

Practical implementation of a Safe-by-Design framework: a proof of concept for early-phase hazard assessment of PFAS-free formulations

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Abstract

Per- and polyfluoroalkyl substances (PFAS) are widely used in various industrial sectors for their thermal stability, chemical resistance and surface-active properties. However, these same characteristics also contribute to their environmental persistence, potential for accumulation and adverse health effects, triggering increasing scrutiny across product life cycles. Within the EU-funded project PROPLANET, a tiered and iterative Safe-by-Design methodology was applied to PFAS-free coatings being developed for technical textiles, metal elements of food-packaging machinery and low-maintenance glass applications, at technology readiness levels (TRL) 3–5. The approach combined regulatory data mining, *in silico* screening and *in vitro* testing to identify potential hazard concerns and inform formulation-development decisions, while accounting for technical-performance requirements. Data mining from Safety Data Sheets (SDS) and the ECHA CHEM database were used to identify and exclude known harmful substances, including carcinogenic, mutagenic or reprotoxic (CMR) compounds and substances of very high concern (SVHCs), during the early stages of formulation development. Data-gap analysis indicated that the existing *in silico* models applied had insufficient coverage of the chemical space relevant to the substances under investigation, encouraging the development of custom-trained models. Two-tiered *in vitro* testing further characterized the toxicity profiles of several formulations throughout the project. One formulation intended for textile applications showed no hazard alerts under the tested conditions, whereas the formulations intended for glass and metal applications raised potential hazard concerns, requiring further investigation before progression to higher TRLs. This study presents the practical implementation of the Safe-by-Design methodology and the main results and inputs for decision making. At the same time, the practical challenges encountered during the implementation of the approach are discussed, together with the methodological gaps and grey areas. In conclusion, the structured, tiered and iterative approach implemented in this study supports the screening and prioritization of candidate chemicals and formulations in the early stage of their development, guiding the selection of PFAS-free chemicals and materials for further development in a SSbD context. In future work, analysing leachates, abraded particles, and chemicals released from coated products could provide an additional approach to assessing consumers and environmental safety during the use phase.

Background

Per- and polyfluoroalkyl substances (PFAS) comprise a large and diverse group of synthetic chemicals that have been widely used for decades in a broad range of products [1, 2]. PFAS are characterized by the presence of fluorinated carbon groups, in which the exceptional strength of the carbon-fluorine (C-F) bond, together with the high electronegativity and low polarizability of fluorine, contributes to their distinctive physicochemical properties, including high thermal and chemical stability, low surface energy, and hydrophobic and oleophobic behaviour [3]. These properties have led to extensive applications across multiple sectors, including food contact materials, textiles, firefighting foams, cosmetics, medical devices, electronics, industrial coatings and others. In coatings, PFAS can provide key functional attributes, such as water and oil repellency, durability, and resistance to degradation, making them attractive from functional and economic perspectives.

However, the same properties that make PFAS useful for many applications also contribute to their persistence in the environment, and potential to bioaccumulate through interactions with proteins and other biological macromolecules [4, 5]. Exposure to certain PFAS has been associated with adverse human health outcomes, including immunotoxicity, developmental and reproductive effects, endocrine disruption, increased risk of cancer, and organ related toxicities [6, 7]. From an environmental perspective, PFAS are often referred to as “forever chemicals” due to their resistance to natural degradation processes, resulting in long-term accumulation and challenges for remediation [1, 6].

These concerns have triggered increasing scientific, regulatory, and societal scrutiny of PFAS and PFAS-containing products use across their life cycle. In response, regulatory initiatives at national and international levels are moving toward stricter controls and, in some cases, broad bans or phase-outs. The Stockholm Convention on Persistent Organic Pollutants (POPs) plays a central role in regulating PFAS. Substances such as perfluorooctane sulfonic acid (PFOS), perfluorooctanoic acid (PFOA), perfluorohexane sulfonic acid (PFHxS) and long-chain perfluorocarboxylic acids (PFCAs), their salts and related compounds are already listed for restriction or elimination, based on their persistence, bioaccumulation, and toxicity [8]. In the European Union, more recent actions such as the proposed Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) restriction on PFAS reflect a paradigm shift from substance-by-substance regulation toward a group-based approach [9]. Although these regulatory actions aim to protect human health and the environment, they also necessitate the rapid development of safe, effective, and sustainable alternatives, to ensure that PFAS-free solutions provide the required performance while avoiding new risks and regrettable substitutions.

In 2022, the European Commission’s Joint Research Centre (JRC) released the Safe-and-Sustainable-by- Design (SSbD) framework under the European Union (EU) Chemicals Strategy for Sustainability (CSS) to guide the development of chemicals and materials toward safer

and more sustainable alternatives across their entire life cycle [10]. Following its application in a series of case studies, the framework was recently revised based on iterative stakeholder feedback [11].

With respect to safety assessment, the SSbD framework aims to guide innovation by integrating hazard assessment and risk characterization from the earliest stages of new chemicals, materials, processes and products development. Achieving this objective relies on the use of new approach methodologies (NAMs), which offer cost- and time-efficient strategies for early-phase chemical screening and prioritization, while supporting the 3Rs principles to replace, reduce, and refine animal testing. The integration of *in silico* and *in vitro* NAMs in SSbD assessments also has an opportunity to further develop and increase confidence in mechanistic, non-animal-based hazard evaluation which can finally support future regulatory approaches, such as, next-generation risk assessment (NGRA) [12] [13].

Although SSbD frameworks incorporating NAM-data have been proposed, the application of NAMs within SSbD remains largely conceptual [14]. Existing studies further highlight significant data gaps and uncertainties at early stages of product development, where evolving chemical compositions and physicochemical constraints challenge conventional hazard assessment approaches [15]. While SSbD case studies are increasingly emerging across different materials and coatings applications, integrated workflows that combine NAM-based hazard assessment with environmental fate, sustainability, and technical performance criteria for the systematic evaluation of PFAS alternatives remain limited [16]. Together, these limitations underscore the need for well-documented, practical case studies that translate SSbD principles into deployable workflows capable of supporting real-world decision-making during chemical and materials innovation.

In response, the EU-funded project PROPLANET aimed at applying SSbD approaches for the development of PFAS-free coating formulations. Within the project, novel coatings were developed from a conceptual stage (technology readiness level (TRL) 3) to validation in a relevant environment (TRL 5) for applications in technical textiles, metal components of food packaging machineries, and low maintenance glass. The main chemistry was based on crosslinked biopolymer microcapsules embedded in a polysaccharide matrix and on hybrid siloxane coatings. The developed products aimed to maintain or improve their mechanical and technical performance while reducing both carbon footprint and toxicity, which were assessed using the SSbD framework. Focusing on safety, here we present a practical proof of concept for implementing a Safe-by-Design (SbD) workflow for hazard assessment. This workflow integrates regulatory data mining, *in silico* screening, and tiered *in vitro* testing to support iterative decision-making during the development of functional PFAS-free coating formulations. More specifically, this publication focuses on the practical implementation of the SbD framework for the early-stage hazard assessment of PFAS-free formulations. It presents the approaches used, evaluates the confidence and uncertainties in the available and newly generated data, and the challenges and solutions to overcome data limitations with particular focus on PFAS-free formulations and coatings.

Methodology

Conceptual framework tailored for PFAS-free formulations

The framework for SbD assessment was designed to be implemented as a continuous and iterative activity throughout the PROPLANET project, with the objective of evaluating the toxicological profile of the coating formulations under development, in accordance with SSbD principles. A tiered evaluation strategy was established, including the definition of hazard criteria and the integration of multiple lines of evidence, namely existing data, *in silico* predictions, and *in vitro* testing.

The main established hazard criteria were:

- - to develop coating formulations without the use of substances classified as carcinogenic, mutagenic, or reprotoxic (CMR), as well as substances of very high concern (SVHC) [10]. These hazardous properties should also be absent from the final formulations.
- - for other hazard endpoints, to achieve reduced adverse effects relative to PFAS-based benchmark formulations, where such comparators were available.

As rationale, CMR effects are here prioritized due to their severity, potential irreversibility, and multi-generational impact. They thus play a crucial role in EU chemicals regulations, including REACH [17] and Directive 2004/37/EC on workers protection from CMR substances.

Figure 1 illustrates the conceptual SbD framework developed in the project, based on a tiered approach consisting of:

1. data mining and review of existing safety information for all the individual substances used in the formulations
2. the use of *in silico* predictions to fill data gaps at the level of individual substances and
3. tiered *in vitro* testing to perform hazard assessment of the developed formulations, beyond the pristine individual substances.

Each of these components and their workflow are provided in detail in the following sections.

