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REVIEW

Analytical approaches to support current understanding of exposure, uptake and distributions of engineered nanoparticles by aquatic and terrestrial organisms

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Abstract Initiatives to support the sustainable development of the nanotechnology sector have led to rapid growth in research on the environmental fate, hazards and risk of engineered nanoparticles (ENP). As the field has matured over the last 10 years, a detailed picture of the best methods to track potential forms of exposure, their uptake routes and best methods to identify and track internal fate and distributions following assimilation into organisms has begun to emerge. Here we summarise the current state of the field, focussing particularly on metal and metal oxide ENPs. Studies to date have shown that ENPs undergo a range of physical and chemical transformations in the environment to the extent that exposures to pristine well dispersed materials will occur only rarely in nature. Methods to track assimilation and internal distributions must, therefore, be capable of detecting these modified forms. The uptake mechanisms involved in ENP assimilation may include a range of trans-cellular trafficking and distribution pathways, which can be followed by passage to intracellular compartments. To trace toxicokinetics and distributions, analytical and imaging approaches are available to determine rates, states and forms. When used hierarchically, these tools can map ENP distributions to specific target organs, cell types and organelles, such as endosomes, caveolae and lysosomes and assess speciation states. The first decade of ENP ecotoxicology research, thus, points to an emerging paradigm where exposure is to transformed materials transported into tissues and cells via passive and active pathways within which they can be assimilated and therein identified using a tiered analytical and imaging approach.

Keywords Environmental Nanosafety · Nanotoxicology · Transformation · Assimilation · Bioimaging · Speciation

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Introduction

Engineered nanoparticles (ENPs) have unique physical and chemical properties that provide significant advances in many fields including medicine, engineering, electronics and consumer goods. Already in use worldwide, many ENPs are made from materials (e.g. metals and metal oxides) or have physical forms (e.g. carbon nanotube) that have been previously linked to adverse effects on biological systems (Lead and Smith 2009). Such features mean that ENPs have the theoretical potential to cause direct toxicity to humans and ecological receptors after their release into the environment (Nel et al. 2006).

Recognising the potential for detrimental effects of ENPs after release, the field of nanoparticle environmental toxicology/ecotoxicology has developed. A key aim has been to underpin the development of appropriate methods for ENP risk assessment and management. In the almost 10 years since the first studies assessed the ecotoxicological effects of ENPs (Moore 2006; Oberdörster 2004; Oberdorster et al. 2005), a steady rise in the number of publications on this topic has taken place (Fig. 1). Initially

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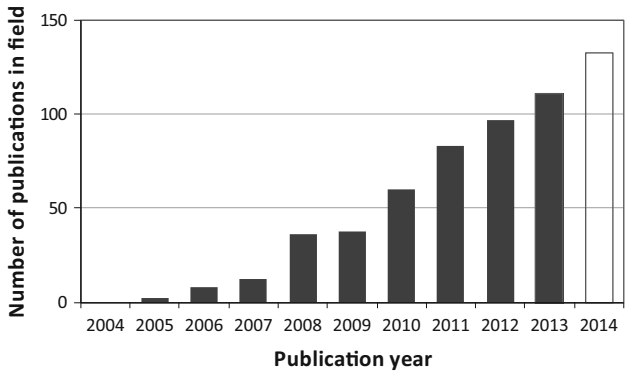


Fig. 1 Number of papers recorded in Web of Knowledge containing the search terms "nanoparticle* and ecotoxicol*" or "nanoparticle* and environmental toxicol*" identified in a search conducted on 11/12/13 (and so not including all 2013 papers), indicating the clear linear trend of increase over the 10 year period from 2004 to 2013 (* = wildcard character)

this published work focussed on assessing the nature of potential ENP hazards often using standardised test systems (for reviews see Handy et al. 2012b; Maurer-Jones et al. 2013). Increasingly, studies have begun to identify forms of environmental exposures and the routes of ENP uptake into organisms, while advanced bioimaging tools have begun to provide a refined picture of their internalised fate and distributions. Such information has contributed significantly to our understanding of a number of pathological effects relating to chronic toxicity and also to ENP transfer within food chains.

As experimental designs advanced, it has become increasingly apparent that integrated and robust multi-method approaches are needed to characterise the exposure and toxicokinetics (adsorption, distribution, metabolism, excretion) of ENPs especially under environmentally realistic scenarios. Here, taking examples mainly from in vivo studies with invertebrates, fish and plants we review current knowledge on the forms of exposure, uptake pathways and methods for tracking the internal concentrations and distributions of ENPs in organisms. Our review focuses primarily on metal and metal oxide, although for certain analyses, carbon based materials are also considered. From this summary of the state of the art, we seek to define the key themes that have characterised the first decade of ecotoxicological hazard assessment of ENPs.

ENP exposure and the importance of environmental transformations

Through advances in nanotechnology, synthesis of a large variety of nanoscale objects with diverse characteristics is now feasible. Assessments have shown that within ecotoxicology studies parameters such as core chemistry (Puzyn et al. 2011), size (Clift et al. 2008; Gorth et al. 2011), surface charge (El Badawy et al. 2011), crystalinity (Clement et al. 2013), oxidation state (Chen et al. 2012b) and shape (Ispas et al. 2009) can influence ENP exposure, assimilation and/or toxicity under certain scenarios. These findings emphasise the importance of characterisation of the starting material and continued analysis of chemistry and form throughout any experimental study.

Analyses of ENP fate and behaviour conducted in environmental systems (air, soils, waters) have commonly found that materials can undergo dynamic changes that alter their form from their starting characteristics. Such transformations fall into five main categories, each with the potential to influence the exposure and subsequent fate of ENPs within the organism (Bone et al. 2012; Pan and Xing 2012; Unrine et al. 2012): (i) Agglomeration; (ii) Dissolution; (iii) Redox reactions; (iv) Surface structural modifications including interactions with organic molecules. Further, these transformations can have implications for the nature of the materials assimilated into organisms that are also relevant for their trafficking and distributions once internalised (see schematic representation Fig. 2).

The ENP transformation that has to date received most attention is *agglomeration*. Collisions between particles result in co-attachment due to electrostatic, steric or hydration forces (Ottofuelling et al. 2011). Since agglomeration affects size distributions and potentially sedimentation rates, it is clearly an important factor governing potential exposure in air, soils and waters. However, at current predicted realistic environmental concentrations (Gottschalk et al. 2013, 2009; Johnson et al. 2011), associations with other particulate materials (hetero-agglomeration) may actually be more often favoured than homo-agglomeration. Hence it is not enough to consider interactions only between ENPs, but also between ENPs and other particulate and colloidal materials (Jarvie et al. 2009). The presence of multiple variant agglomerated (i.e. loosely bound) and aggregated (strongly chemically bound) forms in environmental exposures indicates the importance of understanding uptake mechanisms for a range of particle sizes and attachment states (i.e. associated or chemically bound to other ENPs or colloids). Detection methods need to be able to identify this wide size range (low nano- to micro-metre) of ENPs in environmental media including tissues and biofluids in which other materials may also be present. The presence of these larger agglomerates, however, can facilitate the identification of ENP assimilation using techniques with lower spatial resolution or poorer detection sensitivity than that needed for individual particles.

Dissolution rate is a feature of certain ENPs and can be highly relevant for fate and toxicity, since it determines the life-time of the material and can result in the production of

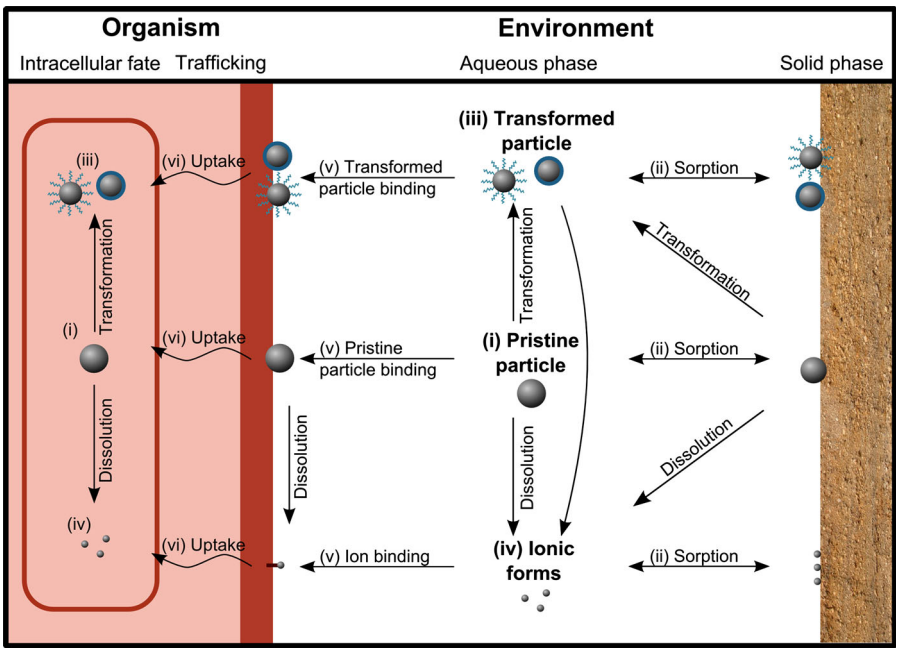


Fig. 2 Schematic representation of the complex behaviour and transformation of single nanoparticles (*grey spheres*) entering the environment in pristine form (*i*) where they can interact with the solid phase (*ii*), undergo surface modification such as changes in speciation and/or the attachment of organic molecules (*iii*) or undergo dissolution to ionic forms (*iv*); each of these forms may then associate with the surface either the organism (*thick red line*) surface via the body wall, respiratory membrane or alimentary epithelium (*v*) via which they may be taken up by passive diffusion, active transport or endocytosis into organisms (*vi*) in which they may then undergo further modification to transformed or ionic forms

potentially toxic ionic species. Dissolution is driven by particle size/surface area/shape as well as chemistry. Metals have long been known to be toxic in their ionic form (Di Toro et al. 2001; Morel and Hering 1983) and the production of ions from ENPs was recognized early as an important determinant of ENP toxicity (Puzyn et al. 2011). In the context of ENP uptake, solubilisation is important because it can result both in changes to particle size and also in the production of ionic species, which may be assimilated through known pathways (Sakar 1999). Even if assimilated as particles, there remains the potential for some ENPs to dissolve within cells and organelles. For example, ZnO nanowires were shown to dissolve very rapidly after entry into the acidic matrix of lysosomes and also in vitro in a surrogate solution of lysosomal pH, but not at circumneutral extracellular pH (Muller et al. 2010). The overall tendency for ENPs to dissolve can add both complexity and opportunity to attempts to track toxicokinetics and internal distributions (Fabrega et al. 2009a; Griffitt et al. 2008). For example, use of bulk elemental analysis for quantification of metal in digested tissue samples does not provide evidence of intact ENP uptake because complete digestion treats accumulated ENPs and ionic forms as equivalents. Thus, while measurement of total concentration can provide insights into overall toxicokinetics, confirmation of intact ENP uptake requires additional evidence centred on imaging of intact particles.

