MACRAMÉ

Deliverable Report D5.1

MACRAMÉ Harmonisation & Standardisation Roadmap Summary Report for MACRAMÉ Methods and Models

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[1] R= Document, report (excluding the periodic and final reports); DEM = Demonstrator, pilot, prototype, plan designs; DEC = Websites, patents filing, press & media actions, videos, etc.; DATA =Data sets, microdata, etc.; DMP = Data management plan; ETHICS = Deliverables related to ethics issues; SECURITY = Deliverables related to security issues; OTHER = Software, technical diagram, algorithms, models, etc..

[2] PU = Public, fully open, e.g. web (Deliverables flagged as public will be automatically published in CORDIS project's page); SEN = Sensitive, limited under the conditions of the Grant Agreement; Classified R-UE/EU-R = EU RESTRICTED under the Commission Decision No2015/444; Classified C-UE/EU-C = EU CONFIDENTIAL under the Commission Decision No2015/444; Classified S-UE/EU-S = EU SECRET under the Commission Decision No2015/444.

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Acronyms Listed in this Document	
AdMas	Advanced Materials, a materials category that includes but surpasses that of 'nanomaterials' (EU, 'Definition of a Nanomaterial')
AMA	Alveolar Macrophage Assay
AOP	Adverse Outcome Pathway
CNFs	Carbon Nanofibers
CNTs	Carbon NanoTubes
CRO	Contract Research Organisation
CSRD	Corporate Sustainability Reporting Directive
DO	Dissolved Oxygen
EC	European Commission
EU	European Union
EUON	European Union Observatory for Nanomaterials
FLG	Few Layer Graphene
GD	OECD Guidance Document
GRM	Graphene-Related Material
GR2M	Graphene and Related two-Dimensional (2D) Materials
HIM-SIMS	Helium Ion Microscopy (HIM) and Secondary Ion Mass Spectrometry (SIMS)
LCA	Life Cycle Assessment
MCL	Material Control Library
NAMs	Non-Animal Methods or New Approach Methodologies
NWIP	New Work Item Proposal
OECD	Organisation for Economic Cooperation and Development
PLGA	Poly Lactic-co-Glycolic Acid (nano)particles
SB4N	SimpleBox4Nano
SME	Small or Medium Enterprise
TG	OECD Test Guideline
WP	Work Package

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Executive Summary

This report describes the strategic MACRAMÉ roadmaps for the harmonisation and/or standardisation of some of the methods developed in WP2 (Development and Advancement of Characterisation- & Test-Methods &-Protocols) and WP4 (Identification, Demonstration & Validation of MACRAMÉ Methods in Use-Cases).

Based on the identified needs of the community, the following five technical 'destinations' have been identified, including:

i) characterisation and sample preparation of carbon-based materials,
ii) aerosol generation for inhalation studies,
iii) *in vitro* / *ex vivo* models for inhalation toxicology,
iv) ecotoxicity studies, and
v) sustainability assessment.

For each of these technical categories, a specific roadmap has been developed, evaluating the potential use of the associated methods under development in the MACRAMÉ Project in the regulatory context. The level of maturity of each method selected has been evaluated by WP5 (Translation to Standards & Policy) and their potential suitability for harmonisation and/or standardisation in the available committees has been considered (e.g. ISO TC/229, OECD, CEN/TC352, VAMAS, ASTM E56) to plan the potential steps towards a successful and timely harmonisation or standardisation.

For each of the five roadmaps, a summary of the discussion and resulting conclusions is presented in this report. These MACRAMÉ roadmap summaries are structured in a first conceptual part (i.e. description of the method for risk assessment and/or testing for regulatory purposes), and subsequently in a second section describing the envisaged strategy (i.e. required steps towards harmonisation and/or standardisation).

1 Introduction to the MACRAMÉ Roadmaps

The preparation of the strategic MACRAMÉ roadmaps for the harmonisation and/or standardisation is the first step for the advancement of methods and standard operating procedures developed by MACRAMÉ into harmonisation and standardisation documents. In this effort, each of the methods or models worked on in WP2 and/or WP4 was considered separately for their use in risk assessment and in a regulatory context. The relevant committees for harmonisation or standardisation have been identified and - where possible – *the 'ISO/TC 229 – IEC/TC113 / JWG2 Metrological Check-List'* for use in the preparation and evaluation of ISO NWIPs (New Work Item Proposal) has been applied, in order to support the metrological maturity assessment of the proposed analytical work.

The resulting prescribed steps towards a successful and timely standardisation or harmonisation are tailored to the specific committee and the relevant applicable method, resulting in the following five roadmaps that address different 'destination' category and that consist of: (a) a conceptual part (i.e. implementation of the method for risk assessment and/or testing for regulatory purposes), and (b) a strategy (i.e. required steps towards harmonisation/standardisation).

1) Characterisation and dispersion protocols for graphene-based materials: This roadmap describes the current status and future priorities for the standardisation of methods pertaining to the characterisation of Graphene and Related two-Dimensional (2D) Materials (GR2M), as a sub-set of the Advanced Materials (AdMas) considered in the MACRAMÉ R&I strategy. By way of an outlook, it describes how the MACRAME R&I strategy aims to harmonise and standardise specific dispersion protocols for GR2M materials to be implemented for *in vitro* toxicological and ecotoxicological testing within the ongoing efforts at the OECD.
2) Aerosol generation for inhalation studies: This roadmap provides advice for aerosol generation protocols in the framework of *in vitro* inhalation exposure studies, i.e. for dose delivery on cells hosted at the air-liquid interface or at surfaces for later usage for cell exposure. The advice is envisioned to become part of an OECD Guidance Document on Aerosol generation for toxicity testing for *in vivo* and *in vitro* studies.
3) MACRAMÉ Roadmap on *in vitro / ex vivo* Models for Inhalation Toxicology: This destination highlights the main steps to harmonise and standardise a step-by-step assay cascade of *in vitro / ex vivo* models for inhalation toxicology, focusing on the strategy to address the major encountered issues of the identified models.
4) MACRAMÉ Roadmap on Ecotoxicity Assessment of Advanced Materials: This destination describes, how the MACRAMÉ R&I strategy aims to widen the development and application of harmonised OECD Test Guidelines (TGs) for ecotoxicological assessment of AdMas, using a series of 5 case studies.
5) MACRAMÉ Roadmap on the Sustainability Analysis for AdMa: This roadmap highlights the major issues encountered, while doing sustainability analysis (focusing on life cycle assessment (LCA)) for AdMa and suggests potential ways to solve these issues and the timeline needed to achieve solutions.

Based on the elaborations described in the roadmaps, different topics for standardisation or harmonisation will be proposed to the relevant bodies identified, e.g. the OECD, as well as VAMAS and EURL ECVAM to promote the organisation of ILC studies for the validation of the MACRAMÉ methods and models.

2 MACRAMÉ Roadmap on the Characterisation & Dispersion Protocols of graphene-based Materials

2.1 Introduction

This section describes the identified needs for the characterisation of graphene-based materials as recently reported by the European Union Observatory for Nanomaterials (EUON) [1]. Furthermore, as part of the roadmap a survey was performed during the 1st MACRAMÉ Harmonisation and Standardisation Workshop (https://macrame-project.eu/macrame-meetings-workshops/#HS-Workshop1); this survey focused on the priorities for standardisation of methods for the characterisation of Graphene and Related Two-dimensional (2D) Materials (GR2M), together with the need for exposure protocols for ecotoxicological and toxicological testing. The survey results are reported in the first part of the chapter.

Among the protocols developed by MACRAMÉ that respond to the identified priorities for material characterisation and sample preparation, specific protocols to generate controlled sample dispersions of GR2M have been identified in complementary activities of the MACRAMÉ Project (i.e. in WPs 2 and 5). These may be regarded as suitable candidates to be included in the revision of existing OECD Guidance Documents. In addition, such protocols may be proposed as potential candidates for new (specific) guidance documents as part of the planned *'Final Recommendations on future Needs for TGs & Standards Developments'* (i.e. MACRAMÉ deliverable report D5.4).

2.2 Problem Framing concerning the Characterisation & Dispersion Protocols of graphene-based Materials

Numerous initiatives to standardise characterisation methods for isolated GR2M (i.e. not embedded in complex matrices) have been taken in recent years at international level (e.g. ISO/TC 229 Nanotechnologies and IEC/TC 113 Nanotechnology for electrotechnical products and systems). These aim to provide tools that will enable GR2M producers to demonstrate the quality of their products. This will eliminate the poor-quality materials that are coming onto the market, whose sub-standard performance is detrimental to graphene's credibility in a wide range of applications, where it could be of great interest and added value. Such methods are furthermore extremely useful for gaining a better understanding of the physico-chemical properties of GR2Ms upstream of risk studies, thereby lending credibility to these studies in line with the philosophy of the ISO Technical Report *Guidance on physico-chemical characterisation of engineered nanoscale materials for toxicologic assessment* (ISO/TR 13014:2012) [2].

The rapid advancement in the development of characterisation methods are due – in particular – to the efforts of the EU Graphene Flagship Standardisation Committee [3] and the European ISO-G-SCoPe project [4].

The Graphene Council, for its part, has developed a strategic tool, the Graphene Classification Framework [5], to list:

- Key graphene material characteristics helping to define the different types of graphene considered within the GR2M family
- Preferred methods of testing
- Range of measurement values
- Syntax to describe forms of graphene
- Technical Data Sheet template

This strategic document is currently being developed into an ISO standard by ISO/TC 229/WG 4 "Material specifications".

ISO/TR 19733:2019 (Matrix of properties and measurement techniques for graphene and related two-dimensional (2D) materials) gives recommendations on the analytical techniques to consider for each key physico-chemical property of GR2M, while a portfolio of standards for the characterisation of materials in the GR2M family is now available (see Table 1). Nevertheless, action is still required for certain properties or to adapt some of the methods to the specific characteristics of certain types of graphene (e.g. Graphene Quantum Dots).

Table 1: Available standards for the characterisation of GR2Ms key physical-chemical properties. (Grey cells represent cases where standard test methods do not exist or are not available, for the associated material or for the associated attribute.)

Type of GR2M	**Graphene Oxide**	**Few Layer Graphene**	**Multi-layer Graphene**	**Reduced Graphene Oxide**	**Graphene Quantum Dots**
Bulk density	IEC TS 62607-6-35 ED1				
Elemental Composition	ISO/CD TS 23359				
Oxygen content	ISO/CD TS 23359				
	IEC TS 62607-6-36 ED1			IEC TS 62607-6-34 ED1	
Impurities	IEC TS 62607-6-30 ED1				
Functionalisation	ISO TS 23359 ED1				
Surface particle charge					
Specific Surface Area	IEC TS 62607-6-31 ED1				
Structural Defect		ISO/TS 21356-1:2021 ISO/AWI TS 21356-2 IEC TS 62607-6-29 ED1			
	IEC TS 62607-6-33 ED1				
Crystallinity					
Number of layers		IEC TS 62607-6-12 ED1 IEC TS 62607-6-28 ED1			
Particle shape		ISO/TS 21356-1:2021 ISO/AWI TS 21356-2			
Lateral Particle dimensions	ISO/AWI TS 23879				
Particle Aspect Ratio					

A systematically structured scientific literature review covering publications, books, research reports, research and review papers, was recently performed by the EUON. The review focused on the potential adverse effects of graphene, graphene oxide and other 2D materials on human health and on the environment [1].

As part of the conclusions of the report, a strong need emerges for implementing a thorough characterisation of the GR2M to be used in toxicological and ecotoxicological assays. The main parameters to be measured and controlled in relevant representative media for toxicological and ecotoxicological studies are:

- the chemical composition;
- the structure;
- the (lateral) size; and
- the number of layers.

Future studies may be improved by considering the successful implementation of definitions and available documentary standards.

Toxic effects identified for specific GR2M on health and environment are mainly dose dependent. For this reason, the doses used for animal and cell exposure during toxicological and ecotoxicological studies should be reliably measured and controlled. In this context sample preparation methods need to be harmonised and standardised for controlling the stability of the tested materials, their aggregation state and thus their dosimetry.

