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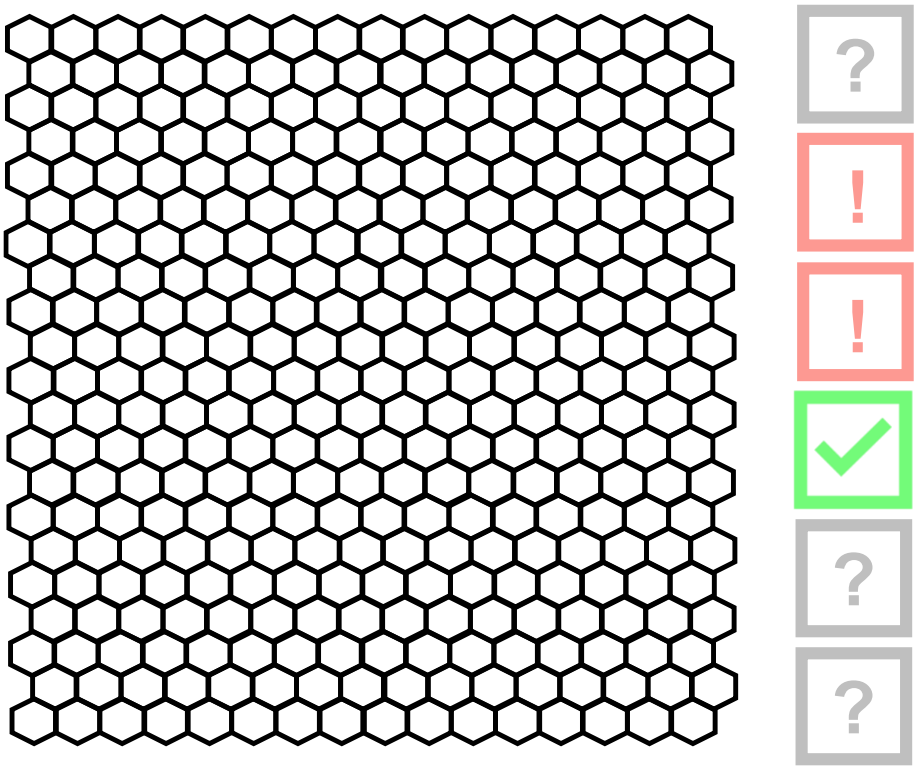

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DEPARTMENT OF TECHNOLOGY
MANAGEMENT AND ECONOMICS
Division of Environmental Systems Analysis

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Tabea Mumberg[1,2], Steffen Foss Hansen[3], Anders Baun[3], and Rickard Arvidsson[1]

[1] Division of Environmental Systems Analysis, Department of Technology Management and Economics, Chalmers University of Technology

[2] Department of Earth Sciences, University of Gothenburg

[3] Section Circularity and Environmental Impact, Department of Environmental and Resource Engineering, Technical University of Denmark

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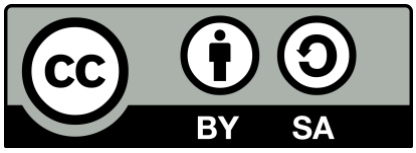
[1] Division of Environmental Systems Analysis, Department of Technology Management and Economics, Chalmers University of Technology

[2] Department of Earth Sciences, University of Gothenburg

[3] Section Circularity and Environmental Impact, Department of Environmental and Resource Engineering, Technical University of Denmark

*Corresponding author: rickard.arvidsson@chalmers.se, +46 31 772 2161



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Department of Technology Management and Economics
Division of Environmental Systems Analysis
Chalmers University of Technology
SE-412 96 Gothenburg
Sweden
Telephone + 46 (0)31-772 1000

Cover: Graphene – the namesake of graphene-based materials, here representing the whole "graphene family" – together with a schematic illustration of the outcome of the evaluation performed in this report. Figure produced by the authors using Microsoft PowerPoint®.

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Summary

Recently, the nanomaterial carbon nanotubes was added to the Substitute It Now! (SIN) List managed by the International Chemical Secretariate (ChemSec). The SIN List considers the same hazard criteria for categorizing chemicals as so-called Substances of Very High Concern (SVHC) as the European chemical regulation REACH. In order to be considered as SVHC under REACH, a compound has to be identified as either: (i) carcinogenic; (ii) mutagenic; (iii) toxic to reproduction; (iv) persistent, bioaccumulative, and toxic; (v) very persistent and very bioaccumulative; or (vi) have properties that give rise to serious effects of an equivalent level of concern as points i-v. In this study, we evaluate another type of nanomaterial, namely graphene and other graphene-based materials (GBMs), and mirror current evidence of hazards and serious effects up against the SVHC criteria. The evaluation is based on a literature review of relevant studies identified in the scientific database Scopus (Elsevier B.V.) and previous review studies. The final corpus consisted of 30 studies that provided relevant information related to at least one of the SVHC criteria. No data was found on carcinogenicity, persistence, and endocrine disruption of GBMs. Studies on these criteria are therefore highly recommended. One study indicates that GBMs are not bioaccumulating, but more studies would be needed before a robust conclusion can be reached regarding this criterion. Several studies on toxicity were identified, with results clearly indicating that GBMs should *not* be classified as toxic. Several studies on reproductive toxicity, were also identified, of which some reported reproductive toxicity in mice. Finally, a number of studies observed genotoxic effects of GBMs, in some cases also explicit mutations. Although there are indications of reproductive toxicity and mutagenicity of GBMs, the current state of knowledge is limited. Detailed assessments of whether some or all GBMs should be classified as toxic to reproduction and mutagenic are therefore recommended. In conclusion, the current scientific evidence is not deemed strong enough to classify GBMs as SVHC, but the toxicological literature should be continuously be monitored, especially with regards to reproductive effects.

Sammanfattning

Nyligen lades nanomaterialet kolnanorör till på den så kallade SIN-listan (Substitute It Now! List) som förvaltas av det Internationella kemikaliesekretariatet (ChemSec). SIN-listan baseras på samma farokriterier för kategorisering av så kallade ämnen som inger mycket stora betänkligheter (SVHC) som den europeiska kemikalielagstiftningen REACH. För att bli kategoriserad som SVHC under REACH måste ett ämne visats vara antingen: (i) cancerframkallande; (ii) mutagent; (iii) reproduktionstoxiskt; (iv) persistent, bioackumulerande och toxiskt; (v) väldigt persistent och väldigt bioackumulerande; eller (vi) ha egenskaper som orsakar allvarliga effekter av motsvarande betänklighet som punkt i-v. I denna studie utvärderar vi ett annat sorts nanomaterial, nämligen grafen och grafen-baserade material, baserat på nuvarande belägg för farlighet och allvarliga effekter enligt SVHC-kriterierna. Utvärderingen baseras på en litteraturgenomgång av relevanta studier identifierade i den vetenskapliga databasen Scopus (Elsevier B.V.) och i existerande litteraturstudier. Det slutgiltiga materialet omfattade 30 studier som innehöll relevant information om minst ett av SVHC-kriterierna. Ingen data om huruvida grafen-baserade material är cancerframkallande, persistenta eller hormonstörande stod att finna. Studier om dessa kriterier rekommenderas därför starkt. En studie indikerar att grafen-baserade material in är bioackumulerande, men fler studier skulle möjliggöra en robustare bedömning av detta kriterium. Flera studier av toxicitet kunde identifieras, som tydligt indikerar att grafen-baserade material *inte* bör klassas som toxiska. Flera studier om specifikt reproduktionstoxicitet identifierades även, av vilka några rapporterade reproduktionstoxicitet i möss. Slutligen rapporterar ett antal studier genotoxiska effekter av grafen-baserade material, ibland även explicita mutationer. Även om det således finns indikationer på reproduktionstoxicitet och mutagenicitet hos grafen-baserade material är den nuvarande kunskapen begränsad. Ytterligare utvärdering av huruvida några eller alla grafen-baserade material bör klassas som reproduktionstoxiska och mutagena rekommenderas därför. Sammanfattningsvis bedöms de nuvarande vetenskapliga beläggen inte tillräckliga för att grafen-baserade material ska klassas som SVHC, men den toxikologiska litteraturen bör följas upp kontinuerligt, i synnerhet gällande reproduktionstoxicitet och mutagenicitet.

