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Hazard characterization of graphene nanomaterials in the frame of their food risk assessment: A review

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ABSTRACT

Different applications have been suggested for graphene nanomaterials (GFNs) in the food and feed chain. However, it is necessary to perform a risk assessment before they become market-ready, and when consumer exposure is demonstrated. For this purpose, the European Food Safety Authority (EFSA) has published a guidance that has been recently updated. In this sense, the aim of this study is to identify and characterise toxicological hazards related to GFNs after oral exposure. Thus, existing scientific literature in relation to *in vitro* degradation studies, *in vitro* and *in vivo* genotoxicity, toxicokinetics data, *in vivo* oral studies, and other in-depth studies such as effects on the microbiome has been revised. The obtained results showed that the investigations performed up to now did not follow internationally agreed-upon test guidelines. Moreover, GFNs seemed to resist gastrointestinal digestion and were able to be absorbed, distributed, and excreted, inducing toxic effects at different levels, including genotoxicity. Also, dose has an important role as it has been reported that low doses are more toxic than high doses because GFNs tend to aggregate in the digestive system, changing the internal exposure scenario. Thus, further studies including a thorough toxicological evaluation are required to protect consumer's safety.

1. Introduction

Graphene is an allotrope of carbon consisting of a one-atom-thick layer of carbon atoms arranged in a two-dimensional hexagonal lattice nanostructure. Graphene exhibits a plane of sp2-bonded atoms. Related materials include few-layer graphene (FLG), ultrathin graphite, graphene oxide (GO), reduced graphene oxide (rGO), graphene nanosheets, nanoribbons, and quantum dots (GQD) (Singh, 2016; Tiwari et al., 2020), and they are usually referred as graphene nanomaterials (GFNs).

Graphene is considered one of the most promising nanomaterials because of its unique combination of distinctive properties: high thermal and electrical conductivity, high elasticity and flexibility, hardness and resistance, transparency, among others (Xu, 2018). All these properties allowed graphene to be used in a wide range of applications in the fields of science, engineering, and technology, such as a promising material for biomedical engineering (gene delivery, tissue engineering, bioimaging), cancer treatment, drug delivery systems, electronics, printing technology, textile engineering or aerospace (Singh, 2016; Dhinakaran et al., 2020). Moreover, the food industry can also benefit from the use of GFNs. In this respect, Sundramoorthy and Gunasekaran. (2014) reported that graphene could be used in food quality analysis such as the detection of chemical contaminants, food composition, or volatile organic compounds. It has additional applications in food safety practices, such as extraction and detection of toxins (i.e. Díaz-Nieto et al., 2018; Yadav et al., 2021; Le et al., 2021), detection of pesticides, and exploitation of its antibacterial properties. Moreover, different studies have emphasized the excellent performance of GFNs in food packaging applications (Goh et al., 2016; Ahmed, 2019; Enescu et al., 2019; Emamhadi et al., 2020; Barra et al., 2020). However, for these potential applications to become a reality in the food sector, GFNs must demonstrate their safety before being delivered to the consumer market. In this regard, the European Food Safety Authority (EFSA) published as early as 2011 the first "Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain" that was later updated in 2018 and 2021 (EFSA, 2018; 2021).

The most recent guidance (EFSA, 2021) covers the application areas within EFSA's remit, including novel foods, food contact materials, food/feed additives, and pesticides. It includes a structured four-step framework for hazard identification and characterisation of nanoparticles. It starts with the identification of materials requiring nano-specific assessment and their physicochemical characterization (Step 1).

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Abbreviations

ADME	absorption, distribution, metabolism and excretion
ALT	alanine aminotransferase
AST	aspartate aminotransferase
EFSA	European food safety authority
FESEM	field emission electron microscopy
FLG	few-layer graphene
FPG	formamidopyrimidine DNA glycosylase
FITC-Dextran	fluorescein isothiocyanate-dextran
GFNs	graphene nanomaterials
GI	gastrointestinal
GO	graphene oxide
GQD	graphene quantum dots
i.v.	intravenous
MN v	micronucleus
NOAEL	no-observed-adverse-effects-level
rGO	reduced graphene oxide
TEM	transmission electron microscope

Step 2 is related to the *in vitro* digestion to determine whether the material quickly and fully dissolves/degrades under the conditions of the digestive tract. In this regard, a review of existing physicochemical and toxicological aspects and/or relevant to grouping/read-across is recommended (Step 2A) as well as the generation of new *in vitro* data (dissolution under lysosomal conditions, genotoxicity, cell toxicity) (Step 2B). If the material proves to be persistent or there is indication of toxicity, it is required to go forward to Step 3: *In vivo* testing. It includes the recommendation of a pilot study for dose finding and assessment of toxicokinetics (Step 3A) and *in vivo* studies (Step 3B). These *in vivo* studies are comprised of genotoxicity testing (if required), a modified 90-day oral toxicity study, and histopathological investigations of gastrointestinal (GI) sites and organs with emphasis on liver, brain, testis, and spleen. The result of this study can be used to identify a reference point (such as the lower boundary of the benchmark dose confidence interval or a no-observed-adverse-effect-level (NOAEL). Moreover, if the results obtained in Step 3 warrant further in-depth targeted tests, Step 4 is reached. This phase covers, for example, additional toxicokinetic studies, reproductive and developmental toxicity, immunotoxicity, neurotoxicity, mutagenicity, carcinogenicity, endocrine effects, or effects on gut microbiome.

Thus, the aim of this review is to establish a general picture of the safety of GFNs in the framework of potential food applications – that is, to compile and analyse reports available in the scientific literature adhering to the main requirements of the EFSA (2021) guidance, particularly *in vitro* digestion data, *in vitro* and *in vivo* genotoxicity assessment, toxicokinetics (absorption, distribution, metabolism, and excretion, ADME) data, oral *in vivo* studies, and targeted in-depth tests, to identify data gaps and research needs. The physicochemical characterization of GFNs is therefore not considered in this review.

To achieve this aim, thorough research of scientific literature using Science Direct, Pubmed, and Scopus databases was performed using as keywords "graphene", "toxicity", "degradation", "genotoxicity", "*in vivo*", "*in vitro*", "oral", "microbiota", etc. No temporary limits were applied in the search.

2. *In vitro* studies

2.1. In vitro degradation studies

2.1.1. In vitro gastrointestinal digestion

The dissolution rate of the tested nanomaterial has to be measured under representative conditions of the GI system (Step 2 in EFSA framework) (EFSA, 2021). The models are considered appropriate if the relevance to the physiological state (fasted or fed) is justified, and it is specified whether the physiological state under study represents worst-case, realistic, or favourable conditions for *in vitro* dissolution (however, worst-case conditions are recommended). Three different concentrations, with the middle concentration representative of human exposure must be tested. Only intestinal phase digestion is considered relevant, and the degradation rate must be determined in at least 4 time points in duplicate (at about 5, 15, 30, and 60 min). Number-based particle size and aggregate distributions and concentration must be analytically determined with a chemically specific method before and after GI digestion. Exposure is considered to occur when the nanomaterial does not degrade or degrades so slowly that more than 12% of the mass of the material is present after 30 min of intestinal digestion. However, a localized exposure in the upper GI tract must be analysed in case of complete digestion.

The following must be also considered for the experimental design: (1) The concentration of the solute and degradation products should also be determined because some materials may degrade in the stomach and then precipitate under the intestinal conditions as salts or nano- or microsized particles (Walczak et al., 2013); (2) The dissolved fraction should not be separated as it may promote the dissolution; (3) Non-nano agglomerates formed in the stomach may deagglomerate in the intestine stage (Peters et al., 2012); (4) When applicable to the material, a comparison to an ionic control should be included.

Regarding the GFNs, Kucki et al. (2016) investigated the *in vitro* effects of acidic conditions during stomach digestion on surface functionalization (C/O ratio), induction of defects and changes in morphology of GO (4 different materials, the smallest 20 nm-1.4 m, and the largest 1–40 μm), and graphene nanoplatelet (GNP) aggregates (1–10 μm). No significant changes in morphology and material properties were found, indicating that GO and GNP are relatively inert materials not affected by the acidic conditions in the stomach.

Guarnieri et al. (2018) used a dynamic *in vitro* digestion assay to study the biotransformation of FLG (0.09 mg/mL) and GO (0.45 mg/mL), both with a similar size of around 400 nm, through the GI tract simulated environments (salivary, gastric, intestinal). They concluded that FLG and GO had a poor and respectively moderate stability in aqueous media while Raman spectroscopy suggested their aggregation in clusters. FLG formed small flakes dispersed and homogeneously distributed in the saliva and intestinal juices, but large cluster collections in the stomach environment. No structural defects nor permanent changes were induced in the two materials in the digestive process. However, FLG and GO interacted with compounds in the juice (proteins, organic molecules, gastric acids, and salts), and charge changes (doping) were observed. This finding differentiates FLG and GO from other nanomaterials characterized in the literature such as silver nanoparticles (Böhmert et al., 2014; Bove et al., 2017).

Similarly, Bitounis et al. (2020) assessed the effect of simulated GI digestion in a fasting model on 2 groups of GO with mean lateral sizes of 0.24 and 1.13 μm, as measured by field emission electron microscopy (FESEM). The samples were suspended in HyClone cell culture with colloidal stability and their stability was tested for up to 10 min. Prior to being used in the experiment, the GO sample showed good dispersibility in aqueous media and only slight agglomeration. Two concentrations were used, 50 and 250 μg/mL for each, with concentrations based on a potential ingestion of GO following the tear of GO- enabled membranes used in water filters. The simulated digestion included the oral phase, the gastric digestion, and a small intestinal phase. After digestion the digested material in each phase was analysed in respect to the thickness and lateral size distributions. Following the experiment, Bitounis et al. (2020) reported a progressive agglomeration of GO especially in the gastric and small intestinal phases. Minimal agglomeration was reported in the oral phase. However, the GO flakes presented a strong tendency to agglomerate in the acidic conditions of the gastric phase. This phenomenon seemed to continue in the intestinal phase most likely because

of the presence of bile salts and other digestive enzymes. Additionally, it was suggested that the agglomerates of GO could be stabilized by the proteins and cations abundant in the GI tract. This could favour associations among different regions of the same sheet, thus causing morphological changes, such as folding and wrinkling. It was also reported that GO was reduced under the GI tract conditions.