Data Mining

The first step of the tiered assessment consisted of collection and review of existing safety information. This was carried out for all individual substances selected as potential formulation components, including, but not limited to, those identified as PFAS replacements. The list of candidate chemicals was continuously updated throughout the project, in parallel with the formulations' development process. The corresponding toxicological information was collected and updated accordingly. To support this work, a data-collection template was developed, which served as a living document. Extracts from this file are reported in Additional file 1. To gather available toxicological information, a tiered-, manual data collection strategy was implemented. In the first tier (TRL 3–4), hazard information was extracted from the materials' Safety Data Sheets (SDS) provided by the developers of the formulations. In the second tier, for chemicals that progressed to TRL 4–5 and other specific cases, additional hazard information was retrieved and evaluated from the European Chemicals Agency (ECHA) CHEM database [18]. This included harmonised classifications and self-classifications under the European classification labelling and packing (CLP) Regulation [19], together with supporting information from REACH registration dossiers, where available. When the PFAS compounds to be replaced were known, their hazard and toxicity data were also compiled for reference comparison.

The main objective at this stage was to identify and, where possible and relevant, exclude from the formulations any substances classified as CMR, and more in general as SVHC. Under the CLP Regulation [19], substances are classified into categories (e.g. 1A, 1B, or 2) for carcinogenicity, mutagenicity, and reproductive toxicity and other endpoints, based on evidence strength. Such substances are further restricted under REACH Annex XVII, which imposes limitations on manufacture, use, and marketing. In line with the project's aim, these substances should be excluded from the PFAS-free alternative formulations.

Another objective was to identify data gaps, to determine whether missing classifications reflected insufficient data or an assessment-based conclusion. For this reason, the underlying toxicological information supporting the classifications was scoped. To support this activity, additional human and environmental hazard endpoints, such as acute toxicity, were extracted from the SDSs and ECHA CHEM database.

The reliability of the collected information was evaluated, to support decision-making processes and gap filling activities. Harmonized classifications under the CLP regulation were considered the most reliable source of hazard information, as they are established through a formal scientific evaluation process at the EU level. Information from self-classifications reported in the ECHA CHEM database and SDS were considered to carry a greater uncertainty. When discrepancies were observed between SDS information and regulatory classifications, regulatory sources were prioritized. Likewise, the absence of information on the toxicological data underpinning a classification was considered an additional source of uncertainty. Notably, from May 2025 the ECHA CHEM database also presents the C&L Inventory [20]. Industry self-notifications are grouped according to the hazard classification reported. When different classifications have been submitted for the same substance, the classifications are ranked according to the quality of the source, from (higher to lower) REACH joint submissions, to individual submission, and finally CLP notifications. A secondary ranking parameter is also provided, consisting of the proportion of the classification, expressed as percentage, against all classifications for the same substance. These aspects were taken into account to evaluate the uncertainty of the classification. In line with a precautionary but proportionate approach, CMR/SVHC alerts reported under conditions of uncertainty did not lead to automatic exclusion of a substance. Instead, such cases were prioritized for further evaluation in subsequent assessment tiers.

Thus, whenever a known/presumed (category 1A/B) or suspected (category 2) CMR compound or SVHC was identified among the candidate components, the finding was highlighted. If the categorization came from a harmonized classification, the candidate component or the associated formulation was excluded from further development, in line with the criteria established. Self-classification reports warranted increased attention and targeted follow-up testing but did not lead to immediate exclusion.

Data gap identification and *in silico* gap filling

in silico

Application of existing in silico models

For individual substances that progressed to TRLs 4–5 having insufficient hazard information, the VEGAHUB tools were first used for data gap filling. VEGA provides more than 100 Quantitative Structure–Activity Relationship (QSAR) models to predict properties of chemical substances including human toxicity, ecotoxicity, environmental properties, physico-chemical properties and toxicokinetics [21]. Simplified Molecular Input Line Entry System (SMILES) for each substance were retrieved from PUBCHEM [22] and used as input in the VEGA platform. Models relevant to the most crucial endpoints, i.e., CMR were selected, and predictions were obtained for the substances with data gaps in these endpoints. VEGA also reports the prediction reliability as low, moderate or high, based on the Global Applicability Domain Index (ADI). The ADI indicates how well the target substance is represented by the model's chemical and response space, thus supporting an assessment of the reliability and uncertainty of the prediction. A higher ADI corresponds to greater confidence in the prediction, whereas lower values indicate increasing uncertainty and a higher likelihood of prediction errors. According to VEGA guidance, an ADI value above 0.85 indicates a good applicability-domain assessment [23]. Predictions with lower ADI values were thus considered to have limited reliability for the present screening exercise. When providing the expert judgement on the conclusion of the prediction (positive, negative, or inconclusive), factors included in VEGA assessment, such as prediction reliability of similar substances and reasoning (e.g., structural alerts) were also considered.

Custom trained mutagenicity models

To further address data gaps left in substances that fall outside VEGA's applicability domain, custom trained predictive *in silico* models were here developed and applied [24]. Mutagenicity based on the Ames test was selected for the development of the new models, as a key CMR endpoint. The derived tool POLIS is an openly available platform [25] and was used to predict the mutagenic potential of the substances. SMILES for each substance were retrieved from PubChem [22] and used as input to the platform. POLIS integrates three complementary predictive models: a guided-kNN read-across model, a graph convolutional neural network (GCNN), and an XGBoost-based QSAR model. For each substance, predictions were generated independently by all three models and integrated through a majority-voting consensus scheme. The reliability of each consensus prediction was characterized as high, moderate, or low based on the combined applicability-domain assessment of the guided-kNN and QSAR models and was considered in the subsequent hazard assessment.

Tiered in vitro testing

In vitro toxicity testing was performed on the formulations under development to characterize their hazard profile. Following the selection of the candidate chemicals (TRL 3–4), multiple formulations were designed, optimized, and characterized, including evaluation of their functional performance (not addressed here). To assess their intrinsic hazard properties, toxicity was evaluated using the actual formulation mixtures rather than the individual parent compounds. Parent chemicals in a formulation may interact, leading to transformation products or other derivatives that can alter the overall hazard profile. Therefore, assessing the hazard properties of the mixtures, rather than those of the parent compounds alone, helps capture potential changes in toxicity arising from chemical transformation, and combined formulation effects, including additive or non-additive interactions among formulation components.

Also at this level, a tiered approach was used, where high-throughput, screening-level models and assays were first applied to a broader set of candidate formulations developed at TRL3-4 phases. More advanced models and assays were thus added to test the final formulations and materials advanced to TRL4-5.

Table 1 presents information about the formulations received at different stages of the project and tested in this study. Due to confidentiality agreements, the exact composition of some formulations cannot be disclosed. In these cases, only the minimum information required to describe the formulation is provided. Few single additives were also tested to clarify some hazard concerns highlighted during the data mining phase, or to fill data gaps (e.g., Bentonite, MoS₂).

Table 1
Tested formulations, their application sector and description (to the extent permitted by confidentiality)

Application	Formulation code/Compound name, CAS nr.	Formulation description/Compound role
Technical textiles	NIC 1.1	Biopolymer-based aqueous formulation containing a film-forming biopolymer, a modified polysaccharide, and an inorganic nanomaterial (ZnO)
	NIC 2.1	Biopolymer-based aqueous formulation containing a hydrophobic additive and a polysaccharide
	NIC 2.2	Biopolymer-based aqueous formulation containing a film-forming biopolymer and a modified polysaccharide
	NIC 2.3	Biopolymer-based aqueous formulation containing a film-forming biopolymer and a bio-based encapsulated functional additive
	NIC 2.4	Biopolymer-based aqueous formulation containing a film-forming biopolymer, a modified polysaccharide, and an inorganic nanomaterial (similar to NIC1.1, different proportions of compounds and ZnO size)
	NIC 2.5	PFAS-based, commercially available
	NIC 2.6	PFAS-based, commercially available
	NIC 3.1	Biopolymer-based aqueous formulation containing a film-forming biopolymer and a modified polysaccharide (similar to NIC 2.2, different proportions of compounds)
	NIC 4.1	Biopolymer-based aqueous formulation containing hydrophobic additive and bio-based nanofibrillar material
Low-maintenance Glass & Food packaging machineries (metal)	TEC 0.0	PFAS-based formulation (EP 4081604 B9)
	FAS17, 101947-16-4	PFAS compound, functional base of TEC0.0
	TEC 0.1	Base formulation of TEC0.0, without the PFAS compound
	GLA	Silane-based formulation containing tetraethyl orthosilicate (TEOS) as inorganic precursor and n-hexyltriethoxysilane (C6) as hybrid precursor in an alcoholic solvent.
	MET	Silane-based formulation containing tetraethyl orthosilicate (TEOS) as inorganic precursor, and trimethoxymethylsilane (TMMS) and diethoxydimethylsilane (DEDMS) as hybrid components in an alcoholic solvent
	MoS ₂ 1317-33-5	Additive of MET formulation
	Bentonite, 1302-78-9	Additive of MET formulation

FAS17: 1H,1H,2H,2H-Perfluorodecyltriethoxysilane; MoS: Molybdenum disulphide

In vitro testing tier 1

In the first tier, high-throughput two-dimensional (2D) *in vitro* cell models, a whole-organism non-vertebrate model, and screening-level assays were selected to evaluate a broad range of biological endpoints, including cytotoxicity, genotoxicity, reproductive toxicity and inflammatory responses, across a relatively large number of alternative formulations and benchmark materials. These models can play a key role in early phases of SSbD, by supporting hazard identification and chemical screening through rapid, cost-effective assessment of the potential toxicological properties of the substances under development. Moreover, they enable the prioritization of the most promising test substances for more advanced testing, facilitate the early identification of safer alternatives, and support informed decision-making within the SbD framework, while reducing reliance on *in vivo* testing during the initial stages of innovation. Figure 2 illustrates the *in vitro* testing scheme performed, highlighting the formulations tested, concentrations, models, endpoints and methods applied.

