Safer life cycle of advanced 2D materials used in energy applications

(Safe^{2}energy)

FINAL REPORT OF THE RESEARCH PROJECT

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ABSTRACT

The development of novel energy technologies has brought new demands for safe and sustainable advanced materials with multifunctional properties. Innovative and efficient novel energy storage and conversion technologies based on bidimensional materials (2DM) are one of the main topics in energy applications. Some 2DM, such as hexagonal boron nitride (hBN) and black phosphorus (BP), are promising materials for energy applications, improving the performances of different kinds of batteries.

In view of the increasing concerns for human and environmental safety posed by novel 2DM-based technologies, Safe^{2}energy aims at anticipating potential emerging issues by characterizing the currently unknown hazard posed by hBN and BP to both humans and the environment along their life cycle, chosen as 2 case studies to investigate the occupational safety of 2DM-based energy technologies. Given that the majority of 2DM-based energy applications are still at the experimental stage, the main concern is associated with an occupational scenario, in which workers, either producing 2DM or developing 2DM-based energy technologies, can be highly exposed. In this frame, Safe^{2}energy focused on cutaneous and inhalational exposures, as the main exposure routes in occupational settings. Regarding ecotoxicity studies, the hazard potential of hBN and BP was evaluated onto freshwater organisms, focusing on species at different organization levels: bacteria, microalgae, invertebrates and vertebrate cell lines. The (eco)toxicological potential was evaluated following New Approach Methodologies (NAMs) to reduce the use of animals, in compliance with the 3Rs principle. Whenever possible, standardized assays, described by specific test guidelines (TG) given by the Organization for Economic Cooperation and Development (OECD), were adopted to increase data robustness and acceptance under a regulatory point of view.

In general, as the final outcome of the Safe^{2}energy project, the (eco)toxicological data have been used to assess the potential risks these materials pose to workers, which, together with the Life Cycle Assessment (LCA) data, have led to an evaluation of environmental impacts and a preliminary guide for occupational risk management.

RIASSUNTO

Lo sviluppo di nuove tecnologie energetiche ha introdotto nuove esigenze per materiali avanzati sicuri e sostenibili, dotati di proprietà multifunzionali. Le tecnologie innovative ed efficienti di accumulo e conversione dell'energia basate su materiali bidimensionali (2DM) rappresentano uno dei principali temi nel settore delle applicazioni energetiche. Alcuni 2DM, come il nitruro di boro esagonale (*hexagonal Boron Nitride*; hBN) e il fosforo nero (*Black Phosphorus*; BP), sono materiali promettenti per applicazioni energetiche e migliorano le prestazioni di diversi tipi di batterie.

Alla luce delle crescenti preoccupazioni per la sicurezza umana e ambientale poste dalle nuove tecnologie basate sui 2DM, Safe^{2}energy mirava ad anticipare potenziali problemi emergenti caratterizzando il pericolo, attualmente poco noto, rappresentato da hBN e BP per l'uomo e per l'ambiente lungo il loro ciclo di vita, scelti come due casi studio per indagare la sicurezza occupazionale delle tecnologie energetiche basate sui 2DM. Poiché la maggior parte delle applicazioni energetiche basate sui 2DM è ancora in fase sperimentale, la principale criticità riguarda uno scenario occupazionale, in cui i lavoratori – sia quelli che producono i 2DM, sia quelli che sviluppano le tecnologie energetiche basate su 2DM – possono essere altamente esposti. In questo contesto, il progetto Safe^{2}energy si è focalizzato sull'esposizione cutanea e quella inalatoria, considerate le principali vie di esposizione in ambiente lavorativo. Per quanto riguarda gli studi di ecotossicità, il potenziale di pericolo dell'hBN e del BP è stato valutato sugli organismi d'acqua dolce, concentrandosi su specie appartenenti a diversi livelli di organizzazione: batteri, microalghe, invertebrati e linee cellulari di vertebrati. Il potenziale (eco)tossicologico è stato caratterizzato seguendo le *New Approach Methodologies* (NAMs) per ridurre l'uso di animali, in conformità con il principio delle 3R. Quando possibile, sono stati utilizzati saggi standardizzati, descritti da specifiche linee guida (*Test Guidelines*; TG) fornite dall'Organizzazione per la Cooperazione e lo Sviluppo Economico (OCSE), al fine di aumentare la robustezza dei dati e la loro accettazione a livello regolatorio.

In generale, come risultato finale del progetto Safe^{2}energy, i dati (eco)tossicologici sono stati utilizzati per valutare i potenziali rischi che questi materiali presentano per i lavoratori, che, insieme ai dati sulla valutazione del ciclo di vita (LCA), hanno portato a una valutazione degli impatti ambientali e a una guida preliminare per la gestione dei rischi occupazionali.

TIIVISTELMÄ

Uusien energiateknologioiden kehitys on tuonut mukanaan uusia vaatimuksia turvallisille ja kestävän kehityksen mukaisille edistyneille materiaaleille, joilla on monitoimisia ominaisuuksia. Innovatiiviset ja tehokkaat energiaa varastoivat ja muuntavat teknologiat, jotka perustuvat kaksiulotteisiin materiaaleihin (2DM), ovat yksi keskeisistä tutkimusaiheista energia-alan sovelluksissa. Jotkin 2DM-materiaalit, kuten heksagonaalinen boorinitridi (hBN) ja musta fosfori (BP), ovat lupaavia materiaaleja energiasovelluksiin, sillä ne parantavat erilaisten akkutyyppien suorituskykyä.

Koska uudet 2DM-pohjaiset teknologiat herättävät kasvavaa huolta ihmisten ja ympäristön turvallisuuden kannalta, Safe2energy-hankkeen tavoitteena on ennakoida mahdollisia uusia riskejä luonnehtimalla hBN:n ja BP:n tällä hetkellä tuntematonta vaaraominaisuutta sekä ihmisille että ympäristölle niiden elinkaaren aikana. Näitä kahta materiaalia käytettiin tapaustutkimuksina 2DM-pohjaisten energiateknologioiden työturvallisuuden arvioimiseksi. Koska suurin osa 2DM-pohjaisista energiasovelluksista on edelleen kokeellisessa vaiheessa, keskeisin huolenaihe liittyy ammatilliseen altistumisskenaarioon, jossa työntekijät — joko 2DM-materiaalien tuottajat tai 2DM-teknologioita kehittävät henkilöt — voivat altistua huomattavasti. Tässä kehyksessä Safe2energy keskittyi ihoaltistumiseen ja inhaloitavaan altistumiseen, jotka ovat keskeisimmät altistusreitit työympäristöissä.

Ekotoksikologisten tutkimusten osalta hBN:n ja BP:n mahdollinen vaara arvioitiin makean veden organismeilla, keskittyen lajeihin eri biologisen organisaation tasoilla: bakteereihin, mikroleviin, selkärangattomiin ja selkärankaisten solulinjoihin. (Eco)toksikologista potentiaalia arvioitiin hyödyntäen uusien lähestymistapojen menetelmiä (New Approach Methodologies; NAMs) eläinkokeiden vähentämiseksi 3R-periaatteen mukaisesti. Aina kun mahdollista, käytettiin standardoituja testejä, jotka perustuvat Taloudellisen yhteistyön ja kehityksen järjestön (OECD) antamiin erityisiin testiohjeisiin (Test Guidelines; TG), jotta tulosten luotettavuus ja sääntelyhyväksyttävyys paranisivat.

Kaiken kaikkiaan Safe2energy-hankkeen yleisenä tuloksena (eko)toksikologiset tiedot on integroitu elinkaariarvioinnin (Life Cycle Assessment; LCA) tietoihin, jotta voidaan arvioida näiden materiaalien aiheuttamat mahdolliset riskit sekä työntekijöille että ympäristölle (riskinarviointi; RA) ja lopulta tuottaa tarvittaessa riskinhallintatoimenpiteitä riskien minimoimiseksi.

RESUMEN

El desarrollo de nuevas tecnologías en energía ha generado nuevas demandas de materiales avanzados, seguros y sostenibles, con propiedades multifuncionales. Las tecnologías innovadoras y eficientes de almacenamiento y conversión de energía basadas en materiales bidimensionales (M2D) son una de los principales aspectos a estudiar actualmente en aplicaciones energéticas. Algunos M2D, como el nitruro de boro hexagonal (hBN) y el fósforo negro (BP), son materiales prometedores para este tipo de aplicaciones, mejorando el rendimiento de diferentes tipos de baterías.

En vista de las crecientes preocupaciones por la seguridad humana y ambiental planteadas por las nuevas tecnologías basadas en M2D, el proyecto Safe^{2}energy tiene como objetivo anticipar los posibles problemas emergentes, caracterizando los peligros actualmente desconocidos que representan el hBN y el BP tanto para los seres humanos como para el medio ambiente a lo largo de su ciclo de vida, siendo elegidos como dos casos de estudio para investigar la seguridad ocupacional de las tecnologías energéticas basadas en M2D. Dado que la mayoría de las aplicaciones energéticas basadas en M2D todavía están en etapa experimental, la principal preocupación se asocia a un escenario ocupacional, en el que los trabajadores, ya sea produciendo M2D o desarrollando tecnologías energéticas basadas en M2D, pueden estar altamente expuestos. En este contexto, Safe2energy se centró en las exposiciones cutáneas e inhalatorias, como las principales vías de exposición en entornos ocupacionales. En cuanto a los estudios de ecotoxicidad, la potencial toxicidad del hBN y el BP se evaluó en organismos acuáticos de agua dulce, enfocándose en especies a diferentes niveles de organización: bacterias, microalgas, invertebrados y líneas celulares de vertebrados. El potencial (eco)toxicológico se evaluó siguiendo metodologías de nuevos enfoques (NAMs) para reducir el uso de animales, en cumplimiento con el principio de las 3Rs. Siempre que fue posible, se adoptaron ensayos estandarizados, descritos por directrices de pruebas específicas (TG) dadas por la Organización para la Cooperación y el Desarrollo Económicos (OECD), para aumentar la robustez de los datos y la aceptación desde el punto de vista regulador.

En general, como resultado final del proyecto Safe^{2}energy, los datos (eco)toxicológicos se han utilizado para realizar una evaluación de los riesgos potenciales que estos materiales representan para los trabajadores y que junto con los datos de Evaluación del Ciclo de Vida (ACV) han dado lugar a una evaluación de impactos medioambientales y a una guía preliminar para la gestión de riesgos ocupacionales.

LABURPENA

Energia-teknologia berrien garapenak propietate multifuntzionalak dituzten material aurreratu, seguru eta iraunkorren beharra handitu du. Energia-arloan, bi dimentsioko materialetan (M2D) oinarritutako energia-biltegiratze eta energia-transformaziorako teknologia berritzaileak ikerketa eremu garrantzitsua bihurtu dira. Zenbait M2D,, hala nola boro nitruro hexagonala (hBN) eta fosforo beltza (BP), energia-aplikazioetarako etorkizun handiko materialak dira, bateria mota desberdinen errendimenduan eragin positibo baitute.

M2Dn oinarritutako teknologia berriek gizakien segurtasunari eta ingurumenari buruz planteatzen dituzten kezkak gero eta handiagoak direla ikusita, Safe^{2}energy proiektuaren helburua sortzen ari diren balizko arazoak aurreikustea da. Horretarako, proiektuak material hauen bizi-zikoan zehar eman daitezkeen, harriskuen (oraindik ezezagunen) identifikaziaoan eta deskrbapen identifikatu ditu eta bera oraindik ezezaunak diren harriskuak hBNak eta BPak gizakientzat eta ingurumenarentzat beren bizi-zikloan zehar dituzten arrisku ezezagunak ezaugarrituz, eta M2Dn oinarritutako energia-teknologien okupazio-segurtasuna ikertzeko bi azterketa-kasu gisa aukeratuz. 2DMetan oinarritutako energia-aplikazio gehienak oraindik fase esperimentalean daudenez, kezka nagusia lan-eszenatokiarekin lotuta dago; izan ere, 2DM ekoizten edo 2DMetan oinarritutako energia-teknologiak garatzen dituzten langileak esposizio handiko egoeran egon daitezke. Testuinguru horretan, Safe2energy proiektuak esposizio kutaneoa eta inhalazio bidezkoa hartu ditu kontuan, lan-inguruneetan esposizio-bide nagusiak direlako. Ekotoxikotasun-ikerketei dagokienez, hBNren eta BPren arrisku-potentziala ur gezako organismoetan ebaluatu da, antolaketa-maila desberdinetako espezieetan arreta jarrita: bakterioak, mikroalgak, ornogabeak eta ornodunen zelula-lerroak. (Eko)toksikotasun-potentziala Animalien erabilera murrizteko New Approach Methodologies (NAMs) metodologien bidez ebaluatu da, 3Rs printzipioarekin bat eginez. Ahal izan den guztietan, Ekonomia Lankidetza eta Garapenerako Antolakundeak (OECD) emandako proba-jarraibide espezifikoetan (TG) deskribatutako saiakuntza estandarizatuak erabili dira, datuen sendotasuna eta ikuspegi arautzaile baten baitako onarpena areagotzeko.

Orokorrean, Safe^{2}energy proiektuaren azken emaitzaren gisa, (eko)toksikologiako datuak material horiek langileentzat izan dezaketen arriskua ebaluatzeko erabili dira, eta, Bizitza-Zikloaren Analisiaren (LCA) datuekin batera, ingurumen-inpaktuen ebaluaziora eta lan-arriskuen kudeaketarako lehen orientazio-gidaliburu batera eraman dute.

INDEX

1 BACKGROUND 10

2 OUTLINE OF THE PROJECT 12

2.1 AIM OF THE PROJECT 12

3 RESULTS 15

4 HAZARD CHARACTERIZATION: HUMAN TOXICITY STUDIES (WP2) 17

4.1 RESPIRATORY TOXICITY 17

4.1.1 Case study 1: hexagonal boron nitride (hBN) 17

4.1.2 Case study 2: black phosphorus (BP) 20

4.2 SKIN TOXICITY 23

4.2.1 Case study 1: hexagonal boron nitride (hBN) 23

4.2.1.1 Hazard characterization in skin keratinocytes 23

4.2.1.2 Hazard characterization on the 3D Reconstructed human Epidermis (RhE) model 26

4.2.1.3 Assessment of skin sensitization properties 28

4.2.2 Case study 2: black phosphorus (BP) 30

4.2.2.1 Hazard characterization in skin keratinocytes 30

4.2.2.2 Hazard characterization on the 3D Reconstructed human Epidermis (RhE) model 32

4.2.2.3 Assessment of skin sensitization properties 33

5 HAZARD CHARACTERIZATION: ECOTOXICITY STUDIES (WP3) 35

5.1 DETERMINATION OF THE INHIBITORY EFFECT OF THE SAMPLES ON THE LIGHT EMISSION OF *ALIIVIBRIO FISCHERI* (LUMINESCENT BACTERIA TEST). 35

5.2 ACUTE TOXICITY TEST IN MICROALGAE 37

5.3 *DAPHNIA* SP. ACUTE TOXICITY TEST 38

5.4 *IN VITRO* ECOTOXICITY USING FISH CELL LINES 39

5.5 OVERALL CONCLUSIONS 40

6 LIFE CYCLE, RISK ASSESSMENT AND RISK MANAGEMENT (WP4) 41

6.1 LIFE CYCLE ASSESSMENT. 41

6.1.1 Goal and Scope – Functional Unit 42

6.1.2 Life Cyle Inventory – Data sources 43

6.1.3 Life Cycle Impact Assessment methodology – nanospecific characterization factors 45

6.1.4 Life Cycle Assessment Results 51

6.2 RISK ASSESSMENT OF HBN PRODUCTION. 53
6.2.1 Raw material weighing and pouring 53
6.2.2 Mixing the raw materials 55
6.2.3 Collection of the 2D-hBN 55
6.2.3.1 Inhalation Risk Assessment using StoffenmanagerNano 56
6.2.3.2 Dermal Risk Assessment. 2D Hexagonal Boron Nitride (hBN) 57
6.3 RISK ASSESSMENT OF PHOSPHORENE PRODUCTION. 58
6.3.1 Production of FL-phosphorene 58
6.3.2 Chemical Risk Assessment of the process 63
6.3.3 Conclusions 67
6.4 QUANTITATIVE *IN VITRO* BASED INHALATION RISK ASSESSMENT OF BLACK PHOSPHOROUS USING HUMAN AIRWAY MODELS 69
6.4.1 Benchmark dose (BMD) analysis of repeated-dose cytotoxicity and barrier integrity 70
6.4.2 Conversion of BMDL values to in vitro surface doses 71
6.4.3 Derivation of Human Equivalent Concentrations (HEC) from the in vitro POD 71
6.4.4 Margin of Exposure (MOE) analysis using literature-based occupational concentrations 73
6.5 GENERAL MANAGEMENT GUIDANCE FOR 2D MATERIALS. 75
6.6 CONCLUSIONS AND RECOMMENDATIONS 81
7 DISSEMINATION 82

1 BACKGROUND

The rapid advancement of next-generation integrated energy technologies is creating an increasing demand for advanced materials that are not only safe and sustainable but also endowed with multifunctional properties, in alignment with the ambitions of the EU Green Deal. In this landscape, two-dimensional materials (2DM) are emerging as a highly promising class of materials, attracting growing interest across multiple technological sectors, particularly in energy storage and energy conversion. Their appeal lies in a unique combination of properties, including large surface-to-volume ratios, high theoretical charge-storage capacities, structural anisotropy, remarkable charge-carrier mobility, and tunable bandgaps.[1]

The rising industrial relevance of 2DM is underscored by market projections: according to Maximize Market Research, the global 2DM market is expected to reach approximately US $2.86 billion by 2027, with an estimated compound annual growth rate of 3.9%.[2]

Beyond the extensively investigated graphene, the first 2DM to be identified and characterized under both a technological and biological point of view, hexagonal boron nitride (hBN), also known as “white graphene”, and black phosphorus (BP, or phosphorene) have become two of the most explored 2DM with demonstrated potential in the energy sector.[3,4] hBN has gained attention for its ability to facilitate ion intercalation when used as an anode material, enhancing lithium-ion (LIB) and lithium-sulfur (LSB) batteries performances.[5] Moreover, its outstanding thermal and dielectric behavior enables its use as an insulating layer in LIBs, where it can reduce degradation and improve operational stability.[3] Similarly, BP is particularly noteworthy due to its exceptionally high carrier mobility and its superior theoretical capacity for metal-ion storage compared with many other 2DM. These characteristics make it an excellent candidate for electrodes and supercapacitors, contributing to performance improvements in lithium-ion (LIBs), lithium-sulfur (LSBs), magnesium-ion (MIBs), and sodium-ion (SIBs) batteries.[4] Hence, these two materials were chosen as case studies based on commercially-available 2DM to derive a roadmap for the assessment of safety issues (occupational and environmental) along their entire life cycle, which could also be valid for other 2DM used in energy applications.

[1] Geim AK, Grigorieva IV 2013, Nature, 499: 419–425.
[2] Maximize Market Research. Two-Dimensional Materials Market – Global Industry Analysis and Forecast (2020–2027).
[3] Zhang Z et al. 2019, Journal of Power Sources, 420: 63–72.
[4] Sun J et al. 2015, Nature Nanotechnology, 10: 980–985.
[5] Pakdel A et al. 2014, Chemical Society Reviews, 43: 934–959.