ENP surface properties are recognised as being critical to their fate and behaviour and hence modifications of these properties can affect the potential for exposure. For example, surface charge can have an important influence on ENP bioavailability (Farkas et al. 2011a). El Badawy et al. (2011) showed that positively charged Ag ENPs have a higher biological activity, and hence toxicity, than their negatively charged counterparts. ENP surface charge is known to be a function of the associated capping agent and the environment (pH and ionic composition) in which the material exists. In the body, physiological pH can vary between body organs encompassing extremes such as the acidic pH of the mammalian stomach and alkaline pH of the insect gut, and also within different sub-cellular compartments, like the acidic lysosome. Such physiological variations may lead to pronounced changes in ENP states as materials move through the cell (Sandin et al. 2012).

Prevailing conditions in environmental matrices can change the chemical *redox state* of materials altering not only surface properties, but also conditions throughout the particle (Kent et al. 2014). For example, analyses of ENP dosed sewage effluent and sludge has identified reaction products including sulphidised and phosphatised particles derived from ZnO (Lombi et al. 2012), sulphidised materials from Ag ENPs (Dale et al. 2013; Kim et al. 2010) and in CeO_2 ENPs a reduction of Ce(IV) to Ce(III) (Barton

et al. 2014). Awareness of such changes is important in the context of exposure and toxicokinetics because it may require tuning of studies to assess and track different chemical species including both the parent and transformed materials. Emerging evidence has begun to relate differences in biological effects to the different toxicokinetic and toxicodynamic characteristics of these different material forms (Ma et al. 2013). Measurements made using elemental and spectroscopic analysis methods, including synchrotron radiation based techniques, are of great potential value for these analyses, as they can help to distinguish the presence of internalised transformed as well as pristine forms.

The attachment of complex *organic molecules* to particle surfaces provides a further aspect of ENP modification that has received attention. The presence of these organic "coronas" has been shown to be a key factor influencing ENP environmental behaviour, uptake, and distribution in tissues and cells (Monopoli et al. 2012). In the environment, the attachment of natural organic materials, such as humic and fulvic acids, has been shown to influence ENP interactions with organisms (Fabrega et al. 2009a). Such "eco-coronas", thus, act as an important factor affecting adsorption (Fig. 2). On entry into tissues and cells, biological macromolecules (particularly proteins) can attach to ENP surfaces influencing how a particle is recognised. The changing nature of these surface modifications provide a further dimension that needs to be considered in respect of ENP uptake and handling by organisms.

Mechanisms of ENP assimilation into biological systems

As the field of ENP ecotoxicology has developed, the role played by different routes for ENP uptake into the body has emerged as a key aspect of toxicokinetics. In terrestrial organisms, major entry routes for ENPs can include inhalation, ingestion, and dermal uptake, while in aquatic organisms uptake over gill surfaces, olfactory organs, the alimentary tract and body wall may all be important (Apte et al. 2009; Moore 2006). From these exposure routes, a number of potential key target organs can be identified, including lungs/gills, skin and the intestine. After initial entry into the body, further organs, such as those known to be involved in detoxification (liver-like tissues, excretory systems) and the immune system, are often implicated as potential targets (Meyer et al. 2010; Moore 2006). There is, thus, an emerging picture of the most important routes of entry for ENPs and potential target organs that can be used for the future development of mechanistic models of ENP uptake.

While the potential major exposure routes for ENPs can be identified, and indeed accord with those for other environmental toxicants, the mechanisms of transport across cell membranes and into cellular matrices are currently less well understood. In cases where ENPs have undergone dissolution, a large knowledge base on the potential routes of uptake of the resulting ionic forms into microbial, plant and invertebrate species does exist that may also have some relevance for ENPs (Sakar 1999; Smolders et al. 2009). For example, ionic silver has been shown to be internalised via Na^+-channels and uptake can, thus, inhibit Na^+/K^+-ATPase activity (McGeer and Wood 1998). Schultz et al. (2012) show then that Ag ENP exposure can also block Na^+ uptake and Na^+/K^+-ATPase activity in juvenile rainbow trout. This suggests that the interactions applicable to ionic silver may also play an important role in Ag ENP toxicokinetics, although whether this is related to particle or ionic silver uptake was not discernible from the study design. What is evident from other work though, is that a range of studies have identified the presence of intact ENPs within cells, clearly showing that transport of ENPs can occur across biological membranes.

For intact pristine or transformed ENPs there are a number of general mechanisms that can potentially lead to cellular uptake including passive diffusion, facilitated diffusion, active transport and endocytosis (Stone et al. 2009). *Passive diffusion* is usually associated with the transport of low molecular weight substances and endogenous metabolites across cell membranes. Currently there are only limited (and mainly in vitro) data to indicate whether this simple process is relevant as a major route of movement of ENPs into organisms and cells (Treuel et al. 2013). Geiser et al. (2005) demonstrated that TiO_2 ENPs internalised by lung cells were not enclosed by a membrane, suggesting the involvement of a non-endocytotic pathway. Further, using a model biomembrane, Ormategui et al. (2012) were able to record the penetration of poly(*N*-isopropylacrylamide) based ENPs through a phospholipid monolayer in a mechanism driven through van der Waals forces (Vakurov et al. 2012). These observations suggest that the ENPs are sufficiently lipophilic to enter the membrane by passive diffusion or that they inflict sufficient damage to the membrane system to cause increased non-specific permeability (Vakurov et al. 2013). Such mechanisms may support redistribution especially in smaller species which rely more on diffusive processes and lack more active transport mechanisms such as a circulatory system.

Facilitated diffusion through membranes is achieved by carrier proteins and channels. These systems provide a potential internalisation pathway for ENPs that matches the size of the membrane pore. Ag ENPs have been shown to enter the chorion space and ultimately the inner cell mass of zebra fish embryos via the chorion pore channel (Lee et al. 2007). However, relevance of this route as a major pathway for ENP active transport into other more general

cell types has been questioned based on the suggestion that transport proteins may be highly specific for certain molecules and are not likely to bind ENPs (Stone et al. 2009). Observations of an interaction between polystyrene ENPs exposure and the activity of the ion channel in isolated epithelial cells suggests that there is a degree of interaction between the particle and components of these channels. Ultimately such effects may have implications for the homeostasis of relevant cell types (McCarthy et al. 2011). Further these results, and also those discussed earlier in relation to Ag ENPs and Na^+/K^+ ATPase activity (Schultz et al. 2012), suggests that ENP interactions with specific transport proteins may be more common than have so far been thought.

There is accumulating evidence that ENPs can be internalised at the cellular level by *endocytosis* and the role of these routes for uptake and distribution has been a key focus for work in the first decade of ENP ecotoxicology (Ding and Ma 2012; Iversen et al. 2011; Kim et al. 2012). Available evidence indicates that different sized ENPs may be taken up into cells through different endocytosis mechanisms that involve differently sized vesicles: macropinosomes 1–5 μm in diameter; clathrin mediated endosomes up to 120 nm; calveolae 50–80 nm in diameter (Patel et al. 2007). Use of pharmacological inhibitors for these respective pathways, coupled with toxicity tests or imaging techniques have provided important tools to investigate the contribution of these mechanisms to toxicokinetics. For example, dos Santos et al. (2011) used a combination of pharmacological inhibition and confocal microscopy to investigate the role of different endocytosis mechanisms for polystyrene ENPs uptake into human cells. Results illustrated the complexity of the mechanisms involved, as the major ENP internalisation pathway varied both between different ENPs and also different cell types. Ultimately, none of the pharmacological treatments tested fully prevented ENP uptake, suggesting that multiple endocytotic mechanisms might be involved.

In vitro endocytosis pathways can also be visualised by comparing the distribution of fluorescently labelled ENPs with the distribution of fluorescently labelled antibodies for the transport proteins under investigation. Employing this multi-labelling approach, ZnO ENP uptake could be attributed, at least in part, to calveolin-mediated endocytosis (Kao et al. 2012). Within the study, specific staining with Zn^{2+}-selective fluorescent dye provided evidence that observed cytotoxicity was associated with ENP dissolution in the late endosome and acidic lysosome following vesicle fusion. Using fluorescence immunolabelling, Shapero et al. (2011) have also identified co-localisation of SiO_2 ENP with lysosomes indicating their importance for ENP accumulation.

Endocytosis pathways have a deep phylogenetic origin (Galletta and Cooper 2009), and thus are well conserved, suggesting these mechanisms may also be important uptake routes in invertebrates that have been exposed to ENPs. The role of endocytosis in transport has, therefore, also been an important focus for studies with lower organisms. For example, work with the protozoa *Tetrahymena* has identified that this cilliate readily endocytoses TiO_2 ENPs and stores them in food vacuoles until the particles undergo exocytosis as larger aggregates. Further, Tsyusko et al. (2012) identified higher survival and altered Au ENP distribution following exposure of *C. elegans* mutant strains that were knockout for endocytosis pathways components including the *chc-1* (clathrin heavy chain) and *rme-2* (receptor mediated endocytosis) genes when compared to wild type animals (Bristol N2 strain). These results point to the importance of endocytosis for in vivo effects. Finally Canesi et al. (2010) working with *Mytilus* mussels identified concentration-dependent lysozyme release, extracellular oxyradical and nitric oxide production that could be linked to high potential levels of endocytosis in this filter feeder when exposed to C60 fullerene, TiO_2 and SiO_2 ENPs. These results, when taken together, firmly establish endocytosis as an important mechanism of uptake at least in some ENP ecotoxicology scenarios.