1. Introduction

1.1 Nanomaterials as a challenge for risk assessment

Concerns about potentially harmful chemicals to human health and the environment have risen during the past decades (Diamond et al., 2015; Persson et al., 2022). A specific reason for concern is the continuous development of increasingly sophisticated materials, which are more challenging to assess regarding their risks and to regulate (Maynard et al., 2011). An example of such sophisticated materials is nanomaterials, which are often defined as substances that are within the size range of 1-100 nm in at least one dimension, while nanoparticles are often required to be within this size range in several dimensions (Boholm & Arvidsson, 2016). In 2018, the European Commission's revised the Annexes of the European Union's chemical legislation (REACH) in order to take nano-specific environmental, health and safety aspects into account. In the revised Annexes, it was specified that materials with a 50% share of the particle distribution (or more) within the size range of 1-100 nm are to be defined as nanomaterials in the EU (Clausen & Hansen, 2018; European Commission, 2022). This definition stems from 2011 and was updated in 2022, with some changes implemented but with the 50% and 1-100 nm values remaining (Hansen et al., 2022).

Nanomaterials can include metals, metal oxides, quantum dots, and carbon-based materials, such as carbon nanotubes (CNTs), fullerenes, and graphene (Klaine et al., 2008). The nanoform of a material can have new physio-chemical properties compared to the bulk form of the same material. In some cases, these can be explained by the increased surface-to-volume ratio that leads to an increased surface reactivity. One the one hand, the physio-chemical properties that materials obtain at the nanoscale could lead to novel applications within, e.g., medical treatment, environmental remediation, and transport. On the other hand, the new physio-chemical properties could also lead to potentially new toxic effects (Skjolding et al., 2016). Due to regulatory latency, scientific uncertainty, high costs, resources, and time requirements of testing, sufficient and robust data on effects of nanomaterials to humans and the environment is currently lacking, which constitutes a challenge for risk assessment and regulation (Grieger et al., 2019; Saldívar-Tanaka & Hansen, 2021). In addition, there are still considerable uncertainties regarding the fate and transport of nanomaterials in the environment (Svendsen et al., 2020).

As a response to these challenges of lacking data and understanding of nanomaterial risks, several approaches to simplified, screening risk assessment have been proposed (Grieger et al., 2018). These are often based on a number of hazard-related parameters, such as (eco)toxicity data, the shape of the nanomaterials, whether they are carcinogenic, and whether they have a high likelihood of becoming released (Arvidsson et al., 2016). Examples of such approaches include NanoRiskCat (Hansen et al., 2013), LICARA nanoSCAN (van Harmelen et al., 2016) and the two proxy measures global production volumes and aquatic ecotoxicity (Arvidsson et al., 2018, 2022). However, to the best of the author's knowledge, none of these screening approaches are currently used regularly for decision making in society.

1.2 The SIN List and REACH Substances of Very High Concern

Screening, or hazard-based, approaches are not limited to nanomaterials, but exist for chemicals in general as well. In 2002, a Swedish non-profit, non-governmental organisation (NGO) called the International Chemical Secretariat (ChemSec) was founded by a number of environmental organisations. After the REACH Regulation entered into force in 2007, ChemSec published the Substitute It Now! (SIN) List in 2008 of substances that ChemSec believes should be restricted or banned in the EU. The SIN List considers the same criteria as the REACH Regulation for classification of Substances of Very High Concern (SVHC). These criteria include whether the

substance is (i) carcinogenic, (ii) mutagenic or (iii) toxic to reproduction (CMR); (iv) persistent, bioaccumulative and toxic (PBT); (v) very persistent and very bioaccumulative (vPvB); or (vi) of an equivalent level of concern, e.g., endocrine disruptive (Table 1). The SIN List is regularly updated and now constitutes one of several tools designed by ChemSec aiming to guide industrial actors on which chemicals should be substituted due to possible hazards and likely future regulation (ChemSec International Chemical Secretariat, 2022), in line with the precautionary principle (Harremoës et al., 2001; Saldívar-Tanaka & Hansen, 2021).

The REACH Regulation generally requires registration of produced or imported chemicals and then the most hazardous chemicals are identified as SVHC during the evaluation process. SVHCs must be substituted unless their use is authorized. A so-called REACH Candidate List of SVHCs that is generated during the evaluation process contained 223 chemicals in January 2022 that had so far been found to fulfil the SVHC criteria. However, proposals for inclusion on the Candidate List as well as the process for categorization of substances as SVHC (or not) is complicated and time-consuming. For this reason, ChemSec's SIN List aims at using the same criteria as the REACH Candidate List based, but making the inclusion of chemicals more efficient, quicker, and less political to allow early substitution and searches for alternative chemicals by the companies (ChemSec International Chemical Secretariat, 2022). This approach has been successful and ChemSec considers their SIN List as "one of the most progressive chemical standards in the world" (ChemSec International Chemical Secretariat, 2022) with a number of companies and labels using the SIN List for selecting chemicals to phase out (Hansen & Lennquist, 2020a). The United Nations Environment Programme (UNEP) refers to the SIN List as one of the most detailed, robust, and justified lists for endocrine disrupting compounds (EDCs) (Hansen & Lennquist, 2020a; United Nations Environment Programme, 2016), although though data scarcity makes the SIN List far from complete (ChemSec International Chemical Secretariat, 2022).

