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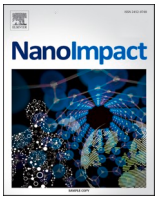

Applicability of OECD TG 201, 202, 203 for the aquatic toxicity testing and assessment of 2D Graphene material nanoforms to meet regulatory needs

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ABSTRACT

Tests using algae and/or cyanobacteria, invertebrates (crustaceans) and fish form the basic elements of an ecotoxicological assessment in a number of regulations, in particular for classification of a substance as hazardous or not to the aquatic environment according to the Globally Harmonised System of Classification and Labelling of Chemicals (GHS-CLP) (GHS, 2022) and the REACH regulation (Registration, Evaluation, Authorisation and Restriction of Chemicals, EC, 2006). Standardised test guidelines (TGs) of the Organisation for Economic Co-operation and Development (OECD) are available to address the regulatory relevant endpoints of growth inhibition in algae and cyanobacteria (TG 201), acute toxicity to invertebrates (TG 202), and acute toxicity in fish (TG 203). Applying these existing OECD TGs for testing two dimensional (2D) graphene nanoforms may require more attention, additional considerations and/or adaptations of the protocols, because graphene materials are often problematic to test due to their unique attributes. In this review a critical analysis of all existing studies and approaches to testing used has been performed in order to comment on the current state of the science on testing and the overall ecotoxicity of 2D graphene materials. Focusing on the specific tests and available guidance's, a complete evaluation of aquatic toxicity testing for hazard classification of 2D graphene materials, as well as the use of alternative tests in an integrated approach to testing and assessment, has been made. This information is essential to ensure future assessments generate meaningful data that will fulfil regulatory requirements for the safe use of this "wonder" material.

Abbreviations: NM, Nanomaterials; OECD, The Organisation for Economic Co-operation and Development; TGs, Test guidelines; GHS, Globally Harmonised System; CLP, Classification, Labelling and Packaging of substances and mixtures; CAS, Chemical Abstracts Service; EC, European Commission; GD, Guidance document; IATA, Integrated Approaches to Testing and Assessment; WoE, Weight of evidence; CAGR, Compound Annual Growth Rate; C/O ratio, Carbon to Oxygen ratio; GO, Graphene oxide; FLG, Few layer graphene; rGO, Reduced graphene oxide; FET, Fish embryo acute toxicity; HO, Hoffman; UV–Vis, Ultraviolet-visible (UV–Vis) spectrophotometry; Chl, Chlorophyll; Chl-a, Chlorophyll a; ISO, International Organization for Standardization; ISO/TS, ISO Technical Specification; ISO/TR, ISO Technical Reports; IEC, International Electrotechnical Commission; NAM, New approach methodology; PPAR-α, Peroxisome proliferator-activated receptor alpha; AhR, Aryl hydrocarbon receptor; HO-1, Heme oxygenase-1; iNOS, Inducible nitric oxide synthase; mRNA, Messenger RNA; ID/IG, Ratio of the intensity of D-Raman peak and G- Raman peak; EtOH, Ethanol; ASTM, American Society for Testing and Materials; HA, Humic acid; EPA, Environmental Protection Agency; NOM, Natural organic matter; DOM, Dissolved organic matter; WNT, Working Group of National Co-ordinators of the TGs programme.

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1. Introduction

1.1. Regulation and environmental hazard classification of graphene

According to the European Commission Regulation (EU) No 2020/878, chemical manufacturers or importers are obliged under REACH to provide specific information requirements and chemical safety assessments for nanoforms of chemical substances (EC, 2020). This information is also used as criterion for the classification, labelling and packaging of substances as hazardous or not according to Regulation (EC) No 1272/2008 on the classification, labelling and packaging of substances and mixtures (CLP), which aligns with the United Nations globally Harmonised System of Classification and Labelling of Chemicals (GHS) and facilitates the entry and safe use of chemicals in the market place (EC, 2009). As well as information on physical and health hazards, environmental hazards must be reported and include hazard classification to the aquatic environment and the atmosphere.

Graphene is a nanomaterial according to the recently updated EC definition, and by its implementation into REACH graphene and its specific nanoforms are subject to regulation within the European Union (EC, 2022). Graphene nanoforms can exist with assembly structures of single, bi-, or few layers (3–10), with external dimensions (thicknesses) of <1 nm, lateral sizes ranging from a few nanometres to a few microns and in the form of nanoflakes/nanosheets/nanoplatelets, ribbons, fibres or quantum dots. They are all characterised by the presence of sp^2 hybridized carbon atoms arranged in a honeycomb lattice, but they can have different assembly structures, distinct edge types/defects, surface states (e.g. oxidations/reductions) and functionalisations according to the vast number of production methods and post processings. Graphene materials have received the CAS number 1034343-98-0, EC number 801-282-5. They have been registered under REACH and classified under the aquatic environment hazard class, as harmful to aquatic life with long lasting effects following chronic exposure (CLP-GHS H412) (ECHA, 2022a, 2022b). However, there is insufficient data to enable aquatic environment hazard classification following acute exposure. There are also distinct EC numbers for different surface treated graphene materials (e.g. graphene oxide: EC 947–768-1 and reduced graphene oxide: EC 922-453-1). Similarly, while the materials are registered under REACH, the data is not sufficient for aquatic environment hazard classification. Remarks have been made that hazard assessment on a case-by-case basis approach is most appropriate, as any available data on the specific tested graphene materials may not necessarily be relevant to all graphene/graphene oxide/reduced nanoforms (ECHA, 2022c). For example, the specific production methods used, post-modifications applied, and post-processing steps introduced to form the final material may contribute significantly to material properties. Furthermore, a single substance may have one or more different nanoforms, based on differences in size distribution, shape, and other morphological characteristics, presence of a surface treatment or functionalisation, and the specific surface area (SSA) of the particles (ECHA, 2022d). Therefore, due to the unique structures and features that nanoforms of graphene materials can have, the extent to which they will fall into a single substance category, and to which grouping or control banding for risk management can be applied, may not be justified (as recently argued in the case of carbon nanotubes (Fadeel and Kostarelos, 2020)).

Current hazard assessments for aquatic toxicity testing include growth inhibition studies with algae and cyanobacteria, short-term toxicity testing on invertebrates and fish lethality tests of short- and long-term. The standardised tests used and heavily relied upon include the OECD TG 201 Algae and Cyanobacteria Growth Inhibition Test (OECD, 2011), the OECD TG 202 Daphnia Acute Immobilization Test (OECD, 2004a), and the OECD TG 203 Fish Acute Toxicity Test (OECD, 2019c), respectively. Such a test battery can be seen as comprehensive and provides the apical endpoints for hazard classification upon which regulation relies (e.g. for CLP hazard classification). While these tests are considered generally applicable for the testing of nanomaterials (NMs) (OECD, 2013a; Rasmussen et al., 2016), they have not been assessed for their applicability when testing specifically 2D graphene materials. Adaptations/procedural modifications to these standardised tests may be needed according to the unique characteristics of the material/nanoform being tested. This has been recognised in the NM community with the establishment of the Malta Initiative to advance the development and amendment of the OECD TGs and guidance documents (GDs) to the unique characteristics of NMs. Also, the aim of the recently published GD for aquatic testing of NMs (GD 317) is to identify how existing OECD TGs can be applied to NMs (OECD, 2022a; Petersen et al., 2021). However, this guidance is not prescriptive for testing of a particular NM type and thus other specific guidance may need to be consulted for testing of certain "difficult to test" materials, for which graphene materials could be considered and for which the OECD GD 23 on Aqueous-Phase Aquatic Toxicity Testing of Difficult Test Chemicals could provide further reference (OECD, 2019a).

Therefore, within this article the applicability of the basic battery of tests for aquatic toxicity assessment (i.e. OECD TGs 201, 202, 203) for testing graphene materials was reviewed. All available information on the testing of 2D graphene materials using the test organisms, and specific taxa specified in the single tests, was collected. The following aspects were taken into account (*i*) if standardised test guidelines were used or followed, (*ii*) if relevant endpoints were reported, and (*iii*) the specific experimental setups used.

Specific considerations unique to this class of NMs were identified and overall comments on the adequateness of data generated and the extent to which traditional protocols were used have been made. In instances where there are information gaps or inconsistencies in data sets weight of evidence (WoE) approaches to testing and assessment have also been proposed ((Annex XI, Section 1.2 to the REACH Regulation) (EC, 2006) (OECD, 2019b)). Specifically, a WoE refers to "a positive expert opinion that considers available evidence from different independent sources and scientific viewpoints on a particular issue, coming to a considered view of the available, oftentimes conflicting data. It is preferred when every source does not provide sufficient information individually" (OECD, 2019b). An example of how such approaches can facilitate classification of NMs, according to CLP criteria for acute aquatic toxicity, into specific hazard classes has been recently presented by Basei and colleagues (Basei et al., 2021). Tests that can be considered in WoE approaches for acute toxicity in fish include fish embryo acute toxicity tests (TG 236, OECD, 2013b) and the recently published *in vitro* fish cell line acute toxicity test (TG 249, OECD, 2021a). Taking into consideration such approaches and the potential value of such data, any information that was available from tests performed using embryos and fish cells to assess hazard of graphene materials were also collected and evaluated for their use in a WoE and integrated approach for testing and assessment (IATA).

1.2. Aquatic toxicity of 2D graphene materials

Aquatic environmental exposure to graphene is particularly relevant according to the potential direct uses of graphene materials in environmental settings (e.g. in environmental remediation (Karthik et al., 2021), water filtration (Buelke et al., 2018), and as an effective antifreeze (Zhang et al., 2021)), and the potential release at the end of life of graphene enabled consumer products such as electronics. This, together with the demonstrated potential for environmental persistence (Candotto Carniel et al., 2021) and mobility in water bodies of aquatic ecosystems for certain types of graphene materials (e.g. GO (Avant et al., 2019; Lanphere et al., 2014)) and FLG (Su et al., 2017)), warrants concern for risks to the environment and emphasises the need for regulation to protect such environmental compartments. There is also a high predicted market growth for graphene materials according to the calculated compound annual growth rate (CAGR) of 39% for the years between 2020 and 2027 (https://www.fortunebusinessinsights.com/graphene-market-102930)).

There are a number of general reviews detailing studies that have assessed ecotoxicological effects and potential environmental risks of graphene based NMs (Guo and Mei, 2014; Arvidsson et al., 2013; Jastrzębska and Olszyna, 2015; Montagner et al., 2017; Fadeel et al., 2018; Evariste et al., 2020). These studies have concluded that there are deficiencies/inconsistencies in the reported toxicity data related to the lack of use of standardized approaches (e.g. TGs) when testing and that additional investigations are needed using standardised approaches. Furthermore, specific reviews on the toxicity of graphene material to aquatic organisms highlight knowledge gaps and the need for more studies to make definite conclusions on the toxic potential of graphene materials to aquatic organisms (Malhotra et al., 2020). Reaching definite conclusions is also hindered by the sheer diversity of graphene materials with distinct sizes, features, and forms. Studies have identified concentration dependent adverse effects for particular GO nanoforms to aquatic organisms (Evariste et al., 2020). However, these need further investigation in order to demonstrate a comprehensive understanding of the possible mechanisms dictating the adverse effects observed.

Studies performed using algae have reported half maximal effective concentration (EC_{50}) values and obvious effects of tested graphene materials related to the reduction of light availability for photosynthesis (shading effect), nutrient depletion effects (Zhao et al., 2017) and membrane damaging effects (Malina et al., 2019). Furthermore, distinct algal interactions for different nanoforms (e.g. GO and GO quantum dots (GOQDs)) have been detailed (Yan et al., 2022). In addition, tests performed using the crustacean *Daphnia magna* with GO (single layer, 200–300 nm) report modest acute toxicity with $LC_{50\ 72h}$ of 45.4 mg/L (Lv et al., 2018) and also distinct effects have been reported for different functionalised GO nanoforms in this test species (Yao et al., 2018).

A specific review on the toxicity of graphene materials in fish has identified that graphene materials have the potential to induce developmental, respiratory, neurobehavioral, inflammatory, and metabolic disorders in fish (Dasmahapatra et al., 2019). A reduction in hatching efficiencies of embryos and Parkinson's disease-like effects have also been evidenced following GO exposure to fish embryos (Ren et al., 2016). Also studies performed hint at possible immunomodulatory effects through perturbations of pathways and signalling, specifically the perturbation of the aryl hydrocarbon receptor (AhR) has been demonstrated for graphene quantum dots in zebrafish (Zhang et al., 2017b). While further studies are needed, this may be caused by a direct binding mechanism, as predicted by recent modelling of binding scores for small graphene fragments to rainbow trout receptors (Connolly et al., 2021). Graphene materials cytotoxicity towards fish cell lines has also been evidenced (Kalman et al., 2019, 2022; Lammel and Navas, 2014; Srikanth et al., 2018; Lammel et al., 2013) associated with oxidative stress responses. A recent review on the *in vitro* toxicity of graphene materials (Achawi et al., 2021), found over 80 publications reporting on the cytotoxicity of graphene materials with no specific trend or agreement for cytotoxicity for the GOs and FLGs tested. However graphene quantum dots (GQD) could be classified as highly ($EC_{50} \leq 30$ mg/L) or moderately cytotoxic ($EC_{50} \leq 100$ mg/L), and rGO ranged from weakly ($EC_{50} \geq 110$ mg/L) to highly cytotoxic ($EC_{50} \leq 30$ mg/L). Again, the overall conclusion was that a lack of standardized assessments makes comparisons of studies and results difficult.

Therefore, to this end and in an attempt to evaluate the applicability of the standardised test battery (i.e. TG 201, 202 and 203), all existing data was collected relating to 2D graphene material testing and the specific endpoints of growth inhibition in algae/cyanobacteria, immobilisation in *Daphnia spp*., and mortality in fish. All the studies and information collected are presented in Tables 1, 2 and 3 respectively. For each test/endpoint, together with available EC_{50} or LC_{50} values, details of the test material specific inherent physico-chemical properties, any processing steps for test dispersion preparation, test protocol/conditions, and any observations regarding potential adverse effects besides the specific endpoint under investigation were recorded. A summary of the findings from specific tests is provided under specific subheadings, and comments on any peculiarities witnessed and any aspects of standard test methodologies that have been modified, either leading to improvement in assay performance or directly affecting results, have been made. Due to the scarce data on fish testing performed using TG 203, studies using fish embryos and fish cell lines were also collected as they can be seen as alternative approaches to the use of juvenile fish and are presented in Tables 4 and 5, respectively. Standardised tests such as the OECD TG 236 fish embryo acute toxicity (FET) test and the OECD TG 249 fish cell line test can contribute to a hazard evaluation in a WoE approach for fish acute toxicity (Belanger et al., 2022; OECD, 2019d).

All of this information provides guidance for the application of standardised tests for aquatic toxicity testing of graphene materials in the future and thus, will improve the quality/reliability of future studies.

2. Results

2.1. Aquatic toxicity test endpoints used in test battery for CLP classification

2.1.1. Freshwater Alga and Cyanobacteria, Growth Inhibition

Dose-dependent inhibitory effects of a test substance on algal/cyanobacterial growth can be monitored using the OECD TG 201 (OECD, 2011). According to this test freshwater algae or cyanobacteria in the exponential growth phase are exposed to the test substance for 72 h and the growth rate is quantified using either cell counts, measurement of cell volume, fluorescence or optical density readings. The concentration causing a 50% growth inhibition is calculated from specific growth rates and reported as an EC_{50} value (usually at the end of the exposure phase: $EC_{50\ 72h}$).

Only thirteen studies were collected assessing the inhibitory effects of graphene materials on algal growth (Table 1). 5 of them explicitly followed TG 201, while the others used similar protocols (e.g. ISO 8692:2004) or modified OECD TG 201 protocols. The species used were *Raphidocelis subcapitata* (6 studies) (one of the recommended species of OECD TG 201), *Scenedesmus obliquus* (5 studies), *Chlorella pyrenoidosa* (1 study), and *Trebouxia gelatinosa* (1 study), all being freshwater green algae, with the exception of *T. gelatinosa* (aero-terrestrial). The cyanobacterial species, *Microcystis aeruginosa*, was also among the organisms used for testing in one of the studies. Test vessels used included conical flasks, micro-centrifuge tubes, 24 and 96-well plates, microbox micropropagation containers, while agitation was introduced in most cases using either magnetic stirring, orbital mixers or simple hand shaking at specific intervals over the test duration.

The graphene material most frequently tested was GO (7/13 studies). Pristine graphene (G), FLG, graphene nanofibers, rGO alone and composites with specific metal ions, and nanoforms with various functionalisations (e.g. -COOH, $-NH_2$) were also tested. Most studies reported dose-dependent inhibitory effects of graphene materials on algal growth (12/13 studies). $EC_{50\ 72\ h}$ values for GO materials ranged from 0.5 to 66.6 mg/L according to the nanoform tested and species used. Differences in the physiology, cell wall composition and structure (e.g. thickness) among the unicellular algae/cyanobacteria affected the way in which the graphene materials tested interacted with the organisms (Yin et al., 2020, Banchi et al., 2019). The reported $EC_{50\ 72h}$ for rGO was 148 mg/L, according to a single study performed (Du et al., 2016). When rGO materials functionalised with Ag, Co_3O_4 or Pd metal NMs were tested, $EC_{50\ 96h}$ values of between 0.2 and 1 were reported (Yin et al., 2020), however, in these composite materials, toxicity is influenced by the metal NMs loadings and also the release of metal ions. Pristine graphene (G) nanosheets tested had a reported $EC_{50\ 72h}$ of 8 mg/L (Zhang et al., 2018), with an increase in EC_{50} values measured for different functionalised graphene nanosheet nanoforms (e.g. 32 and 84 mg/L, for -COOH and $-NH_2$ functionalised G, respectively) (Zhang et al., 2018). The graphene nanofiber tested (GANF®) and nanofibers produced through superheating (GANFg) and a scaled up production process of GANF® (GATam) had $EC_{50\ 72h}$ values of 3.09, 8.48, and 2.12 mg/

Table 1
Freshwater Alga and Cyanobacteria growth inhibition studies.

