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Submission of information and supporting documentation relevant to the trends in new technological developments in synthetic biology (horizon scanning)

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Introduction and summary

This backgrounder was compiled for submission of information on synthetic biology for the Open-ended Online Forums on Synthetic Biology (Ref.: SCBD/CPU/DC/WM/MAQ/MW/90775) within the proceedings of the Convention on Biological Diversity (CBD).

The backgrounder is partially based on a recently published report (Testbiotech 2022). Its terminology has been adapted to the language used in the CBD treaties. The information has also been updated and information added on self-propagating artificial genetic elements (such as gene drives). The backgrounder takes the perspective of the protection goals, such as health and the environment.

The most relevant findings of the horizon scanning are: Soon, large numbers of Synbio LMOs (Living Modified Organisms) of numerous species with a wide range of different traits could be released into the environment within a short period of time. Many of them could spread uncontrollably and complex interactions are expected to occur both between different Synbio LMOs and each other, as well as with their environment. There are multiple pathways to harm and numerous unintended potential interactions with the environment and also between the Synbio Organisms. It is therefore important to maintain control over releases of Synbio LMOs.

It is shown for Synbio LMOs ‘cut-off’ criteria are needed that allow decision-making is required in the face of greater unknowns. In addition, in future, systemic risks and prospective technology assessment additionally deserve a high level of attention by the regulators. We recommend to restrict the scale of environmental releases in regard to numbers of Synbio LMOs, the different traits and species. The concepts of nature conservation and environmental protection are largely based on the principle of avoiding interventions. These should also be applied in the field of genetic engineering and Synbio LMOs.

1. Increase in technical potential and range applications of Synbio applications using CRISPR/Cas

1.1 CRISPR/Cas is the most relevant tool for Synbio applications in plants

Synbio allows new genotypes and traits to be generated in different ways and with different outcomes compared to previous genetic engineering methods or conventional breeding, including random mutagenesis (Eckerstorfer et al., 2019; Kawall, 2019; Kawall, 2021b). So-called site-directed nucleases (SDN), as used in CRISPR/Cas 'gene scissors' (Jinek et al., 2012; Doudna & Charpentier, 2014), are highly relevant in this context: they can be designed to target specific sites in the genome to knock out gene functions (SDN-1), to induce repair mechanisms for specific alterations of particular nucleotides to change specific gene functions (SDN-2), or to insert additional genes (SDN-3).

The nuclease CRISPR/Cas is currently the most relevant Synbio-tool in the development of new plants (JRC, 2021). The nuclease is combined with an RNA that serves as a guide molecule and is designed to be specific for the DNA target site in the genome. After matching the guide RNA with the target region, the nuclease (which is strictly speaking the enzyme Cas) is then activated and typically cuts both strands of DNA. As a result, gene-functions will be disabled or changed.

Other relevant nucleases are TALENs (transcription activator-like effector nucleases) that were already established prior to the introduction of the CRISPR/Cas tool and are still applied in some cases. In addition, some variations on the Cas nuclease have been introduced recently (such as Cpf). There are nucleases which meanwhile appear to be of major importance (see JRC, 2021). All these nucleases can be categorized by using the SDN terminology in this backgrounder.

If the repair mechanisms are left to the process in the cells, this is called 'non-homologous end joining' (NHEJ). In these cases, no specific change in gene function is introduced, the intention is to simply knock out the natural gene functions (SDN-1). Typically, if the cell tries to restore the original gene function, the nuclease CRISPR/Cas can continue to cut until the intended incorrect repair is achieved and no more target sequence is available (Brinkman et al., 2018).

CRISPR/Cas might also be used to achieve specific changes to the gene functions (SDN-2 or SDN-3) via homologous recombination mediated by homology directed repair (HDR). In this case, additional DNA molecules are introduced alongside the Cas nuclease that serve as specific templates for the repair mechanisms which are meant to cause specific genetic alterations. The induced changes at or around the target site can be substitutions, deletions or insertions of one or more base pairs (SDN-2). If additional gene-sequences are inserted, the nucleases are classified as (SDN-3) (Eckerstorfer et al., 2019; Sander & Joung, 2014).

