



Micro- and nano-plastics activation of oxidative and inflammatory adverse outcome pathways

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ABSTRACT

Microplastics (MPs) and nanoplastics (NPs) have attracted considerable attention in the recent years as potential threats to the ecosystem and public health. This review summarizes current knowledge of pathological events triggered by micro- and nano-plastics (MP/NPs) with focus on oxidative damages at different levels of biological complexity (molecular, cellular, tissue, organ, individual and population). Based on published information, we matched the apical toxicity endpoints induced by MP/NPs with key event (KE) or adverse outcomes (AO) and categorized them according to the Adverse Outcome Pathway (AOP) online knowledgebase. We used existing AOPs and applied them to highlight formal mechanistic links between identified KEs and AOs in two possible scenarios: first from ecological, and second from public health perspective. Ecological perspective AOP based literature analysis revealed that MP/NPs share formation of reactive oxygen species as their molecular initiating event, leading to adverse outcomes such as growth inhibition and behavior alteration through oxidative stress cascades and inflammatory responses. Application of AOP on literature data related to public health perspective of MP/NPs showed that oxidative stress and its responding pathways, including inflammatory responses, could play the role of key events. However insufficient information prevented precise definitions of AOPs at this level. To overcome this knowledge gap, further mammalian model and epidemiological studies are necessary to support development and construction of detailed AOPs with public health focus.

1. Introduction

Awareness of general public and scientific community about the scope and amount of plastic pollution has significantly increased in recent years. Global production of plastics reached 348 million tons in 2017, from which over half was produced in Asia, while Europe contributed 64 million tons [1]. Increase in production coupled with chemical stability of plastic materials and shift of consumer preferences toward “single-use” packaging, accelerates generation of plastic waste, leading to contamination of diverse ecosystems and rising concern about environmental and health risks associated with exposure to plastics [2, 3]. Exposure of plastic waste to the elements leads to its degradation through mechanical and chemical processes such as hydrolysis and UV radiation [4], to form microplastics (100 nm–5 mm, MPs) and nanoplastics (<100 nm, NPs) particles [5]. In comparison to larger plastic debris, smaller size of MPs and NPs presents an opportunity for them to enter different environmental compartments, increases efforts required for their removal or clean up and poses higher risk of exposure and

entering food chain.

The number of studies addressing MP/NPs toxicity is rapidly increasing, with focus on polyethylene (PE), polystyrene (PS) and polyvinyl chloride (PVC) which have the highest production and utilization among many species of plastic materials [1]. Rising interest in investigations of MP/NPs is likely related to their ability to translocate through biological barriers while at the same time maintaining large surface/mass ratio, as well as their potential to accumulate in the higher trophic level organisms via the food chain [6,7]. It is therefore of high interest of research and regulatory community to evaluate potential ecological as well as human health hazards of MPs/NPs.

Variety of approaches have been used in toxicological studies of MP/NPs, including *in vivo* (aquatic marine and freshwater, and terrestrial organisms including mammals) and *in vitro* (cell culture) models to investigate effects and behavior of MPs/NPs [8–11]. Reports indicate that MP/NPs are most frequently involved in induction of developmental toxicity, neurotoxicity, cytotoxicity, and oxidative stress [12–15]. Observed toxic effects of MP/NP particles suggest that

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oxidative stress and inflammatory responses are of high importance as critical mechanisms underneath the above listed toxicities. However, hazard assessment outcomes from different studies are variable and sometimes contradictory, probably due to the lack of standardized research methodology such as use of different research models, and not the least due to high diversity of the MP/NPs themselves (different size, shape, surface charging and polymer type). For example, the effects of microplastics in a very high concentration was reported to have no negative effects on crustaceans, which directly contradicted other studies indicating adverse effects on crustaceans after exposure to environmentally relevant MPs concentration [16,17]. Furthermore, observing toxicity endpoints at different biological levels (from molecular to population) can be limited, and sometimes even misleading, based on complexity of the observed model system. For example, majority of *in vitro* model based studies reported toxicity endpoints only on molecular or cellular level, while *in vivo* studies could detect the adverse effects of MPs/NPs in various organs [11,18,19].

In addition to the above, addressing possible differences between engineered and environment derived MP/NPs has several limitations. Studies selected for the review based on common criteria have only rarely differentiated between the two MP/NP sources, and majority of studies have actually used model MP/NPs (by default engineered, rather than derived from the environment). Therefore, there is very limited information about mechanisms of action specific to environmentally derived MP/NPs and most of the available information that is related to environment-derived MP/NPs comes in a form of surveys of wildlife and aquatic organisms, usually only describing current state of, situation, or distribution and relying to laboratory studies to explain observed effects. In this review, information about the source of nanomaterials is included in the text if it was deemed relevant from the aspects of engineered vs environment-derived NP/MP (specifically in the inflammation section). Unfortunately, not enough studies have met the criteria to make a comparison between natural vs. engineered nanomaterials at the selected level of Adverse Outcome Pathway application.

To address the limitations of different model systems and support standardized methodology approaches, it is advisable to implement a mechanism-based framework such as Adverse Outcome Pathway (AOP) to categorize and link these adverse effects in a formal technical manner. AOP concept is introduced as a modular structure that is not substance specific, in an effort to organize the information regarding the linkage between adverse effects from the molecular level of a biological system to an apical endpoint of the perturbation [20]. AOP framework usually starts with a Molecular Initiating Event (MIE), and is followed with events occurring at more complex levels of biological organization. MIE is a special case of a “Key Event” (KE) which describe the changes in molecular function, signaling pathways, cellular function status, and tissue alterations. Ultimately, these KEs lead to the apical adverse effects on organism or population level such as acute toxicity/sickness of an individual, or reduced reproductive capacity and decline of affected population. These apical endpoints that are traditionally used in ecological risk assessment, in AOP system are referred to as “Adverse Outcomes” (AO) [21].

The Key Event Relationships are linkages between adverse outcome pathway modules that provide scientific evidence supporting the relationships between examined substance and its toxic effects [20]. AOP approach for evaluation and assessment of the health risks associated with exposure to a chemical or compound is increasingly used in meta-data analysis of existing knowledge [22,23]. Therefore, we used existing AOPs in an effort to synthesize and present information from systematic review of the reported MP/NPs adverse effects with focus on mechanistic relationships. During this process, we constructed structural schematics depicting current knowledge of MP/NPs triggered potential toxicity endpoints in different levels of biological organization, with an emphasis on oxidative stress and inflammatory responses. Finally, the Adverse Outcome Pathways were applied to both microplastics and nanoplastics, and the results of the synthetic literature review are

presented and described using AOP approach.

2. Molecular initiating events and molecular key events triggered by micro- and nano-plastics

Molecular events caused by noxious/toxic agent effects in many cases form the foundation for explanations of biological phenomena observed on higher levels of biological complexity. In this part of the review, we summarize molecular initiating events and molecular key events (KEs) induced by micro- and nanoplastics including generation of free radicals, oxidative stress metabolism activation, lipid peroxidation, DNA damage, and downstream signaling pathway activation that precede cascade of branching molecular changes and potentially lead to irreparable oxidative damage and exacerbated inflammatory processes.

2.1. Molecular initiating event (KE 1364): reactive oxygen species generation

“Free radical” is an atom or group of atoms with one or more unpaired electrons with high potential to engage in different reactions, including oxidative chemical reactions. In biological systems, many radicals are derived from oxygen and are collectively referred to as the reactive oxygen species or ROS [24]. Reports show that MP/NPs can induce generation of ROS at different sizes, doses, surface-characteristics, and exposure times [25–27]. Observations of MP/NPs triggered ROS generation fall in two distinct compartments: extracellular and intracellular. Extracellular ROS generation induced by the MPs relates to the degree of weathering (aging) process during plastic polymers exposure to the elements in the environment [28–30]. Weathering processes of MP/NPs involves simultaneous or individual action of photo- and thermal oxidation and UV radiation leading to chemical alteration on the surface of plastic polymers [30,31]. Photo oxidation or UV light radiation can lead to formation of free radicals on MP/NPs surfaces as primary products via different pathways: subtraction of a hydrogen atom from the macromolecular chain, or addition to an unsaturated carbon chain group (crosslinking reaction) [32]. Once the free radicals are generated along the polymer chain, they can react with atmospheric oxygen and produce polymer peroxy radicals with further generation of secondary polymer alkyl radicals [33]. These weathering induced extracellular free radicals could be one possible explanation as to why a significant increase of ROS was observed after the cellular entrance of the aged MPs [34].