On another note, Saxena and Sarkar. (2014) tested the effect of chewing and salivary enzymes on GO produced naturally in foods when prepared by barbecuing. The experiment included the actual chewing of charred chicken material for several minutes, and then, the obtained slurry was subjected to simulated gastric digestion. Transmission electron microscope (TEM) and FESEM analysis confirmed the presence of graphene in the slurry obtained by chewing in the form of GO sheets rich in heteroatoms. The simulated gastric digestion supported the hypothesis that an acidic treatment with dilute nitric or hydrochloric acids could separate the layers of disordered (turbostratic) graphene sheets which were distributed in loose bundles. Similar findings were reported for branded Colic Calm baby's gripe water containing charcoal ().

Another study worth considering in respect to the interaction of graphene with the GI enzymes is that by Huang et al. (2020). The authors studied the interaction between GO and trypsin and reported their bonding by van der Waal interaction, hydrophobic interaction, hydrogen bond, and electrostatic interaction to form a ground state complex with molar ratio of 1 to 1. By bounding with the allosteric site of trypsin GO inhibited its enzymatic activity. They also observed that common metal ions could not significantly affect this interaction. Additionally, the GO-trypsin complex seemed to be rather stable which reiterated the biological effect of GO upon this digestive enzyme. These results provide supplementary information for the hazard characterization of GO.

In conclusion, it appears that the graphene and graphene-based materials are roughly morphologically and structurally resistant to the conditions of the GI tract, but they do form clusters of aggregates because of doping. Additionally, they seem to interact with some GI enzymes, such as trypsin, forming stable compounds and impairing their enzymatic activity. However, the scientific data is extremely scarce and of unsuitable quality for a hazard characterization following EFSA nanomaterials framework. Further studies are needed to clarify the GI dissolution of the various graphene-based materials.

2.1.2. Stability in lysosomal fluids

The behaviour of nanomaterials in the lysosomal fluid can be an indicator of toxicity if it is degraded and ions are released, and of biopersistence and intracellular accumulation (EFSA 2018; 2021). Thus, the degradation rate and particle size distribution under lysosomal conditions have to be measured *in vitro* in at least 4 timepoints (up to 72 or 96 h) in duplicate and at 3 different concentrations, similar to *in vitro* GI tract condition testing. A half-life of 24 h, with less than 12% of the mass of the material present at 72 h, and no visible plateau is considered to show a high degradation rate.

Only Liu et al. (2018) and Qi et al. (2018) evaluated the stability in lysosomal fluid of GFNs (a single concentration of 25 μg/mL GO) to study its biotransformation. The GO (hydrodynamic diameter 144.3 ± 6.1 nm) was characterized before and after 5 days incubation at 37 °C and slow shaken, with a later filtration through 0.22 μm membrane filters (Liu et al., 2018). They concluded that GO was significantly affected by the procedure: (1) there was a reduction reaction of GO mainly due to the conversion of epoxy and carbonyl groups to phenolic groups; (2) the morphology was altered as the nanosheets thickened, folded, wrinkled, and developed uneven edges; (3) randomly aggregated edge-to-edge sheets were observed; (4) new graphitic domains smaller in size were formed. Qi et al. (2018) observed that the thickness and lateral size of the GO nanosheets increased upon biotransformation, and demonstrated the importance of organic acids (lactate and tartrate) contained in the artificial lysosomal fluid in the reduction of GO in addition to that of citrates. Their absorption in the surface of GO could explain the increased concentrations in carboxyl groups after the treatment. However, the hydrolysis of acid anhydrides on GO might be another possible mechanism.

Both studies bring detailed information to the degradation assessment of GO in lysosomal fluid by using multiple and reliable analytic methods, but they were focused on lung fluids and biomedical applications. They did not compute a degradation rate and did not consider testing the 3 different concentrations at 4 timepoints to reach the level of detail and quality to be included in a hazard characterization based on EFSA framework for nanomaterials to be applied in the food and feed chain.

In general, and regarding *in vitro* degradation tests, the absence of data makes it mandatory to assume that 100% of the ingested material remains in particulate form (EFSA, 2021).

2.2. In vitro toxicity testing

In vitro tests are included among those suggested by EFSA (2021) in Step 2 of its outline as they can provide insight into the hazardous characteristics of a nanomaterial and their toxic mechanisms. The rapid, precise, and relevant information they provide, along with the reduction of the use of animals they allow, make them a perfect choice for screening purposes. GFNs have been extensively investigated with *in vitro* methods. Thus, a scientific literature search in ScienceDirect using graphene and *in vitro* toxicity as keywords yielded more than 9000 results. There is, moreover, a recent systematic literature review regarding the *in vitro* toxicity of graphene-based materials (Achawi et al., 2021). Therefore, this aspect is out of the scope of the present work.

Most research on this topic deals with cytotoxicity, oxidative stress, and immunotoxicity parameters. Moreover, in the framework of EFSA guidelines (2021), experimental models representative of the gastrointestinal system are to be preferred

Finally, EFSA (2021) pointed out that when *in vitro* methods indicate a lack of toxic effects, and *in vitro* dissolution of the nanomaterial in lysosomal and gastrointestinal conditions is fast, an argument can be put forward to EFSA for waiving *in vivo* studies. However, the research carried out to date on GFNs and reviewed in section 2 does not indicate that possibility.

2.3. In vitro genotoxicity

In vitro genotoxicity assessment plays a key role in the toxicity testing of nanomaterials (Catalán and Norppa, 2017). The evaluation of genotoxicity is based on three critical endpoints: gene mutation, structural, and numerical chromosome aberrations, according to general indications of the EFSA (EFSA, 2018). These endpoints are covered by a battery of 2 tests comprising a bacterial reverse mutation (OECD TG 471 (2020a) and the *in vitro* mammalian cell micronucleus test (MN) (OECD TG 487, 2016a) as indicated by EFSA Scientific Committee (2011). The bacterial reverse mutation (Ames) test is usually recommended for the detection of gene mutations. However, it is not considered appropriate for the analysis of nanomaterials because they may not be able to penetrate the bacterial cell wall, and bacterial cells, unlike mammalian cells, do not have the ability to internalise (Doak et al., 2012; OECD, 2014). Other suitable options are the *in vitro* mammalian cell gene mutation tests using the Hprt and xprt genes (OECD TG 476, 2016b) and the *in vitro* mammalian cell gene mutation tests using the thymidine kinase gene (OECD TG 490, 2016c). Structural and numerical chromosome damage is evaluated i.e. by the *in vitro* mammalian cell micronucleus test (OECD TG 487, 2016a).

Several studies have reported genotoxic effects of GFNs *in vitro* (Table 1). All the studies available in the scientific literature, regardless of the relevance of their experimental models to the GI system, were included because of the scarcity of the reports. Additionally, the current review intends to present the best overview of the genotoxicity of GFNs.

Most of studies used the Comet assay (Akhavan et al., 2012a, b, 2013;

Table 1
In vitro genotoxic effects of GFNs.

Material	Physicochemical properties (size)	Experimental Model	Assays	Concentration range	Exposure time	Relevant Results	Reference
rGONPs	ALDs: 11 ± 4 nm, 91 ± 37 nm, 418 ± 56 nm, 3.8 ± 0.4 μm	hMSCs cell line	Alkaline comet assay Chromosomal aberration	0.01–100 μg/mL	1 h	Concentration- and size-dependent DNA fragmentation in terms of the % of DNA in the tail and chromosome aberrations mainly at low ALDs. The 2 bigger NPs showed a slight effect only at the highest concentrations and only in the comet assay	Akhavan et al. (2012a)
rGONR-PEG, rGO-PEG	Thickness (nm): ~0.9	U87MG cell line	Comet assay	0.01–1000 μg/mL	24 h	Concentration-dependent genotoxicity after exposure to rGONR-PEG (>1 μg/mL). rGO-PEG did not induce genotoxic effects. Genotoxicity could be assigned to the size of the nanoribbons	Akhavan et al. (2012b)
rGONRs	Length (~10 μm), width (~50–200 nm). Thickness (~1.0 nm)	hMSC cell line	Comet assay Chromosomal aberration	0.01–100 μg/mL	1, 5, 24, 96 h	Increased DNA fragmentation and chromosomal aberration in a concentration – and time-dependent manner, even after 1 h exposure	Akhavan et al. (2013)
GO, LA-PEG-GO, PEG-GO, PEI-GO	Lateral width (nm), thickness (nm): GO (200–500; ~1), LA-PEG-GO (100–200; ~2), PEG- GO (50–150; ~1.9), PEI-GO (200–500; ~2.5)	HLF	Comet assay	0, 1, 50 and 100 μg/mL	24 h	DNA damage induced by unmodified GO and PEI-modified GO was concentration-dependent. PEG-modified GO induced less genotoxicity than GO and PEI-modified GO. LA-PEG modified GO decreased DNA damage induced by GO derivatives	Wang et al. (2013)
GO, rGO	Thickness, LSD, Hydrodynamic diameter (nm): GO (6; 40; 297.09), rGO (7; 40; 36.39)	HepG2 cell line	Alkaline comet assay Activation of phosphorylated γ-H2AX	rGO: EC20 (~8 mg/mL) and EC50 (~46 mg/mL) and GO: EC20 (~10 mg/mL) and EC50 (~81 mg/mL)	24 h	Both induced single and double stranded DNA breaks at EC_{20} and EC_{50} in a concentration-dependent way. The concentration dependency was very sharp and significant for GO but not for rGO. Thus, for rGO it was assumed that the damage resulted from physical interaction rather than biological one	Chatterjee et al., (2014)
Micro GO, nano GO	Flake size: Micro-GO (1.32 μm) and nano-GO (130 nm)	A549, Caco-2 and Vero cell lines	Comet assay	10, 50 and 100 μg/mL	24 h	DNA damage directly increased with the concentration of micro-GO. The highest genotoxic effects were observed after nano-GO exposure already with low concentration	De Marzi et al. (2014)
GN, rGO, GO, G	Surface area: from 450 nm to 1.5 μm	U87 cell line	Comet assay	50 μg/mL	24 h	Severe genotoxicity was observed after exposure to GN, rGO and G. GO did not induce DNA damage. GN was the most genotoxic material	Hinzmann et al. (2014)
GO nanosheets	Diameter (nm): 1000–10000. Hydrodynamic diameter (nm): 8276 (in stock) 19000 (in Tris-HCl) and 16896 (in CCM)	T-lymphocyte Jurkat cell line B-lymphocyte cell line (WIL-2NS) Human primary lymphocytes	Micronucleus assay	0–400 μg/mL for (T and B-lymphocytes cell line) 0–100 μg/mL for primary lymphocytes	24 h (T and B-lymphocytes) 44 h (Primary lymphocytes)	The number of micronuclei increased in human lymphocytes after exposure of GO sheets (above 50 μg/mL)	Ivask et al. (2015)
FLG	Primary particle lateral dimension (nm): 160 ± 48.6; ALD (nm): 0.8 ± 0.42; Hydrodynamic diameter (nm): 342 ± 58.4.	HUVEC cell line	Alkaline comet assay	5 and 10 μg/mL	6 h	Increased tail length and tail DNA % with increasing concentration of FLG	Sasidharan et al. (2016)

