Comprehensive Framework for Human Health Risk Assessment of Nanopesticides

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Abstract

Nanopesticides are not only in an advanced state of research and development but have started to appear in the market. Industry and regulatory agencies need a consolidated and comprehensive framework and guidance for human health risk assessments. Our aim was to develop such a comprehensive framework by exploring two case studies from relevant product types: (i) an active ingredient (AI) delivered with a nanocarrier (NC) system and (ii) a nanoparticle as an AI. For a nanocarrier system, three entities are tracked during the assessment: (i) the NC-AI complex, (ii) the empty NC remaining after the complete release of the AI, and (iii) the released AI. For the nanoparticle of pure AI, only two entities are relevant: the nanoparticle and the released ions. We suggest important adaptations of the existing pesticide framework in order to determine the relevant nanopesticide entities and their concentrations for toxicity testing. Depending on the nature of the nanopesticides, additional data requirements, such as those pertaining to durability in biological media and potential for crossing the biological barriers, have also been identified. Overall, our framework suggests a tiered approach for human health risk assessment, which is applicable for a range of nanopesticide products to support regulators and industry in making informed decisions on nanopesticide submissions. Brief summaries of suitable methods including references to existing standards (if available) have been included together with an analysis of current knowledge gaps. Our study is an important step towards a harmonized regulatory-accepted approach for assessing nanopesticides.

Introduction

In order to feed nearly 10 billion people by 2050, food production would need to increase by at least 50% from the 2012 levels[1]. Clearly, innovations in the agricultural sector will continue to be required, which includes the development of effective plant protection products (e.g. pesticides), for achieving this target. The agrochemical industry is constantly seeking novel active ingredients as well as new approaches to formulate and deliver pesticides. Nanoscience and nanotechnology can harness the extraordinary properties of materials at the nanoscale (< 100 nm) to make an important contribution in such innovations [2,3].

Nanopesticides are currently an area of intense interest in nanotechnology and agriculture and food communities, as reflected by several reviews on this topic during the last 5 years[2–6]. Nanotechnology offers new opportunities to facilitate development of novel active ingredients (AIs) and reuse existing chemistries through nano-formulations (e.g. using a nanocarrier delivery system) that enable new pesticide functionalities, such as slow release of AI, increased stability, enhanced penetration (through cell membranes) and a greater efficacy of the AI in controlling the target organisms[4,5], often with a view to reduce application rates through greater efficacy and/or targeted delivery. For example, nanoparticles of metal and/or metal oxides of Ag, Cu, Zn, as active ingredients, have been found to be effective antimicrobial and antifungal agents[3]. In addition, the slow-release (nano-encapsulated) and nanocomposite formulations of metal oxides have been found to be more potent in disease control than conventional formulations[3]. Double-stranded RNA loaded on designer, non-toxic, degradable, layered double hydroxide clay nanosheets not only offered greater stability to the AI (RNA) against plant virus, but also resulted in reduced wash-off in rain , and enhanced systematicity (uptake and transfer inside the sprayed plant)[7]. Nanopesticides (e.g. with nanocarriers or novel AIs) are in an advanced state of research, development and testing and are likely to be presented for regulatory evaluation.
Indeed, some nano-pesticides are already commercially available[2]. For instance, Vive Crop Protection uses polymer-based delivery systems to design nano-formulations for enhancing the stability of the AI in salt solutions to allow their application with fertilizers through irrigation (chemigation) and also as a mixture of various nanopesticides, if needed.
Currently there is no internationally accepted definition of nanopesticides and thus, regulatory agencies may adopt different size ranges and different limits for the fraction of nano-sized particles[8–10]. For our purpose, we use an operational definition of a nanopesticide as a plant protection product in which a nanomaterial is used to enhance the functionality, utility and/or alter the risk profile of a conventional

AI or is presented as a new AI. This perspective does not cover materials that are called “biocides” in the EU, and which include substances used in livestock breeding, food packaging and household kitchen or canteen settings. Some current nanopesticide formulations have sizes larger than the 1 – 100 nm nanoscale size range, similar to the situation with nanomedicines [11,12]. On the other hand, some products (e.g. microemulsions) may contain fractions in the 1–100 nm range and have been on the market for decades without previously being classified as “nano” [13,14].

At the time of introducing pesticides into modern agriculture (at the middle of the last century), it was acknowledged that the use of pesticides potentially presents a risk to human and ecosystem health [15], as they are bioactive agents by design (targeting insects, fungi, weeds etc.) and are applied intentionally in the environment. Ever since, new pesticide products must undergo rigorous safety testing to prevent unacceptable risks as part of a pre-market risk assessment, and this also applies to nanopesticides. While nanopesticides can potentially decrease this risk by reducing the level of exposure (e.g. by reducing the required applied dose), they can also increase the potential for health risks by increasing the bioavailability and/or the bioactivity of the AI, changing its mechanism of action or introducing co-formulants/carriers that may also be bioactive.

Conventional pesticides are evaluated by regulatory agencies primarily based on the AI and its representative formulations. In contrast to the AI, other components of the formulations (co-formulants or excipients) have generally been considered “inerts”; however, safety data may be required by some regulatory agencies (e.g. Europe, Canada), in particular for safeners (chemicals added to a plant protection product to eliminate or reduce phytotoxic effects on certain plants) and synergists (chemicals with no or weak pesticidal activity, that enhance activity of the active substance(s) in a plant protection product). Nevertheless, for nanopesticides, it can be expected that the co-formulants/excipients may contribute to a larger extent to the effectiveness of the pesticide. Therefore, they must be evaluated in all cases for potential risks to humans and ecosystem health. In fact, the European Food Safety Authority (EFSA) guidance for risk assessment of nanomaterials in food and feed [16] stipulates that for nanopesticides all co-formulants/excipients (e.g. surfactants, solvents, carriers, wetting agents) that contribute to the formulation must be considered. Moreover, the safety of all the components of the nanopesticide entity (i.e. AI + co-formulants) must be evaluated, regardless of whether the AI or co-formulants separately have previously been evaluated as safe. A framework for Ecological Risk Assessment (ERA) for nanopesticides has already been developed and published[17], along with some

guidance on its application of specific case studies, especially in problem formulation[18]. This study, therefore, focusses on human health risk assessment only.