However, pristine MP/NPs can also induce excessive generation of ROS intracellularly. This phenomenon was reported using wide array of model systems, from mammalian cell lines to marine invertebrate and living fish models [18,35,36]. MP/NPs can be engulfed by a (usually phagocytic) cell through endocytosis or pinocytosis (<150 nm in case of NPs) processes [37,38]. Once inside a phagocyte, these MPs appear to trigger the innate immune defense mechanisms and are treated as foreign substances [39,40]. During attempts of the cell to neutralize potentially infectious foreign particle, ROS are generated in high quantities as products of NADPH-oxidase or other enzymatic reactions in form of superoxide and hydrogen peroxide. Both O₂⁻ and H₂O₂ have a role in signal transduction and serve as key mediators driving oxidative stress cascades [36,41]. Furthermore, when microplastics degrade into nano-sized particles, their surface-to-mass ratio increases and gives the NPs ability to penetrate directly through lipid membranes. Changes in surface charge potential and mass/surface ratio in NPs also enable easier absorption of free radicals as well as allows for easier translocation through membranes, presenting a strong correlation between particle size and ROS generation potential: the smaller the particle, the higher ROS generation potential [13,27,42]. Size-dependent differences in toxicity of MPs/NPs are supported by several studies [13,26].

2.2. Key event (KE 1392): oxidative stress

The oxidative stress occurs when there is an imbalance between the production of ROS and antioxidant based detoxification/neutralization. The antioxidant systems are complex and include various antioxidant compounds such as vitamins (e.g. C, E, D₃), as well as multiple enzymatic pathways involved in production of antioxidants, or elimination of oxidants including ROS and RNS. Detailed review of antioxidant systems is beyond scope of this review and we point the reader to recent reviews of Piscochi and Pop [43], and Sies et al. [44]. Here, we will focus on oxidative stress pathways most frequently reported in relation to MP/NPs exposure.

Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidases (GPx) play an important role in protecting tissues against ROS damage [45]. For example, complex ROS scavenging system in innate immune cells such as neutrophils utilizes SOD and CAT enzymatic systems to catalyze the reactions of superoxide anion radical (O₂⁻) and hydrogen peroxide (H₂O₂) into water (H₂O) and oxygen (O₂) as the final metabolites, respectively [46]. SOD and CAT based antioxidant mechanisms are important cellular tools against adverse effects of ROS, and their activity has frequently been used as biomarker of xenobiotic-mediated oxidative stress [46,47]. Exposure to MPs/NPs could increase the concentration of ROS in the organism as we previously stated, and the SOD/CAT antioxidation mechanisms would react to such signal.

The activation of antioxidants was found in animal models on different trophic levels following the exposure of MPs including rotifers (*B. koreanus*) [26], water fleas (*Daphnia magna*) [17], fish (*D. rerio*) [15, 48] and mammals (*M. musculus*) [11]. This phenomenon suggests existence of common mechanisms to initiate oxidative stress sensing and scavenging system induced by microplastic exposure. In case of nanoplastic particles, the oxidative stress was also frequently observed (45% publications) both *in vitro* and *in vivo* as indicated by antioxidant system activation. This frequency is placing the oxidative stress as one of most studied adverse effect among all toxicity endpoints studies reported in searched literature databases (Table 1). These phenomena indicate both MPs and NPs can induce oxidative stress as fundamental mechanism for further toxic effects induced by MPs/NPs in a majority of organisms.

2.3. Key event (KE 1238, KE 1279): Activation of oxidative stress pathways

It was clearly demonstrated that ROS can induce or mediate the activation of the MAPK (mitogen-activated protein kinase) pathways [49], but exact mechanisms of MAPK downstream pathway regulations remain unclear. Recent studies have reported that activation of MAPKs signaling cascades could induce ARE-mediated gene expression via the Nrf2-dependent mechanism, and it appears that MAPK activation by ROS can be downregulated by simultaneous increase in Nrf2 activity [50]. In contrast, the prolonged activation of MAPK system with increased ROS can activate various negative consequences (e.g. autophagy), primarily related to the inhibition of ERK and activation of p38MAPK components. It was recently reported that brominated flame retardant induced oxidative stress in two copepod species and activated the MAPK pathway [51], and it was determined that MAPKs pathways play a synergistic role with Nrf2/Keap1 pathway in the oxidative stress response in the scallops exposed to benzo(a)pyrene [52].

As a next step in oxidative stress event series, it was observed that microplastics exposure also activated redox sensitive signaling pathways such as MAPKs. MAPK downstream pathways were initiated post MP exposure in marine copepod *P. nana* and Mitten crab *E. sinensis* [27,53]. Jeong et al. [27] showed that the phosphorylation level increase of ERK

and p38 kinase has positive correlation with intracellular ROS generation level post MP exposure in *P. nana*. Moreover, transcription factor Nrf-2 (nuclear factor erythroid 2-related factor 2) was increased after exposure to MPs suggesting that MPs triggered respiration burst possibly acted via ERK and p38 MAPK pathways by activating activated Nrf-2 as representative of genes involved in regulation of antioxidant enzymes gene expression [27].

The activation of MAPK pathway appears dependent on the particle size, as initiation of MAPK pathway was more frequently reported after Nanoplastics exposure [26,54,55]. It was suggested that plastics with a size of 50 nm could generate more severe oxidative stress when compared to 6 µm plastics, causing higher phosphorylation level of p38 MAPKs in both *P. nana* and *B. koreanus* [26,27]. Moreover, the surface charge of nanoplastic particles appears critical in the activation of MAPK cascades, as plain/native polystyrene nanoparticles (PSNPs) induced the activation of p38 and JNK along with ROS induction, while negatively charged PSNPs activated p38 and JNK independently [55].

2.4. Key event (KE: 1151): Lipid peroxidation

Lipid peroxidation (LPO) is a self-sustaining chain reaction induced by excessive ROS generation (O₂⁻ or others) that results in oxidative damage to cell membranes and other lipid-containing structures [56]. Currently there is a debate whether exposure to MPs would universally induce the LPO reaction and cell membrane damage. Barboza et al. [57] reported significant increase in LPO from brain and muscle tissues of European seabass (*Dicentrarchus labrax*) after the exposure to MPs in concentration of 0.69 mg/L for 24 h. However, another study indicated that the LPO was not significantly induced after 7 days of MPs exposure, along with increased ROS levels, in the hemocytes of the marine mussel *Mytilus spp* [25]. Moreover, in *S. plana*, the LPO levels in gills and digestive gland were either reduced or not significantly increased after 7 days of polystyrene microparticle exposure, and the authors suggested that such result may be explained by sufficient capacity of antioxidant systems to defend the organism from LPO damages [58]. Therefore, based on current literature, it is inconclusive if MPs could induce LPO damage in general, but it is at least prudent to consider this molecular occurrence as a potential key event triggered by MPs. This approach would be further supported with close to 10% of reviewed studies indicating that LPO and subsequent damages were induced by nanoplastic exposure (Table 1), therefore suggesting a possibility that LPO could potentially be a toxicity endpoint shared by the plastic particles in both micro- and nano-size range.

2.5. DNA damage (KE: 1669)

DNA strand damages may occur via the oxidative DNA damages such as 8-Oxo-Guanine damage. There is current controversy whether MPs are inducing DNA damage universally, since only two publications to date reported that DNA damage occurred in aquatic organism models [8, 58]. DNA strand breaks were detected post polystyrene microparticle (20 µm) exposure in the haemocytes of *S. plana*, similarly to mussels treated with polyethylene microparticles [8,58]. Both reports suggested that observed DNA damage could be potentially related to oxidative stress induced by MPs.

Nanoplastics induced genotoxicity, including DNA strand damage, is also under debate. However, a lesson learned from wider nanotoxicology research indicated that some nanoparticles (such as titanium dioxide) can induce the DNA damage through two mechanisms: 1) direct interaction between nanoparticles and DNA; or 2) indirect DNA damage caused by nanoparticle generated ROS or other toxic ions. Furthermore, it was also indicated that DNA strand breaks occur post

Table 1

Nanoplastics induced Key Events (KE) and Adverse Outcomes (AO) summary and their relative distribution in published literature. *Sum of percentages is over 100% because multiple publications reported multiple KEs and AOs.