If one of the six criteria presented in Table 1 is met, the substance may qualify for inclusion in the REACH Candidate List and will then be further evaluated for inclusion in REACH Annex XIV - List of Substances Subject to Authorisation, which in July 2022 contained 59 authorized substances (ChemSec International Chemical Secretrariat, 2022; European Parliament and Council of the European Union, 2007). According to REACH Article 58, assessment of persistent, bioaccumulative, and toxic (PBT), very persistent and very bioaccumulative (vPvB), or widely used substances as well as compounds with high production volumes should be prioritized (European Parliament and Council of the European Union, 2007). Guidelines for assessment of substances meeting the CMR criteria are listed in the Classification, Labelling, and Packaging (CLP) Regulation Annex I, section 3 (European Parliament and Council of the European Union, 2008) and are followed also by the SIN List (ChemSec International Chemical Secretariat, 2022), while information on the categorization of PBT and vPvB substances can be found in REACH Annex XIII (European Parliament and Council of the European Union, 2007; Hansen, 2019).

Although the SIN List mostly follows the SVHC criteria, it also goes beyond these criteria in some regards (ChemSec International Chemical Secretariat, 2022). Since 2014, the SIN List additionally includes:

- substances that show structural similarity to compounds listed as PBT substances,
- substances that show structural similarity to compounds listed as vPvB substances,
- substances that show structural similarity to compounds listed as persistent organic pollutants (POP) under the Stockholm Convention,
- substances with degradation products that are PBT or vPvB according to REACH Annex XIII or structurally similar to those or other POPs.

The same criteria as for (iv) PBT and structurally similar compounds were applied for substances included on the SIN List as persistent, mobile, and toxic (PMT) or very persistent and very mobile (vPvM), falling into the sixth category of substances of equivalent level of concern. Additionally, the criteria for "mobility" from the German Environment Agency (UBA) of a lowest organic carbon-water coefficient log K_{OC} < 4.0 within a pH range of 4 to 9 and "very mobile" with log K_{OC} < 3.0 ($4 \leq pH \leq 9$) were applied (ChemSec International Chemical Secretariat, 2022; German Environment Agency (Umweltbundesamt), 2021). Since 2011, the SIN List also includes endocrine disrupting compounds (EDCs) as substances of equivalent level of concern according to thorough literature studies as well as criteria recommended by European Parliament and Council of the European Union (2017) and European Parliament and Council of the European Union (2018), which include (i) an endocrine mode of action, (ii) possible adverse effects on an organism, and (iii) a plausible link between (i) and (ii) (ChemSec International Chemical Secretrariat, 2022; European Chemicals Agency, 2018).

By these additional criteria, which can be argued to go beyond the SVHC criteria applied within the REACH Regulation, the SIN List can thus be said to operationalize the "substances of equivalent level of concern" criteria, which is otherwise not defined in detail in the REACH Regulation.

1.3 Carbon nanotubes on the SIN List

CNTs is an allotrope of carbon, consisting of hexagonally arranged carbon sheets rolled to cylindrical tubes. While the diameter spans a few nanometers, the tubes can be up to several micrometers in length. CNTs have a wide range of promising applications, such as composite materials, electronics, transparent conductive films, anticorrosion coatings, energy storage, and biosensors (de Volder et al., 2013).

In 2019, CNTs were the first nanomaterials added to ChemSec's SIN List, being classified as carcinogenic (C), possibly toxic to reproduction (R) and very persistent (vP) (Hansen, 2019; Hansen & Lennquist, 2020a). This listing received considerable interest world-wide. At the same time, ChemSec's SIN listing was afterwards criticized for hindering scientific progress and technical development by considering all CNTs as a one compound, which includes different specific types of CNTs that might be more or less hazardous (Fadeel & Kostarelos, 2020; Hansen & Lennquist, 2020b; Heller et al., 2020). ChemSec International Chemical Secretariat (2022) and Hansen & Lennquist (2020b), on the other hand, responded that mixtures of CNTs are commonly present due to costly purification processes and evidence for less hazardous forms is lacking. Additionally, a shift to less studied (thus not yet confirmed hazardous) CNT forms could cause "regrettable substitutions" instead of actual improvement of the product (ChemSec International Chemical Secretariat, 2022). Whether the SIN listing will result in decreased research, development and commercialization of CNTs remains to be seen. Currently, no nanomaterials are listed as SVHC according to REACH, even though additional registration and chemical safety information for nanomaterials are required by REACH since 2020 (European Chemical Agency (ECHA), 2022; Nielsen et al., 2021; Saldívar-Tanaka & Hansen, 2021).

Although the SVHC criteria as operationalized in the SIN List are not nanomaterial-specific, as opposed to some other screening risk assessment approaches mentioned in Section 1.1, the SIN listing of CNTs has so far received notably higher attention than any assessment of CNTs performed using other risk or hazard assessment approaches. This might be because of the established nature and legitimacy of the SVHC criteria as defined in the REACH legislation and their operationalization in the form of the SIN List. Another reason might be the clear link between the six hazard parameters considered in the SVHC criteria and risks to health and the environment. For

several nanomaterial-specific properties applied in screening risk assessment approaches, such as particle size, this link is not as unambiguous (Arvidsson et al., 2018). The inclusion of CNTs in the SIN List raises the question whether more nanomaterials qualify to meet those same criteria?

1.4 Aim of the study

The aim of this study is to assess whether graphene and other graphene-based materials (GBMs) fulfil the SVHC criteria. Graphene consists of one layer of carbon atoms and has several promising applications (Geim & Novoselov, 2007). Companies have expressed concerns about whether graphene would be added to the SIN List in future as well (SweNanoSafe Swedish National Platform for Nanosafety, 2021). If GBMs fulfil any of the SVHC criteria, they might be considered for inclusion on the Candidate List and/or the SIN List in the future. Graphene shares several properties with CNTs in terms of, e.g., chemical composition and envisioned applications – graphene can even be produced by "unzipping" CNTs into sheets (Kosynkin et al., 2009). At the same time, there are also important differences between graphene and CNT properties, such as different shapes and surface areas (Bussy et al., 2013).

Currently, graphene is produced at large scale, but industrial applications remain limited (Ren & Cheng, 2014). The underlying rationale of this study is that an evaluation of whether GBMs constitute a potential environmental or health hazard, likely to become subject to future regulatory attention, is important at an early point in time and before GBM commercialization and use become widespread. On one hand, should the evaluation indicated that GBMs fulfil the SVHC criteria, harm can be avoided in contrast to some historical examples of uses of chemicals and materials (Harremoës et al., 2001). On the other hand, if it can be shown that GBMs *do not* fulfil any of the SVHC criteria based on currently available data, it would send an important signal to researchers and developers that GBMs might be less hazardous than CNTs, and thus preferable from an environmental and health point of view, thereby spurring innovation in that direction. A recent review of environmental and health impacts of graphene, graphene oxide, and other 2D materials was conducted on behalf of ECHA, but it did not evaluate graphene or graphene oxide against the SVHC criteria (Bianco, del Rio, et al., 2022).

In the next Section 2, a brief background to the nanomaterials assessed – GBMs – is first provided. Subsequently, Section 3 describes the method applied to achieve the aim of this study. Section 4 then presents the results of the study, and Section 5 a concluding discussion.

Table 1. Substances of Very High Concern criteria and threshold values. *Obtained from the Regulation (EC) No 1907/2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) article 57 and partly refer to the European Union's Classification, Labelling and Packaging (CLP) Regulation ((EC) No 1272/2008)* (ChemSec International Chemical Secretrariat, 2022; European Parliament and Council of the European Union, 2007, 2008).