As an example, in the case of ecotoxicological studies, protocols included in the OECD Test Guidelines for acute toxicity (i.e. TG 201, TG 202 and TG 203), are not directly applicable for testing GR2Ms. GR2Ms require additional considerations and/or adaptations of the protocols. In this context, Connolly *et al.* have recently assessed the applicability of TG201 to GR2Ms by studying their dispersion stability in the TG 201 medium [6]. Their results show that graphene dispersions are not stable under the standard TG 201 conditions and therefore do not meet the TG requirement of a stable and homogeneous dispersion throughout the test. Currently the OECD TGP Project 2.71 is ongoing to revise GD317 to adapt it for these tests.

2.3 Current Gaps in the Characterisation & Dispersion Protocols of graphene-based Materials

An online polling survey targeting expert stakeholders was organised during the first MACRAMÉ Harmonisation and Standardisation Workshop on 23rd of November 2023. The survey aimed at:

- identifying the remaining priorities for standardisation of methods measuring the pristine properties of GR2Ms
- discussing the minimum set of physicochemical parameters to be measured in the media used for biological or ecotoxicity assays
- discussing priorities for the toxicity and ecotoxicity assessment, including the harmonisation of exposure and dispersion protocols.

The answers reported in this section represent the responses of 42 experts belonging to academia (38%), governmental institutions (17%), industry (including SMEs and specialised CROs) (28%), metrology institutes (10%) and regulatory agencies (7%) (Figure 1).

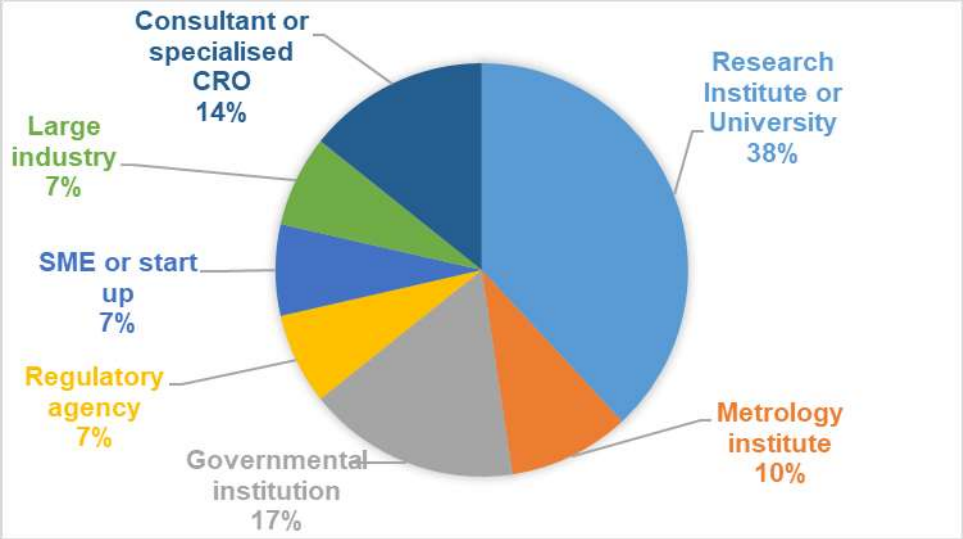

Figure 1: Statistical representation of the institutions represented by the 42 participants of the MACRAMÉ survey, held during the MACRAMÉ Harmonisation & Standardisation Workshop, 23rd November 2023.

2.3.1 Priorities for Standardisation of Methods measuring the Properties of Graphene and GR2M

More than 86% of the participants thought that the standard test methods recently developed for the characterisation of GR2M were not sufficient. However, this step is the essential starting point before considering standardising methods in more complex environments (e.g. those used for performing toxicity or ecotoxicity tests). New standard test methods need to be developed, focusing on measurements of chemical properties of GR2M such as surface functionalisation, impurities and elemental compositions, and of physical properties, including the particle shape and the number of layers of GR2Ms. These outcomes are reflected in Figure 2. In terms of the types of GR2M materials, respondents give priority to graphene oxide and few layer graphene (Figure 3).

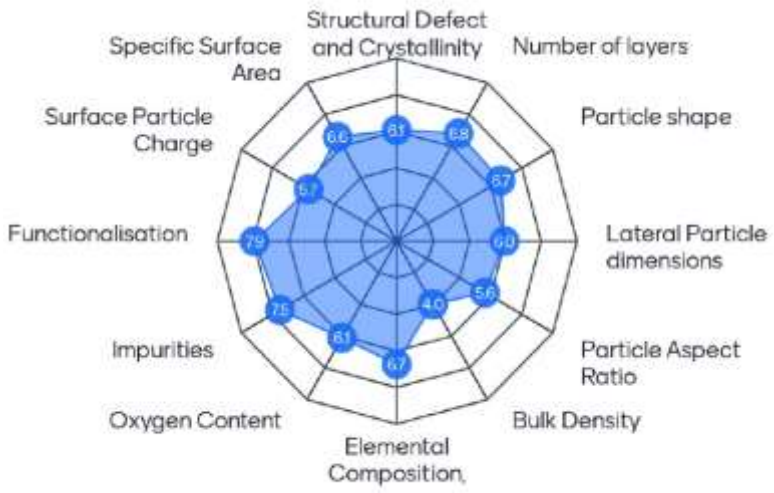

Figure 2: Spider diagram representation of the workshop participants' opinions regarding the pristine attributes of GR2M to be prioritised for the development of standard test methods. The average score 1-10 of each attribute is reported based on the average of 42 answers. (source of the GR2M attributes: Graphene Classification Framework)

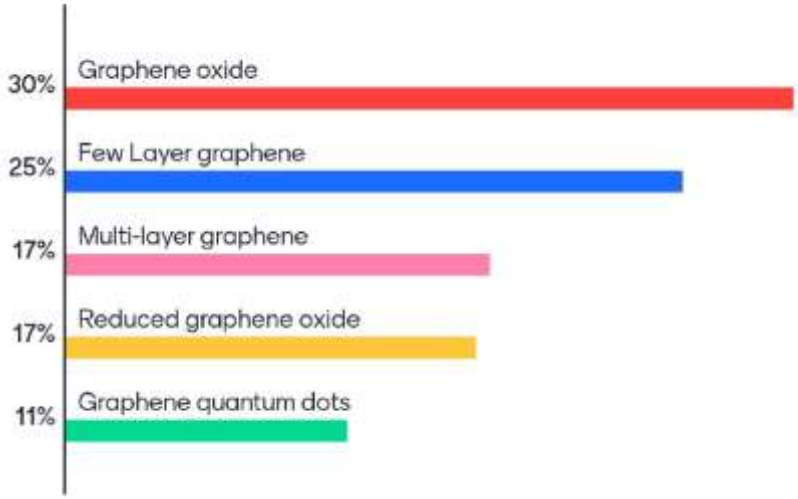

Figure 3: Bar chart representation of the workshop participants' opinions regarding the type of GR2M to be considered as priorities in future characterisation methods standardisation studies and for the development of reference materials. Participants have been requested to evaluate the priorities in terms of development of reference materials, but ranking the proposed materials. The average of 42 answers is reported.

2.3.2 Priorities for the Development of robust Toxicity and Ecotoxicity Assessments

In 2012, the ISO TR/13014 [2] was published with the aim to define and standardise a minimum set of physico-chemical parameters to be measured to support reliable toxicological assessment of engineered nanoscale materials.

74% of the participants responding to the Mentimeter survey stated that a similar guidance document shall be proposed, focusing on the minimum set of physico-chemical parameters to be measured prior to any toxicological assessment and/or ecotoxicological assessment of GR2Ms. In addition to this need, the participants also expressed the needs for developing fit-

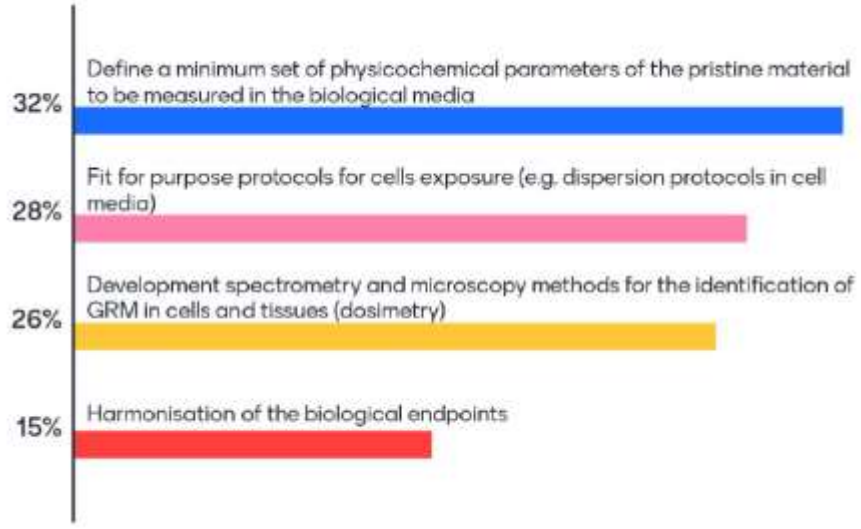

Figure 4: Bar chart representation of the workshop participants' opinions regarding the identified priorities for robust toxicological and ecotoxicological assessment. Participants have been requested to rank the proposed options between 1 and 5. The average of 42 answers is reported.

for-purpose protocols for cell exposure, and for developing spectroscopy and microscopy methods for the identification of GR2M in cells and tissues (Figure 4).

2.4 Implementation of the Roadmap on the Characterisation & Dispersion Protocols of graphene-based Materials

During the preparation of the MACRAMÉ proposal, extensive discussions on the roadmap have been held. Together with the results obtained during the first year of the Project and the outputs of the online polling survey, they formed the basis for an assessment of the need for future standardisation actions (Figure 5).

In the MACRAMÉ material library (D2.1) specific protocols to generate controlled sample dispersions of GR2Ms have been also collected. These have been identified as suitable candidates for harmonisation and standardisation.

By further developing these protocols within WP2 and WP4, MACRAMÉ will contribute to the needs identified.

The protocol(s) under development will contain a suggested workflow for the preparation of dispersions of GR2M in water-based media used for *in vitro* toxicological and ecotoxicological assays. To optimise the protocols, we will focus on the prioritised types of GR2Ms (Figure 3), i.e. few layer graphene (FLG), multi-layer graphene and graphene oxide.

The optimisation will focus in particular on:

1. a protocol to optimise dispersion. This will include indications of the most suitable surfactant or stabiliser to increase the stability of the suspension, and the optimised sonication energy. Stabilisers could be e.g. 0.05-0.1% of Bovine Serum Albumin for *in vitro* toxicological studies and dissolved organic matter (DOM) for ecotoxicological studies.
2. a workflow of measurement techniques (e.g. Dynamic light scattering-DLS, Analytical Ultracentrifugation-AUC, cryo-TEM) combined with preliminary cellular experiments to test the stability and the responsiveness of the dispersion.

The experimental work is still ongoing. Validation of the developed dispersion protocol is envisaged for M18. The experimental details will be reported in the developed SOPs and in D2.2. After validation, planned for M18, the data will be shared and the developed protocols discussed with a few standardisation committees. WP2 and WP5 have identified OECD and ISO/TC 229 as the possible targeted organisations (Figure 5).

In the harmonisation and standardisation approaches, two steps are proposed:

- **Step 1:** contribution with the MACRAMÉ results to an existing project, e.g. including some considerations in the revision of the ENV/JM/MONO(2012)40 (OECD Guidance on Sample Preparation and Dosimetry (GSPD))
- **Step 2:** propose the initiation of a New Working Item in ISO/TC 229 or a project in the OECD Test Guideline Programme with a focus on GR2M and aiming for a dedicated guidance document or test guideline within OECD or a dedicated standard test method within ISO.