The human lung-derived A549 cell line was selected as an inhalation-relevant model because of its demonstrated robustness in mechanistic toxicity testing and pulmonary hazard assessment [26]. A549 cell monolayer were maintained and seeded for exposure in 96-well plates according to previously published cell-culture protocols [27] and subsequently exposed to different concentrations of the formulations for 3h or 24 h under submerged culture conditions. A reporter cell line derived from the human U2-OS osteosarcoma cell line was used to assess endocrine-disrupting activity mediated by estrogen receptor alpha (ER α). The U2-OS cells were similarly cultured in 96-well plates and exposed to different concentrations of the formulations for 24 h under submerged conditions according to the established assay protocol [28]. An LDH assay was performed using culture supernatants collected from U2-OS cells after exposure to confirm that estrogenic activity was assessed only at non-cytotoxic concentrations.

Table 2
List of models and assays applied in the in vitro testing tier 1.

Endpoint	2D model/organism	Assay	Description
Cytotoxicity/cell viability	A549	Alamar Blue	Measures cytotoxicity and metabolic activity based on the reduction of a cell-permeable indicator dye by metabolically active cells.
Genotoxicity (DNA damage)	A549	Comet assay	Identifies single- and double-strand breaks to the DNA, as well as alkali-labile sites. The Fpg-modified assay includes the use of enzyme formamidopyrimidine DNA glycosylase (Fpg) to reveal oxidative damage.
Pro-inflammatory response	A549	ELISA	Quantifies the levels of pro-inflammatory cytokines, e.g. interleukin 6 (IL-6) and interleukin 8 (IL-8).
Endocrine disruption (ED) through estrogen receptor (ER α) activation	U2-OS	CALUX	Measures the ability of test compounds to activate ER α -mediated transcription - an indicator of potential ED via estrogenic activity. Complemented by cytotoxicity assay by LDH release.
Reproductive toxicity	N2 <i>C. elegans</i>	Progenies survival	Non- vertebrate organism, used to assess the F1 survival (the viability and development of the first filial generation) as an indicator of reproductive toxicity.

A549: human lung cell line; U2-OS: human osteosarcoma cell line; ELISA: Enzyme-Linked Immunosorbent Assay; CALUX: Chemical Activated Luciferase gene eXpression; LDH: lactate dehydrogenase; N2: C. elegans strain.

For testing, the as-received liquid formulations were used as stock test substances and considered as undiluted and equivalent to a concentration of 100%. The formulations were added to the culture medium to a maximum concentration determined by solvent content and pH constraints, and then serially diluted by a factor of two to yield final exposure concentrations ranging from 10% to 0.078% (with specific concentration ranges depending on the formulation). Precipitation was not used as a limiting factor, as the formulations either intentionally contained particulate components or included water-reactive constituents that hydrolyze and inherently limit dissolution. However, the presence of precipitates was considered when interpreting the results, evaluating possible effects on the exposure conditions, bioavailability, and assay performance. In the case of nanomaterials or particles (MoS₂ and Bentonite), a standardized dispersion protocol was used [29] and dispersions were prepared accordingly to ensure appropriate and reproducible exposure conditions.

Cytotoxicity was assessed using the Alamar Blue assay, and DNA damage was evaluated using the alkaline and enzyme-modified comet assays, according to established laboratory SOPs and previously published methods [30, 31]. The release of the cytokines IL-6 (prod.no. 88-7066, Invitrogen) and IL-8 (prod.no. 88-8086, Invitrogen) was measured by ELISA, according to the manufacturer's instructions. Estrogen receptor- α (ER α) activation was assessed using the CALUX reporter-gene assay in accordance with OECD Test Guideline 455 [28]. *Caenorhabditis elegans* was used to screen a reproductive toxicity-related endpoint [32]. Specifically, progeny survival was assessed as an early indicator of reproductive toxicity and to identify non-toxic concentration ranges suitable for subsequent higher-tier testing.

The PFAS-based benchmark formulations meant to be replaced were also included in the testing. Commercially available products were available for the textile sectors in aqueous concentrate form, however, limited information was available on the content. The benchmark for the low-maintenance glass and food packaging machineries sectors also presented precipitation when put in water condition, possibly limiting the availability of the compounds to the cells.

The results from the first tier testing informed decisions regarding which formulations should be selected for further development, alongside an evaluation of their functional performance (not addressed here).

In vitro testing tier 2

The formulations that demonstrated expected functional performance and reached TRL4-5 were tested on more advanced *in vitro* models and assays to address higher tier toxicological endpoints, i.e. respiratory toxicity, genotoxicity (chromosomal aberrations), carcinogenicity, and reproductive toxicity. At this stage, standardized and validated methods, e.g. covered by OECD test guidelines (TGs) or guidance documents (GDs), can provide increased regulatory robustness by delivering harmonized, validated endpoints that support hazard identification and enable comparability across studies. Their use facilitates the integration of data into weight-of-evidence approaches and can support scientifically robust and transparent regulatory decision-making. In parallel, advanced *in vitro* models offer enhanced biological complexity and physiological relevance by capturing intercellular communication, barrier function, and tissue-level responses that are not accessible in simplified monocultures. Together, tier 1 and 2 approaches complement one another by implementing efficient screening in simpler systems and more biologically relevant assessment in advanced models, thereby strengthening weight-of-evidence evaluations for regulatory-relevant hazard assessment.

Figure 3 illustrates the tier 2 testing strategy applied to evaluate the toxicological profiles of selected formulations. The approach combines *in vitro* and a whole organism system to address higher tier toxicological endpoints. The list of models and assays that have been used is presented in Table 3. Specifically, to assess respiratory toxicity under more physiologically relevant exposure conditions, a lung triculture model was exposed to formulations or chemicals at the air–liquid interface (ALI). The triculture model was established as previously described in [33] and exposed to formulations or candidate chemicals. Following exposure, cytotoxicity and the release of the pro-inflammatory cytokines IL-6 and IL-8 were assessed as described in the previous section. Cell layer integrity was assessed by transepithelial electrical resistance (TEER) measurements, using an EVOM Manual Epithelial Volt Ohm Meter equipped with an Endohm-24 electrode chamber as described in [34]. Genotoxicity (chromosomal aberrations) was assessed in HepG2 cell cultures through the *in vitro* micronucleus assay (MN) according to the OECD TG 487 [35]. Carcinogenic potential was evaluated in Bhas42 cell cultures using the cell transformation assay (CTA) according to OECD GD 231 [36]. Reproductive toxicity was further examined using the model organism *C. elegans*, this time addressing brood size [37]. Together, these assays provided an exploratory framework for CMR hazard characterization of selected formulations.

In both tier 1 and tier 2 *in vitro* testing, appropriate assay-specific vehicle, positive and negative controls were included in all experiments. Potential assay interference was assessed where applicable. Cell exposure, assay acceptance criteria, data processing and interpretation were applied according to the relevant SOPs, manufacturer’s instructions or OECD guidance. Detailed experimental procedures are provided in the cited references.

Table 3
List of models and assays applied in the in vitro testing tier 2.