Despite the extensive body of research on the technological potential of 2DM in the energy field, knowledge regarding safety aspects, including their (eco)toxicological effects is still extremely limited. Only a small number of studies have addressed their potential impacts on human health and the environment, leaving major gaps in risk assessment. This observation highlights an urgent need to thoroughly evaluate the hazards associated with 2DM exposure, particularly within occupational settings, where workers involved in the production or handling of these materials may experience the highest levels of exposure. Indeed, it should be underlined that the majority of novel 2DM-based energy technologies are still at the experimental stage, and therefore the main concern is currently associated with an occupational scenario, in which workers - either producing 2DM or developing 2DM-based energy technologies - can be highly exposed, mainly through cutaneous contact and/or inhalation.

2 OUTLINE OF THE PROJECT

Safe^{2}energy was financed by the SAF€RA 2022 JOINT CALL (EU ERA-NET; https://www.safera.eu), within Topic #2 of the SAF€RA 2022 call (*Safety of advanced materials in energy conversion and storage applications*). The project had a duration of 2 years and was coordinated by the University of Trieste (UNITS; Italy; Coordinator: Prof. Marco Pelin) and partnered by the Finnish Institute of Occupational Health (FIOH; Finland) and by GAIKER (Spain).

2.1 AIM OF THE PROJECT

In contrast to the large number of existing publications on 2DM applicability in energy technologies, the knowledge about their (eco)toxic effects is currently very scarce, limited to a few studies. Therefore, there is an urgent need to characterize the potential hazards posed by 2DM for both human health and the environment, allowing the identification of the associated risks, especially in the workplace scenario. Hence, the main goal of Safe^{2}energy was the assessment of the potential (eco)toxicity and occupational risks posed by hBN and BP, proposing, if needed, management measures to be taken by industries to mitigate them. For that, Safe^{2}energy generated robust (eco)toxicological data that, together with information provided by the supporting industries, were integrated with Life Cycle Assessment (LCA) information.

Research activities were divided into 5 different work packages (WP), each organized in different tasks (T):

WP1 – Project management [Leader: UNITS; Partners: FIOH, GAIKER]

The WP was aimed at assuring the correct collaborative integration of activities carried out by the different partners, supporting a multidisciplinary and harmonized approach, taking into consideration the industry's needs, to fulfil the goals of the project. Activities were also dedicated to coordinate reporting activities both as project meetings and project reports.

T1.1 Project coordination.

T1.2 Project reporting.

WP2 – Hazard characterization: human toxicity studies [Leader: UNITS; Partner: FIOH]

The WP was aimed at collecting human toxicity data related to hBN and BP suitable for the human health safety assessment carried out in WP4. Toxicological studies were carried out considering the two main exposure routes for humans at workplaces (i.e. inhalation and skin exposure). *In vitro* innovative approaches were adopted to reduce the use of animals following, whenever possible, standardized test guidelines (TGs), such as those given by the Organization for Economic Cooperation and Development (OECD), to increase data robustness and reliability, and to allow their use also for regulatory purposes.

T2.1 Respiratory toxicity.

T2.2 Skin toxicity.

WP3 – Hazard characterization: ecotoxicity studies [Leader: GAIKER; Partner: UNITS]

The aim of WP3 was the characterization of the ecotoxicological profile of hBN and BP suitable for the environmental safety assessment as long as life cycle assessment (LCA) carried out in WP4. The environmental hazard characterization focused on freshwater organisms, considering species at different organization levels: bacteria, microalgae, invertebrates and vertebrate cell lines. Also in this case, standardized TGs (i.e. OECD TGs) were adopted to increase data robustness and reliability.

T3.1 Microtox bioassay.

T3.2 Microalgae acute toxicity test.

T3.3 Daphnia sp. acute toxicity test.

T3.4 In vitro ecotoxicity using fish cell lines.

WP4 – Life cycle, risk assessment and risk management [Leader: FIOH; Partners: GAIKER, UNITS]

WP4 was aimed at assessing the impacts along the life cycle of hBN (life cycle assessment, LCA), chosen as the material with the highest availability of data, and the potential hotspots and associated risks during the production processes (risk assessment, RA), integrating literature data and data obtained by producing companies together with

(eco)toxicological data provided by WP2 and WP3. A very preliminary risk assessment was performed also for phosphorene production considering a lab scale process identified in a literature search as the most promising for scale up

T4.1 Life Cycle Assessment.

T4.2 Risk Assessment.

T4.3 Risk management.

WP5 – Dissemination [Leader: UNITS; Partners: FIOH, GAIKER]

Dissemination activities of WP5 were coordinated to spread the knowledge gained in the Safe^{2}energy project among the scientific fields, through the publication of scientific articles and through communications in international conferences, as well as among relevant stakeholders (companies, occupational safety and health agencies, national/European regulatory agencies and standardization bodies).

T5.1 Scientific dissemination.

T5.2 Stakeholders dissemination.

The timeline of the activities carried out in each WP is depicted in the following Gantt chart.

	Months:	3	6	9	12	15	18	21	24
WP1	T1.1 Project coordination	■	■	■	■	■	■	■	■
	T1.2 Project reporting				■				■
WP2	T2.1 Respiratory toxicity	■	■	■	■	■	■	■	
	T2.2 Skin toxicity	■	■	■	■	■	■	■	
WP3	T3.1 Microtox bioassay	■	■	■	■				
	T3.2 Microalgae growth inhibition test	■	■	■	■	■	■	■	
	T3.3 *Daphnia* sp. acute immobilisation test	■	■	■	■	■	■	■	
	T3.4 *In vitro* ecotoxicity using fish cell lines	■	■	■	■	■	■	■	
WP4	T4.1 Life Cycle Assessment		■	■	■	■	■	■	■
	T4.2 Risk Assessment		■	■	■	■	■	■	■
	T4.3 Risk management				■	■	■	■	■
WP5	T5.1 Scientific dissemination			■	■	■	■	■	■
	T5.2 Stakeholders dissemination	■	■	■	■	■	■	■	■

3 RESULTS

The main scientific results of the Safe^{2}energy project were collected within the activities carried out in WP2 (Hazard characterization: human toxicity studies), WP3 (Hazard characterization: ecotoxicity studies) and WP4 (Life cycle, risk assessment and risk management). At the time of writing this report, most of the results have not yet been published in scientific journals. For this reason, only the main outcomes of each WP will be presented, without disclosing experimental details that could compromise the confidentiality of the findings, while still ensuring full transparency of the final results. To improve clarity, results will be therefore divided among each WP and relevant tasks, considering the two case studies of the project, one involving hBN and the other BP.

In the case-study 1, two commercially-available hBN materials were provided by a company supporting the project (BeDimensional, Italy) to assess the influence of hBN shape and size on its safety profile. Both materials are produced industrially, following the patented wet-jet mill technology of the bulk materials in N-Methyl Pyrrolidone (NMP). These materials were characterized by different sizes but, most importantly, by different shapes: one was characterized by a rounded shape (hBNr), while the other by cornered sharp edges (hBNc).

In the case-study 2, one commercially-available BP was studied, provided by the only company commercializing BP in Europe (Nanochemazone).

Each material was dispersed in 0.1% bovine serum album (BSA) solution to achieve dispersions which were further diluted directly in cell media, allowing cells treatment.

Each material was physico-chemically characterized by different techniques. Elemental analysis was performed to evaluate the atomic composition of each material. The presence of O_2-bearing functional groups on material structures was evaluated by thermogravimetric analysis (TGA). Depending on the studied material, shape was determined by transmission electron microscopy (TEM) and/or atomic force microscopy (ATM). The former was used also to determine the size of each flake.

Endotoxin contamination of each material was assessed by a modified version of the Tumor Necrosis Factor (TNF)-α Expression Test (TET) assay, using macrophages obtained by differentiation of human THP-1 monocytes. The amount of endotoxin was calculated on the basis of TNF-α cell release induced by LPS content in each material.

Once dispersed in 0.1 % BSA, each material was analyzed for the dispersion stability by UV-Vis analysis up to 2 h. The analysis of pH of each dispersion excluded any bias due

to acidic behavior. Table 1 shows the main physico-chemical properties of each of the materials used in both case-studies.

Table 1. Characteristics of the materials used in Safe^{2}energy.

Material	Lateral dimension (nm)*	No. of layers	Shape	Dispersion stability (2h)	Endotoxin content**
Materials case study #1					
hBNc	2079 ± 119	<10	Sharp and cornered edges	Yes	No
hBNr	851 ± 34	<10	Rounded	Yes	No
Material case study #2					
BP	1815 ± 124	10 - 40	Not-sharp-cornered edges	Low	No

* Assessed by TEM on at least 100 flakes
** No: endotoxin content < 0.5 EU/mL (acceptable limit suggested by the U.S. FDA for medical devices)

4 HAZARD CHARACTERIZATION: HUMAN TOXICITY STUDIES (WP2)

4.1 RESPIRATORY TOXICITY

4.1.1 Case study 1: hexagonal boron nitride (hBN)

The main aim of the respiratory toxicity study was to obtain relevant *in vitro* information on biomarkers from long-term cultures chronically exposed to low doses of the selected 2D materials, which could better mimic real-life exposures. After a literature search, the Epithelix MucilAir tissue model was chosen for the study as it is similar in structure and function to the ciliated pseudostratified respiratory epithelium found in the respiratory tract of all mammals. It had been demonstrated to mimic the physiological and barrier functions of airway epithelial cells, including mucociliary clearance. This made it a suitable model for *in vitro* assessment of human respiratory irritation, represented by direct cytotoxicity. In addition, in recent years, multiple papers have been published with this model, testing different types of compounds including nano- and micro-sized particulate materials.

Initially, a preliminary cytotoxicity study to establish the long-term doses to be used in the MucilAir model was carried out. 2D material cytotoxicity was assessed with a preliminary experiment with Calu-3 cells, a non-small-cell lung cancer cell line that displays epithelial morphology, as they grow as a polarized epithelial monolayer, secrete mucins and produce airway surface liquid, similar to MucilAir cells properties.

Calu-3 cells were exposed to a wide concentration range (0.4–100 µg/mL) of the two hBN (hBNr and hBNc) for 4 or 24 hours in a classical *in vitro* setting. After the exposure period, cytotoxicity was assessed from the culture medium with a commercial LDH assay kit, which measures an enzyme the cells release upon lysis. This experiment was performed 3 times. Only hBNr induced significant cytotoxicity on some doses, however this effect was only observable at 4 hours, disappearing at 24 hours. However, based on the results with Calu-3 cells, hBNc was chosen out of the two hBN forms for the MucilAir experiment due to its slightly higher average cytotoxicity at 4 hours as well as the potential role that edge corners could have in the interaction between material and cells. Considering the low cytotoxicity, it was decided to test the following doses in the Mucilair model: 0.1, 1, 10 and 100 µg/mL.

The toxicological impact of hBNc was therefore assessed with the MucilAir model with the objective of generating *in vitro* data specifically on cytotoxic, inflammatory, barrier integrity and genotoxic biomarkers following long-term, low-dose exposures. To achieve this, a series of assays were selected: LDH release to assess cytotoxicity, ELISA cytokine

analysis to analyze inflammation, Lucifer Yellow permeability to evaluate epithelial barrier function, the comet assay to detect DNA damage, and TEM imaging to determine the interaction of the materials with the cells.

Cells were cultured at the air–liquid interface on 24-well plate inserts with 1 µm pores and maintained in the manufacturer's complete medium at 37 °C and 5 % CO_2. Medium was changed three times per week, and an apical HBSS wash was performed before the start of exposures to standardize mucus levels. Cells were treated with hBNc dispersions at 0.1, 1, 10 and 100 µg/mL for 4 h/day, five days per week, over a 28-day period. Vehicle controls received 0.1 % BSA/water, and each concentration was tested using six biological replicates, with inserts reserved for the respective positive and negative controls.

Sampling was performed throughout the study. Basolateral medium was collected at day 0 (both 0 h and 4 h) and on days 7, 14, 21 and 28 for LDH and cytokine analysis (IL-1α, IL-6, IL-8 and MCP-1). LDH maximum-release controls were included at the beginning and end of the study, and LPS (10 µg/mL) was applied 24 h before sampling as a positive control. At day 28, barrier integrity was assessed by Lucifer Yellow assay, with 0.5 % Triton X-100 used as permeability control. Genotoxicity at day 28 was evaluated through the comet assay, with MMS (82.5 µg/mL) as the positive control. Finally, inserts exposed to 10 µg/mL of each material were processed for TEM analysis.

The main results are summarized in Table 2. Exposure to hBNc did not induce detectable cytotoxicity in the MucilAir model throughout the 28-day study. LDH release was comparable to the negative controls at all concentrations and timepoints. No dose dependent viability decrease was observed, indicating that neither acute or cumulative exposure to hBNcorner affected the epithelial viability under the tested conditions.

Lucifer Yellow permeability measurements at day 28 showed that hBNc did not compromise epithelial barrier integrity at any of the concentrations tested. Lucifer Yellow across the epithelium remained comparable with the negative control, with no indication of increased paracellular leakage or dose-dependent effects. All treated tissues maintained low permeability values far below those of the positive control, showing that prolonged repeated exposure to hBNc did not affect tight-junction function or barrier cohesion.

Comet assay analysis at day 28 showed no increase in DNA strand breaks in the inserts exposed to hBNc at any concentration, although damage levels were slightly and with a high degree of variability. However, the positive control did not induce a statistically significant increase in DNA damage. While no genotoxic effect of hBNc was detected, the absence of a robust positive-control response limits the confidence of the findings.

Table 2. Summary of the main results obtained treating the MucilAir model with hBNc (0.1 - 100 µg/mL) for 4 h/day, five days per week, for 28 days.

	hBNc (µg/mL)				
Parameter	**0.1**	**1**	**10**	**100**	**Positive control**
Cytotoxity (LDH release)	no	no	no	no	yes
Barrier integrity (Lucifer Yellow)	no	no	no	no	yes
Genotoxicity (Comet assay)	10%	10%	10%	10%	>20%

Cytokine analysis showed a limited inflammatory response to hBNc exposure (Table 3). IL-6 secretion increased only at the highest concentration (100 µg/mL), with detectable elevations at day 14 that became more pronounced by day 28. IL-8 levels also increased at 100 µg/mL on day 14, although this effect was not observed at day 28. IL-1α and MCP-1 levels were similar to the negative controls across all timepoints. Overall, the cytokine pattern indicates some inflammatory activation exclusive to IL-6 and IL-8 at the highest dose and longer timepoints.

Table 3: Cytokine secretion in MucilAir tissue cells exposed to hBN corner. 4 replicates per timepoint and dose.

	hBNc							
	IL-1α		IL-6		IL-8		MCP-1	
	1 µg/mL	100 µg/mL	1 µg/mL	100 µg/mL	1 µg/mL	100 µg/mL	1 µg/mL	100 µg/mL
4h	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
14d	**-**	**-**	**-**	**+**	**-**	**-**	**-**	**-**
28d	**-**	**-**	**-**	**+**	**-**	**+**	**-**	**-**

The TEM analysis of inserts exposed to 10 µg/mL of hBNc did not show any uptake or intracellular localization. The cell morphology was comparable to the unexposed cells, with no observable aggregates in any cell compartment or in the extracellular space.

Overall, the 28-day repeated-dose exposure of MucilAir tissues to hBNc produced small to none effects in the studied endpoints. No cytotoxicity, barrier disruption or detectable genotoxicity was observed, and TEM imaging did not reveal any interaction of the material with the epithelial cells. The inflammatory response was limited to increases in IL-6 and

IL-8 at the highest concentration, with no changes in IL-1α or MCP-1. Altogether, these results indicate that hBNc induced minor and dose-restricted inflammatory effects and did not induce broad adverse effects under the tested conditions.

4.1.2 Case study 2: black phosphorus (BP)

The same approach described in section 4.1.1 was taken to determine the BP dose range to test in the Mucilair model, but in the case of BP only one material was tested. Again, Calu-3 cells were exposed to a wide concentration range (0.4–100 µg/mL) for 4 or 24 hours in a classical *in vitro* setting. The results obtained showed that none of the doses tested induced a significant increase in cytotoxicity. Interestingly, cytotoxicity was higher at lower doses than at the highest does, which was below the negative control although not statistically significant. Considering the cytotoxicity data, it was decided to test the following doses in the Mucilair model: 0.1, 1, 10 and 100 µg/mL.

The Mucilair model experiment was conducted in parallel with hBNc (as described in section 4.1.1) and the sampling followed the same experimental design. Basolateral medium was collected at day 0 (both 0 h and 4 h) and on days 7, 14, 21 and 28 for LDH and cytokine analysis (IL-1α, IL-6, IL-8 and MCP-1). LDH maximum-release controls were included at the beginning and end of the study, and LPS (10 µg/mL) was applied 24 h before sampling as a positive control. At day 28, barrier integrity was assessed by Lucifer Yellow assay, with 0.5 % Triton X-100 used as permeability control. Genotoxicity at day 28 was evaluated through the comet assay, with MMS (82.5 µg/mL) as the positive control. Finally, inserts exposed to 10 µg/mL of each material were processed for TEM analysis.

The main results are summarized in Table 4. Exposure to BP resulted in a cytotoxic response at the highest concentration tested. While LDH release at 0.1, 1 and 10 µg/mL remained comparable to the negative controls across all timepoints, the 100 µg/mL condition showed a clear decrease in viability beginning at day 14. This effect became more pronounced at day 21 and further increased by day 28, indicating that cumulative exposure to BP at the highest dose progressively affected epithelial viability under the tested conditions.

Lucifer Yellow permeability measurements at day 28 showed that BP affected the epithelial barrier integrity at the highest concentration testet. While permeability values for 0.1, 1 and 10 µg/mL were similar to the negative control, the 100 µg/mL dose induced a significant increase in Lucifer Yellow flux across the epithelium, indicating a disruption of the barrier cohesion after the long-term exposure to BP.

Comet assay analysis at day 28 showed no increase in DNA strand breaks in the inserts exposed to BP at any concentration, although damage levels were slightly and with a high degree of variability. However, again the positive control did not induce a statistically significant increase in DNA damage. While no genotoxic effect of BP was detected, the absence of a robust positive-control response limits the confidence of the findings.

Table 4. Summary of the main results obtained treating the MucilAir model with BP (0.1 - 100 µg/mL) for 4 h/day, five days per week, for 28 days.

	BP (µg/mL)				
Parameter	**0.1**	**1**	**10**	**100**	**Positive control**
Cytotoxity (LDH release)	no	no	no	yes	yes
Barrier integrity (Lucifer Yellow)	no	no	no	yes	yes
Genotoxicity (Comet assay)	<10%	<10%	<10%	<10%	>20%

Cytokine analysis showed a higher inflammatory response to BP compared with hBNc, with effects observed at both 1 and 100 µg/mL (Table 5). IL-6 secretion increased at day 14 for both concentrations although these elevations were not maintained at day 28. MCP-1 levels were also elevated at day 14 at both doses, the increase was still observed at day 28 in the 100 µg/mL condition, suggesting a continuation of monocyte-recruiting signaling at the highest exposure level. IL-1α and IL-8 levels were similar to the negative controls across all timepoints. Overall, BP induced IL-6 and MCP-1 activation, while other cytokines were not affected.

Table 5. Cytokine secretion in MucilAir tissue cells exposed to BP. 4 replicates per timepoint and dose.

	BP							
	IL-1α		IL-6		IL-8		MCP-1	
	1 µg/mL	100 µg/mL	1 µg/mL	100 µg/mL	1 µg/mL	100 µg/mL	1 µg/mL	100 µg/mL
4h	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**-**
14d	**-**	**-**	**+**	**+**	**-**	**-**	**+**	**+**
28d	**-**	**-**	**-**	**-**	**-**	**-**	**-**	**+**

The TEM analysis of inserts exposed to 10 µg/mL of BP did show the presence of aggregated material within the cells. The cell morphology was similar to the unexposed cells except the presence of aggregates, no aggregates were observed in the extracellular space.