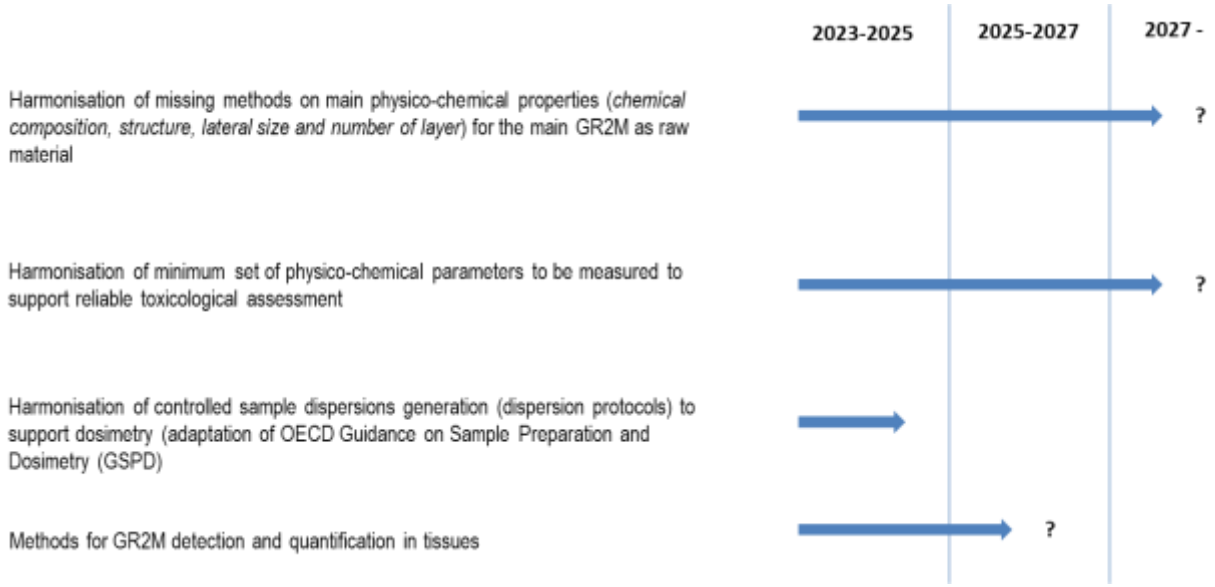

Figure 5: Tentative overview of gaps identified in MACRAMÉ and tentative timeline to move them towards standardisation during and beyond the MACRAMÉ Project.

3 MACRAMÉ Roadmap on Aerosol Generation for Inhalation Studies

3.1 Introduction - Aerosol Generation for Inhalation Studies

This section describes the roadmap towards guidance for aerosol generation in the framework of *in vitro* inhalation exposure studies. The strategy for the guidance is made of a modular approach. Each module comes with a set of standard operating procedures (SOPs) that standardise the specific steps. For most of the SOPs at least the draft version was already existing by the time this document D5.1 was written. Additional SOPs are subject for development within the MACRAMÉ Project.

Modules are sample preparation for aerosol generation, sample characterisation, the aerosol generation, online aerosol monitoring and characterisation (instrument based), aerosol manipulation, application of the aerosols to the cells/surface and – lastly - characterisation of the deposited dose.

3.2 Need for the Method(s) pertaining to the Aerosol Generation for Inhalation Studies

Inhalation studies according to OECD TGs (e.g. OECD TG 412) are lacking specifics dedicated to dose generation, addressing reproducibility of aerosol generation in terms of not only quantity, e.g. particle concentration, but also quality, such as morphological composition.

Current test guidelines generally refer to mass median aerodynamic diameter (MMAD), to check for reparability of the particles, but for high aspect ratio materials like graphene and fibres such diameters are very difficult to interpret. For such high-aspect ratio (HAR) particles, the aerodynamic diameter depends on the alignment of their long axis with the direction of motion. For example, graphene is known to exhibit respirable aerodynamic diameters with lateral diameters of up to 30 µm in the lung context. In aerosol instruments that measure aerodynamic diameter, the measured diameter distribution can be different.

In addition, mass per aerodynamic diameter is very difficult to measure for very light particles such as graphene. Currently, there are no instruments or (cascade) impactors that can provide mass in a measurable range. Further guidance/methodology are therefore needed; to prioritise these, a number of questions have been formulated and answered:

- *How will the work contribute to further international harmonisation of hazard and risk assessment?*
  Reproducibility and comparability of inhalation-toxicological tests is only possible with good control and reproducibility of the dose. Obviously, the latter aspect improves international acceptance of any risk assessment making use of results obtained by the test in question.
  Further test standardisation by giving guidance on aerosol generation improves harmonisation of risk assessment.
- *How will the proposed project address issues and /or endpoints which are of major human health or environmental concerns?*
  Testing of inhalation toxicity requires generation of aerosols. Testing toxicity via the inhalation route is for example the standard route for nanoforms in REACH [7], considered as the most relevant route of exposure. For fibrous materials the inhalation route is also considered as the most relevant exposure route. Aerosols that are not well controlled will lead to test results that are not realistic, relevant and reproducible. In the case of fibrous materials, it is especially important to determine the fraction of

different particle classes in the aerosol, e.g., determining the amount of possible critical fibres. Dose control not only in terms of quantity (e.g., administered mass) but also in quality (e.g. morphological composition) enables a better understanding of possible observed toxicological effects and a clear correlation with a hypothetical harmful aerosol fraction.
Furthermore, advancements in dose control are key to be able to observe similar prevalence of specific toxicological effects (*in vitro* / *in silico*) and observed adverse effects (*in vivo*). However, such correlation is only valid in case administered doses in both tests are considered equivalent quantitatively and qualitatively, preferably produced with the same aerosol generation procedure.
Enhancement of the predictive value of *in vitro* studies is a major step towards reduction, refinement and maybe replacement of animal studies. High control and knowledge of the deposited dose is easier accomplished in *in vitro* studies.

- *Are there existing Test Guidelines/ Technical Specifications or other standards or projects covering the same endpoint?*
  The 'Guidance on Sample Preparation and Dosimetry' lists different techniques for generation of an exposure atmosphere. Possible techniques are for example the venturi [8] and a jet-mill [9] for dry materials or vibrating mesh nebuliser or "chemical printing" for liquid dispersions. These techniques are, however, to a large extent not standardised.
  Challenges differ for testing *in vitro* and *in vivo*. For *in vivo* tests stable exposure conditions are needed to be maintained and monitored over a long period. For *in vitro* tests deposition on cells and (quantitative and qualitative) determination of the dose are challenging. The 'Guidance on Sample Preparation and Dosimetry' is currently under revision. The level of details required to effectively support aerosol generation however also appears to require a separate Guidance Document.
  Standardised aerosol generation used within a testing strategy dedicated for risk assessment can be effective both in the early development stage of new advanced materials (SSbD) and in fulfilling regulatory requirements (e.g. in REACH).

3.3 Description of Method(s) pertaining to Aerosol Generation for Inhalation Studies

3.3.1 Scope of the Method

The aim of the procedure is the production of reproducible aerosol exposure with high control over the dose quantity in terms of aerosol concentration as well as the dose quality in terms of morphological composition, size distribution and properties of the carrier gas. Exact metrics are identified during a later stage of the formulation of the aerosol generation guide.

3.3.2 Physical Principles of the Method(s)

Aerosol generation is based on the dispersion of either dry or liquid-suspended powder in air by various dispersion techniques. Dry powder aerosol generation is implemented by a Venturi nozzle, vibro-fluidisation and the commercial Powder X as well as nebulisation of liquid-suspended powder as part of the commercial CLOUD system.

Various steps are implemented preparing the powder/suspension for aerosolisation, during which both the source material and the prepared form of the material (for aerosolisation) is characterised. The preparation steps determine, apart from technical parameters of aerosol generators, the quantitative and qualitative dose, i.e. mass/number/surface area

concentration, as well as the morphological particle composition of the aerosol. Furthermore, the generated and administered dose is characterised.

A decision-based flow scheme is foreseen to select adequate methods for each step towards, along and after aerosol generation to obtain the desired dose. Each method will come with a standard operating procedure.

At this stage, the focus is on single material doses only. Once tested for single materials, the protocol's applicability for mixtures will be tested.

In the following, the flow scheme is shown. Essentially, the scheme implements a modular approach. Each module slot represents a subsequent process in the procedure of dose generation: Sample preparation/manipulation, sample characterisation, aerosol generation, aerosol monitoring and online monitoring, aerosol manipulation, aerosol application and (deposited) dose characterisation.

Figure 6 introduces and explains the colour scheme used to allocate methods and decisions to these processes.

Figure 7 to Figure 9 show the flow scheme; the steps in all three figures must be taken to ensure a full procedure from source material to (deposited) dose characterisation. SOPs that are not yet existing are labelled (NYE). However, the partner institute that shall develop the SOP over the course of the Project is mentioned.

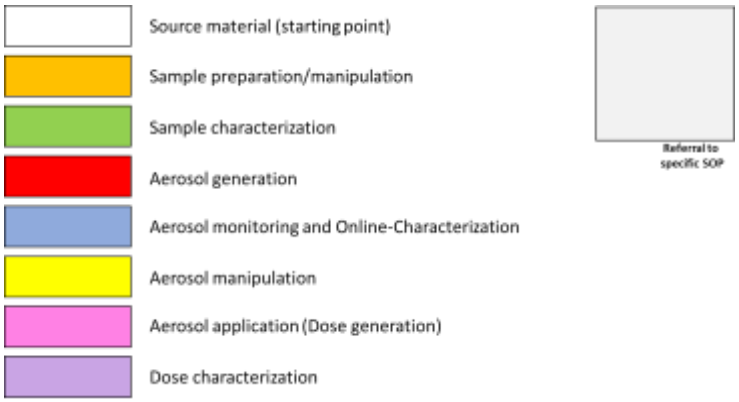

Figure 6: Categorisation of processes and decision-knots in the flow scheme.

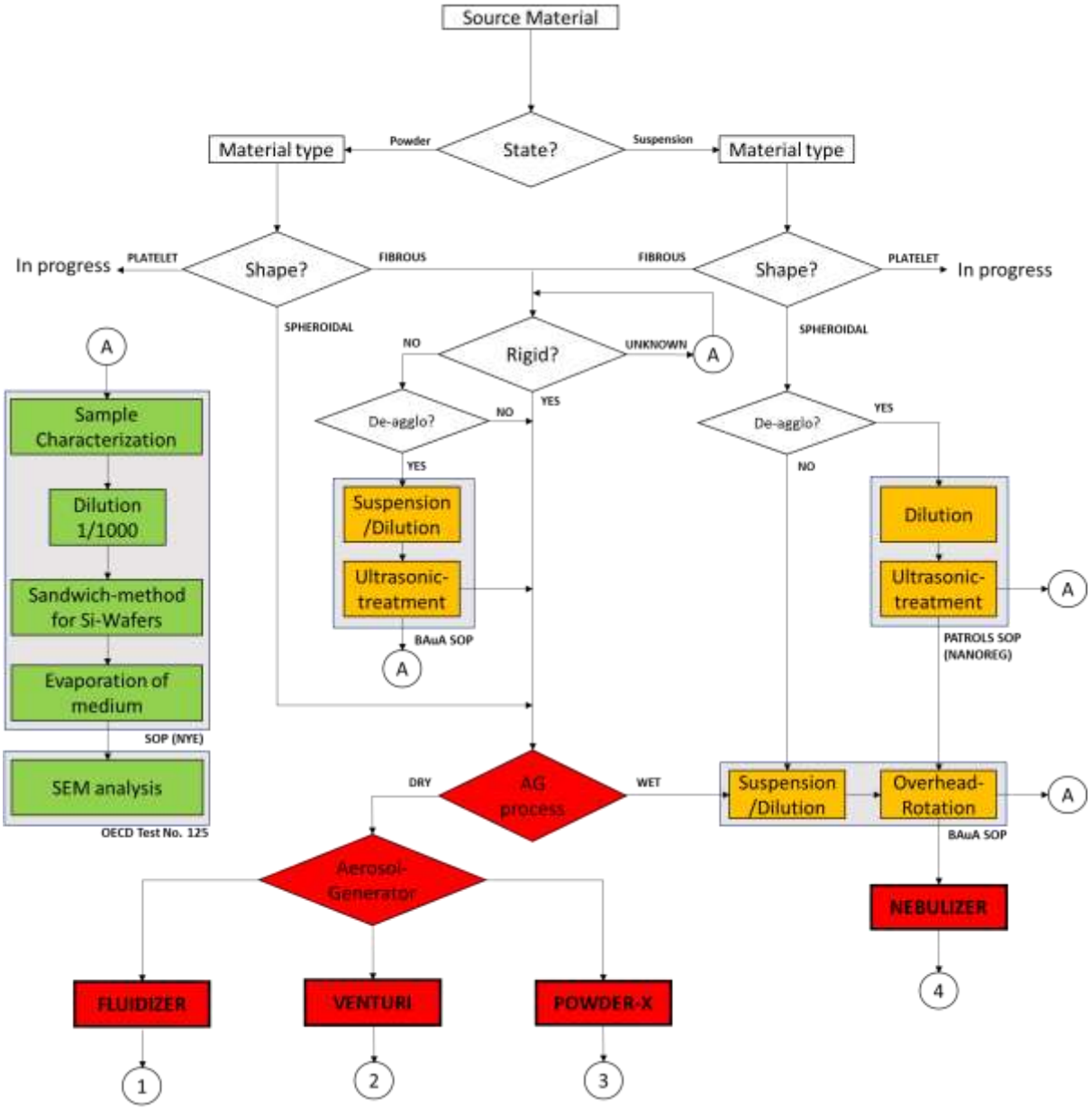

Figure 7: Flow-chart of the material sample preparation, sample characterisation and choice of the applied aerosol generation (AG) process.

