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Evaluation of existing guidelines for their adequacy for the microbial characterisation and environmental risk assessment of microorganisms obtained through synthetic biology

EFSA Scientific Committee,
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Abstract

EFSA was asked by the European Commission to consider synthetic biology developments for agri-food use in the near future and to determine if the use of this technology is expected to constitute potential risks and hazards for the environment. Moreover, EFSA was requested to evaluate the adequacy of existing guidelines for risk assessment and if updated guidance is needed. The scope of this Opinion covers viable synthetic biology microorganisms (SynBioMs) expected to be deliberately released into the environment. The evaluation was based on: (i) horizon scanning of published information, (ii) gap analysis of existing guidelines covering the scope of this mandate, and (iii) future outlooks. A horizon scan showed that SynBioM applications could be ready for deliberate release into the environment of the EU in the next decade. However, extensively engineered SynBioMs are only expected in the wider future. For the microbial characterisation and the environmental risk assessment, the existing EFSA Guidances are useful as a basis. The extent to which existing Guidances can be used depends on the familiarity of the SynBioM with non-modified organisms. Among the recommendations for updated Guidance, the range of uses of products to be assessed covering all agri-food uses and taking into account all types of microorganisms, their relevant exposure routes and receiving environments. It is suggested that new EFSA Guidances address all 'specific areas of risk' as per Directive 2001/18/EC. No novel environmental hazards are expected for current and near future SynBioMs. However, the efficacy by which the SynBioMs interact with the environment may differ. This could lead to increased exposure and risk. Novel hazards connected with the development of xenobionts may be expected in the wider future.

Keywords: synthetic biology, agri-food use, microorganism, environmental risk assessment, microbial characterisation, deliberate release, genetically modified microorganism (GMM), chassis

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Summary

Synthetic biology (SynBio) is the engineering of biology, which aims to develop new biological systems and impart new functions to viable cells. SynBio has potential applications in the agri-food system that would include deliberate release of these microorganisms into the environment, requiring a premarket authorisation in the European Union (EU).

This Opinion addresses four terms of reference (ToR) requested by the European Commission on the safety evaluation of SynBio developments in agri-food use: 1) identification of sectors/advances in the agri-food system considered among SynBioM developments (excluding bioremediation, de-extinction, bioweapons/biopreparedness, medical use, biofuels); 2) identification of potential risks and potential novel hazards SynBioMs could pose for the environment (restricted to wildlife and excluding humans and farmed animals); 3) evaluating the adequacy of existing guidelines for risk assessment of current and near future SynBioMs (arriving to EU market in the next decade; due to the fast research developments also SynBioMs expected in the wider future such as minimal cells, protocells and xenobiology were included); and 4) identification of specific areas where updated Guidance is needed. The scope of this Opinion is limited to viable microorganisms expected to be deliberately released into the environment.

The previous work on SynBio by Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Consumer Safety (SCCS) was considered and complemented with the outputs of a horizon scan, which was commissioned by the European Food Safety Authority (EFSA) to identify the most realistic and forthcoming SynBioM cases of relevance to the terms of reference. In addition, two complementary cases were analysed by the authors of this Opinion to include SynBioM developments expected in the wider future.

It is recognised that information on new SynBioM products for deliberate release may not be made publicly available at early stages in their development, and this limits the predictive capacity of this Opinion.

As a first step, the current Opinion evaluates existing guidelines for the microbial characterisation and environmental risk assessment (ERA) of genetically modified microorganisms for deliberate release into the environment, for their general adequacy for risk assessment of microorganisms obtained through SynBio. Second, with the above-mentioned cases in mind, the existing guidelines were evaluated for their adequacy and sufficiency. As a third step, an overall gap analysis was performed capturing also outlooks for future SynBioM developments. EFSA consulted EU Member States and interested parties during a public consultation and the comments received have been incorporated whenever appropriate.

ToR 1: Identification of sectors in the agri-food system considered among SynBioM developments

No other sectors/advances in addition to the six identified by the SCENIHR, SCCS and SCHER were identified. These were 1) genetic part libraries and methods; 2) minimal cells and designer chassis; 3) protocells and artificial cells; 4) xenobiology; 5) DNA synthesis and genome editing; and 6) citizen science (do-it-yourself biology). For this opinion, citizen science was not considered relevant and therefore excluded.

No clear criteria to differentiate between a GMM and a SynBioM could be identified. From a technical point of view, SynBioM applications could be ready for deliberate release into the environment of the EU within the next decade. However, extensively engineered SynBioMs (e.g. minimal cells and protocells, or xenobionts), falling within the remit of EFSA, are not expected to be deliberately released during the next decade.

ToR 2: Identification of potential risks and potential novel hazards SynBioMs could pose for the environment

Hazards for current and near-future SynBioMs aimed to be deliberately released into the environment do not differ from those for GMMs developed by established techniques of genetic modification. Although no novel hazards have been identified for near future SynBioMs, the efficacy by which SynBioMs interact with their biotic and abiotic environment may differ. This may lead to increased exposure and therefore may result in higher risk. Altered efficacy can be related to levels of exposure by e.g. 1) increased environmental survival and host colonisation, 2) increased invasiveness and, 3) increased competition in naturally evolved microbial communities due to enhanced fitness, thereby displacing beneficial microorganisms or disrupting, 4) altered metabolism, e.g. by changes in substrate utilisation opening new environmental niches;

5) altered lifestyle, e.g. by energy use (aerobic versus anaerobic) opening new environmental niches.

Wider future SynBioMs, including xenobionts, aimed for deliberate release into the environment, may lead to novel hazards compared to microorganisms developed with established genetic modification techniques, e.g. due to 1) new-to-nature organisms/products/constituents possibly with poorly understood interactions with its biotic and abiotic environment, 2) xeno-proteins with new enzymatic properties, i.e. modified substrate specificity or higher environmental robustness, and so opening new environmental niches and 3) substantial reduction of the genome could lead to unexpected interactions with other organisms (e.g. those that lead to evasion of the immune system).

The assessment to identify novel hazards or risks should always be performed on a case-by-case basis.

ToR 3: Evaluating the adequacy of existing guidelines for risk assessment of current and near future SynBioMs

For the microbial (genotypic and phenotypic) characterisation of SynBioMs and the safety of the genetic modification, the FEEDAP Guidance on microbial characterisation (EFSA FEEDAP Panel, 2018), the CEP statement (EFSA CEP Panel, 2019) and the GMM Guidance (EFSA GMO Panel, 2011) are useful as a basis for the risk assessment. The adequacy of existing EFSA Guidances for SynBioMs depends on the degree of familiarity of the SynBioM and chassis with the non-modified microorganism. Whole genome sequencing (WGS) as basis of the analysis is adequate and essential for SynBioMs, irrespective of them being bacteria, archaea, viruses or eukaryotic microorganisms such as protists, fungi and micro algae. WGS can be used for taxonomic identification, identification of antimicrobial resistance genes, searching sequences related to antimicrobial production, toxigenic and virulence/pathogenic characteristics, mobile genetic elements and characterisation of the genetic modifications of the SynBioMs.

For the ERA of SynBioMs, the EFSA GMM Guidance (EFSA GMO Panel, 2011) is a useful basis. The EFSA GMM Guidance is adequate for assessing horizontal gene transfer (HGT) potential for near-future SynBioM cases. Future EFSA Guidance updates would benefit from expanding with descriptions of approaches to test for adverse effects and their likelihood resulting from HGT. The comparative approach is still feasible for near-future SynBioM cases. Given a potentially altered efficacy of near future SynBioMs to interact with their biotic and abiotic environment, it is noted that the risk assessment covering ERA compartments as potential SynBioM habitats beyond the main receiving one may become more relevant than currently foreseen. For wider future cases, the comparative approach may still be sufficient, depending on the familiarity of the SynBioM with non-modified microorganisms with a history of safe use.

For post-market environmental monitoring (PMEM), the EFSA GMM Guidance provides the principles for detection and PMEM, which are applicable for near future products containing viable SynBioM.

ToR 4: Identification of specific areas where updated guidance is needed

For microbial and molecular characterisation of SynBioM, as well as for GMMs, guidance and knowledge is recommended to be developed: 1) for micro-algae: specialised guidance for genomic and phenotypic characterisation; 2) for yeast and fungi: phenotypic testing for antimycotic resistance; 3) for xenobionts: for the new-to-nature components guidance not solely based on history of use and the comparative approach; 4) for xenonucleic acids: guidance for characterisation and detection; and 5) for xenobionts, extensively engineered SynBioM, micro-algae and viruses: suitable model systems for testing virulence and pathogenicity for non-target hosts.

For ERA of SynBioMs as well as for GMMs, future guidance updates should take into consideration all agri-food uses and take into account all microorganisms (e.g. micro-algae, viruses), their relevant exposure routes and receiving environments and should address all 'specific areas of risk' as per Directive 2001/18/EC with definitions of endpoints and descriptions of up-to-date methodologies. For extensively engineered SynBioMs, such as xenobionts, other risk assessment approaches may be considered that are not solely based on the comparative approach for new-to-nature components.

For PMEM for near-future SynBioMs as well as for GMMs, guidance and knowledge is recommended to be developed: 1) descriptions of fit-for-purpose approaches to monitor for

potential adverse effects resulting from the deliberate environmental release and 2) detailed descriptions of detection methods. For PMEM for wider future SynBioMs, suitable detection methods can be challenging to provide, because of the xeno-DNA structure.

The following is recommended: i) research for innovative approaches in the frame of ERA of SynBioMs as well as GMMs, focusing on methods to assess HGT, invasiveness and other areas of risk for SynBioMs deliberately released into the environment; ii) additional research on (functional) gene/genome annotation for all microorganisms, especially for understudied groups like micro-algae; iii) increasing knowledge on microbial interactions, microbiome function and interactions with the receiving environments for the wider understanding of community function and risk assessment/management of the effect of SynBioMs as well as GMMs; iv) development and deployment of system approaches, which should rely on large-scale mathematical and statistical models as well as on semantic technologies and big data analytics to support (environmental) risk assessment; and v) the concept of developing a limited number of engineerable, safe-by-design and reusable SynBioM chassis to create the opportunity to base the risk assessment on the performance of the chassis under prespecified environmental conditions.

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1. Introduction

Synthetic biology (SynBio) is an interdisciplinary field at the interface of engineering and biology aiming to develop new biological systems and impart new functions to viable cells. It uses modern engineering principles supported by mathematical modelling and analytical/biochemical approaches for the design, assembly and deployment of genetic parts.

The principles of standardisation and modularity facilitate the engineering process and iterative engineering cycles of 'design-build-test-learn'. This enhances the progress in developing new designs of genetic parts and their assembly into higher order biological networks with new characteristics and functions. The application of modelling and computer-aided design informs and predicts the outcomes of different engineering strategies to achieve optimal functionality. Subsequently, the models are improved by the inclusion of quantitative data generated from the 'design-build-test-learn' cycles. So, by bridging engineering, life sciences and computational modelling, the range of applications and products that can be developed expands and the predictability of biotechnology is improved.

SynBio has potential applications in the food and feed chain that would require under current legislation a pre-market authorisation in Europe. Some of those applications may include the deliberate release of engineered organisms into the environment (e.g. as SynBio plants or SynBio microorganisms for plant growth promotion or plant protection product (PPP)) and hence will be subject to an environmental risk assessment (ERA). This is also reported by the Scientific Advice Mechanism (SAM) Explanatory note of April 2017 (SAM, 2017) on new techniques in agricultural biotechnology,[1] outlining the agricultural application of new techniques in the fields of SynBio and gene drive.

Previously in 2014 and 2015, the European Commission's Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Consumer Safety (SCCS) had published[2] three opinions on SynBio, addressing six SynBio developments: 1) genetic part libraries and methods; 2) minimal cells and designer chassis; 3) protocells and artificial cells; 4) xenobiology; 5) DNA synthesis and genome editing; and 6) citizen science (do-it-yourself biology). The opinions addressed the definition of SynBio, risk assessment methodologies and safety aspects, risks to the environment and biodiversity and research priorities in the field of SynBio. SCENIHR, SCCS and SCHER concluded that new SynBio developments may be assessed using current methodology used for genetically modified organisms (GMOs) risk assessment. However, the rapidly evolving technologies may require existing methodologies to be revisited at regular intervals and improved when necessary to continue ensuring safety.

Therefore, as a proactive measure, the European Commission requested the European Food Safety Authority (EFSA) for an opinion on GMOs developed using SynBio approaches and the implications, if any, for risk assessment methodologies. EFSA identified a total of six Work packages to be reflected in 6 Opinions to be developed, according to organism group and risk assessment aspects (see Section 1.3).

1.1. Definitions for SynBio for the Terms of Reference

Synthetic biology has been previously defined as follows by SCENIHR, SCCS and SCHER upon request of the European Commission[2]: 'Synthetic biology is the application of science, technology and engineering to facilitate and accelerate the design, manufacture and/or modification of genetic materials in viable organisms'. This definition is used as a starting point for the present Opinion due to the request of the European Commission to build on the Opinions of SCENIHR, SCCS and SCHER.

The Convention on Biological Diversity[3] further clarified that 'While there is no internationally agreed definition of 'synthetic biology', key features of synthetic biology include the 'de novo' synthesis of genetic material and an engineering-based approach to develop components, organisms and products'. This further clarification establishes the link for the request to support the European Union (EU) in the work under the Convention on Biological Diversity and the Cartagena Protocol on Biosafety.

[1] https://ec.europa.eu/research/sam/index.cfm?pg=agribiotechnology

[2] SCENIHR, SCCS, SCHER (2014) Synthetic Biology I Definition, Opinion, September 2014. Available online: http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_044.pdf
SCENIHR, SCCS, SCHER (2015) Synthetic Biology II -Risk assessment methodologies and safety aspects, Opinion, May 2015. Available online: http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_048.pdf
SCENIHR, SCCS, SCHER (2015) Synthetic Biology III –Research priorities, Opinion, December 2015. Available online: http://ec.europa.eu/health/scientific_committees/emerging/docs/scenihr_o_050.pdf

[3] https://www.cbd.int/doc/meetings/cop/cop-12/information/cop-12-inf-11-en.pdf

1.2. Background and Terms of Reference as provided by the requestor

Building on SCENIHR, SCCS and SCHER Opinions and taking into account available literature and previous analyses carried out by EU Member States or at the international level, the Commission asked EFSA, in accordance with Article 29(1) of Regulation (EC) No 178/2002 (European Commission, 2002), for an opinion on GMOs developed through synthetic biology and their implications for risk assessment methodologies. The scope of the present mandate is limited to agri-food uses.[4] In this context:

1) EFSA was asked to consider whether and which **newer sectors/advances** should be considered among SynBio developments, in addition to the six identified by the SCs (ToR1).
2) EFSA was requested to identify, if possible, **potential risks** in terms of impact on humans, animals and the environment that current and near-future SynBio developments could pose; in this respect EFSA was also asked **to identify potential novel hazards** compared with those of established techniques of genetic modification[5] (ToR2).
3) EFSA was requested to determine whether the **existing guidelines** for risk assessment are adequate and sufficient for current and near-future SynBio developments or whether there is a need for updated guidances (ToR3).
4) In the latter case EFSA was requested to identify the specific areas where such **updated guidances are needed** (ToR4).

EFSA was also requested to provide technical and scientific expertise on risk assessment of GMOs obtained through SynBio to support the EU in the work under the Convention on Biological Diversity and the Cartagena Protocol on Biosafety.

1.3. Interpretation of the Terms of Reference and scope

The mandate received from the EC was split in six Work packages by EFSA to be reflected in 6 Opinions to be developed:

1) Microbial characterisation and ERA of genetically modified microorganisms.
2) Molecular characterisation and ERA of genetically modified plants.
3) Food and feed risk assessment of genetically modified microorganisms.
4) Food and feed risk assessment of genetically modified plants.
5) Molecular characterisation and ERA of genetically modified animals.
6) Food and feed risk assessment of genetically modified animals.

The current Opinion is addressing Work package 1.

The following interpretations to the ToRs are made for the development of this Opinion, in agreement with the EC[6]:

- Not all of the six developments previously identified by the SCs were considered relevant and citizen science was excluded.
- 'Near future': for this mandate, this is interpreted as reaching the EU market in the next decade. This is reflected in Section 2.3 when selecting three out of four of the case studies. Due to the specific biology of microorganisms, the wide variety of organisms that can be used (including viruses and algae) and fast research development in this field, also SynBioM developments expected in the wider future as minimal cells and protocells were not excluded and xenobiology was included as a case study.
- 'Agri-food uses': on footnote 5 of the mandate 'For the purpose of this mandate agri-food uses means agri/food/feed products falling within the remit of EFSA', further clarifications were needed to determine which applications fall within the remit of EFSA, within this mandate and within the available time frame. The limited time frame led to the explicit exclusion of bioremediation applications from this mandate. By extrapolation, the following applications are also excluded from this mandate: de-extinction, bioweapons/biopreparedness, medical use, and biofuels (see inclusion Criterion 2 in Section 2.2).