Overall, the 28-day exposure to BP produced clear biological effects at the highest concentration tested. BP induced a progressive decrease in epithelial viability and a measurable disruption of barrier integrity at 100 µg/mL, while lower concentrations did not affect these endpoints. No genotoxicity was detected but the lack of a robust positive control limits confidence in the comet assay findings. The cytokine profile further indicated an inflammatory response, with increases in IL-6 and in MCP-1, particularly at the highest dose, while IL-1α and IL-8 were unaffected. TEM imaging confirmed the presence of intracellular aggregated material in tissues exposed to 10 µg/mL BP, although overall epithelial morphology remained comparable to controls. Altogether, these results suggest that BP can elicit cytotoxic, barrier-disruptive, and inflammatory effects under long-term exposure conditions, with responses mainly in the exposure to the highest dose tested.

4.2 SKIN TOXICITY

4.2.1 Case study 1: hexagonal boron nitride (hBN)

4.2.1.1 Hazard characterization in skin keratinocytes

Preliminary results were carried out on human HaCaT keratinocytes to initially assess the interaction and internalization of hBN particle. To this aim, a research-grade hBN (average lateral dimension: 120 ± 56; n. layers: <10) was initially studied. This hBN was obtained through a mechanochemical approach, using bulk boron nitride as the precursor and glycine as the exfoliating agent, and was provided by the group of Prof. Ester Vazquez (University of Castilla-La Mancha, Spain). Cells treated with hBN (50 µg/mL) for 24 or 72 h were analyzed by super-resolution SIM and TEM. SIM imaging, using DiL or phalloidin plus Hoechst staining, showed that hBN particles interacted with the plasma membrane and were internalized into the cytoplasm, often accumulating around nuclei. Despite this extensive uptake, no morphological alterations were observed in keratinocytes. TEM analysis confirmed massive internalization after 24 h, with hBN located perinuclearly but not inside nuclei, and showing accumulation within lysosomes (Figure 1).

Subsequently, we explored how two of the main physico-chemical properties (i.e. shape and size) of hBN might influence its safety profile at the skin level, in the frame of the Safe-by-Design approach. To this end, two commercially-available hBN samples, one characterized by a cornered sharp edges and larger dimension (hBNc) and the other characterized by rounded shape and shorter dimensions (hBNr), were comparatively analyzed (see Table 1).

The effects of hBNc and hBNr (0.001–100 µg/mL) on HaCaT keratinocyte viability, adhesion, and membrane integrity were assessed after 24 h and 7 days using WST-8, SRB, and PI-uptake assays, respectively. Comparisons between hBNr and hBNc materials were used to evaluate the impact of shape and size on cytotoxicity (Table 6 and 7). After 24 h, neither material altered cell viability, but a 7-day exposure produced a concentration-dependent decrease starting at 10 µg/mL, reaching 49% and 29% at 100 µg/mL. The no-observed-effect-concentration (NOEC) was 1 µg/mL for both materials, whereas the concentrations giving the 50% of the effect (EC_{50}) were 90.3 µg/mL for hBNr and 28.6 µg/mL for hBNc, indicating a 3.2-fold higher cytotoxic potency for the latter.

Similarly, SRB results showed that only long-term exposure impaired cell adhesion. hBNr reduced adhesion from 10 µg/mL (83%) with an EC_{50} of 28.2 µg/mL, whereas hBNc caused stronger effects (63% at 10 µg/mL; 16% at 100 µg/mL) with an EC_{50} of 11.3 µg/mL.

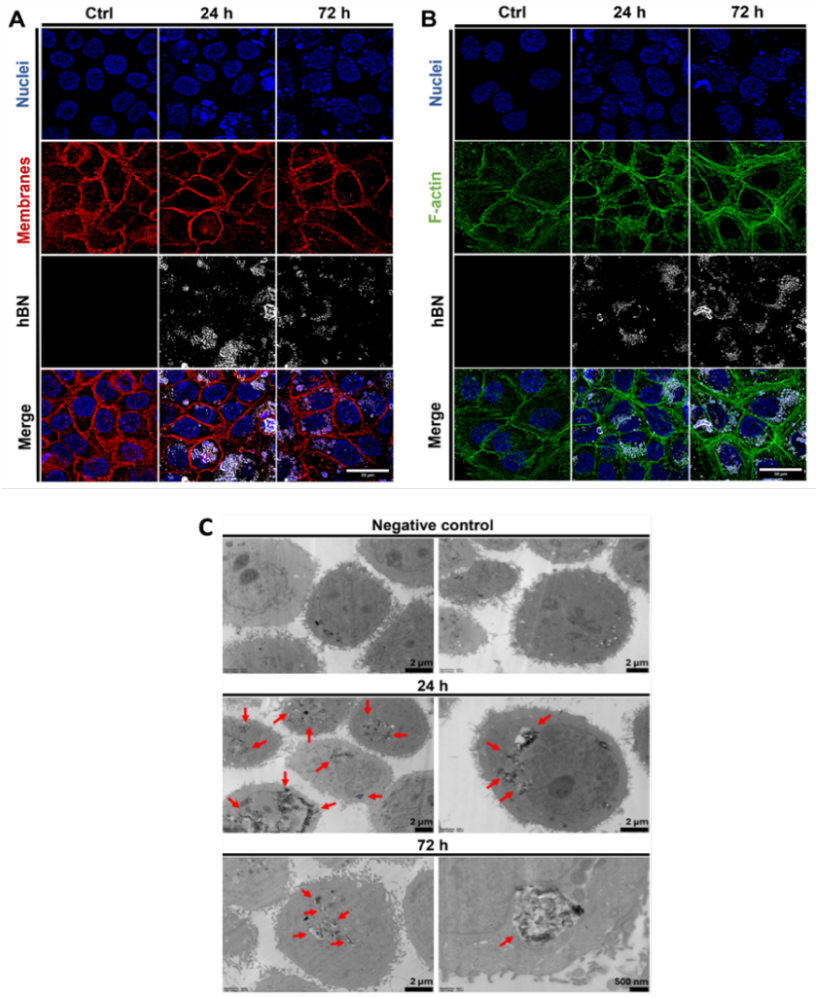

Figure 1. Multimodal imaging of HaCaT cells exposed to hBN (50 µg/mL) for 24 h. (A,B) Representative images obtained by SIM; F-actin filaments are labelled with fluorescent phalloidin (green), membranes are labelled with fluorescence DiL (red); hBN flakes are visualized in white and merged images represent the reconstruction of labelled HaCaT cells with hBN signal. (C) Representative images obtained by TEM. Arrows indicate the presence of hBN inside cells. Modified by Carlin et al. 2025 (J Haz Mat. 494: 138449).

PI-uptake analysis revealed a concentration-dependent loss of membrane integrity after 7 days, starting at 1 µg/mL. At 100 µg/mL, PI uptake reached 47% for hBNr and 67% for hBNc. EC_{50} values were >100 µg/mL and equal to 30.9 µg/mL for hBNr and hBNc, respectively, suggesting that membrane damage was significantly greater for hBNc. Overall, these data show that hBNc exerts consistently higher cytotoxicity than hBNr, likely due to its distinct cornered shape and larger size.

Table 6. Effects of hBNr (0.001-100 µg/mL, dilution factor 10) on HaCaT keratinocytes after short (24 h) and long (7 days) exposures. EC_{50}: concentration giving 50% of the effect; NOEC (No Observed Effect Concentration): highest concentration at which no effect was observed; E_{max}: Maximal effect induced by each material over control considering the complete concentration-response range measured.

	24 h			7 days		
	EC_{50}	**NOEC**	**E_{max}**	**EC_{50}**	**NOEC**	**E_{max}**
Cell viability	>100 µg/mL	100 µg/mL	92.2% of cell viability at 100 µg/mL	79.4 µg/mL	1 µg/mL	48.8% of cell viability at 100 µg/mL
Cell adhesion	>100 µg/mL	100 µg/mL	98.8% of cell adhesion at 100 µg/mL	32.9 µg/mL	1 µg/mL	27.0% of cell adhesion at 100 µg/mL
Membrane damage	>100 µg/mL	100 µg/mL	5.5% of cell necrosis at 100 µg/mL	>100 µg/mL	1 µg/mL	47.2% of cell necrosis at 100 µg/mL

Table 7. Effects of hBNc (0.001-100 µg/mL, dilution factor 10) on HaCaT keratinocytes after short (24 h) and long (7 days) exposures. EC_{50}: concentration giving 50% of the effect; NOEC (No Observed Effect Concentration): highest concentration at which no effect was observed; E_{max}: Maximal effect induced by each material over control considering the complete concentration-response range measured.

	24 h			7 days		
	EC_{50}	**NOEC**	**E_{max}**	**EC_{50}**	**NOEC**	**E_{max}**
Cell viability	>100 µg/mL	1 µg/mL	84.6% of cell viability at 100 µg/mL	19.7 µg/mL	1 µg/mL	29.2% of cell viability at 100 µg/mL
Cell adhesion	>100 µg/mL	1 µg/mL	84.2% of cell adhesion at 100 µg/mL	32.9 µg/mL	1 µg/mL	16.9% of cell adhesion at 100 µg/mL
Membrane damage	>100 µg/mL	10 µg/mL	11.1% of cell necrosis at 100 µg/mL	30.9 µg/mL	1 µg/mL	67.2% of cell necrosis at 100 µg/mL

4.2.1.2 Hazard characterization on the 3D Reconstructed human Epidermis (RhE) model

Subsequent analyses were carried out on the 3D Reconstructed human Epidermis (RhE) model, that mimics all the functional, morphological and biochemical properties of intact epidermis (Figure 2).

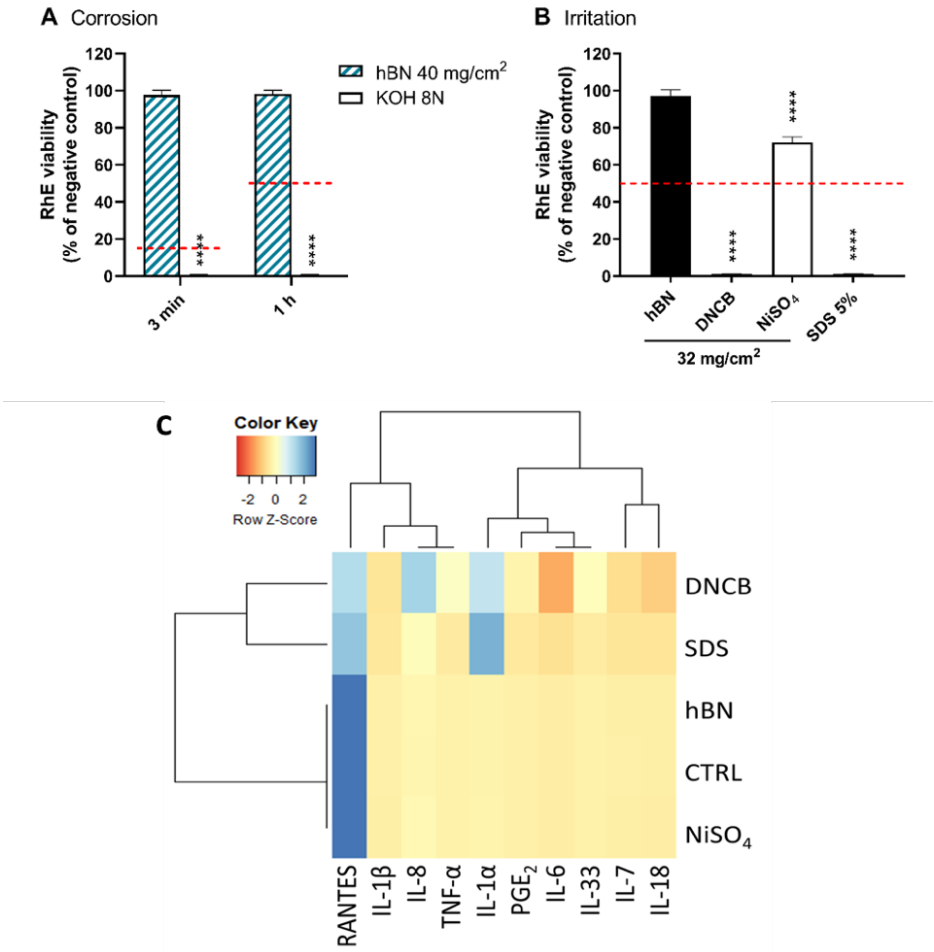

Figure 2. Skin corrosion (A) and irritation (B) properties of hBN, controls and reference substances evaluated as SkinEthic™ Reconstructed Human Epidermis (RhE) viability. Results are the mean ± SE of three independent experiments. (C) Heatmap and relevant hierarchical cluster analysis made on inflammatory mediators' data, released by RhE exposed to hBN, $NiSO_4$ and DNCB (32 mg/cm^2) and the positive control for irritation SDS (5%) after 42 min, followed by 42 h of post-treatment incubation. Modified by Carlin et al. 2025 (J Haz Mat. 494: 138449).

First, skin corrosion properties of the research-grade hBN (40 mg/cm²) were assessed following the procedure described in the OCD TG 431. Exposure for 3 minutes or 1 hour did not significantly reduce the viability of RhE tissue. This was below the OECD TG 431 thresholds (viability <50% at 3 min or <15% at 1 h), classifying hBN as non-corrosive. The positive control (8 N KOH) confirmed the test validity by reducing viability to 0.7%.

Next, hBN (32 mg/cm²) was tested for skin irritation potential following the OECD TG 439, after an exposure of 42 min, followed by 42 h of post-incubation without the material. RhE viability remained above the 50% threshold, indicating hBN as a non-irritant material. The positive control (5% SDS) decreased viability to 1.1%, confirming its irritant nature. Additional reference agents were used: the irritant and sensitizing 2,4-dinitrochlorobenzene (DNCB) reduced viability to 1%, confirming its irritant properties, while the sensitizing $NiSO_4$ reduced viability to 72%, indicating slight toxicity but no irritant effect. These data collectively establish hBN as safe regarding both skin corrosion and irritation endpoints.

To assess the pro-inflammatory properties of hBN, tissue media from treated RhE tissues were collected to quantify a panel of selected pro-inflammatory mediators (IL-1α, -1β, -6, -7, -8, -18, -33, TNF-α, PGE2 and RANTES). Clustering analysis showed that hBN exhibited a mediator release pattern similar to the negative control, indicating a lack of pro-inflammatory properties. Strong irritants and sensitizers showed distinct profiles, with 5% SDS significantly increasing IL-1α, IL-8, and IL-33, while DNCB and $NiSO_4$ weakly increased IL-18.

Subsequent analyses were carried out on the 3D Reconstructed human Epidermis (RhE) model, to evaluate the role of shape and size on hBN irritation and corrosion potential, testing the two commercially-available hBNs, following the specific guidelines given by OECD (Table 8).

In skin irritation tests (OECD TG 439), both hBNc and hBNr (32 mg/cm²) did not lower RhE viability below the 50% threshold, confirming a non-irritant profile. In contrast, the positive control SDS reduced tissue viability to 1.4%, demonstrating strong irritant activity. Regarding skin corrosion (OECD TG 431), exposure to hBN flakes (40 mg/cm²) for 3 minutes and 1 hour slightly decreased RhE viability at values far above corrosive classification limits. Conversely, the positive control KOH dropped viability to 0.7% after just 3 minutes, confirming its corrosive nature. Overall, hBN materials — even those with sharp asperities — did not induce skin irritation or corrosion at the tissue level.

Despite the absence of corrosive and irritative properties, cutaneous bio-interactions and possible penetration of hBN could result of paramount importance for evaluating its safety and biological effects at the skin level. Therefore, histological analyses performed by SEM, TEM as well as by light microscopy on hematoxylin/eosin stained specimen revealed no histological and ultrastructural alterations in RhE tissues treated with hBN, even though both materials (hBNc > hBNr) strongly adhered to the surface of the *Stratum corneum* accumulating on it. However, both materials were ineffective in penetrating the epidermis.

Table 8. Skin irritation and skin corrosion prediction of hBNr and hBNc on the 3D RhE model, through the adoption of OECD TG 439 and 431, respectively.

Parameter	Criteria	hBNr	hBNc
Irritation			
OECD TG 439 - *In Vitro* Skin Irritation: Reconstructed Human Epidermis (RhE) Test Method	RhE viability > 50% after 42 min exposure + 42 h post-incubation time	Non-irritant	Non-irritant
Corrosion			
OECD TG 431 - *In Vitro* Skin Corrosion: Reconstructed Human Epidermis (RhE) Test Method	RhE viability > 15% after 3 min exposure and RhE viability > 50% after 1 h exposure	Non-corrosive	Non-corrosive

4.2.1.3 Assessment of skin sensitization properties

The hazard characterization of hBN at the skin level was completed assessing skin sensitization potential. Skin sensitization properties were evaluated following an *in chemico*/*in vitro* approach able to predict the first three key phases of the skin sensitization Adverse Outcome Pathway (AOP). These are predicted by the adoption of three specific OECD TGs, which combination of the results following the 2-out-3 defined approach is able to predict skin sensitization potential of a substance (OECD TG 497; OECD 2025)[6].

Firstly, the first key event of skin sensitization AOP, namely the reactivity of a substance toward skin proteins, was evaluated through the adoption of the OECD TG 442C (OECD

[6] OECD. (2025). *Guideline No. 497: Defined Approaches on Skin Sensitisation*, Organisation for Economic Co-operation and Development.

2025)[7]. This method evaluates the reactivity of test substances with synthetic peptides containing nucleophilic amino acids cysteine and lysine using high performance liquid chromatography (HPLC). The assay measures the depletion of these peptides after 24 h incubation with the test substance, which is indicative of its potential to cause skin sensitization. A substance can be considered positive to the DPRA if it determines a peptide depletion above 6.38%. After 24 h of incubation, peptide depletions below the 6.38% threshold were recorded for both hBNr and hBNc. Hence, these materials resulted negative according to the OECD TG 442C DPRA prediction model, since they have a minimal reactivity towards cysteine and lysine peptides. In contrast, the positive control cinnamic aldehyde induced 63.64% of peptide depletion, demonstrating its extremely high reactivity (Table 9).

The skin sensitization potential was subsequently assessed *in vitro* using the ARE-Nrf2 Luciferase KeratinoSens™ assay, addressing the second key event of the skin sensitization AOP, namely keratinocyte activation. This method was carried out following OECD TG 442D (OECD 2024)[8] using a human keratinocyte cell line (KeratinoSens™) that contains a luciferase reporter gene under the control of the antioxidant response element (ARE), overexpressed during skin sensitization. The positive control cinnamic aldehyde, hBNr and hBNc induced a significant increase in luciferase activity, above the threshold of 1.5-fold induction at concentrations at which cell viability was >70%, demonstrating their ability to activate keratinocytes. Thus, both hBN materials can be considered positive according to OECD TG 442D (Table 9).

The skin sensitization potential was lastly assessed *in vitro* using the human Cell Line Activation Test (h-CLAT), addressing the third key event of the skin sensitization AOP (activation of dendritic cells). The h-CLAT method was carried out following OECD TG 442E (OECD 2024).[9] Flow cytometry analyses allowed us to quantify the changes in the expression of specific cell surface markers (CD54 and CD86) associated with the activation of dendritic cells derived from the human monocytic leukemia cell line THP-1 after 24 h exposure to a test substance. Flow cytometry analyses demonstrated that both hBNr and hBNc were not able to induce monocyte differentiation to dendritic cells since they did not trigger an expression of the differentiation markers CD54/CD86 at levels

[7] OECD. (2025). *Test No. 442C: In Chemico Skin Sensitisation: Assays addressing the Adverse Outcome Pathway key event on covalent binding to proteins.* Organisation for Economic Co-operation and Development.