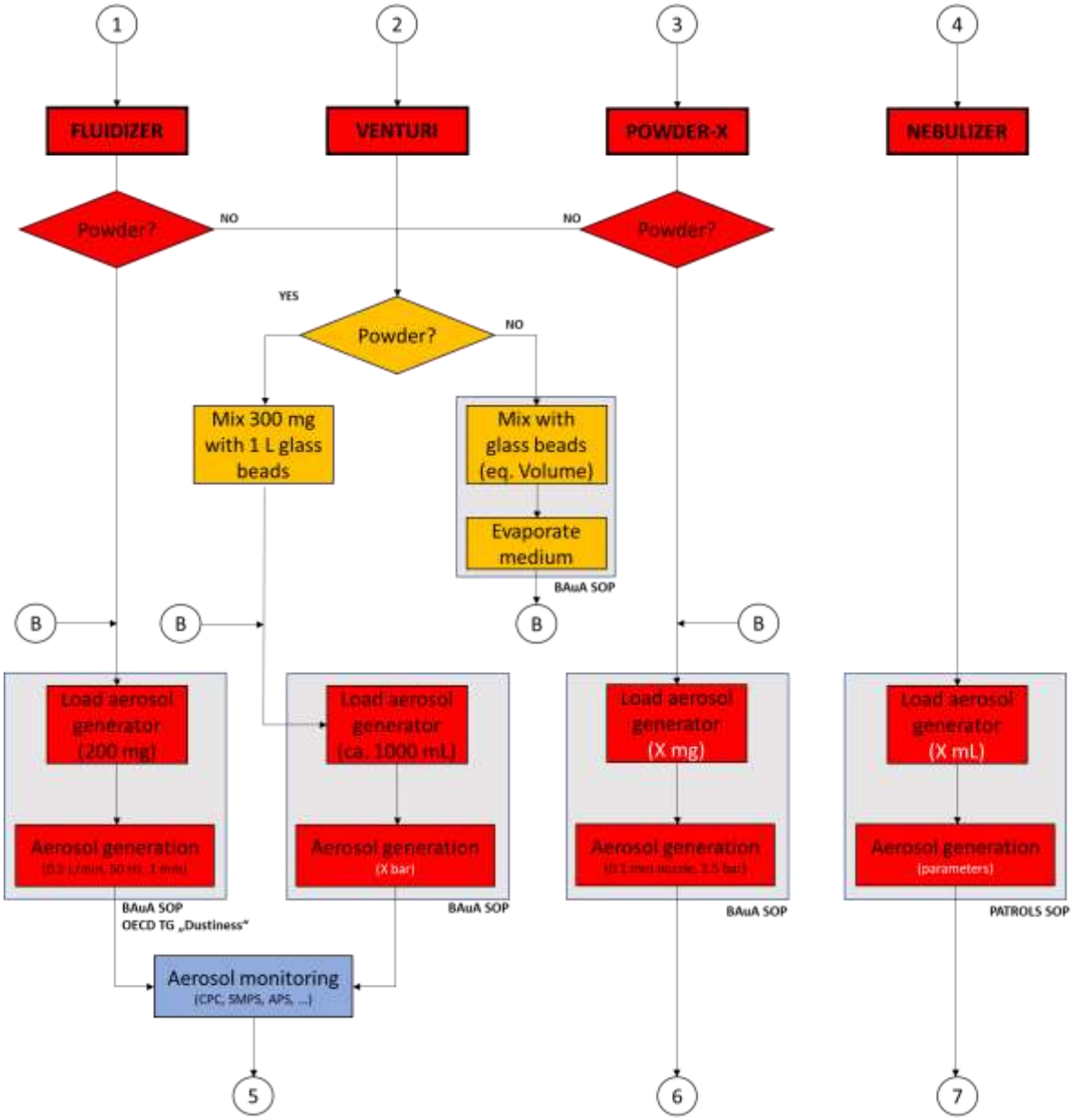

Figure 8: Flow-chart of the sample preparation specific for chosen aerosol generator, aerosol generation and aerosol monitoring steps.

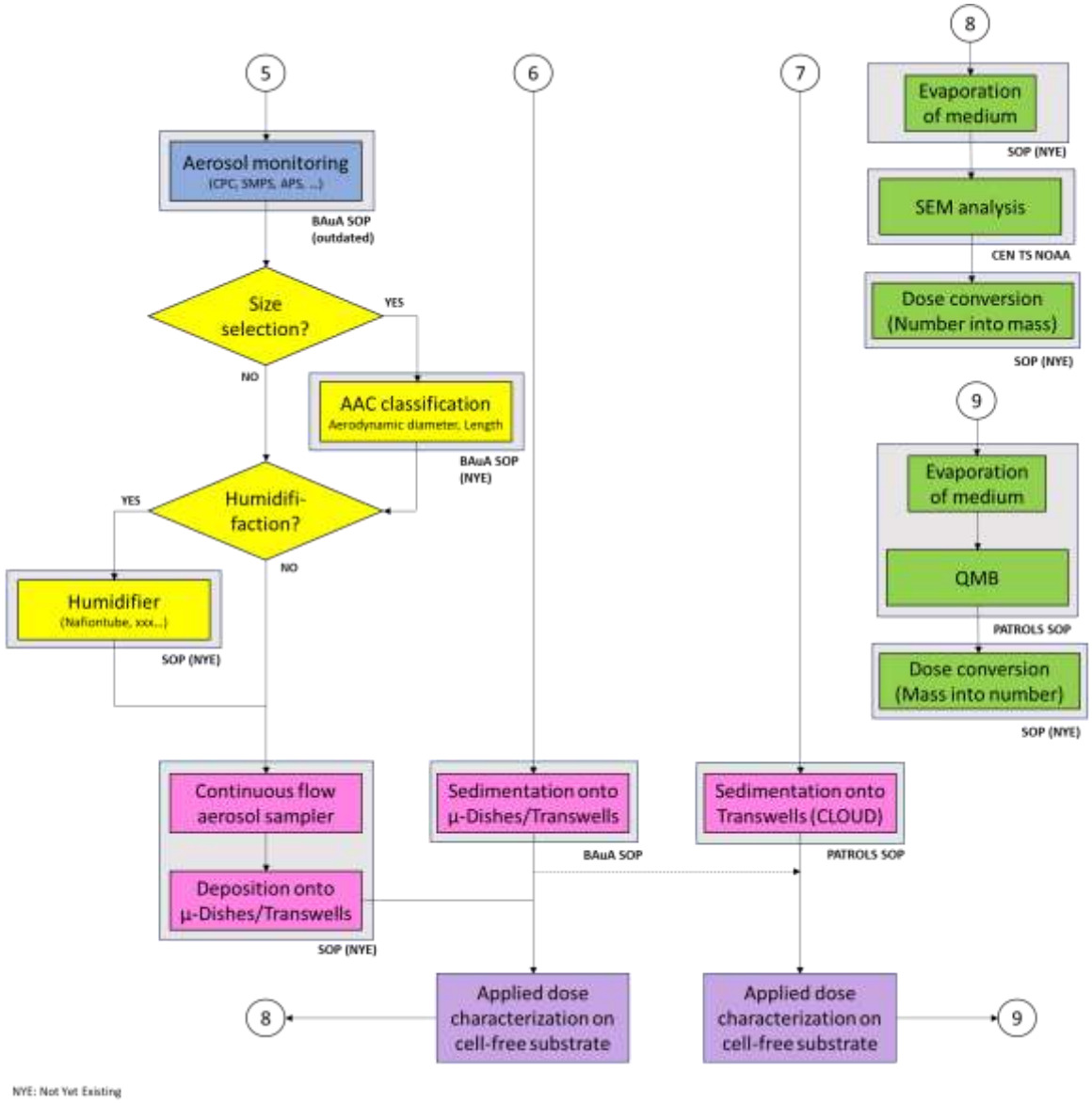

Figure 9: Flow-chart of the aerosol monitoring, aerosol manipulation, dose application and characterisation processes.

3.4 MACRAMÉ Harmonisation & Standardisation Strategy concerning the Aerosol Generation for Inhalation Studies

3.4.1 Type of Outcome, identified Committee and potential Stakeholders interested in the Work

Following the methodological developments in the MACRAMÉ Project, it is envisaged to bring the work to a standardisation body. The identified targeted organisation is the OECD, by proposing a new guidance document.

The stakeholders who may benefit from this work are identified in Table 2.

Table 2: Identified interested stakeholders

Stakeholder identified	Benefits/Impacts/Examples
Industry and commerce – large industry	Benefit : Higher degree of standardisation for tests in the context of regulatory testing. Impact: Higher acceptance of the risk assessment less effort and less costs Examples: Regulatory testing in the context of REACH.
Industry and commerce – SMEs	Benefit: Higher degree of standardisation for tests in the context of regulatory testing. Impact: Higher acceptance of the risk assessment less effort and less costs Example: Regulatory testing in the context of REACH.
Government	Benefit: receiving high quality, interpretable and comparable safety data Impact: enabling risk assessment and regulatory measures based on valid data Example: Regulatory testing in the context of REACH.
Consumers	Benefit: Higher confidence in safety evaluations of advanced materials used in consumer products Impact: Higher acceptance of advanced materials Example: Products making use of nanotubes. When nanotubes are considered safe, despite being fibres, acceptance and application of such new products can be expected to enhance.
Labour	Benefit: Better risk assessment leads to adequate safety measures at work places Impact: Higher confidence in workers safety when handling advanced materials
Academic and research bodies	Benefit: testing according to standards enables high quality, reproducible data Impact: high quality research data

3.4.2 Preparatory Work

The SOPs mentioned in Figures 7-9 are already available. In cases, where the SOPs are still in an exploratory phase, a short description of the necessary steps is available as an institute-internal document.

3.4.3 Validation Status

The procedure outlined in Figures 7-9 has not yet been validated. However, some of the SOPs available for specific methods have been validated and are existing as OECD TGs or CEN standards.

3.4.4 A Metrology Check-List

The following ISO/TC 229 – IEC/TC113 / JWG2 *Metrological check-list* is used to assess the maturity of the proposal as well as actions still to be carried out to develop standards on this topic.

1. *Is the property measured/assessed by the method (= measurand) unambiguously described? Y/N*

Not yet, but a non-exhaustive list of properties that can be measured can be composed of the following elements:

- Cardinality of pre-defined morphology bins, size distribution for particles in each bin, determined for material/aerosol/deposited aerosol samples by electron microscopy (EM):
    - Source material
    - Sample prior to aerosol generation
    - The deposited aerosol;
- Extrapolated total number dose, convertible to average particle number concentration, determined from particle counting results by EM of aerosol samples, differentiated by particle/morphology class;
- Particle number concentration by aerosol monitoring, possibly convertible to mass, volume and surface area concentration (and others); and
- Particle size distribution of the aerosol from size-sensitive aerosol monitors (size represented by the aerodynamic diameter, electromobility diameter).

2. *Are measurement units defined and in accordance with SI rules? Are the tools required to obtain metrological traceability to the measurement units available? Y/N*

Yes

3. *Has it been clearly indicated whether the measurand is 'operationally or method- defined', or whether the measurand is an 'intrinsic' property? Y/N*

As common for aerosol measurements, most measured properties are method-defined. Models can be applied to convert these properties to intrinsic properties (method-independent).

4. *Has the (material / substance / test sample / test system) subject to measurement, clearly been described, including its state? Y/N*

The material subject to aerosol generation must be powdery and, for nebulisation, suitable for suspension in aqueous media. Other than that, the procedure can be applied for all types of advanced materials and high aspect-ratio materials.

