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Challenges and recommendations in experimentation and risk assessment of nanoplastics in aquatic organisms

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ABSTRACT

Nanoplastics (<1000 nm), raise concerns regarding their potential effects and associated risks. These particles exhibit unique characteristics including diverse buoyancy and colloid behaviour, with additional challenges on processing and detection, and on their interaction with aquatic organisms. Consequently, laboratory experiments on nanoplastics can at times lack appropriate experimental controls or quality criteria and may not generate relevant data for conducting reliable risk assessments or capturing environmental realism. This study aimed to review and discuss the methodological challenges involved in assessing the effects of nanoplastics on aquatic organisms and provides recommendations for optimising experimental approaches. We discuss the major challenges and best practices when experimenting with nanoplastics, the current methods for detection of nanoplastics in internal tissues and assess translocation, and the pressing needs for nanoplastics risk assessment. We recommend the development of a rigorous quality criteria framework to advise researchers when designing experimental work, and to ensure suitability of data for risk assessment.

1. Introduction

Contamination of the environment by nanoplastics (<1000 nm) is of concern due to their potential environmental effects [1,2]. Nanoplastics are expected to be ubiquitous in aquatic environments due to their widespread use in consumer products or due to fragmentation of larger plastic litter items, which will persist in the environment because of low degradability rates [3]. Once released into the environment, these particles can be ingested by a variety of organisms, from plankton to fish, and are expected to have far-reaching effects from individuals to ecosystems [1–3]. However, our understanding of the exposure and hazard, and thus the risk associated with nanoplastics, is still limited because of their complex interactions with aquatic media, the limitations for extraction and detection in environmental matrices, and the challenges associated with experimental procedures for assessing effects [3–5]. Notably, a recurrent issue in nanoplastics (eco)toxicity reports is the lack of control for experimental artifacts and the incomplete description of the used methodologies, the non-inclusion of a discussion on the limitations of the study, and the insufficient acquisition of suitable data for risk assessment [3–5]. To better understand the mechanisms by which nanoplastics interact with and impact organisms and the environment, there is a need for improved experimental best practices to assess effects of nanoplastics, but also the inclusion of an objective and factual report of the used procedures. By doing so, the production of suitable effective data, even with the current intrinsic limitations, will contribute to an increasingly improved assessment of the potential risks associated with nanoplastics, which consequently contribute to strategies for plastic pollution mitigation [3].

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1.1. Properties of nanoplastics

Nanoplastics is a particular group of plastic particles which, in aquatic media, differs in physical behaviour and interactions from both microplastics and nanoparticles, i.e., manufactured nanomaterials [1,6]. Nanoplastics, just as other plastic items, are solid and insoluble particles composed of synthetic or semi-synthetic polymers (including rubber), from both oil and biobased origins, which can have diverse shapes (spheric, irregular, fibres, etc) [7]. Nanoplastics can either be intentionally manufactured and unintentionally released to the environment [3], or result from the fragmentation of larger plastics, due to weathering processes [3,8], interactions with biota such as digestive fragmentation from krill [9] or microbial-induced fragmentation [10]. Nanoplastics are therefore part of a continuum of plastic particles in terms of their size distribution, but their definition in terms of size range remains debatable [6,7]. The most broadly used concept considers that nanoplastics are all plastic particles with sizes below 1000 nm (with the largest dimension of the object determining the category) [7]. However, a few other experts limit the term to plastic particles between 1 and 100 nm, as per the definition of nanoparticles, mostly due to their colloidal behaviour in liquid media – i.e., maintenance of these particles in suspension due to Brownian motion, electrostatic repulsion, and van der Waals forces [6,7]. In this work, we consider the broader definition of their size as $<$ 1000 nm [7] as the ecotoxicological mode of action, potential translocation, the experimental manipulation, detection and methodological challenges, as these characteristics are very similar for particles included in this definition [4,5,7].