The human safety testing of nanopesticides thus requires special attention and additional considerations as compared to the safety evaluation of the typical, conventional chemicals. This is primarily because the physicochemical characteristics of nanomaterials (e.g. size, shape, surface area, and surface chemistry) have a strong bearing on their interactions with biological tissues and hence can influence the pharmacology, toxicokinetics and subsequently the potential toxicity [19]. Furthermore, these characteristics may undergo changes in the biological environment, thereby altering the stability and durability of the surface and core of nanomaterials and consequently their toxicological response [19]. Difficulties in evaluation of nanomaterial toxicity that include pharmaco- and toxicokinetic of nanomaterials is well-recognised [20].

The OECD Working Party on Manufactured Nanomaterials concluded that the existing human health risk-assessment paradigm used for chemicals (with a few exceptions) can be adapted to determine the potential for human health risks of nanomaterials [21]. This group also suggested that modified or alternative testing strategies were necessary in some cases for risk analysis to inform human health, ecosystem health, and exposure data needs for manufactured nanomaterials [21]. In this context, our aim was to identify key considerations that are crucial for adaptation of existing human health risk-assessment paradigms to develop a comprehensive framework suitable for human health risk assessment of nanopesticides that is applicable to industry, academic and regulatory agencies. The specific goals were: (i) to identify key additional considerations associated with nanopesticides for human health risk assessment, (ii) to develop a comprehensive framework for testing and assessment of nanopesticides for human health risk assessment, including suggestions for suitable methods and standards (if existing), and (iii) to highlight knowledge gaps (including lack of methodology) that require urgent attention.

In this paper, we have taken a pragmatic approach by building on the existing conventional risk assessment paradigm for pesticides, as well as considering frameworks and guidance that are currently available for nanoparticles in other areas, such as cosmetics [22], and the food and feed chain [16]. Our goal is not to present an exhaustive list of scientific knowledge gaps in the field. Instead, we apply a top-down strategy to support decision making and meet regulatory needs, as recommended by Grieger et al.

[23]. Here, we provide a comprehensive framework for human health risk assessment that will support regulators, researchers, and industry in taking informed decisions on nanopesticide submissions.

Human health risk assessment for conventional pesticides

Human health risk assessment of pesticide AIs and formulated products is a very well-established process that forms an integral part of pesticide regulatory frameworks in many countries. Risk assessment typically consists of three key activities: exposure assessment, hazard identification and characterization, and risk characterization [24]. Human exposure to pesticides may occur from occupational and non-occupational (residential) uses of pesticides as well as via the diet and drinking water that may contain residual traces of pesticides. Figure 1 summarises the three key stages of potential human exposure to pesticides: (i) mixing-loading, (ii) application, and (iii) post application. Each stage involves different forms of the pesticide (e.g. concentrated, diluted, transformed in the field), and exposed population (professional, residential). Occupational exposure can be mitigated through the use of adequate personal protection equipment, whereas mitigation strategies to reduce non-occupational exposure (including bystanders, residents and consumers) are limited. Dermal, inhalation and ingestion are all relevant to each stage of exposure, but according to the type of pesticide and its intended use, one of the routes may become of major concern and require more detailed investigations. Identifying scenarios that may lead to exposure to a specific pesticide is an important stage of the problem formulation.

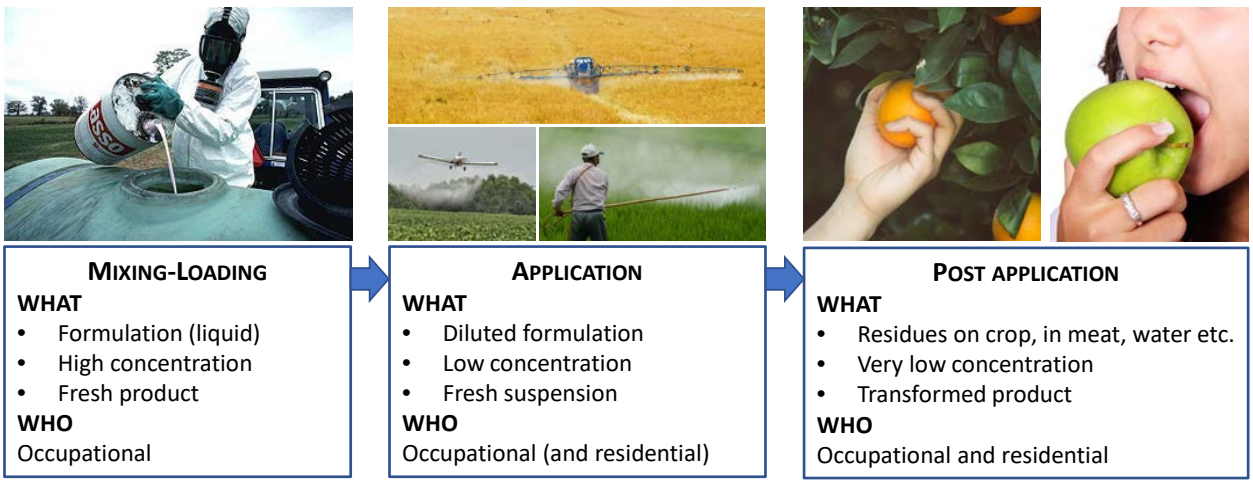

Figure 1. The three stages of human exposure to pesticides considered in risk assessment

Hazard identification and characterization are performed by using results from toxicology studies, typically *in vivo* but increasingly via alternative approaches including *in vitro* and *in silico*. Safety factors are applied to account for uncertainty and variability (e.g. normal vs impaired individuals). The requirements for the AI include toxicokinetic studies (absorption, distribution, metabolism and excretion, both intravenous and oral), acute systemic toxicity (oral, dermal, inhalation), skin and eye irritation, skin sensitisation, short-term and long-term toxicity, genotoxicity (*in vitro* and *in vivo*), carcinogenicity and, reproductive and developmental toxicity as well as other endpoints (e.g. neurotoxicity and immunotoxicity) [16]. Similar data requirements exist in the EU and Canada for some co-formulants (i.e. safeners and synergists) but not for others (e.g. inerts, excipients). For formulations (AI + co-formulants), the toxicity data requirements are currently limited to acute systemic toxicity (oral, dermal, inhalation), skin and eye irritation, skin sensitisation and sometimes dermal absorption [25,26]. The required toxicology studies to support pesticide registrations are broadly similar in Europe [25,27,28], Canada [29] and the US [26]. Finally, the exposure and hazard assessments are used together to characterize the overall risk associated with the use of a pesticide product. If the risk is considered acceptable, the results from the assessment are also used to define a set of detailed instructions appearing on the label (e.g. dose, mode of application, protective equipment) that aim to minimise exposure and ensure safe use.