5. *Are any quality control tools available to enable the quantitative demonstration of a laboratory's proficiency with the method, e.g. in terms of repeatability or bias? Y/N*

Not yet and most probably not manageable over the course of the Project. This would require substantial round robin testing that will not fit within the timelines of the Project.

3.5 Proposed Workplan & Resources

Theoretical work (drafting the flow scheme) can be accomplished in dedicated WP2 - Task 2.2 meetings, every two months following the finalisation of this road map (in M12) and finalisation (by Project month M18). When needed additional teleconferences will be scheduled. Experimental work, i.e. initial testing of the procedure as well as comparative inter-laboratory study is to be conducted from M18 – M30. A dedicated workplan will be worked out after finalisation of the flow scheme (M18).

A draft guidance document based on a limited set of testing results can be generated from M30 until M36. Work after the MACRAMÉ Project needs to focus on continuing inter-laboratory testing in order to finalise formulation of the Guidance Document. The following four milestones are envisaged in this roadmap:

- **Milestone 1:** Draft of the flow scheme for the aerosol generation for inhalation studies applied in this Project (M12) - reached by finalisation of this document.
- **Milestone 2:** Complete flow scheme description, agreed upon by Project partners. All available SOPs collected and approved M18, Identification of SOPs to be developed or validated over the course of the Project.
- **Milestone 3:** Document describing procedure application results in the form of data collection of measured properties of samples/aerosols collected during different stages of the aerosol generation procedure. Flow scheme needs to be applied by three partner institutes, preferably each using equivalent flows in the scheme and materials. Selected flows need to be tested in order to assess reliability and practicability of the procedure – M30.
- **Milestone 4:** By the end of the MACRAMÉ Project, we will approach validation and standardisation/harmonisation bodies (e.g. VAMAS, OECD) to propose our methods and approaches for formal validation and inclusion in official TGs/GDs. Depending on the readiness level reached by the end of MACRAMÉ and in agreement with regulatory bodies, we will explore the possibility of preparing and submitting a Standard Project Submission Form (SPSF). This will be supported by MACRAMÉ's partners who are closely connected with regulatory bodies (e.g. BAuA, LIST, LNE, etc.).

4 MACRAMÉ Roadmap on *in vitro / ex vivo* Models for Inhalation Toxicology

4.1 Introduction - *in vitro / ex vivo* Models for Inhalation Toxicology

MACRAMÉ aims at establishing a framework for *in vitro* inhalation toxicology, relying on an array of *in vitro* and *ex vivo* systems of increasing biological complexity that are representative of the human upper and lower airways. In order to achieve this, MACRAMÉ will stress-test, validate and implement several biological *in vitro* and *ex vivo* models representative of the human respiratory system (Figure 10), including models of upper (bronchial region) and lower (alveolar region) airways, which are commercially available (i.e., Mucilair™, Alveolair™, ALIsense) and the Alveolar Macrophage Model (IBE). At present the amount of data of the applicability of such models to nanomaterials and advanced materials is rather limited. Thus, MACRAMÉ will verify that the proposed methods are applicable to such classes of inhalable pollutants. MACRAMÉ's final aim is to demonstrate the usefulness of such New Approach Methodologies (NAMs) for the regulatory hazard assessment of AdMas.

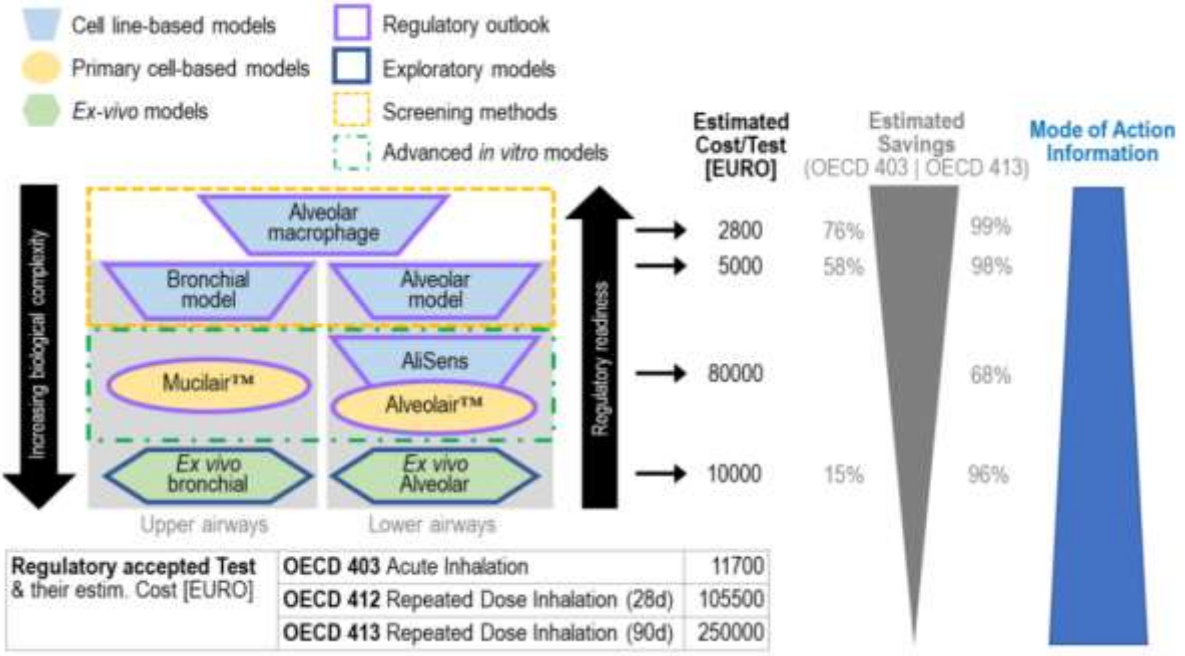

Figure 10: Composite schematic representation of the MACRAMÉ tiered approach to in vitro inhalation hazard assessment. MACRAMÉ will rely on a battery of in vitro / ex vivo biological systems of increasing biological complexity, representative of human upper and lower airways.

The MACRAMÉ's roadmap for the demonstration and validation of the *in vitro / ex vivo* models for *in vitro* inhalation toxicology will include the following steps:

1. Establishment, implementation and validation of *in vitro* and *ex vivo* models representative of the human respiratory system, consisting of the following phases:
   a) All laboratories will establish the respective models at their premises and produce SOPs to be shared across the consortium.
   b) At first, lower tier models, such as the Alveolar Macrophage Assay (AMA) that allow higher throughput, will be used to rank the MCL (MACRAMÉ Control Library) materials

and select a subset of representative control materials to be used for the benchmarking and demonstration of the different models. Based on the ranking from the AMA, a subset of particles will be selected for exposure on the bronchial model (based on a co-culture of CALU- 3 bronchial epithelial cells and macrophages (RIVM)) and the alveolar model (based on a co-culture of A549 alveolar epithelial cells (LIST)).

c) At a later moment, higher tier *in vitro* models of the upper and lower airways (e.g. the ALIsens model (LIST) (alveolar region – tetra-culture including alveolar epithelial cells, endothelial cells and two types of immune cells), the MucilAir-DC (Epithelix) (bronchial region - co-culture between bronchial epithelium and primary human DC), the AlveolAir-Macrophages (alveolar region – co-culture of ATI and ATII primary epithelial cells and resident alveolar macrophages) and *ex vivo* models generated from bronchial tissue will be exposed to the materials selected from the CML in order to understand their regulatory readiness.

d) In the end, using the methods and SOPs harmonised during the establishment and implementation of *in vitro* and *ex vivo* models, the applicability of the assay battery on the materials derived from the Use Cases will be tested.

2. In order to achieve the reproducibility that is necessary in a regulatory framework, approaches for the exposure of the *in vitro* and *ex vivo* biological systems will be harmonised. This will include the evaluation of different exposure devices (and principles), including the continuous flow, ultrasonic nebulisation (Cloud System from VitroCell), "chemical printing" (e.g. TECAN D300e digital dispenser) or the high pressure aerosolisation (e.g. PowderX from VitroCell) at the Air-Liquid Interface or in semi-ALI conditions. A decision tree for the selection of the most appropriate exposure device and biological system depending on the nature of the material and biological endpoint of interest will be developed.
3. To complement the qualitative hazard assessment, the possibility of developing approaches for the detection and the semi-quantitative measurement of AdMas in biological fluids, cells and tissue will be investigated. This is particularly challenging in the case of carbon-based materials, which are difficult to localise and quantify once embedded in a biological matrix.
4. Based on the experimental results obtained by exposing the biological systems to the CML's materials and materials from the Use Cases, the fit of the *in vitro* inhalation framework with the relevant regulatory requirement, such as REACH (REACH Regulation (EC 1907/2006)) and CLP regulations (Regulation (EC) No 1272/2008 on the classification, labelling and packaging of substances and mixtures) will be evaluated. In other words, an assessment will be conducted to establish, if the models are able to provide answers to regulatory needs and to predict regulatory relevant endpoints, such as sensitisation or genotoxicity. In addition, a potential complementing or replacement of the OECD TG 403 (*in vivo* acute inhalation toxicity), TG 412 (*in vivo* 28 days repeated dose inhalation toxicity) and TG 413 (*in vivo* 90 days repeated dose inhalation toxicity) by the proposed integrated approaches will be evaluated.

4.2 Problem Framing concerning *in vitro / ex vivo* Models for Inhalation Toxicology

At present, as a response to changing societal and ethical demands, several NAMs based on *in vitro* methodologies have been proposed to mimic a multitude of biological systems and for the prediction of several biological outcomes. However, despite the high number of developed NAMs, the number of those that reach regulatory validation is still very limited. There are several reasons for this, but the main one is the lack of regulatory readiness of the proposed

NAMs. This means that the majority of the NAMs have shortcomings in several areas, such as reproducibility, a sufficient prediction power, transferability, transparency on the method itself and on the data interpretation, etc.

For this to be solved, it is necessary that method development embraces such principles from the very beginning and include the necessary steps towards harmonisation, robustness and transparency.

4.3 Current Gaps in the *in vitro / ex vivo* Models for Inhalation Toxicology

Several questions are in the process of being MACRAMÉ Project:

Which method or equipment could be used to mimic at best the in vivo conditions while being technically convenient and allowing higher throughput screening of the chemicals/materials?

Several types of equipment that allow the exposure of materials at the air-liquid interface (ALI) are commercially available and will be considered. These include the Cloud system, Powder X and continuous flow from Vitrocell® and the D300e printer from Tecan. In addition, the MACRAMÉ Project will investigate the influence of semi-ALI exposure[3] *vs* ALI exposure and draft guidelines for the selection of the best exposure conditions.

- In regulatory frameworks, it is important to relate the dose to the measured biological effects. However, when dealing with advanced materials, and more specifically with carbon-based materials, it is very difficult to obtain a suitable protocol for the localisation and quantification of materials upon embedment in a biological matrix. Within MACRAMÉ' we will explore the possibility of using advanced imaging techniques such as Helium Ion Microscopy with Secondary Ion Mass Spectrometry (HIM-SIMS), Raman spectroscopy, and dark field hyperspectral imaging for the visualisation and semi-quantitative analysis of the materials' uptake.
- A large part of the efforts will be dedicated to the development of SOPs for the generation of stable dispersions of particles/materials (including aerosol as described in Section 2). The work will include understanding of the advantages and disadvantages of ALI and semi-ALI exposures, what biological fluids can be used to better mimic real life aerosols (i.e. meant as aerosol that mimic the physico-chemical properties of aerosol, as they can be inhaled in occupational or consumer exposure (e.g. thus avoiding addition of non-relevant and non-realistic dispersant/surfactants)), what exposure device can be used and when.
- By the end of the MACRAMÉ Project, validation and standardisation/harmonisation bodies (e.g. ECVAM, OECD WNT, OECD SGTA, and OECD WPMN) will be approached to propose our methods and approaches for formal validation and inclusion in official TGs/GDs. Depending on the readiness level reached by the end of the Project and in agreement with regulatory bodies, the possibility of preparing and submitting a Standard Project Submission Form (SPSF) will be considered. This process will be supported by those MACRAMÉ's partners who are closely connected with regulatory bodies (e.g. BAuA, LIST, LNE).

