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Review

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Nanoplastic toxicity towards freshwater organisms

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Abstract

The fragmentation of plastic litter into smaller fragments, known as microplastics and nanoplastics, as well as their toxicity and environmental distribution have become issues of high concern. Furthermore, the popularization of bioplastics as a greener substitute of conventional plastics represents a challenge for the scientific community in view of the limited information concerning their potential environmental impact. Here, we systematically review the recent knowledge on the environmental fate and toxicity of nanoplastics in freshwater environments, discuss the results obtained thus far, and identify several knowledge gaps. The sources and environmental behaviors of nanoplastics are presented considering *in vitro*, *in vivo*, and *in silico* studies with a focus on real exposure scenarios. Their effects on organisms are classified based on their impact on primary producers, primary consumers, and secondary consumers. This review covers the main results published in the last four years, including all relevant experimental details and highlighting the most sensitive toxicity endpoints assessed in every study. We also include more recent results on the potential environmental impact of biodegradable plastics, a type of material belonging to the category of bioplastics for which there are still scarce data. This review identifies a need to perform studies using secondary nanoplastics rather than synthetic commercial materials as well as to include other polymers apart from polystyrene. There is also an urgent need to assess the possible risk of nanoplastics at environmentally realistic concentrations using sublethal endpoints and long-term assays.

Keywords: Nanoplastics, environmental fate, toxicity, freshwater organisms

INTRODUCTION

Global plastic pollution is a social, political, and scientific cause for concern due to the large amount of plastic litter currently ending up in the environment[1]. Approximately, 9×10^{12} tons of plastics have been marketed since 1950, with a current annual production of > 360 million tons[2]. Despite the recent slight reduction in global plastic manufacturing, the increasing social awareness concerning this type of materials, and the political attempts to regulate single-use plastics, the global trends in plastic use by segments are preserved[3,4]. A considerable amount of these plastics ends up in the environment through different dissemination pathways[5-8]. For instance, the occurrence of microplastics (MPs) in the upper ocean layer has been estimated at $0.8\text{-}5.8 \times 10^5$ tons, equivalent to $> 10^{19}$ items[9]. A major source of ocean plastic pollution comes from rivers, the contribution of which has been estimated to range $0.8\text{-}2.7 \times 10^6$ tons/year[10].

Plastic fragmentation proceeds due to environmental factors such as photodegradation, hydrolysis, or physical abrasion that ultimately result in small fragments, the smallest of which are termed nanoplastics (NPLs)[11-13]. Despite the limited knowledge of the actual role of different aging processes, the potential release of NPLs from MPs raises the possibility of increasing by several orders of magnitude the number of plastic fragments in the environment[14]. The main property defining NPLs is their size, specifically the length of their largest dimension. There is no agreement within the scientific community regarding the upper limit of this size range. Some authors use the limit of 100 nm[15,16], while others prefer 1000 nm[17,18]. There are reasons to support any of these definitions based on analytical limitations or colloidal behavior in water suspension, but a detailed discussion is outside the scope of this review. Overall, NPLs must be considered emerging pollutants with specific properties, different from both larger plastic items, such as MPs, and engineered nanomaterials[19].

Plastic pollutants can be divided into two categories: "primary" refers to plastic items intentionally produced in that specific size and shape that end up in the environment as a consequence of their use or due to waste mismanagement[20] and "secondary" denotes plastic items that are caused by the environmental fragmentation of larger particles[21,22]. This criterion allows policymakers to establish regulations based on their different environmental risks[23]. It is also important to consider the aging processes they undergo because of their influence on reactivity, release of additives, pollutants adsorption behavior, and integrity of plastic particles, among others[24,25]. Furthermore, it is important to include the weathering degree of plastic as an additional criterion for particle characterization. For this propose, standardized methods are needed. The literature provides some approaches that may be useful, such as those based on the oxygen-containing surface groups[26-28]. However, precise characterization criteria for plastic particles are still needed to evaluate the environmental risk of plastic pollution, especially concerning the lower size ranges.

The assessment of NPLs in complex matrices has been hindered by the limited availability of adequate analytical techniques, although new recent tools and methodologies, particularly those based on mass spectrometry, have allowed significant progress in that direction[28,29]. Recent reports concerning NPLs occurrence in the environment have shown their presence in different aqueous and terrestrial compartments, and their widespread presence is generally assumed[30-32]. In parallel, investigation on the potential effects of NPLs to the biota is receiving increasing attention driven by data showing that they are potentially more harmful than larger fragments. NPLs can be internalized by cells through either passively crossing the cellular membrane (promoted by their hydrophobicity and small size) or endocytic processes[33,34]. Furthermore, their large surface area to volume ratio makes them more prone to interact with

environmental contaminants[35]. The capacity to act as a vector for the transfer of pollutants to aquatic organisms has been termed the "Trojan Horse" effect and is the subject of active research[36].

The goal of this review is to discuss the recently reported studies (since 2019) on the effects of NPLs to freshwater organisms. The articles were selected from a first thorough search using the Web of Science citation database with the keywords defining this review (nanoplastics, environmental fate, toxicity, and freshwater organisms) followed by a cross-referencing search in an attempt to identify all relevant articles covering the nanoplastic toxicity towards freshwater organisms. A screening of similar articles from the same groups led to the set of references cited herein. Although most published results refer to polystyrene (PS) NPLs, we also review those obtained with other polymers, with an emphasis on secondary NPLs rather than those specifically produced in that size. Furthermore, as potential replacing material for the traditional oil-based plastics, we include a section focused on the impact of biodegradable plastics in the environment. As the environmental fate of NPLs is widely determined by the stability of their colloidal properties, we reserve one specific section to review the existing body of knowledge on this specific topic. In what follows, studies are classified based on the trophic level of the organisms: primary producers, primary consumers, and secondary producers. Studies concerning the combined toxicity of NPLs and other emerging pollutants are also reviewed. Finally, this review identifies research needs and gives recommendations aimed at minimizing NPLs pollution in the environment.

SOURCE AND OCCURRENCE OF NPLS IN FRESHWATER ENVIRONMENTS

The emission sources as well as the impact of NPLs in the environment remain largely unknown mainly due to the limitations concerning the characterization and identification of small carbon-based particles in complex matrices[15]. In this context, studies are increasingly addressing the potential release of primary and secondary NPLs under relevant conditions. Regarding primary NPLs, it has been shown that the use of facial scrub may release over 10^{13} sub-micron particles per gram of product, mostly discarded with household wastewater[29]. Several studies have addressed the continuous release of NPLs from larger plastic items subject to environmental degradation. Nylon and polyethylene terephthalate (PET) teabags have been shown to release > 10^{12} NPLs (< 100 nm) along with a similar number of MPs into a single cup of tea[37]. Morgana *et al.* determined that a single face mask could release up to 10^{8} NPLs under mechanical stress forces mimicking those encountered in the environment[38]. Zhang *et al.* demonstrated the release of NPLs from the surface of recycled PVC[39]. Sorasan *et al.* reported the generation of up to 10^{10} NPLs per gram of low-density polyethylene (LDPE) after the exposure of MPs to mechanical agitation and the equivalent to one year of solar irradiation[11]. Luo *et al.* estimated a release of up to 3000 polypropylene (PP) items (MPs and NPs) per mm^2 of plastic chopping boards[40]. Munoz *et al.* used in vitro experiments to simulate vaginal conditions and estimated a release of up to 1.7×10^{13} NPLs per tampon after 2 h of use[41]. The available results show the existence of a number of potential sources of NPLs that may eventually end up in the environment, posing a risk to biota and human health.

Obtaining reliable NPL concentrations in environmental samples remains highly challenging despite the considerable efforts paid and the advances currently ongoing. In this regard, Xu *et al.* combined a concentration pretreatment (< 1 μm followed by ultrafiltration through 100 kDa membranes) with pyrolysis gas chromatography-mass spectrometry (Py-GC/MS) to identify and quantify NPLs from surface water and groundwater and reported a total mass concentration reaching 100s ng/L for PP and polyethylene (PE), which were the main polymers identified[42]. It is important to note that mass spectrometry techniques provide mass concentrations but cannot give particle concentrations. Materić *et al.* analyzed the presence of NPLs in freshwaters from the Siberian Arctic tundra and a forest location in southern Sweden using thermal desorption proton transfer-reaction mass spectrometry (TD-PTR-MS). They identified four polymers, PE,

polyvinyl chloride (PVC), PP, and PET, in different lake and stream samples, with a mean concentration of total nanoplastics (< 0.2 μm) as high as 563 μg/L[43]. A study from the same group reported a concentration of PET and PVC in the 5-23 μg/L range in Alpine snow[44]. These experimental data are not only very different from each other but also several orders of magnitude higher than the 0.14-1.4 ng/L range calculated combining the total estimated mass of plastic debris with 3D fragmentation models[45]. The differences may be attributed to the heterogenous distribution of plastics or to a low model accuracy, but they evidence the lack of reliable field data concerning actual environmental concentrations of NPLs. Further efforts should be done in this direction supported by the current development of more accurate and efficient techniques that allow NPL identification and quantification in environmental samples.