Endpoint	Cell line/organisms	Assay	Description
Lung toxicity	tri-culture 3D lung model (A549 + Ea.hy926 + THP-1)	Alamar Blue, ELISA, transepithelial electrical resistance (TEER)	Cytotoxicity and metabolic activity by Alamar Blue; Pro-inflammatory responses by ELISA; Cell layers integrity by TEER
Genotoxicity (chromosomal aberrations)	HepG2	Micronucleus assay (OECD TG 487)	Detects chromosomal damage (damage to the DNA organizational units), as well as cytostasis (slowing of cell growth/division) and cytotoxicity (cell death)
Carcinogenicity	Bhas 42	CTA (OECD GD 231)	Assesses the potential of a substance to induce cell transformation, a key event associated with carcinogenic potential
Reproductive toxicity	N2 <i>C. elegans</i>	Brood size	Measures brood size as an indicator of reproductive toxicity by quantifying total progeny production

A549: human lung cell line; Ea.hy926: human endothelial cell line; THP-1: human monocytic cell line; HepG2: human hepatocellular cell line; Bhas 42: mouse BALB/c 3T3-derived fibroblast cell line; ELISA: Enzyme-Linked Immunosorbent Assay; TEER: transepithelial electrical resistance; CTA: cell transformation assay; N2: *C. elegans* strain.

The tier 2 testing results complemented the evidence collected in tier 1, enabling a more comprehensive characterization of the toxicological profiles of the selected formulations, and providing additional input for decision-making on advancing the candidate formulations.

Results

Data mining

An extract of the hazard information compiled for the candidate chemicals is provided in Additional file 1 (the information is updated at July 2026, changes might occur at later time). Overall, none of the candidate chemicals were classified in CMR categories 1 (known or presumed CMR) or SVHC according to REACH/CLP harmonized classifications (with one exception in the glass application, see below). The benchmark PFAS 1H,1H,2H,2H-Perfluorodecyltriethoxysilane (CAS 101947-16-4), used in glass application, is classified as suspected carcinogen (Category 2) and reproductive toxicant (Category 1B) according to SDS, further highlighting the importance of its replacement. The main findings influencing scale-up decisions or triggering additional follow-up assessments are summarized below, and relevant links to the data sources are provided in Additional file 1, Table S1, S2 and S3.

Technical textiles

For a candidate component, ZnO (CAS 1314-13-2), positive findings have been reported in some mutagenicity and genotoxicity studies, according to the material's SDS. While these findings alone did not warrant exclusion, they were considered alongside the limited technical benefits observed in the ZnO-based formulations. Specifically, the incorporation of ZnO did not result in a sufficiently meaningful increase in the water contact angle (WCA) of the treated textiles (data not shown). Taken together, the available hazard information and the lack of a clear performance advantage led to the decision not to pursue further development of ZnO-based formulations within the project. Interestingly, the *in vitro* tier 1 testing of the formulations here performed (Table 6) showed genotoxic effect of the ZnO-based formulation, further supporting this decision.

Food packaging machineries (metal)

Diethoxydimethylsilane (DEDMS, CAS 78-62-6) was reported as suspected of damaging fertility (reprotoxic Cat. 2, H361f) in a REACH dossier. As this was based on an industrial self-classification, and not harmonized under CLP, a moderate level of uncertainty was assigned to this information. However, the self-classification was accompanied by detailed information on the performed study. The assessment was performed by the OECD TG 422 and in compliance with good laboratory practice (GLP). Overall, the available evidence supports concern for reproductive toxicity, while the confidence in classification is considered moderate, rather than high, due to its self-classified status and the reliance on a screening-level reproductive/developmental toxicity study. Since DEDMS-based formulations presented good technical performance, in terms of the coating's properties desired (data not shown), these were selected for further development in the project. Additionally, as DEDMS is expected to react with other components of the formulations, the final mixture might not present the same hazard concern. The *in vitro* testing aimed at addressing this aspect, as reported below.

Low-maintenance glass

Trimethylolpropane triacrylate (TMPTA, CAS 15625-89-5) was identified as a suspected carcinogen (Carc. 2) according to both its SDS and the ECHA CHEM database (harmonized classification). In parallel, technical performance testing of TMPTA-based formulations yielded only moderate results. Taken together, the identified hazard profile and the limited technical performance led to the decision not to pursue further development of TMPTA-based formulations.

Molybdenum disulphide (MoS₂, CAS 1317-33-5) was reported as suspected carcinogen (Cat. 2) in a minor number (1.43%) of industry CLP notifications on the ECHA CHEM database. The information on the studies supporting this classification was not available on the ECHA CHEM database. Thus, a higher level of uncertainty was here assigned to this information. MoS₂ was not excluded as a component of the formulations under development based on this evidence. The *in vitro* testing aimed at addressing this aspect, as reported below.

As the concerns associated to PFAS often relate to their persistence and bioaccumulation properties, data available on the ECHA CHEM database for these attributes were collected for the final candidate constituents. Among these, no substances were identified as having reported (very) persistent, (very) bioaccumulative, and toxic (PBT/vPvB) or (very) persistent, (very) mobile, and toxic (PMT/vPvM)

classifications. Another critical endpoint is endocrine disruption (ED). Also, in this case no warnings were found in the information available through SDS and the ECHA CHEM database for the chemicals under assessment.

However, several data gaps were identified in the hazard information collected for various investigated chemicals, including in crucial endpoints such as CMR, ED and PBT/vPvB, thus warranting further investigations. Focusing on CMR, any instance where data were lacking or insufficiently conclusive was treated as a data gap, which was addressed through targeted data gap-filling strategies, discussed in the following sections.

In silico predictions

Existing in silico model application

VEGAHUB was used as a screening tool to predict the missing CMR hazard endpoints for the substances that progressed to TRL 4–5. Several models related to different endpoints were used for each substance, and the prediction results are summarized in Table 4.

Table 4
Summary of VEGA predictions for substances progressed to TRL 4–5 with missing hazard information

Chemical	Cas.no	SMILES	Endpoint	ADI range	DoA	Reliability	Conclusion
Tetraethyl orthosilicate	78-10-4	<chem>CCO[Si](OCC)(OCC)OCC</chem>	Carcinogenicity	0-0.344	Outside	Low	Inconclusive
			Genotoxicity	0-0.454	Outside	Low	Inconclusive
n-hexyltriethoxysilane	18166-37-5	<chem>CCCCC[Si](OCC)(OCC)OCC</chem>	Carcinogenicity	0.17–0.346	Outside	Low	Inconclusive
			Mutagenicity	0-0.621	Outside	Low	Inconclusive
			Developmental/Reproductive toxicity	0	Outside	Low	Inconclusive
			Genotoxicity	0.292–0.401	Outside	Low	Inconclusive
Diethoxydimethylsilane	78-62-6	<chem>CCO[Si](C)(C)OCC</chem>	Carcinogenicity	0–0.301	Outside	Low	Inconclusive
			Genotoxicity	0-0.275	Outside	Low	Inconclusive
Trimethoxymethylsilane	1185-55-3	<chem>CO[Si](C)(OC)OC</chem>	Carcinogenicity	0-0.343	Outside	Low	Inconclusive
			Genotoxicity	0-0.346	Outside	Low	Inconclusive
Cellulose nanofibrils	9004-34-6	no structure available	NA	NA	NA	NA	NA
Alkyl keten dimer dispersion	84989-41-3	not available single chemical; no structure available	NA	NA	NA	NA	NA

DoA – Domain of Applicability; ADI – Applicability Domain Index; NA – not applicable; SMILES - Simplified Molecular Input Line Entry System.

For tetraethyl orthosilicate, n-hexyltriethoxysilane, diethoxydimethylsilane, and trimethoxymethylsilane, the ADI indices for the predicted endpoints were below the acceptable threshold (0.85). Looking further into the predictions for similar substances, these were also contradictory, and substances included in the model were missing crucial structural fragments of the target substance. This indicates that none of the predictions were reliable and therefore the results were inconclusive. For cellulose nanofibrils and alkyl keten dimer

dispersion, SMILES were not available due to the heterogeneity and the nature of these compounds, and it was thus not possible to run the models. The list of specific models used for different endpoints and their corresponding ADI are provided in Additional file 2, Table S4.

In house-built mutagenicity models

Table 5 shows the summary of results predicted by POLIS. All the substances run through the models that resulted having high reliable predictions (e.g., diethoxydimethylsilane, trimethoxymethylsilane, n-hexyltriethoxysilane) were predicted as non-mutagens based on the consensus scheme.

Table 5
Summary of POLIS predictions for substances progressed to TRL 4–5 with missing hazard information

Chemical	Cas.no	SMILES	Reliability	Consensus prediction (three models)
Tetraethyl orthosilicate	78-10-4	<chem>CCO[Si](OCC)(OCC)OCC</chem>	High	Non-mutagen
n-hexyltriethoxysilane	18166-37-5	<chem>CCCCCC[Si](OCC)(OCC)OCC</chem>	High	Non-mutagen
Diethoxydimethylsilane	78-62-6	<chem>CCO[Si](C)(C)OCC</chem>	High	Non-mutagen
Trimethoxymethylsilane	1185-55-3	<chem>CO[Si](C)(OC)OC</chem>	High	Non-mutagen
Cellulose nanofibrils	9004-34-6	no structure available	NA	NA
Alkyl keten dimer dispersion	84989-41-3	not available single chemical; no structure available	NA	NA

NA – not applicable

In vitro testing

Under the battery of tests performed for the tier 1 *in vitro* assessment, the different formulations exhibited diverse toxicological profiles. Table 6 summarizes the results obtained for the candidate PFAS-free alternatives and the PFAS-based benchmarks (NIC 2.5 and 2.6, TEC0.0).