[3] A 'semi-ALI exposure' is an ALI exposure with a thin fluid layer of concentrated particle suspension on top of the cells at apical-side of transwell cultures.

5 MACRAMÉ Roadmap on the Ecotoxicity Assessment of AdMas

5.1 Introduction - Ecotoxicity Assessment of AdMas

MACRAMÉ aims to widen the development and application of harmonised OECD TGs and standards to market-relevant AdMas in their complex product matrices, based on five case studies. For the ecotoxicological assessment of these AdMas and the representative products containing each AdMa, a series of additional considerations are required to adapt and extend current tests for AdMas; the ecotoxicology roadmap will focus on the flowing elements:

1. Harmonising approaches for the dispersion of low-solubility AdMas for ecotoxicity testing;
2. Developing and benchmarking exposure approaches that enable assessment of the impacts of the AdMas in their formulations or products, distinguishing material effects from other released components;
3. Proposing adaptations to the standard OECD chronic test for reproduction effects on *Daphnia magna (OECD TG 211)* (i.e. sequential acute exposures at sub-chronic concentrations), building on work performed in the RiskGONE project and extending it towards standardisation; and
4. Exploring the potential of OECD TG 249 for rainbow trout gill cell (RTgill-W1) lines to other rainbow trout cell types (including liver, gut and gonad), and to zebrafish cells (including embryonic cells, gill and liver cells).

The following section highlights the major issues encountered, when assessing the ecotoxicity of poorly water-soluble AdMa, and the MACRAMÉ proposed solutions that will be explored and developed, in order to work around or through the existing challenges. The necessary timelines will also be addressed as well.

The MACRAMÉ ecotoxicity approach, shown in Figure 11: Schematic illustration of the MACRAMÉ ecotoxicity testing approach and outcomes., includes an innovative extension to the existing OECD TG 249 (Fish Cell Line Acute Toxicity: The RTgill-W1 cell line assay) over acute and sub-chronic timescales (24 h - 2 weeks) with the existing acute OECD TG 201 (Freshwater Alga and Cyanobacteria, Growth Inhibition Test) and TG 202 (Daphnia sp. Acute Immobilisation Test) tests , and the subchronic and chronic OECD TG 211 (Daphnia magna reproduction test) utilising a conditioned medium approach to disperse the materials. The approach allows the cells / algae / daphnids to interact with the medium and secrete proteins for a period of 24 hours, which are quantified using a bicinchoninic acid assay (BCA assay) for the total protein content, for fixed times. Subsequently this conditioned medium is used to disperse the materials, allowing more realistic and environmentally relevant environment exposure conditions.

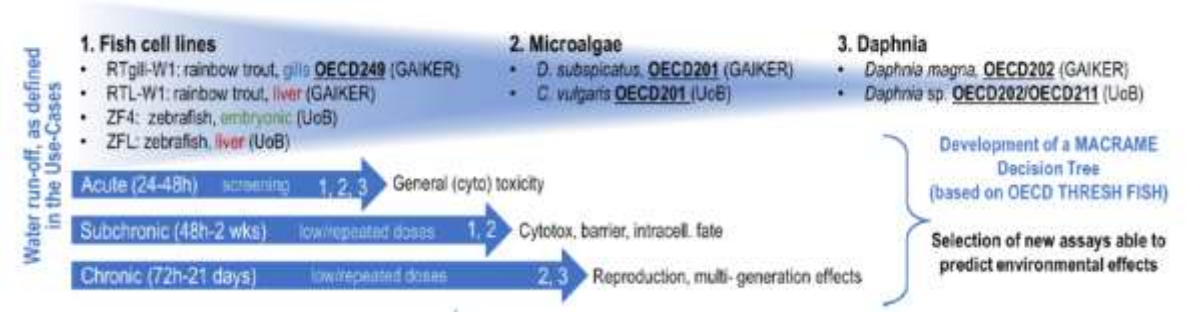

Figure 11: Schematic illustration of the MACRAMÉ ecotoxicity testing approach and outcomes.

The existing OECD TG 249 assay will be extended to enable assessment of accumulation in, and transport across the gill, using transwell systems. In addition, impacts on fish embryonic cells and/or fish liver cells will be incorporated by co-culturing one or the other of these cells on the bottom surface of the basolateral chamber to assess impacts from materials in or crossing through the gill barrier. The OECD TG 201 and TG 202 assays will be extended to include subchronic and chronic multi-generational exposures as well. This aims to provide further parameterisation of the decision tool (based on the OECD THRESH FISH) for when to perform acute fish toxicity testing (OECD TG 203), and to provide mechanistic grouping for AdMas. The OECD THRESH FISH is a tiered-testing strategy based on the comparison of the LC50/EC50 values of three test species (fish, algae and invertebrates) and where the most sensitive species (lower LC50/EC50 value) is commonly used for hazard and risk assessment.

- By the end of the MACRAMÉ Project, we will approach validation and standardisation/harmonisation bodies (e.g. ECVAM, OECD) to propose our methods and approaches for formal validation and inclusion in official TGs/GDs. Depending on the readiness level reached by the end of MACRAMÉ and in agreement with regulatory bodies, we will explore the possibility of preparing and submitting a Standard Project Submission Form (SPSF). This will be supported by MACRAMÉ's partners who are closely connected with regulatory bodies (e.g. BAuA, LIST, LNE, etc.).

5.2 Problem Framing concerning the Ecotoxicity Assessment of AdMas

Ecotoxicological studies of AdMas and their products are hampered by a number of issues; these include the following:

(i) challenges to disperse the materials for aquatic exposures as many AdMas are highly hydrophobic and designed for dispersion and/or use in organic solvents;
(ii) challenges to expose the test organisms to the AdMas and to products containing the AdMas in a manner that represents realistic exposure routes, and that mimics the release forms from the specific product[4];
(iii) the fact that chronic studies have not been applied extensively to 2D and other AdMas or their products, so evaluation of applicability is needed, taking into account proposals for adaptations for nanomaterials by the Horizon 2020 project RiskGONE [10];
(iv) challenges in the available amounts of AdMas which can make longer term aquatic and soil studies complex; and

[4] For example, if an AdMa is dispersed in oil for use as a spray, is the most appropriate exposure form the product sprayed onto a glass slide to assess its release? Or for cells adhesion to the layer? For daphnids, the fact that the organisms can simply avoid the slide adds a further layer of complication that needs to be explored.

(v) ethical issues around the use of animal testing (which includes fish) and the push towards non-animal testing methods or new-approach methodologies (NAMs) in line with the 3Rs directive (replacement, reduction, refinement).

5.3 Current Gaps in the Ecotoxicity Assessment of AdMas

A non-exhaustive list of the current knowledge gaps and resulting questions that MACRAMÉ will address includes:

- Can we implement a common approach for dispersing AdMas for different ecotoxicity exposures that is reproducible yet representative of the real exposures?
    - Could the use of a conditioned medium provide common ground across ecotoxicity studies (e.g. algae, daphnids, fish cell lines) as well as potentially also with toxicity studies?
- Can a simplified eco-corona mimic the complexity of the complete corona, and facilitate realistic assessment of AdMas interactions with living systems?
    - For example, in toxicity studies, sometimes albumin is used as a proxy for serum so whether albumin or another common protein could serve a similar role for ecotoxicity needs to be explored.
- What is the role and impact of direct *versus* indirect effects from AdMas. For example, effects of AdMas on medium pH, dissolved oxygen (DO) and light penetration (which affects photosynthesis ability of algae) are indirect routes of toxicity, but need to be ruled out in order to understand the origins and mechanistic basis of observed effects, and to separate chemical from physical effects.
- How to disentangle the impacts of formulation components versus the AdMas when they are contained in products?
    - For example, in the MACRAMÉ Use-Case 3 (GRM-bearing sprays), the graphene sheets are dispersed in mineral oil, but this has been shown to coat daphnids appendages and result in changes in the frequency of appendage beats, movement, swimming behaviour, carapace shedding to name but a few effects. Figure 12 shows some images of the impact of (crude) oil on daphnids. These include the fact that oil can cause the daphnids to stick together and coat their outer shell (carapace) leading to difficulties with movement and premature shedding of the carapace which impairs development and leaves them vulnerable to predation.

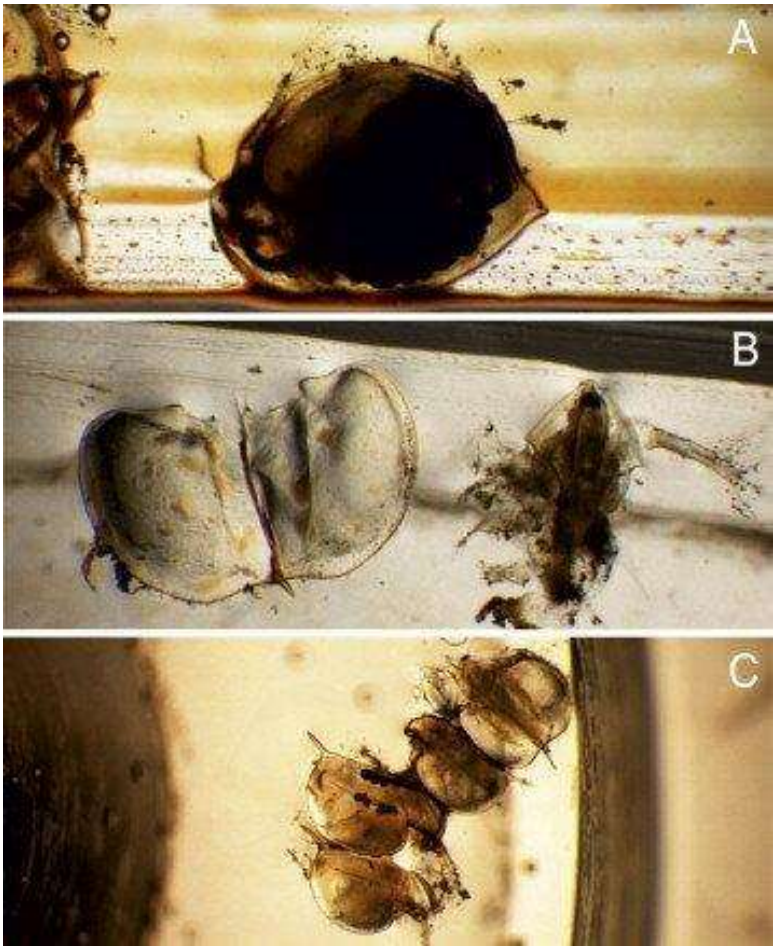

Figure 12: The appearance of Daphnia magna after oil pollution. (A) A specimen immobilised by the insoluble surface layer of crude oil. (B) Whole carapace of a daphnid removed as a result of oil pollution. (C) Cladocerans stick together due to crude oil

- Can we design exposure strategies that mimic the exposures that would arise from usage of AdMa-enabled products, such that there are meaningful exposures?
    - How can we extrapolate from testing the individual components, including the AdMas and the formulation components, to the combined effect of the product?
    - Will a mixture approach work in this case, or does the AdMa become intrinsically inseparable from the matrix, resulting in a new hybrid breakdown material from any weathering and wear-and-tear processes.
- Can we extend the current fish gill cells approach, which utilises a submerged culture approach, and assumes cold freshwater conditions using rainbow trout cells?
    - Do we need to include tropical or salt-water conditions, which will impact the characteristics of the AdMa, and their eco-corona and environmental transformations?
    - To explore this, we are initially planning to extend the OECD TG 249 test to a tropical fish species (zebrafish). In the roadmap we will lay out the considerations needed to extend also to a salt-water fish species, such as silverside or *Menidia spp*. These are fish species that are listed in the USEPA fish acute toxicity test guideline (OCSPP 850.1075; USEPA, 2016).

- Can we add additional AdMas related endpoints into standard tests to provide additional mechanistic information, including localisation of any internalised particles or material fragments and their quantification?
    - This may be assays for oxidative stress and/or genotoxicity for example to provide some mechanistic insights, or other assays linked to a specific key event in a proposed adverse outcome pathway (AOP). Such an AOP may aim to link from a molecular initiative event to an observed adverse outcome, via a set of linked events at the molecular, cellular, tissue and organisms' levels. This may further aim to connect the consequences of this to population level effects. AOPs for nanoscale materials are emerging, typically related to lung overload leading to oxidative stress and fibrosis, as documented in AOP 451 (Interaction with lung resident cell membrane components leads to lung cancer). For an AOP for chronic reproductive failure in daphnids, RISKGONE focused on gut overload in Daphnia.