Depending on the specific SDN-1 or SDN-2 application, more extensive overall changes are possible. For example, multiplexing can target several genes simultaneously in a single application (Raitskin and Patron, 2016; Wang et al., 2016; Zetsche et al., 2017). Repeated applications of SDN-1 or SDN-2 can also be combined (Kawall et al., 2020). Changes involving the insertion of whole genes (including gene-stacking) are also possible (SDN-3) and are mediated by the use of specific donor DNA (Eckerstorfer et al., 2019; Sander & Joung, 2014). If the outcome results in a genetically engineered organism inheriting a gene from another species, it is called 'transgenic'. If

the outcome results in an organism with additionally inserted genes from the same species, it is called ‘cisgenic’.

Further refinements, such as cutting only one strand of the DNA (nickase), the change of base pairs without cutting the strand of DNA (base editing) or specific variations that are meant to increase the efficiency and precision of the nucleases, may be applied. However, with regard to most of the plants (or animals) currently under consideration for being brought to market in near future, the SDN-1 processes as described above, are the ones that are applied in most cases (see JRC, 2021).

1.2 Some characteristics and trends of Synbio applications

The following section provides an overview of some specific characteristics with general relevance for Synbio applications, in particular the CRISPR/Cas nuclease, in order to illustrate their technical potential:

a) Greater precision but a complex multistep process
Synbio can be used to introduce genetic changes with greater precision compared to previous techniques of genetic engineering. Typically, SDNs can be used to directly target the desired sites (Doudna & Charpentier, 2014; EFSA, 2020a; Jinek et al., 2012), whereas previous transformation processes introduce additional DNA sequences only at random sites (see, for example, Forsbach et al., 2003; Gelvin, 2017; Makarevitch et al., 2003). However, Synbio applications are based on processes involving several technical steps that, in case of plants, very often also include the older non-targeted transformation processes (such as biolistic methods[1] or the use of *Agrobacterium tumefaciens*[2]). These non-targeted methods are used to introduce the nucleases into the cells (Kawall et al., 2020) which may lead to unintended effects in many off-target regions (for example, see Yue et al., 2022). As pointed out in some publications, there are additional reasons why higher precision still seems to be challenging in several applications (Eckerstorfer et al., 2019; Kawall et al., 2020). In this context, there are several factors which impact the results of Synbio processes in regard to the intended and unintended effects, such as the species, the trait, the target genes (their site, their function, their number, their similarities with other genes), the gene scissors (or other tools used) and the process of introducing the gene scissors (or other Synbio tools) into the cells (see, for example, Kawall et al., 2020).

b) Overcoming the limitations of natural genome organization
Synbio can be used to achieve genomic changes extending beyond what is known from conventional breeding even without the insertion of additional genes. Compared to methods of conventional breeding (including random mutagenesis), Synbio processes can overcome the boundaries of natural genome organization that have evolved naturally from evolutionary processes. Relevant factors include repair mechanisms, gene duplications, genetic linkages and further epigenetic mechanisms (see, e.g. Belfield et al., 2018; Filler Hayout et al., 2017; Frigola et al., 2017; Halstead et al., 2020; Huang & Li, 2018; Jones et al., 2017; Lin et al., 2014; Monroe et al., 2022; Wendel et al., 2016), thus making the genome much more extensively available for genetic change (Kawall, 2019; Kawall et al., 2020). The resulting genotypes (the patterns of genetic changes) can be vastly different compared to those derived from conventional breeding, both in regard to intended and unintended changes (Kawall, 2021a; Kawall, 2021b), although there may

1 Biolistic transformation is also known as particle bombardment or gene gun. It is a non-targeted method of genetic transformation of plants to deliver DNA into cells/tissues. The DNA to be introduced is coated onto small micro-particles which are ‘shot’ into the tissue at high pressure.
2 *Agrobacterium tumefaciens* is a soil bacterium capable of parasitic growth on plants. The agrobacteria induce a genetic transformation in the host cell via stable integration of a DNA fragment called T-DNA. This mechanism of DNA transfer is a non-targeted method of genetic engineering using genetically engineered agrobacterium.

still be some limitations to the effectiveness of the nucleases (Weiss et al., 2022). This means that it is possible to generate genotypes that are highly unlikely to result from natural processes or traditional breeding techniques, as well as create new phenotypes, including extreme versions of already known traits.

c) Changes in the allelic diversity within populations
Barbour et al. (2022) showed that a higher allelic diversity in plants has an impact on different species within an experimental food web. They may also play a crucial role in the stability of ecosystems and food webs. CRISPR/Cas applications can, in particular, be used to make gene variants within a population more uniform, i.e. the frequency of the abundance of different allelic variants can be reduced, the alleles can be changed or the respective gene (-family) can be blocked in its functions. In this regard, CRISPR/Cas applications are very much more efficient than conventional breeding methods. Therefore, if Synbio-LMOs are released into the environment, their impact on genetic diversity and associated ecosystems can extend far beyond what might be expected compared to natural processes and conventional breeding techniques.