Criteria	**Legislation**	**Definition**
Carcinogenic (**C**)	CLP Annex I, section 3.6 category 1A or 1B; REACH article 57 (a)	Known (human evidence) or presumed (animal evidence) carcinogenic potential for humans
Mutagenic (**M**)	CLP Annex I, section 3.5 category 1A or 1B; REACH article 57 (b)	Known to induce heritable mutations or to be regarded as if they induce heritable mutations in the germ cells of humans (human epidemiological evidence, animal evidence from in vivo heritable germ cell mutagenicity tests or somatic cell mutagenicity tests, or tests showing mutagenic effects in the germ cells of humans, without demonstration of transmission to progeny)
Toxic to reproduction (**R**)	CLP Annex I, section 3.7 category 1A or 1B; REACH article 57 (c)	Known (human evidence) or presumed (animal evidence) to be toxic to human reproduction
Persistent, bioaccumulative and toxic (**PBT**)	REACH Annex XIII and article 57 (d)	**P:** $t_{1/2}$ (marine water) > 60 days, $t_{1/2}$ (fresh or estuarine water) > 40 days, $t_{1/2}$ (marine sediment) > 180 days, $t_{1/2}$ (fresh or estuarine sediment) > 120 days, or $t_{1/2}$ (soil) > 120 days **B:** BCF (aquatic species) > 2000 L kg^{-1} **T:** NOEC (marine or freshwater organisms) < 0.01 mg L^{-1}, EC10 (marine or freshwater organisms) < 0.01 mg L^{-1}, or classification according to CLP as carcinogenic (Annex I, section 3.6 category 1A or 1B), germ cell mutagenic (Annex I, section 3.5 category 1A or 1B), toxic for reproduction (Annex I , section 3.7 category 1A, 1B, or 2), or toxic to specific target organs after repeated exposure (Annex I, section 3.9 category 1 or 2)
Very persistent and very bioaccumulative (**vPvB**)	REACH Annex XIII and article 57 (e)	**vP:** $t_{1/2}$ (water*) > 60 days, $t_{1/2}$ (sediment*) > 180 days, or $t_{1/2}$ (soil) > 180 days **vB:** BCF (aquatic species) > 5000 L kg^{-1}
Substances of equivalent level of concern	REACH article 57 (f) Case-by-case identified substances according to REACH article 59	E.g., endocrine disrupting (**EDC**), persistent, mobile and toxic (**PMT**), or very persistent and very mobile (**vPvM**) compounds

*marine, fresh, or estuarine

2. Background to graphene-based materials

GBMs are a group of nanomaterials that are an allotrope of carbon, having a 2D structure compared to the 1D structure of CNTs. GBMs are commonly present as hexagonally arranged sheets of carbon atoms, ranging from one to few layers with varying degrees of oxidation. Similar to CNTs, GBMs have a range of industrially relevant properties, such as high conductivity and material strength, and are thus envisioned for a range of application, including composites (Stankovich et al., 2006), thin transparent films (Blake et al., 2008), and electronics (van Noorden, 2006). Different types of specific materials exist within the wider "family" of GBMs (Bianco et al., 2013; Wick et al., 2014), which in turn might influence hazard properties (Fadeel et al., 2018). Three common types found in literature are described in the following section and schematically illustrated in Figure 1.

2.1 Graphene

Graphene is, according to ISO/TS 80004-13:2017 (Part 13), a single-atom-thick layer of carbon atoms arranged in a honeycomb-like structure. Estimates of annual global production rates of graphene during the 2010s range between 20 and 2500 ton/year (Arvidsson et al., 2022). Although the term "graphene" strictly only refers to a single layer, arrangements of more graphene layers are often referred to as graphene as well, including bilayer graphene, few-layer graphene, and nanoplatelets, as well as graphene forms with extended dimensions in one or several directions, such as graphene sheets or flakes (Bianco, 2013; Bianco, Prato, et al., 2022; Park et al., 2017). During this literature study, those graphene forms were all classified as "graphene". If sufficient information was available, the number of layers, thickness and lateral size were specified.

2.2 Graphene oxide and reduced graphene oxide

Graphene oxide (GO) is, according to ISO/TS 80004-13:2017 (Part 13), a chemically oxidized form of single-layer graphene with modifications along the basal plane leading to covalently bonded oxidized functional groups and a ratio of carbon (C) to oxygen (O) of around two (Bianco, 2013; de Marchi et al., 2018; Jastrzębska et al., 2012). Reduced graphene oxide (rGO) is the partially reduced form of graphene oxide, produced via different reduction methods, such as chemical, thermal, microwave, photo-chemical, photo-thermal, or microbial/bacterial reduction, all leading to a lower C/O ratios than for GO, but higher than for graphene unless the reduction is complete (Bianco, 2013; Park et al., 2017). Similar to graphene, GO and rGO has several promising applications, including being a precursor material for graphene production (Dideikin & Vul', 2019). Recently, it was noted that a significant share of products labelled as graphene on the market might rather be GO or rGO (Kauling et al., 2018).

2.3 Functionalized graphene-based materials

Additionally, different functionalized versions of graphene, GO, and rGO have been reported in the literature and are especially popular for biomedical applications (Georgakilas et al., 2012). Functionalization may significantly alter material properties such as bioavailability, biocompatibility, stability, and solubility. Both changes in the C/O ratio through addition of carboxyl, hydroxyl, or epoxy groups as well as functionalization with capping agents or coatings, such as polyethylene glycol (PEG) coating, can be found (Park et al., 2017).