Title	Category	Biological level	AOP wiki ID	NPs type	Organism	Reference	Percentage in the literature (%)*
Increase, ROS	Key event	Molecular	KE 1364	PS	<i>D. magna</i> <i>Danio rerio</i> Monogonont Rotifer (<i>Brachionus koreanus</i>) Oyster gametes (<i>Crassostrea gigas</i>) Human <i>Caenorhabditis elegans</i>	[55] [48] [26] [75] [71,104] [95]	19.4%
Oxidative stress	Key event	Molecular	KE 1392, KE 1088	PS, PC	<i>D. magna</i> Fathead minnow (<i>Pimephales promelas</i>) <i>Danio rerio</i> <i>Daphnia pulex</i> <i>Caenorhabditis elegans</i> Oyster gametes (<i>Crassostrea gigas</i>) Monogonont Rotifer (<i>Brachionus koreanus</i>) Mussel (<i>Mytilus galloprovincialis</i>) Human	[55] [39] [48] [101] [15] [93] [13] [75] [26] [64] [54] [85] [14] [60]	45.2%
Activation of oxidative stress pathway	Key event	Molecular	KE 1238, KE 1279	PS	<i>D. magna</i> Mussel (<i>Mytilus galloprovincialis</i>) Monogonont Rotifer (<i>Brachionus koreanus</i>)	[55] [54] [26]	9.7%
Lipid peroxidation	Key event	Molecular	KE: 1151	PS	<i>D. magna</i> Mussel (<i>Mytilus galloprovincialis</i>) <i>Danio rerio</i>	[55] [64] [15]	9.7%
DNA damage	Key event	Cellular	KE 1669	PS	Mussel (<i>Mytilus galloprovincialis</i>) Human	[64] [60]	6.5%
Lysosome disruption	Key event	Cellular	KE 898	PS	Mussel (<i>Mytilus galloprovincialis</i>) Mouse Human	[54] [70] [74]	9.7%
Mitochondrial dysfunction	Key event	Cellular	KE 1483	PS	<i>D. magna</i> Mussel (<i>Mytilus galloprovincialis</i>) Mouse Human	[55] [54] [70] [71,104]	13.0%
Acetylcholinesterase (AChE) inhibition	Key event	Cellular	KE 12	PS	<i>Danio rerio</i> <i>Caenorhabditis elegans</i> Mussel (<i>Mytilus galloprovincialis</i>)	[48] [13] [64]	9.7%
Phase I metabolism enzymes activation	Key event	Molecular	KE 1386, KE 850	PS	<i>Daphnia pulex</i> Mussel (<i>Mytilus galloprovincialis</i>)	[86] [64]	6.5%
Pro-inflammatory cytokines activation	Key event	Cellular	KE 87, KE 1493	PS	<i>Danio rerio</i> Mussel (<i>Mytilus galloprovincialis</i>) Human	[10] [64] [14] [83] [88] [90]	19.4%
Apoptosis	Key event	Cellular	KE 1262	PS	Mussel (<i>Mytilus galloprovincialis</i>) Mouse Human Human	[54] [70] [85] [71,104] [89]	16.1%
Decrease of neuronal network function	Key event	Organ	KE 386	PS	<i>Danio rerio</i> <i>Caenorhabditis elegans</i>	(Brun, van Hage et al., 2019) [13]	6.5%
	Key event	Tissue/Cellular		PS, PC	<i>Danio rerio</i>	[10]	13.0%

(continued on next page)

Table 1 (continued)

Title	Category	Biological level	AOP wiki ID	NPs type	Organism	Reference	Percentage in the literature (%) ^a
Inflammation; Infiltration, Inflammatory cells			KE 1633, KE 901		Rat Fathead minnow (<i>Pimephales promelas</i>) <i>Danio rerio</i>	[15] [90] [39]	
Bradycardia	Key event	Organ	KE 444	PS	<i>Danio rerio</i>	[101] [100]	6.5%
Accumulation, Liver lipid Growth inhibition	Key event Adverse outcome	Organ Individual	KE 455 KE 1521 KE 1467	PS PS	<i>Danio rerio</i> algae (<i>S. obliquus</i>) Monogonont Rotifer (<i>Brachionus koreanus</i>) <i>Caenorhabditis elegans</i>	[15] [9] [26]	3.2% 9.7%
Decreased body size/length	Adverse outcome	Individual	KE 315, KE 864	PS	<i>D. magna</i> <i>Danio rerio</i> <i>Caenorhabditis elegans</i>	[13] [9] [48] [13] [95]	13.0%
Increase, Mortality	Adverse outcome	Individual, Population	KE 350, KE 351, KE 342	PS	<i>D. magna</i>	[55]	3.2%
Locomotor activity, decreased	Adverse outcome	Individual	KE 1389	PS	<i>D. magna</i> <i>Danio rerio</i> <i>Caenorhabditis elegans</i>	[55] [48] (Brun, van Hage et al., 2019) [100] [13] [95]	22.6%
Impaired, Fertility	Adverse outcome	Individual	KE 406, KE 78	PS	Rat Monogonont Rotifer (<i>Brachionus koreanus</i>)	[82] [26]	3.2%
Impaired, Development	Adverse outcome	Individual	KE 577, KE 339	PS	<i>Danio rerio</i> <i>Caenorhabditis elegans</i> <i>D. magna</i>	[101] [48] [13] [9]	13.0%

nanoparticle exposure. An intracellular dynamic imaging study indicated that cationic functionalized PSNPs could result in a prolonged G0/G1 phase in the cell cycle during mitosis in NIH 3T3 cells, therefore indicating potential for DNA damage and the interference with checkpoint control activation [59]. Paget et al. indicated that non-functionalized PSNPs did not induce a general genotoxicity except at the highest tested dose of after 8.1 µg/cm² exposure for 1 h [60]. There is evidence that both MPs and NPs have the ability to induce DNA strands break in a size and surface charge dependent manner. While exact mechanism of DNA damage induction by MP/NPs is currently not fully understood, several studies suggest there is significant role of oxidative stress and physical interaction involved in initiation of DNA strands break by NPs.

2.6. Acetylcholinesterase (AChE) inhibition (KE: 12)

Acetylcholinesterase (AChE) is an enzyme in the choline esterase family that lyses acetylcholine (ACh) into choline and acetic acid, and is critical for proper function of the nervous system [61]. As ACh functions mainly as neurotransmitter controlling motor neuron function, the inhibition of AChE activity is causing accumulation of ACh in the synaptic cleft and the severe disturbance of muscle activity [61,62]. Recently, the inhibition of AChE induced by either MPs or NPs exposure had been identified in multiple studies, and it appears to be shared by a variety of organisms from invertebrates to vertebrates [8,11,13,48]. Oliveira et al. reported that polyethylene microplastics in sizes of 1–5 µm induced the inhibition of AChE alone or in combination with pyrene in the brain of goby (*P. microps*) [63]. The authors also noted that the AChE inhibition rates induced by microplastics and pyrene were not significantly different either as single substances or in combination, suggesting that

enzymatic inhibition mechanisms may be shared by MPs and pyrene [63]. The anti-cholinesterases effects of MPs had been also identified in gills of another marine organism (*S. plana*) after exposure to both polystyrene and polyethylene microspheres [8]. Neurotoxic property of MPs has also been addressed in mammalian models, and reported in mice to include MPs (5–20 µm) induced inflammation and AChE inhibition during a sub-chronic exposure period (28 days) [11]. Taken together, there is growing body of evidence that support that one of toxic characteristics of MPs is the ability to induce neurotoxic responses.

Nanoplastics induced AChE inhibition has been reported by at least three separate studies [13,48,64]. A zebrafish study indicated a decrease in AChE activity in all nanoplastics treatments by 27–40% regardless of their surface modification [48]. Invertebrate model (*C. elegans* and *M. galloprovincialis*) studies also reported a significant decrease in both level and activity of cholinesterase post the polystyrene nanoplastics exposure [13,64].

The mechanisms involved in the micro- and nano-plastics interference with AChE and possibly other neurotransmitters are largely unknown. Studies using nematode (*C. elegans*) based assay system suggested that due to nematode lack of blood-brain barrier (BBB), the NPs may have more opportunities to interact with *C. elegans* neurons causing more neurotoxic effects [13]. This assumption is only partially explaining the toxic effects, as NPs have been observed to cross the BBB and eventually reach the brain, however direct blocking of cholinesterase by NPs has not yet been observed directly or verified in an *in vitro* system [65]. Presence of a blood-brain barrier is likely excluding micro-sized plastic particles crossing, however, neurotoxicity was nevertheless detected after MPs exposure in organisms with BBB. This strongly suggests that an indirect pathway plays a role in the MPs/NPs neurotoxicity potency [11]. Possible explanation for this neurotoxicity

potency is involvement of MP/NPs induced oxidative stress, as anticholinesterases induced neurotoxicity can be caused by it [66]. Simultaneous detection of MP/NPs induced oxidative stress and AChE inhibition further supports such possibility [11,48]. Direct relationship between H₂O₂ and modifications of AChE metabolism and activity was established recently, when it was determined that H₂O₂ decreased AChE levels by allosteric effector action on AChE structure and changes of isoform profile in SH-SY5Y cells confirming that increase in at least one ROS could promote disturbances in cholinergic system of neural cells [67].