	Test Material Raw material (supplier) Production method	No. layers Thickness Later size Oxidation level Defect level	Processing step	Test Species	Protocol Medium used Readout	EC_{50} 72 h (mg/L)	Observations	Reference
1.	**FLG** Graphite (Bay Carbon, USA) Ball-milling treatment **GO** GANF® helical-ribbon carbon nanofibres (Grupo Antolin Ingeniería (Burgos, Spain)) Oxidation	200–400 nm 5.2% O I_D/I_G = 0.49 100 nm 48.8% O	Shaking (30 min) Vacuum-filtration of aqueous suspensions on PTFE membranes Growth in Microbox micropropagation containers	*Trebouxia gelatinosa*	OECD TG 201 (modified to facilitate growth on solid *Trebouxia* Medium) Cell counts, Chl-a content, Chl-a Fluorescence, Membrane damage	FLG: >50 GO: >50	No effects on growth dynamics at concentrations tested (0.01, 1 and 50 mg/L) FLG: down-regulation of HSP70–1 gene expression	Banchi et al., 2019
2.	**GANF®** (Grupo Antolin Ingeniería (Burgos, Spain)) Catalytic vapor deposition **GANFg** (Grupo Antolin Ingeniería (Burgos, Spain)) Superheating of GANF® (1500 °C) **GATam** (Grupo Antolin Ingeniería (Burgos, Spain)) Scaled-up production of GANF®	Fibres 20–80 nm (diam.)	Nanogenotox protocol: EtOH prewetting, Sonication (probe) (16 min) ± BSA 0.05% Continuous stirring	*Raphidocelis subcapitata*	OECD TG 201 OECD 201 Test media Chl-a Fluorescence Magnetic stirring	GANF®: 3.09 GANFg: 8.48 GATam: 2.12	Stability measurement: UV–Vis spectroscopy After 72 h 0–6% nominal conc. Interferences at conc. >12.5 mg/L BSA improved stability but interfered with algal growth Interference with fluorescence readout	Barrick et al., 2019
3.	**GO** (Sigma Aldrich, USA)	Single layer 2 nm 225.1 ± 105.4 nm 32.2% O I_D/I_G = 1.04	Sonication (bath) (30 + 5 min) ± HA (20 mg/L)(Sigma Aldrich)	*Raphidocelis subcapitata*	96-well plates Absorbance	GO: 66.60 GO+HA: 242.78	Stability measured using UV–Vis spectrophotometry (OECD, 2017)After 24 h GO: 60% nominal conc. and GO+HA: >80% nominal conc.	Castro et al., 2018
4.	**rGO** (Hengqiu Graphene Technology Co, (Suzhou, China))	Single layer 0.5–0.6 nm 0.2–5 μm	Ultrasonication (150 W) (30 min)	*Scenedesmus obliquus*	OECD TG 201 (modified) HB-4 medium Cell counts, Absorbance	148	Shaking 1 h intervals Chl-a and Chl- b contents also measured	Du et al., 2016
5	**GO** (Abalonyx AS (Oslo, Norway))		Sonication (probe) (13 min and 45 s)	*Raphidocelis subcapitata*	TG 201 media		Autofluorescence from GO caused an overestimation of Chl-a concentration	Farkas and Booth, 2017
6	**GO** Graphite powder (Sigma Aldrich, USA) Hoffman method (Hofmann and König, 1937): HO-GO Hummers method (Hummers and Offeman, 1958): HU-GO Tour method (	C/O = 2.60, 27.8% O I_D/I_G = 0.75 C/O = 1.95, 33.9% O I_D/I_G = 0.98 C/O =	Sonication (bath) (60 min) Sonication (probe) (pulsed 3×, each 5 s)	*Raphidocelis Subcapitata** *Scenedesmus Elongatus^*	ISO 8692:2012 (modified) 24-well cell culture plate ZBB medium Cell counts, Chl-a Fluorescence	HO-GO: [0.5*, 9^] HU-GO: [14*, 27^] TO-GO: [10.1*, 10^]	No shaking/mixing Interference with fluorescence readout Nutrient absorption and shading effects	Malina et al., 2019

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Table 1 (*continued*)

	Test Material Raw material (supplier) Production method	No. layers Thickness Later size Oxidation level Defect level	Processing step	Test Species	Protocol Medium used Readout	EC_{50} 72 h (mg/L)	Observations	Reference
	Marcano et al., 2010): TO-GO	1.72, 36.8% O I_D/I_G = 1.16						
7.	**GO** Graphite powder (Sigma Aldrich, USA) Improved Hummer's method (Tour method; Marcano et al., 2010)		Sonication (probe) (pulsed: 5 s on, 5 off) (4 h) Centrifugation (4602 *g* for 6 min) Filtration (0.22 μm pore-size) 3- day pre-incubation step in media and continuous orbital shaking until use	*Raphidocelis subcapitata*	MA-MS media OECD TG 201 media Orbital mixing (80 rpm) and shaking by hand 3 times every 24 h Cell counts, Chl-a Fluorescence	9.71 (MA-MS media) 5.82 (OECD media)	UV–vis spectrophotometry for concentration measurement only possible at high conc. (at 16 and 32 mg/L > 90% nominal conc.) Shaking, orbital mixer and by hand shaking Drift in the pH value in MA-MS media Interference with fluorescence readout Cell count decrease due to algae attaching to GO	Marković et al., 2020
8.	**GO** Graphite powder (Nacional de Grafite, Brazil) Modified hummers	Single layer 3.5 nm 120–200 nm	Sonication (probe) (pulsed) (4 h) Centrifugation andd rying step	*Raphidocelis. subcapitata*	Oligo medium Cell counts	20 (96 h)	UV–Vis spectrophotometry for stability Increase in size of GO flakes from 145 nm to 429 nm	Nogueira et al., 2015
9.	**GNPs** (PlasmaChem GmbH, Germany)	Single layer 1–4 nm 2 μm <7% O	Stirring 24 h Sonication (30 s) ± LOA (GA or BA) (1–40 mg/L)	*Scenedesmus obliquus*	OECD TG 201 OECD 201 media	Nd (LOEC 0.5)	Shaking by hand 3 times a day Sedimentation experiments Increased stability with LOAs present but this also lead to increased toxicity	Wang et al., 2016
10.	**GNPs** (PlasmaChem GmbH, Germany) **rGO** (XFNANO Materials Tech Co. (China, Nanjing))	Single layer 1–4 nm 2 μm <7% O 8–1.2 nm 0.5–5 μm	Sonication (bath)(30 min)	*Chlorella pyrenoidosa*	OECD TG 201 (modified) OECD 201 Test media	Nd GNPs: NOEC 0.1 rGO: NOEC 1	Shaking by hand 3 times a day	Wang et al., 2021
11.	**GO** Graphite (supplier not specified) Modified Hummers	1.34 ± 0.071 nm 3–8 μm 32.9% O	Sonication (bath) (30 min)	*Chlorella vulgaris* *Scenedesmus obliquus* *Microcystis aeruginosa* *Chlamydomonas reinhardtii* *Cyclotella sp.*	OECD TG 201 (modified) G-11 media SE media CSI media Cell counts, Cell area, Chl-a content	10 (*Scenedesmus obliquus*)	Shaking by hand 3 times a day Stability measurement: UV–Vis spectrophotometry Sensitivity ranking: *S. obliquus* > *C. vulgaris* > *M. aeruginosa* > *Cyclotella sp.* > *C. reinhardtii*	Yin et al., 2020
12.	**rGO** **rGO-Au** **rGO-Ag** **rGO-Pd** **rGO-Fe_3O_4,** **rGO-Co_3O_4** **rGO-SnO_2** (XFNANO Materials Tech Co., Ltd. (China, Nanjing))	Single layer 0.5–5 mm 5–8 layers 2–4 layers 4–8 layers 3–6 layers 2–6 layers 2–4 layers	Sonication (bath) (1 h)	*Chlamydomonas reinhardtii** *Scenedesmus obliquus^*	OECD TG 201 (modified) Simplified SE medium Cell counts, Chl-a content	rGO-Ag: [<0.2*, <0.2] rGO-Co_3O_4: [1*, Nd^] rGO-Pd:1, Nd^] All other materials: [Nd (>1)*, Nd (>1)^]	Shaking every 8 h by hand Stability measurement: UV–Vis spectrophotometer 30%–55% of nominal conc. (96 h) Increase in Chl-a content evidenced	Yin et al., 2020
13.								

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Table 1 (*continued*)

Test Material Raw material (supplier) Production method	No. layers Thickness Later size Oxidation level Defect level	Processing step	Test Species	Protocol Medium used Readout	EC_{50} 72 h (mg/L)	Observations	Reference
G	Single layer 0.8–1.2 nm 0.5–2.0 μm	Sonication (150 W) (2 h) ±HA 10 mg/L	*Scenedesmus obliquus*	OECD TG 201 BG-11 medium Absorbance, Chl-a content	G: 8 GO: 21 G-COOH: 32 G-NH_2: 84	Shaking 3 times daily by hand HA significantly mitigated the inhibition of cell growth and Chl-a synthesis Shading effect on algal growth and Chl-a synthesis	Zhang et al., 2018
GO **G-COOH** **G-NH_2** (XFNANO Materials Tech Co., Ltd. (China, Nanjing))	Single layer 0.8–1.2 nm 0.5–5.0 μm 0.8–1.2 nm 0.5–2.0 μm 0.8–1.2 nm 0.5–2.0 μm						

GANF®; graphitized carbon nanofibers, GNPs; HSP70–1, heat shock protein 70.1, diam,; diameter, PTFE; polytetrafluoroethylene polymer, EtOH; Ethanol, BSA; bovine serum albumin, HA; humic acids, LOA; low-molecular-weight organic acids, GA; gallic acid, BA; benzoic acid, GNPs; graphene nanoplatelets, G; graphene, rGO; reduced graphene oxide, I_D/I_G; the intensity ratio of defect according to D (ID) and G bands (IG), Chl-a, b; chlorophyll a, b, W; Watt, NOEC; No observed effect concentration, LOEC; lowest observed effect concentration, Nd; not determined, conc..; concentration, G-COOH; carboxyl-modified graphene, G-NH_2; amine-modified graphene.

L, respectively (Barrick et al., 2019). This influence of production process on effects was also evidenced in the case of different production methods used for GO with EC_{50} values of 0.5, 14 and 10 mg/L according to the distinct production methods used (Hoffman, Hummers, and Tour, respectively) (Malina et al., 2019). Thus, most of the graphene materials tested would be classified as slightly toxic (US.EPA, Toxic Substances Control Act of 1976) or in Category: Acute 3 (U.N, GHS, 2022) (EC_{50} values >10–100 mg/L), while the graphene materials tested with EC_{50} > 1 and < 10 mg/L would fall into Category: Acute 2 in the GHS system (GHS, 2022). Only HO-GO tested in *R. subcapitata* with an EC_{50} of 0.5 mg/L would be classified in Category: Acute 1 ($EC_{50} \leq 1$ mg/L) according to CLP classification categories (EC, 2009). This highlights the distinct effects nanoforms have according to different sizes, oxygen contents, functionalisations, shapes and even the different production processes used. In fact, the latter can leave by-products or impurities in the final material that in some cases, especially if they are in significant amounts, can have adverse effects on the target organisms. If not taken into account, their presence can lead to misinterpretation of the results (Petersen et al., 2014). Unfortunately, the possible role of impurities in the GO and rGO materials tested was not considered in the majority of studies but independent studies show that a myriad of metallic elements can be introduced following different synthesis processes of graphene (An Wong et al., 2014). In some, a complete elemental analysis of samples was carried out (Banchi et al., 2019; Malina et al., 2020) and distinct levels of particular elements, e.g. manganese, were identified according to the different production processes (Malina et al., 2020). Filtrate controls should be used as approaches to determine the possible contribution of byproducts to the toxicity and the purification of the materials using distilled H_2O, both to enhance performance and limit any impurities (Barbolina et al., 2016).

Other critical issues that were identified by some of the authors are: *(i)* graphene material hetero-aggregation with algae and precipitation out of the water column (Marković et al., 2020); *(ii)* shading effects of the test dispersions on growth and/or nutrient depletion due to adsorption to graphene materials (Malina et al., 2019); and *(iii)* interferences with fluorescent readouts at high test material concentrations (Barrick et al., 2019; Malina et al., 2019; Marković et al., 2020). These three points are discussed herein.

According to the revised studies the graphene materials tend to agglomerate/aggregate and rapidly settle in TG 201 medium and/or the other standard media used. Importantly, this aggregation and precipitation phenomena could affect the applicability of TG 201, since one of the quality criteria of this TG is that the concentration of the tested substance be satisfactorily maintained within 80% of the nominal or measured initial concentration throughout the test. Only a few studies partially addressed this issue using UV–Vis measurements to monitor the stability of graphene dispersions, obtaining contrasting results. Marković et al. (2020) reported achieving >90% of GO nominal concentrations throughout the test, whereas Castro et al. (2018) reported an absorbance decrease of c. 40%, already after 24 h, for another GO. Similarly, Yin et al. (2020) reported a decrease of 45% to 70% of the nominal concentration in dispersions of rGOs composites with metal ions. This data suggests the importance of the chemical composition of graphene materials on their stability in aqueous media, as large differences can be expected according to graphene material C/O ratio, even though this factor is not the only one affecting the stability of graphene dispersions. Indeed, as mentioned in the OECD GD 317 document, the medium ionic composition is another factor that can strongly influence the stability of NM dispersions. Organic acids have been used as natural dispersants to increase the concentration maintenance time of GO dispersions but this also leads to mitigation of toxic effects (Zhao et al., 2019) (EC_{50} 66.60 to 242.78 mg/L) (Castro et al., 2018). The incorporation of bovine serum albumin (BSA) as a dispersant also appeared to interfere with algal growth (Barrick et al., 2019). Therefore, careful consideration towards the use of dispersants is needed when applying TG 201 to graphene materials.

As graphene materials are often dark coloured and light absorbing, and as algal cells rely on light for photosynthesis, concentration-dependent shading effects are likely to occur in a test system with graphene material dispersions (Malina et al., 2019; Zhang et al., 2018; Zhao et al., 2017). This shading effect was tested by submerging conical flasks with growing algae in beakers filled with the test material. A significant contribution of shading to the growth inhibition was observed (16.4%) when testing graphene at concentrations of 50 mg/L (Zhao et al., 2017).

Interferences with readouts (e.g. direct fluorescence measurement of cell density) at concentrations >12.5 mg/L and when using haemocytometers, as it can be difficult to count individual algae if within graphene material aggregates, can lead to false readings (Marković et al.,

Table 2
Crustacean growth inhibition studies.

	Test Material Raw material (supplier) Production method	No. Layers Thickness Later size Oxidation level Defect level	Processing step	Test Species (life stage) n = number rep = replicates	Protocol Medium used	EC_{50} 48 h (mg/L)	Observations	Reference
1.	**GO** Natural graphite (Týn nad Vltavou, Czech Republic) Modified Hummers	Single layer 63 μm C/O = 2.6 I_D/I_G: 1.18 ± 0.01		*Daphnia magna* n = 5 rep = 4	OECD TG 202 Distilled water	>50 EC_{10}: 50	Stability assessment: UV–vis spectroscopy Instability of GO in the applied test medium (GO floccules formed) Effects on heartbeat, feeding behaviour and oxidative stress Decreased feeding activity	Fekete-Kertész et al., 2020
2.	**GO** Graphite Modified Hummers	Single layer 1 nm 200–300 nm I_D/I_G: 0.96	Sonication (bath) (2 h)	*Daphnia magna* (5 d) *n* = 10 rep = 3	OECD TG 202 (modified) Tap water	72 h: 44.3	Stability assessment: UV–VIS spectroscopy Sedimentation rate constants (ksed) reported 25% of GO suspended in water after 72 h	Lv et al., 2018
3.	**GO** (XFNANO Materials Tech Co., Ltd. (China, Nanjing)) Hummers method	Single layer 0.8–1.2 nm 0.5–5.0 μm I_D/I_G = 1.02	Sonication (30 min) ± HA (25 mg/L)(Sigma Aldrich)	*Daphnia magna* (<24 h) n = 5 rep = 4	OECDTG 202 (modified) Artificial freshwater	84.3 GO+HA: 111.4	Stability assessment: UV–Vis spectroscopy Humic acids used to stabilise and mitigated toxicity (EC_{50} changed to 111.4 mg/L)	Zhang et al., 2019
4.	**^{14}C FLG** $FePO_4$/dodecylamine hybrid nanosheets In house Graphitization and exfoliation	4 layers C:O = 89:6	Sonication (6 h)	*Daphnia magna* (<24 h) n = 3 rep = 3	ISO 6341. 1 Artificial freshwater	Nd NOEC: 0.25	Substantial settling occurred and enhanced settling rates with *Daphnia* spp. present (due to adherence/uptake)	Guo et al., 2013
5.	**FLG** Modified FLG Reaction with TBBPA mediated by HRP In house	4–6 layers 1.05–4.05 nm 3–4 layers 0.7–2.3 nm	Sonication (probe) (6 h)	*Daphnia magna* (<24 h) *n* = 30 rep = 4	OECD TG 202 Artificial freshwater	~10 >10 LOEC: 0.5	Presence of *Daphnia* spp. enhanced settling Pristine FLG was unstable Modified material less toxic	Lu et al., 2015
6.	**GO** Expanded graphite (Nacional de Grafite® (Brazil)) Hummers method	Single layer 1.0 nm	Sonication (bath) (15 min)	*Ceriodaphnia dubia* (<24 h) n = 5 rep = 4	Reconstituted water	1.25	ROS generation at 0.05 mg/L Reproduction inhibition in chronic tests, interference in feeding activity	Souza et al., 2018
7.	**GO** **GO-carboxyl** **GO-imidazole** **GO-polyethylene glycol** (XFNANO Materials Tech Co., Ltd. (Nanjing, China))	Single layer 0.8 nm–1.2 nm 0.5–5 μm C/O ratio = 2.4 I_D/I_G = 1.3 C/O ratio = 2.6 I_D/I_G = 1.2 C/O ratio = 3.9 I_D/I_G = 1.2 C/O ratio = 4.6 I_D/I_G = 1.6	Sonication (bath) (2 h)	*Daphnia magna* (<24 h) *n* = 10 rep = 3	OECD TG202 Simplified Elendt M7 medium (SM7)	GO:~70 LOEC: 8 GO-carboxyl: ~90 LOEC: 8 GO-imidazole: >100 LOEC: 8 GO-polyethylene glycol:>100 LOEC: 32	Stability assessment: UV–VIS spectroscopy >90% remained suspended without *Daphnia spp.*, but with *Daphnia spp.* <80% remained in suspension	Liu et al., 2018
8.	**GO** (Aladdin Industrial Co. (Shanghai, China))	Single layer 0.55–1.20 nm 0.5–3.0 μm	Sonication (bath) (30 min)	*Daphnia magna* (<24 h) n = 5	OECD TG 202 (modified) TG 201 media	21	Stability assessment: UV–Vis spectroscopy GO concentration decrease by 10–12% after 96 h of settling	Ye et al., 2018
9.	**GO** graphite (Sigma-Aldrich) In house Hoffman protocol:	Single layer 1 nm 5 μm C/O ratio = 2.60		*Daphnia magna* (<24 h) *n* = 15	ISO 6341 modified Elendt M4 medium	HO-GO: 31.03 HU-GO: 80.62 TO-GO: Nd	Stability assessment: UV–Vis spectroscopy Test tubes for exposure and aeration from the bottom of test tubes	Malina et al., 2020

(*continued on next page*)

Table 2 (*continued*)

	Test Material Raw material (supplier) Production method	No. Layers Thickness Later size Oxidation level Defect level	Processing step	Test Species (life stage) n = number rep = replicates	Protocol Medium used	EC_{50} 48 h (mg/L)	Observations	Reference
	HO-GO Hummers protocol: **HU-GO**) Tour protocol: **TO-GO**	I_D/I_G = 0.83 C/O ratio = 1.95 I_D/I_G = 1.12 C/O ratio = 1.72 I_D/I_G = 1.18					24 h algae pre-treatment samples also tested Pre-treatment mitigated toxicity	
10.	**GDN** (PlasmaChem (Germany))	4 nm	Sonication (bath)(1 h) 48 h stirring	*Daphnia magna*, (clone K6 neonates) n = 5 rep = 5	OECD TG 202 ASTM hard water medium	7.8 ± 0.3 LOEC: 6.25	Non-stable suspensions	Martín-de-Lucía et al., 2019

GDN; graphite-diamond nanoparticle, ROS; reactive oxygen species, TBBPA; tetrabromobisphenol, HRP; horseradish peroxidase, i_D/I_G; the intensity ratio of defect according to D (ID) and G bands (IG), LOEC; lowest observed effect concentration, Nd: not determined.