When ENPs come into direct contact with biological membranes there is the potential for *targeted membrane damage* (Lai et al. 2013; Nel et al. 2009; Valant et al. 2012; Zupanc et al. 2012). These interactions have been identified as one of the potential routes through which ENPs may enter into cells—particularly when membrane permeability is altered (Mu et al. 2012). For example, histological screening using *Xenopus laevis* larvae exposed to CuO, TiO_2 and ZnO ENPs identified that damage to the intestinal wall was strongly linked to the assimilation of the studied materials into the gut lining and that such uptake was ultimately linked to toxicity (Bacchetta et al. 2012). A range of mechanisms may contribute to any ENP associated membrane injury. Damage could be inflicted by the abrasive nature (i.e. sharp edges and corner defects) of some very hard material types (Apte et al. 2009; Stoimenov et al. 2002). Membrane attachment and contact lesions have also been shown and it has been indicated that these can cause changes in membrane permeability and stability. For example, flow cytometry and transmission electron microscopy (TEM) imaging demonstrated that CuO ENPs disrupt membrane integrity and create holes in the cell envelope of *E. coli* leading to cell leakage (Zhao et al. 2013). Whether such lesions result directly from ENP membrane interactions or are due to localised release of high concentrations of ions from attached particles subject to dissolution remains to be established. These effects do though clearly indicate a need to look beyond classic endocytosis mechanisms as potential routes of uptake in cases where cellular damage is indicated.

ENP distributions and their tracking through in situ characterisation and imaging

The classical paradigm proposes that for a substance to become toxic it must be both assimilated into the organism and also distributed in such a way that it comes into contact with a biological receptor (Fent 2007). Thus, as well as assessing mechanisms for toxicokinetics, the issue of understanding the distribution of ENPs both within target organs and within cells has emerged as an important area of research in ENP ecotoxicology. This has resulted in the application of a range of analytical and imaging-based tools with different spatial resolution and analytical sensitivities to elucidate localisations (Bandyopadhyay et al. 2013; von der Kammer et al. 2012). A non-exhaustive summary of the properties of some of the key imaging/analysis techniques that have been used to date are summarized in Table 1, with key benefits and limitations summarized in Table 2. The salient characteristics of in situ imaging/analysis techniques are also provided in valuable reviews by McRae et al. (2009), Ortega et al. (2009) and He et al. (2013). What has emerged as an important theme has been the notion that there is no ideal technique for imaging and analyzing unlabelled ENPs in biological tissues (Tantra and Knight 2010). The complex 3-D heterogeneous structures that frequently define biological specimens in which particles are 'embedded' do not lend themselves to imaging by a single approach which simultaneously offers high analytical sensitivity and high spatial resolution capability, coupled with ease of access, the capacity to provide metal–ligand speciation chemistry, element quantitation and what might be called 'efficient searching'. Indeed to a rough approximation there is a negative correlation between spatial resolution and analytical sensitivity across the available techniques (He et al. 2013). Additionally, generally techniques that achieve a higher the spatial resolution are also more time consuming, e.g. EM can visualize single particles but samples need to be extensively prepares and only a very limited number of frames can actually be analysed making quantification virtually impossible. Many imaging techniques also do not provide any elemental analysis, thus structures resembling ENPs cannot conclusively be identified without further characterisation (Fig. 2).

In cases where the specific cellular targets are not known, a common case in ecotoxicology studies, the use of a combination of detection methods is recommended. Thus, detection using instrumentation such as inductively coupled plasma mass spectrometry (ICP-MS) and atomic absorption spectroscopy (AAS), can detect the presence of materials at low concentrations. These do not provide detailed morphological information, but can be important for locating broad patterns of accumulation (e.g. identification of major target tissues). Higher resolution analysis can focus on the characterisation of individual ENPs and their interactions with the surrounding biological matrix. Such techniques can potentially be very time- and resource-consuming as well as technically challenging. In reality there is no simple dichotomy between high resolution/low sensitivity versus low resolution/high sensitivity techniques; different techniques offer particular strengths and weaknesses which favours combinatorial or complementary usage (Table 2). Indeed, Bandyopadhyay et al. (2013) advocate a hierarchical or tiered approach, and based on the findings from ecotoxicology studies we offer a potential schema in Fig. 3 to illustrate the principle. Such a generic scheme is not meant to be prescriptive, but rather the basis for question-driven 'decision tree' analogous to that presented by Dudkiewicz et al. (2011) for operational selection among the variants of electron microscopy. Even within a tiered imaging/analysis strategy, pragmatic compromises may still be necessitated with careful judgment needed for interpretation and appropriate selection of procedural controls.

As the need has emerged to understand the distribution of ENPs in organisms, the methods applied have largely been drawn from general biology. In some cases, these have been modified to take account of the specific requirements of ENP

Table 1 Characteristics of the main spatially resolved analytical approaches that can be used for chemical imaging to assess the distributions of elements related to ENP exposures in biological samples (adapted from Lobinski et al. 2006)

Technique	Detection limit (μg/g)	Spatial resolution (μm)	Selectivity	Quantitation	Analytical depth (μm)
LA-ICP-MS	0.01	15–50	Multielement isotopic	Semi-quantitative	200
SIMS	0.1	0.05	Multielement isotopic	Quantitative	0.1
CARS	10	0.5	Single element	Semi-quantitative	>1,000
μ-focus XAS	100	0.1–1	Speciation	N/A	>100
Synchrotron microprobe	0.1–1	0.1–1	Multielement ($z \geq 6$)	Semi-quantitative	>100
Ion beam microprobe	1–10	0.2–2	Multielement (all z)	Quantitative	10–100
μ-PIXE	1–20	0.5	Multielement ($z \geq 6$)	Quantitative	10–50
EELS	1,000	0.001	Multielement ($z \geq 6$)	Semi-quantitative	<0.05
EM-EDX	100–1,000	0.5	Multielement ($z \geq 6$)	Quantitative	0.1–1

Table 2 Key benefits and limitations of analytical and imaging techniques used for ENP in situ tissues localisation and characterisation studies

Analytical techniques	Information	Benefits	Limitations
Elemental analysis	*Quantitative assessment of element concentrations in samples for assessment of broader accumulation and distribution patterns*	*Single and multi-element detection at low concentrations (ppm-ppb); relatively simple sample preparation; wide access to instruments*	*No detailed morphological information; cannot distinguish between particulate or dissolved forms and internalisation or surface attachment; issues when identifying ENPs with core chemistry comprising elements with high natural background*
Atomic absorption spectroscopy	Simple element quantification to low ppm concentrations for traditional analysis and low ppb for graphite furnace	Relatively simple preparation; relatively low machine and running cost; very low level detection by graphite furnace	Single element per analytical run; matrix effects can result in signal interferences
Inductively coupled plasma mass spectrometry (ICP-MS)	Multi-element detection to low ppb concentrations	Efficient approach to multi-element detection; relatively simple sample preparation; commercial services available	Some analytes (e.g. Ag) require use of more complex digestion procedures; matrix effects can lead to interferences
Single particle ICP-MS	Multi-element detection of putative single particles in complex sample matrices	Detection of individual ENPs with information on potential size (peak height) and concentration (peak frequency)	Conventional sample preparation for ICP-MS (e.g. acid digestion of tissue samples) unsuitable for analysis in tissues; identification against natural background can be challenging
Laser ablation ICP-MS	2D quantitative elemental distribution maps, spatial resolution 5–200 μm and detection limits in lower ppb range	Relatively easy sample preparations can be used; multi-element; spatially resolved detection	Trade off spatial resolution with analytical sensitivity
Microscopy and spectroscopy	*Imaging of sample in real time, direct visualisation in vivo*	*Relatively easy sample preparation and se t up; availability of stains and fluorescent probes; instrumentation widely available*	*Limited sample resolution; visualisation of single ENPs in tissue not always feasible unless suitably labelled*
Confocal laser scanning microscopy (CLSM)	100–200 nm resolution; imaging through autofluorescence, fluorochrome tagging/staining or reflectance from highly scattering, unlabelled objects like metal ENPs	Stacks of 2D images can be reconstruct into 3D for internal localisation ENPs; co-labelling of molecular biomarkers possible	Reduced sensitivity and spatial resolution compared to electron based imaging methods; potential need for labelling; potential sample damage through laser beam; artefacts due to photobleaching; interference of background autofluorescence
Dark field microscopy	Detection of ENPs with strong localised surface plasmon resonance (e.g. nobel metal ENPs)	<200 nm resolution; elimination of unscattered beams from microscope images; combination with confocal microscopy: 3D imaging of unlabelled ENPs	Unsuitable for lower Z numbers; no chemical specificity; no visualisation of individual ENPs
Raman microspectroscopy	Sub-μm resolution; mapping of biomolecular distribution	High signal enhancement close to surfaces of specific metals can be used to identify and image ENP distribution	No direct identification of ENPs; no direct elemental analysis; potential sample damage through laser beam
Coherent anti-stokes Raman Scattering microscopy (CARS)	500 nm resolution; ENPs distribution is implied through C–H signal enhancement	Increases Raman scattering efficiency	No direct identification of ENPs; no elemental analysis; potential sample damage through laser beam; signal artefacts; dense inclusions cannot be further chemically identified for confirmation