Some formulations showed clear hazard signals. Cytotoxicity was assessed both as an endpoint and as a basis for selecting appropriate non- or sub-cytotoxic concentration ranges for the evaluation of other endpoints. The ZnO-based NIC1.1 and 2.4 not only induced cytotoxicity, but also genotoxicity at non-cytotoxic concentrations. These results supported the observations collected for ZnO during the data-mining phase. A genotoxic response was also observed for the formulation MET. Reproductive toxicity was detected for NIC 2.3 and GLA, although at higher concentrations compared to one of the PFAS-based benchmarks (NIC 2.5). Some textile formulations (NIC 2.1, 2.4, 3.1), and the single compounds MoS₂ and bentonite exhibited different degrees of pro-inflammatory activity, detected by the release of the markers IL-6 and IL-8. However, none of them induced a response as high as the one of the PFAS-based formulation NIC 2.5. All findings were considered together with other relevant factors, including formulation performance and the availability of suitable alternatives. This integrated assessment supported decisions to either continue developing or deprioritize specific candidates. The formulations selected for further development were NIC 4.1 for the textile sector, based on its good technical performance and lack of hazard signals identified. Although GLA and MET presented some hazard alerts relevant to CMR, their optimal performance in imparting the desired functional properties supported the decision to advance them to the next development level and assessment tier.

Table 7 presents an overview of the results obtained from the tier 2 *in vitro* testing, which included the assessment of the carcinogenic potential by CTA (initiation and promotion), genotoxicity by micronucleus formation assay (assessing chromosomal damage), and reproductive toxicity by brood size assay. None of the formulations showed an effect on the micronucleus formation assay and reproductive toxicity by brood size alterations. These observations added mechanistic information to the assessment, without weakening the previous evidence obtained in the *in vitro* tier 1 step (i.e. in the genotoxicity and reproductive toxicity assays). No firm conclusions regarding the carcinogenic potential of the PFAS-free coating formulations could be drawn, as the study primarily highlighted the need for further optimization and validation of assay conditions to ensure sufficient robustness and reproducibility. The

nature of the formulations, showing precipitation and involving challenging preparation conditions, might have contributed to unstable results. The indicative results obtained are anyway reported, accompanied by an uncertainty indication (#).

The advanced ALI lung model was used to assess cytotoxicity, pro-inflammatory responses and cell layer integrity. The highest non-cytotoxic concentration observed in the 2D lung model was selected. No effects were observed, except a mild, non-statistically significant increase of IL-6 release by cells exposed to NIC 4.1, MoS₂ and bentonite. This seems to contradict the results showing ILs release in the tier 1 2D model. It should be noted that, for particulate components, the experimental setting used in the ALI model possibly results in a lower dose being delivered per cell, because of possible differences in the sedimented particles amounts in the two diverse models format [38]. This suggests that, although the tested substances may have hazard potential (as shown in the tier 1 models), such effects may not be elicited at the delivered doses applied in the ALI model. Therefore, the actual deposited dose-response should be investigated further, if the formulations are progressing to higher TRLs.

Overall, the tests performed highlight no concerns for NIC4.1. GLA showed an effect in the reproductive toxicity assay performed at tier 1, and inconclusive results for carcinogenicity that should be further investigated. MET showed genotoxic effects in tier 1, and positive responses in the carcinogenicity assay in tier 2. These results, despite to be considered as indicative, as part of a screening testing, indicate the need for additional investigations to address the hazard concerns here highlighted.

Table 6
Summary of results from tier 1 in vitro testing of PFAS-free formulations and PFAS-based benchmarks.

App	Formulation	Cytotoxicity (EC50)	DNA damage	Oxidative DNA damage	IL-8 (highest observed FI, triggering concentration in %)	IL-6 (highest observed FI, triggering concentration in %)	ED through ER α activation	Reprotoxicity (EC50)
Textiles	NIC 1.1	0.98%	Positive	Negative	No increase	No increase	Negative	Not tested
	NIC 2.1	Negative	Negative	Equivocal	No increase	No increase	Negative	Negative
	NIC 2.2	Negative	Negative	Negative	Slight increase (1.5, 5%)	No increase	Negative	Negative
	NIC 2.3	Negative	Equivocal	Negative	No increase	No increase	Negative	6.08%
	NIC 2.4	2.4%	Positive	Negative	Slight increase (1.7, 1.25%)	No increase	Negative	Not tested
	NIC 3.1	Negative	Negative	Negative	No increase	Slight increase (1.4, 5%)	Negative	Not tested
	NIC 4.1	Negative	Not tested	Not tested	No increase	No increase	Negative	Negative
	NIC 2.5	0.06%	Negative	Negative	Increase (2.5, 0.02%)	Increase (2.5, 0.02%)	Negative	0.08%
	NIC 2.6	10.09%	Negative	Negative	No increase	No increase	Negative	Not tested
Glass and Metal	GLA	5.2%	Not possible to test	Not possible to test	No increase	No increase	Negative	3.05%
	MET	1.4 µg/ml	Positive	Negative	No increase	No increase	Negative	Negative
	MoS ₂	Negative	Not tested	Not tested	Slight increase (1.9, 100 µg/ml)	Slight increase (1.9, 100 µg/ml)	Negative	Negative
	Bentonite	Negative	Not tested	Not tested	No increase	Increase (7.5, 100 µg/ml)	Negative	Negative
	TEC 0.1	Negative	Not tested	Not tested	No increase	No increase	Negative	Negative
	TEC 0.0	3.4%	Not tested	Not tested	No increase	No increase	Negative	Negative
	FAS17	Negative	Not tested	Not tested	No increase	No increase	Negative	Negative

EC50: half maximal effective concentration, the concentration of a substance that produces 50% of its maximum effect; For ILs release, the indication “slight increase” represents a statistically significant change with respect to the negative control, with a fold increase (FI) < 2; the indication “increase” represents a statistically significant change with respect to the negative control, with a FI > 2. The highest observed FI and the concentration at which it occurred are reported. ED: endocrine disruption; ERa: estrogen receptor α .

Table 7
Summary of results from tier 2 in vitro testing of PFAS-free formulations and PFAS-based benchmarks.

App	Formulation	Cell Transformation		Genotoxicity (Micronuclei formation)	Reprotoxicity by brood size	ALI			
		Initiation	Promotion			TEER	IL-8	IL-6	Cytotoxicity
Textile	NIC 4.1	Negative#	Negative#	Negative	No effect	No effect	No effect	No effect	No effect
	NIC 2.5	Not tested	Not tested	Not tested	No effect	Not tested	Not tested	Not tested	Not tested
Glass and Metal	GLA	Inconclusive#	Negative#	Negative	No effect	No effect	No effect	No effect	No effect
	MET	Negative/ Inconclusive#	Positive#	Negative	No effect	No effect	No effect	No effect	No effect
	MoS2	Equivocal#	Negative#	Negative	No effect	No effect	No effect	No effect	No effect
	Bentonite	Negative#	Negative#	Negative	No effect	No effect	No effect	No effect	No effect

No effect indicates no statistically significant changes observed compared to negative control. TEER, IL-8, IL-6 and cytotoxicity are responses from lung ALI culture. # uncertain conclusion due to insufficient robustness and reproducibility of the experiments. TEER: Transepithelial Electrical Resistance; ALI: Air-liquid interface

Discussion: Progress, challenges and lessons learned

This article presents the development and implementation of a practical hazard assessment strategy within the SSbD framework, aligned with the TRL of the innovation. The proposed workflow follows a tiered and iterative approach in which existing evidence is collected and reviewed, data gaps are identified, and additional information is generated using NAMs where needed. The work also critically examines the challenges associated with operationalizing a conceptual SbD framework, particularly for the assessment and selection of safer chemicals and complex formulations for PFAS-free applications. The outcome of this work indicates that the structured, tiered and iterative approach here presented can support the screening and prioritization of candidate formulations in the early stage of their development, guiding the selection of PFAS-free chemicals and materials with favourable hazard profiles for further development.