Whilst the relative occurrence of nanoplastics in the environment (in terms of mass) and their composition are expected to be similar to other plastic particles, their physical behaviour may vary greatly when compared to microplastics [1,3,6]. For example, nanoplastics will not exhibit the same settling behaviour as other larger particles, even if composed of the same polymer, due to the predominance of Brownian motion over sedimentation, and due to a buoyancy characterized by random movements of the particles in suspension [6]. Nanoplastics will also have a higher proportion of chemical variabilities on their surface, resulting in more frequent surface interactions with, for example, a range of biota, inert substances, organic and inorganic macromolecules/substances, etc) compared to microplastics [1,6]. Nanoplastics may also form agglomerates, aggregates or heteroaggregates, i.e., clusters of different types of particles (plastics or natural) (Box 1), which may have unique properties and interactions compared to single particle types, affecting their fate and toxicity [3,6]. Furthermore, besides having a size range accessible for uptake and ingestion by biota, these particles may also translocate and be transported across biological membranes, which may enhance their toxicity when compared to larger plastic particles [1,3,6,11,12].

The effects assessment of nanoplastics has been building up on previous knowledge acquired on engineered nanomaterials ($<$100 nm) and microplastics assessments (1–5000 μm) [1–3,6]. Nanoplastics have, however, very distinct characteristics when compared to nanoparticles, such as their heterogeneity in terms of shapes, polymer types, densities, and weathering stages [1,6]. Even though nanoplastics in aquatic environments are part of a continuum and will most likely be present in the environment as part of complex particle mixtures [13], their transport and fate, interactions with natural colloids and microorganisms, bioaccessibility and bioavailability, and diffusion times for leaching substances will also differ greatly from microplastics which have different properties [1,3,6]. These challenges have been identified as of major concern in laboratory experiments, as many studies lack quality criteria and suitable experimental controls [3,4,14], suitable results/data for risk assessment [3,5,15] and/or environmental realism [16,17]. A major challenge of experimental work with nanoplastics is to fully characterise both the particles as well the media (biotic and abiotic) in which they are in, as not accounting for this can lead to ambiguous or inconclusive effects and risk assessments. The particular characteristics of

Box 1
Glossary of key terminology used in this work.

Term	Definition	References
Nanoparticle	Particle with all dimensions in the nanoscale and the lengths of the longest and the shortest axes of the particle do not differ significantly ($<$100 nm)	ISO 2015 [66]
Nanomaterial	Material with any external dimension in the nanoscale or having an internal structure or surface structure in the nanoscale ($<$100 nm)	ISO 2015 [66]
Nanoplastics	A plastic material with any external dimension in the nanoscale or having internal surface structure in the nanoscale (1–1000 nm)	Hartmann et al., 2019 [7]
Microplastics	Plastic particles of $<$5 mm in diameter, up to the nano-size range (1000 nm)	Hartmann et al., 2019 [7]
Agglomerate	Formed by weakly bonded particles, can have an active surface area that is the sum of each individual particle surface area. The forces holding an agglomerate together are weak forces, (i.e. van der Waals forces or simple physical entanglement). An agglomerate can more easily release its constituent nano-objects because of its relatively weaker bonding (e.g. by sonication)	ISO 2015 [66]
Aggregate	Is formed by strongly bonded particles, the surface area of the aggregates can be significantly reduced when compared to the sum of each individual particle. The forces holding an aggregate together are strong forces (i.e. covalent or ionic bonds, or those resulting from complex physical entanglement)	ISO 2015 [66]
Bioavailability	Bioavailability is defined as the amount of compound that is absorbed by an organism and available for altering physiological functions at cellular level	Semple et al., 2004 [67]
Bioaccumulation	Is the amount of a contaminant in or on an organism after exposure any sources including water, air, and diet	Newman 2010 [68]
Bioaccessibility	Defined as the quantity of a compound or particle that becomes available for adsorption by an organism in the gastrointestinal tract and it is available for uptake into the circulatory system	Galanakis 2017 [69]
Toxicokinetics	Defined as the rates of absorption, distribution, metabolism and excretion of a chemical or contaminant according to the dose and the exposure time	Richardson 2020 [70]

nanoplastics increase the complexity in effects assessment in aquatic organisms, and some of the major challenges in experimental work with these particles include their detection, characterisation, aggregation or dispersion assessment, administration, and actual concentration assessment (beyond nominal reporting). The goal of this work was to discuss the methodological challenges associated with the assessment of nanoplastics effects in aquatic organisms, and to provide recommendations for optimising experimentation with nanoplastics to generate tangible data that can be used to comprehend toxicity mechanisms, effects, and conduct risk assessments.