[4] For the purpose of this mandate, agri-food uses means agri/food/feed products falling within the remit of EFSA.
[5] For the purpose of this mandate, the term 'established techniques of genetic modification' refers to various genetic engineering techniques that have been significantly used over the last 30 years to produce genetically modified organisms, such as those that have been authorised under Directive 2001/18/EC and Regulation (EU) No 1829/2003.
[6] See correspondence under mandate M-2018-0205 in the EFSA register of questions: http://registerofquestions.efsa.europa.eu/roqFrontend/wicket/page?3

- For this Opinion, ToR2 was limited to deliberate release into the environment (including wildlife). The exposure to humans and farmed animals (accidental or deliberate) will be specifically addressed in a further Work package of this mandate (WP3), covering the food/ feed aspects of microorganisms derived from synthetic biology approaches.
- 'Existing guidelines for risk assessment': see Section 2.1.
- The scope of this Opinion is limited to viable microorganisms expected to be deliberately released into the environment (Category 4 as defined in EFSA GMO Panel, 2011).

The Opinion is produced to support the EC, but is also targeted to the public, scientific community and stakeholders, companies and institutions that were able to comment during the public consultation.

2. Data and methodologies

2.1. Existing guidelines, ad hoc expert Working Group and its strategy

EFSA established an ad hoc expert Working Group[7] (WG) of the Scientific Committee (SC) on the Microbial Characterisation (MC) and ERA of SynBioMs. In delivering its Scientific Opinion, the SC, together with the ad hoc expert Working Group, considered the current GMO legislation and corresponding (EFSA) Guidance documents. The documents that are relevant for MC of SynBioMs are presented in Table 1; those for ERA of deliberate release of SynBioMs are presented in Table 2. Section 4 of this Opinion fulfils ToRs 2, 3 and 4. For missing issues, identified under ToR4 for example, other sectorial EFSA Guidance documents or international guidelines were also considered for their adequacy to the risk assessment of SynBioMs (i.e. applicable guidance for microorganisms used as feed additives, PPPs, novel foods, intelligent food packaging (sensors) or other uses under Directive 2001/18/EC). As agreed with the European Commission, the current mandate was not foreseen to check in detail if all these sectorial guidances are applicable to handle viable SynBioMs.

The WG has reviewed the results of horizon scanning (see Section 2.2) and the available published information. Primary references of relevance to this mandate were identified by the WG members (up to 3 March 2020).

The WG has adopted a strategy based on a three-phase approach:

- **Phase 1**, focused on the analysis of existing EFSA Guidances and underlying EU legislation, was aimed to evaluate section-by-section the adequacy and applicability of available risk assessment approaches for current and near-future SynBioM developments. Although the reference documents in Tables 1 and 2 relate to food/feed use, the evaluations in Phase 1 were carried out having a broader spectrum of microorganisms and a broader spectrum of applications in mind.
- **Phase 2** was aimed to test the adequacy of existing guidance documents in a realistic/most relevant scenario. To reach this goal, four case studies were identified and selected using the criteria described in detail in Sections 2.2 and 2.3. These four cases were used to challenge the existing guidelines and to identify possible limitations. The results of Phase 2 are prevalently presented in table format after each Section.
- **Phase 3** was aimed at an overall gap analysis that could not be captured by the previous phases (i.e. gaps disconnected from the existing guidance documents listed in Section 2.1 or disconnected from the selected cases). In addition, Phase 3 was also used to prepare outlooks for the future in Section 5.

This strategy was applied to analyse the documents listed in Tables 1 and 2. The results of Phases 1 and 2 are reported in Sections 4.1–4.6. In Section 4.7, Phases 1 and 2 are applied to the Directive 2001/18/EC (European Commission, 2001), as amended by Directive 2018/350 (European Commission, 2018), that describes under D.1 the nine so called 'specific areas of risk' to be taken into account in the ERA of GMOs that are deliberately released into the environment.

[7] https://ess.efsa.europa.eu/doi/doiweb/wg/685310

Table 1: Reference documents for the microbial characterisation

Document	Title	Link	Content
EFSA FEEDAP Panel (EFSA FEEDAP Panel, 2018)	Guidance on the characterisation of microorganisms used as feed additives or as production organisms	https://www.efsa.europa.eu/en/efsajournal/pub/5206	This FEEDAP 2018 Guidance details the steps for characterisation of GMMs used as feed additives and introduced the WGS analysis for RA for the first time
Statement of the CEP Panel (EFSA CEP Panel, 2019)	Statement: Characterisation of microorganisms used for the production of food enzymes	https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2019.5741	This statement is similar to the FEEDAP Guidance (EFSA FEEDAP Panel, 2018), although it covers production organisms only, focused on food enzyme applications
In preparation	EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain	https://www.efsa.europa.eu/sites/default/files/consultation/consultation/consultation_EFSA-Statement-WGS-micro-organisms.pdf	This draft (in public consultation) provides recommendations to applicants on how to describe the analysis and results of WGS analysis of microorganisms which should be provided to EFSA in the context of an application

GMM: genetically modified microorganisms; WGS: whole genome sequencing; RA: Risk assessment.

Table 2: Reference documents for the ERA

Reference	Title	Link	Content
EC Directive 2001/18 (European Commission, 2001)	Deliberate release into the environment of GMOs and repealing Council Directive 90/220/EEC	https://eur-lex.europa.eu/resource.html?uri=cellar:303dd4fa-07a8-4d20-86a8-0baaf0518d22.0004.02/DOC_1&format=PDF	Directive 2001/18/EC sets out requirements for the environmental risk assessment (ERA) of GMOs after their deliberate release into the environment. This Directive applies to all GMOs introduced into the environment, including GMMs. This Directive is the basis of the GMO Panel ERA Guidance for GM plants (EFSA GMO Panel, 2010) and the GMO Panel GMM Guidance (EFSA GMO Panel, 2011) Annex II, Section D, states that conclusions on the potential environmental impact in relevant receiving environments from the release of GMOs shall be drawn for each of the nine points (so called 'areas of risk') mentioned, based on an ERA Section D.1 refers to GMOs other than higher plants, such as GMMs. Section D.2 refers to higher plants

Reference	Title	Link	Content
EC Directive 2018/350 (update of Annexes II and III of Directive 2001/18) (European Commission, 2018)	Amending Directive 2001/18/EC of the European Parliament and of the Council as regards the ERA of genetically modified organisms	https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX:32018L0350	This Directive amends Annex II (risk assessment methodology), III (information requirements) and IV (additional requirements for GMOs) of Directive 2001/18/EC Annex II was updated to incorporate the terminology used to describe the six steps in the ERA approach as described in the GMO Panel ERA Guidance for GM plants (EFSA GMO Panel, 2010) Annex II D.2 is updated to reflect the nine areas of risk that are described in the GMO Panel ERA Guidance for GM Plants (2010) Annex II D.1, referring to the specific areas of risk for GMMs, is not updated Annexes II and IV were only updated for GM plants
EFSA GMO Panel, 2011	Guidance on the risk assessment of GMMs and their products intended for food and feed use	https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2011.2193	This guidance focuses on the risk assessment of food and feed consisting, containing or produced from GMMs. It includes ERA for products consisting or containing viable GMMs (Category 4). The aspects on the molecular characterisation of GMMs in this Guidance are superseded by the FEEDAP 2018 Guidance (EFSA FEEDAP Panel, 2018)
EFSA GMO Panel, 2010	Guidance on the ERA of genetically modified plants	https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2010.1879	This GMO Panel 2010 Guidance focuses on the ERA of GM plants, reflecting the 'specific areas of risk' from Directive 2001/18/EC
Commission Regulation (EU) No. 283/2013, Annex part B on microorganisms as PPP, (European Commission, 2013)	Setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market	https://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2013:093:0001:0084:EN:PDF	This Part B provides data requirements for active substances consisting of microorganisms, including viruses, e.g. Data should be supplied on 'persistence and multiplication'
ENV/JM/MONO (2012)1	OECD guidance to the environmental safety evaluation of microbial biocontrol agents (mBCA), Series on Pesticides No 67	http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2012)1&doclanguage=en	This document provides data requirements regarding the characterisation of the mBCA, application type and pattern, fate and behaviour, environmental toxicity to terrestrial and aquatic non-target organisms (NTOs)
ENV/JM/MONO/(2019)8	Report of the 9th Biopesticides Expert Group Seminar on Test Methods for Micro-organisms Series on Pesticides No 100	http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2019)8&doclanguage=en	Experiences are described form OECD countries dealing with assessment of microbial products

Reference	Title	Link	Content
ENV/JM/MONO (2014)2	Report of the OECD/ KEMI/EU workshop on microbial pesticides: assessment and management of risks, Series on Pesticides No 76	http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2014)2&doclanguage=en	Reports on the OECD/KEMI/EU workshop on microbial pesticides assessment and management of risks
ENV/JM/MONO (2010)40	Guidance document on horizontal gene transfer between bacteria	https://www.oecd.org/env/ehs/biotrack/46815958.pdf	This Guidance also provides some direction on how to assess potential risks resulting from HGT are described
EFSA (2016)	Guidance to develop specific protection goals options for ERA at EFSA, in relation to biodiversity and ecosystem services	https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2016.4499	This Guidance describes the methodology to be used during an ERA problem formulation, in order to specify the protection goals that need to be addressed

GMO: genetically modified microorganisms; HGT: horizontal gene transfer.

2.2. Horizon scan for SynBioM cases

ToR1 (and part of ToR2) was addressed with a horizon scanning. SynBio is a rapidly developing research field resulting in new techniques likely to be used for the design of GMOs. To get an overview of the SynBioM developments likely to enter the market in the next decade, EFSA requested a contractor via a procurement call, to perform a horizon scanning process of SynBioM developments in the agri-food sector. The information extracted from the review of the full text publications is presented in the external report (van der Vlugt, 2020). The outcome of this horizon scan addressed ToR1. To create a list of cases that fall within the current Opinion, the following inclusion criteria were used:

- Inclusion Criterion 1: Products consisting or containing viable GMOs, as defined in Commission Directive 2001/18/EC (European Commission, 2001), therefore able to replicate and transfer genetic material. The GMMs covered include archaea, bacteria and eukarya, as previously agreed in the EFSA GMO Panel 2011 Guidance. Eukarya includes filamentous fungi, yeast, protists and microscopic algae. For this mandate viruses, viroids and bacteriophages were also included.
- Inclusion Criterion 2: Products related to 'agri-food uses meaning agri/food/feed products falling within the remit of EFSA'. The following types of uses and products fall within the remit of EFSA and may include viable organisms that can be deliberately released into the environment:
  - human or animal consumption, e.g. food/feed products, probiotics, microbiome engineering (assessed under the food, feed, novel food and health claims regulations);
  - silage and feed fermentation agents;
  - starter cultures, fermentation agents and biocontrol in food;
  - pesticide (PPP and biocides) use, e.g. viruses including bacteriophages, bacteria or fungi PPP inducing resistance or biocontrol agents;
  - biosensors, e.g. used in food contact materials;
  - bacteriophage and bacteria for food decontamination;
  - plant growth promoting microorganisms, e.g. providing and mobilising nutrients or growth factors, in rhizosphere or plant tissue.
- Inclusion Criterion 3: Products deliberately released into the environment for experimental, scaling up or commercial reasons. The product should be meant for deliberate release or its deliberate release is connected to its use, comprising:
  - experimental stages in the laboratory, greenhouse, farm, mesocosm, field trial or food pilot plant. All listed cases must be past the scientific proof of concept phase that merely describe the methodology;

  - placing on the market. This includes greenhouse, farm, pond, field trial or feed and food production plants and products already on the market in territories outside of the EU.

- Inclusion Criterion 4: Product possibly reaching the EU market during the next decade. This criterion could not be derived from the literature sources itself but was scored based on the expert judgement of the contractor.

Relevant SynBio cases were identified using a search strategy including scientific publications and grey literature, websites demonstrating commercial activities in SynBio, databases of regulatory agencies and iGEM projects (International Genetically Engineered Machines, www.iGEM.org). In total, 11 SynBio cases were listed as a result from the search strategy. Five cases fully passed all the inclusion criteria. Six SynBio cases are listed separately as they do not fully match all the criteria, or the available information was insufficient to verify that the case matched all the inclusion criteria.

During the screening, a significant number of publications was found describing the use of SynBioM as therapeutic products for human or animal use. Details revealed that these microorganisms were designed for medical use (e.g. vaccination), not for agro-food use. Unless further use as a novel food or food/feed additive was mentioned, these SynBioMs were not considered.

The listed cases represent agro-food products for human and animal consumption, for use as PPP, biosensor and fertiliser. The readiness of the cases to be introduced into the EU market during the next decade (inclusion Criterion 4) is based on the technical advances of the product and does not reflect the official status of the cases. These SynBioMs were constructed by making use of genetic part libraries (i.e. the use of genetic elements) and genome editing. More advanced techniques like xenobiology or use of minimal cells, artificial cells or protocells are not yet in use for the construction of a SynBioM with a functionality as agro-food product. These techniques are still in an early phase of development and successful use for constructing a SynBioM that can survive outside the laboratory will take more time. Intensive research interest in the development of SynBioM for agro-food products is demonstrated by the many iGEM projects and these research interests will result in more extensively engineered SynBioM in the wider future. The horizon scan did not identify other sectors/advances in addition to the six identified by SCENIHR, SCCS and SCHER[8] (see Introduction).

Based on the horizon scan, two cases (Cases 1 and 3, see below) were selected for assessing the adequacy of the existing EFSA Guidances for the ERA of SynBioM.

2.3. Selection of case studies

As shown in Figure 1, there is no distinct borderline between the microorganisms obtained using existing genetic modification techniques and those derived from SynBio. Considering this lack of clarity, four case studies were selected for Phase 2 of this Opinion, with Cases 1, 2 and 3 being part of a continuum between classical GMM and SynBioM, and with Case 4 at the far end of the spectrum of being least familiar.

These four case studies were considered with the aim to assess the adequacy of the existing guidance documents in a **realistic/most relevant** scenario. While these four case studies helped to focus on the current status of SynBioM developments, SynBio is rapidly evolving and therefore the chosen cases may not be representative for all future applications. Hence the 10 years to market timeline for 'near-future' SynBio developments (see ToR2) was applied with flexibility and this Opinion covers a potentially broader timeframe (e.g. using Case 4). Additionally, the horizon scan used for this Opinion (Van der Vlugt, 2020) was based on published information, rather than other products in unpublished R&D stage.

From the list of cases reported in the external report delivered by the contractor (see Section 2.2), relevant cases were selected to represent:

- different microorganisms, with a possibility to reach the market;
- different routes of exposure (intended use) and the anticipation of hazards for humans, animals and the environment (including plants);
- high extent of genetic modification;
- major phenotypic changes or novel phenotypes.

[8] The SCENIHR, SCCS and SCHER assessed six SynBio developments: 1) Genetic part libraries and methods; 2) Minimal cells and designer chassis; 3) Protocells and artificial cells; 4) Xenobiology 5) DNA synthesis and genome editing; and 6) Citizen science (Do-It-Yourself biology) (see Introduction).

The reason for these selection criteria is that cases resembling (in complexity) GMOs that are already being assessed by the EFSA Guidances, would not require an alteration of such guidance and therefore would not be useful for the development of this Opinion.

The selected cases are as follows:

Case 1 deals with a plant virus, the *Citrus tristeza virus (CTV),*[9] that was redesigned to express a spinach defensin to counteract the citrus greening disease by inhibiting the phloem-limited bacterium *Candidatus Liberibacter asiaticus*. The engineered virus was redesigned to express three copies of the gene coding for spinach defensin in citrus plants while preserving its ability to infect, replicate and spread throughout vascular tissues in citrus trees. No interaction with the *Citrus* genome is expected based on the current knowledge about the CTV This SynBioM was selected from the contractor's list (ID 1, Datasheet A in (Van der Vlugt, 2020)).

Case 2 is selected to focus on the ERA of an organism potentially used as a biofertiliser in soil. This case concerns the soil bacterium *Klebsiella oxytoca*, and its gene cluster encoding the nitrogen fixation pathway. This case was taken from literature (Temme et al., 2012). The cluster of genes involved in the conversion of atmospheric N_2 to ammonia was redesigned and refactored by deleting all the non-coding regions and the non-essential and regulatory genes and sequences. The codons of selected essential genes were optimised, and the new coding regions were organised into operons and placed under the control of synthetic parts (promoters, ribosome binding sites and terminators). The expression of the refactored gene cluster is regulated by genetic sensors and circuits. In the SynBioM no heterologous DNA was introduced. The possibility for market entry is not excluded at this stage, as products sharing very similar features are already on the market outside of the EU.

Case 3 focuses on the route of exposure for humans/animals and was selected from the contractor's list (ID 4, Datasheet B, in (Van der Vlugt, 2020)). This describes a *Saccharomyces cerevisiae* yeast that produces raspberry ketone (Lee et al., 2016) A de novo pathway to produce this aromatic compound was constructed assembling four heterologous genes, resulting in pathway engineering and synthetic enzyme fusion. While the published case was described to be used in wine, the WG assumed the use in beer and assumed the uptake of viable organisms present in the final beer product. This renders the case more useful for the present Opinion.