d) Pervasive changes even without the insertion of additional genes
Even without the insertion of additional genes, changes in genotypes and phenotypes can be pervasive and brought about by, for example, knocking out very many or all copies of a gene family, thus changing several genes in parallel (multiplexing) or altering elements responsible for gene regulation (Kawall et al., 2020; Raitskin and Patron, 2016; Wang et al., 2016; Zetsche et al., 2017). Such technical interventions can lead to major and unprecedented changes in plant composition, which may also be associated with unintended effects (EFSA, 2021; Kawall, 2021a; Kawall, 2021b; Nonaka et al., 2017; Sanchez-Leon et al., 2018).

e) Wide range of species and applications
The range of species that are accessible for Synbio extends far beyond applications of previously used techniques of genetic engineering. While effectiveness may differ from case to case, it does include a wide range of food plants and livestock. It also includes non-domesticated species comprising trees and other plants, insects, vertebrates and microorganisms, thus involving all domains of life (overview in: CBD, 2022; JRC, 2021; Testbiotech, 2021b). There are several specific applications designed for use in wild populations, including gene drives (Frieß et al., 2019; Gantz & Bier, 2015) and the intended release of genetically engineered viruses, also including Horizontal Environmental Genetic Alteration Agents (HEGAA) (Lentzos et al., 2022; Pfeifer et al., 2022). Many of the species targeted in Synbio applications also have the potential to persist and spread over longer periods of time without effective control. This may give rise to next generation effects not observed in the laboratory (Then et al., 2020).

f) Complex interactions also triggered by parallel releases
Large numbers of LMOs, derived from Synbio, including various species with a wide range of different characteristics (intended or unintended), could be released into the same receiving environment within a short period of time (see, for example, JRC 2021). Depending on the scale of the release, its duration over time and the characteristics of the organisms, these Synbio-LMOs may also intentionally or unintentionally interact with each other as well as with the ‘original’ receiving environment. In this context, a number of Synbio-LMOs are designed for complex interactions, such as changes in the microbiome in the soil (Shelake et al., 2019; Shulse et al., 2019; Temme et al., 2012), in plants (Arif et al., 2020; Checcucci et al., 2018; Hettiarachchige et al., 2019; Vorholt et al., 2017), in insects (Bilgo et al., 2017; De Vooght et al., 2014; Fang et al., 2011; Gilbert et al., 2016; Leonard et al., 2018; Lovett et al., 2019; Leonard et al., 2020; Rangberg et al., 2012; Ren et al., 2008) or in corals (Levin et al., 2017). Moreover, some of the applications use a technique

known as ‘paratransgenesis’ which aims to alter the biological characteristics of the host by genetically engineering its microbiome (Wilke et al., 2015).

2. Horizon scanning of Synbio applications in LMOs with relevance for assessment of risks for health and the environment

There are several databases available which show a broad range of Synbio applications on LMOs such as:

https://datam.jrc.ec.europa.eu/datam/mashup/NEW_GENOMIC_TECHNIQUES/index.html
http://euginius.eu/euginius/pages/home.jsf
http://www.eu-sage.eu/genome-search

These databases are based on different criteria and searches can bring different results. In general, the information made available in the databases on specific applications is of mixed quality and largely depends on the availability of relevant publications or entries in other databases. In several cases, the available information is poor, for example, because the data are considered to be confidential business information. No or only limited conclusions can be drawn from these data as to which of these applications will finally enter the market successfully.

However, if their limitations and specificity are taken into account, these databases can nevertheless be useful in that they provide an overview.

2.1 Synbio plants and animals already introduced into markets

The Euginius database (July 2022) lists three animals for food production and two plants which already have market approval in Japan and the US:

- The US FDA (Food and Drug Administration) published its opinion in 2022 on cattle with short, slick coats (meant to be beneficial in higher temperatures), expressing no objections against the marketing of products derived from Synbio beef cattle and their progeny.
- Two Synbio fish ‘events’, sea bream and pufferfish, were approved for sale in Japan in 2021. The fish were developed for faster growth and a higher proportion of muscle or a larger body size compared to conventional fish.
- Japan approved the commercial sale of an Synbio tomato producing a higher amount of GABA (γ-Aminobutyric acid) in 2021. The fruits are supposed to reduce blood pressure if consumed.
- Calyxt was the first company to bring seeds derived from targeted mutagenesis (TALENs) to the US market. The soybean with high-oleic acid oil content was brought to market in the US in 2019. However, the soy failed to produce the desired yields for the farmers and did not meet the expectations of the investors. It appears that the genetic intervention actually resulted in a reduced soybean harvest. Consequently, the company producing the soybean, Calyxt, exited this line of business in 2020. Sales, earnings and the value of Calyxt stock fell dramatically as a result.[3] It appears to be doubtful whether the soybean is actually still on the market.