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Table 1 (*continued*)

Material	Physicochemical properties (size)	Experimental Model	Assays	Concentration range	Exposure time	Relevant Results	Reference
GNP-Prist, GNP-COOH, GNP-NH2, SLGO, FLGO	Thickness (nm); lateral size distribution (μm); layer number: GNP-Prist (877.2; 10; <4), GNP-COOH (735.9; 9.98; <3) GNP-NH2 (945.5, 10; <4), SLGO (21; 10; 1), FLGO (122; 10; 4–8)	BEAS-2B cell line	Comet assay Activation of phosphorylated γ-H2AX	10 and 50 mg/L (comet assay) 10 mg/L (γ-H2AX assay)	24 h	GNPs caused DNA damage at both concentrations. The order of DNA damage by the comet assay was GNP-Prist ≥ GNP-COOH > GNP-NH2 ≥ FLGO > SLGO at 10 mg/L. The order of double strand break potency was GNP-Prist > GNP-NH2 > GNP-COOH > FLGO ≥ SLGO. In general, GNPs possess more genotoxic ability than SLGO/FLGO	Chatterjee et al. (2016)
GO. rGO-s, rGO-l	Layer number; Lateral size (μm); GO (2–3; 2–3); rGO-s (2–3; 1–2); rGO-l (2–3; 1–2)	FE1 cell line	Comet assay	0, 5, 10, 25, 50, 100 and 200 μg/mL	3 and 24 h	No genotoxic effects were observed at any concentration	Bengtson et al. (2016)
GO, rGO	Thickness (nm): ~0.8 for both nanosheets	SSCs cell line	Comet assay	0.1–400 μg/mL	24 h	DNA damage increased by increasing the graphene concentrations	Hashemi et al. (2016)
GO	Length and width (μm): 3–6. Diameter: 1 nm. Single layer	HT20 cell line	Micronucleus assay	10, 15, 25 and 50 μg/mL	48 h	NDI decreased, MN, NBUDs and NPBS increased after exposure of GO in a concentration dependent way	Heshmati et al. (2018)
GO, pure MWCNT, MWCNT-COOH, MWCNT-NH_2	Diameter (nm); GO (117.8 ± 4.12), pure MWCNT (93.02 ± 2.24), MWCNT-COOH (92.69 ± 3.06), MWCNT-NH_2 (91.31 ± 4.02)	L5178Y/Tk $^{\pm}$3.7.2C mouse lymphoma cells	Mouse lymphoma assay	0–250 μg/mL	4 h	No genotoxic effect on L5178Y/Tk $^{\pm}$3.7.2C cells at concentrations up to 250 μg/mL	Demir and Marcos, (2018)
Exfoliated graphene	Diameter (μm): 10.04	*Salmonella typhimurium* strains TA98, TA100, TA1535, and TA1537. *E. coli* mutant WP2uvrA	Bacterial reverse mutation test	0.625, 1.25, 2.5,5.0, and 10.0 μg/plate	72 h	It did not induce genetic mutations	Fujita et al. (2018)
G, GO (small, medium, and large sizes)	Size (nm); S-G (29.31), S-GO (31.25), M-G (307.56), M-GO (321.74)	HEK 293T cell line	Comet assay	5, 25, 50 and 100 mg/L	24 h	Induced DNA damages with a substantial increase in tail length and tail moment in a concentration-dependent manner. Small particles caused more genotoxicity compared to medium and large sizes. GO showed more severe DNA damage than the same sizes of G at the same concentration	Jia et al. (2019)
Pristine GO, GO-NH_2	Average particle size: GO (small fraction 250 ± 68 nm and main fraction 1.5 ± 07 μm), GO-NH_2 (560 ± 300 nm)	Colon 26 cell line	Comet assay	1, 10, 20 and 50 μg/mL	24 h	Aminated GO (50 μg/mL), and pristine GO (10 μg mL^{-1}) caused DNA damage in Colon 26 cells. The absence of DNA damage at higher GO concentrations could be the result of the higher % of cells which were already in their late stages of apoptosis where almost all DNA is degraded and thus unable to be presented as comets	Krasteva et al. (2019)
N-doped GQDs	Size (nm): 10.9 ± 1.3	NIH-3T3 cell line A549 cell line MDA-MB-231 cell line	Comet assay	50, 100 and 150 μg/mL	24 h	N-doped GQDs induced genotoxicity in a concentration-dependent manner in all studied cells. A549 cells were the least sensitive	Şenel et al. (2019)
haGO-NH_2, pristine GO	Size: pristine GO (small fraction 515 nm and main fraction 3.6 μm), haGO-NH_2 (594 nm)	HepG2 cell line	Comet assay	4, 10, 25 and 50 μg/mL	24 h	No DNA damage was detected in HepG2 cells treated with pristine GO for 24 h. A slightly higher genotoxicity was observed	Georgieva et al. (2020)

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Table 1 (*continued*)

Material	Physicochemical properties (size)	Experimental Model	Assays	Concentration range	Exposure time	Relevant Results	Reference
						in haGO-NH_2 treated cells at the lowest concentrations. The mechanisms by which haGO-NH2NPs exert their biological activities were not centered on the stability and maintenance of the genome integrity	
GO, GNPs	Size (nm) GO (249.9 ± 7.4), GNPs (243.4 ± 1.4)	Caco-2/HT29 monolayer	Comet assay Enzyme-modified comet assay (FPG)	5, 15, 50 μg/mL	24 h	Concentration-dependent genotoxic effects by the comet assay. No oxidative DNA damage	Domenech et al. (2020)
Neutral-FLG, Amine-FLG, Carboxyl-FLG, CB particles	Hydrodynamic diameter (nm); thickness (nm); layer number; particle size (nm): Neutral-FLG 290.8 ± 302.6; 94.73 ± 67.94; 50; 101.3 ± 16.8), Amine-FLG (170.1 ± 97.92; 86.20 ± 41.16: 12; 124.4 ± 13.9), Carboxyl-FLG (169.6 ± 76.88; 55.16 ± 42.22; 4; 99.5 ± 15.3), CB particles (513.3 ± 421.2; -; -)	16HBE14o$^-$ cells	CBMN assay	0–100 μg/mL	24 h	CB (>20 μg/mL), neutral-FLG (>10 μg/mL) and amine-FLG (>50 μg/mL) induced genotoxic responses. Only amine-FLG showed significant clastogenic effect. Carboxyl-FLG did not induce genotoxicity at any concentration	Burgum et al., (2021a)
Neutral-FLG, Amine-FLG, Carboxyl-FLG, CB particles	Previously characterized by Burgum et al., 2021	TT1 cell line, TT1/d.THP1 co-culture	CBMN assay	0–100 μg/mL (TT1 monoculture) 0–50 μg mL^{-1} (TT1/THP1 co-culture)	24 h	Primary genotoxicity was observed in all graphene treated cells. All induced secondary genotoxicity in co-culture model of TT1/d.THP1 cell line. DNA damage could be induced by oxidative stress	Burgum et al. (2021b)
GO, RGO (at different times (hours) of reduction; RGO-3, -6, -9 and -12.)	Thickness (nm): ~1.4 ± 0.2 for GO and RGOs.	ARPE-19 (RPE cell line)	Alkaline Comet Assay	100 μg/mL	24 h	GO and RGOs caused DNA damage. RGOs induced much more genotoxic effects than GO. Results suggested that reduction of GO was less biocompatible to ARPE-19 cell line	Ou et al. (2021)
GO, r-GO	Primary particle size (nm); number of layers; GO (2–3; 2–3), rGO (1–2; 2.3)	A549 cell line, THP-1a cell line	Alkaline Comet Assay	0–160 μg/mL (A549 cell line) and 0–80 μg/mL (THP-1a cell line)	6 and 24 h	Only GO exposure after 24 h caused DNA damage in THP-1a cells	Di Ianni et al. (2021)