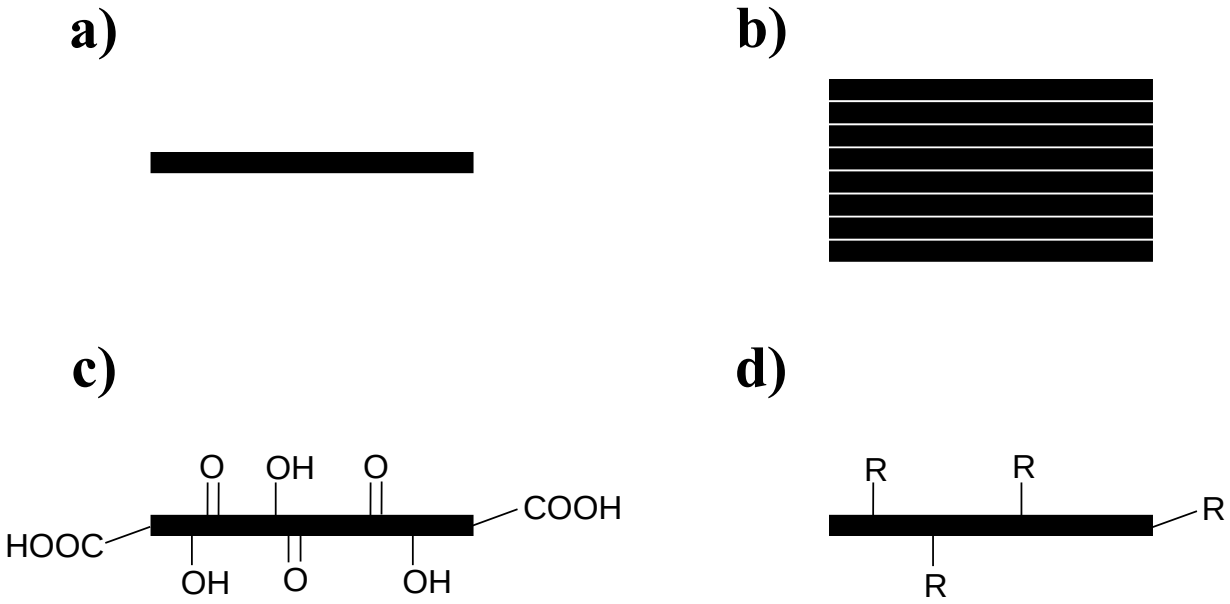

Figure 1. Schematic, two-dimensional illustration of common types of graphene. *a) Single-layer graphene sheet. b) Few-layer graphene. c) Single-layer graphene oxide (GO). d) Single-layer functionalized graphene, with R symbolizing different functional groups. Figure produced by the authors using Microsoft PowerPoint®.*

3. Method

A three-step method was applied to achieve the aim of the study specified in Section 1.4. The first step was a literature review, which was performed to identify scientific data and information of relevance to the SVHC criteria in Table 1. A Scopus (Elsevier B.V.) search was performed, using search strings such as *graphene AND nano AND risk* or combinations of graphene and the SVHC criteria, namely *graphene AND nano AND toxi*, carcinog*, mutagen*, genotoxic*, persiten*, accumulat*, endocrine, hormon** or *graphene AND environmental risk, toxicity, carcinogen*, mutagen. persistence, bioaccumulate, hormone disruptor*. This resulted in approximately 100 potentially relevant studies published since 2010. Moreover, 14 reviews about environmental and health impacts of GBMs were identified:

1. Prospective environmental risk screening of seven advanced materials based on production volumes and aquatic ecotoxicity (Arvidsson et al., 2022)
2. Toxicity of graphene-family nanoparticles: a general review of the origins and mechanisms (Ou et al., 2016)
3. Health and ecosystem risks of graphene (Hu & Zhou, 2013)
4. Health and safety concerns related to CNT and graphene products, and related composites (Sousa et al., 2020)
5. Safety assessment of graphene-based materials: Focus on human health and the environment (Fadeel et al., 2018)
6. An overview of graphene materials: Properties, applications and toxicity on aquatic environments (de Marchi et al., 2018)
7. Safety considerations for graphene: Lessons learnt from carbon nanotubes (Bussy et al., 2013)

8. Graphene: Safe or toxic? The two faces of the medal (Bianco, 2013)
9. Considerations for safe innovation: The case of graphene (Park et al., 2017)
10. Occupational exposure to graphene based nanomaterials: risk assessment (Pelin et al., 2018)
11. Graphene in the aquatic environment: Adsorption, dispersion, toxicity and transformation (J. Zhao et al., 2014)
12. Ecotoxicology of manufactured graphene oxide nanomaterials and derivation of preliminary guideline values for freshwater environments (Markovic et al., 2018)
13. Toxicology of graphene-based nanomaterials (Lalwani et al., 2016)
14. Biological interactions of graphene-family nanomaterials: an interdisciplinary review (Sanchez et al., 2012)

From these 14 review studies, an additional approximately 100 potentially relevant papers were identified, resulting in an initial corpus of about 200 studies.

The second step was the in-depth analysis of the identified studies. For each study, any results related to the six SVHC criteria (Table 1) were noted, whereas irrelevant studies were removed. Many of the identified studies conducted short-term toxicity tests on human cells (e.g., Jaworski et al. (2013)) or tested the toxicity to mice or rats after a single exposure through injection to the blood or lungs (e.g., Duch et al. (2011)). However, the PBT criterion requires either aquatic toxicity data, or that the substance is "toxic to specific [human] target organs after repeated exposure" (Table 1), preferably evidenced by animal studies rather than cell tests. Such studies could therefore not be applied to evaluate GBMs against the SVHC criteria and were thus removed. In addition, a considerable number of ecotoxicological studies were removed since they did not provide NOEC or EC10 values (e.g., Pretti et al. 2014), which are required for the PBT evaluation (see Table 1). In some cases, NOEC values could be derived from studies even though they were not explicitly reported. The approach used to derive these was guided by examples in the supporting information document of the review by Markovic et al. (2018). Finally, only ecotoxicity studies considering the so-called base set of organisms were considered, i.e., algae, crustacean, and fish. The rationale behind this is that studies based on other organisms are rarely considered in regulatory contexts, e.g., in evaluations of substances for inclusion on the Candidate List. In the end, a total of 30 studies were identified as relevant for the scope of this analysis.

The third step was the assessment of the GBM(s) considered in the study according to the SVHC criteria. Regarding the sixth criterion – substances of equivalent concern – ChemSec's operationalization was followed, meaning that only endocrine disruption, PMT, or vPvM properties were considered as being of equivalent concern as the other five criteria (ChemSec International Chemical Secretariat, 2022). The results of each study were colour coded according to the following scheme: Green for "SVHC criteria not met" and red for "SVHC criteria met".

4. Results

The results from this study are summarized in Figure 2 and the Appendix, where the colour coding follows the categorization of studies described in Section 3. Two results stand out: several studies indicate that GBMs are mutagenic, and several studies report low toxicity. A small number of studies report varying results regarding reproductive toxicity, and a single result on bioaccumulation has been found. No studies reporting results for carcinogenicity, persistence, or endocrine disruption have been found. Several studies, such as Duch et al. (2011), report on

inflammation in lungs following exposure to GBMs, but while inflammation in lungs can lead to lung cancer (Coussens & Werb, 2002), it is not a sign of cancer itself. In addition, due to a different particle clearance mechanism in rat lungs compared to human lungs, overloading is likely to cause a pro-inflammatory response in rat lungs that is not necessarily relevant to humans (Borm & Driscoll, 2019). For CNTs, a specific variant called MWCNT-7 was classified as carcinogenic by the International Agency for Research on Cancer (IARC), which contributed notably to its SIN listing (Hansen, 2019; Hansen & Lennquist, 2020a). No similar evidence exists for GBMs.

The criteria for which reported results were available – mutagenicity, reproductive toxicity, bioaccumulation, and ecotoxicity – are described and discussed in detail in Sections 4.1-4.4 below. It can be noted that a majority (21 out of 31) studies considered GO and/or rGO. The other 10 studies considered graphene and functionalized graphene. Lateral sizes varied from a few nanometers to several micrometers, with thicknesses between one and a few nanometers, representing single- to few-layered GBMs, respectively. However, incomplete information about the type of GBM studied was common, even though such information is now required to be reported by producers according to the REACH Regulation (European Chemical Agency (ECHA), 2022; European Chemicals Agency (ECHA), 2019). Because of this limited information, it is difficult to draw conclusions for specific GBM types based on this study. Therefore, in the following sections, all GBMs will be discussed together, on a group basis, in the following sections. This is a limitation of this study, since different GBMs might have different effects on organisms (Fadeel et al., 2018; Fadeel & Kostarelos, 2020).