2. Challenges and best practices when experimenting with nanoplastics

To confidently assess nanoplastics impacts, a thorough characterisation of the particles used in experimental work is fundamental to ensure accurate and reliable results, including detailed information on morphology (size, shape, surface texture, aspect ratio/length-to-width ratio, and surface area), polymer composition, surface chemistry, and, if applicable, a comprehensive description of any particle mixture being tested (Table 1). The size of the particles used requires a full description in addition to nominal size [3], as it is well-established that the size of particles significantly influences their residence time and depuration within aquatic organisms, as demonstrated by Al-Sid-Cheikh et al., 2018 [11]. To obtain information on the size frequency distribution of nanoplastics, the use of flow cytometry has been employed [18], but this technique is limited to the use of fluorescent [18] or stained particles [19], as the analysis depends on the scattered light signals generated by the particles as they pass through a laser beam [20]. The issue of the fluorescence requirement can be solved with the use of Dynamic Light Scattering (DLS), where nanoplastics (and other particles, either fluorescent or not) are analysed by the intensity fluctuations of scattered laser light caused by their Brownian motion, which enables their size frequency distribution to be measured (Table 1). This technique also enables the detection of agglomeration, for instance, which can be a required characterisation step [21,22], but if agglomeration or aggregation detection is not desirable, then a sonication step should precede the DLS analysis for a proper dispersion of the particles [21]. The DLS technique can furthermore determine the surface charge of particles [23–25], a key element in the interaction with other surrounding particles, media, substances and organisms, and which may deeply impact the magnitude of effects in organisms [26]. If particles are purchased, their coating and surface functionalization should be reported according to the supplier information provided [21,27]. In addition to the size of the particles, other techniques that can also inform on morphological characteristics, such as shape and texture, of plastic particles are Transmission and Scanning Electron Microscopy (TEM and SEM respectively) [25,28], with both techniques providing high-resolution imaging of materials at the micro- and nanoscale, but noting that TEM can provide higher resolution for nano-scale observations [25,28,29] (Table 1). Finally, Atomic Force Microscopy (ATM) can further assist in assessing not only particles' size (with a high resolution of as low as 3 nm), but also provide 3D images of the surface structure, stiffness, hydrophobicity, conductivity and magnetization [28]. To obtain information or confirmation of the polymer type or composition, researchers can rely on techniques such as an SEM equipped with energy-dispersive X-ray spectroscopy (i.e., SEM-EDX) which provides information of size, morphology, and surface composition of nano-sized particles [25,28,29]. A complete overview and description of the tested particles will not only improve the reproducibility of the experimental work reported, but also enable sound conclusions on the observed effects.

To prevent contamination from other substances, airborne particles and cross-contamination between samples, it is advisable to adhere to rigorous laboratory practices commonly employed in microplastics or nanomaterials research, including the implementation of quality assessment measures and quality control protocols [30], and controls for experimental artifacts [4]. The manipulation of particles should be done in a clean environment, preferably in a reversed flow cabinet, using glassware when possible, which should be thoroughly washed using a strong dispersant (e.g., sodium dodecyl sulfate (SDS) or Deacon), and rinsed using MilliQ water and acetone [31]. Glassware can also be incubated in a drying oven overnight at temperatures of around 160 °C to burn off any residual plastic. Purchased particles from commercial supplies require an extra purification step, such as a dialysis or (ultra) centrifugation, as the solution in which the particles are provided contains solvating agents, i.e., the dispersants or surfactants (e.g., sodium azide, Tween 20, or SDS), and antimicrobial or preservative agents, which can impact toxicity [26,32,33]. Furthermore, if particles are supplied with a fluorescent dye, in particular if the stain is weakly attached to particles' surface, then either a purification (dialysis, centrifugation) step or an extra control for leaching dyes is also required [4,23,34] in order to remove weakly-associated dye molecules that could otherwise independently interfere with experiments and produce artifacts. Nanoplastics can agglomerate or be incorporated in heteroaggregates, but if these physical forms are not intended, then there is a requirement for dispersing particles, either by using a pre-sonication step, or the addition of a dispersant at a dose below toxicity effects (verified by the addition of an extra positive control). If for any reason the dispersant dose used needs to be higher, controls would need to be integrated into the experimental plan to account for any effects induced by the dispersant.