Table 2 continued

Analytical techniques	Information	Benefits	Limitations
X-ray absorption spectroscopy using synchrotron radiation (XAS)	*Sub-micron imaging (100–200 nm) and chemical speciation of solid samples including biological tissues*	*Minimal sample preparations can be used, imaging of wet samples possible, multi-elemental mapping*	*Sample resolution does not enable detection of individual ENPs; spatial analysis of large samples can require long run times, considerable expertise necessary for interpretation; requires specialised facility time*
Micro X-ray fluorescence (μ-XRF)	Sub-μm resolution and ng/g detection limit; μ-XRF: 2D/3D imaging in situ;	Coupled with XAS provides structural information on ENP interactions with surrounding ligands, and speciation of ENP related domains	No visualisation of individual ENPs; elemental but not speciation mapping
Extended X-ray absorption spectroscopy fine structure (EXAFS)	Quantitative information on chemical environment of a single element: number and identity of neighbour atoms, inter-atomic distances and structural features	Provides detailed information on chemical environment and associated ligands	No imaging of ENPs at subcellular level; generation of artefacts; beam damage; interference of multiple scattering by photoelectron and auger electrons; only feasible in major national synchrotron facilities
X-ray near-edge absorption spectroscopy (XANES)	Quantitative information on chemical environment of a single element: oxidation states; neighbour atom identity, inter-atomic distances and structural features	Can be used to distinguish intact material and reformed biogenic mineralised form based on speciation states	No imaging of ENPs at subcellular level; relatively high concentrations needed for speciation analysis in samples
High resolution microscopy and spectroscopy	*Visualisation and analysis of particles down to sub-nm range using particle beams*	*Precise intracellular distribution; combination with EDX or WDX for chemical identification and (semi) quantitative elemental analysis*	*Can be time-consuming; may need elaborate sample preparation; high concentrations needed for biodistribution anlysis; cannot distinguish NP from other electron dense spots; cannot determine if original particles or biogenic objects without further work*
Micro particle induced X-ray emission (μ-PIXE)	Quantitative elemental analysis of tissues and single cells down to sub micron—nano resolution	Simultaneously detection all elements of $z \geq 11$; Fully quantitative	Inferior spatial resolution compared to electron beam instruments; no visualisation of individual ENPs
Scanning electron microscopy (SEM)	Surface imaging of thick samples to low nm resolution	Detection on individual ENPs in samples and validation of chemical identity using EDX or WDX	Dry (dehydrated) samples unless environmental SEM or wet cells; artefact can occur during sample preparation; quantification time consuming
Transmission electron microscopy (TEM)	Visualization of ENPs within cells and simultaneously assessment of changes of cellular structures	Ultra-high resolution characterisation of ENP structures and chemical identify	Potential sample damage (staining, analysis under vacuum) through prep, sample preparation can be complex; quantification of ENPs time consuming, speciation not possible
Secondary ion mass spectroscopy (SIMS)	Spatial resolution of up to 50 nm; elemental, isotopic or molecular composition of degraded sample	Simultaneous analysis of analyte of interest with other elements and light molecules; detection and analysis of unlabelled ENPs	Relatively insensitive for some metals, but better for metal oxides Background interference reduces resolution; yield of secondary ions depends on ionisation potential of materials

ecotoxicology. With a decade of work now published, it is possible to analyse key requirements for successful deployment for in situ analysis. This includes appropriate sample preparation for ENP imaging and analysis, as well as the salient characteristics and exemplar applications of key imaging/analysis techniques for ENP localisation. Relevant methods used fall into four general categories.

(i) Those that primarily rely on analytical detection of chemical constituents often to low levels, but

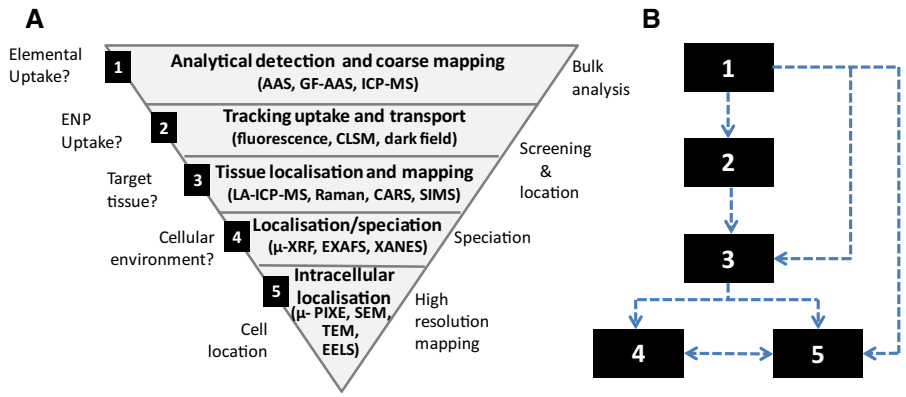


Fig. 3 **a** A tiered relationship of potential approaches for the assessment of ENP assimilation into organism tissues moving from higher throughput analytical techniques, through lower resolution detection and tissue localisation methods onto detailed techniques capable of identifying ENP speciation states and precise intracellular localisations. **b** Flow diagram to indicate how the greatest value for the application of these technique are gained when different types of methods are used in combination; for example elemental detection (*Box 1*) may be augmented by studies to detect target organs (*Box 3*) before detailed speciation (*Box 4*) and high resolution techniques (*Box 5*) are applied

with no information on detailed spatial distributions within the sample.

(ii) Those based on optical microscopy and spectroscopy, including Raman microspectroscopy, that are able to detect unlabelled ENPs (i.e. to visualize them essentially as microdense inclusions) at modest to high resolution.

(iii) Variants of high-brightness synchrotron-based X-ray microprobes with relatively modest imaging capabilities but with the unique ability to determine the chemical environment (e.g. next nearest neighbour).

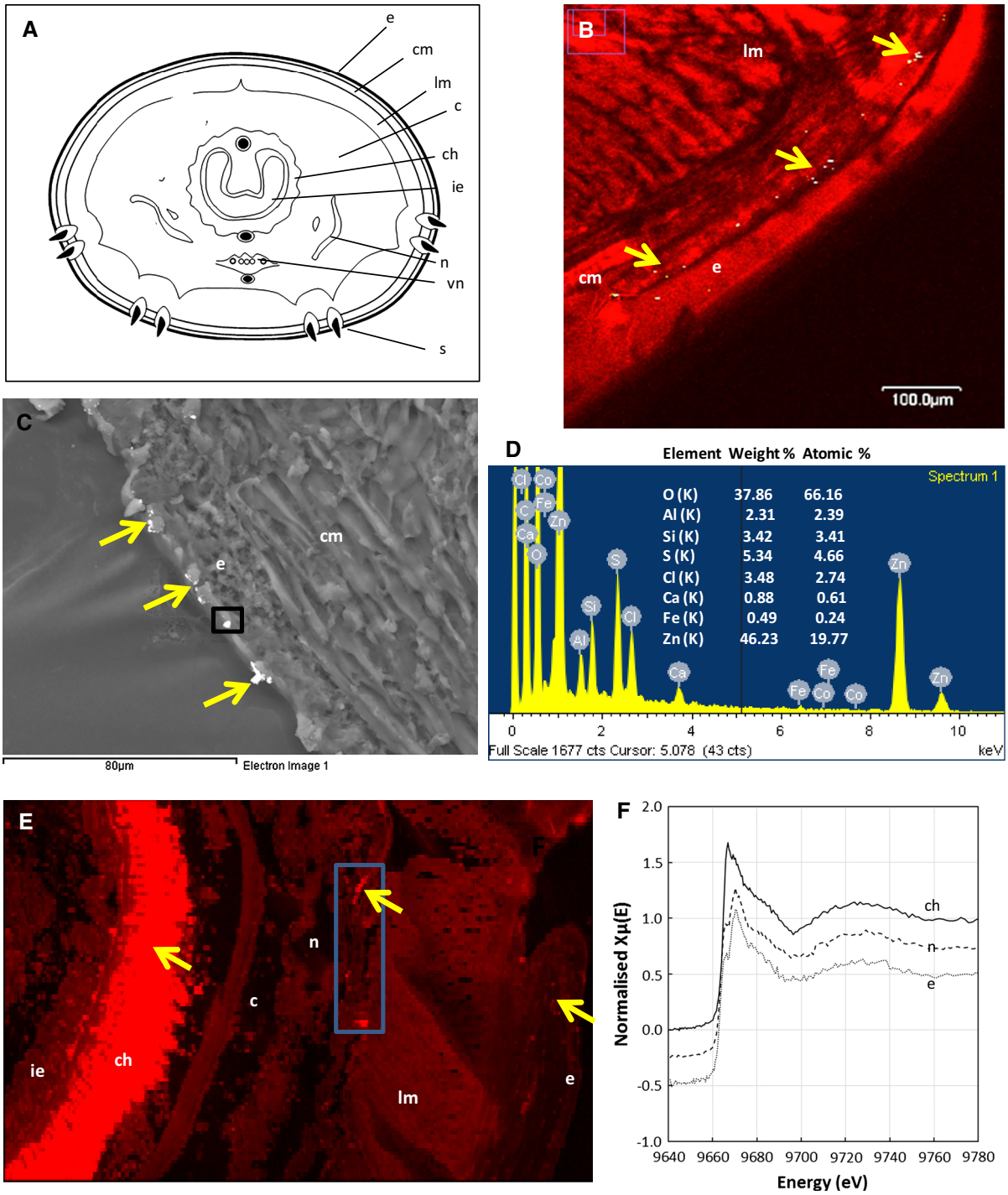
(iv) High resolution analytical methods such as secondary ion mass spectrometry (SIMS) and micro particle-induced X-ray emission, (μ-PIXE) and also electron microscopy and ion beam techniques including energy-dispersive X-ray spectroscopy (EDX) and electron energy-loss spectroscopy (EELS) that can image and analyse local elemental chemistry sometimes to high spatial resolution and high sensitivity.

These four categories cover a range that broadly represents a trend of increasing spatial resolution yet decreasing throughput of imaging. Examples of some of the imaging outputs taken from unpublished work conducted by the authors using invertebrate species can be seen in Fig. 4.