2020). It was also seen that even when using alternative approaches (e.g. chlorophyll (chl) extraction) interferences can be evidenced (e.g. due to adsorption of the extracted chl to the graphene materials) (Malina et al., 2019; Farkas and Booth, 2017).

The depletion of nutrients (e.g. metal ions, which are often essential for algal growth) by adsorption to graphene materials is another witnessed phenomenon raising concerns. This can lead to a reduction in nutrient availability and inhibit algal growth. Approaches to characterise/quantify shading effects and assay interferences have been discussed in GD 317 (OECD, 2022a) along with efforts that can be made to overcome them, for example the use of specialized exposure vessels to maximise light transmission and reduce potential shading effects and alternative methods of chl measurement. One example of a specialised equipment to maximise illumination is the LEVITATT (LED Vertical Illumination Table for Algal Toxicity Tests) system for vertical illumination for testing coloured substances or nanomaterials (Skjolding et al., 2020). Also test setups using double-vial systems to distinguish direct toxic effects and indirect physical effects have been used (Sørensen et al., 2015). Alternative method for chl measurement that have been described include modified versions of ISO 10260:1992 incorporating an algal filtration step (Farkas and Booth, 2017), as well as a locust bean gum separation technique (Hund-Rinke et al., 2022). Such approaches may not be applicable in all cases and thus prior validation studies would be needed. Furthermore the benefit and assurance of including a second endpoint, as used in many of the studies, is evident.

2.1.2. Crustacean toxicity (immobilisation)

The crustacean *Daphnia* spp. is used in TG 202 to determine the acute toxicity of a substance following 48 h exposure to young daphnids (aged <24 h) (OECD, 2004a). The endpoint of the test is immobilisation (loss of ability to move within 15 s under soft agitation) and the concentrations that cause immobilisation of 50% of the daphnids at the end of the exposure period are reported as $EC_{50\ 48\ h}$ values. 10 studies have been collected from the literature related to the acute toxicity testing of graphene materials using *Daphnia* spp. and immobilisation as an endpoint (Table 2). All of them reported using either OECD TG 202 or ISO 6341 (ISO, 2012) or a modified version of these standardised tests.

The materials tested included different nanoforms of GO including functionalised GO, FLG and modified FLG, as well as a graphene diamond nanomaterials (GDNs). In one instance the same GO material (single layer, 0.5–5.0 μm, XFNANO) has been tested using TG 202 by Liu et al., 2018 and Zhang et al., 2019 with consistent $EC_{50\ 48\ h}$ of ~70 and 84.2 mg/L, respectively. This indicates good reproducibility when using the OECD TG 202 for testing graphene materials. EC_{50} values reported following testing of other GO (single layer, ≤5 μm) manufactured with the Hummers method and tested with the standardised ISO 6341 protocol were also comparable ($EC_{50\ 48\ h}$ of 80.62 mg/L) (Malina et al., 2020). GO produced using other methods showed EC_{50} values in the same order of magnitude (Hoffman protocol; 80.62 mg/L) or lower (31.03 mg/L, Tour protocol) (Malina et al., 2020). EC_{50} 48 h values for other GO nanoforms tested with different properties and from other suppliers ranged from 21 mg/L (Ye et al., 2018; GO; 0.5–3.0 μm) to 44.3 mg/L (Lv et al., 2018; GO; 200–300 nm).

These EC_{50} values are based on nominal concentrations and the assumption that organisms were exposed to this concentration for the entire test duration (48 h). Reports of rapid sedimentation in studies which performed stability monitoring (8/10 studies) would suggest that concentrations are likely not maintained in suspension under test conditions (i.e. when organisms were present) (Lv et al., 2018; Malina et al., 2020). According to TG 202, the test should be performed under static conditions to reflect the normal behavioural patterns of *Daphnia spp*., therefore the introduction of any agitation (which may aid graphene material dispersion) is to be avoided. In one of the studies agitation, by means of aeration from the bottom of the test vessels (test tubes), was incorporated in the test design and prolonged the time to sedimentation (Malina et al., 2020). However the authors also observed that the *D. magna* planktonic organisms swam to the bottom of test vessels to feed on any sedimented material. Therefore, any material loss from the water column still appears bioavailable and taken up by the filter-feeder organisms, and thus the maintenance of dispersion in the water column may not influence test results to the same extent as with other tests. However, as the amount of material the organism will take up from feeding cannot be quantified it cannot be assumed that the nominal exposure concertation is the real exposure concentration, and this issue must be taken into account.

Comparative investigations performed within these studies evidenced that functionalisations of GO with carboxyl, imidazole, or polyethylene glycol reduced the acute toxicity (Liu et al., 2018). The toxicity of GO was also mitigated in the presence of humic acid (proposed as a dispersion aid) with a measured $EC_{50\ 48h}$ of 111.4 mg/L (vs. 84.3 mg/L) (Zhang et al., 2019). This highlights the need for testing of different nanoforms of the same material and that care must be taken when using dispersion aids. Testing in another species, *Ceriodaphnia dubia,* produced the lowest $EC_{50\ 48h}$ value reported among the collected studies for GO (single layer) of 1.25 mg/L (Souza et al., 2018). Low $EC_{50\ 48h}$ values (≤10 mg/L) were also reported for graphite-diamond GDNs

Table 3
Fish acute toxicity studies.

	Test Material Raw material (supplier) Production method	No. Layers Thickness Later size Oxidation level Defect level	Processing step	Species (life stage) Weight (g) Length (cm) n = number rep = replicates	Exp. Conc. (mg/L) Setup	Mortality (≥96 h)	Clinical signs/ Observations	Reference
1.	**GO [XF002–1]** (XFNANO Materials Tech Co., Ltd. (Nanjing, China)) Hummers method	Single layer 0.8–1.2 nm 0.5–5 μm 31.4% O	Sonication (bath) (15 min)	*Danio rerio* (6–8 mo.) *n* = 6	0.01, 0.1, 1 Natural salt water (60 mg/L) (rnw. 48 h)	No (upto 21d)	Changes in oxidation-associated genes and proteins in brain associated with the MAPK pathway and regulation of the actin cytoskeleton <Tubulin expression	Sun et al., 2019
2.	**GO** Graphene powder (XFNANO Materials Tech Co., Ltd. (Nanjing, China)) In house Hummers	Single layer 0–1.2 nm 321.74 nm 32.69% O	Sonication (40 min)	*Danio rerio* *n* = 24, rep = 3	0.05, 0.5, 5 (rnw. (50%) 24 h)	No (upto 25 d)	Alteration in developmental indices (<body weight) Alteration of gut microbial composition and cellular intestinal epithelium Inflammatory related gene expression (*il-8, ifn-γ*) in gut tissue	Jia et al., 2019
3.	**GO + PFOS** (Nano Materials Tech Co. (Tianjin, China))	Single layer >1, [0.7–1.8 nm] 0.5–5 μm C/O: 2.14	Sonication and mechanical stirring (30 min)	*Cyprinus carpio* Juvenile (3 mo.) 8.68–11.5 g 10 cm	1 +500 ng/L PFOS Dechlorinated tap water	No (after 28 d)	GO significantly promoted PFOS bioaccumulation in liver, kidney, and intestine Dark faeces reported	Qiang et al., 2016
4.	**GO** Expanded graphite (Nacional de Grafite® (Brazil)) Modified Hummers	Single layer 1.0 nm	Sonication (bath) (15 min)	*Danio rerio (4–6 mo.)* 0.26 ± 0.08 g 2.77 ± 0.33 cm *n* = 22, rep = 2	2, 10, 20 Chlorine-free tap water	No (48 h)	Alteration in antioxidant enzymatic activities in gill but adaptive responses protected against lipid peroxidation	Souza et al., 2019
5.	**GO** Expanded graphite (Nacional de Grafite® (Brazil)) Modified Hummers	Single layer 1.0 nm	Sonication (bath) (15 min)	*Danio rerio* (6 mo.) 0.31 ± 0.09 g 2.34 ± 0.31 cm *n* = 5, rep = 4	2, 10, 20, 100[$] Dechlorinated tap water (rnw. (50%) 72 h) TG 203 (1992)	No (upto 14 d)	Gill and liver (necrotic lesions) Dark spots in gut lumen reported	Souza et al., 2017
6.	**Graphene** (Areej Al furat Bureau Manufacturer: SS nano (Texas, United states))	Platelets 6–8 nm 15 μm	Sonication (bath) (10 min)	*Cyprinus carpio* 66.48 g *n* = 16, rep = 2	10, 20, 100[$] TG 203 (1992)	No (upto 10 d)	Gill hyperplasia and liver necrosis Fusion of gill lamellae	Al-Rudainy, 2019
7.	**GO** (Tanfeng Tech. Inc. Jiangsu, China) Aqueous Suspension (1 mg/mL)	<4 layers 0.7–1 nm 3.0 μm	Sonication (bath) (15 min)	*Danio rerio* (6–7 mo.) n = 22	0.1, 1 Circulating UV sterilized water (rnw. 48 h)	No (upto 14 d)	Altered neurological behaviours; <locomotor activity, <predator avoidance	Audira et al., 2021
8.	**GO** (Graphenea, San Sebastian, Spain) Aqueous Suspension (4 mg/mL)	>2 layers 0.612 ± 0.176 nm 0.5 μm-<13 μm 40% O		*Danio rerio* (7 mo.)	2 [2_{24h}] [0.5_{72h}] Conditioned water (deionised water with commercial salts added) (rnw. (71%) 72 h)	No (upto 21 d)	>catalase activity in gills <AChE activity in brain Structures resembling GO (0.9 to 2.7 μm) in the lumen of the intestine	Martínez-Álvarez et al., 2021
9.	**GO** Natural graphite powder (supplier not specified) Modified Hummers	≥1 <5 μm	Sonication (4 h) Centrifugation (6000 *g* for 5 min)	*Danio rerio* (2 mo.) *n* = 20, rep = 2	1 [0.86_{24h}], 5 [4.7_{24h}], 10 [6.43_{24h}] 50 [3.5_{24h}] Tap water (rnw. (50%) 24 h)	No (upto 14 d)	Alterations in liver and intestine, and effects on hepatocytes and goblet cells Oxidative stress response and > expression of inflammatory cytokines in spleen	Chen et al., 2016
10.	**rGO** Tanfeng Tech. Inc. Jiangsu, China	<3 0.4–1.1 nm 5.4 μm	Sonication (15 min)	*Danio rerio* (6–7 mo.) *n* = 22	0.1, 0.5 Circulating UV sterilized	No (upto 14 d)	<locomotor activity,< predator avoidance, <exploratory behaviour <neurotransmitter levels	Audira et al., 2021

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Table 3 (*continued*)

	Test Material Raw material (supplier) Production method	No. Layers Thickness Later size Oxidation level Defect level	Processing step	Species (life stage) Weight (g) Length (cm) n = number rep = replicates	Exp. Conc. (mg/L) Setup	Mortality (≥96 h)	Clinical signs/ Observations	Reference
	Aqueous Suspension (2 mg/mL)				Water (rnw. 48 h)		(serotonin and dopamine) in brain	
11.	**^{14}C L-FLG** $FePO_4$/dodecylamine hybrid nanosheets In house Graphitization and exfoliation	Few layer 1.4 nm ~500 nm 6% Oˆ	Sonication (probe) (5 min, 4 times a day)	*Danio rerio* (> 3 mo.) 0.0428 ± 0.01 g (dw) 3.42 ± 0.16 cm *n* = 5	0.05 [0.045_{24h}], 0.075 [0.067_{24h}], 0.25 [0.2_{24h}] Freshwater ±10 mg (TOC)/L NOM	No (after 72 h)	>body burdens when NOM present	Lu et al., 2017
12.	**^{14}C S-FLG** $FePO_4$/dodecylamine hybrid nanosheets In house Graphitization and exfoliation	Few layer 1.05 nm ~30 nm	Probe tip sonication (5 min, 4 times a day) to prevent settling)	*Danio rerio* (> 3 mo.) 0.0428 ± 0.01 g (dw) 3.42 ± 0.16 *n* = 5	0.05 [0.047], 0.075 [0.071], 0.25 [0.24] Freshwater ±10 mg (TOC)/L	No (after 72 h)	Distribution to liver and slow elimination from gut Change in gut microbial community	Lu et al., 2017
13.	**M-GQDs;** **r-GQDs** **OH-GQDs** **NH_2-GQDs** (Nanjing Xianfeng Nanotechnology Co., Ltd. (Nanjing, China))	Quantum dots 5–15 nm		*Danio rerio* *n* = 12	2, 10, 50 (rnw. 24 h) Tap water	No (upto 7 d)	Epigenetic effects: high persistent DNA methylation after rGQD and NH_2-GQD exposure in liver	Hu et al., 2019

Modified graphene quantum dots (M-GQDs), reduced graphene quantum dots (rGQDs), hydroxylated graphene quantum dots (OH-GQDs), aminated graphene quantum dots (NH_2-GQDs),ˆ oxygen was introduced by the addition of ^{14}C-phenol not by oxidation, ^{14}C L-FLG; Carbon 14 labelled Large Few Layer Graphene, ^{14}C S-FLG; Carbon 14 labelled Small Few Layer Graphene, <; decreased, >; increased, $; Limit test according to TG 203 (1992), PFOS; perfluorooctanesulfonate, TOC; Total Organic Carbon, NOM; Natural Organic Matter, il-8; interlukin 8, ifn-γ; Interferon gamma, dw; dry weight, AChE; Acetylcholinesterase, rnw.; renewal [] (denote measured values), I_D/I_G; the intensity ratio of defect according to D (ID) and G bands (IG), mo.; months, d; days, $FePO_4$; Iron(III) phosphate.

(4 nm) (Martín-de-Lucía et al., 2019) and FLG materials tested using TG 202 with *D. magna* (Lu et al., 2015). Thus, the GO materials tested in the collected studies can be classified as Category: Acute 3 using standardised TG 202 and *Daphnia* spp. The other graphene materials (GDNs, FLGs) fall into Category: Acute 2 according to the distinct graphene material nanoform and species tested. Also there were evidences of uptake of GO in the gut tract of organisms, that caused decreases in feeding activity in chronic tests (Souza et al., 2018; Fekete-Kertész et al., 2020), and in some cases slow depuration and bioaccumulation of the materials were evidenced (BCF > 5000 L/kg bw) (Lv et al., 2018).

There were no other major peculiarities reported and therefore the evidence would suggest that standardised approaches and tests such as TG 202 can be applied when testing graphene materials without a need for any specific modifications. One approach that could be explored is the use of a renewal of exposure medium techniques (semi-static renewals), to aid in achieving exposure concentration maintenance for the duration of the test. Another approach involves the use of partitioning techniques (e.g. use of meshes) to confine *Daphnia* spp. to exposure to only the test material that remains dispersed and stable in the water phase or indeed to only the sedimented fractions (Sørensen et al., 2015). An interesting approach used by Malina and colleagues was to incorporate a benthic crustacean species in the study to test both bioavailability and potential toxicity of sedimented materials (Malina et al., 2020). The benthic crustacea used was *Heterocypris incongruens,* and it was was exposed to any material sedimented over 6 days. While Raman analysis confirmed that material was taken up and present in the gut of organisms, suggesting a certain degree of bioavailability, viability ≥80% was maintained and an EC_{50} could not be calculated. Thus, in this case, organism sensitivity might also be influencing effects. Also attention to the possible adsorption of materials to the organism body may be needed and considerations for the bioavailability of the attached fraction and any impedance of mobility due to physical effects accounted for. In studies, graphene particle attachment to the organism carapaces as well as in the gut tract was evidenced (Guo et al., 2013), and in some cases mortality (according to loss in heartbeat) was also recorded as an additional endpoint of acute toxicity.

2.1.3. Fish acute toxicity

OECD TG 203 describes a test to determine the acute toxicity (lethality) of a substance following a 96 h exposure in juvenile fish, with the most recent revised guideline published in 2019 (OECD, 2019c). The lethal concentrations to kill 50% of the fish (LC_{50} value) is calculated from cumulative mortalities over the course of the exposure and any sublethal clinical signs and/or visible abnormalities are also reported. While following the same test design as the earlier version (1992), in this revised TG analytical measurement of test concentrations is compulsory to meet test validity criteria.

Following a detailed review of the literature, 13 studies were identified relating to the toxicity testing of graphene materials using fish (Table 3). The materials tested included large sized (15 μm) graphene platelets (Al-Rudainy, 2019), both large (~500 nm) and small sized (~30 nm) FLG sheets (Lu et al., 2015), single layer GO (Souza et al., 2017, 2019; Jia et al., 2019; Sun et al., 2019; Martínez-Álvarez et al., 2021; Chen et al., 2016; Qiang et al., 2016), bi-layer rGO (Audira et al., 2021), as well as quantum dots (5–15 nm) modified through reduction, hydroxylation and amination (r-GQDs, OH-GQDs, NH_2-GQD) (Hu et al., 2019). In 9 out of the 11 studies the fish species used was the zebrafish *Danio rerio* (2–8 months), while both a juvenile and adult common carp *Cyprinus carpio* were also used by Qiang et al. (2016) and Al-Rudainy (2019), respectively.

Only two of the studies referenced explicitly following OECD test guidelines (Souza et al., 2017; Al-Rudainy, 2019), and only one of these mentioned specifically OECD TG 203, albeit the old version (Al-Rudainy, 2019). Following OECD TG 203 (1992) a large sized (15 μm) graphene platelet material was tested using a limit test setup (one concentration, 100 mg/L) and exposure to the adult common carp *C. carpio* (*n* = 7) for a 96 h exposure period. No mortality was reported and no further information on test design was provided, as this study acted as a

Table 4
Fish embryo toxicity studies.