Safety is a foundational and cross-cutting dimension of SSbD, influencing the broader sustainability assessment throughout the life cycle of materials and products [39]. While exposure assessment and risk characterization are essential components of SSbD, this work focuses on operationalization of hazard assessment of the chemicals and formulations under development. The aim is to identify and phase out substances of concern as early as possible in the innovation process, and to prioritize safer alternatives for further advancement. Importantly, a key limitation of the current JRC SSbD framework is that it does not explicitly consider material functionality or technical performance [40] as a part of the assessment. Although the related data are not here presented, within the PROPLANET project, technical performance was a major parameter considered alongside hazard information when deciding whether candidate solutions should progress to higher development stages.

The methodology applied here builds on the JRC SSbD framework, implementing major components, i.e. collection and evaluation of available information, and data gap filling or data generation using *in silico* and *in vitro* methods. While the JRC methodological guidance provides general recommendations on data sources, tools and the assessment of individual substances, it does not explicitly select specific tools or prescribe how these elements should be integrated into SSbD assessments tailored to specific sectors and complex materials, such as coatings and formulations [14]. This reflects a deliberate choice to preserve the flexibility of the SSbD framework, allowing its application to be tailored to different innovation contexts and use cases. In PROPLANET this flexibility was translated into a scientifically robust, tiered and iterative strategy tailored to PFAS-free formulations and their mixture-based nature. Here, the

methodology is described, together with its practical implementation, the challenges encountered, and the remaining methodological gaps and grey areas.

In JRC's SSbD framework, hazard assessment starts with gathering relevant toxicological and hazard information, indicated as possible data sources SDSs, regulatory classifications, and public databases e.g. ECHA/CLP [10] [11]. In this study, SDSs were screened first as they accompany the specific substances acquired, providing product-specific information. The ECHA CHEM database followed as information source in a second tier to complement the SDSs data, fill the data gaps and reduce the uncertainties. A key consideration in this process is the reliability of the information retrieved. In the JRC methodological guidance and revised framework [10] [11], uncertainty is recognized as an inherent aspect of all components of the SSbD assessment, which should be explicitly addressed and transparently communicated. While several SSbD frameworks exist, no harmonised guidance currently available on how uncertainty should be assessed across the different components of the SSbD safety assessment [41]. Here, for the data-mining step based on existing hazard classifications, we adopted an approach grounded in the regulatory strength of the available information. Uncertainty was defined primarily by the availability and reliability of the classification, as described in the Methods section. Harmonised classifications were treated as high confidence evidence, whereas self-notified classifications were used more cautiously, considering elements such as the availability of the toxicological data and the proportion of reports supporting a given classification. Beyond the summary classifications, information from the REACH dossiers was thus retrieved when available. This allowed to determine whether a missing classification reflected a data gap or an assessment conclusion, and to evaluate the level of confidence in the summary classification. This deeper data collection, combined with an explicit reliability assessment, provided a transparent basis for decision-making on formulations advancements and further data-gap-filling activities. In future SSbD applications, this approach could be strengthened through more structured guidance, including the definition of uncertainty classes to support consistent application, easier visualisation and transparent decision making.

Another relevant aspect was that the assessment did not focus only on the substances identified as PFAS replacements, but also on additives, solvents and other formulation components, as these may influence both the functional performance as well as the safety profile of the final product. In the SSbD literature, safety assessments often address one-to-one substitutions, for example replacing a nanomaterial with a functionalized analogue [42] [43]. Unlike these direct substitutions, PFAS-free alternatives, including those developed in PROPLANET, are generally developed as multi-chemical formulations designed to achieve technical performance comparable to that of PFAS-based solutions [44] [45].

The data collection step highlighted the presence of several data gaps. This was not unexpected, considering that among REACH-registered substances, only approximately 0.5% contain extensive hazard data, while around 10% have substantial datasets, 10% have limited information, and another 10% have poor hazard data coverage [46]. Current chemical regulations still heavily rely on animal testing, which is time- and resource-intensive. Consequently, relying solely on regulatory data may leave certain endpoints under-characterized, particularly for emerging or data poor chemicals. Although not implemented here, existing scientific literature can provide additional evidence to support hazard assessment. Such data often originates from non-standardized assays and may not fully support regulatory alignment. Emerging evidence-integration frameworks offer structured approaches to extract, organize and integrate literature toxicological data, including by linking molecular mechanisms to human health hazards [47] [48]. These approaches could be translated into an SbD decision-making context. Still, their application requires substantial toxicological expertise and may be resource-intensive, depending on the endpoints and sectors under investigation, therefore limiting their applicability in early innovation stages. The increasing transition towards mechanistic and non-animal approaches for chemical safety assessment further reinforces the relevance of such evidence-integration strategies. In 2026, the European Commission adopted the Roadmap towards phasing out animal testing [49], moving towards mechanistic based toxicity assessment for chemical safety assessments. In this context, evidence-integration frameworks can support the systematic collection and interpretation of existing information, the identification of data gaps, and the targeted generation and integration of new evidence using NAMs. These developments are also directly relevant to SSbD, where decisions frequently need to be made early in innovation despite incomplete and heterogeneous datasets.

Another aspect to be highlighted is that the manual extraction and assessment of data demonstrated to be resource-intensive. This may limit the scalability and reproducibility of the evaluations, particularly when information must be retrieved and reconciled across heterogeneous sources. The use of artificial intelligence (AI)-based methods such as natural language processing (NLP) or large language models (LLMs) may represent an advancement, automatizing the identification, extraction, and integration of the data needed for the toxicology and SbD assessment [50] [51]. Such approaches could reduce the workload associated with data collection by enabling the rapid screening of multiple information sources and supporting uncertainty analyses. However, their implementation should be accompanied by transparent validation procedures, quality control, and expert oversight to ensure that automated outputs remain reliable and aligned with SSbD principles. These approaches and challenges are being addressed in several ongoing initiatives, including

the EU-funded project ANALYST [52], which is developing expert-supervised NLP tools to retrieve and integrate information from different databases, as well as projects and activities such as ONTOX [51] and JRC/EURL ECVAM initiatives [53] exploring AI-assisted literature screening, evidence extraction and integration for chemical safety and next-generation risk assessment.

For data gap filling, the JRC SSbD framework recommends the use of NAMs, preferably starting with *in silico* approaches, such as quantitative structure–activity relationship ((Q)SAR) models [11]. At early stages of innovation, these tools can support chemicals screening and the rapid, cost-effective identification of potential hazard “red flags”. Here, openly available models from the VEGA HUB platform were used as an initial screening step. However, the assessed chemicals were largely outside the applicability domain of the models, mainly due to structural differences between chemicals in the training data set and target ones [54]. This illustrates a key limitation of existing toxicity-prediction tools: their chemical-space coverage may be insufficient for particular compound classes [55], including the organosilicon structures investigated here. To address this limitation, custom trained *in silico* mutagenicity models were developed within the project. The findings demonstrate that applying existing models in real-world innovation settings is not always straightforward, and that fit-for-purpose models may be needed for specific chemical classes. This requires appropriate data, expertise and resources that might not always be available. Complementary strategies, such as targeted *in vitro* testing or read-across, are therefore important to support robust hazard assessment within the SSbD framework. In the longer term, there is a need to develop prediction models tailored to specific chemical classes that remain poorly represented in current training datasets, such as organosilicon compounds [56].

In the next assessment tier, screening-level *in vitro* testing was applied to the formulations rather than to their individual chemical components. Because formulation components may react to form transformation products and interact in additive or non-additive ways, mixture-level testing can capture changes in toxicity that may not be identified by assessing parent chemicals alone. As overall the formulations may exhibit toxicity comparable to or greater than that of their PFAS-based benchmarks [57], these were tested in comparison, when available and possible.

To support early-phase hazard identification, screening-level *in vitro* assays addressing cytotoxicity, inflammatory responses, and oxidative DNA damage were applied. Together, these endpoints capture general toxicity, early mechanistic events frequently associated with tissue injury and chronic disease pathways [58], and genotoxic potential as key event in further mutagenic processes [59], offering mechanistic insight beyond apical toxicity endpoints. In the final assessment tier, higher-complexity models and assays were applied, providing information on chromosomal damage, malignant transformation, reproductive toxicity, and effects within a more physiologically relevant respiratory model [60], thereby extending the biological relevance and predictive value of the assessment. Together, the tiered testing strategy supported early hazard identification and informed the prioritisation, reformulation, and further development of candidate formulations within the SSbD framework.