PHYSICOCHEMICAL BEHAVIOR OF NPLS IN FRESHWATER

The occurrence of NPLs in freshwater implies their interaction with biota as well as other compounds naturally present such as natural organic matter (NOM), extracellular polymeric substances (EPS), or inorganic compounds (ions, including metals, clay, and other minerals), among others. Such interactions modulate the different mobility, toxicity, bioavailability, distribution, and fate of NPLs[20,46]. The stability/aggregation behavior of NPLs is addressed based on the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory that combines the effects of van der Waals attraction forces and electrostatic repulsion between charged particles, although other non-DLVO interactions, such as bridging flocculation, patch-charge attraction, π-π interaction, or steric repulsion, may be also involved in the aggregation process of NPLs[47,48]. The aggregation kinetics of NPLs is frequently given by the evolution of hydrodynamic size (d_H) with increasing concentration of a given ion, which allow determining the critical coagulation concentration (CCC, or the concentration at which the aggregation rate is maximum). CCC has been commonly used to assess the stability of NPLs under different environmental conditions[49-51]. Table 1 shows CCC values reported in the literature for different NPLs.

The stability of PS-NPL suspensions decreases as the concentration of monovalent ions increases as result of the screening effects of the ions that reduce repulsion forces. The aggregation is higher in the presence of divalent ions (using $CaCl_2$), in agreement with the Schulze-Hardy rule stating that higher valence ions result in faster aggregation due to the compression of the electrical double layer[50,52,60]. This tendency has also been reported for other mono- and divalent ions using K^+, Mg^{2+}, and Ba^{2+}[55]. Indeed, trivalent ions such as Al^{3+} have been proposed as strong coagulants for NPL removal[62]. Similar results were obtained for heavy metal salts[50]. Concerning surface modified NPLs, it has been reported that carboxyl modified PS-COOH displays similar stability as non-functionalized PS-NPLs, while, in the case of amino-modified PS-NH_2, the suspension remained stable even at concentrations as high as 1 and 0.15 M of NaCl and $CaCl_2$, respectively[51]. Similar observations have been reported for irregularly shaped NPLs, which otherwise presented lower CCC values compared with spherical PS-NPLs under similar conditions, indicating less stability of irregularly shaped NPLs aqueous suspensions[49,61]. Weathering is another effect that influences NPL fate in the environment. Recent reports assessed the stability of UV-aged NPL suspensions, showing higher stability of aged NPLs due to the increase in oxygen-containing functional groups that decrease particle hydrophobicity and increase the absolute value of the ζ-potential of negatively charged NPLs[55,59]. The stabilization of irradiated and oxidized PS-NPLs has been attributed to stronger Lewis acid-base interactions that resulted in higher hydration forces. In contrast, UV-aged PS-NPL suspensions displayed less stability than their pristine counterparts when exposed to increasing concentration of divalent cations. This finding has also been attributed to the bridging of oxygen-containing functional groups with Ca^{2+}, thereby promoting the aggregation of UV-irradiated PS-NPLs in $CaCl_2$ solutions[60]. Interestingly, when the weathering is simulated by ozonation, the stability of PS-NPL suspensions increased in the presence of both monovalent and divalent cations attributed to the steric repulsion caused by the attachment of organic

Table 1. Critical coagulation concentration for different NPLs assessed in the presence of mono- and divalent electrolytes as well as different types of natural organic matter or particulate material

NPLs	Size (nm)	Surface, shape, and molecular properties	CCC (mmol/L) $NaCl/CaCl_2$	CCC (mmol/L) $NaCl/CaCl_2$ with (NOM/PM)	pH	Refs.
PS	20	Spherical, NF	311/13	> 300/ > 25 (BSA, 2 mg/L) 10/4.6 (TRY, 2 mg/L)	5	[47]
PS	30	Spherical, NF	540/11	NA	6	[52]
PS	30	Spherical, COOH-	800/10-15	=/= (SRHA, 0.5 mg C/L) =/= (SRHA, 5 mg C/L)	~5	[53]
PS	50	Spherical, NF	264/29	167/20 (CeO_2-NPs), ↓/↓ (CeO_2-NPs+HA 0.1 mg C/L) ↑/= (CeO_2-NPs+HA 5 mg C/L) ↑/↑ (CeO_2-NPs+HA 10 mg C/L)	5	[54]
PS	50	Spherical, COOH-	191/16	60/8 (CeO_2-NPs) ↓/= (CeO_2-NPs+HA 0.1 mg C/L) ↑/↑ (CeO_2-NPs+HA 5 mg C/L) ↑/↑ (CeO_2-NPs+HA 10 mg C/L)	5	[54]
PS	50	Spherical, NH_2-	Stable up to 1000/100	182/27 (CeO_2-NPs) =/↓ (CeO_2-NPs+HA 0.1 mg C/L) ↑/= (CeO_2-NPs+HA 5 mg C/L) ↑/↑ (CeO_2-NPs+HA 10 mg C/L)	5	[48]
PS	50	Spherical, C_2H_2O-	84/10	78/11 (CeO_2-NPs) ↑/↑ (CeO_2-NPs+HA 0.1 mg C/L) ↑/= (CeO_2-NPs+HA 5 mg C/L) ↑/↑ (CeO_2-NPs+HA 10 mg C/L)	5	[48]
PS	80	Spherical, SO_3H-	264/29	47/2 (CeO_2-NPs) ↑/↑ (CeO_2-NPs+HA 0.1 mg C/L) ↑/= (CeO_2-NPs+HA 5 mg C/L) ↑/↑ (CeO_2-NPs+HA 10 mg C/L)	5	[48]
PS	50-100	Spherical, NF	450/33	NA	6	[55]
PS	50-100	Spherical, NF UV-oxidized (5 h)	530/18	NA	6	[55]
PS	50-100	Spherical, NF UV-oxidized (24 h)	760/8	NA	6	[55]
PS	50-100	Spherical, NF	460/32	> 1000/15 (EPS, 2 mg C/L) > 1000/10 (BSA, 2 mg C/L) > 1000/6 (HA, 2 mg C/L) > 1000/NA (SA, 2 mg C/L)	6	[54]
PS	50-100	Spherical, NF	585/NA	1200/NA (HA, 2 mg C/L) 485/NA (DBC, 2 mg C/L)	7	[56]
PS	50-100	Spherical, NF UV-oxidized (12 h)	700/NA	1100/NA (HA, 2 mg C/L) 635/NA (DBC, 2 mg C/L)	7	[56]
PS	50-100	Spherical, NF	270/22	↑/5 (BSA, 2.5 mg C/L) ↑/7 (Col I, 2.5 mg C/L) ↑/↑ (CS, 2.5 mg C/L) =/< 10 (BHb, 2.5 mg C/L) ↑/< 10 (HSA, 2.5 mg C/L)	6	[57]
PS	90	Spherical, NF	159/12	3000/10 (SRHA, 5 mg C/L) 3000/11 (SRFA, 5 mg C/L)	6	[58]
PS	100	Spherical, NF	591/71	↑/NA (EPS)	7.5	[59]
PS	100	Spherical, NF	361/32	> 300/ > 25 (BSA, 2 mg/L) 46/5 (TRY, 2 mg/L)	5	[47]
PS	100	Spherical, NF UV+H_2O_2-oxidized (60 h)	957/NA	NA	7.5	[59]
PS	100	Spherical, NF UV+H_2O_2-oxidized (120 h)	1108/NA	NA	7.5	[59]
PS	100	Spherical, NF	198/21	705/12 (HA, 2 mg C/L) 494/23 (SA, 2 mg C/L) 169/10 (Lz, 2 mg C/L)		[60]
PS	100	Spherical, NF UV-oxidized (12 h)	293/21	480/14 (HA, 2 mg C/L) 200/18 (SA, 2 mg C/L) 74/13 (Lz, 2 mg C/L)	5	[60]
PS	100	Spherical, NF	411/10	NA	5	[60]

		UV-oxidized (24 h)				
PS	100	Spherical, NF O_3-oxidized (15 min)	> 500/28	> 700/20 (HA, 2 mg C/L) > 700/20 (SA, 2 mg C/L) 46/10 (Lz, 2 mg C/L)	5	[60]
PS	100	Spherical, NF O_3-oxidized (30 min)	> 500/37	NA	5	[60]
PS	100	Spherical, NF	349/34	NA	6	[61]
PS	200	Spherical, NF	310/29	410/NA (SRHA, 1 mg C/L) 1138/NA (SRHA, 5 mg C/L)	7.4	[51]
PS	200	Spherical, COOH-	308/28	393/NA (SRHA, 1 mg C/L) 999/NA (SRHA, 5 mg C/L)	7.4	[51]
PS	200	Spherical, NH_2-	Stable up to 1000/150	132/NA (SRHA, 5 mg C/L) 209/NA (SRHA, 10 mg C/L)	7.4	[51]
PS	200	Spherical, COOH-	> 1000/NA	NA		[49]
PS	240	Spherical, NF (dialysed)	140/25	545/6 (HA, 5 mg/L) 83/NA (Clay colloids, 50 mg/L) 73/NA (Clay colloids, 100 mg/L)	6	[50]
PE	250-750	Spherical, NF	~80/0.1	120/0.4 (SRHA, 5 mg C/L)	~ 5	[53]
PS	350	Irregular, mechanically degraded	59/NA	HA 30 mg/L stabilized NPLs at ~530 nm SA 57 mg/L stabilized NPLs at ~820 nm	6.5	[49]
PET	300-700	Irregular, mechanically degraded	54/2	559/12 (HA, 1 mg/L)	6	[61]

NA refers to data not available in the specific study. The symbols "↑", "↓", and "=" refer to increase, decrease, and non-variation, respectively, of CCC in the presence of NOM or PM with respect to the values with the electrolyte only. CCC: Critical coagulation concentration; NPLs: nanoplastics; NOM: natural organic matter; PM: particulate matter; PS: polystyrene; PE: polyethylene; PET: polyethylene terephthalate; NF: non-functionalized; COOH-: surface carboxylate modified; NH_2: surface amino modified; C_2H_2O-: ethylated surface; HA: humic acids; SRHA: Suwannee River humic acids; SRFA: Suwannee River fluvic acids; EPS: extracellular polymeric substances; BSA: bovine serum albumin; SA: sodium alginates; TRY: bovine trypsin; Col I: collagen type I; DBC: dissolved black carbon; CS: bovine casein; BHb: bovine hemoglobin; HSA: human serum albumin; Lz: lysozyme.

matter released from PS degradation[60]. Temperature has also been reported as an important factor determining NPL behavior in aqueous suspensions. It has been shown that temperature increases reduce the CCC values of PS-NPLs, promoting NPL aggregation[50].