Why consider adapting the existing framework to nanopesticides?

The majority of nanopesticides proposed until now consist of reformulations of existing AI that are already authorised for use and that have gone through the human health risk assessment described above. Other nanopesticides aim at delivering novel AI (e.g. RNAi, natural substances) or explore new functionalities of inorganic elements (e.g. Cu, Mg) when they are in nanoparticle form [2]. In all cases, the nano-character of the nanopesticides can significantly affect their fate, biointeractions and effects on human health. Such nano-specific aspects can be easily missed when applying the conventional risk assessment framework. The properties, fate and effects of nanopesticides cannot be assumed to be similar to a conventional pesticide, even when the individual ingredients of the formulation are already considered safe on their own [16]. It is thus essential that additional and suitable tests are conducted to ensure the robust risk assessment of nanopesticides.

Considering the range of nanopesticides currently at various stages of development, two relevant case studies covering a range of properties were considered to develop and test the proposed framework for

human health risk assessment (see illustrations at the top left corner of Figure 2 and Figure 3): the two cases studies represent two of the more commonly discussed types of nanopesticides [2].

Case 1 is a nanocarrier system for the slow release of a pesticide AI e.g. a polymer nanocarrier (NC)[30] releasing an AI (e.g. an insecticide or herbicide) over time after application to soil or foliage. Case 1 (NC-AI complex) includes examples where the AI is either encapsulated within or entrapped by the polymer nanocarrier. The characterisation methods developed up to now for metal and metal oxides nanoparticles may not be applicable for nanoscale polymers. In addition, the degradability of the NC itself has to be determined. There are three entities to track during the assessment: (i) the NC-AI complex, which is likely to dominate in the early stage of exposure (mixing-loading and application), (ii) the empty NC remaining after the complete release of the AI, and (iii) the released AI. In our case study, the latter may be considered to behave similarly to the conventional pesticide AI, keeping in mind that exposure patterns in space and time may be different relative to an AI applied as a conventional formulation, as highlighted in previous work on ecological risk assessment[17,18]

Case 2 is a nanoparticle (NP) made of a pure, nanoscale AI (e.g. metal or metal oxide nanoparticles) stabilised with e.g. salts, surfactants or polymers. The application of similar products has been proposed to suppress pathogen infections [2,31]. For this case study, there are only two entities to track: the metal or metal oxide NP and the released ions. In the environment, the dissolution kinetics of some materials (e.g. copper oxide) is generally considered to be slow compared to zinc oxide or silver nanoparticles. Dissolution may be significantly faster in biological media [32,33].

Nanopesticide may decrease or increase the risk to human health. Often, the quantity of AI applied in the field can be reduced for nanoformulations (Case 1) or nanoscale AI (Case 2), thereby reducing the level of (external) exposure to the AI. However, at the same time, the dose reaching a specific target organ (i.e. internal dose) might increase, e.g. by altering the penetration of the AI through biological barriers (also relevant for Case 1 and Case 2). Thus, both effects must be taken into account carefully. Note that the term “dose” requires careful consideration for nanotoxicology studies since mass, particle number and surface area metrics are used. It is also important to distinguish the characteristics of the nanopesticide at different stages of exposure, as a range of transformation processes resulting from exposure to environmental conditions can significantly affect toxicological responses.

In line with previous conclusions on the ecological risk assessment of nanopesticides [17,18], the existing general human health risk assessment paradigm can be applied to nanopesticides. However, additional

data on novel properties may be required and testing methods may need some adaptations [16]. For instance, one additional requirement when dealing with nanopesticides is related to the simultaneous occurrence of three processes with different kinetics that determine the species of nanopesticides and their concentrations, all of which are relevant for toxicity testing: (i) degradation of the AI, (ii) the degradation of the NC (Case 1) or NP (Case 2) and (iii) the degradation or dissociation of the NC-AI complex (Case 1). These three processes and their kinetics determine the different entities that humans may be exposed to (i.e. the AI, the NC/NP and/or the NC-AI complex) at different stages of pesticide use (Figure 1). Transformation processes of the AI, NC, NP, and NC-AI complex (e.g. dissolution, hydrolysis, formation of corona etc…) should also be considered when characterising exposure and testing toxicity, especially in a post application scenario.

When dealing with AI or NC/NP systems that have already been assessed and are thus data rich, performing comparative exposure assessments may allow some degree of bridging and read across, especially for acute toxicity tests [16,34,35]. In the framework below, we identify a sequence of steps that aim at guiding risk assessors in the identification of necessary additional testing. When possible, existing data should be used to avoid unnecessary animal use for toxicity testing.

Overview of the Framework

The decision trees presented in Figure 2 (Case 1) and Figure 3 (Case 2) were constructed by systematically considering the additional factors and processes that may need to be taken into account when assessing the risk of nanopesticides to human health. The stepwise approach is briefly presented below, while more details on the methods proposed and challenges are presented in the following sections. The vast majority of pesticide formulations being liquid, only nanopesticides supplied as a liquid formulation are considered.

The first step of the decision tree is a decision whether the novel product requires additional assessment relative to a conventional pesticide. The procedure and criteria are expected to differ according to the jurisdiction. In some cases a declaration by the applicant is sufficient to determine whether additional investigations are needed. However, in other cases a decision will be based on whether the material meets the definition of a nanomaterial in the relevant legislation. Nano definitions typically specify a size range but may also include other factors. For example, EFSA[16] requires physical chemical characterisation to determine whether the material meets the nanomaterial definition in the EU regulation and therefore requires a series of additional in vitro and in vivo tests. However, additional

testing may also be required in cases where a material does not meet the definition (for example having size above the order of 100 nm) but does display properties characteristic of the nanoscale. If the initial response to question 1 indicates that additional assessments are warranted then the various questions in Figure 2 and Figure 3 should be considered. Following characterization of the undiluted nanopesticide (Step 2.1), the durability of the NC/NP should be determined (Step 2.2). If degradation/dissolution is very rapid on the time scale of mixing-loading or application, post application assessment will only be required for the AI/ion. For Case 1, the association between the NC and AI should also be characterised to determine whether the NC-AI complex is durable during the mixing-loading, application and post-application stages (Step 2.3). The results from the durability tests will identify the species (AI/ion, NC-AI, NC/NP) that must be considered and characterised for each of the three exposure stages (Step 3). As an example, a NC-AI complex that releases AI rapidly (i.e., complete release during the time required for application) will not require further assessment at the post-application stage. However, for many materials it is likely that a mixture of intact and dissolved or transformed material will have to be considered.