ALDs: average lateral dimensions. A549: human lung carcinoma cell line; BEAS-2B: human bronchial epithelial cell line; Caco-2: human colorectal adenocarcinoma cell line; CBMN: cytokinesis-blocked micronucleus assay; CCM: cell culture medium; FE1: immortalized murine pulmonary epithelial cell line; FLG: Few layers graphene. FPG: formamidopyrimidine DNA glycosylase; G: graphite; GO: graphene oxide; GN: pristine graphene; GNPs: graphene nanoplatelets; GQDs: graphene quantum dots; haGO-NH2: aminated graphene oxide nanoparticles; HEK 293T: Human embryo kidney cell line; HLF: Human lung fibroblast; HepG2: liver hepatocellular carcinoma cell line; hMSC: human mesenchymal stem cell line; HT20: colon cancer cell line; HT29: Human Colon Adenocarcinoma; HUVEC: human primary umbilical vein endothelial cell line; LA-PEG-GO: lactobionic acid-polyethylene glycol functionalized graphene oxide; LSD: laterial size distribution; MDA-MB-231: human breast adenocarcinoma cell line; MSCs: human mesenchymal stem cells; MWCNT; Multi-Walled Carbon Nanotubes; NBUDs: nuclear buds; NDI: nuclear division index; NIH-3T3: mouse fibroblast cell line; NPBs: nucleoplasmatic bridges; PEG- GO: polyethylene glycol functionalized graphene oxide, PEI-GO: polyethylenimine functionalized graphene oxide; rGO: reduced graphene oxide; rGONPs: reduced graphene oxide nanoplatelets; rGONR-PEG: reduced graphene nanoribbons functionalized by amphiphilic polyethylene glycol; RPE: human retinal pigment; SLGO: single layer graphene oxide; SSCs: spermatogonial stem cells; THP-1: Human monocyte cell line; TT1: human-transformed type-I alveolar cell line; U87: glioblastoma multiforme cell line; Vero: kidney epithelial cell line from an African green monkey.

Wang et al., 2013; Chatterjee et al., 2014; De Marzi et al., 2014; Hinzmann et al., 2014; Sasidharan et al., 2016; Chatterjee et al., 2016; Bengtson et al., 2016; Hashemi et al., 2016; Jia et al., 2019; Krasteva et al., 2019; Şenel et al., 2019; Georgieva et al., 2020; Domenech et al., 2020; Ou et al., 2021; Di Ianni et al., 2021) although it is not included among the tests primarily recommended by EFSA. The comet assay or single cell gel electrophoresis is a common tool to measure DNA strand breaks (Collins et al., 2017). EFSA (2021) considered that the *in vitro* comet assay, though not yet validated, may provide complementary information and contribute to an understanding of the nanomaterial genotoxicity mechanisms. The vast majority of the studies (16) that applied the Comet assay reported genotoxic effects for the different graphene materials evaluated and experimental models used. Only Akhavan et al. (2012b) observed that rGO-PEG did not induce genotoxic effects in the U87MG cell line in contrast to rGONR-PEG. GO and rGO also did not induce genotoxicity by this assay when tested up to 200 μg/mL in the murine pulmonary epithelial cell line (FE1) (Bengtson et al., 2016). Georgieva et al. (2020) did not detect DNA damage in hepatic HepG2 cells treated with pristine GO for 24 h up to 50 μg/mL. On the contrary, Chatterjee et al. (2014) observed a concentration-dependent genotoxic effect for GO at lower concentrations in the same cell line. And recently, Di Ianni et al. (2021) reported that rGO did not induce DNA damage either in A549 cells (up to 160 μg/mL) or in THP-1a cells (up to 80 μg/mL) and that GO caused strand breaks only in the monocytic cells.

Apart from the Comet assay, other genotoxicity tests performed were the chromosomal aberration assay (Akhavan et al., 2012a, 2013), activation of phosphorylated γ-H2AX (Chatterjee et al., 2014, 2016), the

Ames test (Fujita et al., 2018), the mouse lymphoma assay (Demir and Marcos, 2018) and the MN assay (Ivask et al., 2015; Heshmati et al., 2018; Burgum et al., 2021a,b). Most of them showed genotoxic effects with the exception of Demir and Marcos (2018) who reported no effects for different GFNs with concentrations up to 250 μg/mL using the mouse lymphoma assay; Fujita et al. (2018), who did not observe genetic mutation in the Ames test (although this method is not recommended by EFSA (2021); and Burgum et al. (2021a) who observed no genotoxicity by the MN test with Carboxyl-FLG in contrast to neutral and amine derivatives that were genotoxic. This test (the MN assay) is among those recommended by EFSA to evaluate structural and numerical chromosome damage. Also, it is important to highlight that although EFSA recommends following the OECD guidelines, only Ivask et al. (2015) and Fujita et al. (2018) followed these internationally agreed protocols.

Some of the studies explored not only a potential concentration-dependent response, but also the influence of the size on the observed effects (Akhavan et al., 2012; De Marzi et al., 2014; Jia et al., 2019). The results suggested that smaller nanomaterials induced higher genotoxicity, and this could be attributed to the internalization of nanoparticles into the nucleus of cells.

Many nanomaterials were shown to induce oxidative stress, which might be related to a secondary mechanism of genotoxicity (EFSA, 2018). However, this aspect has been scarcely investigated *in vitro*. Only Domenech et al. (2020) performed an enzyme-modified comet assay using formamidopyrimidine DNA glycosylase (FPG) and reported no oxidative DNA damage after GO and GNPs exposure. In the modified assay, the oxidatively-damaged DNA bases are recognized by FPG, which excises the damaged base/nucleotide, producing a single-strand break. In this way, the differences between the levels of DNA breaks in the presence/absence of FPG can help to identify the DNA's oxidative damage and, consequently, to assess the oxidative potential of the tested compound (Collins et al., 2017). Several studies in Table 1 evaluated and reported oxidative stress as well (i.e. Chatterjee et al., 2014; Krasteva et al., 2019; Georgieva et al., 2020), but they did not explore further its influence on genotoxicity.

All the studies which focused specifically on cell lines representative for the GI system such as Caco-2 cell line (De Marzi et al., 2014) and Colon 26 cell line (Krasteva et al., 2019) reported genotoxic effects. Moreover, Domenech et al. (2020) used a more complex experimental model: a coculture of differentiated Caco-2/HT29 cells presenting inherent intestinal epithelium characteristics. They also observed genotoxicity induced by GO and GNPs.

Overall, the results reported suggest that GFNs induce genotoxicity *in vitro*, although the complete battery of assays recommended by EFSA (2021) has yet to be completely performed.

3. *In vivo* studies

3.1. *In vivo genotoxicity testing*

In vivo genotoxicity testing should be performed when at least one of the *in vitro* tests suggests genotoxic activity or if the *in vitro* testing of the nanomaterial is not appropriate (EFSA, 2021). EFSA (2021) included the *in vivo* MN test (OECD test guideline 474, 2016d), an *in vivo* mammalian alkaline comet assay (OECD test guideline 489, 2016e), and the transgenic rodent somatic and germ cell gene mutation assay (OECD test guideline 488, 2020b) among the suitable *in vivo* methods.

Although GFNs were reported to induce genotoxicity *in vitro* (section 2.3), the number of *in vivo* studies on oral administration is low. Mohamed et al. (2019) investigated chromosomal integrity by the micronucleus test and DNA damage by the Comet assay on the bone marrow cells of mice exposed to 10, 20, or 40 mg/kg GO nanoparticles (1162 nm hydrodynamic size) by gavage for 1 (acute) or 5 consecutive days (repeated dose). The results showed a significant dose-dependent increase of chromosomal and DNA damage in both administrations. Moreover, they observed histological lesions (apoptosis, necrosis, inflammation, and cell degeneration) in liver and brain.

Later on, the same authors explored further the toxicity observed previously in liver and brain (Mohamed et al., 2020). They used the same treatment schedule to evaluate the genotoxicity and mutagenicity of GO in these tissues. They concluded that GO (≈500 nm) induced genomic instability and mutagenicity in the liver and brain of mice orally exposed. This was probably induced by oxidative stress, as they also observed significant dose-dependent increases in the malondialdehyde level and reductions in both the level of reduced glutathione and activity of glutathione peroxidase. An additional oral study reported, as well, DNA damage in the blood cells of ICR male mice exposed to 1.5 mg/kg of GO after 24 h by the Comet assay (Chatterjee et al., 2014).

All these studies used exposure by gavage as oral route and not by diet (animal feed), and this could influence the outcomes. Currently, there are studies on other types of chemical compounds that suggest eliminating the use of gavage for toxicity testing as it can overestimate toxicity (Turner et al., 2011; Vandenberg et al., 2014). Dietary exposure interacts with the oral mucosa, whereas gavage exposure prevents these interactions, leading to dramatic differences in absorption, bioavailability, and metabolism. Moreover, gavage is associated with complications and can induce stress. In any case, both gavage and diet, together with the exposure through drinking water are considered relevant oral routes in the EFSA guideline (2021), and gavage still remains the method of choice in the case of nanomaterials. Additionally, the exposure times explored in the available studies are short. The evidence of *in vivo* genotoxic effects with these short exposure times might be indicative of genotoxicity after longer time periods as well, but further studies are still needed. Other exposure routes have led to contradictory results. Thus, Liu et al. (2013) observed genotoxic effects by using the MN test in mice exposed to 1, 2, and 4 mg/kg GO (mean diameter 156.4 nm) for 5 consecutive days by intravenous (i.v.) administration. While Fujita et al. (2018) reported no genotoxicity in mice exposed for 5 consecutive days via i.v. to 0.5, 1 and 2 mg/kg exfoliated graphene (mean diameter 10.04 μm).