3. Cellular and organelle key events triggered by micro- and nano-plastics

As the next level, a sum of adverse key molecular events advances into the observable cellular occurrences. Here we will review three adverse cellular effects currently known to be triggered by both microplastic and nanoplastic particles and focus on the mechanistic correlations between cellular key events, and/or between cellular and molecular key events.

3.1. Mitochondrial dysfunction (KE: 1483)

As mitochondria are organelles where most of intracellular production of ROS occurs, an instability in mitochondrial membrane potential can result in excessive generation of ROS through one-electron carriers (e.g. cytochromes and iron-sulphur-protein) and various oxidases [68]. Reports indicated mitochondria membrane dysfunction after MPs exposure in mice and rotifer *B. koreanus*, which requires further investigations as MPs are unable to accumulate directly in the mitochondria due to their size [26,69]. The study in rotifer *B. koreanus* reported that 0.5 µm polystyrene microbeads decreased the mitochondrial membrane potential significantly and suggested that MPs may affect the mitochondrial outer membrane indirectly via increased ROS presence in cellular compartments outside of, but in the vicinity of mitochondria [26]. Exposure of mitochondria to excessive oxidative stress in the cytosol can trigger the opening of several mitochondrial Na/K transmembrane channels. Increased membrane channel ionic flux can further lead to a collapse of the mitochondrial membrane potential and releasing Reactive Oxygen Species through “(ROS)-induced ROS-release” (RIRR) mechanism [68]. As it is established that MPs are capable of inducing oxidative stress (section 2.2), the RIRR mechanism could be responsible as mediator between the MPs intracellular presence and observed hampering of the mitochondrial membrane potential.

Mitochondrial membrane damage induced by MPs has also correlated with particle size (where smaller MPs induced more effects), leading to possibility that NPs could impose a more severe damage towards mitochondria generally [26]. Supportive to such reasoning, NPs induced mitochondria disruption was reported in *D. magna* and in a variety of human cell lines [55,70,71]. It is also possible that reported damage could also be induced by observed direct accumulation of NPs inside mitochondria, and not only by indirect pathways as in case of MPs [71]. Moreover, amine-modified polystyrene nanoparticles induced significantly more severe mitochondrial damages comparing to non-modified ones, suggesting that NPs surface charging plays a role in causing adverse effects of mitochondrial function [71].

3.2. Lysosome disruption (KE: 898)

Lysosome is a single membrane cytoplasmic organelle, with high sensitivity to xenobiotics including environmental pollutants. Thus, lysosomal membrane stability can be used as a biomarker to estimate potential impact of the environmental pollutants [72]. The lysosome function disruption was found in blue mussel (*M. galloprovincialis*) after exposure to both MPs and NPs [8,54]. The severity of the lysozyme function disturbance after MP/NPs exposure suggests high biological

relevance of these events, especially in context of lysosome function and structure being conserved across species [73–75]. Furthermore, the link between oxidative metabolism and ROS generation induced stress has been established [47], including the role of nanomaterials in lysosome function disruption [76]. In summary, two probable MP/NPs induced mechanisms can lead to lysosomal damage: 1) direct damage after ingestion of plastic particles in the cell via endocytosis or permeation (<50 nm) and attempts to digest the foreign body which may result in the lysosome disruption [77–79]; and 2) excessive production of ROS induced by MPs and NPs as lysosomal membranes are highly susceptible to oxidative effects of ROS [80]. Exact mechanisms of lysosome disruption by MP/NPs are not fully understood and this knowledge gap calls for further investigation.

4. Tissue and Organ key events triggered by micro- and nano-plastics

4.1. Decrease of neuronal network function (KE: 386)

Inhibition of AChE (Section 2.6) was found in a variety of organisms post MP/NPs exposure. This toxicity endpoint is considered a type of neuronal dysfunction that can potentially extend into decrease of neuronal network function [81]. In addition to AChE inhibition, MP/NPs may also interfere with other neurotransmitters function. For example, increased concentration of taurine, aspartate, and threonine has been observed in the serum of MPs treated mice, and together with AChE inhibition suggests potential for broader systemic neurotoxicity [11]. Furthermore, several studies illustrate simultaneous suppression of locomotor ability and neurotoxicity [10,13,48,82], providing indirect evidence about a link between MPs/NPs induced changes in neurotransmitters function and behavior alteration of testing model organisms. As the mechanisms of decreased neuronal network function are far from understood, detailed studies are needed to increase our understanding of MP/NPs involvement in this key event.

4.2. Inflammation (KE: 1633, KE: 1496)

Inflammation is a potent mechanism responsible for defenses against pathogenic and other noxious agents, and can be elicited by exposure to, or presence of, xenobiotics or toxicants. In recent years, engineered nanomaterials have entered the environment and can often be found in living organisms and could be considered as a special case of “particulate xenobiotics” that can interact with the immune system [5,39]. Inflammatory processes can be very destructive and must be carefully balanced and controlled at multiple levels of complexity. We will focus on three levels of biological organization (molecular, cellular and tissue) of responses involved in inflammatory responses to MP/NPs. As immune system generally recognizes micro- and nano-plastic particles as xenobiotics, multiple studies include inflammatory response endpoints to measure MP/NPs effects both *in vivo* and *in vitro* [10,11,64,83–86].

At molecular level inflammatory responses triggered by MP/NPs exposure mostly involve activation of pro-inflammatory cytokines, signaling molecules secreted primarily by immune cells (leukocytes) [87]. Available reports clearly demonstrate ability of nano-plastic particles to influence release of cytokines or the alteration of inflammatory response genes *in vitro* cell line models. Pro-inflammatory cytokine IL6, IL8 and IL1β genes are up-regulated in response to 10 µg/ml polystyrene NPs (44 nm) exposure in human gastric adenocarcinoma cells [88] with decreased cell viability [89]. Similarly, Prietl et al. report increased secretion of IL6 and IL8 which was detected after carboxyl polystyrene NPs exposure [14]. However, the authors also noted that IL6 production increased with particle size while IL8 secretion showed the opposite trend in THP-1 monocytic cell line, likely due to regulation of immune responses against pathogens of different sizes [14,26,90]. Details of mechanisms involved in the NPs exposure and cytokines production are not yet clear, however, observed pro-inflammatory responses could

relate to the oxidative stress and its corresponding lysosome membrane disintegration [8,15,38,90,91]. As for the possible effects of MPs, it was reported that 0.5 μm polystyrene MPs increased both mRNA levels of IL1 α , IL1 β and IFN and their respective protein levels in zebrafish gut [84].

The second and third level of inflammatory responses induced by MP/NPs would be cellular responses and tissue damage, respectively. Innate immune cellular effectors such as phagocytic cells appear to interact frequently with MP/NPs, as expected, considering they represent the first line of organismal defenses against particulate matter agents. Increase of degranulation of neutrophil primary granules and neutrophil extracellular trap release was detected after the exposure of both polystyrene and polycarbonate NPs in the fish model [39]. Release of granular contents as well as NETs release, suggested that NPs are capable of phagocyte activation and could function as a potential stressor of innate immunity [92]. Significant influx of neutrophils into the rat lung was observed after inhalation exposure to polystyrene NPs and MPs and the influx was significantly greater for smaller particles (64 nm) compared to larger ones (535 nm), while larger particles caused higher levels of interleukin secretion [90]. These findings demonstrate the potency of MP/NPs to induce inflammatory cellular responses.

Inflammatory tissue damage was detected in different *in vivo* models after exposure to either MPs or NPs [11,15]. Polystyrene microparticles (5 μm) and nanoparticles (70 nm) were both accumulating in the gill, liver and gut of zebrafish after seven days of exposure, causing typical inflammatory damage in liver (vacuolation, leukocyte/neutrophil infiltration, necrosis, and lipidosis) [15]. Liver histopathology findings were supported by increased SOD and CAT activity, indicating possibility of underlying oxidative stress as triggering inflammatory response. Similar hepatotoxicity was histologically detected in mice model when both 5 μm and 20 μm polystyrene MPs induced liver lipid accumulation and inflammation [11]. Published findings support that both MPs and NPs exposure can promote inflammation at all three levels (molecular, cellular and tissue), possibly through oxidative stress and lysosome dysfunction but more evidence is required for better mechanistic understanding.