Figure 2 and Figure 3 also outlines the necessary steps to consider if human exposure to the NC, NP or NC-AI complex is likely to occur. Steps 4-6 should be considered for each relevant entity and stage of exposure identified in Figures 2 and 3 (i.e. AI/ion, NC-AI and/or NC/NP as undiluted, diluted and/or transformed in the environment). First, the durability of the NC/NP (Step 4) is evaluated in appropriate relevant medium for the application route (dermal, inhalation and/or oral). If the NC/NP degrades rapidly, for case 1 a conventional assessment of the AI is sufficient and for case 2, one can rely on data existing for the ions. If the NC/NP does not degrade rapidly, then two additional considerations must be taken into account. The first only applies to Case 1 and is the durability of the NC-AI complex in biological media representing the relevant exposure pathways (dermal, inhalation and/or oral, Step 5a). The second (Step 5b) applies to both Case 1 and Case 2, and is whether the NC/NP can cross a biological barrier (e.g., dermal). Note that here it is assumed that the NC and NC-AI complex will have similar barrier-crossing capability. This is likely to hold true provided the AI does not significantly modify the size, shape or surface charge of the NC, which are the key properties that influence the crossing of particles through dermal barrier[36], intestinal[37] and pulmonary barriers[38]. If no barrier crossing occurs, then a conventional assessment of the AI should be sufficient as one then assumes that only the AI/ion alone will be able to penetrate the barrier. If the barrier crossing occurs and the rate of release/dissolution is slow, then the impact of the NC on the toxicokinetics of the AI needs to be investigated for Case 1 (Step 6, Figure 2). If the toxicokinetics of the AI are not affected by the NC, then

the conventional assessment for the AI must be complemented by a nano-specific assessment of the NC (green box in Figure 2). In cases where the toxicokinetics of the AI are modified by the NC (e.g. increased amount of AI in tissues), the toxicity of the NC-AI complex also needs to be investigated (black box in Figure 2). Both acute and chronic toxicity testing may be required, unless justification for read across to existing toxicological data is available.

It should be noted that Case 2 (metal or metal oxide NP) is slightly simpler than Case 1 (polymer NC). Dissolution tests in biofluids can provide information to estimate the durability of the NP (Step 4) and to inform on release kinetics of the ion (Step 5a). If the NP dissolves rapidly or it does not cross biological barriers, a conventional assessment focussing on the ion toxicity should be sufficient. Barrier crossing combined with slow dissolution require toxicological testing of the NP in relevant acellular or cellular models (green box in Figure 3).

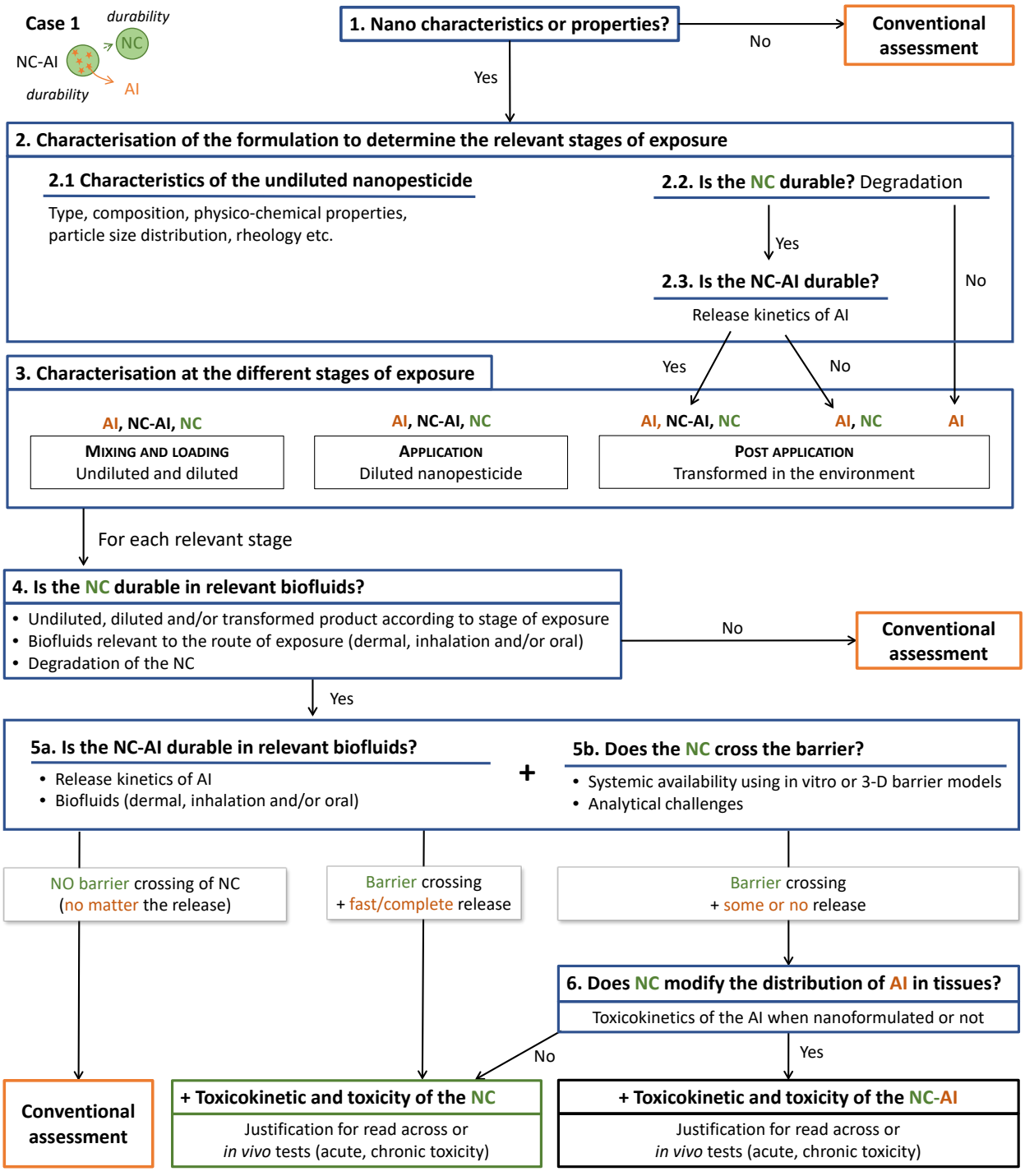

Figure 2. Decision tree considering Case 1: a polymer nanocarrier (NC) associated with a pesticide active ingredient (AI). Step 1-3 aim at determining the characteristics and species of nanopesticides relevant to the three stages of human exposure (Who is exposed to what and when?). For each

relevant stages, Step 4-6 helps identify the requirements for toxicokinetic and toxicity data to support human health risk assessment.