5.4 MACRAMÉ Harmonisation & Standardisation Approaches concerning the Ecotoxicity Assessment of AdMas

Based on extensive discussions during the preparation of the MACRAMÉ proposal, and during the first year of the Project, we have shortlisted a number of the challenges identified above for immediate experimental investigation within MACRAMÉ, with the intention of leading to an updated TG. Other challenges will be addressed as desk studies or a roadmap of evidence and approaches will be laid out to be taken up by other projects/initiatives. The range of needs is far too extensive to be addressed by one project alone. Table 3 and Table 4 lay out the MACRAMÉ approach, considering both existing organism level tests on algae and daphnia (Table 3) and on fish cells lines (Table 4) highlighting the current status, and the foreseen developments within MACRAMÉ.

A key feature of all the MACRAMÉ ecotoxicology work will be the use of conditioned media, whereby the AdMa are dispersed in a medium that has previously been exposed to the test organisms or test cells for a fixed period of time. This will allow the organisms / cells to secrete biomolecules into the medium, thus conditioning it.

The process of conditioning medium is shown schematically in Figure 13, and can be harmonised using a common "conditioning time" (e.g. 6, 12 or 24 hours). Medium conditioning is considered by the MACRAMÉ Project as a way to harmonise across studies whilst also maximising the environmental relevance of the results by tailoring the exposure conditions to the specific organism / cell line. This approach also allows harmonisation with the toxicology work as medium conditioning is an increasingly utilised approach there also. Conditioned medium should also enhance the dispersion stability of the AdMa, and ensure that the acquired biomolecule or eco-coronas have sensible species-relevant compositions (e.g., calf serum is not relevant for fish or daphnia studies). Conditioning of medium is widely used in co-culture approaches (either for sequentially exposed or mixed cell populations). Additional mechanistic insights can be obtained through analysis and comparison of the secretomes from different cells / organisms [11]. This can facilitate determination and eventual prediction of corona compositions and evolution as AdMa interact with cells and living organisms. Thus, the advantages of conditioned medium are several, and MACRAMÉ will produce a detailed paper demonstrating these across its ecotoxicological studies. MACRAMÉ will also provide a comparison to toxicity studies, and a roadmap for validation of the approaches.

Table 3: Planned activities and outputs from MACRAMÉ related to ecotoxicology utilising whole organisms (algae and daphnia).

Method	Scope (measurand, etc.)	Type of materials/use cases (graphene, graphene oxide, composite, material in complex media)	Status (to be developed, SOP internally validate, etc.)	MACRAMÉ output foreseen (validated SOP, guideline, ILC, overview of the applicability, etc.)	What committee(s) foreseen (ISO, CEN, ASTM, VAMAS, etc.)	New item or revision of an existing one	Area (medical, safety, etc.)
Algae (OECD TG 201, ISO 10253)	Acute	Selected materials	NanoHarmony-modified test method (Will become an adapted OECD Guidance Document within MACRAMÉ life)	Extension of applicability of adapted SOP to 2D materials / products.	OECD – e.g., Appendix to revised TG to include different exposure set-ups	Extension of NanoHarmony-revised TG 201	Safety & Medical
Daphnia (OECD TG 202)	Acute	Selected materials	NanoHarmony-modified test method (Will become an adapted OECD Guidance Document within MACRAMÉ life)	Extension of applicability of adapted SOP to 2D materials / products.	OECD – e.g., Appendix to revised TG to include different exposure set-ups	Extension of NanoHarmony-revised TG 202	Safety & Medical
Daphnia (OECD 211, ISO6341-2012)	Subchronic/ Chronic	Selected materials	Existing TG with RiskGONE modifications.	Adapted SOP & test - duration, conditioning of medium (e.g. use of Natural Organic Matter as dispersant), etc.	OECD SPSF – implementation of RiskGONE modifications (ILC)	Revision of existing TG211	Safety & Medical

Table 4: Planned activities and outputs from MACRAMÉ related to in vitro ecotoxicology utilising fish cell lines (rainbow trout and zebrafish).

Method	Scope (measurand, etc.)	Type of materials/use cases (graphene, graphene oxide, composite, material in complex media, etc.)	Status (to be developed, SOP internally validated, etc.)	MACRAMÉ output foreseen (validated SOP, guideline, ILC, overview of the applicability, etc.)	What committee(s) foreseen (ISO, CEN, ASTM, VAMAS, etc.)	New item or revision of an existing one	Area (medical, safety, etc.)
Fish cell line – Rainbow Trout gills (OECD TG 249)	Acute	All 5 use cases	Existing Guideline	Adapted SOP – Different exposure considerations (direct, indirect, release)	OECD - Extension of OECD TG 249 for 2D materials / products & addition of new endpoints e.g. Genotoxicity	Revision of existing OECD TG 249	Safety & Medical
RTL-W1, rainbow trout, liver cells ZF4 zebrafish, embryonic ZFL zebrafish, liver	Acute	All 5 use cases	Application of OECD TG 249 using different cell lines for mechanistic insights.	New SOP including a transwell or co-culture set-up to first pass through gill cells (in transwell insert) and then assess impact on liver cells (bottom of dish)	OECD – Extension of TG 249 to other cell lines (Rainbow Trout & Zebrafish to allow sensitivity analysis) New SPSF for the transwell set-up as either mono- or co-culture to allow a "Systems" analysis	Revision of existing OECD TG 249 to new cell lines New item – OECD SPSF	Safety & Medical

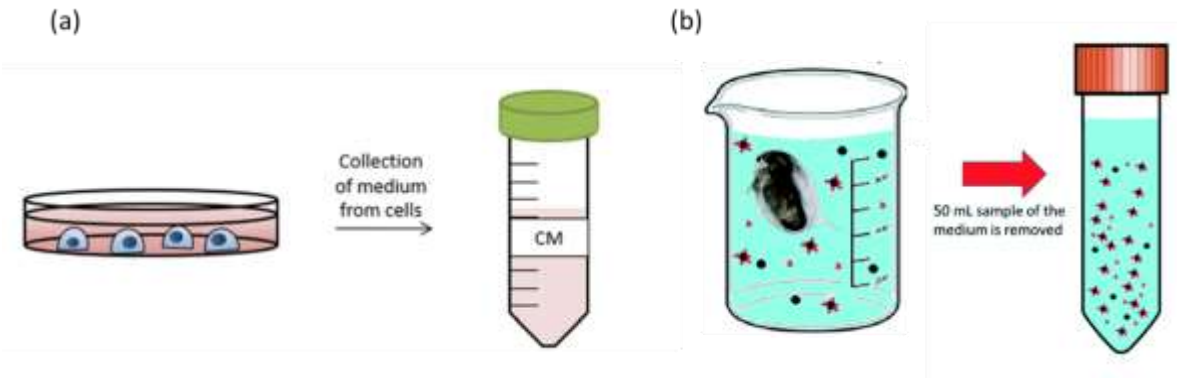

Figure 13: Illustration of the process of conditioning medium, for (a) cells and (b) by Daphnia magna, which can also be applied to algae or other organisms. The secreted proteins and other biomolecules or metabolites in the conditioned medium support the dispersion of AdMa and can provide insights into the metabolic and repair processes underway. Images adapted from (a) [11], and (b) [12].

5.5 Implementation of the Roadmap on the Ecotoxicity Assessment of AdMas

MACRAMÉ will identify on-going activities addressing anticipated gaps highlighted above. The Project will provide a roadmap on what needs to be performed with an estimated timeline (as far as current activities allowed) as shown in Figure 14.

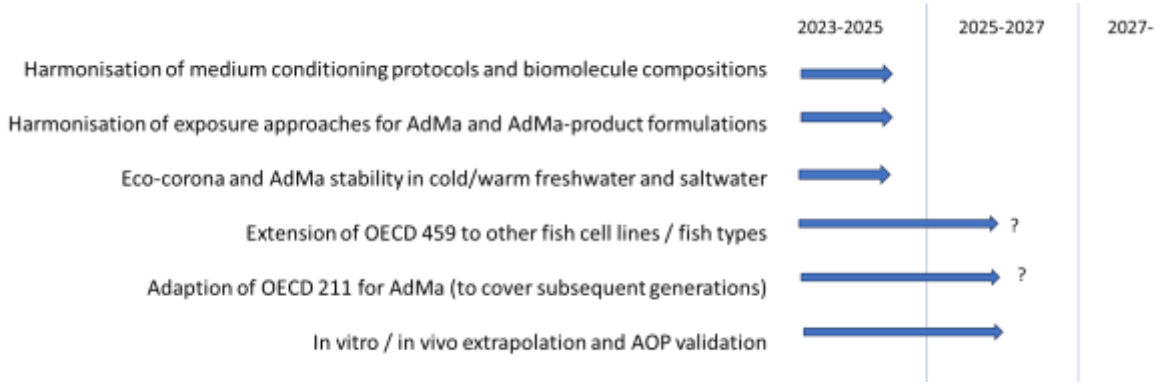

Figure 14: Schematic tentative overview of gaps identified in MACRAMÉ and tentative timeline to move them towards standardisation during and beyond the MACRAMÉ Project.

Each of the concerns and knowledge gaps identified in the 'Current Gaps in the Ecotoxicity Assessment of AdMas' section above is will be mapped, and accompanied by a development, testing and validation plan. It is envisaged that the full deliverable report will consist of three parts, one on the exposure considerations (medium conditioning, AdMa dispersion and corona composition characterisation), one on the *in vivo* ecotoxicity aspects (related to adapting algae and daphnia tests for AdMa), and the third one on *in vitro* approaches (extending the submerged culture approach for gill fish cells to a barrier model, and extending the range of cell types to tropical fish, and potentially even brackish and salt-water fish species).

6 MACRAMÉ Roadmap on the Sustainability Analysis for AdMas

6.1 Introduction - Sustainability Analysis for AdMas

According to the United Nations Brundtland Commission, sustainability may be defined as *'meeting the needs of the present without compromising the ability of future generations to meet their own needs.*[5]

In the past years the European Commission has developed a regulatory strategy addressing sustainability, to help consumers and companies against greenwashing claims and to set a path to achieving the Green Deal Goals in the proposed time frame. In terms of AdMas, the abovementioned definition requires that all potential stages of the life cycle need to be carefully analysed to identify all potentially cumbersome intermediates or waste products along the lifecycle. The life cycle analysis (LCA), therefore, is a mandatory prerequisite to fulfilling the demands on sustainability of any AdMa. LCA is a systematic approach for assessing the potential environmental impacts of products, services and processes across their entire life cycles (ISO 14040)[6]. The LCA methodology may be summarised in the following four iterative steps:

(i) goal and scope definition,
(ii) inventory analysis,
(iii) impact assessment, and
(iv) interpretation. LCA may be used to identify potential life cycle impacts of nanotechnologies and the advanced material sector.

6.2 Current EU Regulations and Directives addressing Sustainability

To achieve Green Deal Goals the EU is driving a number of sustainability regulations which cover the whole value chain, reporting, economics, as well as circularity. Some of the regulations with a higher impact on the implementation of EU sustainability goals are highlighted below:

1. EU Corporate Sustainability Reporting Directive (CSRD)[7]: This directive helps evaluate the sustainability performance of companies, helping investors, consumers and other stakeholders make choices. The regulation requires that companies disclose information on what they see as the risks and opportunities arising from social and environmental issues, and on the impact of their activities on people and the environment. The CSRD drives companies to focus on circularity, supply chain sustainability, decarbonisation and risk of Greenwashing, as they will have to fully substantiate any environmental claims.
2. EU Ecodesign Regulation (currently: Ecodesign Directive 2009/125/EC)[8]: This current directive establishes a Framework to set ecodesign requirements for specific product groups to significantly improve their circularity, energy performance and other environmental sustainability aspects. It focuses on performance and information requirements for almost all categories of physical goods placed on the EU market (with

[5] United Nations Academic Impact (website accessed: 02.06.2024)

[6] ISO 14040:2006: Environmental management — Life cycle assessment — Principles and framework (website accessed: 02.06.2024)

[7] EU Corporate Sustainability Reporting Directive (CSRD) (website accessed: 02.06.2024)

[8] Ecodesign Directive 2009/125/EC (website accessed: 02.06.2024)

the exception of food and feed, as defined in Regulation 178/2002)[9]. The requirements covered by the framework are:

- product durability, reusability, upgradability and reparability
- presence of substances that inhibit circularity
- energy and resource efficiency
- recycled content
- remanufacturing and recycling
- carbon and environmental footprints
- information requirements, including a Digital Product Passport

Since 2022, plans are uynderway to turn the directive into a regulation (EU COM/2022/142)[10].