[8] OECD. (2024). *Test No. 442D: In Vitro Skin Sensitisation: Assays addressing the Adverse Outcome Pathway Key Event on Keratinocyte activation.* Organisation for Economic Co-operation and Development.

[9] OECD. (2024). *Test No. 442E: In Vitro Skin Sensitisation: In Vitro Skin Sensitisation assays addressing the Key Event on activation of dendritic cells on the Adverse Outcome Pathway for Skin Sensitisation.* Organisation for Economic Co-operation and Development.

higher than the thresholds defined in the TG. According to the OECD TG 442E h-CLAT prediction model, they can be considered negative (Table 9).
On the whole, basing on the 2-out-3 defined approach proposed by the OECD TG 497, two concordant results obtained from methods addressing at least two of the first three key events of the skin sensitization AOP (OECD TG 442C, D and E) determine the final classification of a substance. Therefore, the present results suggest that the tested hBNr and hBNc are not skin sensitizers since at least 2 out of the 3 OECD TGs for skin sensitization prediction were reliably negative.

Table 9. Skin sensitization prediction of hBNr and hBNc through the adoption of OECD TG 442C, 442D and 442E, respectively able to predict the first (peptide reactivity), second (keratinocytes activation) and third (dendritic cells activation) phases of skin sensitization AOP. Final prediction was made adopting the 2-out-3 defined approach by the OECD TG 497.

Test guideline	**hBNr**	**hBNr final prediction**	**hBNc**	**hBNc final prediction**
OECD TG 442C - *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay	Negative	**Non-sensitizer** according to the 2-out-3 Defined Approach (OECD TG 497)	Negative	**Non-sensitizer** according to the 2-out-3 Defined Approach (OECD TG 497)
OECD TG 442D – *In Vitro* Skin Sensitisation: ARE-Nrf2 luciferase KeratinoSens™ test method	Positive		Positive	
OECD TG 442E – *In Vitro* Skin Sensitisation: human cell Line Activation Test (h-CLAT)	Negative		Negative	

4.2.2 Case study 2: black phosphorus (BP)

4.2.2.1 Hazard characterization in skin keratinocytes

As done for hBN, the characterization of the hazard posed by BP at the skin level was initially assessed in HaCaT keratinocytes. Different cellular parameters, including cell viability (WST assay), cell necrosis (PI uptake assay), intracellular levels of ATP (ELISA quantification) and mitochondrial depolarization (JC-1 probe), were evaluated both after short (24 h) and a long (7 days) exposures (Table 10).

After a short exposure of 24 h, BP induced only slight cytotoxic effect, reaching a maximum effect for all parameters at the highest concentration of 100 µg/mL, with NOEC spanning from 1 µg/mL (cell viability) to 25 µg/mL (cell necrosis). However, the cytotoxic potential was more evident after an exposure as long as 7 days. Indeed, after a long exposure, cell viability was reduced with an EC_{50} value of 19.7 µg/mL, a NOEC of 1 µg/mL and a maximum effect of 29% residual cell viability at 100 µg/mL. Cell necrosis was induced with an EC_{50} value of 3.2 µg/mL, a NOEC of 0.8 µg/mL and a maximum effect of 78% cell necrosis at 100 µg/mL. ATP cellular production was reduced with an EC_{50} value of 3.6 µg/mL, a NOEC of 1.6 µg/mL and a maximum effect of only 0.8% residual ATP production at 100 µg/mL. Similarly, mitochondrial depolarization, one of the possible causes of ATP depletion, was induced with an EC_{50} of 2.6 µg/mL, a NOEC of 1.6 µg/mL and a maximum effect of 69% mitochondrial depolarization at 100 µg/mL.

Table 10. Effects of BP (0.8-100 µg/mL, dilution factor 2) on HaCaT keratinocytes after short (24 h) and long (7 days) exposures. EC_{50}: concentration giving 50% of the effect; NOEC (No Observed Effect Concentration): highest concentration at which no effect was observed; E_{max}: Maximal effect induced by each material over control considering the complete concentration-response range measured.

	24 h			**7 days**		
	EC_{50}	**NOEC**	**E_{max}**	**EC_{50}**	**NOEC**	**E_{max}**
Cell viability	>100 µg/mL	1 µg/mL	84.6% of cell viability at 100 µg/mL	19.7 µg/mL	1 µg/mL	29.2% of cell viability at 100 µg/mL
Cell necrosis	>100 µg/mL	25 µg/mL	27.8% of cell necrosis at 100 µg/mL	3.2 µg/mL	0.8 µg/mL	78.2% of cell necrosis at 100 µg/mL
ATP production	>100 µg/mL	6.3 µg/mL	52.0% of ATP production at 100 µg/mL	3.6 µg/mL	1.6 µg/mL	0.8% of ATP production at 100 µg/mL
Mitochondrial depolarization	>100 µg/mL	6.3 µg/mL	23.5 % of mitochondrial depolarization at 100 µg/mL	2.6 µg/mL	1.6 µg/mL	68.6% of mitochondrial depolarization at 100 µg/mL

On the whole, these data suggest a slight-to-modest cytotoxic potential of BP in skin keratinocytes, being negligible after a short exposure of 24 hours, but being more evident after a far longer exposure of 7 days. Noteworthy, the cytotoxic potency after 7 days seems to be slightly higher with respect to that previously reported for hBN, at least considering these cellular parameters.

4.2.2.2 Hazard characterization on the 3D Reconstructed human Epidermis (RhE) model

As already done for hBN, subsequent analyses were carried out on the 3D Reconstructed human Epidermis (RhE) model, to evaluate irritation and corrosion potential of BP, following the specific guidelines given by OECD (Table 11).

In skin irritation tests (OECD TG 439), BP (32 mg/cm²) did not reduce RhE viability below the 50% threshold, confirming a non-irritant profile. In contrast, the positive control SDS reduced tissue viability to 1.4%, demonstrating strong irritant activity. Regarding skin corrosion (OECD TG 431), exposure to BP (40 mg/cm²) for either 3 minutes and 1 hour only slightly reduced tissue viability at values far above corrosive classification thresholds. Conversely, the positive control KOH dropped viability to 0.7% after just 3 minutes, confirming its corrosive nature. Overall, despite the mild cytotoxic potential observed in keratinocytes after long exposure times, BP does not induce skin irritation or corrosion at the tissue level.

Table 11. Skin irritation and skin corrosion prediction of BP on the 3D RhE model, through the adoption of OECD TG 439 and 431, respectively.

Parameter	Criteria	BP
Irritation		
OECD TG 439 - *In Vitro* Skin Irritation: Reconstructed Human Epidermis (RhE) Test Method	RhE viability > 50% after 42 min exposure + 42 h post-incubation time	Non-irritant
Corrosion		
OECD TG 431 - *In Vitro* Skin Corrosion: Reconstructed Human Epidermis (RhE) Test Method	RhE viability > 15% after 3 min exposure and RhE viability > 50% after 1 h exposure	Non-corrosive

4.2.2.3 Assessment of skin sensitization properties

The hazard characterization of BP at the skin level was then completed assessing skin sensitization potential as previously reported for hBN (Table 12).
Firstly, DPRA was applied to assess BP capability to react with skin proteins. After 24 h of incubation, peptide depletions below the 6.38% threshold were recorded for BP. Hence, it resulted negative according to the OECD TG 442C DPRA prediction model, since it is characterized by a minimal reactivity towards cysteine and lysine peptides, in contrast to the positive control cinnamic aldehyde.
The skin sensitization potential of BP was subsequently evaluated using the ARE-Nrf2 Luciferase KeratinoSens™ assay, addressing keratinocyte activation. Both the positive control cinnamic aldehyde and BP induced a significant increase in luciferase activity, above the threshold of 1.5-fold induction at concentrations at which cell viability was >70%, demonstrating their ability to activate keratinocytes. Thus, BP can be considered positive according to OECD TG 442D.

Table 12. Skin sensitization prediction of BP through the adoption of OECD TG 442C, 442D and 442E, respectively able to predict the first (peptide reactivity), second (keratinocytes activation) and third (dendritic cells activation) phases of skin sensitization AOP. Final prediction was made adopting the 2-out-3 defined approach by the OECD TG 497.

Test guideline	**BP**	**BP final prediction**
OECD TG 442C - *In Chemico* Skin Sensitisation: Direct Peptide Reactivity Assay	Negative	**Non-sensitizer** according to the 2-out-3 Defined Approach (OECD TG 497)
OECD TG 442D – *In Vitro* Skin Sensitisation: ARE-Nrf2 luciferase KeratinoSens™ test method	Positive	
OECD TG 442E – *In Vitro* Skin Sensitisation: human cell Line Activation Test (h-CLAT)	Negative	

Lastly, h-CLAT method was carried out following OECD TG 442E, addressing the third key event of the skin sensitization AOP, namely the differentiation of monocytes to dendritic cells. Flow cytometry analyses demonstrated that BP was not able to induce monocyte differentiation to dendritic cells since it did not trigger the differentiation markers CD54/CD86 expression at levels higher than the thresholds defined in the TG. According to the OECD TG 442E h-CLAT prediction model, BP can be considered negative.

On the whole, basing on the 2-out-3 defined approach defined by OECD TG 497, the present results suggest that BP is not skin sensitizers since at least 2 of the 3 OECD TGs for skin sensitization prediction were reliably negative.

5 HAZARD CHARACTERIZATION: ECOTOXICITY STUDIES (WP3)

The main objective of this work package (WP) was to characterize the environmental hazard of two 2DM: hBN in two different forms (hBNc-cornered sharp edges and hBNr-round shaped) and Black phosphorous (BP) as indicated in the previous sections. To fulfill this objective, organisms of different complexity regarding their organization level were considered: bacteria, microalgae, microinvertebrates and fish cell lines.

Dispersion of the 2D materials

Before subjecting the corresponding particles to the bioassays indicated in the different tasks, stock suspensions were prepared adding 100 mg of each product in 10 mL of a natural organic matter (Suwannee River NOM, RO isolation, International Humic Substances Society, 2R101N) solution in Milli-Q water (20 mgNOM/L), as shown in NANoREG's validated Enhanced Dispersion Protocol (NOM-water) for ecotoxicological studies. Then, 72 h of continuous magnetic stirring (500 rpm, RT) was applied instead of sonicating to avoid the formation of reactive oxygen species and agglomeration of the particles, based on Lizonova et al.10. The resultant protocol from the combination of these two methods aims to produce highly-dispersed-state suspensions specifically for difficult-to-disperse materials such as the three 2DM used in this study, so that the latter exposure to the test organisms turns out as accurate as possible.

5.1 DETERMINATION OF THE INHIBITORY EFFECT OF THE SAMPLES ON THE LIGHT EMISSION OF *ALIIVIBRIO FISCHERI* (LUMINESCENT BACTERIA TEST).

The luminescent bacteria bioassay was performed to assess the acute toxicity of BP and the two different hBN particles on bioluminescent bacteria *Aliivibrio fischeri* following the ISO 11348-3:2007[11] standard. This *in vitro* test evaluates the acute toxicity (up to 30

[10] Lizonova, D. et al. Nanomaterials 14, no. 7 (2024): 589.

[11] ISO 11348-3:2007. Water quality. Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test) - Part 3: Method using freeze-dried bacteria

minutes) of the analyzed aqueous samples based on changes in the naturally emitted light from the marine bacterium *A. fischeri*, which is highly sensitive to a broad range of toxic compounds. Upon contact with toxic substances, these bacteria typically respond by reducing their luminescence; hence, the light intensity emitted should decrease as the concentration of the toxic substance increases in a dose-response manner.

To conduct the assay, BioLight reagents (Aqua Science, USA) were employed. The bacterial inoculum of A. fischeri was obtained from the reconstitution of the lyophilizate in 1 mL of BioLight Multi Reagent with the BioLight Recon solution. Microtox Acute Toxicity 100% Test (or Whole Effluent Test) was performed to carry this experiment out, as it is the one that fits most with samples which's toxicity is unknown, but expected to be low.

To begin with, a first concentration to 100 mg/L was prepared from the 10 mg/mL stock dispersions (1:100 dilution) using the BioLight Diluent solution and, from there, three more 2-fold serial dilutions were made with the same reagent. Then, Microtox Osmotic Adjustment solution (MOAS) to adjust salinity and the bacterial inoculum were added to each of the dilutions in a 1:10 ratio, so the final true concentrations are 90% of the calculated when making the dilution series. A control sample without product (dose 0) was also tested. Tests were performed in duplicate, at 15°C and adjusting the pH within the operative range (pH 6 – 8). Measurements and calculations were carried out with a Microtox® Model 500 Analyzer luminometer and MicrotoxOmni® software (Azur Environmental), respectively. As endpoint, mean EC_{50} values at 15 and 30 minutes were obtained and expressed as mg/L (with respective 95% confidence limits) by the software. This value indicates the sample concentration that causes a 50% decrease in bacterial bioluminescence compared to the control tube. Samples were analyzed according to the kit manufacturer's instructions.

The potential toxicity of the products was assessed after 30 minutes of exposure as a reduction in bacterial bioluminescence compared to the control sample. The results obtained were as follows:

- No toxicity was observed in any of the samples.
- A slight downward trend in the light intensity of the bacterial inoculum was observed as the concentration of BP increased.
- BP was the only particle for which an EC_{50} value at the first time-point (15 min) could be calculated although it was significantly higher than the highest concentration in the assay (100 mg/L).
- EC_{50} values were greater than the highest concentration tested ($EC_{50} > 100$ mg/L) for the three 2DM.

5.2 ACUTE TOXICITY TEST IN MICROALGAE

The assay was carried out using the ALGALTOXKIT F ™ kit (MicroBioTests Inc., Gent, Belgium), following the manufacturer's instructions. This kit complies both with the OECD TG 201[12] and with the ISO 8692:2012[13] "Water quality — Fresh water algal growth inhibition test with unicellular green algae" guidelines. The microalgal species selected for the test was *Raphidocelis subcapitata*, formerly known as *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata*.

For the reconstitution of the *R. subcapitata* inoculum, the suspension liquid was removed from the tube containing the beads of the immobilized species and 5 mL of dissolving medium were added to dissolve the bead matrix. After a couple steps of Milli-Q water washing and centrifuging, the algal pellet was finally resuspended in 10 mL of the freshly prepared "algal culture medium". Then, the reconstituted inoculum was transferred to a volumetric flask of 25 mL and algal culture medium was filled up to the calibration mark. Lastly, cell density was adjusted to 1×10^6 cells/mL by measuring the optical density (OD) at 670 nm.

Taking the stock solutions of the particles, decimal serial dilutions were prepared for each to obtain the following nominal concentrations: 0.01, 0.1, 1, 10, and 100 mg/L.

The appropriate volume of *R. subcapitata* culture was added to each test concentration to obtain an initial microalgal density of 1×10^4 cells/mL. All concentrations were tested in triplicate. A control consisting of just culture medium inoculated with the same microalgal density (dose 0) was also prepared in triplicate.

The samples were incubated for 72 h at 20 °C ± 2 °C in an incubator under constant illumination (~10,000 lux). The OD at 670 nm was measured at the same time each day to monitor algal growth. For the two highest concentrations (10 and 100 mg/l), a sample from each triplicate was taken and microalgal cells were counted in a Neubauer chamber, as these suspensions were so highly concentrated that they interfered with the optical density measurements recorded by the spectrophotometer. Based on these data, the percentage of growth inhibition caused by the test product was calculated and the EC_{50} value was determined.

[12] OECD (2011), Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264069923-en.

[13] ISO 8692:2012 "Water quality — Fresh water algal growth inhibition test with unicellular green algae"

BP was the only material that could be considered toxic for the microalgae. This material produced a high growth inhibition effect at the highest concentration tested (100 mg/L) after 72 h exposure to the microalgal cells and an EC_{50} was calculated.

Both of the 2D-hBN caused some effect at 100 mg/L and after 72 hours of incubation together with the microalgae, being the one produced by hBNr slightly higher than the hBNc inhibitory effect. But no EC_{50} could be calculated as it was higher than 100 mg/L. Thus, they are considered non-toxic in the conditions of the assay.

5.3 *DAPHNIA* SP. ACUTE TOXICITY TEST.

The *Daphnia* sp. immobilization test was employed to assess the acute toxicity of BP and hBN on the microinvertebrates commonly known as water fleas (*Daphnia magna*), based on the OECD 202 guideline[14]. In accordance with this protocol, the commercial Daphnia magna freshwater immobilization kit, known as DAPHTOXKIT F ™ (MicroBioTests Inc., Gent, Belgium), was used. This bioassay is highly sensitive and allows for the screening of toxicity across a wide range of chemicals, effluents, surface waters, wastewaters, groundwaters, capillary waters and eluates. Furthermore, it has been tested in various environmental laboratories and scientific institutions worldwide and validated through extensive comparison of results under strict control of conditions, ensuring its reproducibility at an international level. The test involves exposing a set number of *D. magna* individuals to different concentrations of the test product suspended in standard freshwater for 24–48 hours, determining the proportion of immobilized organisms relative to the negative control (where the analyte concentration is 0), and calculating the EC_{50} value.

Suspensions of the three particles were prepared at an initial concentration of 100 mg/L from the stock dispersions, and a dilution series of other four 10-fold concentrations were made, leaving a concentration range of 100 – 0.01 mg/l. For each concentration, 4 replicates of 5 individuals were exposed (20 daphnids in total) and counting of the immobilized *Daphnia* is performed 24 h and 48 h after exposure.

The results obtained from the test after 48 hours of exposure were as follows:

- hBNr caused no effects over the exposed *Daphnia*.
- hBNc exerted a subtle toxic effect after 24 hours of exposure at the highest concentration tested (100 mg/L).

[14] OECD (2004), Test No. 202: Daphnia sp. Acute Immobilisation Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264069947-en.

- Similarly, BP showed the same effect over the *Daphnia* as hBNc, but only after 48 hours of exposure at the highest concentration tested (100 mg/L).
- All three materials were considered non-toxic at the concentration interval tested, being the EC_{50} values greater than 100 mg/L.
-

5.4 *IN VITRO* ECOTOXICITY USING FISH CELL LINES

The cytotoxicity assay was conducted according to the OECD TG 249[15] guideline to determine the acute toxicity of BP and the two variants of hBN over the immortalized rainbow trout (*Oncorhynchus mykiss*) gill cell line, known as RTgill-W1. This assay is carried out in 24-well plates, where a specific number of cells (300.000 – 350.000 cells/well) are seeded with the aim of forming a confluent monolayer at the bottom of each well. The cells are then exposed for 24 hours to 6 serially diluted concentrations of the test products ranging from 0.1 to 100 mg/L. Taking the stock dispersions, the serial dilutions of the materials are prepared in the same medium where the cells are seeded, this is Leibovitz's L-15 supplemented with 10% of Foetal Bovine Serum (FBS). After the exposure period, the medium containing the test product is removed and the cells are treated with three different fluorescent dyes to assess cell viability: CFDA-AM, which measures plasma membrane integrity; Resazurin, as an indicator of metabolic activity and Neutral Red, which accumulates in the lysosomal membrane and thus indicates disruption of these organelles. Then, plates are read with a Varioskan™ LUX spectrophotometer and cell viability is evaluated based on the results of the three different parameters.