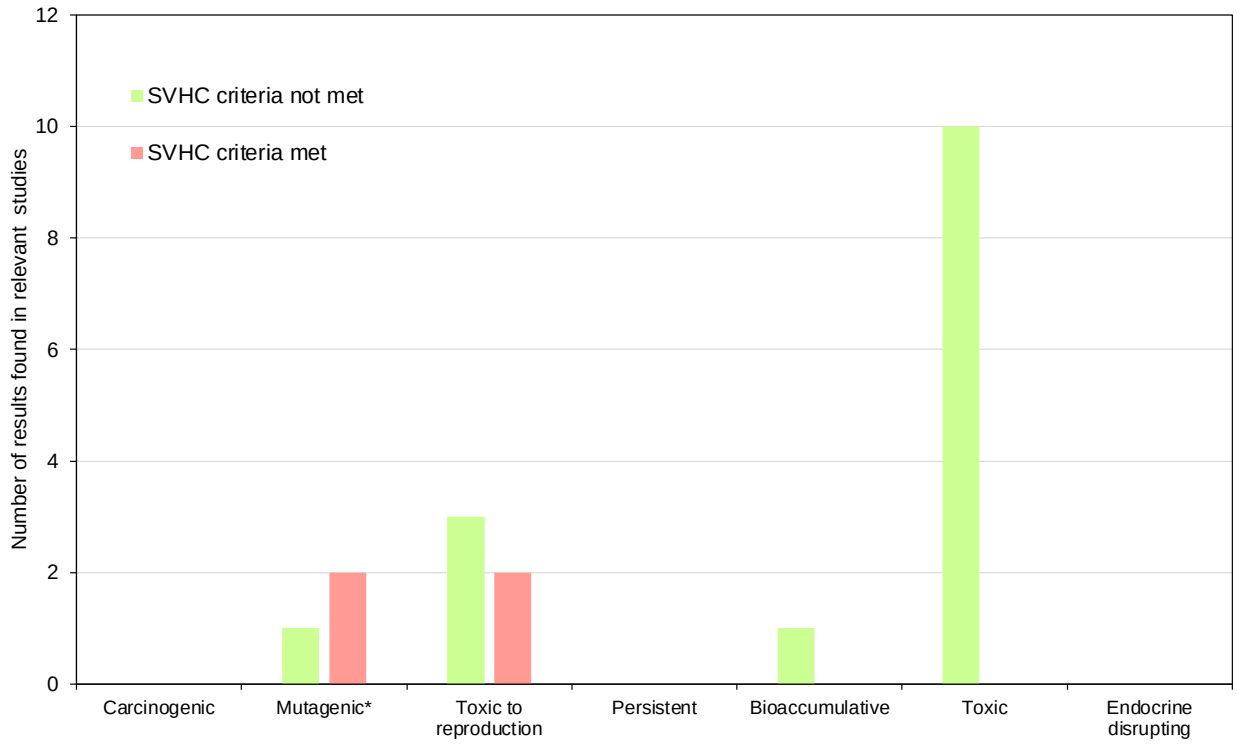

Figure 2. Results for each SVHC criterion reported in the reviewed studies. *Studies reporting results for several criteria were counted multiple times. *In addition to these results on mutagenicity, 12 studies also reported genotoxicity and 1 study reported no genotoxicity, see Appendix. The figure was produced by the authors using Microsoft Excel®.*

4.1 Genotoxicity and Mutagenicity

Genotoxic effects do not unambiguously lead to mutations – a substance can be genotoxic without being mutagenic. However, being genotoxic is an indicator of mutagenicity, since DNA damage is the first step of mutagenesis (DeMarini, 2019). Several of the reviewed studies reported genotoxic effects, most commonly DNA damage. In two subsequent studies, Akhavan et al. (2012, 2013) investigated the genotoxicity of GO and rGO to human stem cells using Comet assays. In both cases, DNA damage in terms of fragmentation and chromosomal aberrations were found. Bengtson et al. (2017) noted DNA damage in bronchial cells of mice after pulmonary exposure to GO and rGO, also using a Comet assay. In a set of sequential studies, Burgum et al. (2021a, b) investigated the genotoxicity of graphene and several functionalized graphene types in human bronchial and alveolar cells as well as macrophages. DNA damage in the bronchial cells was observed for graphene and amine-functionalized graphene, whereas graphene, amine-functionalized graphene, and carboxyl-functionalized graphene all caused DNA damage to both alveolar cells and macrophages. Chatterjee et al. (2016) studied genotoxic effects of single-layer graphene, few-layer graphene, carboxy-functionalized graphene, and amine-functionalized graphene in Comet assays with human bronchial cells. All graphene types caused DNA damage. di Ianni et al. (2021) investigated the genotoxicity of GO and rGO to human alveolar cells and macrophages in a Comet assay. Only the GO caused DNA damage to the macrophages. As part of a large study by the Brazilian Network on Nanotoxicology, GO was evaluated by a number of toxicological tests, including Comet assays with human, hamster, and mouse cells (Durán et al., 2015). The results showed slight DNA damage to the human cells (lymphocytes) and more significant effects on the hamster cells. El-Yamany et al. (2017) found DNA damage in mouse bone marrow and lung cells after injection of GO into the abdomen. This damage reportedly happened both due to direct interactions between GO and DNA, and due to the generation of reactive oxygen species that induced oxidative damage to the DNA. Wang et al. (2015) showed DNA damage from graphene to human fibroblast cells. Some reports of genotoxic effects in non-mammalian animals were also present. In a Comet assay, Fernandes et al. (2017) found DNA damage in the tissue of the shrimp *Litopenaeus vannamei*. Zhao et al. (2021) showed DNA damage from GO in a Comet assay with earthworms. Contrary to these affirmative genotoxicity studies, Souza et al. (2017) assessed the genotoxicity of GO to the zebrafish *Danio rerio* in a Comet assay, which did not reveal any DNA damage.

As described in the CLP Regulation's Annex I section 3.5, mutagenicity can be determined based on human epidemiological studies, mutagenicity tests on mammals, and tests on germ or other somatic cells. Preference is given to germ cell tests and tests on humans or other mammals. Among the reviewed studies, three reported explicit mutagenicity results, rather than mere genotoxicity. In a study by Mohamed et al. (2020), DNA damage was found in Comet assays of the tissue of mice after oral administration of GO, possibly due to oxidative stress. In addition, mutations were observed in two mice genes. Liu et al. (2013) reported that GO could cause mutagenesis in extracted DNA, human cells, and mice. Contrary, Demir and Marcos (2018) tested for gene mutations by graphene in mouse lymphoma cells. They report that no significant changes in gene expression occurred, indicating no mutagenicity.