While providing valuable insight into the hazard profiles of the formulations under development, the case study highlighted some practical limitations of *in vitro* based hazard assessment. In the first place, testing activities struggled to keep pace with the large number of candidate chemicals and repeated formulation iterations generated during formulations development. Therefore, strengthening early-stage hazard screening by identifying the transformation products in the formulation and searching related toxicity information could provide a more agile and resource-efficient way to flag concerns, reserving *in vitro* testing for the most relevant substances and final formulations. Additionally, in this work, the poor solubility of the hydrophobic compounds and formulations has constrained the performance and interpretation of *in vitro* assays. This may lead to ambiguous or misleading results, if not cautiously considered. Such challenges were presented especially by compounds and formulations which hydrolysed and precipitated upon contact with the cell culture media, including MET, GLA and TEC0.0 and TEC0.1. Poor solubility and chemical instability can alter the effective exposure of cells to a test substance. Precipitation can reduce the freely dissolved concentration and interfere with assay readouts, while processes such as hydrolysis may decrease parent-compound concentrations and generate transformation products, potentially masking or altering the observed biological response [61] [62]. Further, particles agglomeration or binding to assay components and/or intrinsic optical or reactive properties of materials may interfere with spectrophotometric, fluorometric or biochemical endpoints, resulting in over- or under-estimation of biological effects [63]. In this study, the poorly soluble PFAS-based benchmark TEC0.0 and the corresponding PFAS compound FAS17 did not elicit a response in the assays performed, except for a moderate cytotoxic effect of TEC0.0. Removing the precipitates and testing the soluble fractions did not show differences in the responses, comparing the results with whole formulations. It cannot be excluded that the lack of responses may be due to limited or absent interaction between the test substance and the biological model. While this possibility was not further investigated, as these formulations were not subject for further development, these challenges underline a fundamental limitation in *in vitro* approaches in addressing complex insoluble mixtures, and formulation-specific interference [63]. Long-term solutions will require the development of strategies capable of evaluating formulations that are reactive or poorly soluble under standard *in vitro* testing conditions or addressing all the necessary interference controls. This

approach might not be compatible with the high-throughput screening need of the early SSbD implementation. The characterization of the specific compounds present in the formulations, hydrolysis products, and precipitated fractions in physiologically relevant media would be useful to support hazard interpretation.

A final important insight from this work was the value of a close collaboration across diverse expertise, i.e. toxicologists, chemists, and formulation developers. Constant communication was critical for the effective design and optimization of safer alternatives, supported by integrating safety considerations early and continuously in the formulation's development process. Besides, the simultaneous evaluation of functional performance and toxicological profiles facilitated informed decisions about which solutions to advance, while clearly identifying trade-offs between efficacy and safety considerations. For example, although some formulations presented some hazards concerns, they were advanced in the development process due to their excellent technical performance. At the same time, further safety assessment was carried out to allow a more solid evaluation of potential hazards and flagging them for more advanced testing, when moving to higher TRLs (5–9). Such integrated workflows not only accelerate the identification of viable safer alternatives but also reduce the risk of unintended toxicological outcomes, supporting a more safe, sustainable and responsible approach to chemical innovation.

Conclusion

In conclusion, the integrated, tiered approach combining data mining and NAMs supported the safety-guided selection of candidate chemicals and formulations. The diverse components of the assessment provided complementary evidence that was particularly valuable during the early stages of development. The study also highlighted practical and methodological challenges related to data availability and reliability, the applicability domains of predictive models, testing of complex and poorly soluble formulations, and the resources required to integrate heterogeneous evidence. Addressing these limitations will be important to further strengthen the operational implementation of hazard assessment within SSbD.

The present assessment focused on candidate chemicals and formulations during development. For the future advancement of the PFAS-free formulations developed, the identified hazard signals could be interpreted in realistic exposure scenarios, which are expected to involve concentrations lower than those producing effects in *in vitro* assays. *In silico* exposure and fate tools developed within PROPLANET, such as *SimpleBox4Planet* multimedia environmental fate model [64] and *LungDepo* for regional particle deposition in the human respiratory tract [65] could complement the hazard assessment presented here, enabling the transition from hazard identification toward risk-based decision-making within the SSbD framework. Finally, the final cured coatings may differ in composition and hazard profile from the original formulations. Future work could therefore also assess the hazard properties of the cured coatings and the potential release of leachates, abraded particles, and other chemicals from coated products during use and end-of-life stages.

Declarations

Ethics approval and consent to participate

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Consent for publication

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CRediT authorship contribution statement

SSbD and hazard assessment: SM: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Visualization, Supervision, Writing—original draft, review & editing. EML: Conceptualization, Methodology, Validation, Formal analysis, Funding Acquisition, Project administration, Supervision, Writing—original draft, review & editing. TS: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Supervision, Writing –review & editing. ERP, AHO: Conceptualization, Methodology, Supervision, Writing –review & editing. MD: Conceptualization, Methodology, Funding Acquisition, Supervision. XM, EMF, TH, AMH: Investigation, Validation, Data curation. SB: Investigation, Validation, Writing –review & editing.

In silico custom-trained models: DDV, DGM, AA: Methodology, Software, Validation. AA: Funding Acquisition, Project administration.

PFAS-free formulations development: AV, ASV: Investigation, Methodology, Writing – review&editing, Project administration. BS: Investigation, Methodology, Writing – review&editing, Project administration, Supervision. FB: Investigation, Methodology, Writing – review&editing, Project administration, Funding Acquisitions, Supervision. UN: Funding Acquisition, Project administration.

Coordination: AJR, JPS: Conceptualization, Project administration, Writing – review&editing.

All authors read and approved the final manuscript.

Appendix A. Supporting information

Author Contribution

SSbD and hazard assessment: SM: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Visualization, Supervision, Writing—original draft, review & editing. EML: Conceptualization, Methodology, Validation, Formal analysis, Funding Acquisition, Project administration, Supervision, Writing—original draft, review & editing. TS: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Supervision, Writing –review & editing. ERP, AHO: Conceptualization, Methodology, Supervision, Writing –review & editing. MD: Conceptualization, Methodology, Funding Acquisition, Supervision. XM, EMF, TH, AMH: Investigation, Validation, Data curation. SB: Investigation, Validation, Writing –review & editing. In silico custom-trained models: DDV, DGM, AA: Methodology, Software, Validation. AA: Funding Acquisition, Project administration. PFAS-free formulations development: AV, ASV: Investigation, Methodology, Writing – review&editing, Project administration. BS: Investigation, Methodology, Writing – review&editing, Project administration, Supervision. FB: Investigation, Methodology, Writing – review&editing, Project administration, Funding Acquisitions, Supervision. UN: Funding Acquisition, Project administration. Coordination: AJR, JPS: Conceptualization, Project administration, Writing – review&editing. All authors read and approved the final manuscript.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

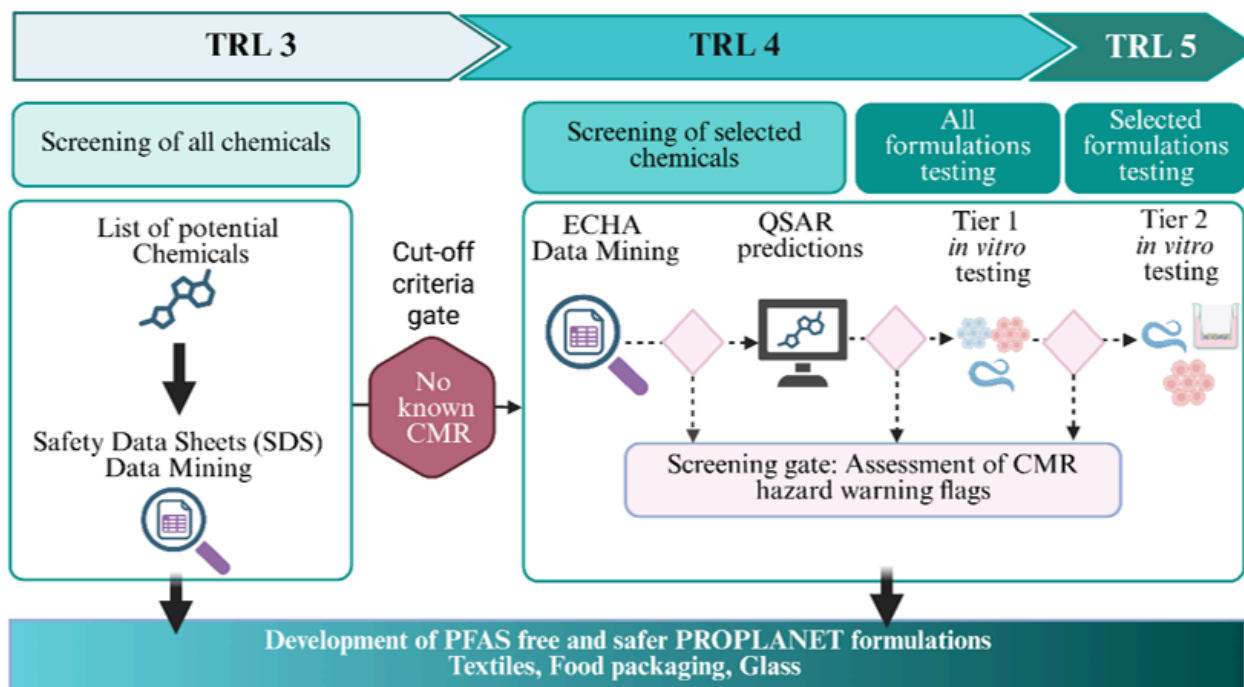


Figure 1

Conceptual safe-by-design framework proposed in PROPLANET project to develop PFAS-free formulations.