After measuring the fluorescence values in the plates exposed to the three 2DM, the following results were observed for the three materials

hBNr

- Integrity of the cell membrane seemed to slightly decrease as the hBNr concentration increased, but from 10 mg/L onwards that toxicity was constant.
- Metabolic activity decreased in a dose-dependent manner.
- Lysosomal membrane integrity remained quite constant except at the highest concentration, where a drop in the viability occurs, and at intermediate concentrations, where lysosomal biosynthesis is hypothesized.

[15] OECD (2021), Test No. 249: Fish Cell Line Acute Toxicity - The RTgill-W1 cell line assay, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/c66d5190-en.

hBNc

- Plasma membrane integrity decreased as the hBNc concentration increased, which is more evident at the two highest concentrations.
- The metabolic activity remained quite unaffected.
- Slight reduction in lysosomal membrane integrity was observed at the highest concentration tested.

BP

- Decreased plasma membrane integrity at the two highest concentrations.
- Increased metabolic activity in a dose-dependent manner.
- Slight downward tendency in lysosomal membrane integrity.

5.5 OVERALL CONCLUSIONS

- A protocol for dispersing the 2DM of this study was successfully established using Natural Organic Matter (NOM), enabling their stable suspension in cell culture media for *in vitro* toxicity studies for a better exposure under more realistic conditions.
- Considering the 4 assays performed in this study, the following classification of the particles could be done, from most to least toxic: BP > hBNc ≈ hBNr.
- Only differing in their morphology, hBNr presented a more pronounced toxicity in the freshwater algal growth inhibition test while hBNc showed more toxicity in the *Daphnia* acute toxicity assay.
- Considering that the freshwater algal growth inhibition test was the only assay in which a particle was determined to be toxic (gave out a known EC_{50} value within the concentration range tested), it can be regarded as the most sensitive bioassay among the set evaluated in this study.
- In this study only acute assays were performed (24 – 72h). For future perspectives, it would be interesting to include subacute or chronic assays with these same particles, with the aim of further assessing their potential toxicity in long-term exposures, as well as other experiments tackling different toxicity parameters/mechanisms of action.

6 LIFE CYCLE, RISK ASSESSMENT AND RISK MANAGEMENT (WP4)

6.1 LIFE CYCLE ASSESSMENT.

In this study, a Life Cycle Assessment (LCA) was carried out for hNB, focusing on a specific use case: its application in lithium-ion batteries (LIBs). More precisely, the assessment concentrates on the integration of hNB into the coating of battery casings, where it is intended to enhance thermal management and extend LIB service life.

Interest in incorporating hNB-based nanomaterials into LIB components has grown substantially in recent years. Their combination of high thermal conductivity with excellent chemical, mechanical, and thermal stability has been shown to improve battery lifetime, safety, cycling performance, and overall efficiency. hNB is considered a promising material for conventional LIB components due to five key characteristics: (1) highly tunable electronic properties; (2) outstanding mechanical stability; (3) suitability for high-temperature operation; (4) chemical stability and inertness; and (5) high ion transfer mobility. These features derive from the nature of the atomic bonding and the distribution of electron clouds within the material[16]

Thermal management is a critical aspect of LIB performance, as electrochemical reactions inside the cell are sensitive to temperature fluctuations. Under high charge or discharge rates, the substantial heat generated within the battery — combined with insufficient cooling — can lead to thermal ageing and potentially thermal runaway[17].

Applying a coating layer to the metal surface of the battery casing can significantly alter its thermal and electrical behaviour. Depending on the coating material, properties such as thermal conductivity, electrical insulation, or thermal resistance can be tailored. hNB, which has a layered structure similar to graphite, offers high thermal conductivity, electrical insulation, a low dielectric constant, and thermal stability up to approximately 1000 °C in air. In addition, it exhibits chemical inertness and excellent resistance to corrosion and erosion. These attributes have led to its widespread use as a release agent and protective

[16] Angizi et al. 2024. Energy & Environmental Materials. 7 (6) e12777

[17] Saw et al., 2014. Applied Thermal Engineering 73. Elsevier. 152-159

coating in applications including moulding, glass manufacturing, metal processing, sintering, welding, and brazing[17].

Although the use of hNB coatings specifically on LIB casings has been explored only to a limited extent, this application is expected to gain importance due to its potential to improve battery safety and longevity. High-energy-density LIBs — particularly those using flammable electrolytes in electric and hybrid vehicles — are vulnerable to abusive operating conditions that may trigger thermal runaway, gas venting, fire, or explosion. Incorporating hNB into casing coatings could help mitigate these risks and support safer and more durable battery systems.

6.1.1 Goal and Scope – Functional Unit

The goal of this study is to assess the environmental impacts associated with incorporating the hBN 2D nanomaterial into the casing of a lithium-ion battery (LIB) and to compare its performance with a benchmark configuration. The LFP battery model was selected for this assessment because it uses an aluminium-based casing — required for the application of the hBN coating — and is widely deployed in electric vehicles.

A cradle-to-grave LCA approach has been applied, covering all stages of the value chain: raw material extraction, production of both the nanomaterial and the battery, the use phase, and the end-of-life phase, including recycling and material recovery.

The outcomes of the LCA will support the following goals:

- Assess the potential environmental benefits resulting from the addition of hBN to the overall composition of the LIB pack.
- Identify the environmental impacts linked to the production of hBN and determine the key hotspots.
- Provide the company with actionable information to reduce the environmental footprint of their product.

It is important to note that the conclusions of this study apply only to the specific battery model analysed, as the quantities and configurations are tailored to this case. Therefore, the results should not be used for comparative purposes with other battery chemistries, designs, or system configurations, nor as evidence for product-specific sustainability claims.

The **functional unit** selected is a LFP model battery with a lifespan of 15 years to be used in conventional electric vehicles.

The study considers the production of the complete battery (incluing the hBN), its use and end of life.

6.1.2 Life Cyle Inventory – Data sources

The Life Cycle Inventory (LCI) captures all relevant inputs and outputs associated with each stage of the system under study. For the environmental assessment of the hBN nanomaterial itself, a production scenario was built with the support of the producer, and with literature-based data. For the modelling of processes related to the application of hBN, the following sources of secondary data were employed:

- Ecoinvent 3.7 LCI database
- PEF 3.1 LCI database
- Product- and process-related information from publicly available industrial sources (e.g., technical datasheets, patents)
- Calculated or model-derived data

The inputs and outputs of the assessed life cycle were determined based on the following assumptions.

Production Phase

Primary data for the last stages of the production of the hBN nanomaterial (excluding the production of the precursors and bulk hBN) were provided directly by the company. For other elements of the production model, the following assumptions were applied:

- Precursors and bulk hBN production: Boric oxide and ammonia were selected as the precursor materials for synthesizing bulk hBN, which is subsequently used in the exfoliation process. Due to the absence of reliable data on the energy requirements of the synthesis process, only the environmental impacts associated with the production of these precursor materials were included.
- Battery model selection: The LFP-based lithium-ion battery pack was chosen as the reference configuration for integrating hBN and comparing it against

the benchmark system. This selection was made because the battery casing is aluminium-based, a necessary requirement for the application of the HBN coating.

Use Phase

To compare the Safe^{2}energy LIB pack with the benchmark, a 50% increase in battery lifespan was assumed for the hBN-enhanced system. This assumption is supported by literature indicating that hBN can improve LIB lifetime, safety, cycling stability, and overall performance.

The battery pack configuration was selected based on typical electric vehicle standards.

For modelling electricity consumption during the use phase, the following assumptions were applied:

- Annual driving distance: 15,000 km
- Electricity consumption: 18 kWh per 100 km
- Vehicle range: 400 km

Under these conditions, the total electricity requirement for a 15-year service life amounts to approximately 40,500 kWh.

End-of-Life Phase

For both the benchmark and Safe^{2}energy scenarios, the recycling of the aluminium battery casing was included. Prior to recycling processes — typically pyrometallurgical or hydrometallurgical treatments — battery packs are assumed to be dismantled to the module level.

Aluminium recycling was modelled in accordance with European Commission guidelines used for the calculation of the Product Environmental Footprint (PEF). Impact allocation at end-of-life was carried out using the Circular Footprint Formula (CFF).

6.1.3 Life Cycle Impact Assessment methodology – nanospecific characterization factors

The Life Cycle Impact Assessment (LCIA) is a critical step in the Life Cycle Assessment (LCA) process, aimed at evaluating the potential environmental impacts associated with a product or service throughout its entire life cycle. In this step, the data collected during the inventory phase (LCI) is analysed and translated into environmental impact categories,

In this study, the Life Cycle Impact Assessment (LCIA) methodology chosen is the Product Environmental Footprint (PEF), which was developed by the European Commission as part of its broader initiative to establish standardized methods for assessing the environmental performance of products and services.

The PEF methodology is designed to provide a robust, transparent, and scientifically grounded framework for evaluating the environmental impacts of products throughout their life cycle, from raw material extraction to disposal. This methodology is based on a set of environmental impact categories that cover a wide range of environmental issues, such as climate change, resource use, toxicity, and ecosystem quality, providing a comprehensive picture of a product's environmental footprint.

PEF emphasizes a life cycle perspective, ensuring that all stages of a product's life are taken into account when assessing environmental impacts. The methodology requires the collection of data on inputs and outputs across the entire life cycle, including manufacturing, transport, use, and end-of-life stages. It incorporates various impact categories, such as Global Warming Potential (GWP), acidification, eutrophication, and water use, which are calculated using a set of characterization factors and models that translate inventory data into environmental impact results. The PEF methodology is structured to be applicable to a wide range of sectors and products, making it a flexible and scalable tool for decision-making.

One of the key advantages of the PEF methodology is its focus on harmonization and standardization across industries and regions. By establishing common guidelines and calculation procedures, the PEF methodology promotes consistency and comparability between different product assessments, facilitating more meaningful comparisons and helping stakeholders identify opportunities for environmental improvements. Additionally, the PEF methodology aligns with existing standards and frameworks, such as ISO 14044 and the Environmental Footprint Guidelines, while offering a more tailored approach for specific product types. This makes PEF an ideal choice for providing clear and actionable

insights into the environmental impacts of products, guiding both industry practices and policy development towards more sustainable consumption and production patterns.

In the PEF methodology, the Human Toxicity and Ecotoxicity impact categories are calculated using the USEtox methodology, a widely recognized model for assessing the toxicity impacts of substances on human health and ecosystems. USEtox evaluates the potential harm posed by chemical releases into the environment by considering factors such as the chemical's persistence, bioaccumulation potential, and toxicity to humans and wildlife. For Human Toxicity, the model calculates the potential for adverse health effects in humans from exposure to hazardous substances, considering factors like toxicity potency and exposure routes (e.g., inhalation, ingestion, or dermal contact). Similarly, for Ecotoxicity, USEtox estimates the potential ecological damage from chemicals released into various environmental compartments, including soil, water, and air, taking into account species sensitivity and exposure pathways.

The USEtox model provides characterization factors (CFs) for different chemicals, which are used in the PEF methodology to quantify the impact of specific substances on the Human Toxicity and Ecotoxicity categories. These CFs are based on extensive environmental and toxicological data, which allows for the integration of both the chemical properties of substances and their environmental fate. By using USEtox in the PEF framework, a more standardized and scientifically grounded approach is applied to assess the toxicity impacts, ensuring consistency and comparability across different product assessments. This approach helps identify substances with the highest potential to cause harm to human health and ecosystems, enabling more informed decision-making in product development and environmental policy.

This model utilizes a multimedia fate-exposure framework and matrix-algebra calculations to determine Characterization Factors (CFs), which represent the contribution of a given mass of an emitted substance to toxicity and ecotoxicity impacts. These CFs are critical for evaluating the environmental and human health effects of different substances, both organic and inorganic.

The process of quantifying characterization factors requires calculating 3 parameters:

- Fate Factor (FF): it describes how a contaminant is dispersed across various environmental compartments. It represents the substance residence time in a given compartment for a given unit of time.
- Exposure Factor (XF): it quantifies the contact between humans or ecological systems and the contaminated environmental media

- Effect Factor (EF): it represents the potential effects of the contaminant per unit mass for human intake or the potential harm to aquatic species, integrated over the water volume exposed to bioavailable chemicals.

The resulting characterization factor (CF) that is required for the impact score for either human health or ecological impacts is generally defined as the combination of these three factors:

$$CF = FF \cdot XF \cdot EF$$

Where the fate factor (FF) the exposure factor (XF) represents the dissolved fraction of the substance and the effect factor.

For chemicals causing human toxicity the fate factor and exposure factor can generally be combined to reflect the intake fraction (iF) for a chemical

$$iF = FF \cdot XF$$

The iF represents the fraction of the quantity emitted that enters the human population. Intake through inhalation and ingestion is commonly considered in iF calculations.

A significant challenge in applying this methodology to nanomaterials lies in the absence of standardized Characterization Factors for nanomaterials' emissions to toxicity and ecotoxicity impact categories. Despite the recognition of their importance for Life Cycle Assessment, the lack of a consistent, comprehensive approach for calculating fate, exposure, and effect factors for engineered nanoparticles (ENPs) remains a limitation[18].

For this reason, this project has developed specific Characterization Factors for the hBN, using up-to-date strategies to overcome existing methodological inconsistencies related to the compatibility of the current models with the specificities of nanomaterials.

In this case, results on human toxicity assays have led to no measurable Human Toxicity, and therefore, no contribution to this impact category has been included, and the calculation of the CFs has been limited to Freshwater Ecotoxicity.

[18] Salieri, B. et al. 2019. Journal of Cleaner Production, 206, 701-712

Fate Factor (FF)

The Fate Model in USEtox accounts for removal and transport process i.e. advection, adsorption/sedimentation, volatilization, degradation and advective transport out of water, all of which influence how a substance behaves in the environment.

The model calculates different rate-constants k [1/d] to build a the rate coefficient matrix, with columns representing media where the emission takes place and files representing the receiving media (air, water, soil and sediment).

The FFs are calculated based on negative and inverse of the exchange-rate matrix:

$$\overline{FF} = -\overline{K^{-1}}$$

Nevertheless, the fate modelling in USEtox takes into account processes such as advection, adsorption/sedimentation, volatilization, and degradation, all of which affect how a substance behaves in the environment. These processes, however, are primarily designed for traditional chemicals and may not be entirely suitable for nanomaterials. USEtox relies on rate constants (k [1/d]) based on properties like vapor pressure and solubility to estimate how long a substance stays in different environmental media. Nanomaterials, on the other hand, have distinct characteristics that might necessitate alternative modeling approaches to accurately reflect their environmental fate [19].

To address these limitations, a Simplebox4nano (SB4N) model has been proposed to model the fate of nanomaterials[20,21,22]), which considers their specific environmental interactions. SB4N accounts for the behaviour of three forms of nanoparticles: free (pristine) nanoparticles, hetero-aggregated nanoparticles, and nanoparticles attached to larger natural particles (aggregation and the attachment are referred to as “collision with natural colloids- <of size 450 nm- and larger natural particles –of size > 450 nm). This model encompasses multiple environmental media, including air, rain, freshwater, soil, and sediment, and incorporates rate constants to represent environmental processes like transformation through aggregation, transport, deposition, and sedimentation.

The environmental fate processes of free, aggregated and attached nanoparticles are represented by pseudo-first order rate constants (k, s-1), describing i) transformation

[19] Praetorius et al., 2014. Environ Sci Nano 1(4):317–323
[20] Ettrup et al. 2017 Environ Sci Nano 1(4):317–323
[21] Salieri, B. Et al. 2018. NanoImpact 10, 108-120
[22] Ke, M. et al. 2025. Environmental Science and Ecotechnology 25 100565

processes of the free nanoparticle as hetero-aggregation with colloidal particles and of the free nanoparticle as attachment to larger particles; ii) transport processes between compartments, dry/wet deposition from rain to soil and water, soil runoff, sedimentation of particles form water to sediment, sediment resuspension, soil leaching and sediment burial.

The output of SB4N is mass concentrations of the three ENPs species (free, aggregated and attached species) in each environmental medium at steady state.

In this study we have further evaluated and tested the approaches for the calculation of Fate Factors based on the SB4N, implementing the necessary adaptations to ensure that the results obtained are consistent with the Effect Factors calculated with the USE tox methodology, but considering previous research.

For the calculation of the Fate Factors in this project, the above-mentioned values (K matrix) have been used to calculate the (day-1), adapting it to the 4x4 matrix used in USEtox. In order to do so, several strategies have been implemented:

- Simplification of soil types: We assume all soil as natural soil, which helps reduce the complexity in fate modelling for nanomaterials.
- Precautionary approach: In the case of hetero-aggregation, we treat aggregated particles as remaining in the system and gradually settling into sediments, rather than being removed through clearing processes. This adjustment ensures a more realistic long-term modelling of nanoparticle behaviour in the environment (Ke, 2025).

As a result of the project, nano-specific FF digital calculation support has been developed, based on the tools previously published[22] but enabling to incorporate outcomes from SB4N to model FFs and extending the scope to cover all emission and reception media.

Exposure factor for freshwater Ecotoxicity Characterization Factor

The exposure factor has been calculated as the bioavailable fraction, based on the proposal from Ke et al[22] the latter one is calculated as:

$$XF = \frac{1}{1+BCF_{free}*[Biota]}$$

BCF is the bioconcentration factor of hBN in freshwater organisms, and [Biota] is the biology concentration in the freshwater environment (value extracted from the USEtox model). No specific information for the assessment of hBN is available, and therefore, literature data for BCF based on a different hBN material has been considered.

In this case we have assumed that the XF of all forms of hBN in the freshwater are equal to the one of the free hBN, since data limitations do not allow going in further detail.

Effect factor for freshwater Ecotoxicity Characterization Factor

The effect factor (EF) for freshwater ecotoxicity was calculated following the procedure described in USEtox model[23]:

$$EF_{eco} = \frac{0,5}{HC_{50}}$$

EFeco: ecotoxicological effect factor for freshwater aquatic ecosystem [PAF m^3/kg].

HC_{50}: geometric mean of chronic EC_{50}s for freshwater species [kg/m^3], considering 3 trophic levels.

HC_{50} has been calculated based on the experimental work carried out in Safe2energy for algae, Arthropoda, and fish. The results obtained within the project correspond to acute toxicity data, and therefore acute-to-chronic ratio (ACR) has been applied: 10 for crustaceans, 20 for fishes and 15 for other trophic levels24. In this case, the highest concentration tested for Ecotoxicity assays has been considered as EC_{50} values, which is a very conservative approach.

[23] Fantke et al. 2017 USEtox® 2.0 Documentation
[24] Fantke et al. 2015. USEtox® 2.0 Manual: Inorganic Substances (Version 2)

6.1.4 Life Cycle Assessment Results

The LCA results indicate that incorporating hBN 2D nanomaterial into the battery casing coating leads to an overall improvement in environmental performance. The comparative life cycle environmental footprint for both systems is presented in Figure 3.