Case 4 includes two xenobiological variants of bacterial origin (**Cases 4A and 4B**). Both represent maximum extents of non-familiarity of the genetic setup and resulting phenotypes and were taken from literature. A general explanation on 'xenobionts' is given in Section 3.5 below. Case 4A describes a bacterium with a modified DNA codon usage, which allows incorporation of the non-canonical amino acid pyrrolysine (Pyl) by utilising the universal 'amber' stop codon UAA as a binding site for a modified tRNA. As a result, the bacterial cell produces new-to-nature ('xeno') peptides and proteins (Acevedo-Rocha and Budisa, 2016). It is envisioned that the organism lacks the capacity for biosynthesis of Pyl and the respective tRNA (Tharp et al., 2018) and would therefore be auxotrophic for both. Case 4B describes a bacterium with the same potential of producing xeno-proteins, but which would carry instead DNA stretches of an artificial genetic polymer, i.e. a xeno-nucleic acid (XNA) with six instead of the common four bases (A, C, G and T). The resulting xenobiont with the additional base pairs d5SICS and dNAM (Malyshev et al., 2014) would lack the capacity to synthesise the xeno-nucleotides, and so their replication would depend on the addition of these two XNA building blocks as growth factors. The XNA organism would also require the growth factors described for Case 4A.

2.4. Consultation

In line with its policy on openness and transparency, EFSA consulted EU Member States and interested parties via an online public consultation. Between April and June 2020, they were invited to submit their comments on the draft SCER Panel Scientific Opinion. Following this consultation process, the document was revised by the SC and the experts of its SynBioM ERA WG. The comments received were considered and have been incorporated when appropriate. The outcome of the online public consultation is reported in an EFSA Technical report that will be published on EFSA's website together with the final this Scientific Opinion.

[9] Non-EU *Citrus tristeza virus* is considered as quarantine virus (EFSA, 2019).

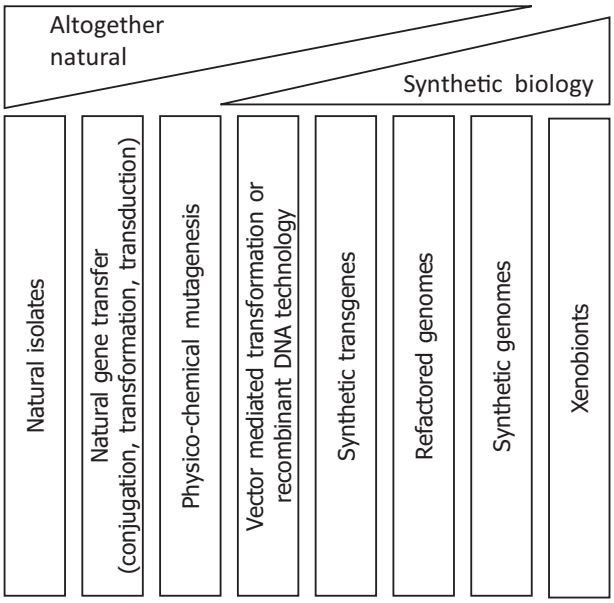
3. SynBioM-specific background

Notwithstanding the definition of SynBio, in practice, as show in Figure 1, there is not a defined distinction between the microorganisms obtained using the established genetic modification techniques and those derived for from SynBio approaches.

The level of genetic modification in SynBio agents or products might range from being very similar to the ones from GM technology assessed so far or can go (far) beyond with unfamiliar characteristics at the genotypic or phenotypic level, such as in the case of xenobionts (see below Section 3.5).

Directed or accelerated evolution, a widely used technique which works through mutagenesis and selection, can also be used in a SynBio design framework.

The term 'Genome editing', although indicated by SCENIHR, SCCS and SCHER as a SynBio development, is not specifically considered in this Opinion. Genome editing refers to a range of techniques that edit the genome in a targeted way by inducing (site-)specific changes with or without targeted insertion of DNA sequences (see SAM, 2017). Although genome editing is increasingly used in SynBio, because of its capacity to 'edit' the genome in a targeted way, it only refers to some of the techniques available to produce a SynBio product and is therefore not a specific topic in this Opinion.



Modified after de Lorenzo, 2010 https://onlinelibrary.wiley.com/doi/full/10.1002/bies.201000099

Figure 1: Continuum going from natural to synthetic biology products

3.1. Chassis concept

The notion of biological chassis is central to contemporary SynBio. Clarification of the terminology and the criteria for chassis is important due to the high numbers of new species and strains proposed as SynBio platforms, and the limited differentiation between organisms used as the recipient for recombinant DNA (i.e. host-vector systems) and those specifically defined as a SynBio chassis.

The concept of chassis is borrowed from mechanical engineering and could be defined as follows: 'A SynBio chassis is an engineerable and reusable biological platform with a genome encoding a number of basic functions for stable self-maintenance, growth and optimal operation but with tasks and signal processing components optionally edited for strengthening performance under pre-specified environmental conditions'.[10]

The quest for the optimal chassis has been addressed from various perspectives. The most common approach reported in the scientific literature is to start with a well characterised bacterium (e.g. *Escherichia coli, Pseudomonas putida* or *Bacillus subtilis*) and then delete the genetic loci from

[10] Note that the key here is optimal performance, not minimized genome size (although deletion of unnecessary functions will cause a degree of genome reduction).

the genome that are not necessary for growth in a given environmental context. The extant genomes of microorganisms are populated with many DNA sequences that are dispensable and even deleterious for the final application of the bacterium. For the time being, some of these minimised *E. coli* (Umenhoffer et al., 2017), *P. putida* (LePrince et al., 2012; Martinez-Garcia and de Lorenzo, 2019) and *B. subtilis* (Reuss et al., 2017) strains are the most efficient available chassis for the implantation of new genetic circuits. The consequence is that a standardised chassis could allow for facilitating the risk assessment.

The definition above implies that specific target environments and tasks may optimally require different chassis. Moreover, the same concept indicates availability of growingly upgraded variants derived from the same initial organism. A list of the most frequently described microbial chassis for SynBioM in published literature is reported by Nora et al. (2019).

An approach like the one for chassis is frequently applied in the current regulated GMMs used for the production of valuable compounds, such as enzymes, fine chemicals, amino acids and vitamins. Most of these compounds are produced in a limited number of bacterial and fungal species (de Lorenzo et al., 2020). It can, therefore, be anticipated that the number of feasible chassis for SynBioM will be limited because of the demand for reusability and interoperability.

3.2. Safe-by-design, firewalls and containment strategies

The design factor in SynBioM may be higher than for established techniques of genetic modification. This offers the possibility to address safety issues already in the 'design–build–test–learn' cycle. Safe-by-design is a well-known principle in mechanical engineering that aims to develop new products by taking into account all safety aspects of the product as well as of the process from the initial ideas through to the final product. Integration of risk assessment and risk reduction questions during the development of the product is a key aspect of safe-by-design. This necessitates safety awareness and a different mindset among scientists and process and product developers. The safe-by-design approach is now also followed in biotechnologies in general and is an interesting concept for further SynBioM developments.

It is also worthwhile considering in the design of SynBioM built-in risk reduction mechanisms that can reduce the likelihood for horizontal gene transfer (HGT) (e.g. genetic firewalls (Schmidt, 2010) and control the spread of synthetic genes into the environment (e.g. by auxotrophy). While these so-called firewalls have potential for gene containment, the efficacy of individual concepts and mechanisms should be assessed on a case-by-case basis, supported by experimental data.

3.3. Barcoding

A unique identifier is a prerequisite for any SynBioM before it may be released into the environment for detection purposes, as required by existing legislation for GMOs. In the design of the SynBioM, it is expected that this unique identifier has been built in as a kind of DNA barcode. Standardised barcoding may be possible e.g. at the chassis level.

3.4. Genetic part libraries

The genetic part libraries are repository of genetic elements, engineered genes, regulatory regions and DNA fragments with characterised properties and functions (see for example http://www.addgene.org/synthetic-biology/). These DNA fragments are units designed for interoperability and can be assembled in novel engineered genetic systems. Specific software tools support the design of complex DNA sequences by combining different genetic parts (McLaughlin et al., 2018).

3.5. Xenobionts

Xenobionts are organisms made up of non-natural products. While such organisms are currently not fully developed and viable, research efforts to generate these have been significant (Kubyshkin and Budisa, 2017). Major targets to replace natural building blocks of cells by new-to-nature products are proteins and nucleic acids (Anasova et al., 2016). The genetic machinery of xenobionts is refactored to allow the insertion of rare, modified or novel amino acids, resulting in xeno-peptides or xeno-proteins (Agostini et al., 2017). Considering the central importance of proteins and nucleic acids for any cellular life on Earth, modifications at these levels can result in organisms with unfamiliar properties. Xeno-proteins acting as enzymes, therapeutic agents, toxins or with other activities could have a

substantial potential for medical applications, industrial biotechnology or environmental engineering. XNA can potentially extend the universal four-base genetic alphabet to a six-base code, so generating a genetic machinery that would not be functional in natural organisms. XNA is regarded as a means of biocontainment or a genetic firewall because it strongly limits an unintended spread and expression of synthetic genes into natural microbiomes, while conversely it bears the potential to produce many xeno-proteins (Acevedo-Rocha and Budisa, 2011).

In context of this document, the new-to-nature cell building blocks mainly considered included xeno-proteins (and peptides) as well as XNAs (Cases 4A and 4B).

4. Evaluation of the adequacy of existing guidelines

This Section describes the evaluation of the adequacy of existing risk assessment guidances listed in Tables 1 and 2. In phase 1 of such evaluation, the relevant parts of such guidances were evaluated for adequacy. In the subsequent phase 2, the adequacy was appraised using the above SynBioM cases 1–4.

4.1. General outline to risk assessment of GMM

The risk assessment of GMMs, seeking an authorisation in EU under specific regulations, as described in the relevant EFSA Guidances, is based on a stepwise approach that can be summarised in three main phases:

- Microbial and molecular characterisation (Section 4.2): aimed to identify the GMM and its parental organism and to identify and characterise related hazards (e.g. antimicrobial resistance (AMR), virulence, pathogenicity, toxin production).
- The safety of genetic modification (Section 4.3): focused on the intended and unintended effects of the GM and potential additional hazards derived from it.
- The ERA (Sections 4.5 and 4.7): targeted to assess potential adverse effects to humans, animals and the environment resulting from the deliberate release of the GMM into the environment. ERA is further complemented with Post Market Environmental Monitoring (Section 4.6).

4.2. Microbial and molecular characterisation

4.2.1. Characterisation of the microorganism and taxonomic identification by WGS

Phase 1: Analysis of the Chapter 2, Section 2.1 'Characterisation of the microorganism' of the EFSA FEEDAP Panel Guidance 2018, and the EFSA CEP Panel Statement of 2019

Whole genome sequencing (WGS) is the preferred approach for the characterisation of the microorganism under assessment and this is mandatory for bacteria and yeast and optional for fungi. The WGS should be used preferentially to identify the microorganism and to document the genetic modification. The WGS will also be searched for the presence of genes of concern as AMR genes and virulence/toxigenic factors. As such, it forms an essential element for the risk assessment of (genetically modified) microorganisms.

This approach is applicable to SynBioMs with some additional considerations needed:

- WGS is considered essential also in the characterisation of SynBioMs: WGS must be performed on all SynBioMs, including those with a fungal chassis.
- It is essential that in all cases the purpose of the development of the SynBioM using a certain chassis design must be explained.
- Guidance on the minimum set of information related to the WGS data is given in 'EFSA statement on the requirements for whole genome sequence analysis of microorganisms intentionally used in the food chain' (in preparation[11]). When SynBioMs would be extensively engineered, a low degree of identity with reference genomes may be expected. For organisms containing extra chromosomal elements such as plasmids, these sequences are also required.

[11] https://www.efsa.europa.eu/sites/default/files/consultation/consultation/consultation_EFSA-Statement-WGS-microorganisms.pdf

- For (SynBioM) micro-algae, the development of specialised guidance(s) for the interpretation of the WGS data is recommended.
- For some SynBioM viruses, it may be required to address the high degree of sequence variability and its relation with biological activity, for example by higher sequence depth than for other microorganisms (further explained in Section 5.3).
- For xenobionts with modified nucleic acids, the standard WGS may not be applied. Similarly, for xenobionts harbouring modified/rewritten genetic code, the interpretation of the WGS data could be misleading (e.g. transformation of a stop codon in a codon for non-canonical amino acids).

Table 3: **Phase 2** testing of WGS on the cases

Case	Specific evaluations	Conclusion on the adequacy	Updates recommended
1–3, 4A	The WGS of the organism is a basis for the risk assessment and the guidances can be used for this	The viral, bacterial or yeast recombinant genome can be determined based on existing guidances. For some SynBioM viruses, a higher sequence depth may be required compared with other microorganisms	None
4B	The standard WGS cannot be applied. Also, the interpretation of the WGS is not yet covered	Sections are not adequate	New tools to be developed

WGS: whole genome sequencing; SynBioM: microorganism obtained through synthetic biology.

Phase 1: Analysis of Chapter 2.1 'Taxonomic identification of GMM' of the EFSA FEEDAP Guidance 2018, and Chapter 1.1 of the EFSA CEP Panel Statement of 2019

The taxonomic identification of the microorganism is a prerequisite for the risk assessment and also the basis for the application of the European Qualified Presumption of Safety (QPS) concept (see Section 4.2.2). Identification of a microbial strain should be based on up-to-date methodologies and current knowledge about the genus and species. It should be based on WGS for bacteria and yeast and WGS is also preferred for filamentous fungi (Section 2.1 of the EFSA FEEDAP Guidance 2018 and Section 1.1 of the EFSA CEP Panel statement 2019). The application of this approach to SynBioM needs some additional considerations:

- Taxonomic identification is only relevant for a SynBioM if there is enough familiarity of the chassis with a non-modified microorganism.
- WGS is mandatory for collecting taxonomic information of all the different building blocks of a SynBioM organisms.
- If possible, the chassis should be taxonomically identified. If the chassis is derived from one non-modified microorganism, the taxonomic identification can be based on the existing guidelines. As a SynBioM chassis can consist of several building blocks, eventually modified, the assessment needs to identify each of these blocks. For this phylogenomics of average nucleotide identity (ANI) can be used for the chassis sequence blocks. Also, depending on the degree of familiarity of the SynBioM with the non-modified microorganism, specific sequences commonly used for taxonomic identification (e.g. 16S rRNA, housekeeping genes, multilocus sequence typing (MLST)) can also be applied if these sequences are present and not or not extensively modified. If the chassis is derived from different microorganisms and/or extensively modified, guidance provided in Section 2.5.2 'Characteristics of modified sequences, Identifying sequences' of the FEEDAP Guidance, and Section 1.5.2 of the CEF statement; from designed organism can be useful. In this Section, the terms 'inserted sequences' and 'donor organism' should be interpreted as building blocks and chassis. In case the data do not allow the assignment of the SynBio chassis under assessment to a known microbial species, its phylogenetic position with respect to the closest relatives should be provided.
- The organism should, in accordance to the guidance on GMM, be deposited in an internationally recognised culture collection and should be kept for the authorised period of the SynBioM.
- No guidance is present in the current EFSA guidance for the taxonomic identification of microorganisms other than bacteria, yeast and fungi. Guidance for the taxonomic identification

of viruses is available in the QPS opinion (EFSA BIOHAZ Panel, 2020). Guidance for (SynBio) micro-algae is recommended to be developed.
- Unequivocal taxonomical identification of most xenobionts may not be established.

Table 4: **Phase 2** testing of taxonomic identification on the cases

Case	Specific evaluations	Conclusion on the adequacy	Updates recommended
1–3	The taxonomic identification is a basis of the risk assessment	Sections are adequate	None
4A, 4B	Taxonomic identification for these xenobionts is not possible	Not relevant	None

4.2.2. QPS approach

Phase 1: Assessment of Chapter 1 'Assessment' of the EFSA FEEDAP guidance 2018, and Chapter 1.4.1 of the EFSA CEP Panel Statement of 2019

The Chapter describes a specific approach for the risk assessment that applies to those species of microorganisms included in the list of recommended biological agents for the QPS status (EFSA, 2007; EFSA BIOHAZ Panel, 2020). The QPS evaluation is based on extensive literature searches to discover the body of knowledge and possible safety concerns for humans, animals and the environment related to their release. Those strains qualifying for the QPS approach are presumed safe for target species, consumer and the environment without the need for specific studies. The QPS status is also applicable to GMMs if the recipient strain qualifies for the QPS status, and if the genetic modification does not indicate a concern (EFSA BIOHAZ Panel, 2020). As the QPS assessment is based on the unambiguously taxonomic identification of the microorganism and on its body of knowledge including the safety for humans, animals and the environment, the concept, as such, is not applicable for SynBioMs.

The QPS concept is worthwhile for considering as a basis for the risk assessment of building blocks of SynBio microorganisms (e.g. chassis, metabolic building blocks). As there are no or limited tests available for assessing the virulence/pathogenicity/toxicity of SynBioM, the option to rely on the body of knowledge of building blocks available in literature is valid when there is sufficient familiarity of the SynBioM/chassis with the QPS organism. However, it should be considered that genome minimisation may lead to new features of concern, e.g. to the loss of antigens that could render the SynBioM invisible to the immune system, therefore altering the safety status of the organism.

Table 5: **Phase 2** testing of QPS on the cases

Case	Specific evaluations	Conclusion on the adequacy	Updates recommended
1, 2	QPS status of these 2 organisms has not been assessed	QPS status for any of the building blocks could be assessed in the frame of an application	None
3	*Saccharomyces cerevisiae* is a QPS organism	QPS is valid for the risk assessment of *Saccharomyces cerevisiae*	None
4A, 4B	These xenobionts have strongly limited familiarity with existing microorganisms	The QPS concept cannot be applied	None

QPS: qualified presumption of safety.