5. Individual and population adverse outcomes triggered by micro- and nano-plastics

5.1. Adverse outcomes in organisms of ecotoxicological concern

The U.S. National Toxicology Program of National Institute of

Environmental Health and Safety (NTP-NIEHS) is assigning an adverse outcome (AO) designations only to individual or population levels which are specifically referred to in the NTP documents (<https://ntp.niehs.nih.gov>), and this approach served as basis for this section of the review. Majority of MP/NPs induced AOs are being discovered using organisms of ecotoxicological concern according to Jeong et al. and our literature summary (Table 1) [23]. Key events “Growth Inhibition” (KE 1521, KE 1467), “Impaired Development” (KE 577, KE 339) and “Decreased body size/length” (KE 315, KE 864) represent the most frequent AOs induced by MP/NPs.

The exposure to both MPs and NPs can result in negative effects on individual growth rate of tested organisms (KEs 315, 864, 1467, 1521). Interestingly, reviewed studies indicating the reduction of growth rates induced by MPs or NPs are mostly based on invertebrate models (10 publications about MPs, three about NPs) (Table 1) [23,93–95], likely due to the fact that growth rate evaluation is a standard toxicological endpoint for invertebrate models according to OECD guidelines [96]. Important factor influencing the growth reduction rate induced by MP/NPs appears to be the size of the particles. Recent rotifer study reported that under similar exposure concentrations to polystyrene particles, clear growth rate decrease was observed for 50 nm and 0.5 μm particle sizes, and growth was not different from control when rotifers were exposed to 6 μm MP [26]. Similar results were reported in microalgae (*S. costatum*), where PVC particles in micro-range (1 μm) induced significant growth inhibition (39.7% maximum) compared to control, while bulk PVC particles (1 mm) showed no effect [97]. It is noteworthy that some studies indicated the growth rate inhibition comes along with the accumulation of MP/NPs due to the persistence of polymers [2]. Their persistence and accumulation within the organisms is one of the main concerns raised about MP/NPs, as both characteristics support a hypothesis that MP/NP particles could transfer within the food web from prey to predator and lead to bioaccumulation and biomagnification. Until now, several laboratory studies demonstrated transfer of plastic particles in low-tier food chains, however, whole system trophic transfers require further investigation [98,99]. For further information regarding trophic level transfers of MP/NPs, we refer readers to Prinz et al. [6] and Prokić et al. [7] who provided detailed overview on this topic.

Development alterations (KEs 339, 577) were found in zebrafish (*D. rerio*), green sea urchins (*L. variegatus*), nematodes (*C. elegans*) and crustaceans (*D. magna*) after exposure to MP/NPs, noting that NPs induced development toxicity were more frequently reported [9,12,13,100,101]. MP/NPs caused different adverse effects during larval

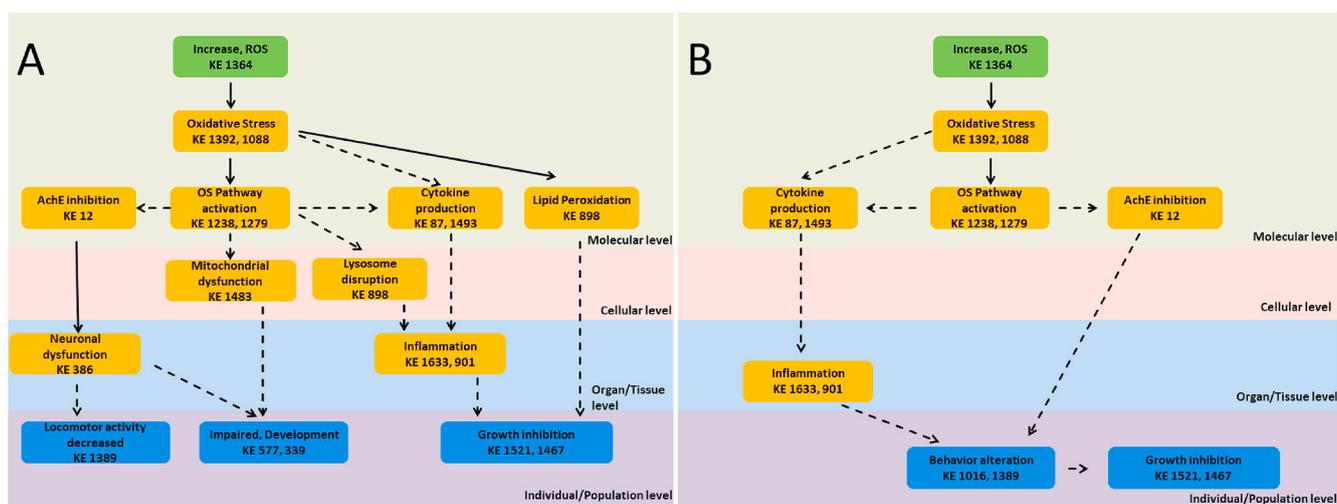


Fig. 1. Adverse Outcome Pathways schematic diagrams related to nanoplastic (A) and combined micro- and nano-plastics (B) adverse effects from ecological perspective with emphasis on the Oxidative stress and Inflammation responses. Green cuboid: Molecular Initiation Event; Orange cuboid: Key Events; Blue cuboid: Adverse Outcomes. Solid lines: Adjacent or strong evidence relationships; Dashed lines: non-adjacent or weaker evidence supporting the relationship.

development even in the same model organism, as two zebrafish larvae studies reported different modes of toxicity. Chen et al. reported that polystyrene MPs exhibited no adverse effects to the development of zebrafish larvae, in contrast to NPs, which induced significant developmental neurotoxicity and disturbance in larval locomotion activity [48]. In study by Pitt et al., polystyrene NPs transferred to offspring and caused bradycardia in the developing zebrafish larvae [101]. Both studies suggest that oxidative stress triggered by NPs could be the reason underlying these toxicities. In other invertebrates, polystyrene NPs were also reported to induce incomplete development of second antennae and cause curved tail spines in *Daphnia* [9]. It appears that MP/NPs have significant potential to interfere with embryonic development in various ways.

5.2. Adverse outcomes related to potential micro- and nano-plastics public health concerns in human beings

Information about formal Adverse Outcomes (AOs) induced by MPs and NPs in mammalian models (*in vivo*) is currently almost unavailable in the literature databases. This section will discuss potential systemic adverse effects of MP/NPs exposure in human beings primarily referencing *in vitro* mammalian cell line studies. In general, possible exposure routes of human beings to MP/NPs are through mucosal surfaces in respiratory system and gastrointestinal (GI) tract, and integument (skin), noting that some of the recently studied drug delivery systems would also expose human beings to MP/NPs via parenteral routes,

including intravenous and intracranial/brain application [102].

Polystyrene MP/NPs can be ingested by alveolar epithelial cells, followed by ROS production and occurrence of corresponding cellular adverse key events [103,104]. Furthermore, crossing of NPs into alveolar capillary vessels was observed, suggesting that large alveolar surface area and thin blood/alveolar barriers allow easier NPs penetration to bloodstream and consequential higher bioavailability [105,106]. Epidemiological studies confirmed possible hazards from airborne exposure to NPs, as adverse effects on health were reported after inhalation of synthetic polymers [107]. Information about presence or abundance of MP/NPs in air is scarce, with recent studies indicating that MPs constitute a non-negligible fraction of airborne particulates in both indoor and outdoor air. However, content percentage is relatively low comparing to the threshold suggested by regulatory bodies [108,109].

Scenario of skin exposure to MP/NPs is mostly related to direct contact with plastic containing cosmetics, clothing, or water. Since primary role of skin, especially top epithelial layer with keratinized cells (stratum corneum) is to serve as protective barrier, it is not expected that a significant uptake of MP/NPs can occur through skin, and this hypothesis was confirmed by several studies. Application of polystyrene MPs/NPs to the porcine skin tissue model illustrated that neither 200 nm nor 20 nm NPs were able to penetrate into deeper skin layers [110]. However, MP/NPs may be able to overcome skin barriers through physiological (hair follicles, via sebaceous or sweat gland openings) or pathological (various skin injuries) discontinuities of integument, but lack of available information prevents definitive conclusions.