NOM, ionic strength (IS), and pH are the most relevant parameters determining the fate of NPLs in real freshwater[49]. The influence of NOM on the stabilization of NPL suspensions depends on the simultaneous presence of electrolytes as well as the concentration and type of NOM[60]. The differences have been attributed to the thickness of the macromolecular layer adsorbed onto the surface of NPLs[54]. Despite the limited number of studies addressing the stability of non-pristine NPL suspensions, it has been reported that mechanically degraded PS-NPL suspensions stabilize in the presence of humic acids (HA) through electrostatic and steric repulsions, as well as with sodium alginate (SA) via hydrogen bonds and van der Waals interactions[49]. The stability of UV-aged PS-NPLs increased in the presence of monovalent electrolytes, although SA yielded less stable suspensions. However, ozonated PS-NPL suspensions displayed higher stability in the presence of HA and SA, in the presence of both mono- and divalent electrolytes[60].

The concentration of mono- and divalent ions reported for freshwater bodies rarely exceeds ~50 mM for Na^+ and ~2.5 mM for Ca^{2+}. Therefore, it is likely that most NPL suspensions are stable in natural freshwater ecosystems since their CCC values are considerably higher[63]. However, freshwater may contain other substances than those reviewed here that could co-occur with NPLs and modify the stability of their suspensions. The results summarized in the Table 2 indicate that most tested NPL suspensions displayed considerable stability. This implies that NPLs in freshwater ecosystems would be bioavailable within the water column and that their fate and distribution would be dominated by their mobility throughout the water column.

Table 2. Stability of NPLs in different natural freshwaters

NPLs	Size (nm)	Surface	Time (min)	River water	Lake water	Groundwater	Refs.
PS	240	Spherical, NF (dialysed)	10	Stable at ~255 nm	NA	Stable at ~240 nm	[50]
PE	250-750	Spherical, NF	60	Stable at ~250-750 nm	NA	Unstable	[55]
PS	25	Spherical, NF	10 days	Increase POM stable at ~4 μm	Increase POM stable at ~4 μm	NA	[46]
PS	90	Spherical, NF	180	Stable at 90-200 nm	Unstable, > 490 nm	Stable at 90-200 nm	[57]
PS	100	Spherical, NF	15	Stable at ~100 nm	Stable at ~100 nm	Stable at ~100 nm	[60]
PS	100	Spherical, NF surface UV-oxidized (12 h)	15	Stable at ~100 nm	Stable at ~100 nm	Stable at ~100 nm	[60]
PS	100	Spherical, NF surface O_3-oxidized (15 min)	15	Stable at ~100 nm	Stable at ~100 nm	Stable at ~100 nm	[60]
PS	50-100	Spherical, NF	120	Stable	NA	NA	[64]

NA refers to data not available in the specific study. NF: Non-functionalized; NPLs: nanoplastics; PS: polystyrene; PE: polyethylene.

It is important to note that all the results listed in the preceding tables (and most of those in the following ones) correspond to spherical PS particles specifically produced in that size and not to incidental secondary NPLs, which would be expected to display a variety of shapes. This is a limitation found in most of the literature concerning NPLs and the reason we also include in this review article some other polymeric NPs such as poly(amidoamine) (PAMAM) dendrimers, which are not conceptually distant from PS latexes.

NPL TOXICITY TOWARDS FRESHWATER PRIMARY PRODUCERS

Freshwater primary producers such as benthic algae and cyanobacteria (periphyton), phytoplankton (suspended algae and cyanobacteria), and macrophytes are crucial for the preservation of freshwater trophic chains. Considering the large amount of plastic litter transported by rivers, NPLs are expected to influence primary producers[65]. Table 3 summarizes the main recently published findings on the single and combined toxicity of NPLs towards freshwater primary producers (excluding macrophytes), highlighting the more sensitive toxicity endpoints as reported by the authors. Thus far, most of the *in vitro* studies have assessed NPL toxicity at high concentrations. This approach may disclose potential biological targets (such as ROS homeostasis alteration and photosynthesis impairment) of NPLs and establish dose-response curves to further understand the toxicological behavior of NPLs and their interaction with other pollutants[91]. It has been shown that both micrometric and nanometric plastic particles may trigger clear effects at high concentrations. In addition, 100 nm PS-NPLs have been shown to cause higher growth inhibition, higher ROS and lipid peroxidation levels, and overproduction of antioxidant enzymes in the algae *Chlamydomonas reinhardtii* compared to 100 μm MPs at the same mass concentration[66]. Furthermore, the internalization of NPLs in algae and cyanobacteria has been reported for sizes between 20 and 100 nm[67,78], as well as in microalgae for sizes up to 2 μm[68]. This process occurs through different potential pathways: (1) direct crossing through the porous structures of cell envelopes for NPLs < 20 nm; (2) direct passage through the cell wall owing to increased cell membrane permeability during cell cycling (up to 140% of the normal permeability); and (3) endocytosis for larger NPLs[92]. These processes, together with NPL attachment onto the cell surface, may result in the ingestion of NPLs by grazers[93]. Apart from their effects at high concentrations, NPL concentrations ≤ 1 mg/L have been reported to cause effects on primary producers. Xiao *et al.* observed a reduction in pigment content (chlorophyll *b*) and an increase in superoxide dismutase (SOD) activity in *Euglena gracilis* exposed to 1 mg/L of 100 nm PS-NPLs[67]. Wang *et al.* reported a growth inhibition of 15.6% in *Chlorella pyrenoidosa* upon exposure to 1 mg/L of 600 nm PS-NPLs[79].

Table 3. Toxicological effects of NPLs on freshwater primary producers

Type of exposure	Size (nm)	Concentration tested (mg/L)	Test organism	Exposure time	Most sensitive parameter	Effects	Refs.
Single exposure							
PS-NPLs	100	50-500	*Chlamydomonas reinhardtii*	0-96 h	POD activity (U/mg of protein)	EC_{50} 300 mg/L (growth inhibition). Decrease in chlorophyll *a* and *b* and carotenoid contents. Decrease in chlorophyll autofluorescence. Promotion of EPS content. Increase SOD, CAT, and POD activity. Increase in cell size, lipid peroxidation (MDA) and cell membrane permeability. Particle internalization through endocytosis	[66]
FL-PS-NPLs	100	0.5-50	*Euglena gracilis*	24 h and 96 h	SOD activity (U/mg of protein)	Growth inhibition rate 35.5% (50 mg/L) and limited internalization. Decreased pigment contents (Chl*b*) and increased SOD and POD activity. Biological pathways "environmental adaptation and glycan biosynthesis" and "metabolism" were altered	[67]
FL-PS-NPLs	1000 and 1000 + 2000 (mix)	10	*Scenedesmus quadricauda*	24-96 h	Cell % containing particles	Mixture growth inhibition 38.0%, 28.1%, 36.5%, and 39.1% at 24, 48, 72, and 96 h, respectively. In average, 43.3% of algae cells contained 1000 nm-PS particles	[68]
PE-R PE-N	Filtered by 0.45 µm	0.001-10	*Scenedemus subspicatus*	48 h	Algae concentration (cell/ml)	PE-R at 10 mg/L growth inhibition 20.6% (48 h). PE-N all concentrations ~50.2% (growth inhibition) related to the accumulation of several trace metals	[69]
$PSNH_2$-NPLs	50	2-9	*Synechococcus elongatus*	48 h	Membrane permeability ($RFU/10^6$ cells)	Growth inhibition EC_{50} 3.81 mg/L. Oxidative stress and membrane destruction. GSH activity decreased. Disruption of glutathione metabolism and damage to membrane integrity	[70]
$PSNH_2$-NPLs	50	3.4 and 6.8	*Microcystis aeruginosa*	48 h and 10 d	Membrane permeability ($RFU/10^6$ cells)	Growth inhibition rate 23.6% and 46.1% exposed to 3.4 and 6.8 mg/L, respectively, for 48 h. Internalization and accumulation (using FL-$PSNH_2$-NPLs). Reduction of chlorophyll *a* content. Oxidative stress and cell membrane permeability increase. Promotion of microcystin synthesis and release. Biological pathways related to PSII efficiency and carbohydrate metabolism were downregulated. Proteins involved in biological transport (ABC) were upregulated	[71]
PS-NPLs	300-600	5-100	*Chlamydomonas reinhardtii*	10 d	Growth inhibition rate (%)	Growth inhibition rates 26.6%, 33.9%, 43.9% and 49.2% exposed to 5, 25, 50 and 100 mg/L, respectively. Fluorescence yield dropped with increasing concentrations. PSII activity (F_v/F_m) inhibited at all concentrations. Increase in lipid peroxidation (MDA) and soluble proteins (osmoregulation). Decrease in EPS and cell settlement	[72]