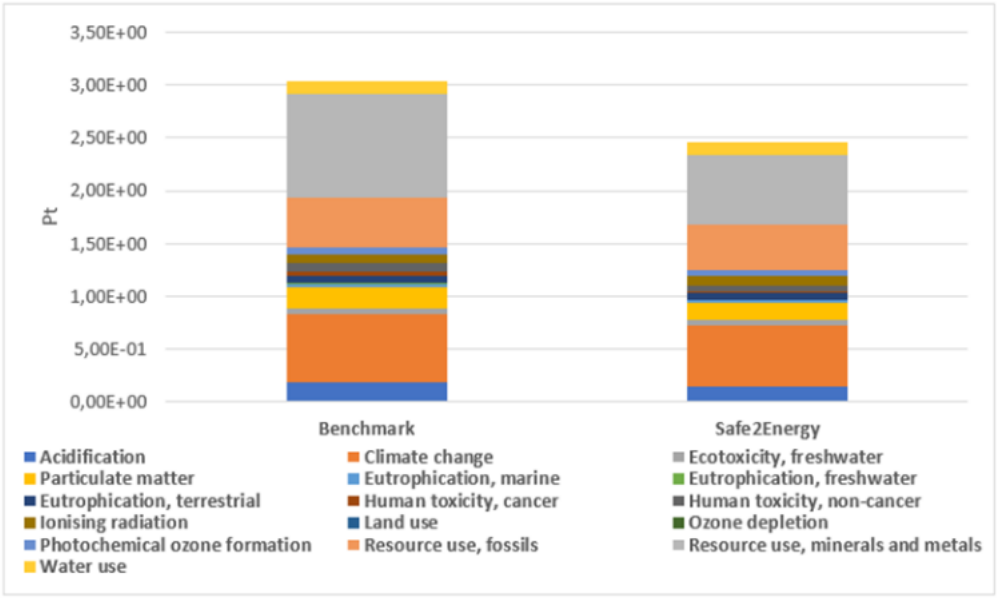

Figure 3. Environmental impacts comparison of the Benchmark and Safe^{2}energy Li-ion battery packs considering all the life cycle of the batteries.

As shown, the overall environmental footprint is substantially reduced, with a total decrease of approximately 19%. The most pronounced improvement is observed in the resource use of minerals and metals, which declines by 33%, followed by freshwater eutrophication and ozone depletion, each exhibiting reductions in the range of 31–32%.

The contribution of individual life cycle stages to the total environmental impacts is illustrated in the following figure, highlighting the specific phases that dominate the overall footprint.

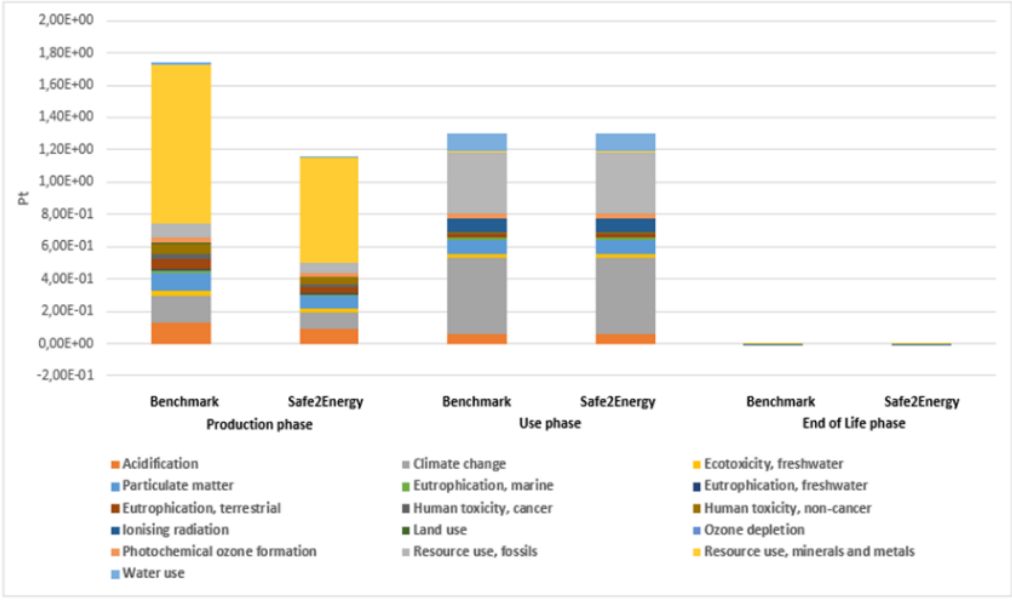

Figure 4. Stage-wise environmental impact comparison: Benchmark and Safe^{2}energy battery pack.

As it can be observed, the most substantial reduction occurs in the production stage, which drives the overall decrease in environmental impacts. In the production phase a significant environmental impact reduction can be observed, reaching a 33% reduction.

Focusing on the value chain of the Safe^{2}energy battery pack, in Figure 4 it can be clearly seen that, contrary to what happens in the benchmark, in this case, given the nano's ability to improve battery life, the impact of the production stage is lower than that required for battery use.

In this case, the use stage accounts for 53% of the impact, 6% higher than the production stage, while the end-of-life stage does not have a significant impact given the recovery of aluminium considered in the recycling of the battery pack casing.

6.2 RISK ASSESSMENT OF hBN PRODUCTION.

The risk analysis of hBN production was carried out using specifc tools designed for this purpose, based on a scenario developed with support from the information provided by the company and the bibliographic analysis conducted in the project. According to this scenario, hBN is obtained through a process that includes several steps. Most of these steps are fully enclosed, although in three of them the conditions could potentially lead to worker exposure, depending on the specific conditions of each process. These steps are those related to the weighing, mixing, and collection of hBN, and the risk assessment focuses on them.

During the weighing and mixing the raw material used is bulk BN whilst in the collection step the 2D-hBN was already obtained

6.2.1 Raw material weighing and pouring

Under the EU's REACH regulation this step is described under the category of PROC 8b that describes the transfer of a substance or mixture (charging and discharging) from or to vessels or large containers at dedicated facilities.

The raw material that is being used in this transfer is hBN (CAS No 10043-11-5). According to its SDS this material has a density of 2.1-2.25 g/cm^3 at 20 ºC and the apparent density is 200-700 kg/cm^3 at 20 ºC. No data is available for the toxicity of this hBN.

In the ECHA (European Chemicals Agency) database[25] some dossiers indicate that it may be an eye irritant category 2 and may cause respiratory irritation (STOT SE category 3) but no DNEL is indicated. BN is very similar to carbon structures. Depending on the pressure and temperature, the BN molecule has different crystal structures such as hexagonal (h-BN), wurstitic (w-BN), rhombus (r-BN) and cubic (c-BN). However, the most stable form at room temperature is the hexagonal form[26]. Since hBN has the same number of electrons as two carbon atoms, it is similar to the graphite structure which features a hexagonal crystal structure. Due to this similarity, hBN is called “white graphite”

[25] https://echa.europa.eu/de/information-on-chemicals/cl-inventory-database/-/discli/details/79734
[26] Nikaido et al 2022. The Journal of Physical Chemistry C 126 (13), 6000-6007

or “white carbon”. Chemically, hBN is highly inert. Graphite is also an inert dust that can cause mechanical irritation by inhalation. A REACH DNEL for workers, long-term, local for graphite is 1.2 mg/m^3 and could be used as proxy for hBN in the absence of a DNEL for the hBN. Alternatively, if no substance-specific DNEL or OEL is available, regulatory authorities recommend applying general exposure limits for “non specific dusts”. For the EU this is 10 mg/m^3 for inhalable dust and 3-4 mg/m^3 for respirable dust. Using the precautionary principle as the DNEL for graphite is the lowest of all those values, this will be used for the RA of this stage.

Weighing of the raw material hBN, is done in an indoor room. Main routes of exposure to workers in this case are inhalation and dermal.

Exposure and Risk estimation

The weighing of input materials in the process can take place in areas where there is potential exposure for workers. Usually, the main routes of exposure are inhalation and dermal contact.

The assessment takes into account the risk management measures implemented in the sector. For this assessment, the ECETOC Targeted Risk Assessment (TRA) tool was used, which calculates the risk of exposure to chemicals for workers, consumers and the environment.

This tool has been identified by the European Commission Regulation on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as a preferred approach for assessing health risks to consumers and workers. The process parameters required have been taken from the production scenario built with support from the company, and are shown in Figure 5.

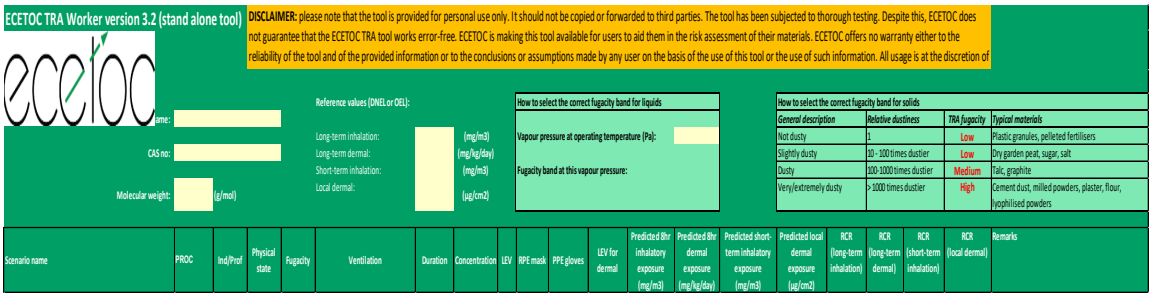

Figure 5. Parameters needed for the calculation of exposure and risk according to ECETOC TRA tool

Using the ECETOC tool in this scenario and with the personal protective equipment (PPE) employed, there is a low risk for both inhalation and dermal exposure routes. The tool can calculate the risk characterization ratio (RCR) for inhalation:

$$RCR = \frac{Exposure}{DNEL}$$

For inhalation this RCR is below 1 indicating a low risk. For dermal exposure, a DNEL is not available, so it cannot be calculated. However, neither the Safety Data Sheet (SDS) nor the ECHA database provide any indication that it may be irritating to the skin, although it can cause eye irritation, so facial protection measures should be used.

6.2.2 Mixing the raw materials

The next point where there may be exposure to workers according to the specific characteristics of the process is the mixing of the raw materials which has the REACH code PROC5 “mixing or blending in batch processes”. At this point, exposure is only possible for bulk BN, because the rest of the reagents are added through a closed system. The process scenario was built with the company, and for the assessment, the protective measures that may be taken are considered. As in the previous step, the ECETOC TRA tool is applied. Using the ECETOC tool in this scenario and considering the PPE used, there is a low risk for inhalation exposure, the RCR is below 1. And, as in the previous stage, since no DNEL is available for dermal exposure, the Risk Characterization Ratio (RCR) cannot be calculated. According to the available data, there does not appear to be dermal toxicity, although there is ocular toxicity, so facial protection measures must be used.

6.2.3 Collection of the 2D-hBN

The final step of the process where worker exposure may occur according to the built scenario is during the collection of the final 2D-hBN product. The exposure route may also be by inhalation or dermal.

6.2.3.1 Inhalation Risk Assessment using StoffenmanagerNano

Not much information is available about the toxicity of the 2D-hBN.

In the bibliography in an *in vivo* study performed in 2023 it was found that hBN nanosheets administrated to mice by oropharyngeal aspiration did not induce inflammation at any of the time points tested and were eliminated from the lung airways in a time-dependent fashion[27].

The result obtained in this project indicate also a low toxicity of hBN. Specifically the project evaluated the potential pulmonary toxicity of hBN using and advanced human 3D airways model, Mucilair (https://www.epithelix.com/products/mucilair), supported by a Calu-3 cytotoxicity assay that was used to select the range of concentrations to be tested in the Mucilair assay. This assay and its results are described in WP2 of this report. Briefly here: evaluated endpoints were,cytotoxicity (LDH release), inflammation (cytokines release), measurement of the integrity of the membrane (Lucifer Yellow assay) and genotoxicity using the Comet assay. Mucilair was performed with the hBNc as due to its shape its toxicity was thought to be greater than the round shaped one. No increased LDH release was seen after 28 days exposure as compared with the negative control, and the genotoxicity assay showed no effect either. The integrity of the membrane was maintained at day 28 and hBN was not internalized into cells as determined by TEM. As for inflammatory effects some cytokines were released after 14 days exposure, increasing in the case of IL-6 after 28 days. Due to this potential inflammatory effect, for a qualitative RA, a category of harmful and/or irritating was given to this material. For a more quantitative evaluation REACH assays that will validate this category are needed.

For the Risk assessment of the inhalation exposure route, the Stoffenmanager nano tool was used and the results are in Table 13.

The risk score given is III meaning a low risk for the task and exposure class 1 indicates a low exposure class. These results are based on the *in vitro* pulmonary assay performed in the project. For a more quantitative RA, those results should be used to estimate a NOEL or DNEL translating them to *in vivo* via an IVIVE tool that although being developed is not yet available. Alternatively, *in vivo* inhalation assays could be performed for REACH registration purposes. The *in vivo* assay found in literature is an oropharyngeal one and though the dose used, 30 µg/animal, could be considered a NOEL its translation into µg/m^3 is very complex.

[27] Visani de Luna et al. 2023. ACS Nano 17 (24), 24919-24935

Table 13. Inhalation risk evaluation using the StoffenmanagerNano tool

General data	
Nanoparticle	hBN
Concentration of the nanoparticle in the product	Main component (50-99%)

Results Risk Assessment	**Task weighed**	**Time and frequency weighed**
Hazard class	B	B
Exposure class	1	1
Risk score	III	III

Meanings of the classification given by this tool:

hazard class (hc)	exposure class (ec)	risk priority (risk)
A low	1 low	III low
B average	2 average	II middle
C high	3 high	I high
D very high	4 very high	
E extreme		
- n.a.		

6.2.3.2 Dermal Risk Assessment. 2D Hexagonal Boron Nitride (hBN)

For the dermal assessment, the project conducted a comprehensive study using the regulatory OECD tests TG 439 (skin irritation), OECD TG 431 (skin corrosion), and OECD TG 442 C, D, E (skin sensitization), as well as a long-term exposure test (7 days) with HaCaT cells. The results that have been described and discussed in WP2 are shown in Table 14.

The effect was higher for longer exposures and more pronounced for hBNc in comparison to hBNr. Dermal exposure was estimated using ECETOC TRA. However, the validated tests for skin irritation and corrosion indicate that the material is neither irritating nor corrosive. Therefore, no dermal hazard classification (H314 or H315) is required. However, the prolonged exposure test with HaCaT cells may indicate some effects that

should be evaluated for a more accurate assessment. Protective measures should be taken to reduce the risk of dermal exposure.

Table 14. Summary of the toxicological profile of hBN at the skin level provided by Safe^{2}energy.

Endpoint	Method	OECD Guideline	Result
Skin irritation	Reconstructed human epidermis	OECD TG 439	Non-irritant
Skin sensitisation	*In chemico* and in vitro assays	OECD 442C, D, E	Non-sensitiser according to the 2-out-3 Defined Approach (OECD TG 497)
Skin corrosion	Reconstructed human epidermis	OECD TG 431	Non-corrosive
Skin cytotoxicity (supporting)	HaCaT cells, 7-day exposure	Non-guideline	NOEC = 1 µg/mL; EC50 varies from 2.6 to 19.7 µg/mL depending on endpoint

6.3 RISK ASSESSMENT OF PHOSPHORENE PRODUCTION.

6.3.1 Production of FL-phosphorene

Although BP exhibits a great potential for wide applications, the synthesis for large-scale industrial applications is still challenging and has not been developed. An important consideration is its instability in ambient conditions. BP films with a few layers degrade in some days whilst single layer BP degrades completely in a few hours28. Therefore, unlike hBN, we do not have real data of production to evaluate the occupational risk for the production of this 2DM. Thus, the first activity was a literature search to determine which methods exist and the most promising one for performing a scale-up. In this one we have done a very preliminary RA that could be used for an SSbD of a future production of phosphorene. A description of the methods found is given below.

Two approaches could be used to produce phosphorene: top-down or bottom-up.

[28] Abellan et al, 2017. Journal of the American Chemical Society 139 (30), 10432-10440

Top-Down Approaches

Top-down methods start with bulk black phosphorus (BP) and break it down into few-layer or monolayer phosphorene through mechanical, chemical, or physical means.

Examples include:

- ✓ Liquid Phase Exfoliation (LPE): Ultrasonication in solvents (e.g., NMP, IPA).
- ✓ Electrochemical Exfoliation: Ion intercalation using an applied voltage.
- ✓ Shear Exfoliation: High-shear mixing in appropriate solvents.
- ✓ Mechanical Exfoliation: Peeling layers using adhesive tape or pressure.

Advantages:

- High-quality material (if protected from air).
- No atomic synthesis required.
- Scalable in some forms (e.g., shear, LPE)

Disadvantages:

- Sensitivity to degradation.
- Inert conditions often required.
- Limited control over flake size

Bottom-Up Approaches

Bottom-up methods build phosphorene from atomic or molecular phosphorus sources. These are less common approaches and remain mostly at the experimental stage.

Examples include:

- ✓ Chemical Vapor Deposition (CVD): Phosphorus vapor reacts with substrates.
- ✓ Molecular Beam Epitaxy (MBE): Atom-by-atom growth in vacuum systems.
- ✓ Hypothetical wet-chemical routes using molecular precursors.

Advantages:

- High control over structure.
- Potential for large-area growth.
- Clean deposition (in vacuum)

Disadvantages:

- Technically complex.
- Very few available working protocols for phosphorus.
- Poor scalability at present

Considerations for Industrial Implementation

- *Oxidation Sensitivity*: All phosphorene production methods must account for its degradation in air and moisture. Inert atmosphere handling or passivation strategies are required.
- *Solvent Recovery & Safety*: Liquid-phase and shear-assisted methods often require high-boiling-point solvents (e.g., NMP), which pose environmental and safety concerns if not properly managed.

Scalability Focus: Among all techniques, top-down exfoliation methods eg. shear-assisted and liquid-phase exfoliation offer the most promise for industrial-scale because they start from readily available bulk black phosphorus and can be run as high-throughput, solution-based processes whilst bottom-up methodologies require harsh control of phosphorus chemistry, special precursors/substrates, high temperatures/pressures and so far give only small-area, low-yield films[29, 30, 31].

A brief description of top-down methods that *a priori* may be more scalable than bottom-up is given below:

Mechanical Exfoliation

This method involves manually peeling layers from bulk black phosphorus using adhesive tape or mechanical tools. It was one of the first techniques used to isolate 2D materials like graphene and phosphorene[32,].

- *Advantages*: Provides extremely high-quality flakes, ideal for studying intrinsic properties without chemical alteration.
- *Disadvantages:* Extremely low yield, not suitable for scale-up, highly labor-intensive.

[29] Woomer et al. 2015. ACS Nano 9 (9), 8869-8884
[30] Tiouitchi et al. 2020. Soc Open Sci. 7(10):201210.
[31] Del Rio Castillo A.E et al.. 2018. Chemistry of Materials 30 (2), 506-516
[32] Woomer et al. 2015. ACS Nano 9 (9), 8869-8884

- *Scalability:* Low – Limited to lab-scale fundamental studies. Yields are in the microgram range.

Liquid Phase Exfoliation (LPE).

LPE employs sonication to exfoliate black phosphorus into few-layer phosphorene within solvents like N-methyl-2-pyrrolidone (NMP) or isopropyl alcohol (IPA). The process relies on breaking van der Waals interactions holding the layers together[33, 34, 35]

Advantages: Simple and adaptable to large volumes; good control over flake size and thickness. Solvent choice enables dispersion stability

- *Disadvantages:* Time- and energy-intensive. Phosphorene is highly reactive with oxygen and water; solvent choice and inert conditions are crucial. Toxic solvents (e.g., NMP)
- *Scalability:* Medium to High – Can reach gram-scale under optimized conditions with inert atmosphere protection.

Electrochemical Exfoliation

BP is used as an electrode in an electrochemical cell. Upon applying a voltage, ions intercalate into the crystal, leading to exfoliation. Carried out in aqueous or ionic liquid electrolytes[36].