3.2. *ADME information*

The toxicokinetics of GFNs is not yet well described and understood as there are a series of contradictory studies. A few reports show that polyethylene glycol (PEG) functionalized GO derivatives (Yang et al., 2013), FLG (Mao et al., 2016), and GQDs (Zhang et al., 2019) are not absorbed in the blood stream and are rapidly excreted by faeces (Mao et al., 2016). However, other studies show rather compelling evidence that GO does enter the blood system, especially when in lower concentrations, and affects the liver (Fu et al., 2015; Mohamed et al., 2019; Li et al., 2021), the kidneys (Patlolla et al., 2016), the lungs (Liu et al., 2021), crosses the blood-brain barrier (Mohamed et al., 2019), the placental barrier (Liu et al., 2021), and may be excreted in maternal milk (Fu et al., 2015). It may be the case that when higher concentrations of graphene nanomaterials are used in experiments, they form large agglomerates under the action of the GI fluids, being less harmful to the intestinal mucosa and less able to cross into the blood stream (Fu et al., 2015; Li et al., 2021). In fact, of these studies only Yang et al. (2013) and Mao et al. (2016) focused on biodistribution aspects and provided data of the presence of the GFNs in the major organs. The other reports are focused on toxicity (a review of oral toxicity studies is available in section 3.3), and from the results described, it can be concluded that GFNs can induce systemic effects and, therefore, they undergo absorption, distribution, and excretion processes.

Yang et al. (2013) reported the *in vivo* systematic biodistribution of GO and PEG functionalized GO derivatives at high doses (up to 100 mg/kg b.w.) using a mouse model and oral administration. Three PEGylated GO were chosen for the experiments: ^{125}I-nGO-PEG (25 nm in dimeter and 1.22 nm in thickness), ^{125}I-RGOPEG (50 nm in dimeter and 4.43 nm in thickness), and ^{125}I-nRGO-PEG (27 nm in dimeter and 5.66 nm in thickness). The PEGylated GO were then labelled by the iodine

isotope ^{125}I, while the pristine GO could not be radiolabelled as it precipitated in the buffer solution. In the first experiment, female balb/c mice were intragastrically injected with the 3 PEGylated GO at 100 mL of 20 mCi per mouse and a dose of 4 mg/kg, sacrificed at 4 h and 1 day post injection, and the major organs assessed for radioactivity. It was reported that the 3 PEGylated GO were not adsorbed in the GI as at 4 h post injection radioactivity was observed only in the stomach and intestine, while after 1 day it was at very low levels (2–3%) in all examined organs of the GI, becoming undetectable at 1 week after the treatment. This has shown the complete excretion of the nanoparticles, with no tissue uptake. In a second 30-day experiment in identical conditions using only nGO-PEG, no apparent abnormality was observed in the serum and blood biochemistry. Thus, the authors concluded that the PEGylated GO could not be absorbed by the body, being rapidly excreted. However, it must be stressed that these studies employ an external radioactive (^{125}I), a method considered less reliable than other analytical methods.

Similarly, Mao et al. (2016) reported that male ICR mice exposed to 10 μg/kg b.w. FLG by oral gavage for up to 3 days did not present detectable absorption in the GI tract. A volume of 100 μL of 0.1 mg/mL FLG suspension with particles in the range of 0.97–3.94 nm and 4 to 6 layers, labelled by ^{14}C, and re-dispersed by sonication was used in the experiment. The results showed that at 12 h post treatment, the FLG were present only in the GI tract: stomach (3%), small intestine (4%), and large intestine (6%), while 85% was located in the faeces. At 48 h, the majority of the FLG (>98%) were excreted in the faeces. These results showed that FLG was not absorbed in the blood circulation via the GI tract at detectable concentrations.

A newer study on GQDs showed that even when administered in high doses (150 mg/kg b.w., 500 μL) by oral gavage in ICR male mice, no effects were observable in the morphologies and histological structures of the major organs, nor in the serum biochemistry and haematology parameters when compared to control groups (Zhang et al., 2019). Additionally, the negatively charged GQDs were not able to penetrate the blood–brain and blood–testes barriers, being rapidly excreted in urine and/or faeces. In this experiment GQDs were labelled with Cu^{2+} ions.

However, Fu et al. (2015) studying female ICR mice fed GO (0.5 mg/mL and 0.05 mg/mL) (average lateral size 0.2 μm, thickness around 1.8 nm) in the drinking water and their litter, reported that GO did significantly affect the activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), blood biochemical indicators of liver damage, in maternal mice at low doses (0.05 mg/mL). Moreover, the histopathological results showed hepatotoxicity after a GO exposure of 21 days at 0.05 mg/mL. Interestingly, no modification in the histopathology was reported at the higher tested concentration of 0.5 mg/mL. This was attributed to the fact that at high doses, the gastric juices produced GO agglomerates with higher dimensions and fewer sharp edges which could not be so easily absorbed, but rather excreted as such. Thus, the smallest nanosheets, including the ones resulted during the interaction with the GI fluids, are distributed in the organism and the subsequent culprits of GO toxicity. Additionally, the new-born mice (up to the 11th day), indirectly exposed to GO through the maternal milk, exhibited severe atrophy in the major organs. This showed that GO was absorbed by the mother and excreted in the milk, negatively affecting the development of the pups.

Similarly, Li et al. (2021) studied, among others, the effect of 2 concentrations (0.025 μg/g diet, 0.25 μg/g diet) of graphene (lateral dimension of 0.5–5 μm, thickness of 0.8 nm) administered with food to C57-BL/6 mice for 6 weeks. No effect was observed in terms of daily food and water intake and body weight. However, it was reported that graphene can produce toxic effects in the intestine and the liver, especially at the lower tested concentration, consistent with Fu et al. (2015). This no-dose effect might be due to the formation of wrinkles, folds, and even aggregates in presence of the GI juices when testing high concentrations of graphene, which leads to an almost complete excretion with the faeces.

Mohamed et al. (2019) reported that GO (hydrodynamic size 1162 nm) administered orally at doses of 10, 20, and 40 mg/kg b.w. produced histological lesions in the liver and brain of Male Swiss Webster mice after an acute (1 day) and repeated-dose (5 days) treatment. This showed that the GO was able to enter the blood stream and was subsequently distributed to the liver and that it was able to cross the blood-brain barrier.

An additional study reported that orally administered GO (up to 40 mg/kg b.w. for 5 days) (40 nm diameter) was able to reach the kidneys of male Sprague-Dawley rats (Patlolla et al., 2016), so they could cross the intestinal barrier.

Liu et al. (2021) orally exposed pregnant ICR mice to 3 concentrations of GO based on the occupational exposure limit of carbon nanotubes: 2, 10, and 40 mg/kg b.w. The average hydrodynamic diameters of the used GO solutions were 236 ± 5 nm in DI water, and 766 ± 179 nm in simulated intestinal fluid, with thicknesses less than 2 nm. The results showed significant alterations in the colon morphology. Additionally, the gut permeability as measured by fluorescein isothiocyanate-dextran (FITC-Dextran) was significantly increased. The results also showed that an oral exposure to 40 mg/kg b.w. led to inflammation in the lungs. An additional negative effect was reported in the case of concentration above 10 mg/kg b.w. which damaged the placenta of the pregnant mice. These data show that the GO can enter the blood stream, reach the lungs, and even cross the placental barrier. In general, all these reports suggest that toxicokinetics of GFNs are highly dependent on their physicochemical properties (size, agglomeration state, etc.). Further research on this topic is required as no studies following agreed test guidelines, such as OECD TG 417 (OECD, 2010) have been performed.

3.3. *In vivo oral toxicity studies*

Oral toxicity studies of GFNs on laboratory animals are very scarce (Ema et al., 2017; Pelin et al., 2018). Only a few *in vivo* studies have been performed to determine the toxic effects of GFNs in rodents by oral route exposure (Table 2). As EFSA (2018, 2021) established, for an ingested nanomaterial, the minimum requirement for *in vivo* toxicity testing is the modified 90-day toxicity test OECD TG 408, 2018). But studies for 90 days or longer exposure periods are nonexistent in the scientific literature. The available studies are not sufficient for the evaluation of potential subchronic toxicity due to their short exposure time. However, these studies can provide hints about appropriate doses and target organs.

None of the studies included in Table 2 followed OECD guidelines. There is a single study that used rats as experimental model (Patlolla et al., 2016), as indicated by OECD TG 408 (2018), but it included only males, rather than both sexes. In all these studies, the exposure was by gavage with the exception of Fu et al. (2015) and Li et al. (2021) who exposed the animals through drinking water and diet, respectively. The studies were mainly focused on the GI system (Chen et al., 2018; Li et al., 2018; Yu et al., 2019; Gao et al., 2021; Li et al., 2021) with some reports studying also the liver (Li et al., 2018, 2021) and kidneys (Patlolla et al., 2016), reproduction and development (Fu et al., 2015; Zhang et al., 2019; Liu et al., 2021), or behaviour (Zhang et al., 2015). Additionally, some explored the alteration of gut microbiota (Mao et al., 2016; Chen et al., 2018; Li et al., 2018, 2021), an aspect that will be further considered in section 4. The results presented are inconsistent as they used different materials and doses. Thus, there are studies that suggested that GFNs induced toxicity in the GI system as evidenced by an increased gut permeability, loss of intestinal crypts, shortened villi, or histopathological lesions, among other effects. The identified toxic mechanisms were inflammation, oxidative damage or apoptosis (Chen et al., 2018; Yu et al., 2019; Li et al., 2021). There are also studies that reported no toxic effects for GFNs per se (Yang et al., 2013; Li et al., 2018; Gao et al., 2021). However, graphene exposure could exacerbate pre-existing colon inflammation (Gao et al., 2021). Moreover, Li et al. (2021) observed that

Table 2
In vivo oral studies with rodents exposed to GFNs.