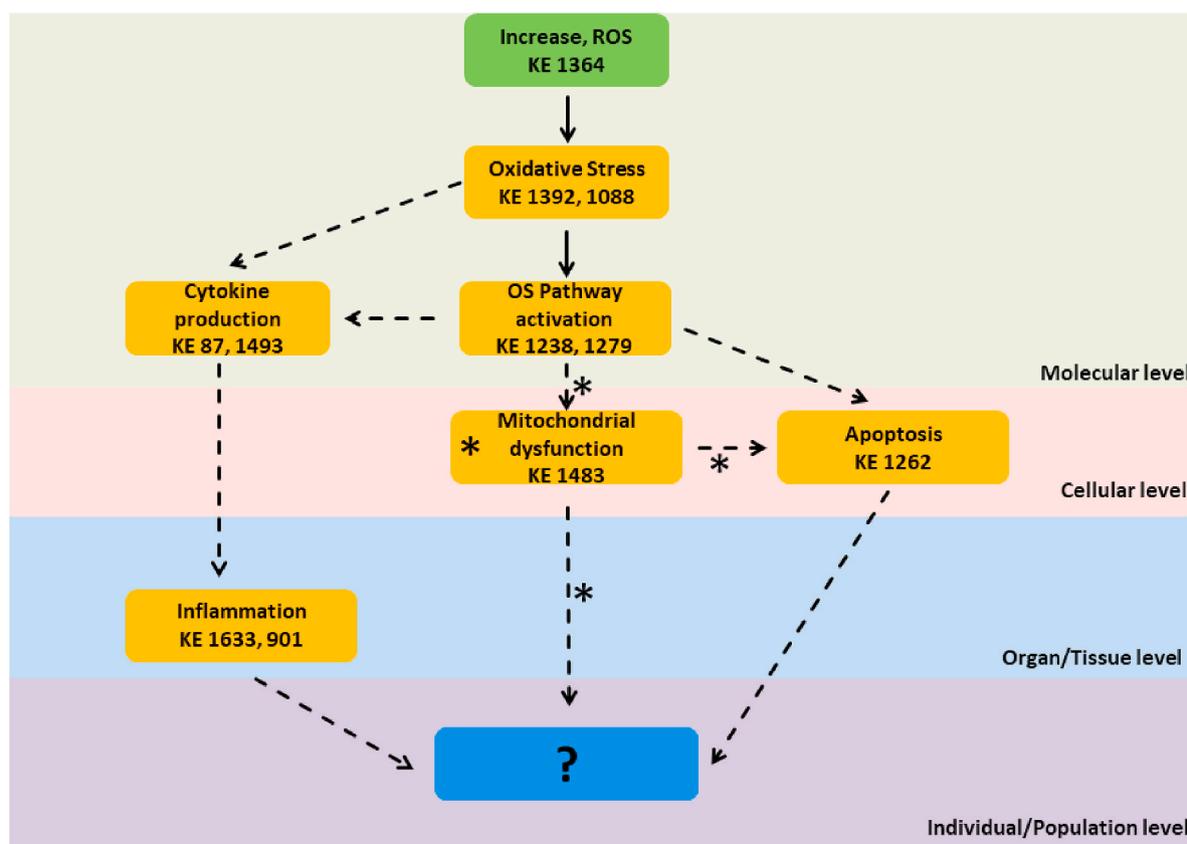


Fig. 2. Adverse Outcome Pathways schematic diagrams related to nanoplasmic and combined micro- and nano-plastics adverse effects from Human/Public health perspective with emphasis on the Oxidative stress and Inflammation responses. Green cuboid: Molecular Initiation Event; Orange cuboid: Key Events; Blue cuboid: Adverse Outcomes. Solid lines: Adjacent or strong evidence relationships; Dashed lines: non-adjacent or weaker evidence supporting the relationship. Asterisk (*) indicates that KE 1483 Mitochondrial dysfunction and related mechanistic relationships apply to nanoplastics AOP only.

Table 2
Nanoplastics induced toxicological endpoints in organisms of ecotoxicological concern.

Organisms		Nanoplastics		Exposure		Endpoints			Other major results	Reference
Species	Type of matrix	Plastic type and size	Surface modification	Concentration	Exposure duration	Molecular and cellular (MIE/KE)	Tissue and organ KEs	Individual or population AO		
algae (<i>S. obliquus</i>) <i>D. magna</i>	Whole body	PS NPs, 70 nm	Pristine, pristine-kairomone, aged, and aged-filtered NPs	0.22–103 mg/L	72 h 21 days	Not stated	Not stated	Growth inhibition Offspring malformation, reduced body size	Increased mortality of <i>D. magna</i> after exposure by aged PS NPs; PS NPs affected several developmental stages	[9]
<i>D. magna</i>	Whole body	PS NPs 50–300 nm	Plain PS, PS-p-NH ₂ , PS-n-NH ₂ and PS-COOH	0–100 mg/L	0–48 h	ROS induction, mitochondrial dysfunction LPO and GSH induction JNK, p38 MAPKs activated	Not stated	Lethality increased Behavior alteration	ROS induction intracellularly most significantly by plain PS NPs as the functional group introduction decreased the toxicity in <i>D. magna</i> . No significant influence on AChE activity by plain PS NPs but by functionalized PS NPs.	[55]
<i>Mytilus galloprovincialis</i>	Hemocytes	PS NPs	PS-NH ₂	1, 5 and 50 µg/mL	10–60 min	Increased lysosomal membrane destabilization Activation of p38 MAPK and PKC (Protein kinase C)	Not stated	Not stated	Disruption of phagocytic or endocytic pathways, formation of PS-NH ₂ -protein corona	[54]
Pacific oyster (<i>Crassostrea gigas</i>)	Larvae	PS NPs, 70 nm–20 µm	Plain PS, PS-NH ₂ , PS-COOH	1000 plastics/mL	24 h	Not stated	Accumulation of NPs in the larval	No significant impact on developmental rate	The aminated PS beads were engulfed in a greater number by larvae than carboxylated and standard PS beads	[35]
Fathead minnow (<i>Pimephales promelas</i>)	Neutrophils	PS 41.0 nm, polycarbonate 158.7 nm NPs	Plain PS and PC NPs	0.025–0.2 µg/µL	2 h	Neutrophils increases in degranulation of primary granules and neutrophil extracellular trap release PC NPs induced increase of respiratory burst	Not stated	Not stated	All PSNP concentrations caused a significant increase of the degranulation of neutrophil primary granules in a dose-dependent manner	[39]
<i>Danio rerio</i>	Larvae	PS NPs, 50 nm	Plain PS NPs	1 mg/L	48, 72, and 120 h	ROS induction detected Nervous system development related genes' expression altered AChE activity related neurotoxicity detected	Not stated	Larval behavioral alteration Body length reduced Locomotion hampered	Nanoplastics significantly inhibited the acetylcholinesterase activity, upregulated rhodopsin and blue opsin gene expression, reduced the length of larvae body and limited the larvae locomotion.	[48]
<i>Daphnia pulex</i>	Whole body	PS NPs, 50 nm	Plain PS NPs	0.1 mg/L 1 mg/L	1–21 days	Antioxidant genes expression altered Genes encoding heat shocking proteins changed energy-sensing enzyme AMPKα, β and γ were significant different	Not stated	Not stated	LC50 values of 1- and 21-day-old <i>D. pulex</i> did not significantly differ from different age groups, suggesting that juveniles and relatively old adults could be equally sensitive to the NPs	[93]

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Table 2 (continued)

Organisms		Nanoplastics		Exposure		Endpoints			Other major results	Reference
Species	Type of matrix	Plastic type and size	Surface modification	Concentration	Exposure duration	Molecular and cellular (MIE/KE)	Tissue and organ KEs	Individual or population AO		
Monogonont Rotifer (<i>Brachionus koreanus</i>)	Whole body	PS 50 nm	Plain PS NPs	0.1, 1, 10, and 20 µg/mL	24 h, 12 days	ROS, MAPK and Oxidative stress (p-JNK, p-p38, GPx, GR, GST, SOD) with highest level by NPs exposure Mitochondrial membrane integrity decreased by NPs exposure	Not stated	Fecundity reduced by NPs Prolonged Reproduction time Growth rate decreased	Nanosized plastic particles cause adverse effects on normal physiological responses on growth, hatching, and reproduction in the rotifer and also indirectly may affect energy flow in the aquatic ecosystem	[26]
Oyster gametes (<i>Crassostrea gi2gas</i>)	Gametes	PS NPs, 100 nm	PS PS-COOH PS-NH ₂	0.1–100 mg/L	1–5 h	PS-COOH induced ROS production in spermatozoa	Spermatozoa aggregation	Not stated	A significant dose-response increase in ROS production by spermatozoa was demonstrated upon exposure to PS-COOH, but not with PS-NH ₂ .	[75]
<i>Daphnia pulex</i>	Whole body	PS NPs, 75 nm	Plain PS NPs	0.1–2 mg/L	21 days	The expression of cytochrome P450 (CYP) family genes were altered	Not stated	Not stated	The transcriptional levels of DpCYP370B, CYP4AN1, CYP4C33, and CYP4C34 were induced by low concentrations of nanoplastics and inhibited at high concentrations of nanoplastics	[86]
<i>Danio rerio</i>	Larvae	PS NPs, 25 nm	Plain PS NPs	10–100 mg/L	48 h	Glucose level during development hampered, insulin expression decreased	PSNPs accumulation in neuromasts thus hamper the HPI-axis, leading to cortisol secretion	Larvae locomotor activity altered	Polystyrene nanoparticles can disrupt glucose homeostasis with concurrent activation of the stress response system during the development of zebrafish larvae	(Brun, van Hage et al., 2019)
<i>Danio rerio</i>	Embryo	PS NPs, 25 nm	Plain PS NPs	10 mg/L	0–120 h	mRNA of pro-inflammatory cytokines (<i>irg1l</i> , <i>il1β</i> , and <i>tnfa</i>) were significantly altered	Inflammatory responses in the tissues affected, particularly the intestine, the skin, and neuromasts	Not stated	Obtained results provide the first evidence that nanoparticles can induce pro-inflammatory responses in the skin and intestine cells.	[10]
Blue mussel (<i>Mytilus edulis</i>)	Whole body	30-nm PS NPs	Plain PS NPs	0, 0.1, 0.2, and 0.3 g/L	4 h	Not stated	Not stated	PS NPs triggered the production of pseudofeces Based on the reduced opening of the valve, the organism's filtering activity was reduced	Long-term exposure to nano PS may therefore harm <i>M. edulis</i> because producing pseudofeces expends energy and reduced filtering activity may eventually lead to starvation.	[94]
<i>Caenorhabditis elegans</i>	Whole body	100 and 500 nm, PS NPs	Plain PS NPs	1 mg/L	3 days	PS NPs enhanced the expression of <i>GST-4</i> and induced oxidative stress AChE transporter encoding genes expression altered	Damage in cholinergic and GABAergic neurons	Motor behavior changed (body bending and head thrashing) Growth inhibition Impaired development	Nanoplastics inhibited the growth and development of nematode individuals, altered locomotor behavior in a manner of size-dependent toxicity, induced significantly oxidative damage, and led to neurotoxicity.	[13]