4.2.3. Antimicrobial and antimycotic susceptibility

Phase 1: Assessment of the Chapter 2; Section 2.2 'Antimicrobial susceptibility' of the EFSA FEEDAP Guidance 2018; and Chapter 1.3 of the EFSA CEP Panel Statement of 2019

The FEEDAP Guidance follows the concept expressed by EFSA (EFSA BIOHAZ Panel, 2008) that AMR is a food risk and the bacteria intentionally introduced into the food chain should not increase the environmental load of AMR genes. This guidance based the risk assessment of AMR on the following topics:

- The difference between acquired and intrinsic AMR. When a strain of a typically susceptible species is resistant to a given antimicrobial drug, it is considered to have an 'acquired resistance' for that compound. In contrast, intrinsic resistance to an antimicrobial is understood

as inherent to a bacterial species and is typical of all the strains of that species. Intrinsic AMR is generally not considered a safety concern.
- The use of a combined approach of phenotypic testing, based on the MIC determination and its comparison with defined cut-off values that discriminate susceptible from resistant strains, and on WGS analysis. The cut-off values are defined for the bacteria species most commonly notified to EFSA as feed additives.

SynBio organisms, as other microorganisms assessed by EFSA, should not add to the pool of AMR genes already present in the gut and environmental microbiomes or otherwise increase the spread of AMR. However, some limitations in the application of the guidance can be foreseen:

- Intrinsic vs acquired AMR (Section 2.2 of the guidance): the concept of intrinsic resistance is strictly related to a single taxonomical unit, mostly the species. For SynBioM, the species concept may not apply, due to wide intervention on the genome. Therefore, in that case any AMR gene harboured by a SynBioM should be considered as a hazard.
- The application of a phenotypic test based on MIC value, as described in Section 2.2.1 of the Guidance, may be of limited use for SynBioM. The cut-off values are determined on distribution of MICs within a defined taxonomical unit, generally the species. For SynBioM, the species assignation, as above reported, may be hampered by the substantial genome synthesis or refactoring. Consequently, reference cut-off values may not be present.

Section 2.2.3 Interpretation of the results does not apply to SynBioM for the reasons above reported.

Assessment of antimycotic susceptibility

Antimycotic susceptibility testing is formulated as a QPS qualification for QPS yeast species used as viable cells in food and feed applications. This qualification is reconfirmed in the latest QPS opinion to be related to a safety concern (EFSA BIOHAZ Panel, 2020). A specific EFSA guidance for applicants to comply with this requirement is not yet available.

Table 6: **Phase 2** testing of antimicrobial and antimycotic susceptibility on the cases

Case	Specific evaluations	Conclusion on the adequacy	Updates recommended
1, 2, 4A	The presence of AMR genes needs to be assessed	Section is adequate	None
3	The presence of the AMR genes in the engineered strain should be assessed. The antimycotic susceptibility should be assessed	Section is adequate	Guidance is recommended to be developed for testing the antimycotic resistance of SynBioM yeast
4B	XNA is not expected to encode for AMR genes functional in environmental microorganisms	Not relevant	None

AMR: antimicrobial resistance; XNA: xeno-nucleic acid.

4.2.4. Antimicrobial production

Phase 1: Assessment of the Chapter 2; Section 2.3 'Antimicrobial production' of the EFSA FEEDAP Guidance 2018

The Regulation on Feed Additive (Regulation (EC) No 429/2008[12]) states that these agents should not produce antimicrobials relevant for use in humans and animals and this applies also to microbial products. The absence of antimicrobial activity should be demonstrated by testing the culture supernatants against reference strains known to be susceptible to a range of antibiotics. If there is a positive outcome in one or more species, the inhibitory substance should be identified.

As for other microorganisms assessed by EFSA, conventional or GMM, SynBio organisms should not produce antimicrobials that may select in microbial environmental communities cross-resistance to critically important antimicrobial (CIA) and highly important antimicrobial (HIA) for human medicine as established by WHO. The approach defined by the FEEDAP Guidance (EFSA FEEDAP Panel, 2018) in

[12] Regulation (EC) No 429/2008 of 25 April 2008 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the preparation and the presentation of applications and the assessment and the authorization of feed additives (text with EEA relevance).

Section 2.3 applies to SynBioM. An exception could be a SynBioM specifically designed to exert an antimicrobial activity against undesirable bacteria.

Table 7: Phase 2 testing of antimicrobial production on the cases

Case	Specific evaluations	Conclusion on the adequacy	Updates recommended
1	Viruses by nature have a biocidal activity on specific organisms/cells. However, viruses do not produce compounds that can induce cross-resistance to clinically relevant antimicrobials	Not relevant	None
2	The production of antimicrobial compounds in *K. oxytoca* should be assessed	Section is adequate	None
3	The considered yeast, *S. cerevisiae*, does not produce antimicrobial compounds, unless specific pathways are introduced during the SynBioM design	Not relevant for yeasts, unless the genetic determinants for antimicrobial compounds are inserted in the SynBioM	None
4A, 4B	Xenobionts can produce both known and/or new compounds with antimicrobial activity	Section is partially adequate. Phenotypic testing against the list of reference bacteria is applicable WGS interrogation for antimicrobial compound production pathways is not applicable due to the limitation in sequencing of xenobionts	Guidance to be developed

SynBioM: microorganism obtained through synthetic biology; WGS: whole genome sequencing.

4.2.5. Toxigenicity and pathogenicity

Phase 1: Assessment of the Chapter 'Considerations of the GMM and/or its product for human health', of the EFSA GMO Panel GMM Guidance 2011

In the guidance, the assessment of toxigenicity and pathogenicity of SynBioM is based on three different approaches for collecting evidence that should be used in a weight of evidence approach. The first is based on the history of use, the body of knowledge of the strain or its close relatives mainly performed by literature searches. The second is based on bioinformatics analysis of the WGS for the presence of known virulence factor or toxic compounds. The third is based on testing of the microorganism in model systems.

- The basic principle of this weight of evidence approach is in principle also applicable for SynBioM. The degree of certainty on the safety of the SynBioM will, however, depend on the degree of familiarity with the non-modified microorganism, because this will determine the applicability of the existing body of knowledge to the SynBioM.
- The 2011 EFSA Guidance for risk assessment of food and feed from GMMs requests that the toxicological assessment considers the presence and levels of newly expressed proteins, the potential presence of other new constituents, the possible changes in the levels of endogenous constituents beyond normal variation and the impact of other changes in composition due to the genetic modification. These general requirements are valid also for the risk assessment of SynBioM.
- Genotoxicity for metabolites produced by SynBioM microorganisms can be tested by several *in vitro* tests using culture concentrates following Organisation for Economic Co-operation and Development (OECD) guidelines, referred to in several EFSA Guidances (e.g. EFSA GMO Panel, 2011; EFSA CEP Panel, 2009): the Ames test (OECD 471, 1997), chromosomal aberration test (OECD 473, 2016a), *in vitro* micronucleus assay (OECD 476, 2016b). If adverse effects are encountered in these *in vitro* tests, *in vivo* tests are necessary, and guidance is available, e.g. in the EFSA CEF enzyme Guidance. These general requirements are valid also for the risk assessment of SynBioM.

- The assessment of the virulence/pathogenicity of a SynBioM will, due to a lack of suitable model testing systems, mainly rely on the body of knowledge and the bioinformatics analysis of the WGS for virulence factors. Even in the absence of known virulence factors it may be difficult to exclude the virulence/pathogenicity of a totally new SynBioM with little or no familiarity to natural microorganisms. Suitable model systems are recommended to be developed to bridge this gap in the assessment. This is especially the case for xenobionts due to their 'new-to-nature' characteristics, for which the body of knowledge will be limited or not existing.
- Guidance is missing for testing the toxigenicity and virulence/pathogenicity of SynBioM micro-algae and for testing of virulence/pathogenicity of viruses (viruses do not produce toxins but are often pathogenic by taking over the cell machinery of infected cells).

The following information may complement the above assessment for completeness purposes. However, the mentioned guidelines will be investigated in depth during Work Package 3 under this mandate. The issue of toxigenicity and pathogenicity (including intracellular replication and interaction with the human genome) is addressed in other guidelines, e.g. the United States Environmental Protection Agency (US EPA[13]) published specific microbial guidances for testing toxicity/pathogenicity/ virulence of microorganisms used as pesticide. The same guidances are also referred to in the EC Regulation for microbial PPPs part B from 283/2013 (for PPPs) and 284/2013 (for active substances used in PPPs) based on requirements documented in the European Commission regulation. In these regulations, the requirements are detailed for assessing the effects on human health in a TIER I and TIER II approach, in which TIER II has only to be conducted if the TIER I has shown adverse effects.

Table 8: **Phase 2** testing of toxigenicity and pathogenicity on the cases

Case	Specific evaluations	Conclusion on the adequacy	Updates recommended
1	The CTV virus must be assessed for toxigenicity and pathogenicity	The Section is not adequate	Guidance for SynBio viruses for the aspects of MC and ERA (including the assessment of impact on plants and insects health)
2, 3	The toxigenicity and pathogenicity can be assessed based on existing guidance	The Section is adequate	None
4A and 4B	Lack of familiarity with existing microorganisms	The Section is not adequate	Suitable model systems for xenobionts

MC: microbial characterisation; ERA: environmental risk assessment.

4.2.6. Impact on gut microbiota

Phase 1: Analysis of the Chapter 4 (Sections 4.1 and 4.2) 'Impact on gut microbiota' of the EFSA FEEDAP Guidance of 2018

This Section is aimed to assess if the feed additive has an impact on gut microbiota, by examining whether its use results in an overgrowth or shedding of potentially pathogenic microorganisms. This requirement applies only to: i) the feed additives that showed an adverse effect related to digestive tract disturbances in the animal studies; ii) to those for which an adverse effect on the gut microbiota can be anticipated (e.g. antimicrobial activity); or iii) to those designed to reduce numbers of enteropathogens. No additional appraisals (e.g. a wider effect on the gut microbiome), other than the colonisation and shedding of microbial pathogens, are required.

This approach is not applicable to SynBioMs, unless the use of these microorganisms falls under the three above provisions.

Moreover, the impact on gut microbiota will be specifically addressed in a further Work package for this mandate (WP3), covering the food/feed aspects of microorganisms derived from SynBio approaches.

[13] Acute oral toxicity/pathogenicity US EPA 712-C-96-315 (US EPA, 1996a) OPPTS 885.3050; Acute pulmonary toxicity/ pathogenicity; exposure by inhalation or intratracheal injection US EPA 712-C-96-318 (US EPA, 1996b) OPPTS 885.3150; Acute injection intraperitoneally or subcutaneously US EPA 712-C-96–318 (US EPA, 1996b) OPPTS 885.3200; Virulence/pathogenicity in cell cultures for assessing intracellular replicating microorganisms US EPA 712-C-96–321 (US EPA, 1996c) OPPTS 885.3500; Subchronic/systemic toxicity/pathogenicity by 28–90 days study US EPA 712-C-96–232 (US EPA, 1996d) OPPTS 885.3600.

4.3. Safety of the genetic modification

4.3.1. FEEDAP Guidance and CEF Statement as basis of the assessment

Phase 1: Analysis of the Chapter 'Purpose of genetic modification and description of WGS as a basis for characterisation – Characterisation of the microorganism' of the EFSA FEEDAP Guidance 2018 (Chapter 2; Section 2.5); and CEF statement Chapter 1.5

The EFSA FEEDAP Panel, (EFSA FEEDAP Panel, 2018) Guidance for the microbial characterisation bases the risk assessment of the genetic modification of bacteria and yeasts on the analysis of WGS. So, this analysed Section of the GMM Guidance focuses on the following topics:

- The purpose of the genetic modification should be described.
- The characterisation of the structure of the genetic modification should be carried out using WGS data for bacteria and yeasts and is recommended for filamentous fungi.
- When the WGS of a fungal GMO is not available, all the steps to obtain the genetic modification should be described and identification of all genetic material potentially introduced into the recipient/parental microorganism is required.

The WGS-based risk assessment of the genetic modification is considered suitable for the risk assessment of SynBioMs, to detect genes of concern and to predict HGT (e.g. location on mobile elements, plasmids, transposons, etc.). However, some modifications in the application of the guidance can be foreseen:

- Characteristics of the modified sequences ((EFSA FEEDAP Panel, 2018) Chapter 2, Section 2.5.1). The Section 'DNA from defined donor organisms' may not be suitable for extensively engineered SynBioM. Therefore, more information should be provided on the sequence design for SynBioM. Particular attention should be paid to genes of concern, such as genes encoding AMR, toxins and virulence factors.
- Structure of the genetic modification based on WGS ((EFSA FEEDAP Panel, 2018); Chapter 2, Section 2.5.2). This Section is relevant for all SynBioM, including eukaryotic organisms.

Additional aspects of SynBioM risk assessment of the genetic modification, such as those dealing with genetic parts and designer chassis, are not completely addressed by the FEEDAP Guidance (2018). So, some Sections are also taken from the EFSA GMO Panel Guidance (2011) analysed below.

4.3.2. GMO Guidance on characteristics of the recipient strain

Phase 1: Analysis of 'Characteristics of the recipient or (when appropriate) parental organism' (EFSA GMO Panel, 2011, Chapter III; Section B, 1.1)

Below are the evaluations of Sub-Sections from the GMO Guidance (EFSA GMO Panel, 2011) that address additional topics not covered above in Section 4.3.1. These are the following Sub-Sections: 1.1.2 on phenotypic and genetic markers for the GMM; 1.1.4 on identification and detection techniques of the GMM; 1.1.5 on source and natural habitat of the parental microorganism; 1.1.6 on transfer of genetic material to other microorganisms; 1.1.7 on genetic stability of the GMM; 1.1.8 on the pathogenicity, ecological and physiological traits and 1.1.9 on the history of use.

Sub-Section 1.1.2 describes guidance for the characterisation of phenotypic and genetic markers. For the ERA assessment of SynBioM, relevant phenotypic features should be determined. For these features, the Sub-Section 1.1.2 of the EFSA GMO Panel 2011 Guidance is adequate and can be extended to features which can be relevant. The relevance and therefore the choice of the parameters to be characterised will depend on the SynBioM to be assessed and in which degree the comparative approach to the non-modified organism can be applied. Therefore, for certain xenobionts, due to their modification with new-to-nature components, this phenotypic characterisation according to the guidance could be of no relevance.

Sub-Section 1.1.5 describes guidance for the required information on the source and natural habitat of the parental microorganism. Information on the natural habitat of the organism from which the chassis of the SynBioM has been derived is useful to include in the ERA assessment if there is still enough familiarity with the non-modified organism. The data could be provided following the guidance described in Sub-Section 1.1.5.

Sub-Section 1.1.6 describes guidance on the required information to assess the transfer of genetic material to other microorganisms and is considered adequate for SynBioMs. The characterisation of the

SynBioM includes the identification of mobile genetic elements and the properties for which they code (e.g. AMR, virulence factors, toxins). These elements, and the open reading frames they contain, need to be identified based on the WGS. The mobile elements and their host range should be documented as indicated in Sub-Section 1.1.6 if the specificity has deliberately changed in relation to the designed purpose of the SynBioM (e.g. genetic material intended to be spread to an extended host range) this should be clearly characterised, eventually experimentally confirmed. The new mobilising properties or newly designed mobile genetic systems need to be incorporated in the existing databases so that they become available for the EU and for the bioinformatics analysis of the WGS of other SynBioM. In Sub-Section 1.4.6 from the guidance from the EFSA GMO Panel (2011), complementary guidance is provided that could be useful for SynBioM, as are the presence of sequences within the SynBioM that could enhance or modify gene transfer or integration of the trait into the genome of other microorganisms, and the presence of genes in the GMM that could provide selective advantage to other microorganisms as a consequence of unintentional gene transfer.

Sub-Section 1.1.7 describes guidance on how to assess the genetic stability that can be applied on the SynBioM. Information on the genetic stability of the SynBioM should be provided for the strain at the premarket phase. This needs to be based on the comparison of the WGS of the SynBioM before and after large-scale fermentation. Other fingerprinting techniques as mentioned in Sub-Section 1.1.7 are insufficiently sensitive. In Sub-Section 1.1.7, several aspects in relation to stability/instability of the SynBioM are missing. If instability is occurring, it would need to be documented how this would affect the phenotype. For SynBioM designed to be intentionally instable (e.g. to go to suicide in certain conditions), the instability changes in relation to environmental conditions should be documented. Conversely, mechanisms specifically designed to enhance the genetic stability of the SynBioM (e.g. modifications in recombination system) should be characterised.

Sub-Section 1.1.8 describes guidance for the required information on the pathogenicity, ecological and physiological traits and is considered adequate for SynBioMs. The pathogenicity, ecological and physiological traits of the SynBioM should be addressed. The toxigenic and virulence/pathogenic potential should be documented with the whole human population in mind. The information of the ability to colonise plants, animals or humans should be provided (following bullet point 3 of this Section) but also the wider environment should be included. The involvement of the SynBioM (chassis) in environmental processes should be provided as mentioned in bullet point 4 of this Section. As for SynBioM organisms with limited familiarity, literature surveys and database searches would not suffice, and experimental work using model systems could be necessary. The information requested in this Section should be extended to the information on the intended effects of the SynBioM that could be the colonisation of a certain environmental niche and the execution of a probiotic or immunomodulatory effect. The guidance for testing the efficacy of the intended effects of the SynBioM is out of the scope of this Opinion and should be addressed by the specific guidances related to the requirements of the specific legislation under which the assessment is performed.