Material	Physicochemical properties (size)	Experimental Model	Assays	Exposure condition	Tissue/Target	Relevant Results	Reference
nGO-PEG, RGO-PEG and nRGO-PEG	Average sheets diameter (nm), sheet thickness (nm): nGO-PEG (25; 1,22), RGO-PEG (50; 4.43) and nRGO-PEG (27; 5.66)	Female balb/ mice	Blood analysis: ALT, ALP, AST, A/G, BUN Histological evaluation	Intragastrical injection at 4 mg/kg for biodistribution analysis. Orally injected with 100 mg/kg of nGO-PEG for blood analysis. Sacrifice at 1, 7, 30 and 90 days post injection	Liver, spleen, kidney, heart, lung, stomach, intestine, skin, muscle, bone, brain and thyroid. Blood.	No gastrointestinal tract absorption or tissues uptake after oral exposure. Blood parameters were not altered in nGO-PEG exposed mice.	Yang et al. (2013)
GO	Average lateral size (μm); thickness (nm): 2; 1.8	Male and female offspring ICR mice	Morphological manifestations, Blood biochemical assay: ALT, BUN, CREA and AST levels. Histological analysis	Free drinking solution at 0.05 mg/mL and 0.5 mg/mL from PND to 21 days	Development. Blood, lungs, heart, kidney, spleen, and liver	The highest dose decreased body weight and caused alterations of the intestinal tract in male and female filial mice. Delayed development. Alterations were observed in hepatic indexes at low dose. Livers of maternal mice were affected after high dose administration.	Fu et al. (2015)
Small and large rGO	Average size (nm): small rGO (87.97 ± 30.83) and large rGO (472.08 ± 249.17)	C57black/6 mice	Behavior studies Kidney, liver functions and blood biochemical assays MDA, SOD and GSH-Px levels AChE and ChAT activities Liver and muscle glycogen Magnetic resonance images Histological assay	Gavage at 60 mg/kg every 24 h for 5 days	Behaviour. Heart, liver, spleen, lung, kidney, and brain	No consequences on body and organ weight, body temperature and instinctive behaviors. Nevertheless, rGO-treated mice exhibited short-term decreased neuromuscular coordination. No anxiety and exploratory conduct were altered. No significant difference in most of the parameters assayed were found.	Zhang et al. (2015)
FLG	Thickness (nm), layers, lateral size distribution (nm): 0.97–3.94; 4–6; 60-540	Male ICR mice	Biodistribution Intestinal flora analysis	Oral gavage at 5 μg for 3 days for biodistribution assay Oral gavage with 0.4 mL (2.5 μg/mL) for 28 days for intestinal flora analysis	Stomach, small intestine, large intestine, blood, heart, liver, spleen, kidney, brain, lung, urine, and faeces	FLG was not absorbed by organs, and it was excreted with the faeces after 48 h of exposure. Intestinal microbial community was altered. The body weight did not change in FLG exposed mice	Mao et al. (2016)
GO	Size (nm): 40	Male Sprague-Dawley rats	Oxidative stress: CAT, SOD and GPx Lipid hydro peroxides assay Serum creatinine and blood urea nitrogen assays Histopathological evaluation	Orally administered by feeding needles at 10, 20, 40 mg/kg every 24 h for 5 days	Kidney	Dose-dependent increase of CAT, SOD, GPx activities and LPO. Administration of GO elevated serum creatinine and blood urea nitrogen. The kidneys of GO exposed rats showed morphological alterations	Patlolla et al. (2016)
SWCNTsMWCNTsGO	Diameter (nm); hydrodynamic size (nm) SWCNTs (1.04–1.17; 114.6 ± 65.54), MWCNTs (6.75–10.88; 61.83 ± 33.38), GO (0.9–1.7; 351.0 ± 68.76)	Male CD-1 mice	Histological and blood biochemical assays Immune response (IL-6, IL-1β, TNF-α) Intestinal permeability assessment and gut bacteria	Acute oral administration (gavage) for 7 days at 0.05, 0.5, 2.5 mg/kg per day	Duodenum and colon	The highest dose caused inflammatory and immune responses, increased gut permeability and changed the structure of gut microbiota in the intestinal tract of mice. SWCNTS induced more	Chen et al. (2018)

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Table 2 (*continued*)

Material	Physicochemical properties (size)	Experimental Model	Assays	Exposure condition	Tissue/Target	Relevant Results	Reference
						severe effects than MWCNTs and GO	
GO	Size (nm); Hydrodynamic size (nm): 200–300; 431 ± 81	High-fat diet (HFD)-induced hyperlipidemic C57-BL/6 mice	Serum and liver levels of TG and TC. Histological analysis. Gut microbes	Gavage for 28 days at 200 mg/kg per day in distilled water	Blood, liver, and intestinal tissues	No consequences on the body weight and histomorphology. It significantly alleviated HFD-induced hyperlipidemia as manifested by the decrease in serum TC and TG. It did not remarkably decrease the total numbers of gut bacteria, but dynamically increased the relative abundance of main SCFA-producing genera	Li et al. (2018)
GQDs	Size: ≤10 nm	Male ICR mice and non-exposed females	Biodistribution. Reproductive capacity. Health of offspring. Blood analysis. Histological analysis of testes and epididymes. Testosterone level and sperm quality. Total protein and major enzymatic activity in testes and epididymes	Gavage every 24h for 10 days at 60, 100 and 300 mg/kg per mouse per day	Reproductive ability and production of litters	Normal sexual behaviours of male mice. GQDs not affected reproductive ability and GQDs-male mice were able to produce healthy litters. The testes and epididymes enzymes were not altered. No sperm quality and male sex hormones were affected. Rapid excretion, mainly via the urine and/or faeces; GQDs, even at high doses, were virtually undetectable in male mouse testis, epididymis, and brain	Zhang et al. (2019)
OH-GQDs	Average D (nm); layer number; hydrodynamic D (nm): ~10; 2–3; 4.9 ± 0.3 in DI water and 13.7 ± 1.5 in PBS	Male C57BL/6J mice	Histological assessment Oxidative stress: 8-OGdG Apoptosis: TUNEL assay	Oral gavage of 0.05, 0.5 and 5 mg/kg per day for 7 consecutive days	Small intestine	OH-GQDs did not alter the weight of the mice. Increased gut permeability, decreased length of the small intestine, crypts loss and shortened villi by the high doses. Oxidative stress in the intestinal crypt cells and apoptosis	Yu et al. (2019)
GO	Average hydrodynamic (nm), thickness (nm): 236 ± 5; 2 (DI water) and 766 ± 179; 5 (Intestinal fluid)	Pregnant ICR mice and faetuses	Histological and biochemical indexes (ALT, AST, ALP, BUN and CREA) Intestinal permeability RNA levels Faecal and microbiome analysis	Oral administration (gavage) of 2, 10 and 40 mg/kg from gestational day 7–16. Sacrifice on day 19.	Blood, reproduction, offspring, placenta, lung, and colon tissues	GO increased the number of abortions, embryo reabsorption, decreased weight of fetus, and it induced placenta barrier alterations. The composition of gut microbiota changed in GO-exposed mice with failed pregnancy.	Liu et al. (2021)
GO sheets	Thickness(nm); 1.5; hydrodynamic size (nm):251.13 in pure water and 201.23 in CCM	Female C57BL/6 mice with sulphate sodium-induced colitis	Histological assay Western blot of microtubule-associated protein 1 light chain 3B and p62	Oral gavage every two days from day 2–8, 60 mg/kg	Colon	GO alone did not induce shortening of the colon and did not increase the level of autophagy-related protein LC3BII. But DSS-GO group mice presented an even worse inflammatory condition in colon than mice receiving GO or DSS alone. These results suggested that GO might be	Gao et al. (2021)

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Table 2 (*continued*)

Material	Physicochemical properties (size)	Experimental Model	Assays	Exposure condition	Tissue/Target	Relevant Results	Reference
Graphene	Lateral dimension (μm); thickness (nm): 0-5 – 5; 0.8	Male C57-BL/6 mice	Histological assessment Oxidative stress: MDA and 8-OHdG. Gene expression by PCR. Gut microbiota	6 weeks by mixing with diet 0.025 μg or 0.25 μg graphene/g diet and with/without 1 mg/L As	Intestine and liver	deleterious only in pre-existing inflammation Graphene alone induced histopathological lesions, inflammation and oxidative damage in the intestine and liver. The low dose upregulated the expression of GPx in the intestine. It significantly reduced the toxicity of As and the high-dose exhibited greater toxicity reduction effects. It significantly decreased the bioavailability of As. It also reduced As toxicity on intestinal microorganisms.	Li et al. (2021)

AChE: acetylcholinesterase; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; CAT: catalase; ChAt: choline acetyl transferase; CCM: complete culture medium; CREA: creatinine; DSS: dextran sulphate sodium; FLG: few layer graphene GO: graphene oxide; GPx: glutathione peroxidase; GQD: graphene quantum dots; IL: interleukin; LPO: lipid hydro peroxides; MDA: malondialdehyde; MWCNTs: multiwalled carbon nanotubes; OH-GQDs: hydroxylated-graphene quantum dots; PEG: polyethylene-glycol PND:: postnatal day; SCFA: short-chain fatty acid; SOD: superoxide dismutase; SWCNTs: single-walled carbon nanotube; TC: total cholesterol; TG: total triglycerides; TNF: Tumour necrosis factor.

although graphene induced toxic effects in the intestine and liver, it could ameliorate arsenic mediated toxicity by decreasing its bioavailability. Additionally, Patlolla et al. (2016) reported the nephrotoxicity of GO. Zhang et al. (2019) did not observed reproductive toxicity in GQDs exposed mice. On the contrary, Fu et al. (2015) concluded that GO induced negative effects on the development of mice in the lactation period, and Liu et al. (2021) observed decreased weight of dam and live foetus, high rate of resorbed embryos, and dead foetus in mice exposed to GO during gestation. Finally, Zhang et al. (2015) reported that rGO nanosheets via oral administration caused a short-term decrease in locomotor activity and neuromuscular coordination, although it did not affect exploratory, anxiety-like, or spatial learning and memory behaviours.

In conclusion, according to the existing literature, toxic effects induced by GFNs through the oral route cannot be discarded.

4. Targeted in-depth studies

4.1. Effects on gut microbiome

The effect of GFNs on the gut microbiome was studied in concentrations up to 500 μg/mL *in vitro* (Chen et al., 2014; Nguyen et al., 2015; Lahiani et al., 2019) and *in vivo* (Xie et al., 2016; Mao et al., 2016; Li et al., 2018, 2021; Chen et al., 2018), but there is also a study assessing this effect *ex vivo*, on faecal rat slurry (Lahiani et al., 2019) (Table 3).