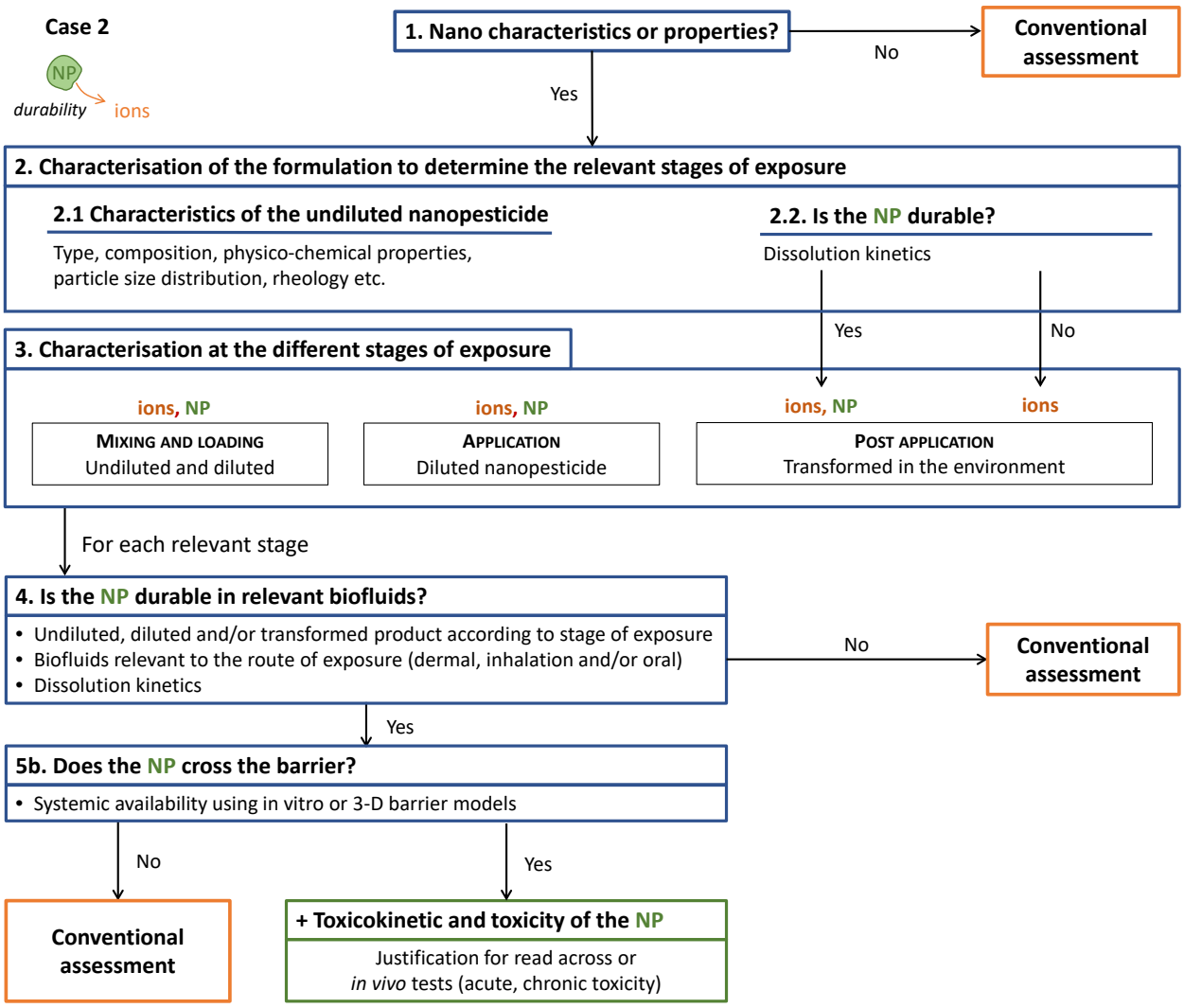

Figure 3. Decision tree considering Case 2: metal or metal oxide nanoparticles releasing ions over time. Step 1-3 aim at determining the characteristics and species of nanopesticides relevant to the three stages of human exposure (who is exposed to what and when?). For each relevant stages, Step 4-6 helps identify the requirements for toxicokinetic and toxicity data to support human health risk assessment.

Key questions to address, methods and limitations

The sections below detail the key steps of the framework presented in Figure 2 and Figure 3, and summarise the methods and approaches required. Further references to the international standards available are presented in the Electronic Supporting Information (Table S1).

Step 2.1. Characterization of the undiluted nanopesticide formulation.

Currently, physico-chemical characterisation is required for the AI but is typically not expected for co-formulants that are not safeners or synergists [16]. For nanopesticides, the undiluted nanopesticide formulation should be characterised in all cases (Step 2.1) and a rationale for developing the novel product is also typically requested at this stage. In addition to describing the type of formulation (e.g. encapsulation, nanodispersion, emulsion), the ingredients and their proportions, the physico-chemical properties of the formulation should be thoroughly characterised. The required properties depend on the type of nanopesticide and its intended use. Characterization will typically include assessment of properties such as chemical composition, crystal structure (where relevant), primary particle size, shape and aspect ratio, which are typically independent of the medium, along with a range of properties such as surface charge, surface chemistry, dissolution, and agglomeration/aggregation level, that depend on the properties of the medium in which they are placed and on time and environmental conditions such as temperature and sunlight. A clearer correlation with exposure and hazard assessment is often observed for medium-dependent properties[39].

Standards developed by ISO and the ongoing development of new (or adaptation of existing) test guidelines by OECD[40] should be applicable for characterisation in some cases. However, it should be noted that many of these methods may require modification when dealing with soft polymer NCs (Case 1). Several recent reviews [20,41–43] summarise the key properties for risk assessment for nanomaterials and the recommended analytical methods, along with a critical evaluation of their range of applicability, advantages and limitations. A different combination of techniques may be required to characterise different nanopesticides [14]. An overview of available methods and relevant standards is provided in Table S1. Some methods have specific concentration requirements that may require dilution of the formulation prior to measurement, and this should be done with a diluent relevant to the mixing-load stage, which may have different composition from the initial formulation (e.g., added salt or dispersants).