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Table 2 (continued)

Organisms		Nanoplastics		Exposure		Endpoints			Other major results	Reference
Species	Type of matrix	Plastic type and size	Surface modification	Concentration	Exposure duration	Molecular and cellular (MIE/KE)	Tissue and organ KEs	Individual or population AO		
Mussel (<i>Mytilus galloprovincialis</i>)	Whole body	110 ± 6.9 nm, PS NPs	Plain PS NPs	0.005–50 mg/L	96 h	(<i>unc-17</i> , <i>unc-47</i> and <i>dat-1</i>) Cell-tissue repair related gene <i>hsp70</i> altered after 50 mg/L PS NPs exposure in GI track <i>cyp11</i> gene expression increase, phase I metabolism activated Immune functional genes activated Oxidative stress and Lipid peroxidation decreased AChE activity DNA damage occurred	Not stated	Not stated	PS NPs, even at low concentrations, led to alterations on biotransformation, DNA repair, cell stress-response and innate immunity genes. Also, the genotoxicity and the oxidative stress were detected in the mussel.	[64]
Mussel (<i>Mytilus galloprovincialis</i>)	Hemocytes	50 nm, PS NPs	PS-NH ₂	1–50 mg/L	0.5 h	lysosomal damage apoptotic processes oxyradical production membrane blebbing and loss of filopodia	Not stated	Not stated	Putative Clq domain containing protein (MgClq6) as the only component of the PS-NH ₂ hard protein corona in <i>Mytilus</i> hemolymph.	[54]
<i>Danio rerio</i>	Adult	42 nm, PS NPs	Plain PS NPs	5 mg/L	7 days	Oxidative stress in the brain of the female, muscle and gonad of the male	N/A	N/A	PS nanoplastics can be transferred from mothers to the offspring via accumulation in the eggs due to interaction of nanoplastics with plasma proteins of oocytes but not affecting the fecundity. PS NPs can penetrate the zebrafish chorion and are taken up by the embryo PS NPs accumulated in gills, liver, and gut of zebrafish	[101]
	Embryo					N/A	bradycardia	Developmental impairment		
<i>Danio rerio</i>	Embryo	51 nm, PS NPs	Plain PS NPs	0–10 ppm	120 h	Not stated	bradycardia	Altered larval behavior		[100]
<i>Danio rerio</i>	Adult	70 nm PS NPs	Plain PS NPs	20 mg/L	7 days	Oxidative stress	Liver	Inflammation lipid accumulation necrosis	Metabolism stress (in lipid metabolism and energy metabolism)	[15]
<i>Caenorhabditis elegans</i>	Whole body	100 nm, PS NPs	Plain PS NPs	10–100 mg/L	Approximately 4.5 days	ROS production	Intestinal permeability increased	decreased locomotion behavior reduced body size	Adverse effect on the function of the intestinal barrier in nematodes was detected. Also the transgenerational toxicity detected	[95]

6. Applying adverse outcome pathways approach to highlight MP/NPs reported toxicity

In previous sections of this review, we focused on mechanical consequential relationships of key events and adverse outcomes induced by either MPs or NPs. Since the adverse outcome pathways (AOPs) initiated by the MPs exposure were developed recently [23], we decided to utilize existing AOPs [111] with focus on literature reports describing the NPs and overlapping MP/NPs toxicity endpoints from ecological and public health perspectives (Figs. 1 and 2). The collection of data to be used in identification of NPs triggered events and assigning them to existing AOPs started with a review of publications reporting the toxicity endpoints induced by NPs in non-mammalian organisms with high ecotoxicological relevance/concern as well as in mammals. Broad literature search was based on following key words: “Nanoplastics”, “Toxicity”, “Ecotoxicology” and “Mammals” using Google scholar search engine. The reports used to generate information to be used in the generation of NP/MP specific oxidative damage AOPs were further evaluated and selected according to following criteria:

1. Studies must be written in English.
2. Studies must be available online before December of 2019.
3. The exposure particles must be polymer plastic particles in a nano-size range.
4. The exposure concentration and exposure duration of NPs must be reported.
5. The exposed organisms must be reported.
6. At least one level of toxicity endpoints (molecular, cellular, tissue, organ, individual, population) should be included.

After literature search, reported toxicity endpoints triggered by NPs including Key events (KE) and Adverse outcomes (AO) were categorized into 3 different levels of biological organization: Molecular and Cellular; Tissue and Organ; Individual and Population (Tables 2 and 3). As a next step, we used the existing AOP framework and applied it to identified and categorized KEs and AOs using KEs and AOs information available from the AOP wiki website (<https://aopwiki.org/>), followed by analysis of KE and AO frequency in searched publications.

In total, 31 publications were selected (as 100%, full text available as of January 2020) according to the above criteria and reported toxicity endpoints induced by NPs were evaluated. KEs with highest reported frequency were “Oxidative stress” (45.2%), “Pro-inflammatory cytokines activation” (19.4%), “Increase, ROS” (19.4%) and “Apoptosis” (16.1%). Based on this data, the oxidative stress and inflammatory responses are considered as major adverse events induced by NPs at all the biological complexity levels except individual and population levels. This NP related finding is in agreement with previously reported AOP summary for MPs [23]. Among the critical key events, we determined that increase of ROS could be designated as Molecular Initiating Event (MIE). ROS generation is critical molecular event that is occurring prior to most frequent KE (Oxidative stress), as stated previously (Section 2.1). Other significant KEs at molecular/cellular level are “Mitochondrial dysfunction” (13.0%), “Acetylcholinesterase (AChE) inhibition” (9.7%), “Lysosome disruption” (9.7%), “Lipid peroxidation” (9.7%) and “Activation of oxidative stress pathway” (9.7%). Mechanistic relationship between these KEs was reviewed and discussed in Sections 2 and 3.

Information regarding Tissue/Organ KEs was found to be limited in reports that fulfill selection criteria, possibly due to high diversity and lack of standardization of research methods and techniques used in studies of MP/NPs effects at this level. In selected literature, most abundantly reported KEs are “Inflammation” (13.0%) and “Decrease of

neuronal network function” (13.0%). Final emerging AOs are listed as “Locomotor activity, decreased” (22.6%), “Impaired, Development” (13.0%), “Decreased body size/length” (13.0%) and “Growth inhibition” (9.7%). It was determined that studies related to individual/population adverse outcomes are almost exclusively available for organisms with ecotoxicological concern. Lack of *in vivo* mammalian studies of NPs toxicity is currently causing a gap in our knowledge and prevents development and possible construction of detailed AOPs (Table 1). The current status and mechanistic relationships between above listed AOs and KEs are discussed in Section 5.