	Test Material Supplier/Raw material Production method	No. Layers Thickness Later size Oxidation level Defect level	Processing step (duration)	Species (life stage) (hpf)	Exp. Conc. (mg/L) Medium Protocol	Mortality	Malformations/ Observations	Reference
1.	**GO-COOH [XF004]** (XFNANO Materials Tech Co., Ltd. (Nanjing, China)) Aqueous dispersion (2 mg/mL)	Single layer 0.8–1.2 nm 50–200 nm 5% COOH	Sonication (1 h)	*Danio rerio* *Transgenic lines*: Tg (elavl3: EGFP) and Tg (mbp:EGFP) 6 hpf $n = 20$	10, 50, 100 6 dpf Zebrafish embryo culture medium	No (72 hpf)	Neurodevelopmental toxicity; <spontaneous tail coiling >AChE activity Disrupted neurotransmitter signalling PD like symptoms-related genes downregulation Locomotor disorder Oxidative stress response	Cao et al., 2021
2.	**GO [XF020]** (XFNANO Materials Tech Co., Ltd. (Nanjing, China)) Aqueous dispersion (0.5 mg/mL)	Single layer 0.8–1.2 nm 50–200 nm	Sonication (1 h)	*Danio rerio* Transgenic line: Tg (fabp10a: dsRed) 6 hpf $n = 30$	0.25, 0.5, and 1 (intravenous microinjection) E3 medium	No (72 hpf)	Reduction in hepatocytes, neutrophils and macrophages Inflammatory responses and oxidative stress Changes in lipid metabolic pathways Liver damage and immunotoxic effects through the ROS and PPAR-a mediated MAPK signalling	Xiong et al., 2020
3.	**GO [XF002–1]** (XFNANO Materials Tech Co., Ltd. (Nanjing, China)) Hummers method	Single layer 0.8–1.2 nm 0.5–5 μm 31.4% O		*Danio rerio* (AB strain) larvae 72 hpf	0.00001, 0.0001, 0.001 120 hpf E3 medium OECD TG 236 rnw. 24 h	Yes (120 hpf) 5% 0.001 mg/L	Malformations: tail flexure (0.00001 mg/L) spinal curvature (0.0001) PD-like symptoms: loss of dopaminergic neurons, lewy bodies (a-synuclein and ubiquitin), reduction in locomotive activity Behavioral and metabolic disturbances	Ren et al., 2016
4.	**O-GNRs** (in house) MWCNT unzipping by $KMnO_4$	1–2.5 μm I_D/I_G: 1.3	Direct addition to embryo media Sonication (bath) (5–20 min) Sonication (probe) 1–10 min Centrifugation 20,800 g (15 min)	*Oryzias latipes* embryos 24 hpf	20 12 dpf rnw. 48 h and gentle agitation (220 rpm) on orbital shaker during exposure	No (12 dpf); bath sonicated O-GNRa Yes (12 dpf) (25–60%); probe-sonicated O-GNRs	Precocious (early) hatching Chorion penetration and structural damage Increased toxicity with longer probe sonication No difference in toxicity of centrifuged versus uncentrifuged suspensions.	Mullick Chowdhury et al., 2014
5.	**GO** Natural Graphite, GRAFINE 99200 (Nacional de Grafite Ltd., Brazil) (in house) Modified Staudenmaier method (Staudenmaier, 1898)	Single layer 5.3 nm	Sonication (bath) 1 h Overnight agitation	*Danio rerio* embryos 2 hpf $n = 30$	5, 10, 50, 100 6 dpf 1% Pluronic F 68 (PF68) water solution rnw. 24 h	No	Dopaminergic system alterations (10 mg/L) >apoptotic associated genes autophagosome formation in brain	Soares et al., 2017
6.	**GQDs** GO (natural graphite powder, in house: using modified Hummers method) (in house) GO Ammonia- H_2O_2 treatment	2–5 nm		*Danio rerio* embryos 4 hpf n = 30	0.012.5, 0.025, 0.050, 0.100, 0.200 120 hpf E3 culture medium rnw. 24 h	Yes (120 hpf) ≥10% (≥50 mg/L))	<heart rate Altered locomotor activity Reduction in hatching rate (200 mg/L)) Malformations including pericardial edema and vitelline cyst, bent tail, and bent spine	Wang et al., 2015
7.	**pG** Graphite (Graphene-supermarket, Graphene Laboratories, Calverton,(New York, USA))	Single layer 0.35 nm 150–3000 nm [170–390 nm] 8.8% O		*Danio rerio* (wild-type AB) embryos 4 hpf $n = 30$	0.005, 0.010, 0.025 96 hpf E3 culture medium rnw. 24 h	Yes (96 hpf) 25% (0.025 mg/L)	Decrease in hatching rate Decrease in heart rate Range of morphological defects, including pericardial edema, yolk sac edema (0.005 mg/L)	Manjunatha et al., 2018

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Table 4 (*continued*)

	Test Material Supplier/Raw material Production method	No. Layers Thickness Later size Oxidation level Defect level	Processing step (duration)	Species (life stage) (hpf)	Exp. Conc. (mg/L) Medium Protocol	Mortality	Malformations/ Observations	Reference
	Dispersion in EtOH (1 mg/L)							
8.	**GO (XF002–1]** (XFNANO Materials Tech Co., Ltd. (Nanjing, China)) Hummers method	Single layer 0.8–1.2 nm [1.02 ± 0.15 nm] 0.5–5 μm [0.3–2.6 μm] 31.4% O	Sonication (bath) 1 h 30 min prior to use	*Danio rerio* (AB strain) embryos 2.5 hpf	0.001, 0.01, 0.1, 1, 10, 100 7 dpf E3 culture medium rnw. 24 h OECD TG 236	Yes (7dpf) >10% (0.01 mg/L)	Developmental and metabolic malformations; pericardial edema, yolk sac edema Skeletal and cardiovascular development gene changes Cardiac toxicity ROS generation	Zhang et al., 2017a
9.	**rGOQDs** GO precursor (graphite powder (10 nm, XF NANO Materials Tech Co., Ltd. (Nanjing, China)) In house (using improved Hummers method and hydrothermal treatment of GO with DMF)	Spherical 1 nm 10 nm 28.2% O		*Danio rerio* (AB line) and Tg (cyp1a:gfp) embryos 4 hpf	25, 50, 100 120 hpf deionized (DI) water rnw. 24 h	Yes (120 hpf) >10% 0.025 mg/L	Pericardial edema, vitelline cyst, bent spine (0.100 mg/L) Heart rate affected (0.100 mg/L) AhR pathway disturbance/ perturbation [cyp1a, cyp1c and cyp7a1 gene upregulation]	Zhang et al., 2017b
10.	**GO** (Sigma-aldrich, USA)		Sonication (bath)(40 min)	*Danio rerio* embryos 2 hpf $n = 50$, rep =3	0.01, 0.1, 1, 10 5 dpf rnw. 24 h	Yes (120 hpf) (10 mg/L)	<Hatching rate (10 mg/L) Developmental toxicity Malformations (1 mg/L) Herat beat (tachycardia) (0.1 mg/L) Disturbed locomotor activity Disturbances to neurotoxicity-related genes Pro-inflammatory immune responseO xidative stress responses (MDA and 8-OHDG levels)	Yang et al., 2019
11.	**GO** (Graphenea (San Sebastián, Spain)) Aqueous dispersion (4 mg/mL)			*Danio rerio* (wild-type and transgenic Tg (fli1a:EGFP) embryos 5 hpf n = 10, rep = 3	100–1000 mg/L 120 hpf	Yes (120 hpf) (300–1000 mg/L) (60–100%)	Decreased yolk sac length (100 mg/L) Delayed hatching (300 mg/L) Pericardia malformations Cardiovascular effects: microvasculature and cardiac looping deformities	Bangeppagari et al., 2019
12.	**^{14}C-labeled FLG** **^{14}C-labeled S-FLG** (in house) Graphitization and exfoliation of $FePO_4$/ dodecylamine hybrid nanosheet precursor		FLG: Sonication (probe) S-FLG: Sonication (probe) (60 h)	*Danio rerio* embryos 2 hpf $n = 100$, rep = 3	0.075 48 hpf aerated artificial water OECD TG 236	No (48 hpf)	Increased uptake for smaller sized FLG	Su et al., 2017
13.	**GO-PEG** Graphite precursor (in house) Hummers method Carboxylation and functionalised with PEG	50 nm	Continues agitation/ shaking until use	*Danio rerio* embryos n = 10, rep = 3	5, 10, 15 96 hpf OECD TG 236	No (96 hpf)	Abnormalities in small percentage (3–5%); cardiac edema, development delay and malformation of the tail	Loureiro et al., 2018
14.	**GO** (Graphenea (San Sebastián, Spain)) Aqueous dispersion **GO(PVP)(** in house) 2% PVP stabilisation **rGO(PVP)(** in house) Chemical reduction of	GO Single layer [0.612 ± 0.176 nm] 500 nm to few μm 40% O GO(PVP) Single layer [1.994 ± 0.319 nm]		n = 10, rep = 3	0.1, 0.5, 1, 5, 10 120 hpf OECD TG 236	No (120 hpf)	Malformations (GO and GO (PVP) (5 mg/L), rGO (10 mg/L):] spinal cord flexure, pericardial edema, yolk sac edema Malformations EC_{50} values (mg/L) GO: 14.7 ± 4.0 GO(PVP): 31 ± 26.7 rGO(PVP): 14.5 ± 3.1	Martínez-Álvarez et al., 2021

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Table 4 (*continued*)

	Test Material Supplier/Raw material Production method	No. Layers Thickness Later size Oxidation level Defect level	Processing step (duration)	Species (life stage) (hpf)	Exp. Conc. (mg/L) Medium Protocol	Mortality	Malformations/ Observations	Reference
	GO by hydrazine monohydrate and 2% PVP stabilisation of rGO	rGO(PVP) Single layer [4.174 ± 0.179 nm]						
15.	**GO** Graphite flakes (Sigma-Aldrich, Lot#MKBW0432V) (in house) Modified Hummers **GO-AgNPs** (in house) using $NaBH_4$ as a reducing agent for $AgNO_3$ when mixed with GO	Single layer 1 nm [180 nm] 31.4% O I_D/I_G: 1.15 I_D/I_G: 1.31	Sonication (bath) 80 min	*Danio rerio* (wild type) embryos 24 hpf Embryos with chorion removed	0.5, 1, 1.5, 3, 2.5, 100 ± NOM (20 mg/L) 96 hpf	GO: No (96 hpf) No deleterious effects LC_{50} > 100 GO-AgNPs: Yes (96 hpf) LC_{50}: 1.4 1.0 (−chorion) 2.3 (+NOM) 1.2 (+NOM,-chorion)	Malformations (1 mg/L): edema, total length, yolk sac size deformations	de Medeiros et al., 2021
16.	**S-GO** **M-GO** **L-GO** (XFNANO Materials Tech Co., Ltd.(Nanjing, China)) Aqueous suspension (2 mg/mL)	S-GO: Single layer 50–200 nm M-GO: Single layer <500 nm L-GO: Single layer > 500 nm		*Danio rerio* (wild-type, AB strain) and transgenic (lyz:DsRed) line embryos 4–6 hpf *n* = 45, rep = 3	0.1, 1, 10, 100 120 hpf E3 culture medium rnw. 24 h	Yes (120 hpf) S-GO:15% (100 mg/L) M-GO:12% (100 mg/L) L-GO: 18% (100 mg/L)	Inhibited hatching rates Immunomodulatory and neurotoxic effects >pro-inflammatory iNOS activity >AChE activity	Chen et al., 2020
17.	**GO** graphite powder (ThermoFisher Scientific) (in house) Modified Hummers method **NanoGO** Prolonged sonication of GO **GO-PF** IPluronic F127 (PF)-functionalized GO **NanoGO-PF** Prolonged sonication of GO-PF	Single layer ~ 768.4 nm 43.6% O I_D/I_G: 0.850 Single layer 86.14 nm 32.9% O I_D/I_G: 0.851 Single layer 766.7 nm 40.6% O 212.8 nm 40.6% O	GO: 1 mg/mL Sonication (bath) (5 min) Overnight stirring nanoGO: Sonication (bath) (24 h) NanoGO-PF Sonication (bath) (24 h)	Danio Assay Kit ((Danio Assay Laboratories Sdn. Bhd, UPM, Malaysia) 24 hpf	0–100 96 hpf	Yes (72 hpf) GO: 90% (100 mg/L) 50% (60 mg/L) NanoGO: 50% (100 mg/L) 30% (90 mg/L) NanoGO-PF: 30% (100 mg/L) GO-PF: No decline in survival	GO: No malformations nanoGO: significant <in heart rate GO-PF: (100 mg/L) NanoGO-PF (60 mg/L)	Shamsi et al., 2020
18.	**GOQD [XF042]** (XFNANO Materials Tech Co., Ltd. (Nanjing, China)) Aqueous dispersion (20 mg/mL)	[5.21 ± 1.32 nm]	Sonication (bath) (30 min)	*Danio rerio* (wild-type Tübingen (TU)) embryos	0.0125, 0.025, 0.050, 0.1 7 dpf rnw. 12 h (twice daily)	No (7 dpf)	Effects on locomotor activities Ca^{2+}-ATPase and Na^+/K^+-ATPase activity inhibition altered the transcription of genes related to locomotor capability and the activity of ATPase	Yan et al., 2020
19.	**N-GQDs** (in house) Modified Hummers Aqueous dispersion (4 mg/mL) **USGO** Method described by Zhang et al., 2013	Single layer ~1 nm 3–7 nm Single layer ~1.5 nm < 50 nm 21.4% O		*Danio rerio* (wide-type AB) and Tg (fli: eGFP) embryos 4 hpf *n* = 5, rep = 3	25, 50, 100 6 dpf	No (96 hpf) For both N-GQDs and USGO	No significant effect on hatching rate Malformation >10% at 100 mg/L (120 hpf): Bent spine and reduced pigmentation Both >expression level of *cyp1a* Both >activation of *ahrr1* and *ahhr2*	Deng et al., 2019

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Table 4 (*continued*)

	Test Material Supplier/Raw material Production method	No. Layers Thickness Later size Oxidation level Defect level	Processing step (duration)	Species (life stage) (hpf)	Exp. Conc. (mg/L) Medium Protocol	Mortality	Malformations/ Observations	Reference
	Aqueous dispersion (2 mg/mL)							
20.	**GO** (Sigma Aldrich, USA)	Single layer [225 ± 105 nm] O: 32.2% I_D/I_G: 1.04	Sonication (bath) 30 min	*Danio rerio* embryos 4 hpf	100 +HA 20 mg/L 5–7 dpf rnw. 4dpf	No (7 dpf)	< in yolk sac size diameter (7 dpf) <expression of AChE Some biochemical, morphological, and behavioural changes in zebrafish larvae Effects abrogated in presence of HA	Clemente et al., 2019
21.	**GO** (in house) Modified Hummers		Sonication 60 min	*Danio rerio* embryos 3 hpf n = 10, rep =3	0.016, 0.08, 0.4, 2, 10 96 hpf OECD TG 236	No (96 hpf)	<in cholinesterase activity	Almeida et al., 2019
22.	**GO** (Aladdin Industrial Co. (Shanghai, China))	Single layer 0.55–1.20 nm 0.5–3.0 μm	Sonication (bath) 30 min	*Danio rerio* (wild-type strain AB) n = 10, rep = 2	20–160 96 hpf OECD TG 236	Yes (96 hpf) LC_{50}: 63	Coagulation of embryos, lack of somite formation, non-detachment of the tail, and lack of heartbeat.	Ye et al., 2018
23.	**GO** (Sigma-Aldrich, USA)	Single layer 1.5 nm 1.5 μm	Sonication (bath) 50 min	*Danio rerio* (wild-type) embryos 2.25 hpf (blastula stage)	5, 25, 50 72 hpf	Yes (24 hpf)	Reduction in survival rate and < hatching rate Up regulation of genes in base excision repair (BER) pathway (25 mg/L)	Lu et al., 2017
24.	**GO** Natural graphite precursor (in house) **rGO** GO (in house) Sonication and hydrazine reduction process	Single and few layers		*Danio rerio* (wild type) embryos 8 hpf *n* = 20	1, 5, 10, 50, 100 96 hpf rnw. 24 h	No (96 hpf)	<Heart rate (GO, 100 mg/L) <Hatching rate (rGO, 5 mg/L)I nhibitory effect on the growth (length of larvae) (rGO)A ggregation of material at surface of chorion	Liu et al., 2014
25.	**GO** (Graphenea Inc.,(San Sebastián. Spain))		Sonication 10 min	*Danio rerio* embryos 4 hpf n = 2, rep = 3	5, 10, 50, 100 rnw. 12 h 120 hpf rnw. 12 h	Yes (48 hpf) 10% (50 mg/L)	<Hatching rates and < heart rate (50 mg/L) <Frequency of larval movement Malformations; fin fold flexure, tail flexure, yolk sac edema, and pericardial edema	d'Amora et al., 2017

GNR; graphene nano-ribbons, GQDs; graphene quantum dots, GOQD; graphene oxide quantum dots, rGO; reduced graphene oxide, RGOQDs; reduced graphene oxide quantum dots, USGO; Ultra small Graphene oxide, N-GQDs; nitrogen-doped reduced GQDs, COOH; carboxylic acid, hpf; hours post fertilisation, dpf; days post fertilisation, AChE; Acetylcholinesterase, PD; Parkinsons disease, ROS; reactive oxygen species, PPAR-a; Peroxisome proliferator-activated receptor alpha, MAPK; Mitogen-activated protein kinase, H_2O_2; hydrogen peroxide, pG; pristine single-layer graphene flakes, rnw.; renewal of exposure medium, PEG; Polyethylene glycol, PVP; Polyvinylpyrrolidone, S-GO; small sized GO, M-GO; medium sized GO; L-GO; large sized GO, iNOS; Inducible nitric oxide synthase, HA; humic acids, cyp1a; cytochrome P450 1A, COOH; carboxylic acid, O-GNR; oxidised graphene nanoribbions, $KMnO_4$; Potassium permanganate, <; decreased, DMF; dimethylformamide, $NaBH_4$; Sodium borohydride, $AgNO_3$; Silver nitrate, $FePO_4$; Iron(III) phosphate.

preliminary test in order to select concentrations to test for sublethal effects (abnormal clinical signs and histopathological changes). Similarly Souza et al. (2017) reported no mortality in the zebrafish *D. rerio* (*n* = 5, 6 months) following exposure to a single layer GO material at concentrations up to 100 mg/L following OECD guidelines in a preliminary acute toxicity study (exact test setup was not stated and no further information on test design was provided). In these studies only nominal concentrations have been reported and therefore they would not meet test validity criteria to be considered performed according to the revised OECD TG 203. Also the use of limit testing, while incorporated in the TG 203, is currently not recommended for NMs due to yet a lack of clear understanding on properties (including concentration) governing effects on particle behaviour (e.g. aggregation and sedimentation) that can directly influence exposure dose and presentation (GD 317, OECD, 2022a).