Sample preparation for imaging/analysis: common considerations

As with all aspects of biological specimen imaging, attempts to establish distributions of ENPs in situ in organisms are strongly dependent on the preparation of appropriate high quality samples. The technical challenge is to ensure that the chosen method of sample preparation and the analysis itself does not significantly alter the chemistry of the assimilated ENPs whilst rendering them visible in their tissue and cellular locations. Different imaging techniques may impose practical constraints on the way a sample is prepared and presented. TEM by definition requires thin samples either mounted on a thin film or suspended across a hole. Scanning electron microscopy can image and analyse the surface of thick samples from which water must either be removed, or immobilized by freezing under high vacuum unless environmental scanning electron microscopy or wet cells are used. Raman microspectroscopy in contrast can image wet stationary samples preferably with uncompromised lipid inclusions.

A biological specimen that has been compromised cannot be 'restored' by imaging optics—however sophisticated and elaborate, hence the vital importance of correct initial preparation (Morgan et al. 1999). This does not, however, mean that specimen preparation always needs to be technically elaborate. For example, live cells grown on glass slides have successfully been used for nano-SIMS applications (Hagenhoff et al. 2013) and the $\sim$ 1 mm long nematode *Caenorhabditis elegans* frozen and freeze-dried on an appropriate substrate was suitable for synchrotron excitation X-ray fluorescence mapping (Gao et al. 2008). *C. elegans* have also been maintained alive in a chambered slide for hyperspectral imaging spectroscopy (Meyer et al. 2010) or for fluorescence microscopy after immobilizing with sodium azide or levamisole on an agar pad sandwiched between glass coverslips (Mohan et al. 2010). Donner et al. (2012) showed that distribution and speciation observations can be made on fresh intact plant material



analysed in bespoke chambers designed to minimise dehydration (as well as on freeze-dried or frozen-hydrated samples) in synchrotron microprobe systems.

Beyond these 'simple' approaches, there are two generic options for sample sectioning prior to imaging. These are ambient and low temperature ("cryo") procedures (Morgan et al. 1999). Ambient methods generally involve fast penetrating fixation to denature enzymes and stabilize structural proteins by crosslinking followed by embedding and sectioning. These 'conventional' methods have been the work-horses of biological imaging for a number of years. They can, however, introduce compositional artefacts characterised by the leaching and redistribution of inorganic and organic constituents that need to be

◀**Fig. 4** Images and analytical outputs from three different bio-imaging techniques applied to the tissues of the lumbricid earthworm, *Lumbricus rubellus* exposed to environmentally relevant concentrations of ZnO NPs in soil. The data are presented to illustrate the complementarity of the selected techniques and how they might be deployed in a hierarchical ENP detection/localization/characterisation scheme. **a** Schematic diagram of a transverse section in the intestinal region depicting the major tissues and organs. **b** CARS image of a thick transverse section of *L. rubellus* exposed to 1,000 mg kg^{-1} ZnO-NPs for 48 h, fixed in 4 % formaldehyde and sectioned by hand. The image was produced by tuning incident lasers to generate vibrational contrast from excitation of lipid CH_2 bonds. The strong scattering signal produced by metallic NPs is independent of the wavelength of the pump beam and can be distinguished by detuning the laser away from the lipid signal. Note that this efficient 'searching' technique locates densities (*arrows*), and can penetrate deeply into tissue, but cannot determine their composition or definitively distinguish ENPs from other metallic inclusions. **c** Back-scattered electron image, taken in a scanning electron microscope, of a region of the body wall in a transverse cryostat section (60 µm, mounted on graphite) of NBF-fixed, sucrose-infused, mouth-unsealed earthworm exposed for 7 days to 4,000 mg kg^{-1} ZnO-NPs; note the electron dense aggregates (*arrows*) on or in the epidermal layer. **d** Energy dispersive X-ray spectrum of the deposit enclosed within the black square in 'C'; the table superimposed on the spectrum provides a quantitative estimate of the **e**lemental composition of the mineralized deposits; note that the Zn counterion is predominantly an oxide (implying that the aggregates probably derive from the NPs to which the earthworm was exposed), not sulphate/sulphide or a phosphate. **e** High resolution Zn fluorescence distribution map obtained on the microfocus beamline at the Diamond Light Source synchrotron, UK; the tissue was sectioned (10 µm, mounted on a 'Spectrosil' glass slide) after alcohol fixation, methacrylate resin-embedding, and no staining; note the occurrence of uniformly high Zn concentration in the chloragogenous tissue, and high focal Zn concentrations in regions of the body wall and the nephridial tubule. **f** Three Zn (K-edge) XANES spectra derived from Zn-rich pixels located in the epidermis, chloragogenous tissue, and nephridium (*blue rectangle*) - see arrows in 'E'; quantitative modelling and speciation (data not shown) confirm that differences in the spectral profiles indicate that the Zn atomic centres within the matrix of the focal deposits imaged in epidermis and nephridium are associated with oxide, a chemical phase distinguishable from the phosphate-associated Zn pool in the chloragogenous tissue. *c* coelomic cavity, *ch* chloragogenous ('liver-like') tissue, *cm* circular muscle layer; *e* epidermis; i.e., intestinal epithelium, *lm* longitudinal muscle layer, *n* nephridial (urine-forming) tubule; s, paired setae (or 'chaetae'), *vn* ventral nerve cord (*e*, *cm*, and *lm* collectively form the body wall of earthworms)

considered in studies with ENPs. Cryo-procedures have been developed to overcome some of the problems with ambient protocols and have been vaunted as providing a more faithfully approximation of in vivo states. However, even with the best available approaches (e.g. plunge or impact freezing using liquid propane or ethane that cool tissue at up to 10,000 °C s^{-1}), the poor thermal conductivities of biological materials and ice can limit the ice layer to no deeper than about 10–20 µm (i.e. approximately one cell diameter). Despite less than optimal preservation, cryostat sections with thicknesses ranging from 10 to 100 µm are acceptable for a range of low- and intermediate-resolution applications in animals (Becker et al. 2011; Morgan et al. 2013; Novak et al. 2012) and plants (Tian et al. 2010). For high resolution applications such as TEM, the well-frozen surface layer can be significantly deepened to around 200 µm in fresh tissues by hyperbaric freezing or alternatively the ice damage can be eliminated by pre-treating specimens typically by mild aldehyde fixation followed by infusion with a cryo-protectant such as sucrose.

Elemental analysis including single particle, isotopic and spatial analyses

Prior to application of imaging techniques, elemental analyses of ENP components (e.g. metal ions) in digests or homogenates of tissues, organs, or whole organisms can represent a valuable approach for quantitative assessments of assimilation and distribution across the body. As methods for the analysis of inorganic ENP components were already very widely available in ecotoxicology, these on techniques such as inductively coupled plasma optical emission spectrometry (ICP-OES), ICP-MS and AAS have already been widely deployed a first tier procedures (e.g. Coutris et al. 2012; Khan et al. 2012; Stewart et al. 2013). A notable limitation of these analytical methods in the context of ENPs uptake is that they are incapable of distinguishing between internalised metal pools that remain in particulate form or that have undergone dissolution to ions either prior to or following assimilation and distribution into target tissues. Thus, these approaches alone cannot provide confirmation of intact ENP assimilation, but rather confirm only that material that is associated with the ENPs under study has been taken up.

A development of the analytical approach that may be useful for putative identification of intact ENPs is single particle ICP-MS (SP-ICP-MS). In this method, a sample of interest is prepared and aspirated in such a way that any ENPs are retained in the sample intact and are then able to enter the ICP-MS plasma individually. Initially used for the detection of ENPs in commercial products (Mitrano et al. 2012; Reed et al. 2012), SP-ICP-MS has since been adapted for use for effluents and receiving water where it has been suggested as a monitoring approach (Farkas et al. 2011b; Mitrano et al. 2012). Conventional ICP-MS sample preparation for biological specimens, i.e. tissue digestion by strong oxidising reagents, such as strong acids, is unsuitable for SP-ICP-MS analysis, since particles will also be digested and solubilised by the acid. With careful use of alternative digestants, suitable preparation of tissues can still be achieved. For example, SP-ICP-MS has been used to identify the presence of Ag ENPs in crude homogenate samples of exposed rats (van der Zande et al. 2012) and in

alkaline or enzymatic tissue digests of the aquatic oligochaete *Lumbriculus variegatus* (Coleman et al. 2013).

At realistic environmental concentrations, it can be analytically challenging to identify the assimilation and distributions of ENPs with core chemistries that include elements that have a high natural background (He et al. 2013). This includes carbon based and also some metallic element based materials (e.g. ZnO, CuO, Fe_3O_4, Fe_2O_3). In these circumstances, stable isotope-doped ENPs have provided notable insights into toxicokinetics and in vivo tissue distributions. For example, stable isotope analysis has shown that ^{67}Zn or ^{68}Zn associated with or derived from ZnO ENPs is bioavailable to aquatic and sediment-dwelling invertebrates including *Corphium volutator*, *Lymnaea stagnalis*, *Scrobicularia plana*, *Hediste diversicolor* and *Peringia ulvae* (Buffet et al. 2012; Khan et al. 2013; Larner et al. 2012; Misra et al. 2012). In these studies, it was feasible to detect ENP derived Zn uptake even when the exposure concentration of the isotope enriched materials was only a minor fraction (<10 %) of ambient Zn background. The toxicokinetic parameters derived from these kinds of studies can provide important information on equilibrium concentrations. However, the constraint that the analytical method cannot discriminate between intact ENPs and ions produced by dissolution remains relevant for this technique.

As a step towards more spatially resolved analysis of ENPs within tissues, laser ablation ICP-MS (LA-ICP-MS) provides an approach capable of producing 2-D elemental distribution maps in a variety of specimens. LA-ICP-MS can be used to simultaneously analyse a range of elements, including isotopes (Becker et al. 2010; Durrant and Ward 2005) with a spatial resolution of 5–200 μm and detection limits in the lower ppb range (Qin et al. 2011). Technical improvements are currently being developed, such as near field LA-ICP-MS (Zoriy and Becker 2009) that may take spatial resolution to <200 nm, although this comes with the potential for some trade-offs in analytical sensitivity (Wu and Becker 2012).