Ecotoxicological assessments of nanoplastics in aquatic environments require careful planning if the experimental design and set-up will lead to data acquisition that is suitable for risk assessment. Major issues include the insufficient reporting of environmental (and other) relevant parameters [17,27], and the limited use of best practices such as quality assessment and quality control measures. All these additional challenges for assessing ecotoxicological effects of nanoplastics compared to microplastics may require extra experimental controls to distinguish between artifacts, distinct behaviour of the particles and to provide accurate information on actual effect in biota [4]. Toxicity testing following conventional methodologies built for soluble chemicals without taking into consideration the unique physicochemistry of the nanoplastics can lead to inaccurate results and misleading information. To establish the aqueous dispersion of engineered nanoparticles [35] and microplastics [36] and ensure the continuous exposure of organisms, there have been developed semi-isolated chambers inside exposure vessels, coupled with magnetic stirrers. Exposure systems such as these dispersion chambers should be however carefully tested before implementation, as exposure depends on the chamber mesh size and highly agglomerated particles can block pores. Additionally, these systems can be unsuitable to organisms highly sensitive to turbulence. To our knowledge, no custom-made dispersion chambers have been employed nor tested for nanoplastic toxicity assessments, but if implemented could assist in avoiding the use of additional dispersants. Risk assessment [see below section 4] requires the obtention of exposure concentration-response curves, which will enable the calculation of (eco)toxicity parameters (effects thresholds, such as EC50, LC50, NOEC/LOEC etc), i.e., quantitative measures of the toxicity of nanoplastics and their potential effects on aquatic organisms [1,3,37]. Therefore, the goal of the assessment will dictate the exposure of organisms, both in terms of the concentrations (nominal concentrations, actual concentrations, and doses), duration (acute, chronic, or multigenerational), and pathways (diet-borne exposure, water borne, static immersion, or injection, Table 2) [3,38–40].

When organisms are exposed via aquatic media, nanoplastics will agglomerate, aggregate or disperse in aquatic media, depending on environmental factors such as salinity, pH, organic matter presence, and the interaction with other organisms or contaminants [41,42], which will affect their fate and probability of being ingested and, therefore, their exposure [3]. The use of dispersants in waterborne experimental set-ups can assist in guaranteeing uniformity of exposure in aquatic media, but as dispersants are known to also induce toxicity in organisms

Table 1
Best practices and recommendations for common experimental challenges in experimentation and risk assessment of nanoplastics in aquatic organisms.