In fact, the primary aim of the reviewed studies was the determination of sublethal effects rather than the determination of a 96 h lethality endpoint according to OECD 203. However in almost all of them fish were exposed to graphene materials over a period of time ≥ 96 h. Thus, they serve to evaluate testing setups and if OECD TG 203 validity criteria was/could be met.

The lack of analytical measurement of exposure/test concentrations is a critical issue in the majority of studies collected (9/13 studies). Thus, assessments are based on assuming that the nominal exposure

Table 5
Fish cell line toxicity studies.

	Test Material Raw material (supplier) Production method	No .Layers Thickness Later size Oxidation level Other	Processing steps	Cell line	Exp. Conc. (mg/L) [duration]	Assay/ Endpoint EC_{50} (mg/L) or LOEC	Observations	Reference
1.	**CNF GANF®** (Grupo Antolin Ingeniería (Burgos, Spain)) Catalytic vapor deposition **GO GRAnPH®** CNF GANF® (Grupo Antolin Ingeniería, SA (Burgos, Spain)) Chemical oxidation **CNF GAtam** (Grupo Antolin Ingeniería, SA (Burgos, Spain)) **CNF GAqUA** (Grupo Antolin Ingeniería, SA (Burgos, Spain))	*GANF®* Helical ribbon nanofibres Graphitization 70% 10.5% Ni *GRAnPH® GO* Single or few layers ≥1 um CNF GAtam Helical ribbon nanofibres *Graphitization 60%* *11.1% Ni* CNF GAqUA Helical ribbon nanofibres 2% Ni	CNF GAqUA: Sonication (bath) (10 min) GO GRAnPH®, CNF GAtam, CNF GANF®: Sonication (probe) (20 min)	PLHC-1 topminnow fish, *Poecilipis lucida,* hepatoma cell line CLC carp, *Cyprinus carpio*, leukocyte cell line	1.56–200 24 h, 72 h PLHC-1: EMEM+ (5% FBS) CLC: EMEM-EB (10% FBS)	GO GRAnPH® PLHC-1: 24 h /72 h AB:122/ 107 CFDA-AM: >200/>200 NR: >200/ 191 CLC: 24 h/ 72 h AB: >100/ >25 CFDA-AM: >200 /139 NR: >200 /153 CNF GAqUA PLHC-1: 24 h /72 h AB: >100/ >100 CFDA-AM: >200/>200 NR: >200/ >200 CLC: 24 h/ 72 h AB: >25/83 CFDA-AM: >200/139 NR:>200/ 153 CNF GAtam PLHC-1: 24 h/72 h AB: >25/ >25 CFDA:AM: >200/>200 NR:>200/ 156 CLC: 24 h/ 72 h AB: >25/ >12.5 CFDA-AM: >200/74 NR:>200/ 172 CNF GANF® PLHC-1: 24 h/72 h AB:>50/ >50 CFDA-AM: >200/>200 NR: >200/ >200 CLC: 24 h/ 72 h AB: >100/ >12.5 CFDA-AM: >200/103 NR:>200/ >200	Interference; fluorescence quenching at conc. ≥12.5 mg/L in AB assay Lowest EC_{50} 24 h: GO GRAnPH®: 122 mg/L Lowest EC_{50} 72 h: CNF GANF®: 103 mg/L CNF have higher toxicity compared with GO Contribution of free Ni metal impurity tested; contribution of Ni to cytotoxicity at highest tested concentration of CNF GAtam in CLC cells	Kalman et al., 2019

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Table 5 (*continued*)

	Test Material Raw material (supplier) Production method	No .Layers Thickness Later size Oxidation level Other	Processing steps	Cell line	Exp. Conc. (mg/L) [duration]	Assay/ Endpoint EC_{50} (mg/L) or LOEC	Observations	Reference
2.	**GO1** (Grupo Antolin Ingeniería, SA (Burgos, Spain)) **GO2** (Graphenea (San Sebastián, Spain)) **CNF** (Grupo Antolin Ingeniería. SA (Burgos, Spain))	GO1 Single or few layers GO2 Single layer CNF helical-ribbon nanofibre Graphitization 90%	GO1,CNF Sonication (bath) (30 min) Centrifugation (1300 x g for 30 min) GO2 Nanogenotox protocol (Jensen et al., 2011)	Rainbow trout, *Oncorhynchus mykiss*, primary hepatocytes	GO1: ≤80 GO2: ≤128 CNF:≤40 L-15 medium ± 10% FBS	GO1 LOEC 24 h/ 72 h AB: 5/2.5 CFDA-AM: 5/5 GO2 LOEC 24 h/ 72 h AB:16/16 CFDA-AM: 8/8 CNF LOEC 24 h/ 72 h AB: 10/10 CFDA-AM: 5/5	Exposure in FBS free medium increased cytotoxic potential and influenced uptake of CNF materials	Kalman et al., 2022
3.	**GO** Graphite Modified Hummers method (Kovtyukhova et al., 1999) Aqueous suspension	Single layer 100 nm	Sonication (bath) (1 h)	BF-2 bluegill sun fish, *Lepomis macrochirus*, cell line	10–100 EMEM	24 h MTT:~20 NR: ~40	Dose dependent lipid peroxidation, reduction in GSH levels,i ncreased activity of SOD, and ROS and 8-OHdG levels	Srikanth et al., 2018
4.	**GO** (ACS Material (Ames, IA, USA)) **CXYG** carboxyl graphene (ACS Material (Ames, IA, USA))	Single layer C/O ratio:1.67 C/O ratio:1.59	Sonication (bath) (30 min) Centrifugation (1300 x g for 30 min)	PLHC-1 topminnow fish, *Poecilipis lucida*, hepatoma cell line	GO: 0.125–16 CXYG: 0.25–32 α-MEM+ (5% FBS)	GO LOEC CFDA-AM: 0.125 MMP: 16 CXYG LOEC CFDA-AM: 0.125 MMP: 16	Increased metabolic activity according to AB assay Increased ROS levels Evidence of graphene sheets piercing cell membrane	Lammel and Navas, 2014
5.	**CNF GANF®** (Grupo Antolin Ingeniería (Burgos, Spain)) Catalytic vapor deposition **CNF GANFg** (Grupo Antolin Ingeniería (Burgos, Spain)) Superheating (1500 °C) GANF® **CNF GATam** (Grupo Antolin Ingeniería (Burgos, Spain)) Scaled-up production of GANF®		Nanogenotox protocol: EtOH prewetting, Sonication (probe) (16 min) ± BSA 0.05% Continuous stirring	PLHC-1 topminnow fish, *Poecilipis lucida*, hepatoma cell line CLC carp, *Cyprinus carpio*, leukocyte cell line	≤ 256 Eagle's minimum essential medium (5% FBS) ≤ 256 Eagle's minimum essential medium (10% FBS)	GANF® PLHC-1: 72 h (+BSA) AB: 175.8 (>64) CFDA-AM: >256 (>256) NR: >256 (>256) CLC: 72 h (+BSA) AB: 36.9 (18.9) CFDA-AM: 51.4 (37.6) NR: >256 (>256) GANFg PLHC-1: 72 h (+BSA) AB: (>256) (>32) CFDA-AM: >256 (>256) NR: >256 (>256) CLC: 72 h (+BSA) AB: 101.2	Interference checks showedi nterference with fluorescence readouts at high conc.	Barrick et al., 2019

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Table 5 (*continued*)

Test Material Raw material (supplier) Production method	No .Layers Thickness Later size Oxidation level Other	Processing steps	Cell line	Exp. Conc. (mg/L) [duration]	Assay/ Endpoint EC_{50} (mg/L) or LOEC	Observations	Reference
					(>32) CFDA-AM: 220.9 (252) NR: >256 (>256) GATam PLHC-1: 72 h (+BSA) AB: 160 (>128) CFDA-AM: >256 (>256) NR: >256 (234.4) CLC: 72 h (+BSA) AB: 26.1 (46.7) CFDA-AM: 49.7 (89.9) NR: 215.6 (>256)		

CNF; carbon nanofibre, AB; alamar Blue, CFDA-AM; Carboxyfluorescein Diacetate, Acetoxymethyl Ester, NR; Neutral Red, MTT; 2,5-diphenyl-2H-tetrazolium bromide assay, MMP; mitochondrial membrane penetration, FBS; fetal bovine serum, EMEM; Eagles minimum essential with non-essential amino acids, EMEM+; Eagles minimum essential medium with non-essential amino acids and Na Pyruvate without l-Glutamine, and supplemented with 1% l-glutamine, 1% penicillin/streptomycin, EMEM-EB; Eagles minimum essential medium with Earle's Balanced Salt Solution without l-Glutamine and supplemented with 1% l-glutamine, 1% penicillin/streptomycin, 1% non-essential amino acids, L-15; Leibowitz's 15, α-MEM: Minimum essential medium supplemented with 1% l-glutamine, 1% penicillin/streptomycin, LOEC; Lowest observed effect concertation, MMP; Mitochondrial membrane potential assay, GSH: Glutathione, ROS; reactive oxygen species, SOD; Superoxide dismutase, 8-OHdG; 8-hydroxydeoxyguanosine, BSA; bovine serum albumin, C/O; carbon/oxygen, ROS;Reactive Oxygen Species.

concentrations are achieved and maintained in the experimental setup and during the exposure period. This can lead to uncertainties and a reduction in reliability of the data. In studies which monitored the exposure concentration (using UV–Vis spectroscopy) rapid settling and sedimentation of large sized GO was evidenced at concentrations >2 mg/L over time with complete loss of concentration over 72 h (Martínez-Álvarez et al., 2021; Chen et al., 2016). Authors have made reference to the observation of "megascopic precipitates" immediately after preparation of concentrations of 50 mg/L GO (Chen et al., 2016). Thus, in exposures performed with concentrations of 100 mg/L (with lack of measurement/renewal) it is likely that graphene test materials were not maintained in suspension and thus fish were not exposed to nominal concentrations for the duration of the exposure phase.

According to TG 203, a series of five concentrations should be used. Concentrations tested for GO nanoforms ranged from 0.01 to 100 mg/L (nominal) and 0.86 to 6.43 mg/L (measured), with concentration series of only two to four concentrations being used. In some cases, the actual concentrations measured in the water column were either similar to the nominal concentrations or radically different depending on the different concentrations prepared. In a test concentration series prepared at 5, 10 and 50 mg/L, concentrations of 4.7, 6.4, 3.5 mg/L, respectively were measured. Therefore, the loss of material in the water column due to rapid precipitation in the higher concentrations led to fish actually being exposed to similar concentrations (Chen et al., 2016). This highlights how testing of unstable dispersions can lead to erroneous conclusions and may explain why in some cases a lack of concentration dependent effects have been evidenced. According to TG 203 there must be evidence that the concentration of the chemical being tested has been satisfactorily maintained, and it should be at least 80% of the nominal concentration throughout the test. In the only examples of maintenance of exposure concentrations within the collected studies FLGs were tested at concentrations of 0.05–0.25 mg/L (nominal) and 0.045–0.24 mg/L (measured), with direct probe tip sonication affording the maintenance of nominal concentration in suspension, and with no mortality after 72 h exposure (Lu et al., 2017). Nominal concentrations of 5 mg/L of a GO material tested were also maintained according to measured concentrations of 4.7 mg/L 24 h after exposure and a renewal of 50% of exposure medium daily was used to maintain concentrations. No mortality was evidenced over 14 days of exposure to this tested GO (Chen et al., 2016).

In a number of studies, renewal of exposure medium daily (Hu et al., 2019) or at distinct timepoints (e.g. every 48 h or 72 h) (Jia et al., 2019; Qiang et al., 2016; Souza et al., 2018; Audira et al., 2021) was performed in what has been described as a renewal of exposure medium protocol. However, without prior information on material sedimentation rate and the concentration that will remain in the water column over time, the frequency of renewal of concentration needed would be unknown. Despite this, very few studies incorporated (preliminary) assessments of particle stability (concentration maintenance) that would aid in establishing an experimental setup to ensure the maintenance of a constant exposure concentration. For example a series of quantum dots were tested (2–50 mg/L) (nominal) using a renewal of exposure medium approach every 24 h, and while no mortality was reported up to 7 days of exposure, stability (concentration maintenance) tests were not performed (Hu et al., 2019). Also it is clear that stability is likely influenced by the presence or absence of fish. For example, Lu et al., 2017 performed a time course stability experiment monitoring at 4, 12, 24, 48, and 72 h and showing distinct settling behaviours for different sized single and few layer graphene, both in the presence and absence of fish.

In summary, lethality was not reported in fish for any of the graphene materials at any of the concentrations tested. However, this information must be interpreted with caution due to a lack of measurement of actual exposure concentration and/or maintenance of 80% of nominal concentrations during the exposure period in most cases. Abnormalities/

clinical signs evidenced included alterations in locomotor activity, exploratory behaviour, and predator avoidance associated with a change in neurological behaviour in *D. rerio* exposed to GO (few layer, 3.0 μm) and rGO (few layer, 5.4 μm) at nominal concentrations of 0.1 and 1 mg/L and 0.1 and 0.5 mg/L, respectively (Audira et al., 2021). Effects in the brain including a reduction in acetylcholinesterase (AChE) activity and loss of dopaminergic neurons in the brains of *D. rerio* exposed to GO (few layer, 0.5 μm- <13 μm) at concentration of 2 mg/L were also reported (Martínez-Álvarez et al., 2021). This, together with gill and liver histopathological and biochemical alterations following exposure to other GO nanoforms, may indicate potential chronic effects that warrants further investigation and testing (Souza et al., 2017; Chen et al., 2016). Furthermore, specific investigations showing epigenetic effects for quantum dots and different effects according to specific functionalisations were evidenced (Hu et al., 2019).

2.2. Weight of evidence (WoE) approaches to toxicity assessment and hazard classification

Testing with fish embryo's using the OECD TG 236 Fish Embryo Acute Toxicity (FET) test and also the newly published OECD TG 249 for acute toxicity assessment using fish cell lines both represent alternative standardised tests that can be used in a weight of evidence (WoE) approach for fish acute toxicity and hazard assessment (Sobanska et al., 2018). They also represent new approach methodologies (NAMs) for fish acute toxicity assessment that would save time and resources (Mittal et al., 2022). Incorporating such standardised NAM tests into an integrated approach to testing and assessment (IATA) contributes to increasing certainty/reliability while also advocating for 3Rs and alternatives to animal testing (Paparella et al., 2021). However, as with OECD TGs 201, 202 and 203, these approaches have not been formally adopted for NM testing and thus an evaluation of their applicability/suitability when testing NMs is required. Specific concerns for their use when testing NMs relate to the additional considerations regarding exposure dose, as in both cases cells/embryos residing at the bottom of culture well plates will be exposed to materials that sediment in unstable systems. Also, an additional consideration when testing embryos is that they have a chorion, which acts as a protective layer but also may limit NM uptake. Following a review of published literature all studies related to the testing of graphene materials using fish embryos were collected and are presented in Table 4. Using a similar approach, all studies which used fish cell lines and report on the cytotoxicity of graphene materials were gathered and are presented in Table 5. A review of the available data has been provided in the following sections focusing on the value of the specific tests in a WoE approach for graphene material hazard assessment.

2.2.1. Fish embryo acute toxicity (FET) testing

The standardised test used for testing fish embryos for acute toxicity is the TG 236 FET test which was published as an OECD test guideline in 2013 and while not considered as a stand-alone test for hazard classification purposes it is seen as a useful test in a WoE framework for fish acute toxicity assessment (OECD, 2013b). According to this test, embryos are exposed to a test substance for 96 h, and acute toxicity is determined based on the positive identification of any of the four apical effects: (*i*) coagulation of fertilised eggs, (*ii*) lack of somite formation, (*iii*) lack of detachment of the tail-bud from the yolk sac, and (*iv*) lack of heartbeat. Observations are made for these apical effects at 24, 48, 72 and 96 h of exposure. Hatching is also monitored from 48 h onwards until the end of the test to allow the derivation of hatching rates. LC_{50} values are then calculated according to percentages of embryos scoring positive for apical effects at a specific observation timepoint (e.g. 48 or 96 h).

A total of 25 studies have been collected from the literature that have used fish embryos (24 with *D. rerio*, and one with Japenese medaka, (*Oryzias latipes)*) to test the acute toxicity of graphene materials (Table 4). Seven of these studies specifically have referred to the use of TG 236 when performing testing. In one of these seven studies a commercialised testing kit (Danio Assay™) and 24 h post fertilisation (hpf) embryos have been used for exposures (Shamsi et al., 2020), while in the rest general methodologies consistent with OECD TG 236 test setups have been followed. For example embryos 2–6 hpf have been used in all but two of the studies, which in these specific cases either 24 hpf embryos (Mullick Chowdhury et al., 2014) or 72 hpf embryos were used for exposures (Ren et al., 2016). Observations for signs of mortality were made following exposure durations of 96 hpf in most cases. This observation period was extended sometimes to 120 hpf and in some cases embryos were exposed for durations up to 5–7 dpf (days post fertilization, larval stages) (Yan et al., 2020; Deng et al., 2019; Chen et al., 2020; Zhang et al., 2017a, 2017b). In one particular study embryos were injected with the material through an intravenous microinjection (Xiong et al., 2020), while in another the chorion was removed from embryos prior to exposure (fish embryo dechorionation) (de Medeiros et al., 2021). In all other studies embryos were exposed in appropriate test medium (e.g. E3 medium, deionized (DI) water, artificial (AF) water) in cell culture plates (6, 24, 96-well) under appropriate test conditions (pH, temperature, photoperiod) with even the testing of a positive control, 3,4-dichloroaniline in one of the studies (Ye et al., 2018). Despite an emphasis on the maintenance of exposure concentration in TG 236, overall, tests for stability were missing in most of the collected studies. However, a proportion of studies (12/25) did use a semi-static dose renewal system, as detailed in TG 236, which likely contributes to aiding the maintenance of nominal concentrations of dispersed graphene materials in the water column. One author has reported modest losses of 10–12% of GO concentrations after 96 h under test conditions (embryo culture medium, up to 160 mg/L) (Ye et al., 2018). However, stability is likely to be distinct for different materials and concentrations tested. This also has potential implications for the exposure dose as embryos may be exposed to higher concentrations of materials which have aggregated and settled out of suspension. Therefore the complete lack of attempts to measure exposure concentrations must be noted and identified as a critical issue that has been neglected in test setups to date. According to the OECD TG 236 it is strongly recommended that results be based on measured concentrations. Instead effects (e.g. LC/EC_{50} values) reported in studies were based on nominal exposure concentrations. Further considerations are also needed when testing NMs, to characterise the contribution of the concentration of material that may settle out of dispersion between renewals to the exposure dose and toxic effects. This was not considered in any of the collected studies. However, in exposures using a semi-static multi-well test setup and renewals every 24 h, graphene material aggregates were evident at the bottom of wells and adhered to embryos chorions (Liu et al., 2014).