Step 2.2. Durability of the NC/NP in environmental media

The durability of the NC (Case 1) or NP (Case 2) should be determined using degradation or dissolution assays. It should be noted that it is unlikely that the NC/NP will be unstable in the initial formulation. However, there may be cases where the NC/NP is designed to facilitate mixing-loading and application but to then degrade rapidly after application. Assessing durability in the diluted formulation can minimize testing at the post-application stage and in biological media.

For Case 1, NCs may be made of naturally occurring polymers such as polysaccharides and proteins that are easily degraded in the environment or biological tissues [5,30,44]. The use of these materials is likely to be of minimal concern for adverse side effects. Other NCs are made of synthetic polymers that are used for encapsulation and these NCs may persist for an extended time period after application. The degradation of many of these polymers will have been studied in some environmental media and possibly also in biological fluids, although not necessarily in their nano form [45]. Options for bridging and read-across from existing data should be considered when possible. For polymer NCs for which the persistence is unknown, studies of carbon dioxide release from isotopically labelled polymer can be used to estimate persistence, as for example in studies of biodegradation of polyacrylates in soil [46]. Many available studies have not focused specifically on polymer NC and some adaptation of existing methods used for larger polymer particles and films may be required.

For Case 2, measurement of dissolution kinetics will be required (Table S1). Methods for assessing dissolution rates for metal and metal oxide NPs are well-established and generally rely on measuring ionic concentration in the aqueous phase using inductively coupled plasma atomic emission spectroscopy (ICP-AES) or mass spectrometry (ICP-MS) [19,47,48]. The use of a continuous flow through system is preferable to a static system which has a restricted volume of fluid that may lead to saturation of the sample with one of the solutes, preventing further dissolution. Considerable literature data are available for metal and metal oxide NPs. It is important to note that dissolution rates are sensitive to the concentration of the material, the presence of s additives (e.g., salt) and pH, potentially requiring measurement under conditions relevant to the formulation, as well as environmental and biological samples. Dissolution measurements will be more challenging under post-application conditions where the ion concentration may be low, the composition of the environmental sample (soil, water, etc) is not well-known and contamination by naturally occurring ions may be an issue. In such cases the use of simple soil models may prove adequate (e.g. OECD 2000); otherwise indirect lines of evidence may be necessary. It is important to note that exposure to environmental conditions is more likely to cause degradation and transformations for pesticides than for other nano-enabled products.

Step 2.3. Durability of the NC-AI complex (Case 1 only)

For Case 1, determination of AI release kinetics will indicate whether the NC-AI complex is durable during the mixing-loading, application and post-application stages. Release kinetics from a nanoformulation before and after dilution can be assessed by adapting methods currently employed to measure release from polymer NCs used for drug delivery. Most methods rely on separation of the free and complexed drug by methods such as ultrafiltration, centrifugation, dialysis, or continuous flow, followed by quantification of residual free drug in the filtrate or supernatant using standard analytical techniques such as High Performance Liquid Chromatography (HPLC) or Gas Chromatography-Mass spectrometry (GC-MS)[50–52]. A separation method that does not disturb the integrity of the NC or perturb the equilibrium between free and bound AI should be selected. Careful method validation and well-designed controls are essential to account for the effect of dilution on the release of the AI and the potential adsorption of the AI on the filtration/centrifugation device. Analytical ultracentrifugation is a promising alternative to separation methods that does not require a prior separation step and can detect and quantify the unbound material by changes in UV-visible absorbance or refractive index [52].

The methods described above can also be adapted to examine NC-AI durability in some simple soil models which are needed in Step 3. However, measuring release kinetics of AI under realistic field conditions (post-application) is very challenging and indirect methods should also be considered to demonstrate whether the association is durable or not (e.g. [53,54]). Examination of release of AI in biological media should be feasible with the same tests, taking into account the possibility of complications due to any of the components (see Step 5a). As noted above it is important to consider that rapid release kinetics upon dilution or application means that the NC-AI complex as a whole will not require assessment in the post-application scenario. Such examples would be cases where the NC is used to facilitate mixing-loading or application but not to further modify the AI behaviour.

Step 3. Comprehensive characterisation at the three stages of human exposure

The characterization methods described for Step 2.1-2.3 can generally be applied to determine the key characteristics of the nanopesticides at the first stage of human exposure (i.e. undiluted and diluted nanopesticide). Additional aspects relevant at the mixing-loading and application stages include the formation of degradation products and aerosols, that require assessment of exposure through inhalation. For the post-application stage, the nanopesticide transformed in the environment (residues

on crop surface, soil, groundwater, possible transfer to meat, fish etc.) must also be characterised, and investigations into the formation of dislodgeable residues on crops or dust may be required.

It should be noted that if the durability (i.e. kinetics of degradation/release/dissolution) is sufficiently short during the mixing-loading and application stages, post-application investigations are only needed for the AI or ion, leading to a conventional assessment and read across if relevant data already exist . For nanopesticides with slow transformation kinetics, investigations into environmental fate of the NC-AI complex and the NC itself may be necessary to determine the relevant exposure levels and characteristics. The detection, characterisation and quantification of nanopesticides after application in the field is currently limited by the lack of adequate analytical techniques, especially for Case 1. While the total residues of AI can be determined with conventional methods, determining whether the AI is still associated with the NC or is in a nanoform, and whether transformation processes have occurred, is very challenging [55].

Step 4 and 5a. Characterisation of nanopesticides in relevant biofluids

The biodurability of the NC/NP (step 4) and NC-AI (step 5a, for Case 1 only) refers to the dissolution, enzymatic degradation or chemical disintegration of the nanopesticide [47]. It can be assessed with acellular *in vitro* assays that utilize simulated biological fluids to identify cases for which it will be necessary to obtain toxicokinetic and toxicity data for the nanopesticide. These measurements will identify cases for which the durability of the NC/NP and NC-AI is sufficiently short that one can rely on pre-existing data for the AI or ion. Many of the analytical challenges mentioned earlier for environmental media (Step 2.2, 2.3 and 3) are also relevant for biofluids [56].