Topic	Challenge	Best practices and recommendations	References
Particle characterization			
Morphology, including size, shape, surface texture, aspect ratio (length-to-width ratio), and surface area	Morphological aspects of particles can affect agglomeration and aggregation, interactions with biota and the sorption of substances	• Morphology should be thoroughly assessed using Transmission or Scanning Electron Microscopy (TEM and SEM respectively) and reported • Morphology can also be assessed using Atomic Force Microscopy (ATM)	[25,28,29]
Size frequency distribution	Only nominal sizes are reported	• Assess the actual measurement (e.g. Ferret diameter) of particles and report mean values, including standard deviation • Techniques such as flow cytometry (fluorescent particles only), dynamic light scattering (DLS), ATM, or TEM and SEM can be useful in assessing particle sizes	[3,18,19, 21,22]
Agglomeration and aggregation	Exposure and particle concentrations altered in experimental set-up due to clumping	• If intended can be characterized via DLS • If not intended, particles can be dispersed via sonication procedures or using dispersants	[21,22]
Particle coatings and functionalization	Coatings and particle functionalization alters surface charge and impacts interactions with media substances and organisms	• Assess the actual surface charge of the particles (zeta potential analysis) and report it • Particle surface charge can be observed using DLS • ATM can provide an overview of particles' stiffness, hydrophobicity, conductivity and magnetization	[21,23–25, 27]
Polymer type/surface composition	Surface chemical characteristics affect interactions with media substances and organisms	• Reporting polymer composition is essential for results interpretation • Polymer surface composition of particles can be assessed using a SEM equipped with energy-dispersive X-ray spectroscopy (EDX)	[25,28,29]
Quality Assessment/Quality Control (QA/QC)			
Sample contamination by other particles (airborne, residual or other)	Contamination can introduce additional particles in the sample, which interfere in quantification, create additional background noise in the data and lead to inaccurate results or distinguishing between specific effects of the target particles	• Follow rigorous laboratory practices guidelines used in microplastics and nanomaterials research, including working in a clean environment and/or in an inverted flow cabinet while manipulating samples, covering samples, thoroughly wash glassware and any contact materials, oven-dry glassware (160 °C), etc	[4,30,31]
Associated solvating agents, fluorescent dyes or additives to commercial nanoplastics	Solvating agents, dyes, additives and dispersants may have their own ecotoxicological properties that could interact with nanoplastics or other media parameters, which can lead to false positive/negative results	• Dialysis or ultra-filtration to purify particles • Use of a leachates solution control (after dialysis for example) • Use the dye itself as a control if available	[4,23,26, 32–34]
Dispersant		• Use of dispersant concentrations below known toxicity • Report actual concentrations used to avoid misinterpretation of results • Use of a control for the dispersant (treatment with same dose of dispersant, but no particles present) • Use of dispersion chambers as an alternative of chemical dispersants	[26,35,36]
Particle control	Due to the nature of nanoplastics, ecotoxicological effects can either be induced by the particle itself, the chemical characteristics of the tested polymer or both	• To disentangle the mechanistic effect of the target nanoplastic tested, it is recommended to use an extra particle control with similar buoyancy (e.g. silica beads)	[43]
Certified Reference Materials	Reproducibility of tested ecotoxicological effects of nanoplastics can be difficult to achieve	• The use of certified reference materials (as a positive control) enables reproducibility of studies and results and validates analytical measurements	[3,49–51]
Experimental set-up			
Media parameters	Media parameters (e.g. pH, temperature, organic matter) may affect how nanoplastics aggregate and/or interact with biota	• Media characteristics should be carefully assessed and reported (e.g. pH, temperature, salinity, particulate organic matter content, presence of suspended sediment, etc).	[3,17,27, 41,42]
Full report of experimental procedures	Insufficient methodology reporting can hinder the reproducibility and transparency of the work	• Proper and thorough reporting of the methodology used to enable future work to build upon and validate research findings effectively, crucial for risk characterization	[4]
Experimental design	Insufficiency of suitable data for establishing concentration-response curves, which are essential for estimating ecotoxicological parameters	• The calculation of concentration-response curves is key for the computation of (eco)toxicity parameters, including effects thresholds such as EC50, LC50, NOEC/LOEC, etc. Include both high and low concentrations, i.e. a range of nanoplastic concentrations starting from low-no toxicity effect levels to highly toxic concentrations, as well as several intermediate concentrations, to capture the full concentration-response relationship	[1,3,37]
Quality criteria	The absence of specific quality criteria guidelines results in a knowledge gap concerning best practices for experimental effect assessments involving nanoplastics	• Establishment of quality criteria to evaluate hazardous effects based on OECD, CRED and NanoCRED	[5,14, 44–48]

[26], it is recommended that low concentrations of dispersants are used (and reported) after careful verification of currently known Predicted No Effect Concentrations (PNECs) provided by the supplier in the safety data sheet (SDS) or via compiled information available in official registries [e.g. registration dossier submitted to the European Chemicals Agency (ECHA)], but also that a control treatment with only dispersant is included. Furthermore, key media parameters are frequently under-reported in published studies [3], making it difficult to evaluate results

Table 2
Exposure techniques of aquatic organisms to nanoplastics, and corresponding advantages and disadvantages of their application (e.g. Refs. [38–40]).