While there is an element of conflicting results regarding the mutagenicity of GBMs, most studies indicate that graphene, GO, and some functionalized types of graphene are genotoxic. Some studies even report mutations. This serves as an indication that the "M" criterion in CMR might be fulfilled.

4.2 Reproductive toxicity

In total, five studies related to the reproductive toxicity of GBMs were identified. Three of these showed no effects, while the other two did. Fu et al. (2015) investigated developmental effects of GO on mice offspring, showing reduced increase in body weight, body length, and tail length, as well as dysfunctions in the intestinal tract. Xu et al. (2015) also investigated effects on mice offspring but considered the reproductive ability of female mice as well. The results from this study show that the effects of rGO depend on when during pregnancy exposure through injection happens. Exposure before pregnancy did not affect the mating behaviour or the health of the offspring, but malformed foetuses were observed. Injection of rGO at a late stage of pregnancy resulted in abortions in all mice as well as death of all pregnant mice given a high enough dose. However, all surviving rGO-injected mouse mothers could give birth to another set of healthy pups.

Contrary to these studies showing effects related to reproductive toxicity, Liang et al. (2015) found no adverse effects from GO injection in male mice – they showed no significant changes in sex hormone levels, testicles, and reproductive behaviour, and mating with untreated female mice resulted in healthy pups. Skovmand et al. (2018) investigated the effect on sperm quality from GO pulmonary exposure. They found no significant changes in the sperm. Nirmal et al. (2017) received somewhat conflicting results from GO exposure to rats – reduced sperm count, sperm abnormalities, and damage to testicular tissue were noted, but the male fertility was not affected by the GO exposure. Also, there was a significant recovery after 30 days.

In summary, while there was no evidence of reproductive toxicity in male mice, two studies show evidence of reproductive toxicity in female mice and offspring. This constitutes an indication that the reproductive toxicity criterion might be fulfilled for GBMs.

4.3 Bioaccumulation

Only one study with reported bioaccumulation of GBMs in the aquatic environment was found. In a study of the toxicity of graphene to the crustacean *Daphnia magna*, Fan et al. (2016) reported that the bioaccumulation of graphene in *Daphnia magna* was relatively low. At a concentration of 1 mg/L of graphene in the medium, the concentration in the test species was 90.7 mg/kg, which corresponds to a 90 times higher concentration in in the animals compared to the concentration in the water phase. This is considerably below the threshold of 2000 times required for a SVHC classification. However, since only one single value could be identified, more research into the bioaccumulation of GBMs is strongly recommended.

4.4 Toxicity

Regarding ecotoxicity, NOEC values were reported in several studies, and for some studies they could be deduced despite not being explicitly reported. Additionally, one EC10 value was reported. Included is also one EC20 value (Fekete-Kertész et al., 2020), even though this measure cannot strictly be used for SVHC assessments. However, its high value is a strong indication that the corresponding EC10 and NOEC values are likely higher than 0.01 mg/L.

Castro et al. (2018) investigate the toxic effects of GO to several organisms, including algae (*Raphidocelis subcapitata*) and two different crustaceans (*Daphnia magna* and *Artemia salina*). They report log(NOEC) values in one of their figures, the lowest being approximately -1 (for *Artemia salina*). This translates to a NOEC value of about 0.1 mg/L. Chen et al. (2016) tested the toxicity of GO to zebrafish embryos for several endpoints (hatching rate, heartbeat, and incidence of malformations). No effects were seen at 0.1 mg/L or below. The above-mentioned large study by the Brazilian Network on Nanotoxicology on GO also conducted toxicity tests on two crustaceans: *Daphnia smilis* and the shrimp *Palaemon pandaliformis* (Durán et al., 2015). The

NOEC values reported were 3 mg/L for the shrimp and 100 mg/L for *Daphnia smilis*. Fan et al. (2016) investigated the chronic toxicity of graphene to *Daphnia magna* and found no adverse effects at 0.1 mg/L. In the study by Li et al. (2014), the phototoxic effects of graphene combined with titanium dioxide nanoparticles on *Daphnia magna* and the fish *Oryzias latipes* were tested. However, for graphene alone, no toxicity was observed at values as high as 100 mg/L. Liu et al. (2014) tested the toxicity of GO and rGO on zebrafish embryos regarding body length, movement, hatching rate, and heart rate. While no NOEC value could be derived for rGO, the lowest concentration showing no effect for GO was 1 mg/L. Nogueira et al. (2015) reported results for GO toxicity to the green algae *Raphidocelis subcapitata*. No toxic effects were seen at 5 mg/L or below. Souza et al. (2018) reported an EC10 value for GO and the crustacean *Ceriodaphnia dubia* at 0.26 mg/L. Finally, Fekete-Kertész et al. (2020) reported and EC20 value at 4.78 mg/L in the study of GO effects to *Daphnia magna*.

Overall, all NOEC, EC10, and EC20 values found are higher than the threshold for toxicity of 0.01 mg/L (see Table 1), sometimes much higher. Thus, GBMs cannot be considered as "T" in the PBT criterion.

5. Concluding discussion

Table 2 summarizes the conclusions of this assessment of GBMs when mirrored up against the SVHC criteria. A colour coding for the aggregated result of all reviewed studies is applied. Green here stands for "indication that SVHC criteria are not met", red stands for "indication that SVHC criteria are met", and grey stands for "not enough data to conduct an assessment according to the SVHC criteria".

More data is clearly needed for carcinogenicity, persistence, and endocrine disruption of GBMs. In addition, more data on bioaccumulation and reproductive toxicity would be useful, since there was only one study of the bioaccumulation of a GBM, and relatively few reproductive toxicity studies. However, in the case of reproductive toxicity, existing studies indicate adverse reproductive effects to female mice and offspring. This constitutes an indication that the "R" in the CMR criterion might be fulfilled. A detailed assessment on whether some or all GBMs are toxic to reproduction is therefore strongly recommended. In addition, several studies report genotoxic effects of GBMs in human and mammalian cells, as well as in earthworms and a shrimp. In two studies, mutations were explicitly reported. A detailed assessment of whether some or all GBMs should be classified as mutagenic is therefore also recommended.

For toxicity as evaluated under the PBT assessment of REACH, the available results clearly indicate that GBMs should *not* be classified as toxic according to the SVHC criteria. Since the toxicity criterion is not fulfilled, this means that the PBT criterion as a whole, requiring all three of persistence, bioaccumulation, and toxicity, is not met.

In conclusion, although there are indications of mutagenicity and reproductive toxicity, the limited number of studies means that the current evidence is not deemed strong enough to classify GBMs as SVHC. Instead, more studies are recommended, in particular addressing the concern about "M" and "R" and the scientific literature should be monitored continuously for new findings related to these effects.