3https://www.bizjournals.com/twincities/news/2022/09/22/calxyt-considering-sale-of-assets-merger.html

2.2 Synbio applications in food plants

With regard to plants, the focus is on a broad range of species including cereals, oil and fiber crops, vegetables, fruit plants; trees and others are also being targeted in the research and development of Synbio applications. See Figure 1 for a list (JRC database, July 2022).

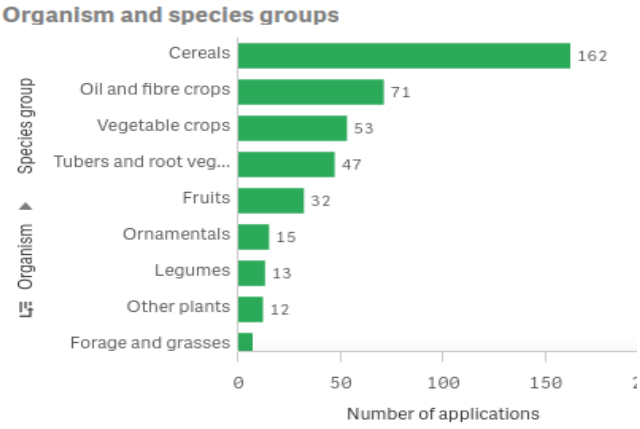

Figure 1: Plant species being used in Synbio (also called new genomic techniques, NGT) applications up until the end of July 2022. Source: https://datam.jrc.ec.europa.eu/datam/mashup/NEW_GENOMIC_TECHNIQUES/index.html

With regard to potential traits, categories such as modified composition, stress tolerance (biotic and abiotic), yield, herbicide tolerance, storage performance and others are used. See Figure 2 for a list (JRC database, July 2022).

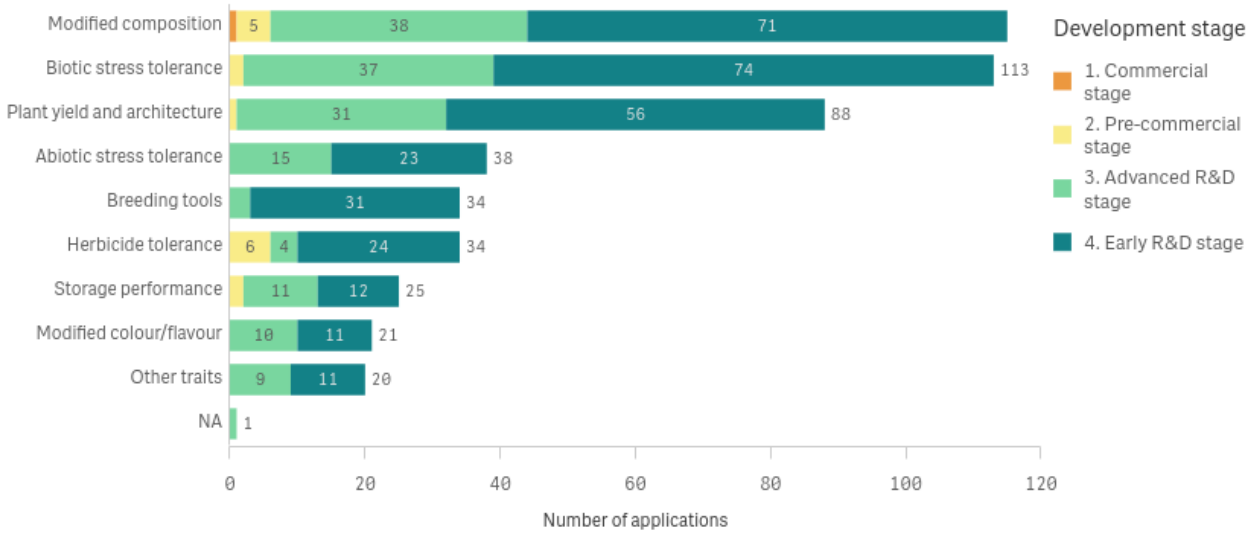

Figure 2: Traits that are assumed to be under development with the help of Synbio- up until the end of July 2022. Source: https://datam.jrc.ec.europa.eu/datam/mashup/NEW_GENOMIC_TECHNIQUES/index.html

Some indications as to potential products that may enter the market within the next few years can be derived from the JRC database (see Figure 3). Most of those applications concern herbicide-tolerant crops.