Exposure technique	Description	Advantages	Disadvantages
Waterborne exposure	Exposure to nanoplastics through direct contact with the media, which can be renewed	Used in a wide range of aquatic organisms as it is easy to administer, allows for a broad range of exposure concentrations to be tested	May not reflect how organisms are actually exposed (encounter and ingestion) to nanoplastics in environmental conditions, may not account for agglomeration/aggregation and interaction with other particles which may induce artifacts in actual exposure concentrations
Static exposure	Exposure to nanoplastics through direct contact with unrenewed media	Allows to assess long term effects	Nanoplastics concentrations will fluctuate overtime, and may be difficult to control and/or assess
Diet-borne exposure	Food items will be spiked with a known concentration of nanoplastics, or organisms will feed on pre-exposed prey	Can more accurately reflect how organisms encounter nanoplastics in their environment, enables to assess trophic effects across various levels	The actual ingestion concentrations may be difficult to assess, as organisms may not ingest all the available food items
Injection	Direct injection directly into the organism's tissues, with a delivery of a precise concentration of nanoplastics	Allows for precise dosing, and to assess effects in specific organs or tissues	The injection process may induce extra stress, and therefore require extra control to procedure, and may not be representative of how organisms encounter nanoplastics in their environment

across publications or extrapolate laboratory findings to real world scenarios [17]. Besides the actual exposure concentration, the distinction between particle or chemical effects needs to be disentangled, which is possible with the addition of an extra treatment using inert and natural particles (e.g., silica-based) [43]). The addition of the extra control for particle treatment will enable conclusions to be drawn on the "particle" or "chemical" related effects of the tested nanoplastics.

The current effect assessments of nanoplastics need to comply with rigorous methods and data reporting, but are currently missing international well-established procedural guidelines. For example, well developed guidelines for ecotoxicological assessment of substances have been adapted and adjusted to establish procedural guidelines of ecotoxicological assessments of nanomaterials by international organisations, such as The Organisation for Economic Co-operation and Development (OECD) Guidelines for the Testing of Chemicals [44]. Also, to evaluate the risk of nanomaterials, previous assessments have used comprehensive quality criteria to establish if reported data concerning dose-response effects would be suitable based on frameworks such as CRED (Criteria for reporting and evaluating ecotoxicity data) [45], adjusted to NanoCRED [46]. These frameworks have assisted in ensuring the quality, comparability, and reliability of ecotoxicity data of nanomaterials, as established ecotoxicological tests for substances are not applicable due to the specific characteristics of these particles. Using a similar logic, quality criteria to assess the suitability of microplastics effects assessment data have recently been suggested, considering a points system to evaluate existing studies [5,47,48], and have assisted in identifying priority adverse mechanisms to be used in risk assessment [47]. For nanoplastics, there have been some initial steps towards the establishment of quality criteria to evaluate hazardous effects, such as those by Kokalj et al., 2021 [14], where authors have mentioned that their criteria catalogue was intended as a "starting point for further elaborations", but from which have not yet been any developments. The use of certified reference materials (as a positive control) in nanoplastics studies can further increase the credibility level, as these materials, which are intended to assist in validating analytical measurements (e.g., see Seghers et al., 2022 [49] for microplastics, and Hildebrandt and Thünemann 2023 [50] for nanoplastics reference materials), can enable the validation and benchmarking of toxicity effects [3], as in the case of nanomaterials [51]. The inclusion of reference materials in future quality guidelines for nanoplastics studies is thus recommended.

3. Detection of nanoplastics in internal tissues

The assessment of nanoplastics translocation and toxicokinetics in organisms is a challenging task due to the complex nature of these particles and their interactions with biological matrices. However, understanding the mechanisms of nanoplastics uptake, translocation and biotransformation is crucial for evaluating their potential impacts [3], both at a molecular and individual level. One of the main challenges is the lack of suitable methods to quantify and characterise nanoplastics, which are carbon-based materials, in complex matrices, such as tissues or organs [1]. While sample extraction or purification techniques may apply for nanoplastics analysis and detection using for example pyrolysis Gas Chromatography-Mass Spectrometry (GC-MS), the use of these techniques is limited as they require a large number of particles or high mass for quantification above the current instruments' detection limits [1]. However, this assessment would be highly relevant to complement the chemical and physical properties characterisation of nanoplastics, which significantly influence their uptake and distribution in tissues, resulting in unique behaviour compared to other types of nanoparticles. For example, polystyrene (PS) and silver (Ag) nanomaterials in *Pecten maximus* (great scallop) showed distinct accumulation patterns in different organs [3,11], and this may lead to distinct ecotoxicological effects. Also, while microplastics commonly host a "plastisphere" [52] and biofilms on their surfaces, nanoplastics however, being smaller than the average prokaryotic cell (2×0.5 μm), exhibit distinct interaction characteristics and can be taken up by microorganisms [53], which can result in intracellular stress [54].