- *Advantages:* Faster than LPE. Avoids use of organic solvents. Tunable surface properties by electrolyte choice.
- *Disadvantages:* Possible defect generation. Requires tight process control. Non-uniform yield
- *Scalability:* Medium

[33] Hanlon et al., 2015. Nat Commun 6, 8563
[34] Brent et al. 2014. Chem. Commun., 50, 13338–13341
[35] Del Rio Castillo A.E et al.. 2018. Chemistry of Materials 30 (2), 506-516
[36] Zeng et al., 2021. iScience, 24(10), 103116

Shear Exfoliation

Bulk BP is exfoliated by high-shear mixing (e.g., in rotor–stator mixers or blenders). Solvents help prevent reaggregation and oxidation.

- *Advantages:* Simple and cost-effective. No ultrasound needed. - Scalable using industrial mixers
- *Disadvantages:* Lower yield of monolayer phosphorene. Limited thickness control. Requires inert conditions
- *Scalability:* High

Solvent-Stabilized LPE

This is a refinement of standard LPE in which specific solvent systems are used to increase the chemical stability of exfoliated phosphorene in dispersion. Some approaches use solvent mixtures or stabilizing additives to reduce degradation[37,38,].

- *Advantages:* Produces dispersions with enhanced flake stability and longer shelf-life; suitable for ink formulations and device integration.
- *Disadvantages:* Requires systematic solvent optimization; may involve costlier chemicals.
- *Scalability*: High – Particularly suitable for industrial applications like energy storage, where large, stable batches are necessary.

For scaling up phosphorene production the most promising method, based on the reviewed references and practical criteria, may be the Solvent-Stabilized Liquid Phase Exfoliation (LPE) due to[39]**:**

- *Stability:* It improves phosphorene's notoriously poor ambient stability by using optimized solvent systems or additives (e.g., NMP + IPA or ionic liquids).
- *Yield:* Demonstrated gram-scale production potential.
- *Application-Ready*: Produces dispersions suitable for inks, coatings, and composite integration, key for industrial and energy storage applications.
- *Flexibility:* Can be integrated into continuous-flow systems or large-batch sonication/shear setups.

[37] Baboukani et al. 2021. Small Structures. Volume2, Issue 5, 2000148
[38] Hanlon et al., 2015. Nat Commun 6, 8563
[39] Tiouitchi et al. 2020. Soc Open Sci. 7(10):201210.

Thus, we will use this process for the Risk assessment

Description of the process

Preparation of Bulk BP. Black phosphorus crystals are ground into fine powder before exfoliation.

Dispersion in Solvent. The powdered BP is dispersed in anhydrous N-cyclohexyl-2-pyrrolidone (CHP) under ambient conditions.

Ultrasonication Exfoliation. An ultrasonic bath (~37 kHz) is applied for 24–48 hours. The solution is cooled to keep the temperature below 30 °C.

Centrifugation for Size Selection. After sonication, the mixture is centrifuged at ~1,000 rpm (~106 g) for 180 minutes. This removes unexfoliated bulk material and yields a stable colloidal dispersion of few-layer phosphorene.

Size Control via Centrifugation. Centrifugation speed and duration are tuned to isolate flakes of specific thicknesses, ranging from few-layer to multilayer phosphorene.

Solvent-Based Stabilization. CHP acts as a stabilizing solvent, forming a solvation shell around the flakes, significantly enhancing ambient stability and reducing degradation.

6.3.2 Chemical Risk Assessment of the process

Liquid-phase exfoliation of black phosphorus using CHP and ultrasonication. As the process is at a laboratory scale yet, a quantitative risk assessment cannot be performed. A very preliminary qualitative Risk assessment will be done that could be considered for a safe design of the scale-up of the process. The chemicals involved in the process are: black phosphorous (CAS No: 7723-14-0) that is the precursor for exfoliation and the solvent that in this case is CHP (CAS No 6837-24-7) and the final product that is the Few layers Black Phoshorous (FL-BP) or phoshorene (CAS#: 7723-14-0).

Hazard identification of the raw materials

Black Phosphorous (CAS No 7723-14-0). According to the SDS of ACS Material LLC it may cause respiratory irritation by inhalation, and may be Flammable (Category 2) H228, Acute aquatic toxicity (Category 3) H402 and Chronic aquatic toxicity (Category 3) H412.

CHP (N-Cyclohexyl-2-pyrrolidone): According to a REACH dossier in ECHA the hazard classification is as follows: Acute Tox. 4 (Dermal), H312; Acute Tox. 4 (Oral) H302; Skin Corr. 1B H314; Eye Dam. 1 H318.

FL-Black phosphorous. There is not much information about the toxicity. It has been tested in the project and the results are the following:

Skin toxicity: FL-BP was non-irritant and non-corrosive on the 3D reconstructed human Epidemis model conducted according to OECD TG 431 used. Therefore, no dermal hazard classification (H314 or H315) is required. Similarly, using the OECD TG 442C, 442D and 442E the phosphorene was classified as non-sensitizer according to the 2-out-3 defined approach (OECD TG 497).

However, using HaCaT keratinocytes with different endpoints [cell viability (WST assay), cell necrosis (PI uptake assay), intracellular levels of ATP (ELISA quantification) and mitochondrial depolarization (JC-1 probe)] and longer exposures (till 7 days) more effects could be identified. After this time of exposure, a lowest NOEC (0.8 µg/mL) and a maximum effect of 78% of cell necrosis at 100 µg/mL were identified. All the other endpoints were similarly affected. A more detailed description of the Skin irritation assays is given in WP2.

Inhalation toxicity. In the project, the potential pulmonary toxicity of the phosphorene was assessed using and advanced human 3D airways model, Mucilair (https://www.epithelix.com/products/mucilair), supported by a Calu-3 cytotoxicity assay that was used to select the range of concentrations to be tested in the Mucilair assay. This assay and its results are described in more detail in WP2 of this report.

The evaluated endpoints included cytotoxicity (LDH release), inflammation (cytokine release), membrane integrity (Lucifer Yellow assay), and genotoxicity (Comet assay). The analysis indicates that BP produces concentration-dependent effects on the epithelial barrier integrity and, at higher doses, also affects cell viability. Additionally, it induced some cytokine release at 14 and 28 days of exposure at the highest concentrations tested, suggesting a potential inflammatory response. In Table 15 a summary of the hazards identified for the materials in the production of FL-BP are given

Table 15. Summary of the toxicological profile of BP provided by Safe^{2}energy.

Chemical	CAS Number	Role	Hazards
Black Phosphorus (BP)	7723-14-0	Precursor for exfoliation	Reactive in air; forms phosphorus oxides; respiratory irritant; possible flammability*
CHP (N-Cyclohexyl-2-pyrrolidone)	6837-24-7	Solvent/stabilizer	Skin corrosion, eye damage and harmful in contact with skin or if swallowed.
FL-BP	7723-14-0**	Final product	Subacute damage for dermal exposure in an *in vitro* experiment Inflammatory effects for subchronic inhalation exposure and possible irritant and corrosive

*This has not been studied in this project as it is mostly focused in biological effects. **The CAS No of the FL-BP in the SDS of the tested material is the same as the one for the Black Phosphorous

Exposure routes

- Inhalation: Risk from fine BP particles or CHP vapor/mist during handling or ultrasonication.
- Dermal Contact: Skin contact with CHP, or BP during preparation and cleaning steps and with phosphorene when collecting the product after the centrifugations.
- Eye Contact: Splash risk during weighing, pouring, and centrifugation.
- Ingestion: Unlikely in a controlled lab, but possible via poor hygiene or hand-to-mouth contact. This route has not been considered in this project

Qualitative Risk Analysis

As real data for phosphorene production were not available, it was not possible to apply risk analysis tools as was done for hBN. Instead, a preliminary qualitative risk assessment was performed based on the identified exposure routes and hazards in this project. This assessment could inform the safe design of a future scale-up of the process. During the design phase, various scenarios could be simulated with different parameters (e.g., facility size, production quantities per cycle, number of cycles per year, equipment design), and by using risk estimation tools, such as those applied for hBN (e.g., ECETOC TRA, StoffenmanagerNano) or other available tools (e.g., Swiss Precautionary Matrix, SUNDs., the safest parameters and the most appropriate risk management measures could be determined.

The results of this qualitative evaluation for the different stages of the production are given in Table 16.

Table 16. Qualitative risk characterization of BP production.

Task	Chemical(s)	Risk Level	Notes
Weighing black phosphorus	BP	Medium	Dust generation, fire hazard in air. Inhalation of the powder may lead to respiratory irritation
Mixing/sonication in CHP	CHP, BP, FL-BP	Medium–High	Prolonged sonication may generate aerosol or heat; flammable solvent risk. Aerosols may be inhaled causing respiratory irritation or corrosion. Dermal contact could be possible.
Centrifugation of dispersion	CHP, BP, FL-BP	Low–Medium	Sealed containers reduce vapor, but breakage risk exists. Risk of dermal and inhalation exposure when collecting the final product with the potential to cause respiratory irritation or corrosion and some dermal problems with repeated exposure

Risk mitigation measures

The guide recently published by HSE: "Working Safely with Nanomaterials in Research & Development (3rd Edition- 2025) gives indications to work with Nanoamaterials even in the case of unknow properties. A summary of measures that can be taken in the case of phosphorene is given below.

Engineering Controls

- Fume hood for handling CHP and BP.
- Inert atmosphere (argon or nitrogen) for handling black phosphorus.
- Temperature-controlled ultrasonic bath.
- Proper ventilation (≥5 ACH).

Personal Protective Equipment (PPE)

- Nitrile gloves (chemical resistant)
- Safety goggles or face shield

- Lab coat or chemical-resistant apron
- Respirator (P3 or organic vapor filter) during sonication or powder handling

Safe Handling Procedures

- Minimize open handling of powders.
- Store BP in sealed containers under inert atmosphere.
- Use sealed centrifuge tubes.
- Avoid skin contact and inhalation of solvent vapors.

Waste Management

- Contain liquid waste in solvent-compatible containers.
- Dispose of CHP-contaminated materials as hazardous waste.
- Follow institutional hazardous material disposal protocols.

6.3.3 Conclusions

The phosphorene production method presents moderate risks primarily due to:

- CHP solvent toxicity and volatility,
- Air sensitivity of BP,
- Sonication-related aerosolization.
- Skin contact when weighing, mixing and collection of the nanoform

Proper ventilation, PPE, and inert handling protocols may make the process manageable and safe under controlled lab or pilot conditions.

In the previous process, CHP was used as the solvent following the protocol described by Hanlon et al. (2015) [40], A modification proposed by del Río Castillo et al. [41] replaces CHP with acetone. The hazard classifications for acetone are: flammable liquid (category 2), eye irritant (category 2, H319), and specific target organ toxicity from single exposure (category 3, inhalation, H336). This suggests that acetone may present lower risks than CHP (Table 17).

[40] Hanlon et al., 2015. Nat Commun 6, 8563
[41] Del Rio Castillo A.E et al.. 2018. Chemistry of Materials 30 (2), 506-516

Table 17. Comparison of the toxicities of the two solvents used in the BP production process.

Acetone	**CHP**
Flammable liquid (cat 2) Eye irritation cat 2: H319 STOT-SE cat 3 (inhalation): H319	Acute Tox. 4 (Dermal), H312; Acute Tox. 4 (Oral) H302; Skin Corr. 1B H314; Eye Dam. 1 H318

6.4 QUANTITATIVE *IN VITRO* BASED INHALATION RISK ASSESSMENT OF BLACK PHOSPHOROUS USING HUMAN AIRWAY MODELS

The main aim of the quantitative risk assessment was to translate the long-term *in vitro* data generated in MucilAir cultures into human-relevant points of departure for inhalation exposure to the selected 2D materials, BP and hBNc. To achieve this, a structured approach was followed, based on the OECD guidance document on *in vitro–in vivo* extrapolation (IVIVE) and benchmark dose (BMD) modelling using Epithelix airway tissue models (https://one.oecd.org/document/env/cbc/mono(2022)31/en/pdf). This document served as the methodological reference to define each step of the process, establishing a line from the initial concentration–response data to the derivation of Human Equivalent Concentrations (HEC) and, ultimately, Margins of Exposure (MOE).

Several biological endpoints were evaluated in the 28-day MucilAir study described in section 4.1.1, including cytotoxicity (LDH release), barrier integrity (Lucifer Yellow permeability), pro-inflammatory cytokines, genotoxicity via the comet assay, and TEM imaging. Among these, only two endpoints, LDH viability and Lucifer Yellow permeability, exhibited statistically significant concentration-dependent response for BP. Importantly, no genotoxicity was observed for either BP or hBN in the comet assay on day 28.

Because this approach requires a measurable dose–response, only endpoints showing a detectable biological effect with sufficient tested doses were taken forward into BMD modelling. Endpoints showing no effect, even if mechanistically relevant, could not provide a benchmark dose and therefore cannot contribute to a quantitative point of departure.

The steps taken for the assessment were as follows. First, the concentration response data for LDH and Lucifer Yellow were analyzed using PROAST (https://www.rivm.nl/en/proast). PROAST fits mathematical dose–response models and estimates both the Benchmark Dose (BMD) and its lower confidence bound (BMDL) for a defined Critical Effect Size (CES). Second, these BMDL values, initially expressed in µg/mL, were converted into surface doses (mg/cm²) on the apical surface of the MucilAir inserts, generating *in vitro* points of departure (POD), these POD values were translated into Human Equivalent Concentrations (HEC, mg/m³) by applying regional lung deposition fractions, airway surface areas and human ventilation rates. Finally, the HEC values were compared with airborne concentrations reported for workers handling graphene material, which allowed the derivation of Margins of Exposure (MOE).

In this study, only BP showed a concentration-dependent effect, allowing the calculation of BMD and BMDL values. On the other hand, hBNc did not produce significant changes in the relevant endpoints. Therefore, hBNc did not fulfill the fundamental criteria for quantitative benchmark dose analysis, and no BMD-derived points of departure were generated for this material.

6.4.1 Benchmark dose (BMD) analysis of repeated-dose cytotoxicity and barrier integrity

The cytotoxic and barrier-disrupting potential of BP and hBN after long-term, low-dose exposure was assessed through benchmark dose analysis of the 28-day MucilAir data described in section 4.1.1 The modelling was carried out using PROAST version 71.1, a tool that allows to quantify the dose at which a biological effect becomes relevant. PROAST is useful to identify the BMD and its confidence limits (BMDL and BMDU). In this system, the Critical Effect Size (CES) defines how large a response must be to be considered biologically meaningful.

Two endpoints were suitable for quantitative analysis. Lucifer Yellow permeability, used to evaluate barrier integrity, was modelled with a CES of 1.5, corresponding to a 50 % increase in paracellular leakage. LDH viability was modelled with a CES of 0.5, representing a 50 % reduction in cell viability.

For both endpoints, BP showed a concentration response relationship, which allowed PROAST to derive BMD and BMDL values. For Lucifer Yellow, BP showed increasing permeability with dose, leading to a $BMDL_{90}$ of 34 µg/mL. For LDH viability, the decline at the highest concentration resulted in a $BMDL_{90}$ of 102 µg/mL. In contrast, hBNc showed no meaningful changes in either permeability or viability across all concentrations, resulting in extremely large (over tested dose) or unbounded BMD estimates. Therefore, hBNc did not provoke detectable barrier disruption or cytotoxicity under the tested conditions could not be used for quantitative modelling.

Overall, the benchmark dose analysis indicated that BP induces both barrier dysfunction and cytotoxicity, with barrier impairment being the more sensitive endpoint. Following the document of reference, the Lucifer Yellow-derived BMDL (34 µg/mL) was selected as the primary *in vitro* point of departure, while the LDH-derived BMDL provided supportive information.

6.4.2 Conversion of BMDL values to in vitro surface doses

The BMDL values obtained from the *in vitro* concentration response modelling are expressed in volumetric terms (µg/mL). However, for comparison with human lung dosimetry, it is more appropriate to work in mass per unit surface area (mg/cm²), which reflects how inhaled material deposits on airway epithelium. Following the OECD workflow for airway models, each BMDL was therefore converted into a surface dose, POD_surf.

In this context, the Point of Departure (POD) represents the dose at which a biologically relevant effect first happens, in this case, either a 50 % loss of barrier integrity or a 50 % loss of viability.

The conversion used the apical volume applied to the tissue and the known surface area of the MucilAir insert. The general relationship is:

$$\mathrm{POD_{surf}}\ (\mathrm{mg/cm^2}) = \frac{\mathrm{BMDL\ (mg/L)} \times V_{\mathrm{apical}}\ (\mathrm{L})}{A_{\mathrm{insert}}\ (\mathrm{cm^2})}$$

Where:

- BMDL (mg/L) is the benchmark dose lower confidence limit converted from µg/mL (1 µg/mL ≡ 1 mg/L),
- V_apical = 20 µL = 20 × 10^{-6} L,
- A_insert = 0.3318 cm².

BP surface-dose PODs

- LDH viability ($BMDL_{90}$ = 102 µg/mL) → POD_surf = 0.00615 mg/cm²
- Lucifer Yellow permeability ($BMDL_{90}$ = 34 µg/mL) → POD_surf = 0.00205 mg/cm²

Therefore, a cumulative surface dose of 0.00205 mg/cm² was sufficient to trigger a 50 % barrier increase, while 0.00615 mg/cm² was required to induce a 50 % loss of viability after 28 days.

6.4.3 Derivation of Human Equivalent Concentrations (HEC) from the *in vitro* POD

To place the *in vitro* POD for BP into a human inhalation context, the next step was to derive Human Equivalent Concentrations (HEC, mg/m³). The purpose was to determine the airborne concentration of BP that would deposit, over a standard 8-hour working day, the same surface dose (mg/cm²) on human airway regions as the dose that produced an effect in the MucilAir model.

Because no new computational fluid dynamics (CFD) simulations were generated in this study, regional deposition fractions were obtained from the literature. CFD models are commonly used to predict how particles move and deposit within the human respiratory tract, and these deposition fractions are essential for calculating Human Equivalent Concentrations (HEC). However, no CFD or experimental dosimetry data are currently available for BP. Therefore, deposition fractions were taken for 1 µm graphene nanoplatelets using validated morphometric and MPPD inhalation models[42]. These values were selected because graphene nanoplatelets share similar features with BP, making them an appropriate alternative for inhalation dosimetry.

Deposition fractions were taken for the extra thoracic (ET), tracheobronchial (TB), alveolar (AI) regions, and for total deposition. Standard human ventilation and surface areas were applied (ET ≈ 0.016 m², TB ≈ 0.32 m², AI ≈ 7.0 m²; ventilation 7.5 L/min).

For each airway region *r*, the HEC was calculated as:

$$\mathrm{HEC}_r = \frac{\mathrm{POD}_{\mathrm{surf}} \times SA_r}{VE \times DF_r \times t}$$

Where:
– POD_surf = 0.00205 mg/cm², the selected *in vitro* point of departure (Lucifer Yellow),
– SA_r is the surface area of region *r* (m²),
– VE = 0.0075 m³/min,
– DF_r is the deposition fraction for region *r*,
– t = 480 min, representing an 8-hour workday.

Using deposition fractions for 1 µm graphene platelets (Total ≈ 0.17; ET ≈ 0.05; TB ≈ 0.03; AI ≈ 0.07), the following region-specific HECs were obtained for BP:

- ET (head airways):
  HEC = 0.000182 mg/m³ (0.18 µg/m³)
- TB (tracheobronchial):
  HEC = 0.00607 mg/m³ (6.1 µg/m³)
- AI (alveolar):
  HEC = 0.0569 mg/m³ (57 µg/m³)
- Total deposition:
  HEC = 0.0234 mg/m³ (23 µg/m³)

Among them, the alveolar HEC (~0.057 mg/m³) is the most relevant for deep-lung effects, while the ET HEC (~0.00018 mg/m³) represents a conservative portal-of-entry metric due to the small surface area and low deposition fraction. The total deposition HEC (~0.023

[42] Gao H et al.. 2021. NanoImpact 21, 100292

mg/m³) represents a systemic reference often used in occupational exposure assessments.