A range of model biological fluids have been used for dermal, inhalation and oral exposure routes[19]. For inhalation, two lung compartments should be considered: the fluid in the extracellular airway lining to which the particles are initially exposed and the phagolysosomal fluid found in alveolar macrophages which rapidly scavenge inhaled particles. Models for both are based on the original Gamble's solution (a neutral electrolyte solution with added glycine) and an acidified analogue that better mimics the phagolysosomal environment. Oral exposure can be assessed using saliva, gastric fluid (acidic) and intestinal (neutral) fluid models that typically contain a mixture of salts and several enzymes), although it should be noted that the composition of each of these fluids varies considerably as a function of time and diet. Dermal exposure models include simulated sweat formulations and simulated sebum

formulations. A compilation of simulated biological fluids that have been used for durability studies is available in an ISO technical report [19]; this report summarizes several examples from the literature where model biofluids have been used to assess nanomaterial dissolution. A more detailed summary of available studies on nanomaterial dissolution in model biofluids is provided in an OECD report [47]. It is important to keep in mind that although these simulated models have been shown to provide reasonable mimics for human biofluids, they have defined compositions that do not match the dynamic *in vivo* conditions and typically do not include the range of *in vivo* enzymes and proteins that may modify nanomaterial behaviour.

Step 5b. Does the NC/NP cross the barrier (systemic availability)?

If the NC/NP does not degrade/dissolve (for instance >12% persistent[16]), it is important to establish whether it can cross biological barriers at the site of exposure and enter the human body. Human biological barriers, particularly those that are directly exposed to the surrounding environment (skin, lungs and gastrointestinal tract) have evolved to protect us from infection, bacteria and parasites. However, entities on the nanoscale have the potential to penetrate deeper into the human body and persistent nanomaterial may remain in biological compartments for extended periods of time [57]. Thus, NCs/NPs that are both biopersistent and able to enter the systemic circulation require more rigorous hazard evaluation to understand their long-term impact on human health. Evaluation of bioavailability is a priority for assessing potential toxicity, independent of the exposure route and endpoint [58](ESI).

Measurement of the absorption or penetration of an exogenous agent across the skin is evaluated according to the OECD Test Guideline 439: Skin Absorption *In Vitro* Method [59]. This approach is used for the evaluation of pesticides, biocides, and other industrial chemicals applied as formulations to human or animal skin preparations. The guideline is very broad and additional guidance on the application of this method and reduction of variability in data sets has been provided by EFSA[60]. However, this document specifically states that “the issue of nanoformulations in plant protection products is not addressed” and thus, evaluation of nanopesticides should be performed on a case-by-case basis.

Three dimensional (3-D) and advanced cell culture systems for a variety of human tissues are commercially available or can be grown in standard tissue culture laboratory facilities. These *in vitro* tools exhibit more natural cell-cell contacts, improved metabolic activity and transcriptomic profiles that more closely represent the *in vivo* situation[61]. The importance of 3-D reconstructed skin models for risk assessment is prominent for cosmetics hazard assessment, where testing *in vivo* is prohibited. [61]. Skin

equivalent models typically consist of a fully differentiated and stratified epidermal barrier that closely resembles normal human skin with a dry surface stratum corneum. Thus, exogenous test materials are applied to the skin model in a similar manner to human dermal exposure. The skin irritation and corrosion endpoints are now evaluated *in vitro* using 3-D reconstructed skin-based protocols (e.g. OECD Test Guidelines 439 [62] and 431[63]). International validation efforts to facilitate genotoxicity testing in 3-D reconstructed skin models for chemicals have also been expanded to nanomaterials [61,64]. Interestingly Wills and colleagues [64], demonstrated the utility of the 3-D reconstructed skin model for evaluation of dermal barrier penetration of silica nanoparticles using Transmission Electron Microscopy (TEM).

Other biological barriers of importance are those presented by the pulmonary system and gastro-intestinal tract, following inhalation and/or ingestion of the nanopesticide, respectively. For the lung, advanced co-culture systems which include multiple pulmonary cell types are grown at the air-liquid interface supporting exposure to aerosols [65,66]. More complex 3-D culture systems and 3-D *in vitro* respiratory tissue models are also commercially available [67,68]. These models have been applied for characterising nanomaterial toxicity and present advantages over standard two-dimensional cell culture, as they allow for the detection of damage mechanisms such as those associated with chronic inflammation that usually only arise *in vivo* [69]. Similarly, triple culture models of the gastro-intestinal tract have been developed, which actively produce mucins [70,71]. Barrier penetration can be assessed in these pulmonary and gastro-intestinal tract *in vitro* co-culture systems, by exploring the passage of materials across the model system when applied to the top surface using imaging (e.g. TEM) or chemical analysis techniques (e.g. ICPMS) [66,69,72,73].

While the above methods for assessing barrier crossing are suitable for studies of many NPs (Case 2), NCs made of polymers (Case 1) are very challenging to detect and track in biological tissues using conventional imaging techniques (TEM, SEM), mainly due to the lack of contrast i.e. both carrier and tissues are carbon-rich materials. The Wills study[64] that used advanced TEM imaging modes to study uptake of silica nanoparticles in 3-D dermal models illustrates the challenges, even for nanoparticles that are detectable by TEM without staining. Radiolabelling approaches (e.g. ^{14}C) can also be used to detect NC in biological matrices, but they must be used with care and are expensive and time-consuming. Producing labelled NC is often impossible at an industrial scale as this implies an excessive use of radioactive label. Smaller scale production processes can lead to NC that have different properties than the one for which regulatory authorisation is required. Fluorophore labelling and fluorescence imaging are attractive alternatives, but it is important to ensure that the added

fluorophore remains attached to the NC during the barrier crossing study. Furthermore, labelling the NC may alter its fundamental physico-chemical characteristics, thereby modulating its barrier penetration capacity. In some cases, Step 5b may thus be best addressed using indirect lines of evidence to indicate whether barrier crossing occurs.

It is important to understand if NC/NP have the ability to cross biological barriers at the potential sites of exposure using *in vitro* approaches, such as those described above. If Step 5b demonstrates that there is no barrier crossing of the NC (Case 1) or NP (Case 2), then, a conventional assessment of the AI or ion(s) should be sufficient, regardless of the release/dissolution kinetics. If the NC/NP can cross biological barriers, then the conventional assessment must be complemented by characterisation of the toxicokinetics and toxicity of the NC or NP.