The infrequent, or even absent, reporting of several criteria (like carcinogenicity, persistence, and bioaccumulation) could be due to several different reasons. For studies on carcinogenicity, the costs of such studies probably play as significant role and for other toxicological endpoints the tendency in toxicological research not to report negative results (Boorman et al., 2015) might contribute to this. It might also be that if GBMs due to their chemical structure are considered likely to be degraded, the interest in conducting scientific studies on their persistence will be low. For novel materials like GBMs, technical challenges in testing may also make some parameters challenging to measure. Still, considering their regulatory relevance, a strong recommendation from this review is to perform more investigations of the carcinogenicity, persistence, endocrine disruption, bioaccumulation, and reproductive toxicity of GBMs.

The consideration of GBMs as one single group of materials is a generalization of a group of compounds with different functional groups and properties (Wick et al., 2014). This is a methodological limitation of this study. At the same time, waiting for detailed assessments of specific GBMs might lead to industry actors missing the opportunity for early substitutions, which can be beneficial (ChemSec International Chemical Secretariat, 2022). Grouping chemically and physically similar compounds in early risk assessments can thus be justified from a pragmatic point of view. More detailed reporting of GBM forms in studies deriving experimental results would allow for more detailed compound resolutions in future assessments.

In this study, published papers reporting results relevant for the SVHC assessment have been accepted as reliable sources of information. However, such studies can sometimes include methodologically questionable or unclear approaches, which limit their relevance for risk assessment and regulation. An option for future research would be to scrutinize the reviewed studies by different sets of quality criteria, such as Science in Risk Assessment and Policy (SciRAP) (Roth et al., 2021) and nanoCRED (Hartmann et al., 2017).

Finally, this assessment considers GBMs only. However, the number of ENMs is increasing. This is true for other carbon-based nanomaterials, such as nanodiamonds and nanocellulose, but also for other types of ENMs, such as metal nanoparticles and MXenes (Arvidsson et al., 2018, 2022). Especially MXenes appear to show high toxicity (Arvidsson et al., 2022) and might thus be relevant for future studies. Additionally, using different screening methods, such as the NanoRiskCat tool (Hansen et al., 2013), to complement the SVHC criteria would enable a comparison between risk screening tools and an analysis of the robustness of the results.

Table 2. Summary of SVHC criteria and results for graphene-based materials. *Green stands for "indication that SVHC criteria are not met", red stands for "indication that SVHC criteria are met", and grey stands for "not enough data to conduct an assessment according to the SVHC criteria".*

Criteria	**Assessment results**
Carcinogenic (**C**)	No relevant studies, no assessment possible
Mutagenic (**M**)	Two studies report mutagenicity and even more studies report genotoxic effects – detailed assessment recommended
Toxic to reproduction (**R**)	Several studies show reproductive toxicity to female rats and offspring – detailed assessment recommended
Persistent, bioaccumulative and toxic (**PBT**)	**P:** No relevant studies, no assessment possible **B:** One single study, no confirmed transgression **T:** Several studies, all showing low toxicity
Very persistent and very bioaccumulative (**vPvB**)	**vP:** No relevant studies, no assessment possible **vB:** One single study, no confirmed transgression
Substances of equivalent concern (e.g. **EDCs**, **PMT**, **vPvM**)	No relevant studies, no assessment possible

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7. Conflicts of interest

There are no conflicts of interest to declare. In particular, none of the authors are involved in research and development of graphene technology, nor in the catering of the Candidate List or SIN List.

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9. Appendix

Detailed results from the review of the 42 relevant studies. *The different graphene-based material (GBM) types are explained in Section 2. Letters show the studied criteria with C = carcinogenic, M = mutagenic, R = toxic to reproduction, P = persistent, B = bioaccumulative, and T = toxic. Colours code the following results: green for "SVHC criteria not met" and red for "SIN List criteria met". In addition, results from studies reporting genotoxicity are noted within parentheses in the M column, since genotoxicity is an indicator of potential mutagenicity.*

Study	GBM	Organism	C	M	R	P	B	T
Akhavan et al. (2012)	Graphene	Human stem cells		(DNA damage)				
Akhavan et al. (2013)	Reduced graphene oxide	Human stem cells		(DNA damage)				
Bengtson et al. (2017)	Reduced graphene oxide and graphene oxide	Mice		(DNA damage)				
Burgum et al. (2021a)	Graphene and functionalized graphene	Human bronchial cells		(DNA damage)				
Burgum et al. (2021b)	Graphene and functionalized graphene	Human alveolar cells and macrophages		(DNA damage)				
Castro et al. (2018)	Graphene oxide	Raphidocelis subcapitata (algae), Daphnia magna (crustacean), Artemia salina (crustacean)						NOEC ≈ 0.1 mg/L
Chatterjee et al. (2016)	Graphene and functionalized graphene	Human bronchial cells		(DNA damage)				
Chen et al. (2016)	Graphene oxide	Danio rerio (fish)						NOEC = 0.1 mg/L
Demir & Marcos (2018)	Graphene	Mouse lymphoma cells		No mutagenic effects				
di Ianni et al. (2021)	Graphene oxide	Human alveolar cells and macrophages		(DNA damage to macrophages)				
Durán et al. (2015)	Graphene oxide	Daphnia smilis (crustacean), Palaemon pandaliformis (shrimp), human cells		(DNA damage)				NOEC = 3 mg/L, NOEC = 100 mg/L
El-Yamany et al. (2017)	Graphene oxide	Mice		(DNA damage)				
Fan et al. (2016)	Graphene	Daphnia magna (crustacean)					BCF<<2000	NOEC = 0.1 mg/L
Fekete-Kertész et al. (2020)	Graphene oxide	Daphnia magna (crustacean)						EC20 = 4.78 mg/L

Fernandes et al. (2017)	Graphene	Litopenaeus vannamei (crustacean)	(DNA damage)			
Fu et al. (2015)	Graphene oxide	Mice		Effects on offspring		
Li et al. (2014)	Graphene	Daphnia magna (crustacean), Oryzias latipes (fish)				NOEC = 100 mg/L
Liang et al. (2015)	Graphene oxide	Mice		No reduced fertility		
Liu et al. (2013)	Graphene oxide	Human cancer cells, mice	Mutagenesis			
Liu et al. (2014)	Graphene oxide	Danio rerio (fish)				NOEC = 1 mg/L
Mohamed et al. (2020)	Graphene oxide	Mice liver and brain tissues	Genomic instability and mutations			
Nirmal et al. (2017)	Graphene oxide	Rat		No reduced fertility		
Nogueira et al. (2015)	Graphene oxide	Raphidocelis subcapitata (algae)				NOEC = 5 mg/L
Sanchís et al. (2016)	Graphene	Daphnia magna (crustacean)				NOEC = 2 mg/L
Skovmand et al. (2018)	Graphene oxide	Mice		Sperm and semen unaffected		
Souza et al. (2017)	Graphene oxide	Danio rerio (fish)	(No genotoxicity)			
Souza et al. (2018)	Graphene oxide	Ceriodaphnia dubia (crustacean)				EC10 = 0.26 mg/L
Xu et al. (2015)	Reduced graphene oxide	Mice		Effects on offspring		
Wang et al. (2015)	Graphene	Human fibroblast cell	(DNA damage)			
Zhao et al. (2021)	Graphene oxide	Earthworms	(DNA damage)			

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