Currently, the methodologies to track particles and to detect translocation and nanoplastics accumulation in organisms rely on the use of fluorescent [4,23,34], radiolabelled [11,55] or metal-doped particles [12,56]. The use of fluorescent particles has been a popular technique so far to demonstrate translocation of particles, but without an extra purification step or additional controls, this has been shown to potentially lead to confounding effects, as dyes can leach from particles, and observations based only on fluorescence can lead to misinterpretation of results [23,34]. In what concerns both labelled particles (radiolabelled and metal-doped), these methods offer the possibility to demonstrate translocation, assessing biokinetics, biodistribution, and trophic transfer, but a major challenge is still the use of low (and therefore more environmentally relevant) exposure concentrations [55]. The use of radiolabelled particles is suitable for low dose detection [11], but the extra costs or the complexity of the methodologies may limit their use [55]. The use of metal-doped nanoplastics offers greater potential, and at a lower cost, to assess their translocation [12], detection and accumulation in particular tissues or organs, and to provide an initial overview of toxicological mechanisms [1]. Current advances in this area will be useful to improve our understanding of nanoplastics translocation and (toxico)kinetics, as besides size, shape and morphology could further play a significant factor in determining particle uptake and (bio) accumulation, but there have not been any studies so far exploring morphological differences in nanoplastics' translocation [3]. Therefore, a standardised framework for tracking nanoplastics in aquatic organisms is crucial for reliable effects and risk assessment.

4. Nanoplastics risk assessment

The challenges posed by the exposure of aquatic organisms to nanoplastics, including the potential translocation and mechanisms of action, highlight the pressing need for risk assessment. Ingested nanoplastics by aquatic organisms can mostly be eliminated via egestion [9,11], but due to their small size range, a fraction can cross epithelial barriers in the gut and translocate to other parts of the organism [11,12]. Therefore, nanoplastics exposure poses several challenges beyond gut obstruction or starvation induction/food dilution [5,47], and it is crucial to further investigate and clarify their mechanisms of action within environmentally realistic scenarios [17], and to inform on risk [3,5]. However, there is a major lack of data on the potential environmental exposure of aquatic organisms to nanoplastics, which limits the assessment of risk. This is because the observation and quantification of these particles still remains challenging, mostly due to current methodological limitations [1], leading to both the abundance and the characteristics of environmental nanoplastics being largely unknown [5]. To overcome the fact that there is limited environmental data for nanoplastic exposure, studies on this could, for instance, rely on modelling [57] or estimating the size frequency distribution of plastic particles below 1000 nm [5]. For example, models based on continuous cascading fragmentation enable the simulation of particle size distributions in oceanic environments [58]. For example, the log-linear particle size distribution of particles resulting from the fragmentation of polystyrene has been assessed to nanoparticle scale [8], which can assist in calibrating existing models. Authors such as Lenz et al., 2016 [16] have also estimated the concentration of nanoplastics expected in marine environments based on the observation of available size distribution of microplastics. Besides recent studies on extrapolation of size frequency distributions, multimedia models have been suggested as a model-based alterative to quantify the fate of nanoplastics in the environment, and as a potential solution to circumvent the methodological issues when sampling and analytically processing environmental samples [59,60].