						with increasing concentration.	
FL-PS-NPLs	100	10-100	*Scenedesmus obliquus*	24 h and 72 h	Relative growth rate	NPLs damage reduced under climate change mimicking conditions (elevated CO_2 concentration and warmer temperatures)	[73]
FL-PS-NPLs	100 and 1000	5	*Microcystis aeruginosa*	0-96 h	ROS level (%)	1000 nm particles promoted algal growth (12.4% at 96 h), increased intracellular microcystins content but inhibited their release. 100 nm particles promote microcystins production	[74]
PS-NPLs	80	1-10	*Chlorella pyrenoidosa*	0-21 d	CAT activity (U/mg of protein)	Maximum growth inhibition rate 7.55% at 10 mg/L after 9 d. Slight growth promotion at the lowest concentration after the 15th day of exposure. Decrease in chlorophyll *a* and *b* and carotenoid contents. Increase SOD, CAT and POD activity. The most affected biological pathway was aminoacyl-tRNA. biosynthesis pathway	[75]
PS-NPLs	80	5-50	Chlorella pyrenoidosa	0-6 h	MDA content (%)	NPLs at concentrations 5-50 mg/L induced growth inhibition after 48 h of exposure (maximum 27.7 % at 50 mg/L). Inhibition in algal photosynthetic pigment and photosynthetic efficiency (F_v/F_m). ROS and MDA increase along with increase of SOD and CAT activities. NPLs inhibition ascribed to the blockage of the gene expression of aminoacyl tRNA synthetase	[75]
PS-NPLs	60	25-100	*Microcystis aeruginosa*	0-30 d	Lipid peroxidation (MDA)	Maximum growth inhibition rate 60.2% at 100 mg/L after 8 d. Increase in aggregation rate of algal cells. Photosynthetic efficiency inhibition and alteration of pigments content. Increase in lipid peroxidation (MDA).	[76]
PS-NPLs	100	10-100	*Planktothrix agardhii* (strain NIVA-CYA 630)	0-7 d	Infection prevalence (%)	100 mg/L of NPLs caused a growth inhibition regardless nutrient load (low/high) while controls growth was higher under high nutrients conditions. Prevalence and intensity of infection by *Rhizophydium megarrhizum* (strain NIVA-Chy Kol2008), an obligate fungal chytrid parasite, was significantly lower in presence of 100 mg/L NPLs, while sporangial size was not affected by NPLs	[77]
Combined exposure							
FL-PS-NPLs + G7 PAMAM	30	1-200 (NPLs) 1-30 (PAMAM dendrimers)	*Nostoc* sp. PCC7120	72 h	Lipid peroxidation (MDA) (%)	Growth inhibition EC_{50} 64.4 mg/L. NPLs induced ROS overproduction, lipid peroxidation, increased cell membrane permeability and depolarization, intracellular acidification, and reduction of photosynthetic efficiency (oxygen evolution). NPLs internalization was observed. Several biological pathways were altered. Combined exposure triggered aggregation and resulted in antagonistic effects, except in the	[78]

						case of lipid peroxidation	
FL-PS-NPLs + IBU	600	1 (NPLs) 5-100 (IBU)	*Chlorella pyrenoidosa*	0-96 h	Growth inhibition rate (%)	Growth inhibition 15.6% at 1 mg/L after 4 d. Inhibitory effect of IBU on growth decreased in the presence of NPLs. Co-exposure led to a total antioxidant capacity increase. NPLs led to a decrease on cell bioaccumulation of IBU and accelerated its biodegradation	[79]
PS-NPLs + Cd	100	0.05-5 (NPLs) 1-50 μg/L (Cd)	*Euglena gracilis*	0-96 h	ROS production (%)	Growth inhibition 4.8% at 0.05 mg/L and 34.6% at 5 mg/L after 96 h. Inhibition of photosynthetic efficiency (F_v/F_m) and increase in ROS and SOD activities. Combined exposure increased inhibition rate. FL-PS-NPLs growth inhibition 9.8% at 0.05 mg/L and 38.4% at 5 mg/L after 96 h, toxicity significantly higher than that observed for PS-NPLs	[80]
PSCOOH-NPLs + HA	50 and 350	0.1-100 μg/L (NPLs) 25 (HA)	*Gomphonema Parvulum*, *Nitzschia palea*, *Nostoc* sp. PCC7120, *Komvophoron* sp. and *Scenedesmus obliquus*	96 h	Photosynthetic efficiency (F_v/F_m) (%)	The algal species exhibited very low sensitivity (growth and photosynthetic efficiency); planktonic algal growth increased > 150% with presence of heteroaggregates at 1 μg/L. 50 nm NPLs formed 100-500 nm heteroaggregates with HA. NPLs alone or heteroaggregated with HA marginally affected the photosynthetic efficiency (F_v/F_m)	[81]
PSCOOH-NPLs + Cu + EPS	87-106	0.5-50 (NPLs) 1-200 μg/L (Cu)	*Raphidocelis subcapitata*	0-72 h and 7 d	Protein content (mg/cells)	Maximum growth inhibition ~10%. Cell morphology and protein content alterations. No adsorption of Cu ions was observed onto the NPLs. EC_{50} 84 μg/L (Cu) and 86 μg/L (Cu combined with NPLs)	[82]
PS-NPLs + Ag-NPs	20	3-30 (NPLs) 1-300 μg/L (Ag-NPs)	*Chlamydomonas reinhardtii* and *Ochromonas danica*	0, 12 and 24 h	Cellular Ag content variation under combined exposure (%)	*C. reinhardtii* growth inhibition rate EC_{50} ~30 mg/L (NPLs). *O. danica* growth inhibition rate ~40% at 100 mg/L (NPLs). NPLs internalization in *O. danica*. *C. reinhardtii* and *O. danica* growth inhibition rate EC_{50} 62 μg Ag_{Total} /L and 225 μg Ag_{Total}/L, respectively. These values were reduced in presence of NPLs meaning synergistic effects	[83]
FL-PS-NPLs + Cd	100	1 (NPLs) 0.5 (Cd)	*Euglena gracilis*	96 h	POD activity (U/mg of protein)	Growth rate inhibition ≤ 10 % at 1 mg/L (NPLs) or 0.5 mg/L (Cd). Combined exposure induced growth inhibition rate of ~25%. Combined exposure induced increase SOD and POD activities. Metabolism-related biological pathways hindered by combined exposure, resulting in higher toxicity	[84]
PS-NPLs, $PSNH_2$-NPLs, PSCOOH-NPLs + EPS	200	1 (NPLs) ~9.5 (EPS)	*Scenedesmus obliquus*	72 h	Hydroxyl radical generation (%)	The three types of NPLs decreased cell viability (30%) and photosynthetic efficiency (F_v/F_m), and an increase of ROS, cell membrane permeability, and SOD and CAT activities. The more aged time with EPS (0, 12, 24 and 48 h) the more reduction in the effects of pristine NPLs was observed. This effect was ascribed	[85]

						to the aggregation promoted by the EPS	
PS-NPLs + Cu	500	48-100 (NPLs) 66-200 µM (Cu)	*Chlorella* sp and *Pseudokirchneriella subcapitata*	96 h and 16 d	Chlorophyll *a* concentration (mg/L)	NPLS increased the toxicity of Cu (EC_{50}) after 16 d. NPLs increased the toxicity of Cu at EC_{50} in both microalgae, only in chronic exposure. NPLs altered chlorophyll *a* concentration	[86]
FL-PS-NPLs + TiO_2-NPs	100-200	1 (NPLs) 0.025-2.5 (TiO_2-NPs)	*Scenedesmus obliquus*	72 h	CAT production (%)	NPLs reduced cell viability by ~50% at 1 mg/L NPLs, increased different ROS levels and lipid peroxidation, and modified the SOD and CAT activities. Decreased photosynthetic efficiency (F_v/F_m) and esterase activity. TiO_2-NPs led to similar damages than NPLs. The combined exposure with NPLs increased the effects of TiO_2-NPs	[87]
$PSNH_2$-NPLs + HA	200	25-400 (NPLs) 5 and 10 (HA)	*Chlorella vulgaris*	0-72 h	Chlorophyll *a* content (ng/ml)	Growth inhibition was dose dependent reaching ~57% at 100 mg/L after 72 h NPLs induced a decrease of photosynthetic pigments, reduction of algal size and formation of cellular aggregates in a dose dependent manner. HA mitigated NPLs toxicity in a dose dependent manner in terms of biomass chlorophyll *a*, and morphological alterations. The mitigation was ascribed to aggregation of NPLs in the presence of HA	[88]
PS-NPLs + WW	30	12.5-200 (NPLs) 1:16-1:1 (WW-dH_2O)	Recombinant bioluminescent *Anabaena* sp. PCC 7120 CPB4337	24 h	Bioluminescence inhibition (%)	Bioluminescence inhibition EC_{50} for NPLs 58.3 mg/L after 24 h. Combined exposure reduced toxicity probably due to the sorption of WW micropollutants onto de NPLs and heteroaggregation processes	[89]
PS-NPLs + MWCNTs	50-100	5-50 (NPLs) 5-50 (MWCNTs)	*Microcystis aeruginosa*	15 d	SOD activity ($U/10^8$ cells)	Maximum growth inhibition 22.8% at 50 mg/L after 15 d. NPLs increased SOD activity and lipid peroxidation (MDA). Combined exposure resulted in antagonistic effect due to heterogeneous agglomeration. Translation and membrane transport were the most altered biological pathways upon NPLs exposure	[90]

NPLs: Nanoplastics; PS: polystyrene; PE-R: reference polyethylene; PE-N: polyethylene collected from North Atlantic gyre; FL: fluorescent; COOH-: surface carboxylate modified; NH_2: surface amino modified; EPS: extracellular polymeric substances; IBU: ibuprofen; HA: humic acids; G7 PAMAM: amine-terminated poly(amidoamine) dendrimers of generation 7; NPs: nanoparticles; WW: waste water; MWCNTs: multi-walled carbon nanotubes; ECx: effective concentration of the pollutant that inhibits the toxicity endpoint by x percent; GSH: reduced glutathione; PSII: photosystem II; MDA: malondialdehyde; SOD: superoxide dismutase; POD: peroxidase; CAT: catalase; ROS: reactive oxygen species; RFU: relative fluorescence units; U/mg: units per mg for enzyme activity.