The basis of LA-ICP-MS is the use of a focussed laser to precisely ablate areas of a sample in an orderly raster pattern that are then transported by a carrier gas (usually argon) into the ICP-MS instrument. When coupled with analysis of matrix matched standards, these measurements can produce quantitative spatial elemental maps. Specimen preparation for LA-ICP-MS is relatively easy, partly because imaging is performed at atmospheric pressure. Whole tissue or 20–30 μm thick cryosections are ideal (Becker et al. 2010; Wu and Becker 2012), as these dispense with the need for elaborate fixation, dehydration and resin or wax embedding which might alter the distribution, concentration, or chemical form of analytes. Overall quantification using LA-ICP-MS is generally easier to achieve than with other mass spectrometry techniques such as SIMS and Matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) (Santos et al. 2009), hence the method can act as a good first tier spatial distribution analysis tool.

LA-ICP-MS had already found limited, but expanding application in metal ecotoxicology research. For example, LA-ICP-MS has been successfully employed to measure heavy metal, notably Zn, accumulation in *Arion* slugs (Becker et al. 2007), tissue metal distributions in the marine prosobranch snail *Nassarius reticulatus* collected from polluted sites (Santos et al. 2009) and to identify naturally occurring iron-rich ENPs in worker bees (Wang et al. 2013). The majority of ENP ecotoxicological studies conducted so far using LA-ICP-MS have taken advantage of the relative stability and insolubility of Au ENPs to investigate potential uptake into plants (Judy et al. 2011, 2012; Koelmel et al. 2013), insects (Judy et al. 2011) and earthworms (Unrine et al. 2010a). These studies have all revealed that Au ENPs of different sizes and surface chemistry can be assimilated into organisms where they are distributed often into tissues known to be involved in metal sequestration. These sites include plant roots and also liver like tissues, such as the hepatopancreas of arthropods, molluscs and fish and chlorogogenous tissue that surrounds the earthworm gut. An important finding developed from this initial work was an understanding of the potential of these accumulated materials for further trophic transfer (Judy et al. 2011).

Light, confocal, dark field and spectroscopy detection and analysis

Light, confocal and dark field microscopy methods are well established in biology and can be used with a range of relatively simple sample preparation methods. These characteristics have encouraged their use in ENP ecotoxicology studies at an early stage, despite a relatively limited sample resolution of approximately half the wavelength of light ($\sim$200 nm in conventional systems, or $\sim$100 nm under certain optical arrangements) making it impossible to visualise individual ENPs in brightfield and phase contrast modes. Although resolution does not allow individual nanoparticle imaging, detection of fluorescent ENPs, such as quantum dots, can be achieved (Feswick et al. 2013; Rosenkranz et al. 2009). Coupled imaging of individual fluorescent-tagged ENPs with fluorescent co-labelling of molecular biomarkers of cellular stress and apoptosis has been widely used as an approach to study the relationship between ENP distribution and cellular status (Mohan et al. 2010; Pluskota et al. 2009; Qu et al. 2011).

Confocal laser scanning microscopy (CLSM) allows tissue component imagery at 100–200 nm resolution through autofluorescence, fluorochrome tagging or staining, or reflectance from highly scattering objects like metal

particles (Cox and Sheppard 2004). The ability of CLSM to optically section specimens to yield a stack of in-focus, perfectly in-register, 2-D images from which three-dimensional reconstructions can be made provides a useful approach to assessing internal localisation of assimilated ENPs (Tantra and Knight 2010). In reflectance mode, CLSM has convincingly detected the internalisation of unlabelled Ag ENPs and CeO_2 ENPs by cultured fish and human cells (Gaiser et al. 2012). Further, CLSM has also been used to demonstrate the attachment of TiO_2 ENPs to micro-algal cell surfaces (Chen et al. 2012a) and the transport of quantum dots across the gut epithelium of exposed *Daphnia magna* and *Ceriodaphnia dubia* (Feswick et al. 2013). Detailed imaging using CLSM of resin sections of exposed cladocerans has further shown that ENPs are predominantly located inside the digestive tract, but not within the gut epithelia or the brood chamber of the exposed organisms (Li et al. 2011). These results show that these kinds of relatively accessible lower resolution methods can be used to provide information on potential target tissues that can underpin later more detailed analyses.

Dark field microscopy is an alternative and again relatively accessible technique that can be used to image relevant biological specimens. The technique relies on elimination of unscattered beams from light (and electron) microscope images so that the field around the scattering specimen appears dark. The technique is not reliant on fluorescent labelling or staining and is applicable to living cells for which its contrast-enhancing capability facilitates detection below the 200 nm detection limit possible with conventional light. It does not, however, provide any form of chemical specificity. Using dark field optics, noble metal nanoparticles, such as Ag and Au in particular can be detected in biological matrices due to their specific optical properties (strong localised surface plasmon resonance) (Stebounova et al. 2011). The interfacing of dark field imaging with confocal microscopy can now also allow effective 3-D imaging of unlabelled ENPs in cells and tissues as an approach that clearly can be relevant to ecotoxicology studies (Gibbs-Flournoy et al. 2011).

Dark field microscopy has so far found niche applications in ENP ecotoxicology studies (Harutyunyan et al. 2010). Using the technique in single particle imaging mode, Lee et al. (2007) tracked the movement of Ag ENPs into and out of living zebrafish embryos via chorion pore canals, identifying this biological structure as a potential route of embryo exposure. Weinkauf and Brehm-Stecher (2009) also used dark field to investigate binding of thirteen differently cored ENPs by *Candida albicans* in relation to anti-fungal properties, while Roh et al. (2009) also used dark field microscopy to study Ag ENP internalisation and aggregation in the uterine area of *C. elegans* and the link to reproductive failure. Combining dark field microscopy with visible and near-infrared hyperspectral imaging offers higher sensitivity imaging (Hutter et al. 2010). Meyer et al. (2010) used these complimenting approaches to detect the internalisation of uncoated Au ENPs into a number of cell types in *C. elegans*, including the eggs of a mutant strain with an egg laying defect. Similarly, Leclerc and Wilkinson (2013) used dark-field hyperspectral imagery to show the presence of Ag ENPs inside green algal cells, although in this case it was concluded that these inclusions were more likely to be due to the internalised rereduction of Ag^+ than to the internalisation of the Ag ENPs in an intact form.

Combining microscopy with laser light derived spectroscopy can provide sample analysis at moderately high resolution. Raman microspectroscopy is one such approach. The technique is based on the inelastic scattering of monochromatic laser light to give information on characteristic low frequency (e.g. vibrational) transitions of molecules. The resulting spectra can be used as a "fingerprint" of molecular composition in both processed (dried, fixed and/or sectioned) and live tissue samples. Due to the relatively high spatial resolution (sub-micron) when combined with a microscope, imaging of Raman signals is possible by rastering either the laser or the microscope stage to produce maps of biomolecule distribution in cells and tissues (Donfack et al. 2010; Schie and Huser 2013). An additional feature of Raman scattering is the extremely high level of signal enhancement (as much as 10^{10}) derived as Surface Enhanced Raman Spectroscopy (SERS) when molecules are in close proximity to the surfaces of specific metals showing nano-scale features or nanoscale roughness (plasmonic enhancement). This feature has been used in ENP ecotoxicology to simultaneously identify and image ENP distributions including TiO_2 ENPs in biofilms (Battin et al. 2009) and individual cells of the cyanobacterium *Anabaena variabilis* (Cherchi et al. 2011) and also to locate and map the distribution of polystyrene ENPs (Dorney et al. 2012).

Since the Raman spectroscopy signal is quite weak, with only a small proportion (10^{-8}–10^{-10}) of photons subject to Raman scattering, coherent anti-stokes Raman scattering microscopy (CARS) has been applied as a means of increasing scattering efficiency. The CARS method relies on a non-linear four-wave mixing technique to irradiate the sample, which significantly enhances the Raman signal when tuned to a Raman-active vibrational band. At high energy, the laser can also cause significant sample damage, resulting in notable signal artefacts, which can be an issue for data interpretation.

In fish, CARS has shown that ZnO ENPs (Moger et al. 2008) and TiO_2 and CeO_2 ENPs (Johnston et al. 2010) are accumulated within gill tissues and that ZnO ENPs (Scown et al. 2010) are internalised by cultured fish hepatocytes in vitro. However, not all CARS studies conducted have necessarily indicated potential for ENP uptake. For

example, intact Ag ENPs were not found to traverse the chorionic membrane of exposed zebrafish embryos (Osborne et al. 2013). This finding is not consistent with the results from other studies that have indicated that the embryo may be exposed to Ag ENPs via this route (Lee et al. 2007; Nallathamby et al. 2008; van Aerle et al. 2013) suggesting that the potential for ENP transport into a biological system is governed by unique properties of the specific ENPs involved.

For invertebrates, Galloway et al. (2010) used CARS to locate TiO_2 ENPs in the gut lumen and on the outer epidermis of the marine polychaete *Arenicola marina*, although no evidence of intracellular uptake of intact ENPs was found. Similarly, CARS imaging in conjunction with scanning transmission electron microscopes (STEM) with energy-dispersive X-ray (EDX) spectroscopy detected Zn aggregates in the gut lumen and hepatopancreas of the marine amphipod *Corophium volutator*, but again cellular internalisation was not evident (Larner et al. 2012). Such results provide an illustration of the need to conduct detailed studies of distributions to ascertain not just whether materials are consumed into the gut or attached to external body surfaces, but also whether they are then assimilated across the gut lining or body wall and further translocated to other organs and tissues. One shortcoming of the CARS technique is that the method does not offer elemental analysis to confirm the chemical identity of putative nanoparticles. Complimenting CARS assessments with other approaches, such as electron microscopy with X-ray techniques can overcome this issue. However, without supporting evidence, CARS can only offer circumstantial evidence of the presence of intact ENPs in any given sample.

Synchrotron radiation microprobe X-ray analysis

Synchrotron radiation is used for sub-micron imaging and chemical speciation of solids and can be used to map detailed elemental distributions by measuring the emitted fluorescence from a rastered analysis of the sample after excitation with an incident X-ray beam at resolution in the range $\sim$100–200 nm. In this respect, the synchrotron acts as a high intensity radiation source. To further elaborate on the surrounding chemical environment, micro X-ray fluorescence (μ-XRF) approaches can be coupled with X-ray absorption spectroscopy (XAS) to provide structural information on ENP interactions with surrounding ligands in the relevant matrix. Combining the two techniques can be used to speciate ENP related nanomaterial domains that are initially mapped out and putatively identified using μ-XRF.