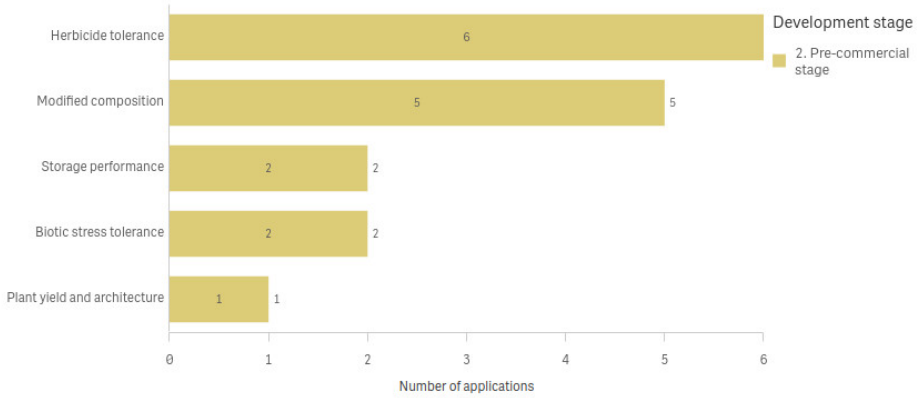

Figure 3: Traits that are claimed to be under development with the help of Synbio - up until the end of July 2022. Source: https://datam.jrc.ec.europa.eu/datam/mashup/NEW_GENOMIC_TECHNIQUES/index.html

2.3 Synbio applications in animals (vertebrates)

Most applications of Synbio in animals concern pharmaceutical research. There are, however, also several applications aimed at using animals in food production, such as cattle, pigs, poultry and fish. Again, data availability is in many cases poor. For example, no data are available for pigs listed in the Euginius database which were developed by the University of Edinburgh for disease resistance (GE-CD163 Pig), or developed by Revivicor with allergen reduction (GalSafe pig). A further example are hens developed by researchers in Israel which are supposed to not produce male offspring, where data only seem to be available from patent applications (such as WO2020178822) but not from any peer reviewed research. Some of the Synbio animals are already approved for food production in the US (cattle) and Japan (fish) and are described below.

2.4 Synbio applications involving microorganisms and viruses

Applications of Synbio may involve microorganisms such as bacteria, archeae, fungi, yeast, and in some cases, viruses. EFSA published the results of a horizon scanning in 2020 which mentions more than 700 relevant publications, 45 cases and a selection of 11 examples (EFSA, 2020b). Starting with this overview, but also by taking into account other publications from ongoing horizon scans, we compiled the following, non-comprehensive list. Various Synbio-tools were applied in these examples as well as other tools (EFSA, 2020b). In here the various applications are summarized as genetic engineering (GE) for greater clarity.

- Potential uses of GE microorganisms could include the engineering of ecosystems and microbial communities for purposes such as changing biodegradation, waste treatment and bioremediation (Mee et. al., 2014; Qian et al., 2020; Wang et al., 2013).
- Several projects aim to change gut microbiota in animals and humans (Kim et al., 2018; Mimee et al., 2015; Ronda et al., 2019). Some of these approaches are under discussion for therapeutic concepts (Bober et al., 2018; Hwang & Chang, 2020; Mimee et al., 2015; Ozdemir, 2018; Sheth et al., 2016).
- Other applications directed at food and feed aim to change the composition of diets and products for human consumption (Lee et al., 2016; Mertens et al, 2019).
- GE applications to change gut microbiota are also under discussion, e.g. for insects such as flies (De Vooght et al., 2014; Gilbert et al., 2016) mosquitoes (Bilgo et al., 2017; Fang et al., 2011; Lovett et al., 2019; Ren et al., 2008) and bees (Leonard et al., 2018; Leonard et al.,