Baudrimont *et al.* reported significant growth reduction of the green alga *Scenedemus subspicatus* exposed to 1 mg/L of PE-NPLs, which was greater when using PE-NPLs gathered from the North Atlantic gyre than reference PE-NPLs. The difference attributed to the accumulation of trace metals in plastics exposed to environmental pollutants[69]. Similarly, it has been described that the photosynthetic activity of the alga *E. gracilis* was impaired by 100 nm PS-NPLs at concentrations as low as 0.5 mg/L[80]. In contrast, low

concentrations of NPLs have been reported to promote algal growth at concentrations < 1 mg/L, sometimes in long-term exposure assays[81,94]. This discrepancy may be related to the physicochemical conditions that alter the colloidal status of NPLs suspensions and, consequently, their toxicological behavior. Finally, it is important to stress that, in combination with other pollutants, low concentrations of NPLs may induce considerable effects. For instance, co-exposure to PS-NPLs and Cd^{2+} at the same concentration (0.05 mg/L) resulted in significant and synergistic inhibition of algal growth[80]. Further efforts should be made to reach a deeper understanding of the effects of NPLs on primary producers at realistic concentrations and environmental conditions in order to accurately assess their environmental fate and risk. The use of real secondary NPLs is highly recommended.

TOXICITY TOWARDS FRESHWATER PRIMARY CONSUMERS

Freshwater primary consumers, organisms that feed on primary producers, include invertebrates, some fishes, and a few amphibian larvae. Among the primary consumers, invertebrates are the dominant grazers in the freshwater ecosystems of temperate latitudes. In this regard, most of the NPLs toxicological studies have been carried out using different species of *Daphnia*, one of the preferred organisms for toxicity assessment[95]. Table 4 summarizes the main findings reported since 2019 concerning single and combined toxicity of NPLs towards freshwater primary consumers, highlighting the most sensitive assessed toxicity endpoints. EC_{50} for negatively charged NPLs (more common than positively charged NPLs) has been reported to range between 30 and 300 mg/L, depending on the plastic used and the organism assessed[96,102]. However, harmful effects, such as ROS overproduction or the reduction in the number of neonates per brood, have been reported at lower concentrations (≤ 1 mg/L)[108,109]. The advantage of behavioral endpoints is the possibility to observe sublethal effects, which are generally undetectable for tests based on global or lethal endpoints, although their findings may be difficult to interpret[95]. *D. magna* swimming behavior has been reported to change by the exposure to PS-NPLs at concentrations > 16 mg/L[98]. However, such alterations do not seem to appear at concentrations < 1 mg/L, despite a clear accumulation of NPLs in the gut and external body appendages[110,111]. It is worth noting that some of the effects observed in daphnids at low concentrations only appeared during multigenerational or long-term assays, revealing that the interaction between these organisms and NPLs may occur through parental transfer, and their effects may span the entire lifetime of the organism[97,98]. The effect of NPL ingestion by *D. magna* through feeding has been tracked using algae pre-exposed to 270 and 640 nm metal-doped NPLs (4.8×10^{10} particles/L, 1-7 mg/L). The results show that both types of NPLs became attached to the cell surface of algae and are ingested by the daphnids and excreted without any effects on daphnids growth were observed, but the smaller ones increased the reproduction time, reduced the number of neonates, and induced higher offspring mortality[112]. These finding reveal that low concentrations of NPLs may damage primary consumers not only through direct exposure but also through feeding on NPL-polluted algae. Finally, it is important to note that the effects described above may be enhanced by the co-occurrence of NPLs with other chemicals. For instance, it has been described that glyphosate (the active compound of several herbicides) in combination with PS-NPLs exerts synergistic and multigenerational effects on *D. magna*[103]. The interaction between NPLs and potential co-occurring contaminants should be studied to better understand their environmental risk under realistic conditions. Furthermore, given the relatively long lifetime of higher organisms and the data showing multigenerational effects, the long-term fate and toxicity of NPLs on freshwater biota should be addressed.

TOXICITY TOWARDS FRESHWATER SECONDARY CONSUMERS

Secondary consumers are also crucial for the equilibrium of freshwater ecosystems. Located at the top of the trophic chain, any alterations to them may cause a potential cascade of interactions through the food web. Furthermore, human health may be jeopardized due to the consumption of organisms such as fish or

Table 4. Toxicological effects of NPLs on freshwater primary consumers

Type of exposure	Size (nm)	Concentration tested (mg/L)	Test organism	Exposure time	Most sensitive parameter	Effects	Refs.
Single exposure							
PE-NPLs	< 0.8 μm (~110) and < 10 kDa	0.53 (< 0.8 μm) and 2 (< 10 kDa)	*Daphnia magna*	134 d	Survival (%)	Mechanical breakdown of high-density polyethylene, followed by filtration through 0.8 μm filters, produced toxic material. The toxicity was attributed to the fraction < 10 kDa (~3 nm). Both size fractions were toxic in terms of mortality and number of offspring, but NPLs without < 100 kDa fraction were non-toxic	[16]
PS-NPLs	75	10-400	*Daphnia pulex*	24 h, 48 h and 21 d	Total offspring per female (number)	Growth inhibition EC_{50} 76.7 mg/L after 48 h. Chronic exposure reduced body length in a time- and dose-dependent manner. The expression of stress defense genes (*SOD*, *GST*, *GPx*, and *CAT*) was first induced and then inhibited. Induced gene expression of heat shock proteins (*HSP70* and *HSP90*)	[96]
$PSNH_2$-NPLs PSCOOH-NPLs	53 (NH_2), 26 and 63 (COOH)	0.0032-7.6	*Daphnia magna*	103 d	Long-term survival (%)	$PSNH_2$-NPLs were lethal at concentration of 0.32 mg/L (lifetime of individuals was shortened almost three-fold). PSCOOH-NPLs, were toxic at all concentrations used during long-term assessment	[97]
PS-NPLs	75	0.1-2	*Daphnia pulex*	21 d	Relative expression of *GSTs2* (%)	The expression of DP-GSTs1, GSTs2, and GSTm1 was higher in older daphnids compared to neonates. Exposure of mothers to NPLs (1 μg/L) elevated GSTs2 level in neonates	[98]
PS-NPLs	75	1 μg/L	*Daphnia pulex*	21 d	Relative expression of *GSTD* (%)	Growth rate, number of clutches, and total offspring per female were reduced in the F2 (2nd generation). Content of H_2O_2, expression of *CAT*, *GSTD*, *MnSOD*, *CuZn SOD*, *GCL*, and *HO1* genes, and enzyme activity of GST, CAT, increased in F0 (parental generation) and F1 (1st generation). NPLs have stimulative effect for F0 and F1 but are toxic to F2	[99]
PS-NPLs	71	1	*Daphnia pulex*	96 h	CYP450 drug metabolism (enrichment score)	Biological processes, cellular components and molecular functions affected. Biological pathways related to immunity (drug, xenobiotics and glutathione metabolisms, hippo signaling pathway and adherents junction) and oxidative stress (arachidonic acid, glutathione, porphyrin and chlorophyll metabolisms) were altered	[99]
PS-NPLs	75	0.1-2	*Daphnia pulex*	48 h	ROS level (RFU in %)	Dose dependent ROS overproduction. Low NPLs concentrations increased the expressions of MAPK pathway genes. The activities of CAT and SOD decreased	[100]
PS-NPLs	72	0.1-2	*Daphnia pulex*	21 d	GSH content	Population fitness (estimated	[100]