Regarding the different graphene materials tested, GO was the most frequently tested (18/25 studies). In fact the same GO material [XF002–1] was used in two studies (Zhang et al., 2017a; Ren et al., 2016), otherwise the materials varied widely and included a range of different GO from different suppliers as well as prepared in house. Different sized materials of GO (nanoGO, USGO, S-GO, M-GO, S-FLG) and GO with specific functionalisations (e.g. –COOH functionalised, AgNP functionalised, PVP, PEG, Pluronic F127 (PF)-functionalized GO) were all assessed for embryo toxicity. rGO was tested to a much lesser extent and only in two studies (Liu et al., 2014; Martínez-Álvarez et al., 2021). Other materials studied included a pristine single-layer graphene flake (pG) (Manjunatha et al., 2018), graphene nanoribbons (GNR) (Mullick Chowdhury et al., 2014) and in one particular study radiolabelled FLG materials (Su et al., 2017) were investigated. GO quantum dots (Yan et al., 2020; Wang et al., 2015) and also rGO quantum dots (rGOQDs) (Zhang et al., 2017b; Deng et al., 2019 (nitrogen doped)) were also among the materials tested (Table 4).

In most studies a concentration series was included with a wide range of concentrations from as low as 0.0001 mg/L to 1000 mg/L

tested. Endpoints of mortality/survival and apical endpoints of malformations were included. The lowest concentration at which mortalities (>10%) were reported for GO was at 10 mg/L with associated malformations at 1 mg/L 120 hpf (Yang et al., 2019). For all other GO materials tested, mortalities and malformations occurred at higher concentrations (20–100 mg/L), with a reported LC_{50} of 63 mg/L for GO 96 hpf (Ye et al., 2018) consistent with the 50% mortality reported by other authors at 60 mg/L GO (Shamsi et al., 2020). GQDs also showed significant toxicity at these concentrations (>10% mortality at 50 mg/L (Wang et al., 2015)), that appeared to be abrogated when testing reduced forms (significant effects only at concentrations ≥100 mg/L).

The most toxic material tested was a pristine graphene flake (pG) with mortalities of 25% reported 96 hpf following exposure of embryos to concentrations as low as 0.025 mg/L and malformations, including pericardial/yolk sac edema, evidenced at even lower exposure concentrations (0.005 mg/L) (Manjunatha et al., 2018). Also it is worth noting that when the same GO [XF002–1] material was tested using 72 hpf embryos, mortality (~6%) was evidenced at concentrations as low as 0.001 mg/L and malformations at even lower concentrations (0.00001 mg/L) at 120 hpf (Ren et al., 2016), while in embryos exposed through embryonic and larval stages (2.5 hpf-7dpf) only concentrations ≥0.01 mg/L caused mortality (~6%) and malformations (Zhang et al., 2017a). Such differences in sensitivity were also evidenced when testing other NMs (e.g. AgNMs and MWCNT) with different Zebrafish life stages and when applying the OECD TG 210 Fish, Early-life Stage Toxicity Test (Shaw et al., 2016).

In studies where mortality was not evidenced, there were observations of sublethal effects, in particular associated with neurodevelopmental toxicity. There were also evidences of crossing of the chorion barrier (Mullick Chowdhury et al., 2014), metabolic disturbances, perturbations of specific pathways (AhR, PPAR-a mediated MAPK signalling) (Xiong et al., 2020) that warrant further investigation.

The data collected would suggest that fish embryo testing is a sensitive test to aid in assessing the acute toxicity of graphene materials and that TG 236 is a useful tool for hazard assessment. The obvious critical issues that would increase the reliability and ensure TG 236 validity criteria is met when testing graphene materials is the introduction of stability monitoring and measurement of exposure concentrations taking into consideration also the fraction that may have sedimented and adhered to organisms during exposure. This also brings into question the extent to which these materials are available and the sensitivity of embryos with chorions to toxic effects of NMs. However, according to the effects evidenced from the collected studies, fish embryos appear as a more sensitive test organism to the toxic effects of graphene materials compared to fish, and thus act as a sufficiently sensitive and protective test model for use in a WoE approach.

2.2.2. *Fish cell lines*

The newly published OECD TG 249 (OECD, 2021a) details a standardised fish cell line acute toxicity test performed using the RTgill-W1 rainbow trout gill fish cell line, and by assessing the levels of viability following 24 h exposure to a test substance. The test uses three cytotoxicity assays to monitor a reduction in metabolic activity (alamarBlue™), disturbance in plasma membrane integrity (5-carboxyfluorescein diacetate acetoxy methyl ester (CFDA-AM)) and lysosomal disruption (Neutral Red dye). Its use for the generation of toxicity information for hazard assessment in combination with other lines of evidences for acute toxicity in fish has recently be reviewed (Belanger et al., 2022). It also incorporates an analytical measurement step, in which exposure concentrations are analysed. as well as checks for potential interferences of test materials with the assay/assay readouts (e.g. fluorescence measurements). To date there is no published data on the use of the RTgill-W1 cell line for testing graphene. Instead tests have been performed using a fibroblastic cell line derived from the caudal fin of bluegill sunfish (*Lepomis macrochirus*) (BF-2) (Srikanth et al., 2018), a topminnow fish (*Poeciliopsis lucida*) hepatoma cell line (PLHC-1) (Kalman et al., 2019; Lammel and Navas, 2014; Barrick et al., 2019), a common carp (*C. carpio*) leucocyte cell line (Kalman et al., 2019) and primary hepatocytes isolated from rainbow trout (*Oncorhynchus mykiss*) (Kalman et al., 2022) (Table 5). While in these studies no standardised protocols were followed, general cell culture techniques for culturing, seeding and exposures of cells to graphene materials were used according to the cell lines necessities (specific medium, temp. etc.). In all of these studies the cells were exposed for a period of 24 h (some 72 h) and viability was assessed using all or some of the assays selected for use in TG 249. The materials tested included GO from different suppliers as well as a GO synthesised in house, a functionalised (carboxy) GO and helical-ribbon carbon nanofibers (CNFs) graphized to varying degrees (60–90%). Considerations for the presence and contribution of any impurities in the materials tested following the various synthesis processes were made by Kalman and colleagues (Kalman et al., 2019). In this study cells were also exposed to filtrate controls to assess the contribution of Ni residues to the toxicity of CNFs. Interference checks from tested materials with assays were performed and reported at high concentrations for certain materials with fluorescence readouts (Kalman et al., 2019; Barrick et al., 2019). $EC_{50\ 24\ h}$ values of 20 and 40 mg/L according to the tetrazolium-based MTT and neutral red (NR) (TG 249 specific assay) assays, respectively were reported for GO using the BF-2 cell line (Srikanth et al., 2018). While an $EC_{50\ 24\ h}$ value of 122 ± 21 mg/L was measured following GO (GRAnPH®) exposure in the PLHC-1 cell line (Kalman et al., 2019). All other $EC_{50\ 24\ h}$ values could not be calculated as they were above the tested concentrations or at concentrations at which interferences could not be distinguished from effects. However, dose dependent effects were evidenced with LOECs of 0.125–16 mg/L depending on the cells used, assay employed, and GO material tested (Kalman et al., 2022). In one of the studies, longer exposure durations were used and served to enable the calculation of $EC_{50\ 72\ h}$ values which ranged from 74 to 191 mg/L for the graphized CNFs (Kalman et al., 2019).

One must be aware that reported exposure concentrations and EC_{50} values from these studies are based on nominal concentrations prepared in working suspensions. Quantification of test concentrations is prescribed in TG 249 and involves taking a sample of the exposure medium at the start and end of exposure to allow calculation of the geometric mean of the measured test chemical concentrations. Such an approach has not been performed in any of the studies presented in Table 5. The stability of these materials under culture conditions will influence the exposure dose, as in such an experimental setup any loss of the material through settling/sedimentation will lead to higher concentrations of material in direct contact with cells seeded at the bottom of culture well plates. If this occurs, it creates an uncertainty and inconsistency in exposure levels. There are indications from some of the studies which performed a characterisation of the hydrodynamic size distribution of graphene materials in cell culture media over time that there is a strong tendency for some GO materials to aggregate and they have a low stability in cell culture medium (e.g. L-15 medium) (Kalman et al., 2022). Also differences in behaviour/stability when using serum free medium was evidenced, with direct observations of graphene material covering the cell layer using optical microscopy in serum free culture media, providing direct evidence that settling/sedimentation occurred (Kalman et al., 2022). Thus, considerations for the contribution of this settled fraction to toxicity are needed and must be accounted for. Differences in sensitivities between different cell lines and increased toxicities were evidenced in some cases using longer exposure periods (e.g. from 24 h to 72 h). However, as factors such as the distinct culture media used with different serum protein levels may have a direct influence on stability, this also may cause differences in exposure levels making direct comparisons and overall conclusions difficult.

In summary, based on the collected studies, albeit scarce (5 studies in total), testing using fish cell lines represents a very useful approach to data generation to aid in hazard assessment and should be availed of when testing graphene materials in the future. The use of TG 249 will aid

in standardising the approach to testing using fish cell lines and if followed will ensure that the exposure concentration is quantified. However, the use of approaches to accurately measure or estimate the effective dose of NMs delivered to cell cultures will need to be validated (Botte et al., 2021). Also while all of the limitations/interferences posed by NMs (e,g. reactivity, inherent fluorescent/absorbance) when using cell culture testing platforms apply to tests with graphene materials, further considerations may be needed. Such considerations relate to the unique graphene material-cell culture medium interactions (corona formations) controlled by the laminal materials structure (e.g. sheet size) and surface chemistry that to date are not fully understood (Franqui et al., 2019). These interactions can lead to cellular deprivation of nutrients (nutrient depletions) or contrastingly intracellular enrichment of certain nutrients (through material uptake) that could have a knock on effect on cellular bioactivity. However, as to date no studies have been published using this TG for testing graphene, its real value and applicability for testing such 2D NMs remains to be seen. Advanced characterisations of processes and interactions of these materials in the TG 249 L-15 ex exposure medium will provide valuable information. Overall, further investigations on this TGs applicability will shed some light on how it can contribute to the generation of data that are meaningful and that can be used in WoE approaches for graphene and other 2D material fish acute toxicity assessments.

2.3. Specific factors to consider when applying standardised OECD tests for testing graphene materials

The possible need for adaptations to standard ecotoxicity tests for testing NMs was first eluded to in early publications (Crane et al., 2008). Progress has now been made in identifying areas in which changes, developments, and standardisations may be needed (Boros and Ostafe, 2020; Nasser and Lynch, 2019). OECD GD 317 has been published to aid in the application of existing OECD TGs on aquatic (and sediment) toxicity testing to allow more reliable and reproducible data generation for NMs, and it includes a section with advice on specific modifications for specific TGs when testing NMs (OECD, 2022a). Among the particular TGs mentioned, and for which it has been recognised that additional considerations are needed, are TG 201 and TG 236. In addition there are also publications dedicated to the specific considerations which may be needed when testing certain types of NMs (e.g. Nanobiomaterials; Amorim et al., 2020), albeit with no specific reference to TG 201, 202, 203 or 236.

In the following section, based on the observations made via this comprehensive review, a number of factors to consider when performing acute aquatic toxicity testing of graphene materials using these TGs have been identified. These specific factors will be detailed under the relevant subheadings concerning test dispersion preparation, the conduct of tests and data analysis and reporting. Specific guidance tailored to the needs of acute aquatic toxicity testing of graphene materials is highlighted in Table 6 and 7 and 8. This specific guidance goes beyond the generic guidance given in GD 317 by drawing on evidence from all the studies performed with 2D graphene materials. This additional information contributes to the generation of a more prescriptive guidance to facilitate the increased use of OECD TGs for testing NMs such as graphene materials in the future.

2.3.1. Test stock dispersion preparation

For aquatic toxicity testing, normally aqueous (stock) dispersions of graphene materials are applied to the test systems/vessels of respective tests, by dosing test media to generate the test concentrations required for testing. The majority of the graphene materials tested were supplied/produced in powder form and therefore needed to be dispersed prior to testing. The dispersion method heavily relied upon to prepare aqueous (stock) dispersions of graphene in the studies reviewed was ultrasonication. The protocols used were highly variable, and included the use of different sonication techniques (probe or bath sonication, continuous or pulsed modes) and different time scales (from 10 to 15 min up to 4–6 h). Use of ultrasonication, and particularly probe sonication, may cause a significant change in material properties. For example, breaks in flakes have been evidenced after probe sonication (Baig et al., 2018) and strikingly different size distribution profiles were evidenced when using bath or probe sonicators to prepare graphene nanoribbon stock dispersions (Mullick Chowdhury et al., 2014) and after short and prolonged sonication times (30 min vs. 2.5 h) (Jiang et al., 2021). In fact, prolonged sonication times were used to achieve dispersions of materials with smaller sizes for comparative testing. These distinct dispersions, produced using prolonged sonication times, have shown different interactions with organisms including enhanced membrane penetration ability (Su et al., 2017), increased cytotoxicity to cells (yeast and lung epithelial cells) (Jiang et al., 2021), and increased fish embryo mortality (Mullick Chowdhury et al., 2014). Aggressive sonication techniques have also been shown to increase defects (detected according to increased ratios of the intensity of D- Raman peak and G-Raman peak (I_D/I_G) of 1.3 to 2.3) (Mullick Chowdhury et al., 2014), and likely also contributes to an increased toxicity as evidenced in the case of graphene nanoribbons, which, due to their form, may prove particularly susceptible to material transformations under such conditions. Besides considerations for sonication type and time, one must consider the power and total ultrasonication energy (kJ) applied. This is rarely reported in studies making comparisons problematic. Occasionally the electrical input and output power were specified, however it is the effective acoustic power that is delivered to the suspension that is critical and that can be measured using calorimetry. An excellent attempt towards a standardisation of ultrasonic dispersion of NMs has been made by Taurozzi and colleagues describing the calorimetric method and detailing the parameters and material characteristics recommended for reporting purposes (Taurozzi et al., 2013). The total power delivered will also determine the dispersion state, with an alteration of chemical structure (exfoliation and destruction of surface groups) being shown to occur at sonication energies of 100 kJ (Dimou et al., 2021). At these high energies there is also an increase in temperature and this must be controlled as it may also influence a change in material form due to heating. Therefore careful consideration is needed regarding initial dispersion method selection and a thorough characterisation of the dispersed material, including the characterisation of any potential changes in nanoform caused by the processing, is essential and must be assessed (see section 2.3.3). On one hand, according to GD 317, the

Table 6
General and specific factors to consider towards a prescriptive guidance for aquatic testing of graphene materials related to stock dispersion preparation.

	Recommendation	
Dispersion Preparation	GD 317	Graphene material specific consideration
Method/ Protocol selection	Begin with evaluation of material stability in ultrapure water Use of dispersion method described in OECD TG 318 as a starting point	Dispersion method described in TG 318 may not be suitable. Use of less aggressive approaches (e.g. bath sonication) that may cause less defects and breaks
Use of sonication	Beyond the GD scope but its use should not alter the test material or medium and an optimum sonication time and energy should be considered	Sonication time and energy should be optimised to achieve minimum agglomerate/particle size distribution without altering material properties (e.g. introducing breaks or defects)
Use of dispersants	It is preferred to avoid the addition of stabilizing substances The stability of the stock dispersion should be evaluated if it is to be reused for media renewal during the test	To avoid the need for use of dispersants, freshly prepared stock dispersions can be generated for each renewal

Table 7
General and specific factors to consider towards a prescriptive guidance for aquatic testing of graphene materials related to the conduct of tests.

Test Conduct	Recommendation	
	GD 317	Graphene material specific consideration
Exposure concentration/ dosimetry	Should be measured in the highest and lowest test concentration at the start and end of test, and before and after water renewals (if used).	Limitation in analytical sensitivity (UV–Vis) in measuring low concentrations (<1 mg/L). More robust analytical techniques required.
	Should be maintained at ≥80% of the nominal chemical concentration throughout the test. Averaging, time-weighted averaging, or geometric mean approaches can be used if concentrations not maintained at 80%.	Low stability under test conditions but such approaches to date have not been used in studies
	Considerations needed for contribution of any settled fractions to exposure dose.	Both overlying and sedimented dose must be considered if performing renewals (e.g. TG 236).
		In static systems using submerged cells (e.g. TG 249) effective dose during exposures should be considered
Methods for concentration maintenance	Agitation can be used in specific tests (TG 201, 203) but such protocols must be optimised using pre-tests.	Agitation protocols need to be developed on a graphene material and test specific basis.
	Semi-static water renewals can be used in specific tests (e.g. TG 203, TG 236) but renewal frequency must be dictated by concentration measurements.	Renewals can be applied, but there are limitations in analytical sensitivity (UV–vis can be used for approximations). Water renewals may also be an approach for use in TG 202.
	Potential settling effects should be considered (e.g. fish embryo testing). Caution embryos not sensitive to drying effects when performing concertation renewals.	Agitation may also be appropriate in other tests (e.g. TG 236 and TG 249), to address uncertainty and inconsistency in exposure levels due to test materials settling and improve dispersion.
Media supplementation/ modification	General considerations for performing test media adjustments are provided. Environmental realism issues are beyond the scope of this GD. DOM/NOM can be used on case basis but minimum amount to achieve stability and controls needed.	Use of standardised media (when described) in tests to ensure comparisons between studies (e.g. TG 201 medium, L-15/ex medium). NOM has a stabilising effect but also reduces the toxicity of the graphene material and therefore it is advisable to avoid its use. Include controls and also tests with materials without NOM.
Media interaction	Possible adsorption of media components and depletion of nutrients in test dispersions need specific consideration when testing NMs. No guidance provided.	Nutrient depletion effect evidenced and caused by the adsorption of Ca^{2+} and Mg^{2+} to graphene materials. Approach using materials pre-conditioned in media proposed to negate effects. Pre-tests should be used to assess media interaction.
Interferences	Shading effects in particular with TG 201. Efforts to reduce or quantify these effects should be made in pre-tests or parallel tests.	Specialised experimental setup should be used to assess and quantify shading effect (see Zhao et al., 2017).
	Measurement interference of the tested NM in the test dispersion on the toxicity endpoint to be measured should be evaluated.	Fluorescence quenching evidenced for graphene materials with TG 249 assay readouts.
	Physical interferences (e.g. hetero-aggregation may cause a reduction in algal cell number in TG 201)	Hetero-aggregations evidenced to cause a reduction in algal cell number. Pre-tests or parallel tests should be used to assess physical interferences.

method used to disperse NMs should be optimized to achieve the smallest agglomerate size, narrowest distribution and adequate dispersion stability, yet the use of sonication should not alter the test material or medium. Therefore, careful selection of a dispersion method should be made for graphene materials. However, to date, to our knowledge, no standardised protocol specifically for graphene material dispersion has been developed. Dispersion protocols such as those described in TG 318, and advocated for as a starting point in GD 317, use probe sonication, however a less aggressive dispersion technique (e.g. bath sonication) may be more appropriate for graphene material dispersion preparation. For example, using probe sonication and the generic nanogenotox dispersion protocol, which has been developed within the EU project NANoREG to facilitate a standard operating procedure (SOP) for NM dispersion (Jensen et al., 2011), graphene material dispersions were prepared with 7056 J of delivered acoustic energy (Barrick et al., 2019). While not characterised in this study, increases in defect ratios (I_D/I_G of 0.07 to 0.2) and fragmentation of FLGs have been evidenced when such energies were applied (Baig et al., 2018). Another approach applied when preparing graphene material stock dispersions was the use of dispersants. For example, and again following the SOP of the nanogenotox protocol, bovine serum albumin (BSA) was used as a dispersant (Barrick et al., 2019). While it improved the stability of dispersions, it also led to changes in the interaction between graphene and organisms in *Daphnia* studies (e.g. decreased adherence to body and lower toxicity). Also distinct EC_{50} values were observed in fish cell lines depending on the addition of BSA in the stock dispersions (Barrick et al., 2019) (Table 5). The addition/use of dispersants for stock dispersion preparation must be considered in the context of the test design and can be differentiated from their use in working test dispersions (discussed later in section 2.3.2.2). Fresh stock dispersions serve to prepare working test dispersions at the start of the test, thus stability of these dispersions over an extended timeframe is only relevant if the same stock dispersions are going to be used for water renewals. Also it is preferred that the use of stabilising agents is avoided (GD 317) and not advocated for (GD 23). In this particular example BSA has been used as a dispersant, and in the context of environmental relevance this material is not representative or found in aquatic environments.