The risk assessment of nanoplastics in aquatic environments is a key step to inform regulators of the potential impacts of these particles in the environment. However, so far, the risk of nanoplastics has not yet been assessed [3], mostly due to the large uncertainty associated with the quantification of environmental exposure [5]. A solution to assess risk could be to employ a probabilistic risk assessment, which is an approach used to evaluate the likelihood and magnitude of adverse effects associated with exposure [5] to, in this case, nanoplastics. This approach involves the use of statistical and probabilistic methodologies to characterise exposure scenarios and the magnitude of effects in aquatic organisms, as well as the uncertainties associated with these estimates, as applied previously for microplastic risk assessment [57,61–64]. This approach considers both the hazard (i.e., the intrinsic properties of the nanoplastics that could induce toxicity) and exposure aspects (i.e., the concentrations of nanoplastics to which aquatic organisms are exposed) of risk, and could be used to identify the most significant sources of uncertainty and variability in the assessment. The probabilistic risk assessment has been used successfully to estimate the risk of microplastics in aquatic environments (e.g., see Refs. [57,62]) and has provided a quantitative estimate of the likelihood and magnitude of adverse effects associated with exposure to plastic particles, after the screening of existing literature, thanks to rigorous quality criteria [47,57]. The reported risk assessments used the estimate of species sensitivity distributions (SSDs), which enabled authors to determine the affected fraction of a series of species at a given concentration, i.e., the hazard concentration for 5% of the species (HC_5), which is considered the "safe" concentration in ecological risk assessment. So far, online tools, such as the "Toxicity of Microplastics Explorer" (ToMex, sccwrp. shinyapps.io/aq_mp_tox_shiny), which is an open access database and web application that enables users to visualise and analyse plastic particles toxicity data [37], is limited in what concerns nanoplastic toxicity information that would otherwise enable the calculation of SSDs. Using alternative approaches, such as Bayesian hierarchical modelling techniques, can assist in estimating nanoplastics HC_5, as proposed by Takeshita et al., 2022 [65], who used chronic lowest-observed-effect concentrations (LOECs) to define micro-and nanoplastics SSDs, while considering particle size, polymer type, and media. Also based on Bayesian modelling, an alternative approach for nanoplastics to the current risk assessment methodologies used for microplastics has been suggested by Cunningham et al., 2023 [3]. These authors recommended the use of Bayesian Network Relative Risk Model (BN-RRM), which considers the complex nature of nanoplastics and their interactions with ecological factors, other particles, and chemical contaminants, and which integrates current data and is flexible to include new data as the field evolves and considers the uncertainty inherent in our current level of understanding of nanoplastics. However, to our knowledge this approach has not yet been applied for any plastics particles risk assessment.

5. Conclusions and outlook

The presence of nanoplastics in aquatic environments and their potential risks requires a comprehensive understanding of exposure, hazards, and impacts, from individuals to ecosystems, and therefore standardised experimental practices, transparent reporting, and reliable effect data are crucial to enhance risk assessment and develop effective strategies for mitigating plastic pollution. However, nanoplastics present unique challenges due to their heterogeneous nature and distinct characteristics, and their comprehensive characterisation is essential, including size, shape, polymer composition, and surface chemistry, using complementary techniques such as flow cytometry, DLS, TEM, and/or SEM(-EDX). Furthermore, the inclusion of additional controls (including the use of reference materials), and the implementation of rigorous laboratory practices and QA/QC measures can confound the accurate interpretation of results and ensure reproducible outcomes. There are current important knowledge gaps regarding, for example, the mechanisms of nanoplastics toxicity to organisms, and research should go beyond the use of spheric pristine particles of limited polymer types and employing metal-doped and radiolabelled particles to improve knowledge on the translocation, effects and (toxico)kinetics of nanoplastics of multiple sizes, shapes and types. Ecotoxicological assessments must consider concentration-response curves, exposure parameters, and environmental factors to obtain reliable data for robust risk assessment, and therefore we strongly recommend that guidelines should be developed and established based on current frameworks for microplastics and nanomaterials effects assessment (e.g., OECD, CRED and NanoCRED, etc), to ensure suitability and comparability of nanoplastics ecotoxicity data. Risk assessment of nanoplastics is challenging due to uncertainties in quantifying environmental exposure, but the use of probabilistic risk assessment methodologies and Bayesian modelling is recommended to assist in advancing this field, which is crucial to inform regulators and to prioritise research and as mitigation measures.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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