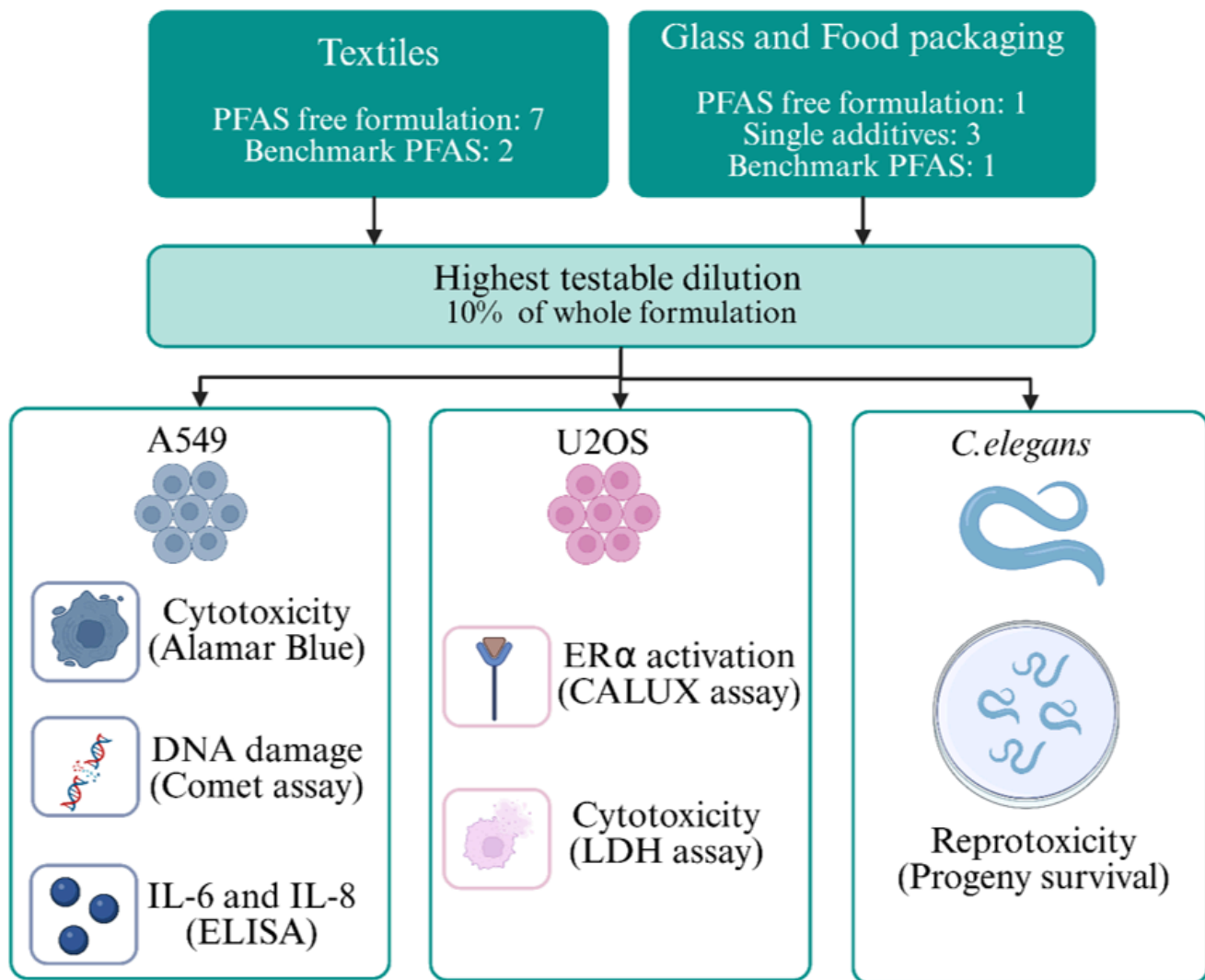


Figure 2

Overview of tier 1 in vitro testing performed using high-throughput, screening-level models and assays. A549: human lung cell line; U2-OS: human osteosarcoma cell line; C. elegans: Caenorhabditis elegans; ELISA: Enzyme-Linked Immunosorbent Assay; ER α : estrogen receptor alpha; CALUX: Chemical Activated Luciferase gene eXpression; LDH: lactate dehydrogenase.

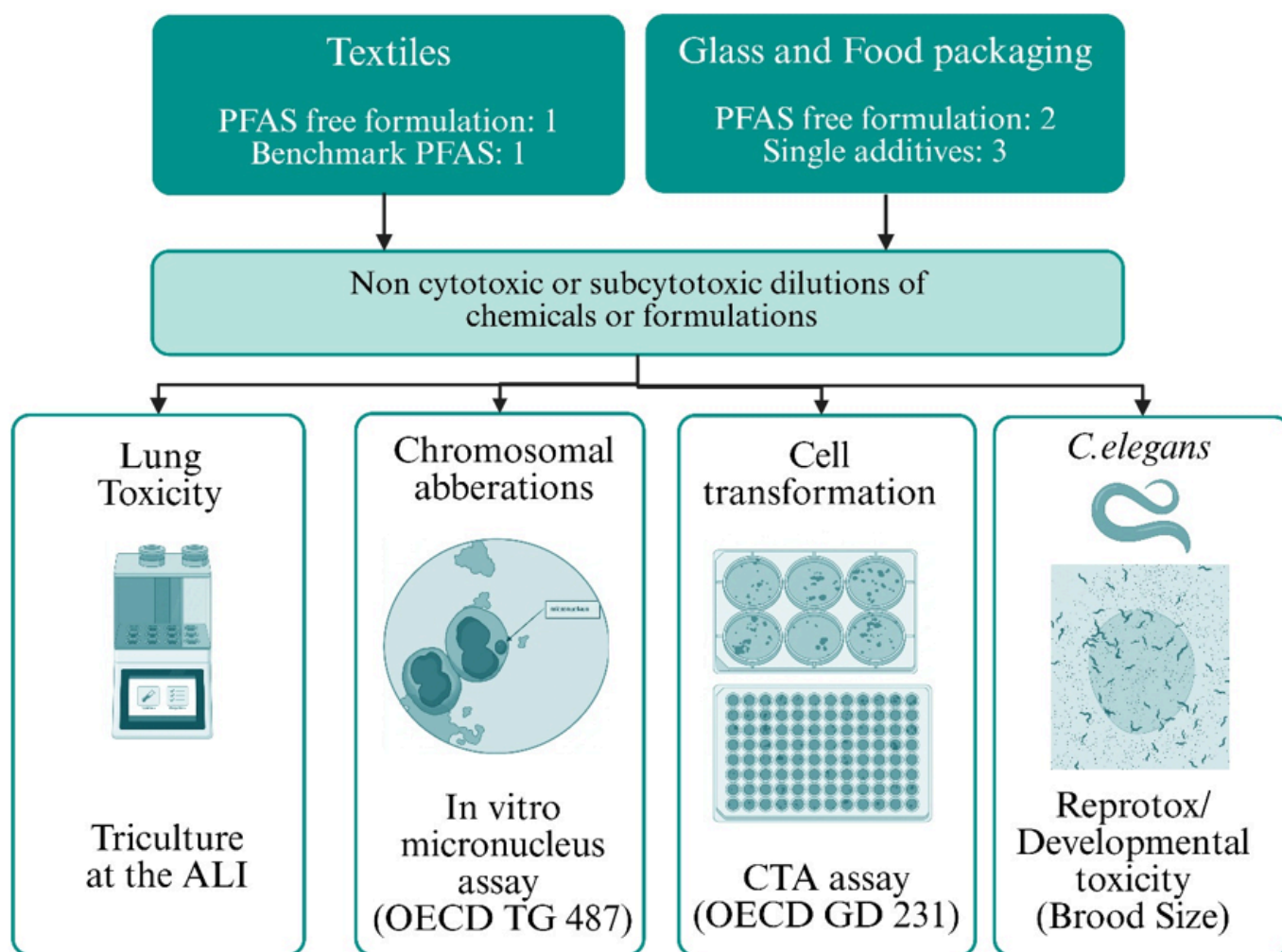


Figure 3

Overview of tier 2 in vitro testing performed using advanced models and standardized assays. ALI: air-liquid interface; CTA: cell transformation assay.

Supplementary Files

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