2020; Rangberg et al., 2012). Some of these approaches are known as ‘paratransgenesis’, which means that the biological characteristics of the target host are changed by genetically engineering its symbiotic bacteria, for example, to eliminate a pathogen from insects via the expression of effector molecules (Wilke et al., 2015).
- Similar approaches are under discussion for corals (Levin et al., 2017).
- In agriculture, there are ongoing discussions in regard to applications to change the microbiomes of plants, e.g. mycorrhiza or endophytes (Arif et al., 2020; Checcucci et al., 2018; Hettiarachchige et al., 2019; Ke et al., 2022; Vorholt et al., 2017).
- In agriculture, GE applications targeting soil microorganisms are also being discussed (Shelake et al., 2019; Shulse et al., 2019; Temme et al., 2012).
- Further potential uses include the usage of GE microorganisms as pesticides (Azizoglu et al., 2020; Fang et al., 2014; Leclère et al., 2005; Scheepmaker et al., 2016; Tseng et al., 2005; Wang et al., 2011).
- Several projects are looking at using GE microorganisms (such as cyanobacteria or algae) in energy production (Motomura et al., 2018; Nozzi et al., 2013; Wang et al., 2013).
- Other applications include viral systems, such as bacteriophages (Citorik et al., 2014; Lemire et al., 2018), and even the dissemination of genetically engineered/GE viruses via insects (‘insect allies’) for potential military purposes (Lentzos et al., 2022; Reeves et al., 2018).

3. Technical and biological characteristics of selected examples

The following section contains some selected examples with relevance for risks to health and the environment. These examples include plants and animals recently given market approval in the US and Japan.

In addition, some further cases were selected:
- Synbio mushrooms were the first ‘CRISPR-food’ product meant for the US market.
- Hornless cattle were the first Synbio product to be withdrawn from market application in the US (and Brazil).
- Herbicide-resistant maize is the first Synbio plant for which an application has been filed for market approval in the EU.
- CRISPR laying hens which are under discussion to be brought onto the market in the EU.
- Synbio camelina was chosen because of its relevance to both environmental and health risks.
- The examples of wheat and tomatoes were chosen because of their relevance for food production and some specific comments made by European Food Safety Authority (EFSA).

3.1 Examples of Synbio plants and mushrooms

The following section contains short technical case studies describing examples of Synbio plants for use in food production. Some of them have already been applied for and/or have marketing approval.

3.1.1 CRISPR-mushrooms

This example was the first Synbio food product derived from CRISPR/Cas (SDN-1) that was declared to be safe by US APHIS (Animal and Plant Health Inspection Service) and ready for market introduction in 2016 (Waltz, 2016). However, the CRISPR/Cas mushrooms are so far not

available to consumers. Edible mushrooms were created using CRISPR/Cas to stop cut surfaces from turning brown by blocking the function of the polyphenol oxidase gene; the non-browning mushrooms were meant to have a longer storage and shelf-life. This was achieved by destroying the structure of the target gene that is present in the fungus in several copies. This meant that the fungus was changed in several locations on the same gene. Such a pattern of genetic change is unlikely to appear spontaneously. The responsible US authority, APHIS, approved the mushrooms in April 2016,[4] because it was, in their view, sufficient that the developers said that no additional DNA had been inserted. At this stage, no further investigations were required to check whether other substances in the mushrooms had changed. No data on unwanted changes in the genome were available. It seems there is also no peer reviewed scientific publication on how exactly the properties of these mushrooms were intentionally or unintentionally changed. The likelihood of these mushrooms ever really being brought to market still seems not decided.

3.1.2 Herbicide-resistant maize

The first Synbio plant for which an application for market approval was sought in the EU is a maize variety developed by Pioneer/Corteva (previously owned by DowDuPont).[5] The plant was generated with the help of CRISPR/Cas (SDN-3). Maize DP915635 is resistant to the herbicide glufosinate and produces an insecticidal toxin found in specific ferns growing on trees.[6] The maize was generated with a combination of old and new genetic engineering methods: to deliver the CRISPR/Cas ‘gene scissor’ into the plant cells, they are first bombarded with small particles (‘gene gun’). The cells then produce the enzyme for the gene scissors which is subsequently inserted as a DNA-sequence into the maize genome. This additional DNA-sequence is meant to facilitate the insertion of other genes. It is therefore is known as a ‘landing pad’. In the next step, a further gene construct is inserted into the ‘landing pad’ in the maize genome, thus conferring resistance to the herbicide and producing the fern toxin. The company has filed several patent applications for the plants, some of which have already been granted in Europe.