The characterisation of pathological, ecological and physiological traits as mentioned in Sub-Section 1.1.8 should be better addressed in the ERA Section and not in the characterisation of the organism.

Sub-Section 1.1.9 describes guidance how to describe the history of use. It is significant that any information on previous uses or releases of the SynBioM or organisms based on the same basic chassis should be provided, including literature references or other documentation. Emphasis should be placed on information that relates to possible impacts on human or animal health or the environment.

4.3.3. GMO Guidance on the origin of inserted sequences

Phase 1: Analysis of the Chapter 'Characteristics of the origin of the inserted sequences [donor organism(s)]' (EFSA GMO Panel, 2011; Chapter III; Section B; 1.2); the Chapter 'Characteristics of the modified sequences – Inserted sequences' (EFSA FEEDAP Panel, 2018, Chapter 2.5.2; Section 2.5); and CEF statement Chapter 1.5.2

The Chapters are subdivided into sequences from defined donor organisms, synthetic DNA or nucleic acids from environmental samples. These Sections are applicable when sequences are inserted (or implanted) in a chassis of a SynBioM. The source and function of the inserted sequences are important to determine potential toxicity, virulence or allergenicity of the gene product for humans, animals, plant health and the environment, but also to determine other traits that are significant for survival and competition of the SynBioM in the environment in the niches where it is introduced or can spread to. The part on designed sequences would be most applicable for SynBioM. For xenobionts, the text should include also the term XNA instead of only referring to DNA.

Table 9: **Phase 2** for combined Sections 4.3.1, 4.3.2 and 4.3.3

Case	Specific evaluations	Conclusion on the adequacy	Updates recommended
1–2-3	The chassis is well known, origin and function of the insert is known; there is familiarity with the sequence of the non-modified organism	Section is adequate	None
4A and 4B	Xenobionts with new-to-nature proteins or XNA	Not adequate	Guidance for characterisation of xenobionts

XNA: xeno-nucleic acid.

4.3.4. GMO Guidance on information on the GMM

Phase 1: Analysis of 'Information on the GMM' (EFSA GMO Panel, 2011; Chapter III; Section B; 1.4)

The GMM Guidance focuses on the rate and level of expression of gene products as results of the genetic modification. Three different topics are considered:

- the condition affecting gene expression;
- the cellular localisation of the recombinant proteins (e.g. intracellular, secreted);
- for enzymes, the function and mode of action.

This approach has some limitations for the assessment of SynBioM. While most of the GMM are designed to express a single trait (e.g. enzyme production, vitamin pathway, production of a single amino acid), the SynBio approach is generally targeted to re-design metabolic pathways, substantially affecting many cellular functions and not a single gene product. Therefore, the approach of the guidance may not fully be adequate because a comparative approach could for specific cases no longer be sufficient.

Table 10: **Phase 2** testing of information on the GMM on the cases

Case	Specific evaluations	Conclusion on the adequacy	Updates recommended
1, 2, 3	The inserted gene(s) can be assessed for their function as separate traits	Section is adequate	None
4A and 4B	Xenobionts based on DNA coding can still be sequenced by conventional methodology and the sequence can, based on the degree of novel amino acids to be built in, related to biological functioning, e.g. by searching for genes of concern as AMR genes and genes potentially coding for toxins or virulence/pathogenicity	This approach is partly applicable	New approaches for xenobionts with novel genetic coding based on XNA

XNA: xeno-nucleic acid.

4.4. Concluding remarks for microbial and molecular characterisation and safety of the genetic modification

Concluding remarks for Sections 4.2 and 4.3:

- For the microbial (genotypic and phenotypic) characterisation of SynBioM and the safety of the genetic modification, the FEEDAP Guidance on microbial characterisation (EFSA FEEDAP Panel, 2018), the CEP statement (EFSA CEP Panel, 2019) and the GMM Guidance (EFSA GMO Panel, 2011) are useful as a basis for the assessment.
- Adequacy of the existing EFSA Guidances for SynBioM depends on the degree of familiarity of the chassis with the non-modified microorganism. The extent to which the existing body of knowledge on the microorganism can be used in the risk assessment will be higher when there is a high degree of familiarity with the chassis. The following guidance is adequate and sufficient in this context:
  - WGS analysis, essential for SynBioM, irrespective of them being bacteria, archaea, viruses, viroids or eukaryotic microorganisms such as protists, fungi and micro-algae.

  - Explaining the purpose of the development of the SynBioM using a certain chassis is essential.
  - WGS for taxonomic identification, identification of antimicrobial resistance genes, searching sequences related to antimicrobial production, toxigenic and virulence/pathogenic characteristics, mobile genetic elements and characterisation of the genetic modifications of the SynBioM.
  - QPS concept for the risk assessment of building block of SynBio microorganisms (e.g. chassis, metabolic building blocks);
  - phenotypic testing for the production of antimicrobial compounds;
  - weight of evidence approach for the assessment of toxigenicity and pathogenicity based on bioinformatics analysis, body of knowledge and the use of model systems;
  - need for collecting information on the source and natural habitat of the recipient microorganism;
  - possibility of transfer of genetic material to other microorganism;
  - genetic stability;
  - pathogenicity, ecological and physiological traits;
  - history of use;
  - rate and level of expression of gene products resulting from the genetic modification;
  - need for a unique identifier.

- The following guidance and knowledge is recommended to be developed for microbial and molecular characterisation of SynBioM as well as for GMMs:

  - for micro-algae: specialised guidance for genomic and phenotypic characterisation;
  - for yeast and fungi: phenotypic testing for antimycotic resistance;
  - for xenobionts: guidance not based on history of use and not solely based on the comparative approach for the new-to-nature components;
  - for XNA: guidance for characterisation and detection;
  - for xenobionts: extensively engineered SynBioM, micro-algae and viruses, suitable model systems for testing virulence and pathogenicity for non-target hosts.

4.5. Environmental risk assessment

The ERA focuses on potential adverse effects resulting from the interaction between the SynBioM and its receiving environment, including biotic and abiotic components. The genotypic and phenotypic characterisation of the SynBioM are significant elements for the ERA as these data can be used to make assumptions on the behaviour of the SynBioM in the receiving environment and its potential interactions.

The following Sections take into consideration the original EFSA Guidance documents, specifically developed for food/feed uses, as well as Directive 2001/18/EC (European Commission, 2001) which deals with deliberate release of GMOs in the environment. This Directive is amended by Directive 2018/350 (European Commission, 2018) to reflect the terminology used in the EFSA Guidance on ERA of GM plants (EFSA GMO Panel, 2010) such as the use of the term 'area of risk' (Table 1). These 'specific areas of risk' described in Directive 2001/18/EC have not been explicitly addressed in the GMM Guidance of the GMO Panel in 2011. This is because this document was drafted to cover food feed uses of viable microorganisms (Category 4) only and does not cover the wider range of uses to be covered within the scope of this current Opinion.

Hence, for this Opinion, the text of the guidance has been considered also addressing 'specific areas of risk' as mentioned in Directive 2001/18/EC, Annex II (D.1) (European Commission, 2001) and taking in consideration, for each 'specific areas of risk', the five steps in ERA as described by the EFSA Guidance on ERA for GM plants, GMO Panel, 2010 (problem formulation including hazard identification, hazard characterisation, exposure characterisation, risk characterisation, risk management strategies). For each 'specific areas of risk', several pathways to harm are described, considering all the receiving environments that a SynBio organism may enter or spread to.

4.5.1. Principles and strategies for risk assessment of genetically modified microorganisms

Phase 1: Assessment of Chapter II of EFSA GMO Panel, 2011

The Chapter deals with:

- objective of the ERA
- categorisation of the GMMs and their products (Categories 1–4)
- comparative approach
- unintended effects.

All the elements mentioned are equally applicable to SynBioM as for viable GMMs, with the possible exception for the comparative approach (see Section 4.5.2). As for GMMs, unintended effects of SynBioM may occur as a side-effect of the newly introduced trait(s) and/or of the genetic modification techniques applied.

This Chapter is applicable to SynBioMs, subject to the following considerations:

- Objective of the risk assessment: The definitions of risk assessment should be checked if they are in line with those of Directive 2001/18/EC (European Commission, 2001).
- Categorisation of the GMMs and their products: Only the part on viable GMM (Category 4) is relevant for this Opinion as the GMM Categories 1–3 do not concern products containing viable GMMs.

Table 11: **Phase 2** testing of principles and strategies for risk assessment of GMMs (as per the EFSA GMO Panel Guidance of 2011), on the cases

Case	Specific evaluations per case	Conclusion on the adequacy	Updates recommended
1–4	All are Category 4 GMMs	Partly adequate, only text with respect to general principles of ERA, text on Category 4 organisms and on (un) intended effects is adequate	Broaden the scope beyond food and feed use (and refer to Directive 2001/18/EC as relevant legislation) (European Commission, 2001)

GMM: genetically modified microorganisms; ERA: environmental risk assessment.

4.5.2. Comparative approach

Phase 1 Assessment of the Chapter on 'Principles and strategies for risk assessment of GMMs' (EFSA GMO Panel, 2011; Chapter II)

The Section of the Chapter focuses on the comparative approach as a key general principle in the ERA of GMMs. As SynBioMs may be extensively modified or redesigned, the comparative approach may not equally be sufficient for SynBioMs. Therefore, a revision of the Section is recommended to include the following aspects:

- According to the GMM Guidance, effects of the GMM are compared with those of the non-GMM that is applied under similar conditions. A comparator for the ERA of GMMs is therefore generally considered to be the non-GM microorganism. For SynBioMs, this may no longer be sufficient in specific cases with new-to-nature components. In those cases, other comparators could be used, such as strains derived from the same chassis with similar traits or functionalities or comparable GMMs/SynBioMs with a history of (safe) use for similar applications (familiarity). Comparators should be selected on a case-by-case basis and depending on the purpose of the test. The choice of the comparator should be explained.
- The lack of a comparator may trigger requirements for extra data to conclude on potential adverse effects on human and animal health, and the environment according to the aspects mentioned in Annex II of the Directive 2001/18/EC (D.1) (European Commission, 2001).

Table 12: **Phase 2** testing of principles and strategies for risk assessment of GMMs (comparative approach) on the cases

Case	Specific evaluations	Conclusion on the adequacy	Updates recommended
1–3	For all cases, a comparator exists in nature	The comparative approach is still feasible	None
4	Semi-synthetic organisms comprise cellular components that do not exist in nature	The comparative approach is not sufficient for such components	Risk assessment of new-to-nature SynBioM or components that do not rely on data of non-modified counterparts

SynBioM: microorganism obtained through synthetic biology.

4.5.3. Potential environmental impact of GMMs and their products

Phase 1: Analysis of the EFSA GMO Panel 2011, Chapter III, Section B, 4

The Chapter describes that the ERA of GMMs and its products should be performed in line with the requirements laid down in Directive 2001/18/EC (European Commission, 2001). This part is equally applicable to viable SynBioMs:

- Evaluation of GMM/products belonging to Categories 1–4 is described in Sections 4.1, 4.2 and 4.3 of the EFSA GMO Panel (2011) Guidance. Only aspects related to Category 4 GMMs are applicable for the scope of this Opinion as SynBioMs, like Category 4 GMMs, are living organisms. Aspects related to Category 3 are only relevant with respect to potential transfer of DNA (or XNA) from the SynBioMs to the receiving environment.
- As applications of SynBioMs are expected to be broader than only for food/feed, the description of this Section should be more general to also include other applications of SynBioMs in the area of the EFSA remit. Therefore, also other exposure routes and receiving environments need to be considered in the pathways to harm, such as the compartment air and water (aquatic systems).
- The text should be revised to specifically address all the 'specific areas of risk' as mentioned in Directive 2001/18/EC, Annex II (D.1) (European Commission, 2001) and make use of the concept of ecosystem services to derived operational protection goals, as mentioned in EFSA Scientific Committee (2016).

In addition, the text should be updated to include terminology for ERA according to the amended Annex II of Directive 2001/18/EC.

Phase 2: Analysis of the four case studies confirmed the outcome of Phase 1.

4.5.4. Environmental hazard identification and characterisation

Phase 1: Analysis of the Chapter 'Evaluation of products belonging to Category 3' (EFSA GMO Panel, 2011; Chapter III; Section B; 4.2)

The Section focuses on the ERA of HGT, e.g. recombinant DNA released from GMMs, on the potential for transfer of this recombinant DNA to other (micro-)organisms in the receiving environment and potential adverse effects resulting from this transfer.

This Section is also applicable to SynBioMs, released into the environment, containing DNA but not to genetic elements containing xeno-nucleotides (see Phase 2 table below).

Adverse effects resulting from HGT is one of the areas of risk as mentioned in Directive 2001/18/EC (European Commission, 2001) (see below) and is further addressed in Section 4.7.3 'Horizontal gene transfer'.

Table 13: **Phase 2** testing of potential HGT of the SynBioM cases

Case	Specific evaluations	Conclusion on the adequacy	Updates recommended
1	This general Section is also applicable to viruses	Adequate	None
2–3	None	Adequate	None
4A	The organism contains a new reprogrammed codon in the DNA: Instead of 'Stop' it instructs the insertion of a new-to-nature amino acid. The chemical composition of the DNA is not changed	If stretches of homologous DNA sequences occur in natural microorganisms, the DNA of the xenobiont can be subjected to double homologous recombination (DHR) as foreseen for other GMMs. However, due to the reprogrammed codon usage, such genes from the xenobiont would not be equally functional in natural recipients and so, the likelihood of providing a selective advantage to recipients is generally lower for xenobionts of this type	None
4B	The organism contains XNA, i.e. nucleic acids with two additional new-to-nature bases, so extending the genetic alphabet from a four-letter to a six-letter code	XNA will not find natural counterparts for homologous recombination in natural environments. So, the probability of gene transfer by DHR should be zero, as proposed by the 'genetic firewall' concept. However, if the XNA also includes genomic regions of > 200 bp sequences with only the natural four bases, such genetic regions may have a potential for DHR. If these flank regions with XNA, XNA elements may transfer to natural recipients. In the recipient, XNA would not be replicated due to the requirement for the new-to-nature bases as building blocks. So, the transfer would be without consequences. Uncertainties remain for the potential of a natural recipients to assimilate XNA sequences to DNA by replacing the unnatural bases by A, C, G or T, so generating new genes that could replicate in the natural hosts and provide a new function The guidances on HGT are applicable	Detection of XNA and assessing the efficacy of a genetic firewall.

GMM: genetically modified microorganisms; XNA: xeno-nucleic acid.

Phase 1: Analysis of 'Evaluation of products belonging to Category 4' (EFSA GMO Panel, 2011; Chapter III; Section B; 4.3)

The Section focuses on the aspects to be considered in the ERA of viable GMMs of Category 4:

- characterisation of GMM-receiving environments;
- potential of the GMMs to survive and proliferate in receiving environments;
- possible interactions of GMMs with their abiotic and biotic environments including indigenous microorganisms, plants and animals;
- horizontal gene transfer (HGT).

This Chapter of the guidance on Category 4 is equally applicable to SynBioMs.

Phase 2: Analysis of the four case studies confirmed the findings of Phase 1.

4.5.5. Exposure – information related to the product

Phase 1: Assessment of 'Principles and strategies for risk assessment of genetically modified microorganisms' (EFSA GMO Panel, 2011; Chapter II)

No details are provided in the existing guidance on how to perform exposure assessments of GMMs after their environmental release. In case of deliberate release, as is the topic of this Opinion, the release rate, the way and frequency of application and formulation will determine initial exposure levels. The subsequent exposure levels will be affected by the capacity of the microorganisms to survive, persist and invade. This equally applies to SynBioMs.

Due to limited knowledge about the specific performance of microorganisms in high diversity of environmental microhabits, it is often difficult to make predictions about their environmental fate. Under Directive 2001/18/EC (European Commission, 2001) the potential of persistence or invasiveness of the GMM must be assessed (see Section 4.7.1) and is likely to include measurements of GMM population levels over time. This is also applicable to SynBioMs.

Phase 2: Analysis of the four case studies confirmed the outcome of Phase 1.

Phase 1: Analysis of 'Information on the product' (EFSA GMO Panel, 2011; Chapter III; Section B; 2)

The type of product will determine the route and the level of exposure of the environment to the product. This Section of the GMM Guidance deals with the characteristics of the products introduced in the market and mainly focuses on GMMs belonging to Categories 1, 2 and 3. Therefore, several parts of this Section do not apply to SynBioMs intended to be deliberately released as viable cells in the environment. The topics considered pertinent for the risk assessment of SynBioMs are the following:

- Information on the production and product preparation process: the details on these processes, the possible related hazard and risk mitigation measures should be described.
- Description of the product and designation of the product.
- Intended use and mode of action.
- Composition and physical properties: this information, including the number of viable cells/g, should be given.

The paragraph 'Considerations of the GMM and/or its product for human health' addresses the subject discussed in Section 4.2.5 Toxigenicity and Pathogenicity. This Section applies to SynBioMs.

Phase 2: Although such information is only available for case number 1, and not for the others, we consider the existing guidance adequate.

4.6. Post-market monitoring

The development of a specific detection method needs to be carried out for any viable GMM that will be introduced into the environment, as a requirement under Directive 2001/18/EC (European Commission, 2001). Detection of the GMM may also be necessary for post-market monitoring, which is aimed to detect potential adverse effects on human health, animal health and the environment as a consequence of the release. This will equally apply to SynBioMs that will be released into the environment.