					(mg/g prot.)	based on the intrinsic rate of increase) decreased at 2 mg/L and the total number of neonates was reduced by 26.8% and 41.89% at 0.5 and 2 mg/L, respectively. GSH and GSSG content increased in a dose-dependent manner. Processes involved in detoxification, metabolism, assembly, and development were impacted	
$PSNH_2$-NPLs	20, 40, 60 and 100	0.5-100	*Daphnia magna*	48 h	Immobilization (%)	EC_{50} for 20 and 40 nm were < 2 mg/L; for 60 nm < 4 mg/ L; and for 100 nm ~8 mg/L. Synthetic water mimicking natural water reduced toxicity	[101]
Combined exposure							
PS/PS/PP/PVC-NPLs + BaP	50 (PE/PP), 200 and 600 (PS), 200 (PVC)	3×10^{10} part. /L (NPLs), 10 μg/L (BaP)	*Daphnia magna*	21 d	Neonates per brood (number)	Mortality ranged from 10 to 30%. Significant variation in the number of the produced neonates appeared in broods 4 and 5. The number of neonates in brood 4, exposed to PE-NPLs 50 nm and PS-NPLs 200 nm, reached the highest level, whereas, in brood 5 decreased to zero. Combined exposure induced earlier alterations in neonates. BaP with PS-NPLs impairs daphnids reproduction to a larger extent than the combination of BaP with PE, PP or PVC-NPLs	[17]
Fe-PS-NPLs + BaP	270	10 (NPLs) 5 (BaP)	*Anodonta anatina*	72 h	SOD activity In digestive tract (%)	SOD activity induced in the digestive tract by exposure to NPLs alone. SOD and CAT activities in digestive tract and gills preferably induced by co-exposure. The sorption of BaP to aged NPLs was lower than to pristine NPLs, but co-exposure increases the accumulation of NPLs in mussel tissues	[33]
PS_NPLs + wastewater (WW)	30	12.5-200 (NPLs) 1:16-1:1 (WW-d H_2O)	*Daphnia magna*	48 h	Immobility (%)	NPLs EC_{50} in terms of mobility was 32.4 mg/L; WW caused no effect. Combined exposure decreased the toxicity of the NPLs. NPLs aggregates accumulated onto thoracopod with loss of body integrity. Combined exposure induced adhesion of NPLs aggregates to the body of daphnids, but to a lower extent compared with single exposure to NPLs	[72]
$PSNH_2$-NPLs, PSCOOH-NPLs + SRHA or alginate	200 (NH_2 and COOH)	10-400 (NPLs) 2 (SRHA or alginate)	*Daphnia magna*, *Thamnocephalus platyurus* and *Brachionus calyciflorus*	24 h and 48 h	Lethality (%)	*D. magna*: $PSNH_2$-NPLs EC_{50} 36.2 mg/L, PSCOOH-NPLs EC_{50} 111.4 mg/L. *T. Platyurus*: $PSNH_2$-NPLs EC_{50} 194.8 mg/L, PSCOOH-NPLs EC_{50} 318.2 mg/*L. B. calyciflorus*: PSN H_2-NPLs EC_{50} 49.9 mg/L, PSCOOH-NPLs EC_{50} 263.6 mg/L. In all cases the combined exposure with SRHA o alginate reduced NPLs toxicity. NPLs in the organism body (mainly in the gut) increased with concentration suggesting dose-dependent accumulation	[102]
PS-NPLs + Gly	73	16-500 (NPLs) 6-200 (Gly)	*Daphnia magna*	48 h and 21 d	ROS level (RFU)	EC_{50} (48 h) for NPLs and Gly individually were 244 mg/L and	[103]

						89.3 mg/L, respectively. NPLs and Gly induced dose-dependent ROS overproduction and decreased swimming distance. NPLs reduced the reproduction and age of the first brood of both F1 and F2 at < 15 mg/L. Based on Abbott's model, the combined exposure increased toxicity (synergism)	
$PSNH_2$-NPLs + HA	100-120	1-400 (NPLs) 1-50 (HA)	*Daphnia magna*	96 h	Relative expression of *P-GP* (%)	NPLs induced a dose- and time-dependent response (65% of mortality after 96 h exposed to 10 mg/L). Toxicity drastically decreased in the presence of HA in a dose dependent manner. The expression of genes related to stress response (*GST*, *CAT* and *HSP70*) and detoxification (*P-GP*) were strongly up-regulated (between 2 and 14-fold). Most of the NPLs were found trapped within the filter combs rather than ingested	[104]
PS-NPLs + PCBs	100	01-75 (NPLs) 0.1-1.5 (PCBs)	*Daphnia magna*	48 h	Lethality (%)	EC_{50} in terms of mortality were 5 mg/L for NPLs and 0.64 mg/L for PCBs. Combined toxicity decreased up to 1 mg/L (NPLs) due to PCBs sorption onto the NPLs; at higher concentrations the toxicity was due to NPLs	[105]
$PSNH_2$-NPLs + IgG or BSA	50, 200 and 500	1.4 and 2.7 (NPLs)	*Daphnia magna*	48 h	Alive organisms (number)	50 nm NPLs caused ~80% of mortality at 48 h (1.4 mg/L) and 100% < 24 h (2.7 mg/L). Similar effects were caused by 50 nm NPLs in 100-600 nm aggregates (IgG) or BSA coated. No effects were observed by IgG, BSA and 200/500 nm NPLs	[106]
PS-NPLs + PAHs + HA	100	1 (NPLs) 100 (HA)	*Daphnia magna*	0-36 h	Bioaccumulation (modeling)	Bioaccumulation depended on dermal uptake (≥ 99.3% of the total). NPLs retarded intestinal PAHs uptake; while the HA and HA-NPLs facilitated the transfer of PAHs to gut lipids	[107]

NPLs: Nanoplastics; PS: polystyrene; PE: polyethylene; PP: polypropylene; PVC: polyvinyl chloride; COOH-: surface carboxylate modified; NH_2: surface amino modified; HA: humic acids; SRHA: Suwannee River humic acids; ECx: effective concentration of the pollutant that inhibits the toxicity endpoint by x percent; BSA: ovine serum albumin; Gly: glyphosate; PCBs: polychlorinated biphenyls; IgG: human immunoglobulin G; PAHs: polycyclic aromatic hydrocarbons; BaP: benzo(a)pyrene; WW: waste water; MDA: malondialdehyde; SOD: superoxide dismutase; GST: glutathione S-transferase; GPx: glutathione peroxidase; CAT: catalase; GCL: glutamate cysteine ligase; ROS: reactive oxygen species; MAPKs: mitogen-activated protein kinases; GSH: glutathione S-transferase; GSSG: oxidized glutathione; RFU: relative fluorescence units.

crustaceans that could constitute an important route for NPL transfer to humans[25]. Table 5 summarizes the main findings reported since 2019 on the toxicity of NPLs towards freshwater secondary consumers. The exposure of *Danio rerio* (zebrafish) to 1 mg/L of 500 nm fluorescent PS-NPLs revealed particle translocation from the gut epithelium of the digestive tract to different tissues where they activated enzymatic responses against oxidative stress[113]. Small NPLs (70 nm) have been reported to accumulate in zebrafish gonads, intestine, liver, and brain causing oxidative stress, metabolic alterations, and neurological impairments, including the decrease in acetylcholine esterase, acetylcholine, or gamma-aminobutyric acid, as well as neurobehavioral alterations, at concentrations as low as 0.5 mg/L[114]. Important effects have also been found for 20 nm PS-NPLs, which resulted in increased fish mortality, occurrence of abnormalities, and excessive

Table 5. Toxicological effects of NPLs on freshwater secondary consumers

Type of exposure	Size (nm)	Range tested (mg/L)	Test organism	Exposure time	Most sensitive parameter	Effects	Refs.
Single exposure							
PE-NPLs	55	6.8×10^8 (particles/mL)	*Danio rerio*	48 h	Apoptotic cells (%)	NPLs accumulated during zebrafish embryogenesis throughout a passive skin diffusion process. NPLs induced cell apoptosis to a higher extent than MPs (1650 nm)	[29]
FL-PS-NPLs	500	1	*Danio rerio*	48 h	COX activity (U/mg prot.)	NPLs uptake was observed in the digestive tract, and also translocated to other tissues through the gut epithelium. COX activity decreased and SOD activity increased. Behavioral tests revealed variation in turning angle of the exposed embryos	[113]
PS-NPLs	70	0.5-5	*Danio rerio*	7, 30 and 49 d	VTG content (ng/μg prot.)	NPLs accumulated in gonads, intestine, liver, and brain, inducing alterations in lipid metabolism and oxidative stress. NPLs induced strong behavioral alterations in locomotion activity, aggressiveness, shoal formation, and predator avoidance along with dysregulated circadian rhythm locomotion activity after chronic exposure	[114]
FL-PS-NPLs	20	~270	*Danio rerio*	0-120 h	Apoptotic cells (RFU)	For NPLs injected into the yolk sac of the embryos the survival rate was ~70%. Injected NPLs induced malformation, ROS overproduction (especially in the head), overall cellular death and bioaccumulation in brain	[115]
PMMA-NPLs	40	0.001-1	*Xenopus laevis*	0-96 h	Body mass daily incrmt. (mg/day)	NPLs induced alteration in the daily increase of body weight/length, and anatomical changes in the abdominal region (gut externalization) was observed in 62.5% of the tadpoles	[116]
PMMA-NPLs	40	1-640	*Hydra viridissima*	96 h	Mortality (%) during regeneration	NPLs induced EC_{50} (mortality) of 84 mg/L and several morphological and physiological alterations were detected at concentrations ≤ 40 mg/L like partial or total loss of tentacles. Regeneration rate was reduced and the EC_{50} (mortality)	[117]
PS-NPLs	50 and 100	1-80	*Hydra attenuata*	96 h	Lipid peroxidation (ug TBARS /mg prot.)	EC_{50} were 3.6 and 18 mg/L for morphological changes and 14 and 28 mg/L for biomass, both for 50 and 100 nm, respectively. NPLs accumulated in concentration-dependent manner for both sizes. NPLs led to decreased biomass, lipid peroxidation (MDA), increased polar lipid levels, viscosity, and formation of liquid crystals at the intracellular level	[118]
PS-NPLs	75	20-1280 (acute) 5-40 (chronic)	*Macrobrachium nipponense*	0-96 h (acute) 0-28 d (chronic)	GSH-ST activity (U/mg prot.)	EC_{50} in terms of mortality was 396 mg/L after 96 h. As NPLs concentration increased, the activities of antioxidant enzymes generally decreased, except at low concentrations at which they were strongly induced; the contents of	[119]

