmersson, K. 2020. Effects of Microplastic Leachates on Phytoplankton: A Laboratory Study on *Nodularia spumigena* and *Phaeodactylum tricornutum*. Luleå University of Technology, Department of Civil, Environmental and Natural Resources Engineering. Collaborator: Stockholm University. Independent thesis Advanced level (professional degree).

Sjöberg, S. 2019. Behavioural changes of *Corophium volutator* in response to microplastic exposure (Bachelor Thesis). Department of Environmental Science and Analytical Chemistry, Stockholm University, Sweden.

5.6 Outreach and popular science publications

5.6.1 Popular Science Lectures

FN-förbundet UNA Sweden; E. Gorokhova: Lunchseminarium Fred med Naturen i samband med International Mother Earth Day. Temat: Mikroplaster; https://fn.se/stockholm/2022/04/29/lunchseminarium-fred-med-naturen/ Sändes även live via Zoom.

5.6.2 Appearances in media

Rapport, SVT 17/4-2022 ”Mikroplast hittad i lungor”, Hanna Karlssons kommentar om mikroplaster och effekter i lunga, SVT Nyheter.

Tidskriften *Forskning och Framsteg*. Läsarfråga om hälsoeffekter av mikroplaster i mat. Andrea Montano Montes svarar på frågor och kommenterar.

Kudos Showcase by Zandra Gerdes, Markus Hermann, Martin Ogonowski, Elena Gorokhova *Particularly harmful particles? Sifting out the dirt to find the microplastic effect* https://www.growkudos.com/publications/10.1038%25252Fs41598-019-47160-1/reader

ACES webpage; News and Features: Microplastics: friends or foes of contaminant transport? Zandra Gerdes contribution; https://www.aces.su.se/news/microplastic-friend-or-foe-of-contaminant-transport/

Web Magazine Baltic Eye. Effects of microplastics on marine life; Zandra Gerdes contribution; https://www.su.se/stockholm-university-baltic-sea-centre/web-magazine-baltic-eye/hazardous-substances/effects-of-microplastics-on-marine-life-1.614008

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MIXiT: Towards quantifying impacts of microplastics on environmental and human health

The current environmental concern is that plastic debris, particularly microplastics, transfer through the food web and affects aquatic consumers with potential effects on humans as the top consumers. However, the evidence for the biological effects of microplastics is scarce, and the mechanisms of these effects in aquatic biota and humans are largely unknown. To assess the possible impacts of microplastic pollution on the environment and human health with scientific rigour, we need a sound methodology, which is currently missing.

The primary motivation for the MIXiT project has been to establish the principles for hazard assessment methods targeting microplastics as a case of solid waste type.

This research project was focused on five main issues: (1) gathering global evidence for adverse effects of microplastics using meta-analysis of published data, (2) developing a test system for evaluating the effects of solid particles and the leachates, (3) preparation and standardisation of environmentally relevant microplastics for (eco)toxicological testing with considerations of particle morphology and physicochemical properties, (4) demonstrating approaches for microplastic effect studies using ecotoxicological test organisms and human cell lines with an emphasis on understanding the effect mechanisms and deriving effect concentrations suitable for regulatory work, and (5) modelling food-web transfer of microplastics using trophic guilds in the pelagic system of the Baltic Sea.

The critical focus of the research is on designing appropriate animal and human tests and providing methodological recommendations on testing the hazardous properties of anthropogenic particles and their leachates.

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