Selection of existing AOPs or their components related to nanoplastics toxicity was based on most prominent KEs and AOs. Developed schematic was presented as ecologically relevant AOP (Fig. 1A), and human public health perspective AOP (Fig. 2). The NPs AOP indicate that at the ecosystem level, NPs could cause toxicity based on increased ROS formation through activation of oxidative stress pathways, followed by cytokine production, mitochondrial dysfunction and AChE inhibition, that in turn would further induce inflammation and neuronal dysfunction to cause changes in activity/behavior, and interfere with normal development and growth.

Use of AOPs to highlight public health perspective of NPs toxicity was limited with the scarcity of available data from publication fulfilling the listed criteria, and the outcome is therefore simpler. In short, generation of ROS can lead to oxidative stress and inflammatory responses that induce apoptosis. However, final AOs from a public health perspective are largely unknown. In an effort to partially overcome this problem, we used MPs related AOPs as reported by Jeong et al. and modified it as AOP schematic to include both MPs and NPs (Fig. 2). Overall, the results of applying the AOP approach to evaluate MP/NP toxicity indicate that oxidative stress and inflammation responses initiated by ROS production represent two cascades of critical adverse key events that are shared by MP/NPs in both ecological and public health perspectives. Main difference between the two AOP schematics is that from ecological perspective, the relevant pathway involves inhibition of AChE followed with locomotor or feed behavior changes leading to growth retardation (Fig. 1). From public health perspective, apoptosis induced by oxidative stress pathway activation may be the relevant AOP cascade (Fig. 2).

7. Conclusion

Toxicity mechanisms induced by both microplastics and nanoplastics were reviewed with an emphasis on oxidative stress and inflammation. These adverse effects were categorized into different levels of biological complexity from both ecosystem and human health perspectives. Mechanistic relationships between different toxicity endpoints have been discussed using the concept of Adverse Outcome Pathways. The information about key events and adverse outcomes was collected and matched with the online knowledgebase to utilize existing AOPs and apply them to highlight possible effect mechanisms of nanoplastics alone and cumulative micro- and nano-plastics on ecosystems and public health. Development of AOP schematics based on AOPs knowledge base and literature search revealed that MP/NPs both share the ROS formation as their initial molecular occurrence, causing AOs such as growth inhibition and behavior alteration through oxidative stress cascades and inflammatory responses in the ecotoxicological context. From public health perspective, AOP of MP/NPs toxicity showed no clear adverse outcomes probably due to limited information, however it also supported that events of oxidative stress and its responding pathways, including inflammation play a critical role.

Table 3
Nanoplastics induced toxicological endpoints based on mammalian studies.

Organisms		Nanoplastics		Exposure		Endpoints			Other major results	Reference
Species	Cell/Tissue type	Plastic type and size	Surface modification	Concentration/Dose	Exposure duration	Molecular and cellular (MIE/KE)	Tissue and organ KEs	Individual or population AO		
Wistar rats	Whole body	PS NPs 38.92 nm	Plain PS NPs	1, 3, 6, and 10 mg PS-NPs/kg of body weight/day	35 days	Not stated	Not stated	may exacerbate behavioral effects	The uptake of pristine nanoparticles did not affect behavior of adult rats	[82]
Wistar rats	Whole body	25 and 50 nm, PS NPs	Plain PS NPs	1, 3, 6, and 10 mg PS-NPs/kg of body weight/day	35 days	high-density lipoprotein level blocked	Not stated	thyroid endocrine disruption	PS treatments significantly increased serum LDL, cholesterol, GOT, and GPT levels notably	[19]
Human and mouse	human bronchial epithelium (BEAS-2B), mouse monocyte macrophage (RAW 264.7)	PS NPs 60 nm	Plain PS, PS-NH ₂ , PS-COOH	10–25 mg/L	1–8 h	lysosomal permeability increased in RAW 264.7 after PS-NH ₂ exposure Ca ²⁺ influx increased in RAW 264.7 after PS-NH ₂ exposure Apoptosis Mitochondrial disruption Cell viability hampered Oxidative stress leading apoptosis	Not stated	Not stated	The cationic PS nanospheres had no effect on cellular toxicity in HEPA-1, HMEC, and PC-12 cells	[70]
Human	colon carcinoma cells (Caco-2)	PS NPs 20–40 nm	Plain PS, PS-NH ₂ , PS-COOH	0.3, 0.9, 2.0, 4.0, and 6.6 nM	4–16 h	PS-NH ₂ induced apoptosis in all cell types All NPs induced ROS induction PS-NH ₂ induced mitochondrial disruption and release of cytochrome C	Not stated	Not stated	the uptake efficiency is surface charging and size dependent	[85]
Human	alveolar epithelial type 1, 2 cells (TT1, AT2), primary alveolar macrophages (MAC)	PS NPs 50–100 nm	Plain PS, PS-NH ₂ , PS-COOH	1–100 mg/L	4 and 24 h	PS-COOH induced cytokine production (IL-8, IL-6) 20 nm PS-COOH induced Oxidative stress PS-COOH stimulated myeloperoxidase release of granulocytes and nitric oxide generation in macrophages	Not stated	Not stated	Plain PS, PS-COOH exhibited little cytotoxicity or mitochondrial damage, although they induced ROS; TT1 and MAC cells internalized all NP formats, whereas only a small fraction of AT2 cells internalized PS-NH ₂	[71,104]
Human	monocytic leukemia cell line THP-1, histocytic lymphoma cells U937	PS NPs 20, 100, 200, 500, 1000 nm	PS-COOH	10, 20 and 50 mg/L	30 min to 24 h	PS-NH ₂ nanobeads induced DNA double strand breaks GSH depletion, antioxidant hamper	Not stated	Not stated	Twenty nanometers CPS were cytotoxic to all phagocytes, ≥500 nm CPS particles only to macrophages. 20 nm particles were taken up passively, 100–1000 nm actively and passively.	[14]
Human	Calu-3 epithelial cells, monocytic leukemia cell line THP-1	PS NPs 50 nm	Plain PS, PS-NH ₂ , PS-COOH	1–100 mg/L	1–24 h	Induction of apoptosis by PS-NH ₂	Not stated	Not stated	Particles partly adsorbed and internalized then released by Calu-3 cells; THP-1 macrophages quickly incorporated all nanobeads. Surface modification matters in the nanotoxicology.	[60]
Human	colon carcinoma cells (Caco-2, LS174T, HAT-29)	PS NPs 57 nm	Plain PS, PS-NH ₂ , PS-COOH	20, 50, and 100 µg/mL	72 h		Not stated	Not stated	Positively Charged Polystyrene NPs Reduce Cell Viability; binding of mucin	[89]

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Table 3 (continued)

Organisms		Nanoplastics		Exposure		Endpoints			Other major results	Reference
Species	Cell/Tissue type	Plastic type and size	Surface modification	Concentration/Dose	Exposure duration	Molecular and cellular (MIE/KE)	Tissue and organ KEs	Individual or population AO		
Human	Monocyte macrophages	PS NPs; 120 nm	Plain PS, PS-NH ₂ , PS-COOH	100 µg/ml	24 h	Nanoplastics impaired expression of scavenger receptor (CD163 and CD200R) in M2 Nanoplastics impaired the release of cytokines (IL-10) in M2 Frustrated phagocytosis by PS-NH ₂ PS-COOH increased ATP level in M2	Not stated	Not stated	The nanoparticles did not compromise macrophage viability nor did they affect the expression of the M1 markers CD86, NOS2, TNF-α, and IL-1β.	[83]
Human	Gastric adenocarcinoma (AGS) cells	PS NPs, 44 and 100 nm	Plain PS	2, 5, 10, 20 and 30 µg/ml	1–24 h	Cytokine genes expression increase (IL-6 and IL-8) Up Regulation, TGFβ1 expression	Not stated	Not stated	NPs in smaller size accumulate rapidly and more efficiently in the cytoplasm of AGS than bigger size; energy dependent mechanism of internalization and a clathrin-mediated endocytosis pathway	[88]
Human	ovarian cancer cells	PS NPs 10–30, 50 nm	Plain PS, PS-NH ₂ , PS-COOH	10–75 µg/ml	1–8 h	PS-NH ₂ accumulated within lysosomes cell death	Not stated	Not stated	Polystyrene nanoparticle uptake occurred via a caveolae-independent pathway, and was negatively affected by serum	[74]
Human	lung adenocarcinoma cells (A549)	PS NPs 64, 202, 535 nm	Not stated	250 µg/ml or 2 mg/ml	2–24 h	Increased cytokine production lactate dehydrogenase increase	Not stated	Not stated	Ultrafine polystyrene particles also stimulated the entry of extracellular calcium on treatment with thapsigargin	[90]
Rat	Whole body				24 h		Neutrophil influx (Infiltration, Inflammatory cells) Inflammation			

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