The *in vitro* studies usually assessed the effect of graphene materials on bacteria relevant for the gut, such as the Gram-positive *Lactobacillus acidophilus* (Chen et al., 2014; Nguyen et al., 2015; Lahiani et al., 2019), *Bifidobacterium longum* (Lahiani et al., 2019), *Bifidobacterium adolescentis* (Chen et al., 2014), *Enterococcus faecalis* (Chen et al., 2014), *Staphylococcus aureus* (Chen et al., 2014), *Bifidobacterium animalis* (Nguyen et al., 2015), and Gram-negative *Escherichia coli* (Chen et al., 2014; Nguyen et al., 2015; Lahiani et al., 2019). However, the information reported in these studies is rather limited, as only these 7 bacteria are presented, and the majority are standard ATCC cultures and not strains isolated from the gut. The conclusions on the studies are contradictory. On one hand, no effect on the bacterial cell membranes or morphology was reported for graphene and GO against *B. longum* and *E. coli* in a static culture exposed to 10 and 100 μg/mL graphene (Lahiani et al., 2019); or *L. acidophilus*, *E. faecalis*, and *S. aureus* exposed to up to 100 μg/mL GO (Chen et al., 2014); or *E. coli* and *B. animalis* exposed to up to 500 μg/mL GO (Nguyen et al., 2015). On the other hand, it was reported that GFNs enhanced the growth of bacteria, such as *L. acidophilus* in both static and dynamic culture conditions and *B. longum* at 4 h after a treatment with 1 and 10 μg/mL graphene (Lahiani et al., 2019). This might be because the dynamic cultures do not allow the graphene sediment, keeping the bacteria under constant contact with the nanomaterial.

Additionally, Chen et al. (2014) reported that GO sheets had the highest stimulating effect on the strict anaerobe *B. adolescentis*, compared with the aerobe or facultative aerobe tested bacteria (*L. acidophilus*, *E. coli*, *E. faecalis*, and *S. aureus*). A thin GO membrane was found to cover the surface of *B. adolescentis* as soon as 2 h after the exposure. This GO scaffold seemed be responsible for the enhanced adhesion and proliferation of anaerobe bacteria. However, other authors suggest that this observation is specific to certain anaerobes only (Lahiani et al., 2019). Apart from the enhanced growth, the *B. adolescentis* pre-cultivated with GO – thus having a GO scaffold – showed a significantly higher antagonism with the tested pathogens (*E. coli* and *S. aureus*).

An *ex vivo* experiment assessed the effect of graphene exposure (up to 100 μg/mL) on the natural microbiota of rats, using fresh faecal slurry (3%) extracted from the colon of healthy male Sprague−Dawley rats (Lahiani et al., 2019). The most common phyla in the gut microbiome were, as expected, *Firmicutes* and *Bacteroidetes*. Overall, the abundance of *Firmicutes* and *Bacteroidetes* was not significantly affected by any treatment. However, the ratio of *Firmicutes*:*Bacteroidetes* did change after an exposure to 100 μg/mL graphene in favour to *Firmicutes*. A significant increase in the abundance of *Enterobacteriaceae* (Gram-negatives including pathogens such as: *E. coli*, *Klebsiella*, *Enterobacter*, *Citrobacter*, *Salmonella*, *Shigella*, *Proteus*, *Serratia*) was also reported at this high concentration. A significant dose-effect relationship was observed at the genus level: a concentration of 1 μg/mL had minimal to no effect on the microbiota, especially for *Bifidobacterium*, *Lactobacillus*, and *Bacteroides*, while at 100 μg/mL *Bifidobacterium* were more abundant and *Lactobacillus* significantly less. Another important factor was the duration of the treatment. The aerobic and anaerobic bacterial counts significantly

Table 3
Effects of GFNs on gut microbiome.

Material	Physicochemical properties (size)	Experimental Microorganism/ Model	Assays	Exposure condition	Samples	Relevant Results	References
GO	Thickness (nm); lateral dimensions (μm^2) 0.9–1.7; 0.8–14:3 μm^2	Bacterial strains L. *acidophilus* (ATCC 4357), *B. adolescentis* (ATCC 15704), *E. faecalis* (ATCC, 19433), and *S. aureus* (ATCC 25923)	*In vitro* bacterial growth measurements; survival rates %; plate colony counting; 16S rRNA gene sequencing; morphological observation of bacteria; cell imaging; bacterial membrane potential assay	20, 50, and 100 μg/mL suspension solutions in culture solution or bacterial suspension	–	GO sheets formed effective, anaerobic, membrane scaffolds and enhanced the antagonistic activity of *B. adolescentis* against the *E. coli* and *S. aureus*. It promoted the proliferation of *B. adolescentis*. No effect was observed against cell membranes or morphology.	Chen et al. (2014)
GO	Average flake: 20 nm	Bacterial strains *E. coli* K-12, *L. acidophilus* ADH, and *B. animalis* Bif-6	*In vitro* effect on the growth curve of the tested bacteria (up to 24 h)	10–500 μg/mL in broth cultures	–	No toxicity was reported against the selected bacteria.	Nguyen et al. (2015)
Graphene	NA	4-week-old male ICR mice	High-throughput sequencing: 16S rRNA in DNA samples amplified by PCR before Illumina Miseq sequencing	Oral gavage of 1 μg/d, 10 μg/d, 100 μg/ d graphene for 4 weeks	Faeces	The exposure increased the gut microbial diversity. The highest effect was observed for the lowest concentration (1 μg/ d). *Prevotella*, *Anaeroplasma* and *Paraprevotell* were more abundant, while *Lactobacillus* and *Mycoplasma* decreased. Overall, Gram-negative bacteria were less affected than Gram-positive. It increased the types and abundances of genes related to membrane-damage and antibiotic resistance.	Xie et al. (2016)
FLG	Thickness (nm); interlayer distance (nm); layer: 0.97–3.94 nm, with 72% in range 1.2–2.1; 0.35 ± 0.01; 4-6	4-week-old male ICR mice	High-throughput sequencing using Illumina's Miseq platform	Oral gavage, 0.4 mL of 2.5 μg/mL FLG suspension in 0.1% Tween 80 saline, for up to 28 days	Germ free faeces of each set collected from the mice rectums	The quantity of *Proteobacteria* was similar with the control group in mice oral gavage. In mice treated for up to 3 days the relative abundance of *Firmicutes* community was lower by 10 ± 1.2%, while that of *Bacteroidetes* increased. The body weigh was not affected.	Mao et al. (2016)
GO	Size (nm); hydrodynamic size (nm) 200–300; 431 ± 81	8-week-old high-fat diet (HFD)-induced hyperlipidemic C57BL/6 male mice	Real-time PCR analysis	Oral gavage of 200 mg/kg b.w. per day, suspended in distilled water, for 28 days	Fresh faeces collected on 0, 3, 7, 14 and 28 day	The exposure did not decrease the total number of gut bacteria. It increased the relative abundance of SCFA-producing bacteria (i.e. *Clostridium clusters* IV, *Allobaculum* spp.) and enhanced the copying of bacterial butyryl coenzyme A transferase at 7 days after into the treatment.	Li et al. (2018)
GO SWCNTs; MWCNTs	Lateral dimensions (μm); thickness (nM) GO: (0.8–14.3; 0.9–1.7). Diameter (nm) length (μm): SWCNTs: (1.04–1.17; 1–5) MWCNTs: (8.4 ± 0.9; 0.5–2)	7-week old male CD-1 (ICR) mice	clinic-like colitis: body weight, stool consistency index, and fecal bleeding index RNA gene sequencing by PCR	Oral 0.05, 0.5, and 2.5 mg/kg b.w. per day for 7 days	Faeces, cecum, colon, and rectum	No obvious colitis-like symptoms were observed. The exposure affected the microbe by upregulating *Bacteroidetes* and downregulating *Firmicutes*. It also increased the abundance of proinflammatory bacteria such as *Alitipes* uncultured bacterium and *Lachnospiraceae* bacterium A4. The GO treatment decreased the most the gut bacterial diversity.	Chen et al. (2018)
Graphene	NA	*In vitro*: bacterial strains *Lactobacillus acidophilus* (ATCC 4356), *Bifidobacterium longum* (ATCC 35183), and *Escherichia coli* (ATCC 10798) *Ex-vivo*: fresh faecal slurry (3%) from the colon of 3 healthy	*In vitro* measurement of bacterial growth and survival *Ex vivo* count of viable aerobic and anaerobic bacteria; RT-PCR of bacterial groups; V3–V4 Based 16S Next-Generation Sequencing; quantification of SCFAs	1, 10, and 100 μg/mL for 22 h and for 2, 4, and 24 h 1, 10, and 100 μg/mL for 3, 6, and 24 h	– Faeces	The growth of *L. acidophilus* was promoted at 1 and 10 μg/ mL in a stationary culture, with no significant effect on the other 2 bacteria. An up to 5-fold increase was observed for *L. acidophilus* in dynamic culture, while the effect on *B. longum* was evident only at the 4 h interval, and no effect was observed for *E. coli*. The *in vitro* effect of graphene varies	Lahiani et al. (2019)

(*continued on next page*)

Table 3 (*continued*)