Two types of XAS are extended X-ray absorption fine structure (EXAFS) and X-ray near-edge absorption spectroscopy (XANES) (Handy et al. 2008). Both techniques can be used to define the chemical environment of a single element in relation to the number and type of neighbour atoms, inter-atomic distances and structural features. In ecotoxicology, synchrotron based X-ray mapping and chemical speciation analysis have been used to define intracellular distributions of trace metals in a range of invertebrates exposed to elevated concentrations. These have identified the different coordination of Zn and Pb with phosphate and oxygen atoms respectively (Morgan et al. 2013) and of Cd with sulphur ligands likely to be associated with metallothionein in earthworm tissue sections (CotterHowells et al. 2005; Stürzenbaum et al. 2004). Identification of these different ligand environments can provide valuable information for ENP ecotoxicology studies because it can be used to assess whether materials remain intact or are reformed into other biogenic mineralised forms with different co-ordination.

Servin et al. (2012) applied μ-XRF and μ-XANES to track the presence and chemical speciation of TiO_2 ENPs in cucumber (*Cucumis sativus*) tissue. Results demonstrated that titanium was translocated from the roots into the leaf trichomes and that the absorbed TiO_2 ENPs were not biotransformed. μ-XANES has also been employed to assess uptake and biodistribution of Au ENPs in tobacco plants (*Nicotiana xanthi*). Here internalisation was found to be size selective, such that of the two tested particle sizes (3.5 nm and 18 nm) only the smaller ones were taken up and subsequently transported into the vasculature (Sabo-Attwood et al. 2012). Unrine et al. (2010a) also imaged the uptake and distribution of Au ENPs in the earthworm *Eisenia fetida* verifying their presence outside the gut lumen. In another study led by the same author, Cu ENPs were located in the muscle and gut of *Eisenia fetida*. Analyses indicated that these had been transformed through oxidation, with this process occurring more rapidly in the organism tissues than in the soil exposure medium (Unrine et al. 2010b). This later case, thus, highlight the particular value of the combination of elemental mapping with material speciation as it could allow identification of the key processes of handling and chemistry that define a materials ultimate biological fate.

Secondary ion mass spectrometry, Micro Particle Induced X-ray Emission (μ-PIXE) and electron microscopy with XAFs

Secondary ion mass spectrometry uses a focused primary ion beam (e.g. Ga^+, In^+, Cs^+) with a diameter which can be $<$10 nm to bombard and etch the surface of a sample, resulting in the release of secondary ions. The mass/charge ratio of these secondary ions is then measured to determine elemental, isotopic, or molecular composition of the degraded sample. The sensitivity of SIMS can depend on the ionization potential and sample matrix. It can be relatively

insensitive for some metals, but is often better for metal oxides. An advantage of the SIMS method is that an analyte of interest (such as a metal ion) can be analysed simultaneously with other elements and light molecules, including metabolites (Lobinski et al. 2006). Mapping endogenous elements, such as Na, K, P, and S, as well as low molecular weight constituents is particularly useful to delineate tissue and cellular structure as well as to monitor physiological changes and cytotoxic perturbations linked to ENP exposure (Moore et al. 2010). As an example, Ag ENP induced changes in membrane lipids in human macrophages were detectable using a ToF–SIMS based approach (Tentschert et al. 2013). Quantitation in SIMS can be challenging because the yield of secondary ions varies according to the ionisation potential of the materials to form positive (e.g. metals) and negative ions and also due to matrix and topographical effects (Moore et al. 2012). Thus while SIMS is capable of detecting, locating and analysing directly unlabelled ENPs in sectioned biological tissues this is best achieved when the background signal is negligible. Under such circumstances, spatial resolution of up to $\sim$50 nm is achievable given high quality sample preparation and instruments designed with high sensitivity in mind (Goodwin 2012; Kaletas et al. 2009).

Initial applications of SIMS have included detection of the penetration of TiO_2 and ZnO ENPs into human skin epidermal cells (Monteiro-Riviere et al. 2011) and detection of individual 150 nm silica particles within mammalian epithelial-like NRK cells maintained on glass microscope slides (Hagenhoff et al. 2013). In ecotoxicology, SIMS has recently been used to show that Ag ENPs not only enter the gut lumen of *Daphnia magna*, but also that they seem able to pass across the epithelial barrier and accumulate within specific organs including the ovaries where there is potential for reproductive effects (Audinot et al. 2013; Georgantzopoulou et al. 2013). In contrast, SIMS analysis suggests that SiO_2 and TiO_2 ENPs are probably retained within the gut lumen (Audinot et al. 2013). These studies highlight both the value of the SIMS approach for detection and also a more general biological point that ENP distributions and mobility between organ compartments can be highly dependent on the composition and other characteristics of the material under study.

Micro particle induced X-ray emission spectroscopy (μ-PIXE) is a spatially resolved technique that allows elemental analysis of tissues and single cells down to a sub micron or even nanometer resolution (Lobinski et al. 2006). The technique, where the ion beam is scanned over the specimen surface, is analogous to electron microprobe analysis, but use of a proton rather than an electron beam yields approximately 100-fold higher analytical detection sensitivity. Like SEM-EDX and TEM-EDX, μ-PIXE can simultaneously detect all elements of $Z \geq 11$ as for conventional energy-dispersive X-ray detectors. It is a fully quantitative method, however, spatial resolution is inferior to that of electron beam instruments. Hence while the approach is well suited to study of elemental distributions, localisation of single intact ENPs is not achievable with current technologies.

The analytical potential of μ-PIXE in conventional metal ecotoxicology has been illustrated by Collins et al. (2010) who used the method to described cobalt distribution in the leaves of exposed wheat and tomato. By combining quantitative mapping with XAS observations it was possible to show that Co did not transform from the Co(II) state within plant tissues, and formed a complex with carboxylate-containing organic acids. Larue et al. (2012) then used a combination of synchrotron-radiation micro-X-ray fluorescence mapping, TEM and quantitative μ-PIXE to investigate TiO_2 ENP distribution in wheat. Results showed that 14 and 25 nm TiO_2 ENPs accumulate in roots and are also distributed through the whole plant without dissolution or crystal phase modification. Particles with diameters $\geq$140 nm were not found to accumulate in roots, indicating a size dependent control of uptake that may be linked to the endocytosis pathway used or the capacity of the materials to pass biological membranes.

In invertebrates, Tkalec et al. (2011) used μ-PIXE to identify high concentrations of Ag sequestered preferentially within the 'S-cells' of the hepatopancreas of the terrestrial isopod *Porcellio scaber* exposed to Ag ENPs in food. Supporting TEM analysis failed to locate intact ENPs, thus, suggesting that observed cytotoxicity was mediated by Ag^+ ions released after ENP dissolution in the gut lumen or after cellular uptake, rather than by intact particles. Similar effects related to potentially assimilated dissolved ions have also been reported in isopods exposed to diets containing a range of heavy metals (Golobič et al. 2012; Novak et al. 2013). These findings are consistent with a hypothesis suggesting that dissolved ions play a key role in ENP ecotoxicology. TiO_2 ENPs are largely insoluble and a μ-PIXE study with woodlice failed to identify accumulation in S-cells of the hepatopancreas at lower concentrations, although biomarkers of toxicity were found (Novak et al. 2012). At high exposure concentrations, μ-PIXE analysis was ultimately able to confirm the presence of titanium in hepatopancreas cells which correlated with severe damage to cell membranes. This indicates both the assimilation of intact ENPs to this target organ and also more generally the value of conducting assessments at multiple exposure concentrations to provide a more complete picture of physiological responses across the dose dependent effect range.

Electron microscopy in its various forms can allow the visualization and analysis of particles down to sub nanometer range. Hence when uptake of ENPs into cells is anticipated, then these high spatial resolution imaging

techniques may be the method of choice for detailed analyses to ascertain precise intracellular distributions. Two main types of electron microscopes are most widely used: Scanning electron microscopes (SEM) mainly collect signals from the electron/sample interaction at the surface of materials (secondary electrons, back scattered electrons and X-rays) and have a lateral resolution down to low nanometer range. Transmission electron microscopes (TEM) mainly collect signals arising from the interaction of electrons transmitted through the sample with resolutions to sub Angstrom level. Both instruments can be combined with EDX (energy dispersive X-ray spectroscopy) detectors or WDX (wavelength-dispersive X-ray spectroscopy) as electron probe microanalysis methods that can be used for chemical identification and (semi) quantitative elemental analysis.

SEM-EDX/WDX and TEM-EDX/WDX techniques are particularly useful to locate, identify, and spatially resolve elemental domains and can indicate potential complexation between the ENP surface and the matrix (Handy et al. 2012a; Morgan 1985). For this reason, EM-EDX methods have already found widespread application in understanding the trafficking and cellular distribution of trace metals in ecotoxicology studies (CotterHowells et al. 2005; Hopkin 1989). There is, therefore, an obvious potential to use this group of techniques to map metal distributions that are associated with ENP exposures and to identify material association with other ligands (Hooper et al. 2011). The simplest applications of electron microscopy lie in the imaging of ENPs in bacteria for which the lower complexity and smaller size allows use of simpler sample preparations methods (Bae et al. 2011; Duran et al. 2007; Fabrega et al. 2009b; Hwang et al. 2008). For most multicelluar organisms, more elaborate preparation including sectioning following the approaches previously outlined is usually necessary. Despite these additional requirements, there are an increasing number of studies that have used to SEM and TEM, often with EDX, to evaluate whether there is accumulation of intact ENPs.