6.4.4 Margin of Exposure (MOE) analysis using literature-based occupational concentrations

To contextualize the HEC values estimated for BP, a Margin of Exposure (MOE) analysis was carried out. As no occupational exposure measurements are currently available for black phosphorus, representative graphene airborne concentrations were taken from Lovén et al (2021)[43]. These materials are handled in similar downstream operations. The study measured breathing zone concentrations range from background levels below 1 µg/m³ to handling peaks around 5–6 µg/m³, with the highest time-weighted averages (TWA) near 1.2 µg/m³. These two values, the 8-hour TWA (0.0012 mg/m³) and the short peak (0.0056 mg/m³), were selected as the conservative exposure scenarios for calculating MOE.

For each respiratory region *r*, the MOE was defined as the ratio between the corresponding HEC value and the assumed exposure concentration:

$$\mathrm{MOE}_r = \frac{\mathrm{HEC}_r}{C_{\mathrm{exp}}}$$

Where HECr is the region-specific Human Equivalent Concentration (mg/m³) and Cexp is the representative occupational exposure (mg/m³). Because the point of departure originates from a human-relevant *in vitro* system, no interspecies uncertainty factor is required. The only uncertainty factor applied is the standard one for intra-human variability (UF ≈ 10). Therefore, MOE values equal to or above 10 indicate low concern, values between 3 and 10 fall within an intermediate or borderline range, and values below 3 indicate potential concern.

HEC values were converted into MOEs under both exposure scenarios (Table 18). Under the 8-hour TWA scenario, the alveolar region presents an MOE of approximately 47, clearly above the threshold for low concern. The total deposition MOE is also above 10. Under short-term peak exposure, the alveolar MOE is around 10, at the acceptance threshold and still compatible with low concern for short events. In contrast, the tracheobronchial region shows an MOE of about 5 under TWA conditions and close to 1 under peak conditions, reflecting conservative assumptions regarding deposition

[43] Lovén K et al.. 2021. J Expo Sci Environ Epidemiol 31, 736–752

distribution rather than true toxicological sensitivity. The extra thoracic MOEs remain well below 1, although these values are influenced by normalizing to a very small surface area.

Table 18. MOE values calculated from HEC and C_{exp}

Respiratory Region	HEC (mg/m³)	MOE – 8 h TWA	MOE – Short Peak
Extrathoracic (ET)	0.000182	0.15	0.033
Tracheobronchial (TB)	0.00607	5.06	1.08
Alveolar (AI)	0.0569	47.4	10.2
Total deposition	0.02345	19.5	4.19

When interpreting these results, it is important to take into account that the alveolar region is considered the most relevant for chronic inhalation risk. Unlike the extrathoracic and tracheobronchial regions, the deep lung has no mucociliary clearance mechanisms and there is no mucus layer or ciliary transport to remove deposited particles. Therefore, respirable materials that reach the alveoli may persist for extended periods, interact directly with alveolar epithelial cells or macrophages, and contribute to long-term effects. On the other hand, particles deposited in the upper and airways are efficiently removed by mucociliary clearance and contribute far to long-term local or systemic effects.

For these reasons, the alveolar and total deposition MOEs provide the most meaningful base for risk interpretation. Both indicate that realistic exposures in the low microgram-per-cubic-metre range are associated with low concern for BP, as long as good industrial hygiene practices are maintained to minimize handling peaks. The relatively low MOEs calculated for the extra thoracic and tracheobronchial regions are a reflect anatomical normalization and conservative deposition assumptions.

The quantitative *in vitro* based risk assessment conducted in this study integrates long-term MucilAir data, benchmark dose modelling, surface dose conversion and literature-based inhalation dosimetry to derive human-relevant points of departure for BP. The analysis shows that BP induces concentration-dependent effects on epithelial barrier integrity and, at higher doses, on cell viability. These effects allow the derivation of BMDL values and their surface-dose points of departure. When translated to human equivalent concentrations and evaluated through margin of exposure analysis, the results indicate that realistic occupational exposures in the low µg/m³ range are associated with a low level

of concern for BP, especially when focusing on the alveolar region, which is the most relevant target for chronic inhalation exposure. The findings also show that hBN did not produce measurable cytotoxic, barrier-disrupting or genotoxic effects under the conditions of the study, suggesting the material as non-hazardous for the endpoints examined.

However, several limitations must be taken into account when interpreting these results. The PODs were derived from a single *in vitro* model, and although the MucilAir model recreates a physiologically relevant human airway epithelium, it does not capture all aspects of lung physiology, including systemic interactions, immune responses and long-term particle retention. Also, no computational fluid dynamics (CFD) simulations or material-specific deposition models are currently available for BP, therefore, a read-across approach was applied using deposition fractions for graphene nanoplatelets. While this is justified based on aerodynamic similarity, the absence of BP-specific inhalation dosimetry introduces an uncertainty. Also, no occupational exposure measurements exist for BP, requiring the use of surrogate exposure data from graphene-handling workplaces. Although this approach is reasonable, it may not fully represent exposure scenarios for BP, depending on how it is manufactured or processed. Finally, the study did not evaluate potential transformations of BP during ageing or interaction with lung fluids, which could modify its behavior in real-world conditions. Altogether, these limitations do should be considered when applying the results in a regulatory or industrial context.

6.5 GENERAL MANAGEMENT GUIDANCE FOR 2D MATERIALS.

Based on the combined evidence generated in the project, no additional risk-mitigation measures beyond established good practices for engineered nanomaterials appear necessary for hBN or BP under realistic occupational exposure scenarios. Across the project, progress has been made in understanding the toxicological behavior of the selected 2DM. For hBN, both inhalation and skin exposures have been evaluated: the material did not induce cytotoxic, barrier-disrupting or genotoxic effects in the long-term inhalation model, and dermal testing showed no adverse effects in acute OECD skin exposure assays. Also, both hBN and BP tested negative in two of the three OECD skin-sensitisation assays (OECD TG 442C, 442D and 442E). These findings, along with the low cytokine changes observed only at high inhalation concentrations, may indicate that hBN presents a low hazard for both dermal and inhalation routes under occupational conditions. For BP, the concentration-dependent effects observed at 100 µg/mL enabled the derivation of biologically relevant points of departure; however, the resulting human-

equivalent concentrations and margin-of-exposure analysis show that typical workplace airborne levels, generally in the low µg/m³ range, stay below thresholds of concern. Therefore, established good practices for nanomaterial handling should be enough to ensure worker protection.

Despite the overall low concern under realistic exposure conditions, some research gaps identified in the project should be taken into account when interpreting the current risk mitigation needs. For hBN, further work addressing potential chronic effects on both skin and lung would strengthen the hazard assessment. Although acute OECD dermal assays showed no effect, using HaCaT cells under 7day exposure conditions indicated subtle responses that might require additional investigation. For BP, uncertainties remain in inhalation dosimetry since deposition was estimated using graphene nanoplatelet data due to the absence of BP-specific CFD or deposition models. In addition, no direct occupational exposure measurements exist for BP, requiring read across of workplace data from graphene. Finally, neither material has been evaluated for long-term transformation, ageing or interaction with lung fluids, factors that may modify behavior under real-world conditions. Future studies may also support the derivation of DNEL or NOAEL values to enable a more refined quantitative risk assessment.

Considering the information obtained for hBN and BP in this project, in bibliography and in the SDS and the gaps that have been identified, a general management guidance has been developed and is included here.

Even if many 2DM (like hBN) appear chemically inert, their nanoscale form and handling methods can create respiratory and dermal risks. Proper containment, PPE, solvent substitution, and monitoring are essential for safe management across the sector.

a) General Principles

- Always work under the precautionary principle for all 2DM treating all 2DM as potentially hazardous dusts/nanomaterials unless proven otherwise and minimizing exposure
- Main exposure routes: inhalation of dusts/aerosols and dermal contact with powders/solvents.
- Keep records of training, exposure monitoring, and PPE fit testing.

b) Engineering controls

- **Ventilation**:

- Local Exhaust Ventilation (LEV) with ≥50–80% capture efficiency for powder handling.
- ≥3–5 air changes/hour in rooms where open handling occurs.

- **Containment**:

  - Use enclosed systems (closed funnels, sealed centrifuge tubes, gloveboxes) wherever possible.
  - Prevent re-aerosolization of dried powders (avoid sweeping; use HEPA-filtered vacuum systems).

- **Atmosphere control**:

  - Handle air-sensitive materials (e.g., phosphorene) under inert atmosphere (argon/nitrogen).
  - Use sealed storage to prevent degradation and oxidation hazards.

c) Personal protective Equipment (PPE)

- **Respiratory Protection**:

  - Full-face respirators with P2/P3 filters or APF ≥ 20 for nanopowder handling.
  - Organic vapor filters if solvents (e.g., CHP, NMP, acetone) are involved.

- **Dermal Protection**:

  - Chemical-resistant gloves (nitrile or equivalent); change frequently.
  - Full-body protective clothing with ≥95% effectiveness against dust penetration.

- **Eye/Face Protection**:

  - Safety goggles or face shield.

- **Footwear:**

  - Dedicated work shoes or overshoes to prevent tracking dust outside controlled areas.

d) Safe Handling Procedures

- **Powder handling (hBN, phosphorene, other 2D nanomaterials):**
    - Weigh and transfer only in designated powder rooms or fume hoods.
    - Keep containers sealed when not actively dispensing.
- **Mixing/processing in solvents:**
    - Use sealed reactors, mixers, or sonicators with cooling to avoid aerosol generation. Avoid open funnels
    - For solvents: work in fume hood and prevent splashes
    - Ensure spill kits are available and absorbents are compatible with solvents.
- **Collection of dry products:**
    - Collect dry powders outdoors or in well-ventilated areas;
    - Seal containers immediately after filling.
- **Cleaning:**
    - Wet wiping or HEPA vacuum; never dry sweeping.

e) Chemical & Process Risks

Should be determined for each 2DM. For the ones under study a summary could be:

- **hBN**:
    - Inert dust but may cause mechanical irritation to eyes/respiratory tract.
    - Still no DNEL available so graphite DNEL could be used as proxy (1.2 mg/m³ inhalable dust).
- **BP:**
    - Sensitive to oxidation; stability issues.
    - Possible dermal and inhalation effects at longer exposures.

- Production solvents (e.g., CHP) may be corrosive/toxic; alternatives like acetone have lower toxicity but higher flammability.

- General rule**:** Always check the SDS and risk assessment of solvents, additives, and by-products as they can also present a high hazard

f) Waste Management

- Powders**:** Collect waste material in sealed, labeled containers.
- Solvents (e.g., CHP, NMP, acetone)**:** Collect in solvent-compatible, clearly labeled containers; dispose as hazardous waste.
- Contaminated PPE and wipes**:** Dispose in double bags as hazardous waste.

g) Emergency Procedures

- Inhalation**:** Move to fresh air, seek medical advice if symptoms persist.
- Skin Contact**:** Wash thoroughly with soap and water; remove contaminated clothing.
- Eye Contact: Rinse with water ≥15 minutes, seek medical attention.
- Spill Response**:**

    - Powders → HEPA vacuum or damp cleaning.
    - Liquids → absorbent pads, collect for hazardous waste disposal

h) Monitoring & Review

- Conduct airborne dust/nanoparticle exposure monitoring**.**
- Test and maintain LEV and ventilation systems annually.
- Regularly review new toxicological data on 2D materials, updating DNELs and procedures as knowledge evolves.
- Train workers every 6–12 months on updated procedures.

A summary as a leaflet is given below:

Safe Handling of 2D Materials
(hBN, Phosphorene, etc)

Before work

Wear required PPE:
- Respirator (P2/P3) or organic vapor filters (for solvents)
- Chemical-resistant gloves
- Goggles or face shield

Ready containers, waste bins and spill kits

After work

Remove PPE carefully. Dispose of contaminated gloves/wipes in **hazardous waste bags**

Store powders and solvents in sealed labelled containers

Clean area using wet cloth or HEPA vacuum. **NO sweeping**

Golden Rule

If dust or solvent smell present outside the work zone → stop work, check ventilation, inform supervisor

Icons from: https://www.flaticon.com/free-icons/fresh-air
https://www.freepik.com/

During work

Weigh/transfer powders in fume hood or room with LEV ≥ 50-80% efficiency

Keep **containers closed** when not dispensing

Avoid open funnels. Use enclosed systems

Collect dry powders outdoors or in ventilated areas

Never sweep dust, use HEPA vacuum or wet cloth

Emergency

Move to **fresh air,** seek medical attention if symptoms persist

Skin contact. Remove contaminated clothing. Wash with soap and water

Eye contact. Rinse with water ≥ 15 mins. Seek medical help

Spills:
Powders: damp clean or HEPA vacuum

Solvents: absorb with pads, hazardous disposal

6.6 CONCLUSIONS AND RECOMMENDATIONS

From an environmental perspective, the Life Cycle Assessment (LCA) of the application of 2D hBN in LIB batteries, specifically in casing coatings, points to a significant reduction in environmental impact over the battery's lifetime. The ability of hBN nanomaterial to enhance battery performance substantially reduces, among other things, the risk of overheating that could lead to thermal runaway, thereby extending battery life.

In the risk analysis, risk estimation tools were applied to the 2D material production processes using the toxicity information obtained in the project and production scenarios established with the company. This information was used to develop a basic risk management guide.

Identified gaps and research needs: for a more precise risk assessment, the potential chronic effects of hBN on both skin and lungs should be studied. In the skin, OECD acute exposure assays show no effect, but effects are observed in HaCaT cells with a 7-day exposure. Similarly, for inhalation, a 28-day exposure produces increases in some cytokines, which may indicate a potential inflammatory process that should be studied in more detail. If necessary, a DNEL or NOAEL should be calculated to allow a quantitative risk assessment to support informed decisions on risk management.

From an environmental standpoint, since this study focused only on acute exposures (24–72 h), future work should include subacute or chronic assays with the same materials to deepen understanding of their long-term toxicity, as well as other experiments addressing different toxicity parameters and mechanisms of action.

Regarding the life cycle assessment, the study focuses on evaluating an innovative application of 2DM that is currently in the early stages of development. It will be necessary to perform further analyses and validate results as technological advances provide data from real industrial applications.

7 DISSEMINATION

The project has been extensively disseminated through newsletters and social media, including those of the Department of Life Sciences of the University of Trieste (https://dsv.units.it), FIOH website (https://www.ttl.fi/tutkimus/hankkeet/kehittyneiden-kaksiulotteisten-materiaalien-elinkaaren-turvallisuuden-parantaminen; https://www.ttl.fi/en/research/projects/safer-life-cycle-of-advanced-2d-materials-used-in-energy-applications-safe2energy) as well as GAIKER web page (https://www.gaiker.es/en/proyectos_destacados/safer-life-cycle-of-advanced-2d-materials-used-in-energy-applications-safe2energy/) Linkedin and Instagram pages (https://www.linkedin.com/feed/update/urn:li:activity:7236284218706673664; https://www.linkedin.com/feed/update/urn:li:activity:7254398974965534720; https://www.instagram.com/p/DBa0JP6NPbv/?utm_source=ig_web_copy_link&igsh=MzRlODBiNWFlZA==). The results of the project have also been made publicly available through the SAF€RA partnership (*https://www.safera.eu*).

In addition, dissemination of the results with stakeholders was made inviting members of the Advisory Board [composed by representatives of Funding Authorities (INAIL, TUKES, OSALAN) and Companies interested in the project (Bedimensional, Avanzare Innovación Tecnológica SL, Graphenea SA, Smena Catalysis AB)] at the project meetings.

Dissemination of the results was made also through publication of articles in international peer-reviewed journals and through oral communications/poster presentations at national and international conferences, as follows.

Publications:

- Carlin M, Sosa S, González VJ, Tubaro A, Vázquez E, Prato M, Pelin M. Skin biocompatibility of hexagonal boron nitride: an *in vitro* study on HaCaT keratinocytes and 3D reconstructed human epidermis. J Hazard Mater. 2025, 494: 138449. DOI: 10.1016/j.jhazmat.2025.138449.
- Carlin M, Pavan G, Sosa S, Crosio L, Mantero E, Bonaccorso F, Pelin M. Hexagonal boron nitride bio-interfacing the skin: role of shape and size in its safety profile assessed on 2D and 3D epidermal models. J Hazard Mater Adv. 2025, *Under review*.

Scientific contributions at conferences and seminars:

- Carlin M. Safety evaluation of two-dimensional nanomaterials: overcoming challenges in skin sensitization testing. Future Materials 2025 (Tenerife, 27-29 October 2025). *Invited oral communication.*
- Carlin M et al. Hexagonal boron nitride: safety assessment at the skin level. INSTM Young Researchers' Forum (Napoli, 9-10 October 2025). *Oral communication.*
- Pelin M. Are 2D materials safe for our skin? A toxicological perspective. NanoInnovation 2025 (Roma, 15-19 September 2025). *Invited oral communication.*
- Carlin M et al. Safety assessment of hexagonal boron nitride at the skin level: impact of physico-chemical properties. EUROTOX 2025 (Athens, 14-17 September 2025). *Poster.*
- Pelin M. 2D materials bio-interfacing the skin: safety assessment and hazard characterization. 7th International Congress on Advanced Materials Sciences and Engineering (Krakow, 29-31 July 2025). *Invited oral communication.*
- Katsumiti, A. OECD: Extending the use of standardised *in vitro* ecotoxicity models to support neurotoxicity testing. Joint Regulatory Risk Assessors Summit – Advancing Safety & Sustainability Assessments of Advanced Materials (Paris, 19-20 June 2025). *Poster*
- Carlin M. Safer life cycle of advanced 2D materials used in energy applications (Safe2energy). SAF€RA Symposia (Roma, 11-13 March 2025). *Oral communication.*
- Carlin M et al. Effetti del nitruro di boro esagonale a livello cutaneo: studio *in vitro* su cheratinociti HaCaT ed epidermide umana ricostruita. 22nd National Congress of the Italian Society of Toxicology (SITOX) (Bologna, 10-12 February 2025). *Poster and Flash oral communication*.
- Venäläinen M et al. Effects of long-term exposure to 2-dimensional materials in an *in vitro* lung 3D model. Kansallainen Kemikaalifoorumi (Helsinki, 12 November 2024). *Oral communication.*
- Carlin M et al. Hazard characterization of hexagonal boron nitride at the skin level. Future Materials 2024 (Athens, 21-23 October 2024). *Oral communication.*
- Katsumiti, A et al. New Approach Methodologies (NAMs) based on *in vitro* ecotoxicity models to support the development of Safe and Sustainable by Design (SSbD) Advanced Materials. Future Materials 2024. (Athens, 21-23 October 2024). *Oral communication*

- Pelin M. OECD-based hazard characterization of 2D materials at the skin level. Graphene 2024 (Madrid, 25-28 June 2024). *Oral communication.*
- Carlin M et al. Safety assessment of hexagonal boron nitride at the skin level: an in vitro study on 3D reconstructed human epidermis and HaCaT keratinocytes. SETAC Europe 2024 (Seville, 5-9 May 2024). *Poster.*
- Carlin M et al. Adoption of the OECD TG 439 and 431 for the assessment of skin irritation and corrosion properties of 2D nanomaterials. ESTIV 2024 (Prague, 3-6 June 2024). *Oral communication.*