Step 6. Does the NC modify the distribution the amount of AI in tissues? (Case 1 only)

At this stage of the framework, we have demonstrated that the NC can cross relevant biological barriers, while it is still associated with some AI (no or incomplete release as determined in Step 5a). Toxicokinetic tests should be conducted to investigate whether the nanoformulation modifies the distribution of the AI (Case 1) in different organs and the possibility that it may increase the concentration of AI in certain tissues. Change in the toxicokinetic behaviour was previously recognised by the EFSA as an important trigger for nano-specific assessment for nanopesticides with size range above 100 nm [16]. The current OECD Test Guideline 417 on *in vivo* toxicokinetics is not applicable to nanomaterials [74]. The underlying processes determining the toxicokinetics of NPs require a dedicated test design[75] and several projects are currently developing a new guideline adapted to nanomaterials [40,58].

When considering the AI in Case 1, however, using TG 417 [74] can already be very informative at this stage. For instance, comparing the toxicokinetics of the nanoformulated AI and a conventional formulation (or the unformulated AI) will indicate whether the nanoformulation modifies the distribution of the AI in animals. If not, toxicity tests carried out on the NC and AI separately may be sufficient. If yes, toxicokinetic and toxicity tests (acute and chronic) for the NC-AI complex are also needed, unless read across from existing data is deemed acceptable.

Toxicity testing

According to the results obtained from Step 1-6, three levels of toxicity assessment may be required for nanopesticides.

1. The simplest case corresponds to the conventional toxicity assessment currently applied to pesticides and focussing on the AI or ion (orange box in Figure 2 and Figure 3). The current assessment is warranted for products that do not fall within the regulatory definition of nanopesticides, and nanopesticides whose NC/NP do not cross biological barriers or NP (Case 2 only) that are not durable (i.e. rapid dissolution).

2. Additional toxicokinetic and toxicity testing of the empty NC or NP using protocols adapted to nanoparticles are required when barrier crossing occurs.

3. In cases similar to Case 1 where the nanoformulation modifies the toxicokinetics of the AI, toxicological studies of the NC-AI complex are also required.

For (2) and (3), toxicokinetics and toxicity testing need to consider nanospecific aspects (e.g. dispersion, agglomeration/aggregation) and this may require adaptation of the protocols that have been developed and validated for substances that can be solubilised. Some tests (e.g. toxicokinetics) are currently being adapted through OECD programs and a new toxicokinetics guideline is being developed. For others, guidance with respect to possible modifications of existing tests are presented in Hardy et al.[16] and they often require case-by-case considerations based on expert judgement. For *in vitro* studies, data on stability are essential to ensure that exposure levels are maintained during the test to avoid false negative results due to e.g. sedimentation or agglomeration.

Knowledge gaps

As discussed under various sections above, there is a range of limitations in the current state of knowledge for human health risk assessment of nanopesticides. Some of these limitations are briefly described below to provide an impetus to future work in this area. Several organisations (e.g. OECD, EFSA) active in this space may have ongoing work to address these gaps, to some extent. For example, new OECD test guidelines for nano-relevant properties are currently being developed[40].

- The framework has been largely developed based on two large classes of nanopesticide products (i.e. polymer NC-AI complexes and inorganic metal or metal oxide nanoparticles). In

due course, different types of nanopesticides (e.g. dendrimer technology, solid formulations) may emerge, warranting additional considerations.

- Many nanopesticides are based on complexation of an AI with a NC (e.g., a polymer). Such nanopesticides will require characterization of the amount of AI incorporated in the carrier and the kinetics for its release. Methods for the reliable measurement of release kinetics are currently limited. The methods employed for nanomedicines (polymer-drug complexes) may allow adaptation for nanopesticides.
- Tests and procedures employed in risk assessment of conventional pesticides, while being useful, may be insufficient for nanopesticides. New tests are likely to be needed depending on the nature of nanoproducts and as the experience with such products grows. Current adaptations of toxicokinetic and toxicity tests for nanomaterials are largely based on inorganic materials (Case 2) and thus may not be suitable for nanopesticides represented by Case 1.
- Many formulations currently contain relatively large amounts of “inerts”, including nano forms of silica (typically used as rheology modifier). These and other non-nano "inerts" can potentially (unintentionally) alter the bioavailability of the pesticide.
- While it is recognised that the biological environment can alter the physicochemical characteristics of nanomaterials and consequently their toxicological response, such potential changes are currently not well understood for a range of nanopesticides.
- Validated approaches to detect and track nanomaterials in biological matrices are currently lacking and the detection of polymer NC complexes will present even more challenges than inorganic NPs. Potential use of indirect approaches to collect evidence should be explored. For example, a comparative assessment of a nanopesticide with non-nano or unformulated product may help discern the effect of excipients (inerts) on the fate of the chemical. An early dialogue between applicants and regulators may help design scientifically sound and relevant experiments for such purposes.

Concluding remarks

We noted that regulatory agencies use different definitions for nanopesticides, and some also take into account parameters in addition to size for identification of nanopesticides. Independent of the criteria applied to decide whether a nano assessment is needed, the framework that we have developed can be applied to all cases where nanotechnology has been harnessed to offer novel formulation properties

thatlead to desirable outcomes (e.g. targeted delivery, greater efficacy, lower environmental footprint – to name a few).

Certainly, new guidelines and guidance documents will have to be established to deal with nanopesticides. However, existing knowledge on assessing conventional products plays a valuable role in their development. In some cases, conventional AIs are being reformulated (e.g. hydrophobic pyrethroids in a hydrophilic carrier to facilitate their targeted delivery) and significant knowledge about the fate and behaviour of the AIs may thus already exist. In such cases, some of the data and information required to employ the above framework may already be available. Additionally, using bridging arguments or read-across from conventional to nano formulations can help to speed up the regulatory process.

One of the key benefits intended from this framework is a better understanding of the portfolio of data and information required for sound assessment of human health risk that regulators will expect industry to provide. A clear communication between the two parties is therefore expected to be mutually beneficial. We hope this framework will facilitate an early dialogue between the regulators and industry to develop strategies to adequately address the regulatory requirements for emerging nanopesticides. In addition to focussing on the technical potential of novel nanopesticides, developers should consider the contextual applicability of the products at the earlier stages of development; this includes how the products can be assessed for regulatory authorisation [76]. The application of more holistic evaluation tools such as life-cycle assessment [77] or one-health approaches [78] can also help maximise the benefits of nanopesticides relative to conventional products.

Electronic Supporting information

Table S1 presents a summary of physico-chemical properties of nanomaterials that should be assessed for regulatory purposes and international standards for their measurement. Additional information and references about bioavailability and bioaccessibility concepts are also provided.

Conflicts of interest

There are no conflicts of interest to declare.

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Author contributions

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