						H_2O_2 and lipid peroxidation products increased	
FL-PS-NPLs	42	0.5-5 (aqueous exposure) 52 nL of 1, 3 and 5 ×10^3 (injection exposure)	*Danio rerio*	0-72 h	Bent tail malformation (% of organisms)	The comparison between both exposure routes (aqueous and microinjection) revealed that despite both exposure routes led to NPLs accumulation in the yolk sac followed, during larvae stage, by brain, eyes, gut and swim bladder, the aqueous exposure induced higher NPLs concentrations in the brain and eyes while the injection exposure caused NPLs accumulation mainly in the trunk area. Only the aqueous exposure provoked a decreased body length, increased tail flexure in a dose-dependent manner and alterations in locomotor activity. An overall downregulation of several enzymes was observed under both route of exposure	[120]
FL-PS-NPLs	100	100 µg of NPLs (1.6% of the food)	*Procambarus clarkii*	0-72 h	VTG gene expression (TPM)	NPLs altered expression of genes involved in immune response, oxidative stress, gene transcription and translation, protein degradation, lipid metabolism, oxygen demand, and reproduction, and, in females, strong downregulation of vitellogenin expression	[121]
FL-PS-NPLs	23	0.04, 34 and 3400 ng/L	*Ctenopharyngodon idella*	20 d	Comet assay (% of DNA in the tail)	DNA damage (comet assay) increased in a dose-dependent manner. NPLs induced changes in erythrocyte shape and size, oxidative stress (NO levels, lipid peroxidation, H_2O_2), antioxidant system (GSH) inhibition and particle accumulation in liver and brain	[122]
PS-NPLs	50 and 1000	10	*Danio rerio* (cells and whole organism)	0-24 h (cells) 72-120 h (larvae)	ROS overproduction (ROS intensity)	*In vitro* assay (cells): 50 nm FL-PS-NPLs were more internalized than 1 µm NPLs independently of the internalization method studied (natural internalization, transfection, and electroporation) and dynamic dependent for 50 nm NPLs while through phagocytosis for the 1 µm ones. Smallest NPLs upregulated antioxidant gens while the biggest induced membrane depolarization. In vivo assay (larvae): Internalization was higher for 50 nm NPLs. Both sizes were internalized in the gut and induced ROS overproduction. PS-NPLs exposure to immunocompromised *D. rerio* infected with *Aeromonas hydrophila* the presence of NPLs of both sizes increases the effects of the infection	[123]
PS-NPLs	500	0.04-40	*Macrobrachium nipponense*	21 d	GST activity (U/mg prot.)	NPLs decreased molting rate (from 4 mg/L) and the expression of molting-related gene (from 0.04 mg/L). ROS overproduction was observed (H_2O_2) along with higher SOD and GSH-Px activity at low concentration and CAT at high ones. GSH content increased	[124]
PS-NPLs	75	5-40	*Macrobrachium nipponense*	0-28 d	Pepsin activity (U/mg prot.)	NPLs caused concentration dependent effects on hepatopancreas. Digestive enzymes (lipase, trypsin and pepsin) were	[125]

						initially activated and then inhibited along with the response of molting-associated genes	
PS-NPLs	75	5-40	*Macrobrachium nipponense*	0-28 d	ATPase activity (U/mg prot.)	Cell apoptosis increased with NPLs concentration. Ion levels (Na^+, K^+, Ca^{2+}, and Cl^-) in the gills decreased in a concentration dependent manner. Ion transport-related genes in the gills were first induced and then downregulated	[126]
FL-PS-NPLs	23	50 μg/L	*Poecilia reticulata*	30 d	Immature/mature eggs (%)	NPLs affected pregnancy rate, the number of embryos per female and the percentage of matured eggs. The levels of triglycerides and carbohydrates were altered by NPLs. ROS levels (general ROS and H_2O_2), and SOD and CAT activities increased	[127]
Combined exposure							
PS/PS/PP/PVC-NPLs + BaP	50 (PE/PP), 200 and 600 (PS), 200 (PVC)	3×10^{10} part. /L (NPLs), 10 μg/L (BaP)	*Danio rerio*	0-12 h	The number of hatched embryos per day	PE 50 nm, PS 200 nm, and PS 600 nm NPLs led to hatching delay and PP 50 nm NPLs caused embryos failure to develop the normal morphology and led to spine curvature malformation in 18% of the larvae. The presence of PS 200 nm and PVC 200 nm NPLs counterbalanced the effect of BaP on the hatching rate of zebrafish	[17]
PS-NPLs + PHE	44	0.015-150 (NPLs) 1.5-20 (PHE)	*Danio rerio*	0-120 h	Total swimming distance (mm)	NPLs altered swimming behavior at the lowest tested concentration (0.015 mg/L) and increased CAT activity at 1.5 mg/L. Combined exposure increased both CAT and GSH activities but no clear synergism or antagonism was observed	[18]
PS-NPLs + PAH	44	0.1-10 (NPLs) 5-25 μg/L (PAH)	*Danio rerio*	0-96 h	Oxygen consumption rate (pmol/min × embryo)	NPLs individually and combined with PAHs disrupt mitochondrial energy production, affecting two important mitochondrial functions (NADH and ATP synthesis). During combined exposure, NPLs aggregation increased, and the bioaccumulation of PAHs decreased. Induction of EROD activity was detected in animals exposed to PAHs with or without NPLs	[128]
PET-NPLs + BSA or SDS	20, 60-80 and 800	1-50 (NPLs) 0.0001% BSA or SDS	*Danio rerio*	6-144 h	ROS level (%)	Size dependent distribution and the size- and concentration-dependent toxicity observed for NPLs in terms of hatching rate, heart rate, and ROS generation (mainly in the head and in the spine). NPLs combined exposure with BSA caused higher effects than combined with SDS (which allowed higher NPLs aggregation)	[129]
PS-NPLs + Au	50, 200 and 500	50-200 (NPLs) 0.1-10 μg/L (Au)	*Danio rerio*	0-24 h	ROS level (%)	The smallest NPLs readily penetrated the chorion and accumulated throughout the whole body, mostly in lipid-rich regions such as in yolk lipids. Effects were synergistically exacerbated by Au in a dose- and size-dependent manner. ROS and proinflammatory responses increased in the presence of NPLs	[130]

NPLs: Nanoplastics; PS: polystyrene; PE: polyethylene; PET: polyethylene terephthalate; PP: polypropylene; PVC: polyvinyl chloride; PMMA: polymethylmethacrylate; FL: fluorescent; COOH-: surface carboxylate modified; NH_2: surface amino modified; ECx: effective concentration of the pollutant that inhibits the toxicity endpoint by x percent; PAHs: polycyclic aromatic hydrocarbons; BaP: benzo(a)pyrene; BSA: bovine serum albumin; PHE: phenmedipham; PCBs: polychlorinated biphenyls; PAHs: polycyclic aromatic hydrocarbons; SDS: sodium dodecyl sulfate; COX: cyclooxygenase; VTG: vitellogenin; TPM: transcripts per million; MDA: malondialdehyde; CAT: catalase; EROD: ethoxyresorufin-O-deethylase; SOD: superoxide dismutase; ROS: reactive oxygen species; GSH: glutathione S-transferase; RFU: relative fluorescence units.

ROS formation and apoptosis, particularly in the brain[115]. Other studies reported physical abnormalities found in different freshwater organisms such as *Xenopus laevis* or *Hydra viridissima* at concentrations as low as 1 mg/L, highlighting the importance of using approaches that overcome the limitations of traditional toxicity tests[116,117]. Interestingly, several recent studies focused on the potential effects induced by NPLs in the intestinal microbiome of freshwater secondary consumers. It has been found that both MPs and NPLs may cause dysbiosis in the zebrafish gut at very low concentrations (1 μg/L), but NPLs can also increase the presence of pathogenic genera, such as *Aeromonas*[131]. It has also been found that PS-NPLs at concentrations ≤ 0.1 mg/L affected the brain-intestine-microbiota axis of zebrafish, causing reduced growth, inflammatory responses, and altered intestinal permeability, even inducing transgenerational effects such as NPL accumulation in the gastrointestinal tract of the offspring[132]. Microbiome alterations have been described in the freshwater crustacean *Procambarus clarkia* (crayfish) exposed for 48 h to 75 nm PS-NPLs, which resulted in a reduced abundance of *Lactobacillus* and an increase in the number of pathogenic bacteria, probably linked to a lower immunity[133]. Concerning the co-occurrence of NPLs with other pollutants, the results are still limited. No clear toxicological interactions have been described in zebrafish exposed to polycyclic aromatic hydrocarbons or the herbicide phenmedipham (PHE) in the presence of NPLs[18,128]. However, the combined exposure of PE-NPLs with bovine serum albumin (as a model of a naturally occurring protein) induced more toxic effects on zebrafish than their co-exposure with an artificial surfactant such as sodium dodecyl sulfate, which was attributed to the higher colloidal stability provided by the first[129]. Considering the complexity of this type of organisms, the combination of *in vitro* studies with different cell lines together with *in vivo* studies using the whole organism are deemed necessary for understanding the potential adverse outcome pathways of NPLs to secondary consumers. Finally, attention should also be paid to artifacts when using labeled plastic particles due to the leaching of fluorochromes or metals.