Material	Physicochemical properties (size)	Experimental Microorganism/ Model	Assays	Exposure condition	Samples	Relevant Results	References
		male Sprague–Dawley rats aged 4–6 months				with the type of culture (static or dynamic). Overall, the abundance of *Firmicutes* and *Bacteroidetes* was not significantly affected by any treatment. The time of exposure has a major effect on the microbiota. There was a significant increase in abundance during the first 6 h, followed a decrease to near control levels at 24 h in some bacteria (i.e. *C.hylemona*, *C. symbiosum*, and *C. fimetarium*), while for others (i.e. *S.wadsworthensis*, *S. stercoricanis*, *P. excrementihominis*, *R. champanellensis*, and *A. putredinis*) a significant decrease in abundance was observed at 6 h, followed by a recovery at 24 h. The exposure caused an increase in the abundance of butyrate-producing bacteria (*C. fimetarium*, *C.hylemona*, and *Swadsworthensis*).	
Graphene	Lateral dimension (μm); thickness (nm):0.5–5; 0.8	6-week-old male specific-pathogen-free C57-BL/6 mice	Quantitative real-time PCR	0.025 μg graphene/g diet and 0.25 μg graphene/g diet	urine and faeces collected from the metabolic cage housing the mice	Exposure to 0.25 μg graphene/g diet significantly decreased the abundance of *Bacteroidetes*. The lowest concentration upregulated the abundance of *Bacteroides* (from 55% to 75%) and downregulated *Firmicutes* (from 45% to 25%). It increased the ratio of Gram-negative bacteria.	Li et al. (2021)
GO	Hydrodynamic diameters (nm); thickness (nm): (236 ± 5 in DI water, and 766 ± 179 in simulated intestinal fluid; < 2	Pregnant ICR mice	16s RNA gene sequencing and microbiome analysis	40 mg/kg b.w. in drinking water	fresh faecal samples collected in the 19th day of gestation	The gut microbiome presented a significant decreased α- and β-diversity. The ratio of *Firmicutes/Bacteroidetes* was upregulated. *Euryarchaeota, Methanobrevibacter, Lactobacillus, Sporosarcina, Archaea,* and *Sellimonas* were significantly more abundant, while *Cyanobacteria, Chliroflexi, Fusobacterium, Phascolarctobacterium, Latescibacteria, Aneurinibacillus, Gammaproteobacteria, and Corynebacterium* significantly less.	Liu et al. (2021)

FLG: few layer graphene; G: graphene; GO: graphene oxide; GQD: graphene quantum dots; MWCNTs: multiwalled carbon nanotubes; NA: not available; OH-GQDs: hydroxylated-graphene quantum dots; PCR: polymerase chain reaction; SCFA: short-chain fatty acid; SWCNTs: single-walled carbon nanotubes.

increased (by 2-fold) in the first 3 h for the 10 and 100 μg/mL concentration. Additionally, after a 24-h treatment with 100 μg/mL, pristine graphene resulted in the 120% decrease of aerobic bacteria, with no effect on the anaerobics. No significant changes in the total count of *Bifidobacterium* and *Lactobacillus* were reported after 24 h. At the species level, there was a significant increase in abundance during the first 6 h, followed a decrease to near control levels at 24 h in some bacteria, while for others a significant decrease in abundance was observed at 6 h, followed by a recovery at 24 h. Additionally, the exposure caused an increase in the abundance of butyrate-producing bacteria. Lahiani et al. (2019) concluded that gut bacteria seemed to be resilient and able to overcome short-term exposure to graphene.

The conclusions observed *ex vivo* are not so evident *in vivo*. Six *in vivo* studies tackled the effect of oral exposure to graphene materials, all in mice of which 5 were in male mice (Xie et al., 2016; Mao et al., 2016; Li et al., 2018, 2021; Chen et al., 2018) and 1 in pregnant females (Liu et al., 2021). There are reports concluding that graphene exposure increased the gut microbial diversity (Xie et al., 2016); others showed a significant decreased in gut diversity caused by GFNs (Chen et al., 2018; Liu et al., 2021); while others stated that GO exposure did not decrease the total number of gut bacteria (J. Li et al., 2018). However, all the studies do present significant effects upon phyla, classes, or genera. In this sense Xie et al. (2016) reported that Gram-negative bacteria were less affected by graphene than Gram-positives when the mice were exposed to doses up to 100 μg/d for 4 weeks. They explained this finding as the outer membrane of Gram-negative bacteria could reduce the membrane damage induced by graphene and make them more tolerant to graphene. Similarly, H. Li et al. (2021) reported that the ratio of

Gram-negative bacteria increased in the case of a 0.025 μg graphene/g exposure in the diet. However, *Proteobacteria*, a phylum of Gram-negative bacteria, was reported to remain unchanged after an exposure to 2.5 μg/mL FLG (Mao et al., 2016).

As expected, the most abundant reported phyla were *Firmicutes* and *Bacteroidetes;*however, it is not clear how GFNs affect them. The majority of the studies concluded that the lowest tested concentrations of graphene (0.025 μg/g diet.), FLG (2.5 μg/mL), and GO (0.05 mg/kg b.w.) had the highest impact upon the two phyla by upregulating the abundance of *Bacteroides* and downregulated *Firmicutes* by up to 20% (Mao et al., 2016; Li et al., 2021; Chen et al., 2018). However, in the case of pregnant ICR mice the ratio of *Firmicutes/Bacteroidetes* was upregulated (Liu et al., 2021), and a concentration of 0.25 μg graphene/g significantly decreased abundance of *Bacteroidetes* (Li et al., 2021). *Cyanobacteria*, *Chliroflexi* and *Latescibacteria* phyla were also reported to decrease as a result of GO exposure, while *Euryarchaeota* increased (Liu et al., 2021).

In terms of specific genera, the lowest tested concentration of graphene (1 μg/d) had the highest effect on specific genera: *Prevotella*, *Anaeroplasma* and *Paraprevotell* were significantly more abundant, while *Lactobacillus* and *Mycoplasma* significantly decreased (Xie et al., 2016). Other reports concluded that 40 mg/kg b.w. GO in drinking water caused a significant increase of *Methanobrevibacter*, *Lactobacillus*, *Sporosarcina*, *Archaea*, and *Sellimonas*, and a significant decrease of *Fusobacterium*, *Phascolarctobacterium*, *Aneurinibacillus*, and *Corynebacterium* (Liu et al., 2021). The most relevant is the contradictory report of the effect on *Lactobacillus*.

Other relevant observations were that the lowest tested concentration of graphene (1 μg/d) increased the types and abundances of genes related to membrane-damage and antibiotic resistance (Xie et al., 2016). A concentration of 200 mg/kg b.w. per day GO caused an increase in the relative abundance of *Clostridium* clusters IV, *Allobaculum* spp. (short--chain fatty acid-producing bacteria) (Li et al., 2018). An exposure to GO produced an increase in the abundance of proinflammatory bacteria (i.e. *Alitipes* uncultured bacterium and *Lachnospiraceae* bacterium) (Chen et al., 2018). Additionally, Liu et al. (2021) reported a significant effect of GO on the increased abundance of *Euryarchaeota* especially in the case of failed pregnancies caused by GO exposure, thus proposing this community as a biomarker.

It seems that the higher used doses for exposure produce aggregations of graphene materials that are more easily eliminated with the faeces, and thus less toxic toward the bacterial cells (Xie et al., 2016), supporting the *ex situ* observations.

In conclusion, the effect of graphene materials on the gut microbiota is not yet clear. It is, however, obvious that they do impact the relative abundance of the most representative phyla *Firmicutes* and *Bacteroidetes*, and many important bacteria genera. Additionally, it seems that lower concentrations of thinner and sharper nanomaterials affect more severely the gut microbiota because they do not aggregate and can penetrate the bacterial cell and membrane by piercing. Thus, further *in vivo* studies are needed to fully understand the complexity of these effects.

4.2. Other

The analysis of the toxic effects induced by GFNs in oral *in vivo* experiments (Table 2, section 3.3) revealed that there are 3 studies (Fu et al., 2015; Zhang et al., 2019; Liu et al., 2021) dealing with reproductive and developmental toxicity. GQDs (5.25 ± 1.63 nm) did not adversely affect the reproductive activity of male mice exposed by gavage up to 300 mg/kg for 10 days (Zhang et al., 2019). Moreover, female mice housed with GQD-exposed males produced healthy litters. The authors explained these results due to the low toxicity of GQDs in germ cells and their rapid excretion after exposure in mice, with undetectable levels in testis, epididymis, and brain. However, results reported for GO were totally different. Thus, Fu et al. (2015) exposed maternal and filial mice to a drinking solution containing 0.5 and 0.05 mg/mL GO (average lateral size 0.2 μm, thickness 1.8 nm) in the suckling period from postnatal day 1–21, and they concluded that GO could induce many problems to the filial mice, such as evident dysfunctions of the intestinal tract, abnormal blood biochemical index, and reduction of body weight. Also, Liu et al. (2021), found that orally administrated GO (10, 20, 40 mg/kg) (average hydrodynamic diameter 236 ± 5 nm) daily during gestational day (GD) 7–16 caused dose-dependent pregnant complications of mice on the endpoint, including decreased weight of dam and live foetus, high rate of resorbed embryos and dead foetus, and skeletal development retardation, among other effects.

These studies, although limited in number, suggested that GFNs could be related to developmental toxicity, so this aspect should be carefully investigated.

5. Conclusions

This work reviews the main toxicological aspects recommended by EFSA in its guidance on risk assessment of nanomaterials to be applied in the food and feed chain, in relation to GFNs. The studies performed by far do not cover all EFSA's requirements and do not follow agreed test guidelines. From a general point of view, after oral exposure GFNs seem to be resistant to GI conditions with very scarce data regarding their lysosomal stability. Moreover, it has been evidenced that they can be absorbed, distributed along the organism, and be excreted, and that they can elicit not only local, but also systemic effects, including genotoxicity. Therefore, potential food and feed applications of GFNs that lead to a demonstrated human exposure will require a thorough toxicological evaluation in order to guarantee the consumer's safety.

CRediT authorship contribution statement

Óscar Cebadero-Domínguez: Investigation, Writing – original draft, Visualization. **Angeles Jos:** Conceptualization, Resources, Writing – original draft, Writing – review & editing, Project administration, Funding acquisition. **Ana M. Cameán:** Resources, Writing – review & editing, Supervision, Funding acquisition. **Giorgiana M. Cătunescu:** Investigation, Writing – original draft, Visualization, Writing – review & editing, Supervision.

Declaration of competing interest

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

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