3. Packaging and Packaging Waste Directive (EU Directive 94/62/EC)[11]: This directive was set to handle the large amounts of packaging that get wasted and are not recirculated back into the supply chain. Hence the EU has set rules that tackle packaging design and packaging waste management to avoid further environmental issues and misused of finite resources.

4. Regulation for Waste Shipments[12]: Entered into force on the 20th May 2024, this regulation aims to contribute to the environmentally sound management of waste. It seeks to strengthen enforcement to prevent illegal shipments of waste occurring within the EU, as well as from the EU to third countries, whilst increasing the traceability of shipments of waste within the EU and facilitating recycling and re-use.

6.3 Problem Framing concerning the Sustainability Analysis for AdMas

AdMas represent a wide group of materials that has been purposely designed to possess new or improved technical (i.e. structural or functional) properties or environmental features compared to materials traditionally used to perform the same functions. AdMas are used in different sectors, in which a variety of sectoral regulations will apply. Regarding environmental sustainability, what remains relevant to every sector is the evaluation of the impact of a given AdMa or AdMa-containing product or process throughout the product's life cycle; this assessment is generally performed through an LCA. Unfortunately, LCAs of AdMas are currently hampered by gaps of knowledge regarding the quantities and effects of components released into the environment during the life cycle.

[9] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety (website accessed: 02.06.2024)

[10] COM/2022/142 - Proposal for a REGULATION OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL establishing a framework for setting ecodesign requirements for sustainable products and repealing Directive 2009/125/EC (website accessed: 02.06.2024)

[11] EU Directive 94/62/EC on packaging and packaging waste (website accessed: 02.06.2024)

[12] Regulation (EU) 2024/1157 of the European Parliament and of the Council of 11 April 2024 on shipments of waste, amending Regulations (EU) No 1257/2013 and (EU) 2020/1056 and repealing Regulation (EC) No 1013/2006Text with EEA relevance (website accessed: 02.06.2024)

6.4 Current Gaps in the LCAs of AdMas and AdMa-containing Products and Processes

The MACRAMÉ Project has identified the following list of gaps hampering the LCA of AdMas and AdMa-containing products and processes:

- There is a lack of Live Cycle Inventory (LCI) data; flows for bulk materials (standard materials or components) only are accounted for;
- There is a scarcity of specific characterisation factors for (eco)toxicity-related potential impacts [13,14];
- Information on the behaviour and forms, in which AdMa are released during their life cycle (especially during use and end-of-life), is generally lacking;
- Conventional Life Cycle Inventory and Assessment (LCIA) methodologies may require some adaptation in order to model the fate, exposure and/or effects of AdMa;
- Circularity has not been evaluated yet (or only for few cases), because industrial scale recycling facilities that recover AdMa are only few or non-existent. In the addition, the following challenges apply:
    a) materials other than AdMas are often of higher economic interest;
    b) there is not enough information to understand if AdMa could re-enter the product life cycle; and
    c) there are no instruments to detect if AdMa pollute secondary materials, in order to evaluate if AdMa can enter in different product life cycles and persist in closed loops.

Furthermore, recycling processes are becoming more chemical-heavy and energy-intensive [15], meaning that recycling does not always translate to sustainable life cycles;

- It is unclear how to use proxy or other strategies to cover for data gaps in a harmonised way is yet unclear; and
- There is a high degree of uncertainty on the applicability of OECD Test Guidelines / Guidance Documents for AdMas, resulting in uncertainties on safety (link here with MACRAMÉ safety roadmaps).

6.5 Approaches taken by MACRAMÉ pertaining to the Sustainability Analysis for AdMas with a Focus on LCA

To contribute to characterisation data of the pristine materials, MACRAMÉ is developing technologies to address physico-chemical properties in complex matrices.

To contribute to the lack of data regarding transformed materials, MACRAMÉ is focused on the characterisation at the end-of-life stage (incineration) and toxicity of the transformed material both at the occupational setting and in the environment.

To better understand the behaviour of AdMa in their lifecycle MACRAMÉ will look into materials flow analysis (MFA). Fate and exposure characterisation factors of AdMa will be modelled using SimpleBox4nano (SB4N). Literature data and physico-chemical data from MACRAMÉ WP2 and MFA results from MACRAMÉ WP4 will be used as input to the SB4N.

To address one of the gaps highlighted above (i.e. scarcity of specific characterisation factors for (eco)toxicity-related potential impacts) MACRAMÉ will develop a decision tree in order to understand which conditions and parameters contribute in the LCA for nanomaterials/AdMa and to determine when a nano/AdMa-specific correlation factor (CF) is needed (Figure 15).

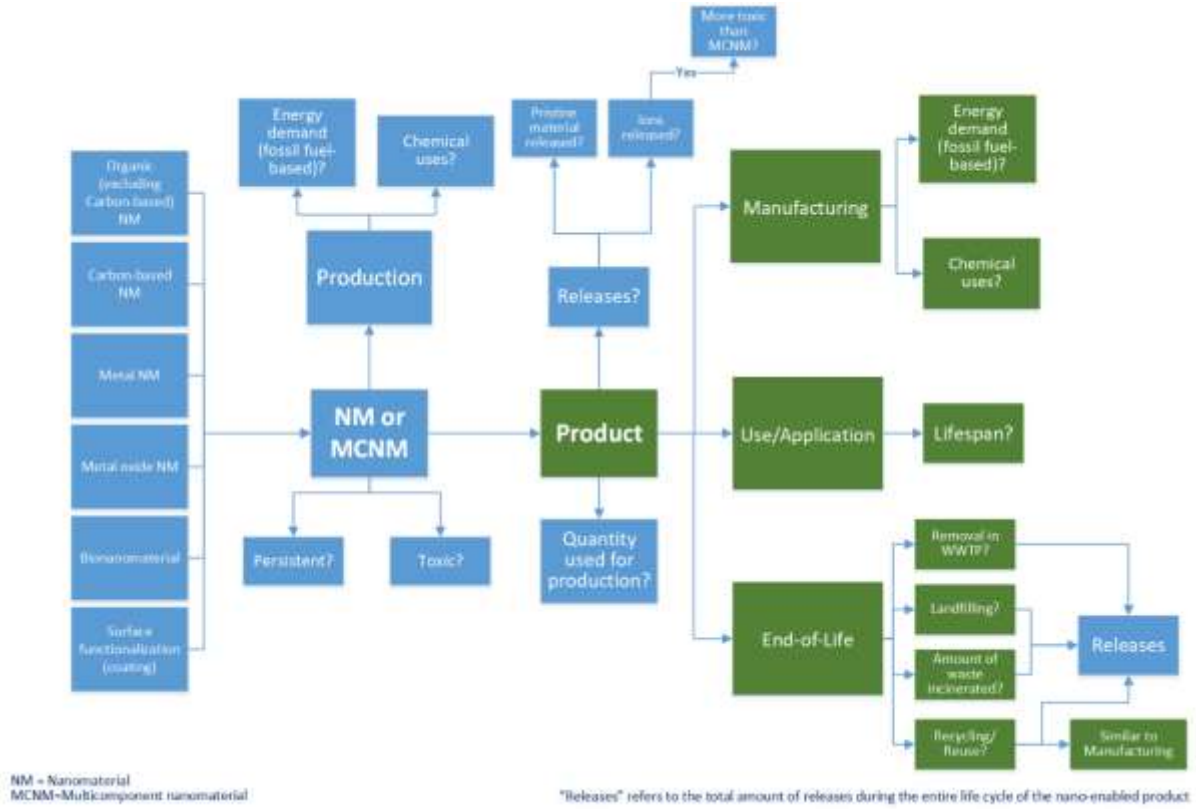

Figure 15: Decision tree defining the conditions associated to the LCA for AdMa and to determine when a nano-specific CF is needed. Green blocks refer to those parameters related to the life cycle of the product (which integrates the NM/MCNM) as well as the product itself. Blue refers to the material and its corresponding life cycle flows.

To cover for current gaps regarding safety (effects), MACRAMÉ will make use of the vast amount of *in vitro* and *in silico* data available from different sources and will develop a CF to ensure such data will be adequate to feed LCIA. *In vitro* | *in vivo* extrapolation approaches will also be developed to correlate as much as possible *in vitro* toxicological data produced in the Project with input relevant for risk assessment and LCA.

6.6 Implementation of the MACRAMÉ Roadmap on the Sustainability Analysis for AdMas with a Focus on LCA

The MACRAMÉ Project will identify on-going activities addressing anticipated gaps highlighted above. The Project will provide a roadmap on what needs to be performed with an estimated timeline (as far as current activities allowed) as shown in Figure 16.

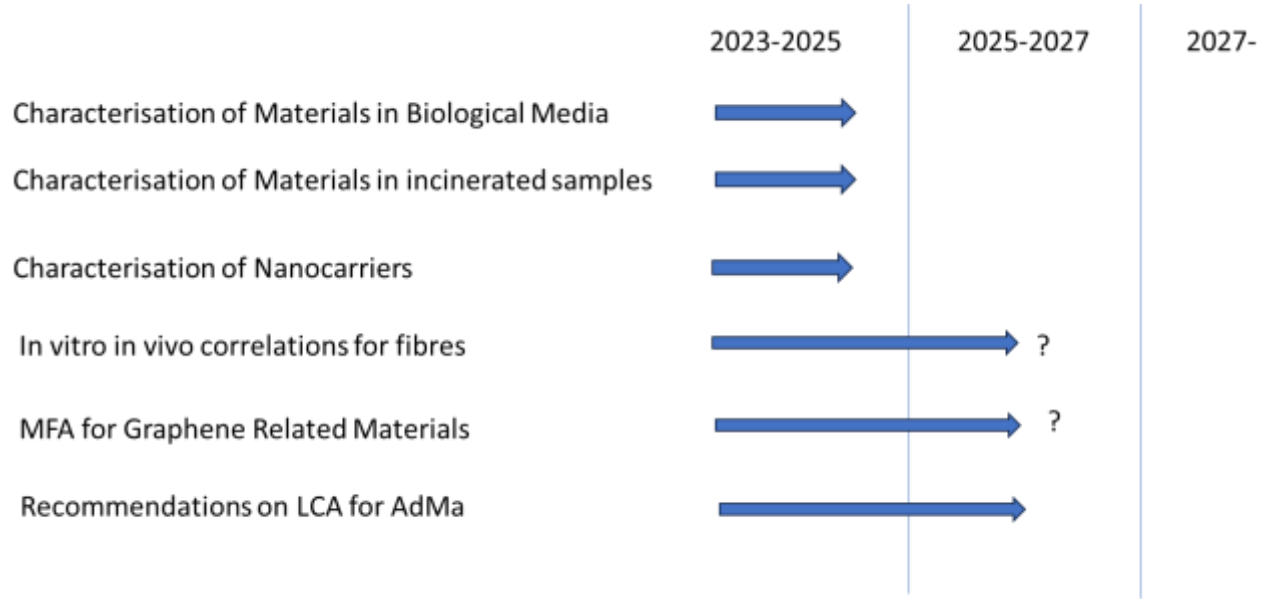

Figure 16: Schematic tentative overview on gaps to be identified in MACRAMÉ and tentative timeline.

7 Conclusion

This report provides a summarising introduction to five technical destination of the MACRAMÉ R&I approach:

i) characterisation and sample preparation of carbon-based materials,
ii) aerosol generation for inhalation studies,
iii) *in vitro* / *ex vivo* models for inhalation toxicology,
iv) ecotoxicity studies, and
v) sustainability assessment.

For each of the destinations, a roadmap has been elaborated, consisting of the following elements:

- an elaboration of the methods pertaining to the relevant area, including a prioritisation, where deemed necessary;
- needs analysis to advance the relevant methods for each destination;
- identification of the problems and hurdles hampering the methods development in each destination; and
- a gap analysis concerning the methods developments for each destination.

This roadmap summary will serve as the basis to further elaborations and reports in each on of the destination areas.

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