BIODEGRADABLE NPLS

The materials produced to replace the traditional petroleum-based plastics are ambiguously referred to using several terms such as biodegradable or biobased plastics. A wide denomination for all these types of plastic material is "bioplastics". Table 6 summarizes the information concerning the main types of bioplastics developed for replacing the traditional non-biodegradable petroleum-based ones. Among them, the category of biobased plastics refers to plastic materials manufactured using renewable resources. It is important to note that biobased plastics are not free from environmental issues. The life cycle assessment of biobased plastics shows that they may reduce carbon emissions, but other characteristics, such as their persistence, are not necessarily better than those of conventional plastics. Furthermore, there is an important problem concerning the occupation of agricultural land for their production. The materials based on conventional plastics supplemented with additives that allow their rapid degradation are not a realistic solution since this process enables only their fragmentation into smaller pieces but not their complete degradation. Accordingly, oxo-degradable plastics have been restricted in the EU and Switzerland. Regardless of the type of plastic, recycling is difficult due to the presence of additives in almost every finished plastic product. However, it is important to note that recycling is clearly the most environmentally friendly option for end-of-life plastic management, even better than composting. Accordingly, the preferred bioplastics would be those both biobased and biodegradable. This is the case of bioplastics such as

Table 6. Classification of the different types of bioplastics

Type of plastic	Definition	Example	Refs.
Biobased	Plastics made from renewable resources	PEF or PLA	[134]
Biodegradable	Plastics susceptible of biological degradation by total/partial assimilation	PLA or PCL	[134, 135]
Compostable	Plastics recyclable through organic recovery (biodegradable by composting and anaerobic digestion)	PBAT	[136]
Home-compostable	Plastics recyclable through organic recovery (biodegradable by composting and anaerobic digestion at ambient temperature)	PLA-PCL blend	[137]
Hydro-biodegradable	Plastics in which biological assimilation is preceded by hydrolysis	Starch blends	[138]
Photo-degradable	Plastics whose degradability is induced by additives that initiate oxidation reactions or by incorporating a photosensitive degradable chromophore into the polymer backbone	E-CO	[139]
Oxo-degradable	Plastics whose degradability is induced by additives that initiate oxidation reactions	Oxo-PP	[134]
Hydro-degradable	Plastics whose degradability is induced by the polar groups susceptible to hydrolysis	PA	[134]

PLA: polylactic acid; PHA: polyhydroxyalkanoates; PEF: polyethylene furanoate; PBAT: poly(butylene adipate-co-terephthalate); PCL: polycaprolactone; E-CO: ethylene-carbon monoxide copolymers; Oxo-PP: oxo-degradable polypropylene; PA: polyamide.

polylactide or polylactic acid (PLA) and polyglycolide (PGA) along with those obtained from bacteria or algae that do not imply the use of lands for agriculture, such as polyhydroxyalkanoates (PHA).

The largest plastic demand by segment in 2015 was in packaging (36%), which is considered the greatest source of waste, globally accounting for 146 million tons that year, of which > 95% was not recycled[140]. Bioplastics are mainly being developed for single-use products, such as packaging, in order to reduce the environmental burden of plastic wastes[141]. The global production capacity for biodegradable plastics is still modest, 2.24 million tons in 2021, but it is expected to expand up to 7.5 million tons by 2026 (source: European Bioplastics). Thus, in the near future, bioplastics are expected to reach the aquatic ecosystem following similar routes as petroleum-based materials[142]. However, their potential impact on organisms of freshwater environments has been shown to be similar to that from conventional plastics, or even larger due to their more rapid degradation[143,144]. Furthermore, during their degradation, bioplastics may release millions of MPs and billions of NPLs per gram[145]. Table 7 summarizes the main findings reported on the toxicity of biodegradable NPLs (including some carbon-based nanoparticles) towards freshwater organisms. Secondary biodegradable NPLs have been shown to consist of short polymeric chains (< 1600-3000 kDa) produced during the degradation of larger items that will continue to release as long as the source (any biodegradable plastic litter) remains in the environment.

There is a considerable lack of information concerning the colloidal stability of bioplastics, but the knowledge gathered during the last decade with other NPLs suggests that their higher degradability could lead to a more oxidized surface (probably along with a more negative surface net charge) and higher stability in aqueous suspension. This colloidal stability could be comparable to that observed in artificially aged petroleum-based NPLs (see Table 1) but in considerably less time and under softer weathering conditions. The information on the toxicity of biodegradable NPLs is also scarce but points towards a non-negligible biological impact on freshwater organisms. For instance, unlike the studies summarized in Table 7, Tong *et al.* did not find that PBAT- or PLA-NPLs affected the survival of the copepod *Tigriopus japonicas*[150]. Likewise, Götz *et al.* did not report any adverse outcomes to the freshwater invertebrate *Gammarus roeseli* exposed to different particle sizes of PS- and PLA-NPLs at concentrations up to 430 ng/mg of food[151]. Overall, the environmental fate and risk of biodegradable nanometric plastic remains poorly understood and needs further scientific efforts to be properly assessed. As the substitution of petroleum-based plastics by biodegradable materials is accelerating, a thorough risk assessment is urgently

Table 7. Toxicological effects of biodegradable NPLs (including NPs) to freshwater organisms

Bio-NPLs type	NPLs size (nm)	Range tested (mg/L)	Test organism	Exposure time	Most sensitive parameter	Effects	Refs.
Chitosan-NPs	200 and 340	10-40	*Danio rerio*	0-96	Hatching rate (%)	Chitosan-NPs caused decrease in hatching rate and increased mortality at the highest concentrations. NPs of 200 nm caused malformations (bent spine or pericardial edema) and an opaque yolk. Both tested NPs caused ROS overproduction	[146]
PCL-NPs	200-300		*Pseudokirchneriella subcapitata* and *Daphnia similis*	0-96	Inmobilized *D. Similis* individuals (%)	EC_{50} for *P. subcapitata* after 96 h 2410 mg/L of PCL-NPs. EC_{50} for *D. similis* after 24 and 48 h 32 and 13 mg/L of NPs, respectively	[147]
Secondary PHB-NPLs	75-200	0-200	*Anabaena* sp. PCC7120, *Chlamydomonas reinhardtii* and *Daphnia magna*	0-72 h	Cytoplasmatic membrane potential (% of control)	PHB-NPLs EC_{50} 139, 54 and 107 mg/L for *Anabaena*, *C. reinhardtii* and *D. magna*, respectively. Damages related to oxidative stress, membrane integrity and intracellular pH were reported at the EC_{50}	[148]
Cellullose-NPs	< 1 μm	0.01-10	*Scenedesmus obliquus*, *Daphnia magna* and *Danio rerio*	0-96	ROS overproduction (% of control)	No growth inhibition or mortality were observed. Cellulose-NPs induced ROS overproduction to the three aquatic organisms at 0.01 mg/L	[149]
Secondary PCL-NPLs	10-150	90	*Anabaena* sp. PCC7120 and *Synechococcus* sp. PCC 7942	0-72 h	Cytoplasmatic membrane potential (% of control)	PCL-NPLs (90 mg/L) caused growth inhibition of ~40% and ~50% after 72 h on *Anabaena* and *Synechococcus*, respectively. Damages related to oxidative stress, membrane integrity, intracellular, metabolic activity and cell size and internal complexity were reported. The oligomeric fraction released was also considerably toxic	[12]

NPLs: Nanoplastics; NPs: nanoparticles; PCL: polycaprolactone; PHB: polyhydroxybutyrate; ROS: reactive oxygen species; ECx: effective concentration of the pollutant that inhibits the toxicity endpoint by x percent.

needed to ensure a sustainable replacement for petroleum-based plastics.

REMARKS AND FUTURE RESEARCH NEEDS

The current knowledge on the distribution of plastic litter and the information available on the effect of the weathering processes suffered by plastic debris suggest a widespread presence of NPLs in all freshwater compartments. The first attempts to measure the environmental concentration of NPLs in freshwater systems, along with the estimations obtained from mathematical models, point to probable environmental concentrations in the parts per billion (< 1 μg /L) range, similar to other anthropogenic pollutants. The development of techniques for the routine monitoring of NPLs in environmental samples is urgently needed.

Most environmental fate and toxicity studies have been performed using commercially available or synthetic NPLs, especially PS-NPLs (PS latexes). This approach allowed gaining a considerable body of knowledge on the colloidal stability of NPLs in water bodies and insight into their main toxicity drivers, but it does not represent the variety of shapes and chemical compositions of real secondary NPLs that can be found in the environment. Special attention should be paid to possible artifacts due to the leaching of the substances used to label plastic particles.

The available data show clear damage upon NPL exposure at concentrations as high as tens or even hundreds of milligrams per liter. The use of high concentrations clarifies potential biological targets, but efforts should be made to assess the possible effects upon exposure to realistic environmental

concentrations. The effect of NPLs is expected to be enhanced at low concentrations due to higher colloidal stability and possibly triggered by the release of oligomeric fractions detached from larger particles. Long-term assays and mesocosm studies using low concentrations of secondary NPLs would be needed to perform realistic risk assessments for regulatory purposes.

Although biodegradable plastics are considered environmentally friendly substitutes for traditional petroleum-based polymers, their risk must be assessed in the same way it is being performed for their non-biodegradable counterparts. Thus far, there is very limited information regarding the physicochemical behavior of biodegradable plastics in relevant conditions and their impact on biological organisms and ecosystems. This is a particularly urgent need as bioplastics are already replacing conventional plastics in various segments of the global plastic market.

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Authors' contributions

Contributed to the conceptualization of ideas presented in the manuscript and revising the manuscript: Tamayo-Belda M, Pulido-Reyes G, Rosal R, Fernández-Piñas F
Drafted the manuscript and generated the tables: Tamayo-Belda M

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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