In a study with cell lines, Garcia-Alonso et al. (2011) used TEM to identify electron-dense particles associated with the apical plasma membrane, in endocytotic pits and in endosomes that were confirmed to contain Ag using EDX detection. For whole organism in vivo studies, Kim et al. (2012) used simple TEM and SEM techniques to confirm that Ag ENPs inflicted damage to biological surfaces of *C. elegans* following exposure. Focussing more directly on detection of the ENPs themselves, TEM analysis has been used to demonstrate the presence of Au ENPs in the gut epithelia of *Eisenia fetida* (Unrine et al. 2010a); the presence of CuO ENPs in the gut lumen and in association with gut epithelial cells in *Daphnia magna* (Heinlaan et al. 2011) and ZnO dominantly associated with ingested sediment particles in *Corophium volutator* in a form that suggested dissolution and associations of ions with sediment material rather than the presence of intact ENPs (Larner et al. 2012). In the later study, the electron microscopy analysis followed assessment of distributions using lower spatial resolution methods (LA-ICP-MS and Raman microscopy) to identify target tissues prior to detailed imaging. The success of this study emphasises the value of a combined approach to ENP detection that links lower resolution techniques for initial gross localisation with high spatial resolution methods that can provide chemical information on ENP environments.

Some TEMs are equipped with a scanning unit and a high angle annular dark field detector (HAADF) where inelastically scattered electrons are collected (Cheng et al. 2009). This gives an advantage in terms of signal collection efficiency. Morones et al. (2005) have convincingly demonstrated that this technique can determine the presence of small (1 nm) Ag ENPs directly interacting with different microbial species to cause bactericidal activity. HAADF-STEM has also been used to investigate the dynamics of the association and disassociation of Fe_3C ENPs that were initially attached to single wall carbon nanotubes within exposed murine macrophages. This later study, like others discussed previously with respect to cellular targeting following assimilation, highlighted the potential importance of the lysosome in the internal fate of assimilated ENPs. In particular it was noted that blockage of intracellular lysosomal acidification prevented the Fe_3C ENPs detachment from CNT bundles and protected cells from CNT downstream toxicity (Bussy et al. 2013).

A further development of the use of electrons for analysis of biological samples is through Electron energy loss spectroscopy (EELS). This technique is complementary to EDX, with the particular value that it allows analysis of the valence states of elements within a given material. This characteristic gives a number of potential advantages for ENP studies. For example, different carbon forms (diamond, graphite, mineral carbon) can be readily separated (Baalousha et al. 2012) and functional groups on surfaces also identified (Wepasnick et al. 2010). The method has also been used to a limited extent in toxicology. For example, Hardas et al. (2010) used EELS to detect the presence of both Ce(III) and Ce(IV) valency states in the livers of rats exposed to 5 nm CeO_2 ENPs. This ability through EELS can provide an indication of the transfer of materials between forms that may occur in situ (Xin et al. 2012).

Overview and conclusions

The focus on ENP toxicity has arisen from the realisation that the development and commercialisation of ENP applications will inevitably result in the release of these materials to air, soils and water. As the range of nanotechnology products and applications increases, the need to understand the fate and hazard of ENPs in the environment has been identified. Research conducted to develop the scientific basis for the risk assessment of ENPs started initially by assessment of the release, fate and effects for the initial pristine materials. Such analyses were central to initial efforts to support the derivation of predicted environmental concentrations (Gottschalk et al. 2013, 2009; Johnson et al. 2011); establish the suitability of existing test methods for ENPs (Handy et al. 2012b, c); and develop initial QSAR and categorisation approaches (Puzyn et al. 2011; Rallo et al. 2011). The maturation of the field has extended these studies to include an understanding of the mechanisms of exposure and effect for both pristine and transformed materials.

Even at the outset of ENP ecotoxicology work, the importance of good metrology to characterise the starting material to provide a sound basis for tracking and in situ detection of materials assimilated into organisms was already recognised (Klaine et al. 2008; Royal Commission on Environmental Pollution 2008). Research in the following years has reinforced/confirmed this requirement. With an increasing focus on more realistic in vivo exposures in water, soils and sediments, the need to include analyses of transformation processes as potential arbiters relevant to determining the real nature of environmental exposures has become apparent. Key aspects in relation to fate of natural colloids, such as agglomeration, dissolution and changes in surface properties were already established and a decade of ENP studies has confirmed that these mechanisms define ENP exposure. Agglomeration and changes in surface properties such as the attachment of organic molecules might decrease rates of uptake and further toxicokinetics and toxicodynamics, while ENP dissolution can shift the focus to the study of classical metal toxicity. A consequence of the highlighted role for transformation in exposure is that studies of assimilation, internal distribution and ecotoxicity should now seek to include (and indeed prefer) studies with these modified materials and use appropriate methods for tracking distributions and analysis chemical forms. Such studies can be supported by in situ analysis that allows speciation (e.g. microprobe X-ray analysis) which provides information on the chemical state of the accumulated material not achieved by elemental analysis methods alone.

In the early phases of ENP ecotoxicology, it was not established whether materials of different sizes and forms would traverse biological barriers (e.g. respiratory and gut surfaces) and from there to which internal locations they would be transported. Potential mechanisms for cellular endocytosis of foreign material were known and a natural focus for early studies. Initial in vitro studies identified a role for the known endocytosis mechanisms in the uptake of different sized ENPs and subsequent research in ecotoxicology, including imaging studies, gene expression analysis and studies with mutant strains have confirmed this apparent role. Additional to these known pathways, uptake has also been linked to direct transfer across lipid membranes due to their damage or other direct interactions (e.g. with transmembrane proteins and ion channels).

The identification of uptake pathways highlighted the need for in situ analysis of ENPs in their hazard assessment as a means to determine the levels of internal exposure, target tissues and the link of exposure to effects on key traits (e.g. mortality, reproduction) as observed in ecotoxicological tests. Development and use of a range of quantitative and qualitative imaging techniques has helped to address a number of key questions in relation to the nature of toxic effects. Identification of the major routes of uptake, target organs and routes of elimination and inactivation has progressed quickly. Such studies have attributed routes of uptake to observed effects, for example, starvation due to physically blocking digestion or ENP induced malformations of the gut or intestine; respiratory effects due to damage to gill or body surfaces and physiological effects associated with accumulation in particular tissues. Further, when levels and forms of accumulated material are identified, these can be traced to assess the potential for trophic transfer.

To analyse uptake and internal distributions, the requirement for appropriate sample preparation is no less pertinent in studies of ENP ecotoxicology than for any other type of assessment. This does not mean that all sample preparation needs to be elaborate—indeed for some techniques even living organisms can be used. However, in cases where detailed structural and chemical information is needed, attention to detailed requirements is necessary to ensure that appropriate structural integrity is maintained without affecting ENP morphology and chemistry. Generally, it is those techniques where sample preparation is least technically demanding (e.g. elemental analysis, Raman spectroscopy) that are most suitable for initial screening assessment for large sample sets, while techniques requiring more elaborate sample preparation are better suited to higher tier assessment of key samples (see Table 2 for information on advantages and disadvantages of techniques including sample preparation). The methods requiring more elaborate sample preparation (e.g. the various electron microscopy approaches) are then best suited to more detailed assessment relations to key samples and target tissues identified within the initial assessment.

The demand of sample preparation, coupled with other key consideration such as spatial resolution, time and cost etc. have underpinned the frequent use of a combination of approaches in the most comprehensive studies of ENP distributions conducted to date. Bulk chemical analysis methods are often used to provide an initial assessment of toxicokinetic parameters and broad tissue distributions. Spectroscopy and spectrometry methods can provide an intermediate resolution analysis able to map distribution to the cellular levels, with electron microscopy techniques as the methods of choice for follow up high resolution studies (Fig. 3). The most powerful analyses combine low and high resolution methods to establish tissue and cellular distribution and to determine the chemical environments of ENPs at target sites (Unrine et al. 2010a).

Whilst major analytical and experimental advances have been made in understanding ENP exposure, uptake and tissue distributions, a number of key areas remain to be addressed. As outlined, fate assessments of ENPs in waste streams and natural environments have identified a range of ENP transformations that occur under relevant conditions. So far only limited exposure and tissue localization studies have been conducted with these transformed materials. These have generally indicated a lower toxicity of the transformed compared to pristine materials (Levard et al. 2013; Reinsch et al. 2012). Tracking how the assimilation and the intracellular fate of transformed ENPs compares to that for pristine materials will provide understanding of how these identified mechanisms relate to toxicity. Such studies need to include not only elemental analysis and imaging, but also more detailed in situ characterisation and speciation analysis. The relationship between these assimilated materials and the mechanisms associated with the trafficking of related chemicals and trace elements will also be a key area to further evaluate (Polak et al. 2014). Further, the translocation of ENPs to subsequent generations and further across trophic levels needs to be investigated at environmentally realistic concentrations.

Future study of ENP hazards also needs to consider exposure routes, pathology and potential future trophic transfer under realistic exposure condition including consideration of low concentration/dose effects. This type of study requires researchers to make decisions about the best techniques to use to track uptake and in situ tissue distribution at the lower concentrations. Central considerations will include sample type and size, anticipated concentration, material core chemistry and characteristics; as well as practical considerations such as prior information available, instrument availability and costs. The hierarchical strategy presented in Fig. 3 coupled with the summary of major strengths and weaknesses in Table 2 can provide a framework to select relevant analytical methods. Elemental detection methods like ICP-MS and AAS will always be valuable as a first pass method to confirm the element from a metal or metal oxide nanoparticle is present in the relevant sample. These methods provide often exquisite sensitivity, but no information on material form (especially for material with high background). Studies using isotope enriched ENPs can, however, provide further added value to such studies and are likely to remain a value aspect of exposure studies. Mapping techniques such as LA-ICP-MS, Raman spectroscopy and μ-XRF provide information on target and storage organs supporting the development of adverse outcome pathways and suitable tissues for their further investigation. The challenge for these methods will be detection at the lower accumulated concentrations anticipated from realistic environmental concentration studies. Focussing analysis on key target organs is an important prerequisite to address this potential issue. Ultimately, confirmation of presence as intact ENPs, however, requires the use of techniques such as SIMS and electron microscopy that can operate both at the highest spatial resolution and at relatively low detection limits within samples. Only the use of a combination of these methods will ultimately lead us to a better understanding of uptake under likely real world exposure conditions.

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