4.6.1. Detection

Phase 1: Assessment of Chapter 'Description of identification and detection techniques' (EFSA GMO Panel, 2011; III; Section B; 1.1.4)

Chapter 1.1.4 describes guidance for the development of identification and detection techniques for GMMs that can be applied to SynBioMs. The SynBioM needs to be easily detectable in samples from the environments in which it is released. The guidance provided in Section 1.1.4 can be used for this. For this, a unique genetic signature should be available in the SynBioM. The detection method should reach a sufficient sensitivity and should be validated for different relevant environmental samples.

For xenobionts, it can be challenging to provide a suitable sensitive detection method because of the xeno-DNA structure. If the organism cannot multiply in the environment because of a lack of suitable nucleotides/enzymes, the detection method can be of no practical relevance.

Further information will not be provided on detection, as this is outside the remit of EFSA.

Traceability is outside the remit of EFSA.

Table 14: **Phase 2** testing of "detection" on the cases

Case	Specific evaluations	Conclusion on the adequacy	Updates recommended
1 to 4A	Unique genetic signature can be derived from the recombinant DNA fragments spanning the junction of the inserted gene. Suitable detection techniques may need to be adapted for these cases in relation to the relevant environments	The Section requesting a unique genetic signature and suitable detection methods for the different relevant environments is applicable	None
4B	The XenoSynBio organisms contain unnatural nucleotides. Methods developed for detection at the DNA level may not be fully applicable to XNA to detect a unique genetic signature	The Section requesting a unique genetic signature is applicable	Detection methods should be made available but can be challenging to provide because of the xeno-DNA structure

XNA: xeno-nucleic acid.

4.6.2. Post-market environmental monitoring for use of the GMM

Phase 1: Assessment of the Chapter EFSA GMO Panel, 2011; III; Section E

This Section describes the obligation to implement a post-market environmental monitoring plan (PMEM) under Regulation 1829/2003 according to Annex VII of Directive 2001/18/EC (European Commission, 2001) for products consisting or containing viable GMMs. PMEM is meant to identify any direct or indirect, immediate and/or delayed adverse effects of GMMs of Category 4 on human health and the environment after the GMM has been placed on the market. Two types of monitoring are described, e.g. case-specific monitoring and general surveillance, the ways to perform this monitoring and to report on the results. As SynBioMs are viable microorganisms, this Section on PMEM also applies to SynBioMs. The PMEM is focused on potential adverse effects of any viable GMM, irrespective of the extent of modification or re-design. The text is recommended to be revised on one aspect: Reference to Regulation EC No 1829/2003 should be broadened to also include a wider range of uses of viable SynBioMs, other than food/feed uses alone.

The aspects of uncertainties as flagged up during product-specific risk assessments and according to the guidance of the EFSA SC to report uncertainties is tightly linked to PMEM. A case-specific monitoring plan needs to be supplied in case of uncertainties linked to the risk hypotheses used or linked to effects that may occur only after large-scale application. General surveillance is a prerequisite for all GMO applications. This surveillance is meant to detect potential adverse effects of the GM application that are not foreseen in the ERA and therefore addresses uncertainties related to, for example, indirect or long-term effects of GMOs.

Table 15: **Phase 2** testing of PMEM on the cases

Case	Specific evaluations	Conclusion on the adequacy	Updates recommended
1–4	None	Adequate, it concerns Category 4 organisms, so a PMEM plan can and must be supplied	None, but as there are no prior PMEM plans under the Directive, Part C for microorganism deliberately released in the environment, this may still raise practical questions from the applicant

PMEM: post-market environmental monitoring.

4.7. Analysis of 'specific areas of risk' from Directive 2001/18/EC

One of the general suggestions for updates in the ERA part of the EFSA GMM Guidance (EFSA GMO Panel, 2011) for SynBioMs, as well as for GMMs, was to broaden the scope beyond food and feed uses (see Section 4.5). To address this, reference is made to Directive 2001/18/EC (European Commission, 2001), as amended by Directive 2018/350 (European Commission, 2018), that describes 'specific areas of risk' to be taken into account in the ERA of GMOs that are deliberately released into the environment for all uses.

In the below Sections, for each area of risk as defined in this Directive, an overview is given of the problem formulation, examples of pathways to harm and, if applicable, recommendations for updating ERA guidance or test methods are indicated. These are equally applicable for SynBioMs as for GMMs. The same methodological approach (phases 1 and 2) used for the appraisal of EFSA guidance was applied in this Section.

4.7.1. Persistence and invasiveness, including selective advantage

Upon release, SynBioMs may survive and persist in a receiving environment or invade new environmental niches where they may exert biotic or abiotic interactions.

4.7.1.1. Problem formulation and examples of pathway to harm

A SynBioM may have adverse effects on microbiologically mediated ecosystem services by displacing native microorganisms in ecological niches to which they spread. This displacement can be a result of utilising more efficiently nutrients for growth or producing metabolites that inhibit indigenous microorganisms, e.g. releasing antimicrobial agents or changing the pH. Conversely, the SynBioM may not provide the ecosystem functions of the organisms they displace, so losing the potential, for example, to degrade a pesticide, transform a nitrogenous compound such as ammonium efficiently, lose plant symbiontic partners such as mycorrhiza or rhizobia, or inhibit suppressors of plant pathogens.

4.7.1.2. Adequacy of guidance and methods

Potential risks as a consequence of persistence and invasiveness are not explicitly addressed in the EFSA GMM Guidance (EFSA GMO Panel, 2011). Information on how to fill this gap may be derived from the Chapter 'Fate and survival' in the OECD guidance to the environmental safety evaluation of microbial biocontrol agents ENV/JM/MONO (2012)1.[14]

The GMM Guidance (EFSA GMO Panel, 2011) also does not mention methods to measure potential persistence and invasiveness of GMMs, nor does it give endpoints to measure. This also applies to SynBioMs.

In Commission Regulation (EU) No. 283/2013, Annex part B on non-GM microorganisms as PPP (European Commission, 2013) is stated that data should be supplied on 'persistence and multiplication'. If the application of a microbiological biocontrol agent is not expected to increase the natural 'background' levels of the species or related species, risks may be considered acceptable or 'not deviating' from 'normal'. In the OECD document on ERA of (non-GM) microbial biocontrol agents ENV/JM/MONO (2012)1, the key aspect of the assessment is the determination of background level of the biocontrol agent. Considering there is no background level of indigenous SynBioMs present in the environment, the relevance of this document in the context of SynBioM risk assessment is limited.

No official OECD tests/methods are available to measure persistence and invasiveness of microorganisms. In ENV/JM/MONO(2012)1 is stated that it is not feasible to develop standardised methods specifying the minimum number of different conditions, soils, application timings and samplings. This approach is considered to be not practicable (ENV/JM/MONO(2012)1).

Taking into account the specific features and extended applications of SynBioMs (traceability, diversity of receiving environment, potential for invasion), future risk assessments would benefit from new methods to test, model and so predict the survival, persistence and invasiveness of a SynBioM under a range of environmental conditions. These may include high-throughput laboratory testing methods, standardised microcosms and integration of complex environmental models.

Phase 2: For Cases 1–4, no additional aspects need to be mentioned with respect to risk assessment associated with persistence and invasiveness.

4.7.2. Selective advantage or disadvantage

Any selective advantage or disadvantage conferred to the SynBioM and the likelihood of this occurring under the conditions of the proposed release(s). This area of risk is taken together with area of risk 'persistence and invasiveness', in accordance with the EFSA GM ERA plant Guidance (EFSA GMO Panel, 2010).

[14] See http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2012)1&doclanguage=en. In ENV/JM/MONO/(2019)8, Experiences are described form OECD countries dealing with assessment of microbial products. See: http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2019)8&doclanguage=en. ENV/JM/MONO(2014)2, Reports on the OECD/KEMI/EU workshop on microbial pesticides assessment and management of risks. See http://www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2014)2&doclanguage=en

4.7.3. Horizontal gene transfer

According to the Directive, the potential for gene transfer to non-related organisms under the conditions of the proposed release and selective advantages (or disadvantages) that potential recipients may gain, must be assessed.

4.7.3.1. Problem formulation and example of pathway to harm

The transfer of genetic information from the released organism into other organisms in the environment may have consequences for human and animal health as well as for plants and more general ecosystem services. The diversity of a microbiome may be reduced, and its natural balance may be disturbed by providing a selective advantage to one or some of their members, reducing or displacing other organisms with beneficial properties. SynBioMs may affect human, animal or plant health by transfer of DNA encoding a harmful trait, to recipient microorganisms.

The first step in a potential pathway to harm would be that DNA of the SynBioM is transferred to members of the natural microbiomes through mechanisms such as conjugation, transformation, or transduction (EFSA, 2009). Microbiomes with a high diversity and cell density as well as abundant nutrients (as they occur in the gastrointestinal tract) increase the likelihood as HGT compared to low diversity environments, e.g. a sandy soil with low organic carbon. SynBioM DNA is from this point forward expressed in the recipient microorganism, resulting in a trait that negatively affects environmental microorganisms as they occur, e.g. in soils, surface waters, or gut ecosystems, including the gastrointestinal tract of humans and farmed animals. In addition to this, SynBioMs may pose further risks related to the 'new-to-nature' features of these organisms (e.g. new synthetic DNA fragments coding for non-natural gene products or XNA).

4.7.3.2. Adequacy of guidance and methods

Potential risks as a consequence of HGT are specifically addressed in the EFSA GMM Guidance (EFSA GMO Panel, 2011) for microorganisms, and no gaps were identified for SynBioMs with respect to this area of risk. Therefore, potential risks as a consequence of HGT can be assessed by using the current EFSA GMM Guidance (EFSA GMO Panel, 2011), with the exception of xenobionts.

With respect to methodology, the EFSA GMM Guidance (EFSA GMO Panel, 2011) does not mention methods how to measure HGT of GMMs, nor does it give endpoints to measure. This also applies to SynBioMs. For assessing HGT of GM plants to microorganisms by means of double homologous recombination (DHR), EFSA has issued an Explanatory note (EFSA, 2017a). The use of bioinformatic analysis for measuring HGT potential of GM plants is equally applicable to GMMs and SynBioMs. The OECD has issued a guidance document in 2010 that addresses the risk assessment related to HGT from GMMs (ENV/JM/MONO(2010)4[15]). This document, developed before the wider application of 'omic' techniques, describes no specific tests, but gives some direction on how to assess potential risks resulting from HGT.

Testing methods, which comprise high-throughput experimental studies, the identification of pathways to harm (gene transfer mechanisms, including conjugation, transformation or transduction), and the application of bioinformatic tools as well as environmental modelling, are not available. Such methods would enable testing of the HGT potential of the SynBioM and the impact of environmental conditions on transfer rates and possible adverse effects in the main receiving environments and beyond.

Phase 2: For Cases 1–3 and the hypothetical xenobiont described in Case 4A, there are no additional case-specific aspects to be mentioned for HGT. It can be predicted for Case 4A that the non-natural building block of xeno-proteins, i.e. the non-canonical amino acids and their corresponding tRNA, are not present in the receiving environment. Therefore, the likelihood that a natural gene transfer recipient would gain a selective advantage by acquiring a gene coding for a xeno-peptide or protein would be extremely low if not zero. However, for Case 4B, there is a gap with respect to evaluating the efficacy of genetic firewalls intended to be functional with the use of XNA as a means for gene containment, and also for the specific detection of XNA e.g. from environmental material.

The EFSA GMM Guidance for assessing HGT potential (EFSA GMO Panel, 2011) would remain valid for such SynBioMs producing xeno-peptides, xeno-proteins and new-to-nature carbohydrates or lipids, as long as they are encoded by DNA.

[15] See https://www.oecd.org/env/ehs/biotrack/46815958.pdf

4.7.4. Effects on target organisms

SynBioM may exert potential immediate and/or delayed environmental impacts due to the direct and indirect effects/interactions with target organisms (if applicable).

4.7.4.1. Problem formulation and example of pathway to harm

SynBioMs may reduce ecosystem services (such as biocontrol) or biodiversity, for example through loss of a food source for organisms that feed on the target organism.

An example for a pathway to harm with respect to this indirect effect is the following. The SynBioM may suppress target organisms by means of a specific mechanism of action, leading to its reduction. Consequently, this may result in loss of food source for other environmental organisms that are dependent on this organism. Biodiversity may therefore be reduced and consequently may affect e.g. natural biocontrol.

4.7.4.2. Adequacy of guidance and methods

Potential risks as a consequence of (in)direct effects on target organisms are not explicitly addressed in the EFSA GMM Guidance (EFSA GMO Panel, 2011) and no information could be found in other guidances.

With respect to methodology, the GMM Guidance (EFSA GMO Panel, 2011) does not mention examples of methods to measure (in)direct effects on target organisms, nor does it give endpoints to measure. This also applies to SynBioMs.

No validated methods exist to measure adverse effects of microorganisms resulting from (in)direct effects on target organisms. Most methods focus on adverse effects on non-target organisms (NTOs) (see Section 4.7.5).

New methods may be developed to predict effects resulting from the suppression or removal of target organisms, including e.g. the implications on the ecologically important food webs to which the target organism naturally belongs. Such methods and their modelling require sufficient knowledge on food webs in the receiving environments, strategies to assess such implications and to determine endpoints of measurements.

Phase 2: For Cases 1–4, there are no additional aspects to be mentioned for interactions with target organisms.

4.7.5. Effects on non-target organisms

SynBioMs may have potential immediate and/or delayed environmental impacts due to direct and indirect interactions with NTOs, including impacts on population levels of competitors, prey, hosts, symbionts, predators, parasites and pathogens.

4.7.5.1. Problem formulation and example of pathway to harm

The SynBioMs can negatively affect NTOs. NTOs may include protists, insects or other organisms in ecosystems. A SynBioM may for example deplete a nutrient source or produce a metabolite with unintended adverse effects on organisms that are important for ecosystems functions, agricultural productivity or the health of animals. They may also disrupt food webs by suppressing organisms that are part of these food webs with a consequence of losing natural biocontrol of pathogens.

4.7.5.2. Adequacy of guidance and methods

Potential risks as a consequence of adverse effects on NTOs are not explicitly described in the EFSA GMM Guidance (EFSA GMO Panel, 2011). The NTO testing described in the EFSA GMO Panel guidance for plants ('Scientific Opinion on the assessment of potential impacts of genetically modified plants on non-target organisms') is not designed for GMMs and therefore not adequate for SynBioMs.

For PPP in general (chemicals and non-GM microorganisms used as PPP), the current assessment takes into account a series of legal requirements[16] and EFSA documents that mention **which** NTOs should be tested. Methods are however not detailed. The EFSA PPR Panel Guidance for risk

[16] Documents on the approval of active substances are listed on https://ec.europa.eu/food/plant/pesticides/approval_active_substances/eu_rules_en. Of particular interest for assessment are 'Regulation EU 283/2013 - setting data requirements for active substances' and its 'Communication - list of test methods and guidance documents' (Part A for chemicals, Part B for microorganisms including virus); as well as 'Regulation EU 284/2013 – setting data requirements for PPPs' with its 'Communication - list of test methods and guidance documents'(Part A for chemicals, Part B for microorganisms including virus).

assessment of PPP for aquatic organisms, for example, includes a Chapter on 'Specific protection goal proposal for algae (e.g. green algae, diatoms, blue-greens) in edge-of-field surface water' (EFSA PPR Panel, 2013). For exposure through soil, the EFSA Guidance (EFSA, 2017b) includes a Chapter on the 'Applicability of the tiered assessment scheme for microbial active substances' that might be useful for predicting environmental concentrations of active substances of PPP and transformation products of these active substances in soil.

At OECD level (ENV/JM/MONO(2012)1 guidance is given on NTO testing for potential adverse effects by non-GM microorganisms used as PPP. In ENV/JM/MONO(2012)1 OECD tests and guidelines from US EPA, Canada and OECD are given for assessing adverse effects of microbial plant protection agents on NTOs present in terrestrial and aquatic compartments. Guidance is also given on the types of NTOs to be tested, depending on the type of application of the microbial plant protection agent (e.g. spray, soil drench). This document also provides methodologies based on a tiered approach that may be applicable for assessing SynBioMs.

Phase 2: For Cases 1 to 4 there are no additional aspects to be mentioned for interactions with non- target organisms.

4.7.6. Effects on humans

SynBioMs may have potential immediate and/or delayed effects on human health resulting from potential direct and indirect interactions with people working with, coming into contact with or in the vicinity of the SynBioM release(s).

4.7.6.1. Problem formulation and example of pathway to harm

The SynBioM may be harmful to people who come into contact with the SynBioM, for example because SynBioMs are pathogenic or toxic for humans or cause allergies in humans. This issue will be addressed in a subsequent Work package 3 under this mandate (see Section 1.3).

4.7.7. Effect on animals

SynBioMs may have potential immediate and/or delayed effects on animal health and consequences for the feed/food chain resulting from consumption of the SynBioM and any product derived from it, if it is intended to be used as animal feed.

The effect of the feed on the animals will be addressed under a subsequent Work package 3 under this mandate (see Section 1.3).

4.7.8. Effect on biogeochemical processes

SynBioMs may have potential immediate and/or delayed effects on biogeochemical processes resulting from potential direct and indirect interactions in the receiving environments and beyond.