3.1.3 GABA tomato

Japan approved the first Synbio plants for consumption in Japan in 2021.[7] These are tomatoes with a much higher concentration of a specific plant compound (GABA) compared to conventionally bred tomatoes. Several previous attempts to achieve a permanently higher level of GABA in the plants through conventional breeding failed. GABA (γ-Aminobutyric acid) is an inhibitory neurotransmitter in the central nervous system which may, amongst others, reduce blood pressure. The tomatoes will therefore be introduced as a modern ‘lifestyle’ product. At the same time, it is known that GABA has a multifunctional role in tomato plants: it influences, for instance, plant growth, resistance to plants pests and diseases as well as several other metabolic reactions. Due to the multifunctional role of GABA, it should be assumed that the genetic intervention will affect plant metabolism on several levels. These changes can also cause unintended health effects at the stage of consumption. In addition, the plants can show unexpected reactions to environmental stress conditions, which can again have an impact on the safety of food products (Nonaka et al., 2017). As far as is known, no data are available on the potential benefits or on potential adverse effects.

4 www.aphis.usda.gov/aphis/ourfocus/biotechnology/am-i-regulated/Regulated_Article_Letters_of_Inquiry
5 www.testbiotech.org/pressemitteilung/erster-zulassungsantrag-fuer-crispr-pflanzen-in-eu
6 www.testbiotech.org/content/application-authorisation-maize-dp915635-pioneer
7 http://euginius.eu/euginius/pages/home.jsf

3.1.4 CRISPR-camelina

Many scientists in the US and the EU are interested in genetically engineering camelina (*Camelina sativa*). One focus is on the production of agro-fuel. Camelina plants, in which 18 sites on the genome were changed using CRISPR/Cas gene-scissors, were developed in the US (Morineau et al., 2017). The multistep process also involved the application of 'old' non-targeted methods of genetic engineering known as transformation by *Agrobacterium tumefaciens*. Since *C. sativa* is an allohexaploid plant composed of three sub-genomes, conventional breeding faces substantial limitations where homozygous mutations of homeologous genes is required. As a result, the Synbio plants show patterns of genetic change and altered oil quality that would not be possible or at least very unlikely to achieve with conventional breeding even without inserting additional genes. In 2018, APHIS declared the plants to be safe for the market.[8] Camelina is one of the oldest cultivated plants in Europe and is an important plant species for pollinating insects. The plants can survive and multiply in the environment as well as cross into natural populations. Experts are warning that risks can arise from the cultivation of the genetically modified plants due to their altered oil quality and potential uncontrolled spread (see Kawall, 2021a). For example, the oleic acids formed in genetically modified plants can change the growth and reproductive rate of wild animals feeding on them. Problems could also arise if the oil seeds are accidentally introduced into food and feed.

3.1.5 De-novo domesticated tomato

In 2018, researchers succeeded in using CRISPR/Cas to change several genes at the same time in non-domesticated wild tomatoes. Six genes were knocked out with the result that small fruits growing on bushy plants were changed into tomatoes that look similar to the ones currently being marketed (Zsögön et al., 2018). This was intended to show that the outcomes of years of conventional breeding are replicable within a very short period of time using Synbio processes. Even though no additional genes were inserted, the impact was extraordinary: the number of fruits, their size, form and composition, as well as the architecture of the plants, were changed in just a few working steps and within a short period of time. EFSA (2022a) also analyzed this Synbio plant.

3.1.6 Wheat 'events'

The following section contains four examples taken from species of wheat (*Triticum aestivum*) with different traits. They were all developed with Synbio (SDN-1) using multistep processes (involving old GE such as biolistic methods). Bread wheat (*Triticum aestivum*) is characterized by its huge genome, comprising six sets of chromosomes (Guan et al., 2020). This causes some difficulties in conventional breeding since in many cases a high number of gene duplications are involved in a specific trait.

As explained above, Synbio processes offer the potential to overcome the limitations of previous breeding methods by introducing genetic changes in all gene copies at the same time. However, in the case of the selected Synbio traits, there are also reasons to assume that the intended (on-target) genetic alterations are associated with unintended biological characteristics (see below).