Therefore regarding the preparation of stock dispersions of graphene materials, some specific factors to consider relate to the dispersion method or protocol selection, the effective acoustic power delivered when using sonication and the need for use of dispersants. Such factors are detailed in Table 6.

2.3.2. Conduct of the test

2.3.2.1. Exposure concentration. According to the OECD standardised TGs, working test dispersions should be analysed to measure exact exposure concentration, as a minimum, at the highest and lowest test

Table 8
Inherent and system dependent physico-chemical properties for graphene material characterisation and reporting.

Physico-Chemical property	Graphene material specific reporting	Monitoring Approach (Standardised TG and guidance documents available and/or being developed)
Chemical composition	Elemental composition [*identification based on sp^2 bonded carbon detection*] Oxygen content Raw material Production method Material form (e.g. powder, solution)	Raman spectroscopy (tip enhanced) XPS, ICP-MS, TGA, FTIR, XRD
Impurities	**%,** μg L^{-1} *e.g.* N-doping which is a substitutional impurity, S, and potentially toxic elements (Cd, Cr, Cu, Mn, Pb)	ICP-MS, ICP-OES
Surface treatment/ functionalisation	Edge or Surface chemical groups Oxidations	XPS/EDX OECD WNT project 1.6: Guidance document on identification and quantification of the surface chemistry and coatings on nano- and microscale materials (OECD, 2021b)
Particle size	No. of layers Layer thickness Lateral size **Size distribution [Number based distribution**; D10, 50, 90]	Raman spectroscopy/AFM//XRD OECD TG 125 (OECD, 2022c) Laser diffraction (ISO, 2020)
Shape/Aspect ratio	e.g. platelet, spherical, ribbon, fibre, quantum dot	TEM, rheo-SAXS
Crystallinity **Assembly structure**/ Orientation	(%) crystallite size (diameter (L_a)) e.g. stacking; Bernal, Rhombohedral, Turbostratic	XRD/Raman Raman spectroscopy
Defects (type and distance)	Zero, one or two dimensional defect type I_D/I_G ratio as measure of defect distance	
Surface area, including porosity	**Specific surface area by mass (m^2/g), or volume**	BET method (ISO 9277 (2010)) OECD TG 124 (OECD, 2022b)
Density	Bulk density (tapped) (graphene council framework)	Helium pycnometry TG 109 Density of Liquids and Solids (2012) (OECD, 2012); ISO 12154:2014 (ISO, 2014)
Hydrophobicity attachment efficiency (α)	Surface hydrophobicity	OECD WNT Project 1.7: New test guideline on determination of surface hydrophobicity of manufactured nanomaterials (OECD, 2021b) Maximum particle dispersion (MPD) (Li et al., 2022) Dark-Field microscopy (Valsesia et al., 2018) Dye adsorption method (e.g. rose bengal or nile blue dye partitioning) Contact angle hydrophobic interaction chromatography (HIC)
Solubility: Rate of dissolution / Equilibrium solubility*	Test for leaching of components/ production products	OECD WNT Project 1.5: Guidance document on determination of solubility and dissolution rate of nanomaterials in water and relevant synthetic biological media (OECD, 2021b) OECD WNT Project 3.10: New test guideline on dissolution rate of nanomaterials in aquatic environment ongoing (OECD, 2021b)
Dispersion stability	Concentration maintenance in dispersion under test conditions Zeta potential measurement **Aggregation/ agglomeration state**	OECD TG 318 (OECD, 2017), GD 318 (OECD, 2021c) Zetasizer folded capillary cells Dynamic light scattering
Surface reactivity (redox reactions)		TG 495 OECD (OECD, 2019e) Electron spin resonance (ESR) Ferric reduction ability of serum (FRAS) assay (Gandon et al., 2017)
Degradation/ Transformation /Surface chemistry changes	Physical breakage, enzymatic reactions, oxidation, hydrolysis, sulfidization, photocatalytic **Degradation or transformation products**	Dichlorodihydrofluorescin diacetate ($DCFH_2$-DA) assay Protein carbonylation ISO 20814:2019 (ISO, 2019a, 2019b); NADH monitoring Rhodamine-B dye degradation OECD TG 316 (OECD, 2008) OECD WNT Project 3.16: Guidance Document Environmental abiotic transformation of nanomaterials (OECD, 2021b) Degradation halftimes ($t_{1/2}$) (Kümmerer et al., 2011) OECD TG 309 (OECD, 2004b), TG 303 (OECD, 2001) OECD TG 301 (OECD, 1992) BIOLOG MT2 assay (adapted) (Cross et al., 2022) OECD TG 86 (OECD, 2018)

Note: properties in bold reflect those specifically required to fulfil obligations under REACH (Annex VI) (EC, 2006).

concentrations. In a static system, measurements should be taken at the beginning and end of the test, whereas in a semi-static system before and after renewals. This is used to verify the initial concentration and to assess if the exposure concentration can be maintained. Concentration measurement and stability assessment, related to the maintenance of 80% of nominal (measured) exposure concentrations is prescribed in TG 201, 202 and 203 as well as TG 236 for embryo testing. Despite this, in the studies collected overall there was a lack of analytical determination of graphene material test exposure concentrations and instead effective values were reported in the majority of studies based on nominal concentrations. While this could be explained by the poor availability of robust/available analytical techniques and procedures for graphene material quantification, effects based on nominal concentrations can only be relied upon in cases where stability and concentration maintenance is assured. UV–vis has been used by a number of authors for approximate estimations of initial concentrations. However, it has specific limits of quantification ((LOQ) (e.g. 3.21 mg/L for graphene materials (Marković et al., 2020)). On a number of occasions UV–Vis

spectroscopy was used to monitor any alterations in absorbance spectra in test dispersions over the exposure duration and any decreases were expressed as % loses of initial nominal concentrations. Results from these studies, as well as visual observations of precipitates, indicate that often in the tests performed nominal concentrations were not maintained and therefore uncertainties surround actual exposure concentrations in these studies.

The loss/change in the initial nominal concentration is related to the materials physical and chemical characteristics as well as to the molecules/compounds released by the organisms and specific media compositions. The materials tend to spontaneously aggregate/agglomerate because of (*i)* their hydrophobicity (as in the case of FLG, graphene and other very reduced graphene materials) or (*ii)* because of the presence of functional groups negatively or positively charged (as in the case of GO, rGO, and functionalised materials) that can coordinate ions in the medium and create cross-flakes bonds (drove especially by polyvalent ions). Furthermore, organisms such as algae can release molecules and compounds, such as extracellular polymeric substances (generally polysaccharides), that can agglomerate with the graphene materials causing their precipitation. These substances can be produced as a defence response to toxicants (Andrade et al., 2010) or for other physiological functions. In the case of graphene materials it was observed that these substance can help to slough off the materials from the algae surface (Garacci et al., 2017).

Any losses in the water column concentration, as well as reducing the exposure dose to free swimming test organisms (e.g. algae, fish), also may lead to increases in exposure doses of fish cells or test organisms such as fish embryos that reside at the bottom of test systems. In fact, often in these particular test setups and when testing materials susceptible to sedimentation, there is a particular contribution of this sedimented fraction to exposure that can be referred to as a delivered dose. In this field of NM *in vitro* dosimetry, models and approaches have been proposed and are being developed to estimate this dose (DeLoid et al., 2014; Botte et al., 2021). While very few studies report this delivered dose, once methodologies are developed and validated this will become very important information for interpreting NM *in vitro* test results. According to TG 249 the exposure concentration of a test substance to fish cell lines is quantified by measuring the concentration in suspension at the start of the test and at the end and a geometric mean is calculated to express the exposure concentration. While such an approach has been standardised for chemicals, further consideration may be needed when testing NMs to include also consideration for the delivered dose.

Therefore, in the context of NM and graphene material testing, the validity criteria in OECD TGs concerning the maintenance of exposure concentrations becomes increasingly important and even a special consideration of this issue is needed that may be extended to proposing specific suitable methods to achieve this criteria.

2.3.2.2. Methods for concentration maintenance. To maintain nominal exposure concentrations throughout testing, approaches already proposed by GD 317 for testing NMs include media modifications (pH, ionic strength/composition), adding turbulence (agitation/shaking), addition of stabilising substances (e.g. NOM/DOM) or introduction/modification of water renewals. However feasible and acceptable approaches differ depending on the specific TG: TG 201 allows agitation while TG 202 should be a static system, and in TG 203 both agitation and an exposure renewal can be used. According to TG 236 water renewals can be used, while none of the approaches are detailed in the test procedure of TG 249 and instead shorter (e.g. 4 h) exposures are prescribed for unstable test substances. In the collected studies agitation (hand or orbital shaking and magnetic stirring) of exposure dispersions of graphene materials was often used in algal tests, however, despite this, unstable dispersions and loss of concentrations during the 72 h exposure period were often reported. Specific modifications described in GD 317 for TG 201 when testing NMs relates to agitation of dispersion and the use of pre-tests to determine optimum mixing methods. In one of the collected studies (Barrick et al., 2019) choice of magnetic stirring over other approaches was based on a study by Manier et al., 2016 which compared orbital shaking and magnetic stirring regimens and showed that the magnetic stirring minimized the agglomeration and sedimentation of TiO_2 NMs. However the result is likely to be different for different NMs. In fact in the study of Barrick et al. (2019) magnetic stirring was applied to the three different types of graphene materials being tested and the differences in concentration maintenance for each ranged from 0 to 94%. In all other studies the rationale for selection of specific agitation methods (e.g. shaking by hand 3 times a day) was not provided, stability was not maintained and pre-tests as prescribed by GD 317 may have aided in optimising maintenance of the graphene materials in dispersion.

Such agitation approaches are not advocated for in TG 202, in order to maintain the organism's natural static environment and indeed sedimentation and losses of concentrations were often reported. However, concentration maintenance may not be as critical when testing using TG 202 as *Daphnia,* the test organism, is a filter feeder and it has been observed that any material which sedimented to the bottom of the exposure vessels were still bioavailable and taken up by the organisms (Malina et al., 2020). While not explored in any of the studies using TG 202 to date, an exposure water renewal approach could also be used to facilitate the maintenance of a constant exposure phase. Exposure concentration renewal approaches were used in the majority (8/13) of studies in the fish acute toxicity tests that were performed with graphene materials (Table 3). Exposure renewal approaches were also used in a large number of the studies (10/26) performed using embryos (TG 236) (Table 4). In both of these tests renewals were more commonly performed every 24 h, however in some cases longer or shorter (e.g. twice daily) intervals were used. In order for such an approach to ensure consistent exposures, pre-tests for stability assessment should be performed and will inform on the frequency of renewal that may be necessary to maintain exposure concentrations of at least $\geq$80% of the nominal starting concentration to be tested. These pre-tests were missing in the studies performed with graphene materials to date and if incorporated into future study designs water renewals with increased frequency may prove a feasible approach for use in TG 203 and TG 236. However, careful considerations must be given to the role of any material that has attached to the organisms during these renewal phases and its contribution to the overall exposure dose.

A standardised test guideline for NM stability assessment in simulated environmental media is available (TG 318, OECD, 2017). According to this test guideline, stability assessments are performed in media representing natural surface waters at specific pHs, concentrations of divalent ions and NOM and specific particle concentrations. However, the methodology, while not standardised or validated for stability assessment under specific ecotoxicity test conditions, can be adapted and serve also for testing stability in a range of different test media and at different concentrations. Also to be as representative as possible of test conditions, not only freshly prepared specific test media but also test media which has been held under test conditions and with organisms present (matured media) could be used. Such an approach would introduce also secretions from organism into the systems, which have been shown to have a direct impact on graphene material stability (Lu et al., 2017; Lv et al., 2018). Also these pre-tests can be performed in the same test vessels as prescribed in respective tests to represent test conditions.

Another approach for test concentration maintenance is the use of stabilising substances. According to GD 317, while this approach should be avoided, natural organic matter (NOM)/dissolved organic matter (DOM) may be permitted in certain cases where there is a strong desire to stabilise the test material. Humic acid (HA), the principal constituent of NOM, is used in some protocols for fish acute toxicity (e.g. EPA, 1996) while recently a standardised NOM (Suwannee River NOM (2R101N)) composed of various constituents (including HA) has been used in an

approach to allow the characterisation of NM stability under such conditions (OECD TG 318) (OECD, 2017). However NOM/DOM use has not been prescribed specifically in any of the OECD TGs discussed in this review for aquatic toxicity assessment. In the collected studies, organic matter has only been used on a few occasions (6 studies) and overall promoted exposure concentration maintenance in the various test media but also mitigations of toxic effects were evidenced. In algal tests the use of HA (20 mg/L) promoted GO colloidal stability and concentrations remained within (or close to) the limit of the 20% reduction of the nominal concentration. However $EC_{50\ 72\ h}$ values for GO increased from 66.60 to 242.78 mg/L when tested in the presence of HA (Castro et al., 2018). Other low molecular weight organic acids (LOAs), specifically benzoic acid (BA) and gallic acid (GA), also promoted colloidal stability of graphene materials in a concertation dependent manner, but to different degrees and with distinct effects on algal growth and toxicity (Wang et al., 2016). In tests using TG 202 and incorporating different concentrations of HA (5–25 mg/L) to GO test dispersions, reductions in agglomeration and defects were evidenced in the presence of HA and EC_{50} values for acute toxicity to *D. magna* increased from 84.2 to 111.4 mg/L in a HA concentration dependent manner (Zhang et al., 2019). In fish tests, the addition of Suwannee River NOM (10 mg/L) facilitated the maintenance of exposure concentrations $\geq$90% over 72 h when testing small FLG, however exposure concentrations of large FLG $\geq$75 μg/L could not be maintained at or above 80% (Lu et al., 2017), highlighting that specific considerations and adaptations for different graphene material forms may be needed. Similarly, in fish embryo tests, de Medeiros et al. (2021) have tested GO and GO-AgNPs in zebrafish embryo medium supplemented with 20 mg/L Suwannee River NOM and only evidenced the promotion of maintenance of exposure concentrations of GO over the exposure period (72 h). The presence of NOM did not improve maintenance of GO-AgNPs exposure concentrations and also mitigated the toxic effect of these materials to embryos (LC_{50} from 1.4 to 2.3 mg/L). Clemente et al. (2019) reported also the maintenance of GO exposure concentrations in reconstituted water, and a reduction in toxicity of the GO to zebrafish embryos when HA (20 mg/L) was used. According to GD 317, in cases where distinct EC_{50} values are obtained the most conservative EC_{50} values should be used for hazard assessment.

2.3.2.3. Media interactions. Working test concentration ranges are usually prepared from initial stock dispersions, in relevant test exposure/growth medium that vary in chemistry (e.g. ionic strength and ionic species, nutrients etc.) according to the requirements of the organisms/cells used in the specific TGs. There are already excellent articles on the influence of solution chemistry on NM dispersion and toxicity (Gao et al., 2009). Thus, the distinct media formulations used, as well as any supplements, are likely to influence material behaviour (stability) and results. For example, Castro et al. (2018) have provided a detailed characterisation of the stability of GO in a range of standard media used for testing aquatic species including algae and *Daphnia spp.*, demonstrating distinct dispersions and behaviours. Marković et al. (2020) showed distinct size distributions of the GO test material and toxicity when using MA-MS medium vs. the OECD TG 201 standard medium in tests with algae. The other media used in the TG 201 algal tests performed included: HB-4, ZBB, Oligo, G-11, SE, CSI, Simplified SE, and BG-11 (Table 1). In tests that exposed *Daphnia spp*. the media used included: distilled water, tap water, artificial freshwater, reconstituted water, ASTM hard water, Elendt M4 medium or a simplified Elendt M7 medium (SM7), as well as the standard TG 201 medium recommended for use in algal studies (Table 2). Similarly, in fish studies, the reported exposure media varied from freshwater, tap water (chlorinated or dechlorinated; UV-sterilised or non-sterilised), natural salt water or conditioned water (salts added) (Table 3). While in embryo testing, E3 culture medium was often used, and also deionised and artificial freshwater was utilised (Table 4). Thus in this wide range of different media used, the graphene materials tested likely have distinct and media-composition/condition dependent behaviours that must be fully characterised and understood.

One particular graphene material-media interaction reported in algal toxicity tests was a nutrient availability decrease by the tested GO materials (Malina et al., 2019). The nutrients Ca^{2+} and Mg^{2+} were almost completely removed (88–89% respectively) from the TG 201 medium and adsorbed to GO (Malina et al., 2019). The lower bioavailability of Ca and Mg caused a growth decrease in the algae in algal tests (Zhao et al., 2017), interpreted as evidence of "indirect toxicity" of the tested material. Approaches that have been used to alleviate this effect include pre-conditioning the materials in medium prior to preparation of working suspensions in an "absorption to saturation approach" (Marković et al., 2020).