4.7.8.1. Problem formulation and example of pathway to harm

SynBioMs may reduce biodiversity and so alter biogeochemical processes, e.g. by negatively affecting microorganisms or invertebrates involved in the decomposition of organic materials or the transformation of nitrogenous compounds, sulphur or other elements. Therefore, the productivity or ecosystems services may be negatively affected. If the SynBioMs are able to survive in the receiving environment and produce, for example, antimicrobial compounds or metabolites that lower soil pH, they may have adverse effects on other microorganisms or ecologically important invertebrates (e.g. nematodes, springtails or earthworms). This may disrupt biogeochemical processes and negatively affect plant growth.

4.7.8.2. Adequacy of guidance and methods

Potential risks as a consequence of adverse effects on biogeochemical processes are not explicitly addressed in the EFSA GMM Guidance (EFSA GMO Panel, 2011).

OECD document ENV/JM/MONO(2012)1 gives some guidance on testing earthworms and on microorganisms involved in nutrient cycling. The EFSA GMM Guidance (EFSA GMO Panel, 2011) does not mention specific methods to measure the effects on biogeochemical processes, nor does it give endpoints to measure. This also applies to SynBioM.

In ENV/JM/MONO(2012)1, it is mentioned that in the EU, OECD tests on nitrification and respiration have been used to measure effects of microbial products on soil microorganisms. These tests,

however, are designed for chemical PPPs and are not validated for microorganisms. For the US, the US EPA does not support testing for effects of (non-genetically modified) microbial PPPs on soil microorganisms. ENV/JM/MONO(2012)1 describes test guidelines for earthworms as contributors to biogeochemical processes.

There is no methodology by which generally effects on biogeochemical cycles can be tested, nor is there a definition of measurable endpoints. New methods could be developed for this area of risk. It is envisaged that high-throughput technologies and modelling could be the right means to evaluate the risk associated with a potential impact on biogeochemical processes.

Phase 2: For Cases 1–4, there are no additional aspects to be mentioned for effects on biogeochemical processes.

4.7.9. Effect on managements techniques

Possible immediate and/or delayed, direct and indirect environmental impacts of specific techniques used for the management of the SynBioM that may differ from those used for current management systems.

4.7.9.1. Problem formulation and example of pathway to harm

SynBioMs can have an environmental impact due to a change in the way management techniques are used, for example because the mode of action of the SynBioM is changed, the SynBioM is applied using a different method, or is applied in a different receiving environment.

The SynBioM, for example, developed as an improved biocontrol agent due to combining different mechanisms of action in the organism, can lead to another regime of spraying with chemical plant protection agents. This other regime of spraying with chemicals may have an impact on the environment other than the original spraying regime. This impact can be positive, neutral or negative for ecosystems or health.

4.7.9.2. Adequacy of guidance and methods

Potential effects resulting from a change in management related to the SynBioM are not explicitly addressed in the EFSA GMM Guidance (EFSA GMO Panel, 2011).

In the EFSA ERA Guidance for GM plants (EFSA GMO Panel, 2010), there is some guidance on how to address environmental effects resulting from a change in management for GM plants, that may also be applicable to SynBioMs.

The EFSA GMM Guidance (EFSA GMO Panel, 2011) does not mention examples of methods to measure (in)direct effects as a result of a change in management, nor does it give endpoints to measure. This also applies to SynBioMs.

Methods to measure these effects resulting from a change in management, e.g. use of scenario analysis, are described in the EFSA guidance on ERA of GM plants (EFSA GMO Panel, 2010).

On a case-by-case basis, new methods may need to be developed and applied in ERA.

Phase 2: For Cases 1–4, there are no additional aspects to mention for effects on management techniques.

4.8. Concluding remarks for ERA and PMEM

For ERA, Section 4.5:

- The EFSA GMM Guidance (EFSA GMO Panel, 2011) is a useful basis for the ERA of products containing viable SynBioMs, because living GMMs were also foreseen in this Guidance. However, the points raised below are suggested for future adjustments of this guidance and equally apply to viable GMMs and SynBioMs.
- The EFSA GMM Guidance is adequate for assessing HGT potential for near-future SynBioM cases. Future updates would benefit from expanding with descriptions of approaches to test for adverse effects and their likelihood resulting from HGT.
- The comparative approach is still feasible for near-future SynBioM cases. For wider future cases, the comparative approach may depend on the familiarity of the SynBioM with known microorganisms with a history of use.
- Given a potentially altered efficacy of near future SynBioMs to interact with their environment, it is noted that the risk assessment covering environmental compartments as potential SynBioM habitats beyond the main receiving one may become more relevant than currently foreseen.

Future guidance update for ERA of SynBioMs, as well as for GMMs, should take into consideration the following aspects:

- The scope of the EFSA GMM Guidance covers the use of GMMs for food and feed. Given the mandate of this Opinion, it is recommended that future updates of this GMM Guidance should cover all agri-food uses, all types of microorganisms (including micro-algae, viruses), their relevant exposure routes and receiving environments.
- Future updates of the EFSA GMM Guidance, with a scope beyond food and feed use, should address all 'specific areas of risk' as per Directive 2001/18/EC.
- For extensively engineered SynBioMs, such as xenobionts, other risk assessment approaches may be considered that are not solely based on the comparative approach for new-to-nature components.

For PMEM, Section 4.6:

- The EFSA GMM Guidance (EFSA GMO Panel, 2011) provides the principles for detection and PMEM, which are applicable for near future products containing living SynBioMs.

For PMEM of SynBioMs, as well as for GMMs, guidance and knowledge are recommended to be developed as follows.

- Future updates would benefit from including descriptions of fit-for-purpose approaches to monitor for potential adverse effects resulting from the deliberate environmental release.
- Detailed descriptions of detection methods are recommended for current and near-future SynBioMs as well as GMMs.
- For wider future SynBioMs, suitable detection methods can be challenging to provide, because of the xeno-DNA structure.

5. Phase 3 and outlook

The following Sections cover an overall gap analysis that could not be captured by the previous phases (i.e. gaps disconnected from the existing guidance documents listed in Section 2.1 or disconnected from the selected cases). In addition, Phase 3 was also used to prepare outlooks for the future.

5.1. Environmental interactions

Interactions between SynBioMs and natural microbial communities include for example competition for energy sources (including light for micro-algae), nutrients or inhibition of each other through the production of biocidal compounds or parasitism (virus). For SynBioMs, the levels of interactions as well as the types of interactions may have been altered that could affect the exposure assessment during ERA. These include for example synergy by cooperatively accessing new nutrient and energy sources, cross-feeding of metabolites or exchanging genetic material by HGT and so optimising their environmental performance. Survival and invasiveness can strongly depend on the properties of the specific SynBioM. Requirements for growth factors may limit survival and invasiveness, while photo-autotrophy as for micro-algae could increase both.

An altered exposure can relate to higher abundance of SynBioMs in a given environmental compartment (e.g. rhizosphere, lake sediment, leaf surfaces) or to a wider range of such compartments in which a SynBioM can survive. Therefore, the efficacy by which the SynBioMs interact with the environment can differ from GMMs made by established techniques of genetic modification. Such altered efficacy can be related to levels of exposure as described above. For example, altered efficacy can be related to levels of exposure, such as:

- increased environmental survival and host colonisation;
- increased invasiveness;
- increased competition in naturally evolved microbial communities due to enhanced fitness, so thereby displacing beneficial microorganisms or disrupting food webs;
- altered metabolism, e.g. by changes in substrate utilisation;
- altered lifestyle, e.g. by energy use (aerobic versus anaerobic).

Therefore, given a potentially altered efficacy of **near future** SynBioMs to interact with their environment, the presence in environmental compartments beyond the main receiving compartment may become more relevant during ERA.

Consequently, the hazards for current and near-future SynBioMs when deliberately released into the environment, do not differ from those for GMMs made by established techniques of genetic modification. Although no novel hazards have been identified for near future SynBioMs, the efficacy by which the SynBioMs interact with their biotic and abiotic environment may differ. This may lead to increased exposure and therefore may result in higher risk.

Wider future SynBioMs, including xenobionts, aimed for deliberate release in the environment, may lead to novel hazards compared to microorganisms developed with established genetic modification techniques, for example because of:

- new-to-nature organisms/products/constituents possibly with poorly understood interactions with its biotic and abiotic environment;
- xeno-proteins with new enzymatic properties, i.e. modified substrate specificity or higher environmental robustness, and so open new niches;
- substantial reduction of the genome that could lead to unexpected interaction with other organisms (e.g. those that lead to evasion of the immune system).

5.2. ERA Guidance for micro-algae

The considered case studies do not include micro-algae. The photoautotropic lifestyle of these organisms is a crucial point for risk assessment and distinguishes micro-algae from most other GMMs that have been previously considered in EFSA Guidances. Micro-algae occur predominantly in habitats that are not usually considered in detail in ERAs so far. Hence, this constitutes a lack of RA experience, but the same ERA framework and principles (as for viable GMMs) may apply.

In general, microalgal research is usually focused on improving and optimising native existing production pathways (i.e. photosynthetic efficiency, lipid, pigments, among others) rather than introducing new pathways (Wijffels, 2015). In fact, there are species capable to produce toxins affecting human and possible animal health (Van Dolah, 2000), and a risk assessment to determine the level of risk of such species may be necessary.

A limited number of natural micro-algae species have received the QPS status for production purposes. When these micro-algae species would be used as a chassis for the construction of SynBio algae, this status can be used as a basis for the risk assessment. The value of this status in risk assessment depends on the familiarity of the SynBio micro-algae with the natural micro-algae. The MC of the SynBio micro-algae can be established using the existing guidances, based on WGS and phenotypic characterisation, but they should be complemented with specific guidance for the taxonomic identification of algae and for the interpretation of WGS data. As gene annotation is far from complete for micro-algae, the obtained information will be more limited compared with that obtained for bacteria and fungi and, therefore, the conclusions would include higher degree of uncertainty. This uncertainty will be increased by increasing complexity of the introduced genetic modifications. In this regard, it is recommended that knowledge be collected to correlate gene sequences to biological functions so that future guidance can be updated for assessing the toxigenicity and virulence/pathogenicity of these organisms.

Some experience in the ERA of GM micro-algae has been build. The US EPA, in the USA, developed a draft guidance 'Draft Algae Guidance for the Preparation of TSCA Biotechnology Submissions' for assessing the environmental risks related to open-pond cultivation of GM micro-algae (US EPA, 2016). Moreover, there are documents already highlighting some of the possible issues in the risk assessment of the use of micro-algae for production purposes (Segal and Yang, 1986; Gressel, 2014; US EPA, 2016; Beacham et al., 2017).

5.3. ERA Guidance for viruses

SynBio viruses are viruses generated from a (partly) synthetically made DNA sequence, either as such (for DNA viruses (Myhr and Traavik, 2012)) or from infectious RNA transcripts (RNA viruses, e.g. influenza (Cox et al., 2015)). The DNA sequence designed is a (sometimes partly) synthetic copy of the consensus sequence from the collection of genotypes present in a natural virus isolate, more specifically viruses from plants, insects and bacteria (bacteriophages). The genotypic variation in RNA viruses is much higher than in DNA viruses. Because of the high replication and error rate (e.g. RNA

viruses 1/10^4 and DNA viruses 1/10^8) genotypic variation is a typical characteristic of viruses. The issue is whether the genetic variation of SynBio viruses will be ultimately similar to that of natural virus equivalents.

The MC of the SynBio viruses is the first step of the ERA. The genetic assembly of SynBio viruses can be analysed by WGS and compared with the genotypic assemblies of natural viruses, if needed. Therefore, the existing microbiological EFSA Guidances (EFSA FEEDAP Panel, 2018; EFSA CEP Panel, 2019) may be used for the risk assessment of SynBio viruses. Alphaflexiviruses, potyviruses and baculoviruses, used as PPPs, were included in the QPS evaluations and received QPS status (EFSA BIOHAZ Panel, 2020). Therefore, the QPS approach may be applied as a basis for risk assessment of some SynBio viruses, using a case-by-case approach.

While the biological characteristics of SynBio viruses can be tested by conventional methodologies (e.g. biological activity, host range, pathology), there is limited information on methodologies to assess the impact of released SynBio viruses with respect to all the 'specific areas of risk' in ERA. An example of GM virus ERA is Case 1, for which United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA APHIS, 2019) performed an analysis of environmental impact. However, the approach used by these regulatory bodies did not address all the 'specific areas of risk' mentioned in Directive 2001/18/EC (European Commission, 2001).

5.4. ERA Guidance for xenobionts

Due to their new-to-nature composition, xenobiotic microorganisms (xenobionts) require specific consideration when assessing their environmental risks. Approaches developed for GMMs, e.g. the QPS concept and the WGS-based microbial characterisation, may not be directly applicable. The development of specific risk assessment approaches for xenobionts should consider that WGS can be used when the genetic information is still coded in DNA, but not in SynBioM with XNA.

Xenobionts producing xeno-peptides and xeno-proteins present novel hazards, triggering a case-by-case risk assessment that may require testing procedures that may differ from those used for non-modified microorganisms or GMMs. For example, xeno-peptides could act as novel inhibitors of enzymes or a new type of signal molecules. Xeno-proteins may act as enzymes with new-to-nature substrate specificities or catalyse a novel type of chemical reaction (Agostini et al., 2017). Xeno-proteins could be more resistant to heat or other means of inactivation, or they could represent structural macromolecules less accessible for microbial decomposition. Xeno-peptides and xeno-proteins could also act as novel antigens. Beyond that, xenobionts of the future may also contain or produce new-to-nature carbohydrates or lipids that may alter cell permeability or cell structure (hypothetical case). Other xenobionts may grow with up-to-date unused chemical elements, e.g. fluorine or boron (Schmidt et al., 2018). This could result in a change of lifestyle and ecophysiology allowing these to enter new niches or become more competitive in already colonised niches (e.g. in a host organism or a soil microcompartment). Such organisms could also trigger an immunological response of host organisms because of novel antigens. So, SynBioMs with new-to-nature carbohydrates or lipids may in fact pose new hazards to human and animal health, as well as to the environment.

For xenobionts with novel genetic coding based on XNA, no guidance for assessing the safety of the genetic modification for human health and the environment is yet available. While the potential to produce new-to-nature carbohydrates, lipids and xeno-proteins involves new hazards for human health and the environment, the replacement of DNA by XNA as a means for storing genetic information should decrease hazards for the uncontrolled spread of modified genes into receiving environmental and below, and the expression of XNA-encoded functions in recipients is extremely low, if not zero. Replication of XNA in natural microbial recipients is inhibited by the absence of xeno-nucleotides as building blocks of XNA in natural environments. Therefore, it has been suggested that XNA can act as a firewall to prevent HGT resulting in the modification of natural microbiomes (Acevedo-Rocha and Budisa, 2011). For the ERA, it should however be noted that the genetic firewall concept, as of today, is only a theoretical concept, leaving out the potential that segments/fragments of XNA molecules may gradually be transformed into DNA by host-specific DNA repair. The efficacy as a firewall in the environmental containment of XNA should be tested and specific methodologies are recommended to be developed. Equally, detection techniques for the presence of XNA, comparable with the use of polymerase chain reaction or gene probes for DNA, would be an essential tool for monitoring of xenobionts with XNA.

5.5. Outlook for new approaches to ERA of SynBioM

The specific features of SynBioMs and the limitations identified in the existing guidances support the need for new approaches for ERA of these microorganisms. These new risk assessment strategies should take in consideration the continuous and rapid advancement in methodologies to study the biological systems. This Section applies equally to GMMs.

5.5.1. Systems approaches

Systems approaches address ERA from a holistic point of view, by taking into account the many interactions following a specific intervention, being it SynBioMs released into the environment or microbiomes in soils, water, plants or animals. Systems approaches make extensive use of a variety of modelling and data analysis technologies to allow for assessment of factors such as exposure, persistence, invasiveness, (horizontal) gene transfer, genetic stability, pathogenicity, environmental fate, etc. Such approaches include: genome-scale constraint-based models of microbiome (van der Ark et al., 2017); network analysis of microbiomes/microbiota within their environments; models of microbial interactions and functioning and fate of genetic material; and data modelling using probabilistic models and evolutionary models. However, these approaches are not yet tested and validated for the ERA of microorganisms.

5.5.2. New testing methods for microbial interactions

SynBioMs released into the environment will inevitably come into contact with the resident microbial communities. Therefore, when there is deliberate introduction into an open environment it is expected that SynBioMs affect the existing microbiomes, e.g. for feed additives the microbial communities colonising the gastrointestinal tract or for biological fertilisers, soils and rhizospheres. Accordingly, the existing ERA guidance for GMMs requires an assessment of interactions of SynBioMs with microbiomes, e.g. when evaluating environmental survival, persistence over time and invasiveness, HGT, interactions with target and NTOs, or impact on biogeochemical processes.

To assess these effects, information can be achieved by high-throughput DNA sequencing, either by metagenomics, studying the total DNA, or by PCR amplicon sequencing that analyses the diversity of specific genetic markers. The suitability of applying either one of these methods or both may depend on the hazards and risks identified for a particular organism, i.e. on a case-by-case basis. This may also trigger additional analyses that evaluate the activity or gene expression products, by means of metatranscriptomics and metaproteomics, respectively. Even with the complete genetic information of a synthetic microorganism, it is beyond the capacity of any existent bioinformatic analysis to fully predict the capability of a synthetic organism to survive, colonise and interact with other organisms under natural conditions, given the uncountable diversity of potential microhabitats and their temporal variability. Therefore, the knowledge obtained by WGS of the SynBioM introduced in the natural environments should be complemented with studies targeted to appraise the interactions of SynBioM with environmental microbiomes on a case-by-case basis.