Also substances (e.g. biomolecules) excreted by organisms during the testing can contribute to the media composition and influence effects. For example, when using TG 203, fish are held in exposure tanks/test water for an acclimatisation period of a minimum of 7 days and thereafter depending on the test setup/dosing protocols/water renewals the condition of the water (including biomolecules) will be modified, influencing particle-media interactions and the nanoforms of particles the fish are exposed to. Studies have begun investigating these interactions further and the influence of what has become known as "eco coronas" to reach a deeper understanding of how they may influence NM-organism interaction (Nasser et al., 2020; Ekvall et al., 2021; Nasser and Lynch, 2019; Fadare et al., 2020) or even bio-coronas *in vivo* (Abdolahpur Monikh et al., 2021). This may also explain the wide variability in ecotoxicity data and the need for thorough characterisations of materials and of any changes in properties/nanoforms under controlled test conditions when testing.

Also an influence of serum protein (e.g. for instance those present in fetal bovine serum, FBS) used in cell culture medium (fish cell lines included) likely also contributes to the differences in sensitivities among *in vitro* assays used for testing graphene materials (Table 5). While such proteins aid stability (dispersion maintenance), they may also influence cellular interactions. In the newly published TG 249 fish cell line acute toxicity test, a defined, protein and serum-free exposure medium (Leibovitz L-15 medium/ex) is used when preparing working test concentrations, to avoid any possible protein interfering effects. To date this TG has not been followed when testing graphene materials, and thus the behaviour of graphene materials in this standardised TG 249 exposure medium has yet to be characterised. The use of such a standardised medium will facilitate future comparative studies, without media specific influences complicating interpretation of results.

2.3.2.4. Interferences. The final specific factor to take into account when performing tests with graphene materials is related with the interferences of the graphene test materials with standard testing procedures. As graphene material dispersions are coloured and light absorbing, in tests which rely on absorbance or fluorescence measurements (e.g. TG 201 and TG 249) interferences with measurements were evidenced (Kalman et al., 2019; Barrick et al., 2019; Malina et al., 2019; Marković et al., 2020). This led, in some cases, to impediments in the characterisation of toxic effects (even to the inability to interpret effects) at high concentrations. The necessity to use other and alternative approaches to determine effects (e.g. cell counting) became evident and the need for modified methods for extraction and measurement of chl a to overcome interferences (see section 2.1.1). Also in tests with algae, which rely on the penetration of light for algal growth, what has been described as a shading effect was highlighted. It will be important to characterise the contribution of this effect to any growth inhibition and to distinguish it from inherent toxicity. In most studies this was not performed, however reference to specific protocols for testing this effect have been provided (Zhao et al., 2017). Lastly physical interferences resulting from the interaction of aggregated material with algae, inhibiting direct counting of algal cells as a measure of reduction in

growth were reported (Marković et al., 2020), as well as adsorption of aggregates to organism bodies (e.g. *Daphnia*) that through a physical effect may contribute to a reduction in immobilisation.

2.3.3. Data analysis and reporting

A thorough characterisation and specific reporting of physico-chemical properties is essential to any toxicological evaluation/hazard assessment. Also, from a regulatory perspective any particle characteristics that impact the safety of the substance must be indicated and this includes system dependent properties such as dispersion stability, dissolution, reactivity (biological, photo-reactivity) and potential transformations/degradations (EC, 2020). A framework for the classification of the many different forms, or "grades" of graphene materials and a syntax has been recently developed by the Graphene Council (Graphene Council, 2021) to aid in distinguishing distinct forms and types, and it facilitates a certain degree of standardisation in reporting. Specific properties that have been identified as important for classification as well as describing the unique behaviour of graphene materials include number of layers, lateral size, carbon-to oxygen (C/O) ratio (oxygen content), and structural defects (Wick et al., 2014; Fadeel et al., 2018). The material can be positively classified as graphene through the identification of sp^2 bonded carbon detection. However, the complete chemical composition of the material must be considered and accounted for, including any residual impurities or byproducts from production processes. Toxicological effects have also been evidenced to vary as a function of these distinct properties or their alterations (Liu et al., 2012; Sydlik et al., 2015; Jiang et al., 2021; Lu et al., 2017; Chen et al., 2021). For example lateral sheet size seemed to influence adverse effects towards zebrafish embryos in a comparative study, with larger sheets (>200–600 nm) showing increased toxicity through physical interactions and smaller sheets (<200 nm) not showing any effects (Moreira et al., 2021).

In Table 8 all relevant material properties, both inherent and system-dependent, are presented building on work by Wick et al. (2014), considering *(i)* information requirements to fulfil reporting obligations by REACH (Annex VI) (EC, 2006), *(ii)* particle properties that are relevant for ecotoxicological hazard assessment in the aquatic environment, and *(iii)* those defined by the graphene classification framework Graphene Flagship and IEC/ISO. Methods that can be used to measure the respective properties are also listed, identifying those which have been applied/validated/developed for NM testing and those that are currently under development. The structural property headings used include chemical composition, impurities, surface treatment/functionalisation, particle size, shape/aspect ratio, crystallinity, assembly structure/orientation/rigidity, defects (type and distance), surface area (including porosity), density, and hydrophobicity/attachment efficiency (α). The ISO/IEC standards, ISO/TS 21356-1:2021 and ISO/TR 19733:2019 can be consulted for methods that can be used to measure structural properties of graphene materials. Such methods include Raman spectroscopy (for defects, exfoliation degree, number of layers characterisation), atomic force microscopy (AFM) and transmission electron microscopy (TEM) (lateral size, thickness characterisation), BET (Brunauer, Emmett and Teller) method for specific surface area measurement and X-ray photoelectron and/or Fourier Transform Infrared spectroscopy for surface chemistry (ISO, 2019a: ISO, 2021). The importance of such standards for the graphene community has been highlighted by Clifford et al. (2021). The Graphene Material Classification framework details also standardised test methods for measuring definitive properties for a minimum set of relevant characteristics of graphene materials to be reported on material specification sheets (Graphene Council, 2021). These characteristics include, among the 19 identified: oxygen content, structural defects, specific surface area, shape, surface charge, bulk density (tapped) and crystallinity. While developed principally for manufacturers for the purpose of fulfilling registration requirements of substances, such a framework will also greatly aid in establishing toxicological profiles and help link intrinsic material properties with apical toxic effects or specific system dependent properties (property-effect relationships).

For some of the properties, standardised guidelines/protocols/methodologies are still under development (e.g. hydrophobicity, OECD WNT project 1.7). For example, a maximum particle dispersion (MPD) methodology (and dye absorption method, Li et al., 2022) has been successfully used to measure the hydrophobicity of a graphene nanoplatelet (30.9 mJ/m^2 and 0.8 according to respective methods). Other methods used include a water contact angle method with values of 93 and 79 for a single and multiple layer graphene (Bahl et al., 2020). However, before these values can become meaningful the most appropriate metric scale would need to be developed to classify levels of hydrophobicity and identify thresholds. The attachment efficiency related to the affinity of NMs to attach to other particles or surfaces (hetero-agglomeration) has also been proposed as a proxy for hydrophobicity characterisation and has been identified as an important parameter that can determine fate and be used in fate modelling. There are still challenges in calculating a precise attachment efficiency value taking into consideration all the possible interactions (e.g. heteroagglomerations (Praetorius et al., 2020)). However currently attachment efficiency can be derived from dispersion stability testing according to OECD TG 318 and its guidance document (OECD, 2017, 2021c), although also specific test guidelines are being developed.

Another information requirement under the REACH regulation (Annex VII, 7.4) is on material density, which is a property not widely reported for the range of graphene materials tested. While perfect graphene sheets have a density the same as crystalline graphite (2.267 g/cm^3), this changes with stacking order. Methods to measure density include helium pycnometry (ISO, 2014).

Defects are an important graphene material specific property. While visual inspections using TEM can be used to identify, for example, wrinkles/folds/breakages in physical structure (Meyer et al., 2008). The different types of defects at an atomic level that can be evidenced in graphene materials have been discussed in the review paper by Ahmad et al., 2021. The ratio between the intensities of the D and G peaks of a Raman spectra (I_D/I_G) correlate with the mean distance of two defects in graphene (Lucchese et al., 2010). The distance between defects, grain size and relative density of defects can be measured using the shift in the relative position and width of the G-peak, D-peak and 2D-peak of the Raman spectrum (Ferrari, 2007: Cançado et al., 2011), and further developments combining techniques are emerging (Raman spectroelectrochemistry, Raman-μSEC). An I_D/I_G value was in fact provided in a number of the collected studies and ranged from 0.49 to 1.31 according to the specific material being tested, with higher ratios representing higher defect levels.

Characterisation of the manufactured material should be distinguished from characterisation of the material post-processing/manipulation (e.g. dispersion preparation). Any processing/manipulation is likely to re-arrange the symmetry, could change the charge, stacking, and can create defects and disorder in the carbon lattice (hetero-structures, grain boundaries, vacancies, and interstitial impurities) as well as irregular/sharp edges. Ultimately, all of this leads to testing of a very dissimilar material to the starting pristine material. These post-processing manipulations can also directly affect toxicity assessments. For example high-energy sonication of graphene material can create smaller fragments with increased toxicity (Mullick Chowdhury et al., 2014: Jiang et al., 2021), while the presence of irregular sharp edges can lead to direct cell membrane destruction (Li et al., 2013). Also any residual impurities could be released from the material through such post processing (Kalman et al., 2019).

The system-dependent properties include solubility, stability, surface reactivity and degradation/transformation and these will be dictated by the specific conditions under which the various aquatic tests are performed. In an environmental setting these properties will directly determine fate and behaviour and what compartments are most likely to be exposed to the NMs (e.g. benthic vs. pelagic systems) and may be at

greater risk. However when testing, a controlled system is required/desirable. A dispersion is considered stable, for the purposes of meeting test assay validity criteria, when the concentration of graphene in medium is maintained at least at 80% of the nominal value, however further considerations may be needed as the understanding of stability can be extended to maintenance of a constant hydrodynamic diameter and polydispersity index with ≤10% deviation. TG 318 outlines a general methodology for stability assessment for NMs which could be adapted, while not prescribed for or described according to the standard operating procedures, to assess the stability of NMs under specific test conditions (e.g. in test medium, and for a specific test exposure duration). For example, an extended test monitoring period for the duration of the exposure, according to a specific test (e.g. 96 h), could be applied and testing in complete exposure medium at higher concentrations (e.g. 100 mg/L). For measuring graphene dispersion stability, approaches based on UV–Vis spectroscopy have been used in the collected studies and reported as % of initial concentration during the experiment. Other approaches for dispersion stability assessment focused on size distribution and include measurements of particle size by dynamic light scattering (DLS) and reporting of the size distributions contained in dispersion in percentiles (90%, 50% and 10%) (i.e. D90, D50, D10 values) (ISO, 2020). Also light scattering of a dispersion can be measured using Turbiscan instruments which generate Turbiscan stability indices (Dai et al., 2015). These can be categorised into scales according to stability as proposed by Dai and colleagues, for example; (1–5 = well dispersed and stable), (5–20 = can be redispersed), (>20 = precipitated). Also an approach has been explored and applied for GO characterisation of the mass and number of individual particles or aggregates in suspension using resonant mass measurements (RMM) that can be used to analyse 2D materials in suspension without the assumptions of spherical models (Crica et al., 2021).

Particular attention must also be given to any changes (degradations/transformations) that may occur under aquatic testing conditions (Zhao et al., 2020; Zhao et al., 2021). Such transformations can be physical or chemical, abiotic or biotic, and can lead to the creation of new nanoforms with distinct hazard profiles. To highlight the importance of such an assessment, transformed graphene materials and by-products (low molecular weight aromatic compounds) that show higher toxicity following photo-reduction under simulated sunlight irradiation have been detected (Zhao et al., 2020). This transformation aspect is particularly relevant considering that certain naturally occurring enzymes can biodegrade graphene (Kotchey et al., 2011; Liu et al., 2015) and it can be degraded naturally by fungi and bacteria (Candotto Carniel et al., 2021). Specifically it has been seen that *Shewanella oneidensis* MR-1 (a normal component of the surface flora of fishes) have the ability to reduce GO to rGO (Wang et al., 2011a, 2011b). This has important implications for potential transformations that may occur under environmental and testing conditions.

Various methods that can be used to investigate (bio)degradation have been detailed by Chen et al. (2017). However standardised testing strategies to monitor this property are still needed (Baun et al., 2017) and currently approaches rely on direct visualisations using TEM-EDX or AFM, while other standardised approaches are being developed (e.g. OECD, Project 3.16 GD on transformation of nanomaterials in aquatic environmental media will be developed to provide advice on ways to determine abiotic transformations of nanomaterials in the environment (OECD, 2021b)).

Raman spectroscopy can be used to monitor degradation according to the appearance of a D-band ($\sim 1350^{-1}$) which can serve as an indicator of the material structural disintegration. Also already standardised tests that may be used for graphene biodegradation assessment include OECD 301F (manometric respirometry). Any possible transformations occurring *in vivo* are also useful for biopersistence/bioaccumulation assessment and may be assessed *in vitro* by monitoring transformation in biological fluids that mimic biological compartments and intracellular environments.

Overall limitations in analytical techniques for material quantification (e.g. UV–Vis spectroscopy limits of detection of 0.5 mg/L reported by Martínez-Álvarez et al. (2021)) and a lack of standardised protocols for degradation/transformation likely explains the lack of reporting of degradation/transformation in collected studies and is causing an information gap and uncertainties which need to be addressed in future studies.

3. Conclusion

3.1. Future perspective and overall conclusions on applicability of environmental related OECD TGs for aquatic toxicity hazard classification of graphene materials

The existing OECD TGs 201, 202, 203 that form a test battery for CLP classification and for ecotoxicity assessment under a variety of regulations (e.g. REACH) have been and can be applied for graphene material testing. The overall methodology, procedural approaches, and conventional endpoints of TG 201, 202 and 203 used are sufficiently generic to be applicable to a wide range of substances including 2D graphene materials. However, the studies reviewed here highlighted also some drawbacks, especially for TG 201 that make this TG less robust when applying it to graphene related materials. Indeed more emphasis must be put on the need for additional assessments (safe guards, quality checks) both prior to conducting and during testing. The absence of this in tests to date has impeded conclusions to be made on the hazards of certain "challenging to test" substances such as graphene materials. These assessments are required to ensure there is no evident change in nanoform under, or directly caused by, pre-processing/experimental conditions, that a certain degree of stability can be maintained under general conditions (or following already detailed adjustments (e.g. agitation/aeration/renewal approaches), and that graphene materials properties (e.g. opacity, inherent fluorescence) or behaviour (absorptive capacity/media interaction) do not interfere with the performance/readout of the test. Additional assessments include preliminary stability tests extended to the assessment of agglomeration state, transformations and specific media interactions, interference checks and then, if deemed necessary, the use of appropriate adaptations to test design to meet test validity criteria and to overcome specific factors as detailed in this review. Often it is not the performance of the testing itself but other aspects such as the lack of a thorough characterisation of physico-chemical properties, including system dependent properties, that reduce the quality of performed studies. Also in most cases any characterisation of, or contribution from, impurities that may be introduced through the various production processes was not considered and must be in future studies using filtrate controls or purified materials.

A critical aspect that must be addressed in future studies is the overall lack of concentration measurements. These are compulsory in the updated TG 203 and therefore to meet future test validity criteria, measurements must be performed and more sophisticated analytical techniques developed for carbonaceous based materials such as graphene.

Also the large number of studies which have used fish embryos and applied TG 236, that can be regarded as new approach methodologies (NAMs) to test graphene materials, points to a valuable source of data that can be used in a WoE approach for fish acute toxicity assessment for graphene materials. Such alternative NAM tests, together with the standardised test battery, deemed applicable for testing graphene materials according to this review, will play a major role in the testing strategies and future IATA's to meet regulatory needs.

Glossary

Two-dimensional material/2D material: material, consisting of one or several layers with the atoms in each layer strongly bonded to neighbouring atoms in the same layer, which has one dimension (*i.e.* the

thickness) in the nanoscale or smaller and the other two dimensions generally at larger scales.

Graphite: allotropic form of the element carbon, consisting of graphene layers stacked parallel to each other in a three dimensional, crystalline, long-range order (ISO, 2017).

Graphene/single-layer graphene/monolayer graphene: single layer of carbon atoms with each atom bound to three neighbours in a honeycomb structure (ISO, 2017). Graphene layers can be classified as a two-dimensional material up to 10 layers thick for electrical measurements, beyond which the electrical properties of the material are not distinct from those for the bulk [also known as graphite] (ISO, 2017).

Few-layer graphene (FLG): two-dimensional material consisting of three to ten well-defined stacked graphene layers (ISO, 2017).

Graphene oxide (GO): chemically modified graphene prepared by exfoliation and oxidation of graphite, causing extensive oxidative modification of the basal plane. Graphene oxide is a single-layer material with a high oxygen content, typically characterized by C/O atomic ratios of approximately 2.0 depending on the method of synthesis (ISO, 2017).

Reduced graphene oxide (rGO): reduced oxygen content form of graphene oxide. It can take the form of several morphological variations such as platelets, fibres and worm-like structures (ISO, 2017).

Hummers' method: method for production of graphene oxide from graphite in a sodium nitrate and sulfuric acid solution after the addition of potassium permanganate (Hummers and Offeman, 1958).

Lateral size: lateral dimensions of a 2D material flake. If the flake is approximately circular then this is typically measured using an equivalent circular diameter or if not via x, y measurements along and perpendicular to the longest side (ISO, 2017).

Nanoform: term used to distinguish forms of a substance that fulfil the EC Recommendation on the definition of the term 'nanomaterial' but differ with regard to size distribution, shape and other morphological characterisation, including surface treatment and functionalisation and specific surface area of the particles. Variation of one or several of the defined characteristics results in a different nanoform, unless such variation results from a batch-to-batch variability (ECHA, 2022).

CRediT authorship contribution statement

M. Connolly: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **G. Moles:** Conceptualization, Methodology, Investigation, Data curation, Visualization, Writing – review & editing. **F. Candotto Carniel:** Methodology, Data curation, Writing – review & editing. **M. Tretiach:** Methodology, Data curation, Writing – review & editing. **G. Caorsi:** Methodology, Data curation, Writing – review & editing. **E. Flahaut:** Methodology, Writing – review & editing. **B. Soula:** Methodology, Writing – review & editing. **E. Pinelli:** Methodology, Data curation, Writing – review & editing. **L. Gauthier:** Methodology, Data curation, Writing – review & editing. **F. Mouchet:** Methodology, Data curation, Writing – review & editing. **J.M. Navas:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare no competing financial interest.

Data availability

Data used is availabel in already published articles

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