While the spatial and temporal complexity of environmental conditions cannot all be mimicked under laboratory, or otherwise standardised conditions, the lack of prediction about potential interactions could require test systems in which the survival and interactions between synthetic organisms and natural microbial communities can be assessed. The goal of such systems is ultimately to predict environmental risks, i.e. adverse effects on ecosystem services provided by environmental microbial communities. As an example, there are now technical options for high-throughput testing at a small, miniaturised scale, to mimic a wide range of environmental conditions. Miniaturised platforms could deliver standards of reference for a wide range of situations, including long-term persistence, biological status of the cells at endpoints, worst case scenarios. However, these approaches have not yet been tested and validated for the ERA of microorganisms.

6. Overall conclusions

6.1. Identification of newer sectors/advances

ToR1: EFSA was asked to consider whether and which newer sectors/advances should be considered among SynBio developments, in addition to the six identified by the SCs. In response, a horizon scan based on a literature search and under the conditions at the start of this Opinion was

performed for products over the next decade. The WG experts furthermore added to that scan during the full duration of this Opinion with further knowledge. The conclusions reached are as follows:

- No other sectors/advances were identified in addition to the six identified by the SCs.[17]
- There are no clear criteria to differentiate between a GMM and a SynBioM.
- From a technical point of view, there are SynBioM applications (e.g. Cases 1, 2 and 3) that could be ready for deliberate release into the environment of the EU in the next decade. However, extensively engineered SynBioMs (e.g. minimal cells and protocells, xenobionts), falling within the remit of EFSA, are not expected for deliberate release into the environment of the EU in the next decade.
- Information on new SynBioM products for deliberate release may not be made publicly available at early stages of their development. This situation limits the predictive capacity of this Opinion.

6.2. New hazards/risks

ToR2: EFSA was requested to identify, if possible, potential risks in terms of impact on humans, animals and the environment that current and near-future SynBio developments could pose; EFSA was also asked to identify potential novel hazards compared with established techniques of genetic modification.[18] This Opinion is focused on the ERA, whereas the risk assessment for humans and farmed animals will be addressed in Work package 3 under the current mandate.

It was concluded that the hazards **for current and near-future** SynBioMs when deliberately released into the environment, do not differ from those for GMMs made by established techniques of genetic modification. Although no novel hazards have been identified for near future SynBioMs, the efficacy by which the SynBioMs interact with their biotic and abiotic environment may differ. This may lead to increased exposure and therefore may result in higher risk.

Wider future SynBioMs, aimed for deliberate release in the environment, may lead to novel hazards compared to microorganisms developed with established genetic modification techniques, e.g. due to new-to-nature components or (substantially) reduced genomes with poorly understood interactions with its biotic and abiotic environment.

The assessment to identify novel hazards or risks should always be performed on a case-by-case basis.

6.3. Adequacy of existing guidelines

ToR3: EFSA is requested to determine if the existing guidelines for risk assessment are adequate and sufficient for current and near-future SynBio developments or if there is a need for updated guidance.

Concluding remarks for microbial characterisation (genotypic and phenotypic):

- For the genotypic and phenotypic characterisation of SynBioMs and the safety of the genetic modification, the FEEDAP Guidance on microbial characterisation (EFSA FEEDAP Panel, 2018), the CEP statement (EFSA CEP Panel, 2019) and the GMM Guidance (EFSA GMO Panel, 2011) on characterisation of the recipient strain are useful as a basis for the assessment.
- Adequacy of existing EFSA Guidances for SynBioMs depends on the degree of familiarity of the SynBioM and chassis with the non-modified microorganism. The extent to which the existing body of knowledge on the microorganism can be used in the risk assessment will be higher when there is a high degree of familiarity with the SynBioM and chassis. The following guidance is adequate and sufficient in this context:
  - WGS analysis is essential for SynBioMs, irrespective of them being bacteria, archaea, viruses, viroids or eukaryotic microorganisms such as protists, fungi and algae.
  - Explaining the purpose of the development of the SynBioM using a certain chassis is essential.
  - WGS, which can be used for taxonomic identification, identification of antimicrobial resistance genes, searching sequences related to antimicrobial production, toxigenic and

[17] Six SynBio developments: 1) genetic part libraries and methods; 2) minimal cells and designer chassis; 3) protocells and artificial cells; 4) xenobiology; 5) DNA synthesis and genome editing; and 6) citizen science (do-it-yourself biology).

[18] For the purpose of this mandate, the terms 'established techniques of genetic modification' refers to various genetic engineering techniques that have been significantly used over the last 30 years to produce genetically modified organisms, such as those which have been authorised under Directive 2001/18/EC and Regulation (EU) No 1829/2003.

virulence/pathogenic characteristics, mobile genetic elements and characterisation of the genetic modifications of the SynBioM.
- QPS concept for the risk assessment of building block of SynBioMs (e.g. chassis, metabolic building blocks);
- phenotypic testing for the production of antimicrobial compounds;
- weight of evidence approach for the assessment of toxigenicity and pathogenicity based on bioinformatics analysis, body of knowledge and the use of model systems;
- need for collecting information on the source and natural habitat of the recipient microorganism;
- possibility of transfer of genetic material to other microorganisms;
- genetic stability;
- pathogenicity, ecological and physiological traits;
- history of use;
- rate and level of expression of gene products resulting from the genetic modification;
- need for a unique identifier.

Concluding remarks for ERA:

- The EFSA GMM Guidance (EFSA GMO Panel, 2011) is a useful basis for the ERA of products containing viable SynBioMs, because living GMMs were also foreseen in this Guidance. However, the points raised below are suggested for future adjustments of this guidance and equally apply to viable GMMs and SynBioMs.
- The EFSA GMM Guidance is adequate for assessing HGT potential for near-future SynBioM cases. Future updates would benefit from expanding with descriptions of approaches to test for adverse effects and their likelihood resulting from HGT.
- The comparative approach is still feasible for near-future SynBioM cases. For wider future cases, the comparative approach may depend on the familiarity of the SynBioM with known microorganisms with a history of use.
- Given a potentially altered efficacy of near future SynBioMs to interact with their environment, it is noted that the risk assessment covering environmental compartments as potential SynBioM habitats beyond the main receiving one may become more relevant than currently foreseen.

Concluding remarks on PMEM:

- The EFSA GMM Guidance (EFSA GMO Panel, 2011) provides the principles for detection and PMEM, which are applicable for near future products containing living SynBioMs.

6.4. Need for new guidance

ToR4: In the latter case, EFSA was requested to identify the specific areas for which such updated guidance is needed.

For microbial and molecular characterisation of SynBioMs, as well as for GMMs, guidance and knowledge is recommended to be developed:

1) for micro-algae: specialised guidance for the genomic and phenotypic characterisation.
2) for yeast and fungi: phenotypic testing for antimycotic resistance;
3) for xenobionts: guidance not based on history of use and not solely based on the comparative approach for the new-to-nature components;
4) for XNA: guidance for characterisation and detection;
5) for xenobionts, extensively engineered SynBioMs, micro-algae and viruses: suitable model systems for testing virulence and pathogenicity for non-target hosts.

For environmental risk assessment of SynBioMs, as well as for GMMs, guidance and knowledge are recommended to be developed:

1) The scope of the EFSA GMM Guidance covers the use of GMMs for food and feed. Given the mandate of this Opinion, the future update of the GMM Guidance should cover all agri-food uses, all types of microorganisms (incl. micro-algae, viruses), their relevant exposure routes and receiving environments.
2) Future updates of the EFSA GMM Guidance, with a scope beyond food and feed use, should address all 'specific areas of risk' as per Directive 2001/18/EC.

3) For extensively engineered SynBioMs, such as xenobionts, other risk assessment approaches may be considered that are not solely based on the comparative approach for new-to-nature components.

For PMEM of SynBioMs, as well as for GMMs, guidance and knowledge are recommended to be developed:

1) Future updates would benefit from including descriptions of fit-for-purpose approaches to monitor for potential adverse effects resulting from the deliberate environmental release.
2) Detailed descriptions of detection methods are recommended for current and near-future SynBioMs as well as GMMs.
3) For wider future SynBioMs, suitable detection methods can be challenging to provide, because of the xeno-DNA structure.

7. Recommendations

The following is recommended:

- Research for innovative approaches in the frame of ERA of SynBioMs as well as viable GMMs, focusing on methods to assess HGT, invasiveness and other areas of risk for SynBioMs deliberately released into the environment. In this respect, advantage could be taken of recent technological advancement in methodologies for investigating complex microbial communities and microbiomes.
- Additional research on (functional) gene/genome annotation for all microorganisms, in particular for understudied groups like micro-algae.
- Increasing knowledge on microbial interactions, microbiome function and interactions with the receiving environments for the wider understanding of community function and risk assessment/management of the effect of SynBioMs as well as GMMs.
- Development and deployment of systems approaches. These should rely on large-scale mathematical and statistical models as well as on semantic technologies and big data analytics to support (environmental) risk assessment.
- The concept of developing a limited number of engineerable, safe-by-design and reusable SynBioM chassis to create the opportunity to base the risk assessment on the performance of the chassis under prespecified environmental conditions. This may offer the opportunity to evaluate a presumption of safety concept for a specific chassis.

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Glossary

Barcoding	DNA barcoding is a method of identifying organisms based on a short, standardised fragment of genomic DNA. It has been developed for use by taxonomists, ecologists, conservation biologists, regulatory agencies, and others
Cell-free systems	subsets of biochemical reactions that happen within cells, made *in vitro* apart from a full cell system
Chassis	A naturally derived or highly engineered organism repurposed to build, maintain and amplify the components necessary for deployment of synthetic biological systems and their applications. For the purpose of this opinion the meaning of the term deals with live cells containing an editable genome. It is noted that cell-free systems, reconstructed vesicles and nucleoid-dissolved cells (i.e. with no DNA) have also been occasionally described as chassis
Comparative approach	Analysis of potential adverse effects resulting from a GMM when compared with a counterpart with familiarity
Deliberate release	Any intentional introduction into the environment of a GMM or a combination of GMMs for which no specific containment measures are used to limit their contact with and to provide a high level of safety for the general population and the environment
Design-Build-Test-Learn (DBTL)	A workflow for synthetic biology applications that entails an iterative cycle of designing the system, building it, testing it and learning from the results of testing, often with the help of machine learning and artificial intelligence. This workflow mirrors those for engineering and computer sciences
Environmental risk assessment	is defined as the evaluation of risks to human health and the environment, whether direct or indirect, immediate or delayed, which the deliberate release or the placing on the market of GMMs may pose and carried out in accordance with Annex II of Directive (2001/18/EC)
Extensively engineered	Organisms where either a large proportion of the chromosome has been genetically modified or central metabolic pathways (e.g. for energy conversion

	or anabolism) of an organism have been modified. Also Xenobionts are considered to be extensively engineered
Familiarity	The concept of "familiarity" refers to the fact that most GMMs to be used for food or feed purposes belong to well-characterised microbial species. This "familiarity" allows the risk assessor to draw on previous knowledge and experience with the introduction of similar microorganisms into food and the environment. "Familiarity" will also derive from the knowledge and experience available from the risk/safety analysis conducted prior to the scale-up of the microorganism in a particular environment (OECD, 1993a,b and EFSA GMO Panel, 2006)
Genetic circuit	An assembly of biological parts including regions encoding RNA or protein that enables individual cells to respond and interact with each other to perform some logical functions (such as signal processing and decision making)
Genetic Firewall	Result of an engineering approach to biologically control the unintended environmental spread of the SynBioM or its genetic material via gene transfer to other organisms
Genome Editing	Technology in which DNA is inserted, deleted, modified or replaced in the genome of a viable organism. Genome editing targets the modifications to site-specific locations. As explained in the Scientific Advice Mechanism (SAM) Explanatory note of April 2017 (SAM, 2017), genome editing aims to achieve a precise alteration of a DNA sequence in a cell, or to achieve random changes at precise locations
Hazard	A biological, chemical or physical agent in, or condition of, food or feed with the potential to cause an adverse health effect (From Regulation (EC) No 178/2002, (European Commission, 2002)). According to the EC Council Decision of 2002[19], hazard is the potential of an organism to cause harm to or adverse effects on human health and/or the environment.
Metabolic engineering	Metabolic engineering is generally defined as the redirection of one or more enzymatic reactions to produce new compounds in an organism, improve the production of existing compounds, or mediate the degradation of compounds. Metabolic engineering can also be used to expand the eco-physiology of SynBioM
Micro-algae	A polyphyletic group of unicellular photosynthetic eukaryotes, typically found in freshwater and marine systems
Microbiome	Microbiome refers collectively to communities of microorganisms and their combined genomes in a defined environment
Microorganism	A definition of microorganism is provided in Article 2 of Directive 2009/41/EC of the European Parliament and of the Council of 6 May 2009 on the contained use of genetically modified microorganisms (European Commission, 2009): 'microorganism' means any microbiological entity, cellular or non-cellular, capable of replication or of transferring genetic material, including viruses, viroids, and animal and plant cells in culture
Minimal cells	A cell whose genome only encodes the minimal set of genes necessary for the cell to survive and autonomous grow under specified conditions
Post-market environmental monitoring	A risk management tool that provides a mechanism to monitor possible adverse environmental consequences of the GM product e.g those that are included in the risk assessment, in accordance to Annex VII of the Directive 2001/18/EC
Problem formulation	The process including the identification of characteristics of the GMM capable of causing potential adverse effects to the environment (hazards) of the nature of these effects, and of pathways of exposure through which the GMM may adversely affect the environment (hazard identification). It also includes defining the assessment endpoints and setting of specific hypotheses to guide

[19] European Commission, 2002. Council Decision of 3 October 2002 establishing guidance notes supplementing Annex VII to Directive 2001/18/EC of the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal of the European Communities L, pp. 27–36.

	the generation and evaluation of data in the next risk assessment steps (hazard and exposure characterisation)
Protocells	An approach to engineering novel biological systems working strictly from the 'bottom up' and attempting to construct new simple forms of living systems, using chemical and physical processes and employing as raw ingredients only materials that were never alive. Currently, the systems constructed by bottom-up approaches are not viable organisms, but are chemical vesicles, called 'protocells'
Quantitative presumption of safety (QPS)	This is a harmonised generic pre-assessment approach applied by EFSA for the safety of biological agents used in food and/or feed. This approach is based on extensive reiterative scientific literature review and absence of reported hazards or risks
Receiving environment	The immediate environment into which microorganisms (including SynBioM) will be released
Risk	A function of the probability of an adverse health or environmental effect and the severity of that effect, consequential to a hazard (From Regulation (EC) No 178/2002 (European Commission, 2002)). According to the EC Council Decision of 2002,[20] risk is defined as the combination of the magnitude of the consequences of a hazard, if it occurs, and the likelihood that the consequences occur.
Safe-by-design	A principle aiming to develop safe new products (e.g. SynBioMs) by taking into account all aspects of the product as well as of the process, from the initial ideas of the project, up to the well-characterised final product
Synthetic Biology	An interdisciplinary field at the interface of engineering and biology aiming to develop new biological systems and impart new functions to viable cells with potential applications (for the purpose of this Opinion) in the food and feed and environment system
Systems approaches	The systems approach principle places individual system elements in their environments and observes the relationships between them. This approach relies on large-scale mathematical and statistical models as well as on semantic technologies, big data analytics and artificial intelligence
Xenobiology	A branch of SynBio that started to design alternative biochemical components for bioengineering other than DNA or the 20 canonical amino acids

Abbreviations

AMR	antimicrobial resistance
ANI	average nucleotide identity
CEF	Panel on Food Contact Materials, Enzymes and Processing Aids
CIA	critically important antimicrobials
CTV	*Citrus tristeza virus*
DHR	double homologous recombination
ERA	environmental risk assessment
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
GM	genetically modified
GMM	genetically modified microorganisms
GMO	genetically modified organisms
HGT	horizontal gene transfer
HIA	highly important antimicrobials
iGEM	International Genetically Engineered Machine
MC	microbial characterisation
MIC	minimum inhibitory concentration
MLST	multilocus sequence typing
NTOs	non-target organisms

[20] European Commission, 2002. Council Decision of 3 October 2002 establishing guidance notes supplementing Annex VII to Directive 2001/18/EC of the European Parliament and of the Council on the deliberate release into the environment of genetically modified organisms and repealing Council Directive 90/220/EEC. Official Journal of the European Communities L, pp. 27–36.

OECD	Organisation for Economic Co-operation and Development
PMEM	post-market environmental monitoring
PPP	plant protection products
QPS	qualified presumption of safety
RA	risk assessment
SAM	Scientific Advice Mechanism
SC	Scientific Committee
SCCS	Scientific Committee on Consumer Safety
SCENIHR	Scientific Committee on Emerging and Newly Identified Health Risks
SCHER	Scientific Committee on Health and Environmental Risks
SynBio	synthetic biology
SynBioM	microorganism obtained through synthetic biology
ToR	Term of Reference
WG	Working Group
WGS	whole genome sequencing
WHO	World Health Organization
XNA	xeno-nucleic acid