Trait 1 - reduction in gluten: Gluten proteins in wheat are thought to trigger several gluten-related disorders, including celiac disease (Gatti et al., 2020). It is known that alpha-gliadin peptides contribute to the overall concentration of gluten in bakery products (Verma et al., 2021). These

8 www.aphis.usda.gov/aphis/ourfocus/biotechnology/am-i-regulated/Regulated_Article_Letters_of_Inquiry

genes occur within a large family of genes that are present in multiple copies at different locations in the genome. With the help of the CRISPR/Cas nuclease, scientists succeeded in 2018 in switching off a large number of these genes: 35 of 45 genes that are necessary to produce alpha-gliadins were knocked out (Sanchez-Leon et al., 2018). This resulted in a new genotype and also resulted in a greater degree of complexity for risk assessment (EFSA, 2021). However, gliadins are, for example, also known to play an important role in the plant responses to stress conditions, including drought and heat (Blumenthal et al, 1995; Marín-Sanz et al., 2022; Phakela et al., 2021). Therefore, larger reductions in the content of alpha-gliadins may also unintentionally impact the heat and/or drought tolerance of this trait.

Trait 2 - reduction in acrylamide: CRISPR/Cas9 was used to reduce the content of the free amino acid asparagine in wheat (Raffan et al., 2021). Free asparagine is present in higher concentrations in wheat grain. It is a precursor of acrylamide, which forms during the baking, toasting and high temperature processing of foods made from wheat. Acrylamide has been shown to have carcinogenic properties. The relevant gene (asn2) occurs a total of six times in the wheat genome. In some of the wheat plants, the asparagine content was reduced by 90% compared to the wild type. Other methods have not previously achieved such a strong reduction of the asparagine content in wheat grain. While it seems that a gene function involved in the production of the amino acid asparagine was to some extent successfully blocked, this also creates problems since asparagine is also involved in seed germination, plant growth, stress response and defense mechanisms. It was found that some lines of this CRISPR-wheat almost lost capacity to germinate (Raffan et al., 2021). This wheat is about to be tested in field trials in the UK, first preliminary data were published in 2023 (Raffan et al., 2023) which exhibit changes in weight and number of grains derived from the Synbio wheat.

Trait 3 - reduction in susceptibility to powdery mildew: The mildew resistance locus o (mlo) gene in barley is of interest for several projects. There are three different mlo genes involved in resistance to powdery mildew which is found in natural populations. One of the studies used TALENS to target the mlo gene in hexaploid wheat (Wang et al., 2014). The nuclease introduced alterations in all three homoeoalleles of mlo in wheat, enabling their parallel knock-out. This was not previously possible with either chemical mutagenesis or other breeding methods. The simultaneous knock-out of the three homoeoalleles conferred a broad-spectrum resistance to powdery mildew in these lines. In addition, unintended effects were described in the wheat (i.e., leaf chlorosis under growth conditions), which were also not observed in randomly mutated plants (Acevedo-Garcia et al., 2017). Growth aberration, accelerated senescence, induced necrosis, increased susceptibility to other fungal pathogens are all unintended effects described in the context of this trait – which may, however, also be overcome (Spanu, 2022).

Trait 4 - increased immune response to fungal diseases: In the German PILTON[9] project, researchers are aiming to block the gene function of a gene (CPL3) in wheat that is known as a regulator in the fine tuning of immune responses in the plants (Koiwa et al., 2002; Li et al., 2014). The intention is to block the function of the CPL3 gene by using the CRISPR/Cpf1 nuclease variant. The plant might thus be able to prolong or enhance its immune response to plant diseases, such as wheat leaf rust (*Puccinia triticina*), which is a fungal disease affecting leaves and grains. With the help of Cpf1, it may be possible to knock out all gene copies on each of the six sets of chromosomes. However, as preliminary results show, the loss of the gene function is associated with fitness costs for the plants: they are likely to show slower growth and earlier flowering which indicates reduced fitness. The start of the project has already been announced, the initial data were

9 https://pilton.bdp-online.de/?lang=en

meant to be published in 2021, however, as of August 2022, it seems no results have been published yet.

3.2 Examples of Synbio animals

The following section of the backgrounder contains short technical case studies of Synbio animals for food production that were either approved for the market or for which applications have been filed.

3.2.1 Cattle with short, slick coats

This is the first Synbio animal for food production deregulated in the US[10], but not yet on the market. In March 2022, the US FDA has decided to issue approval for CRIPSR/Cas cattle with short, slick coats for agricultural purposes.[11] CRISPR/Cas was used to alter the genes of a receptor for the hormone prolactin (SDN-1). The aim was to generate cattle with shorter hair, a trait called SLICK which is already known from traditional breeding. Animals with this conventionally bred trait are, according to various studies, better able to cope with higher ambient temperatures (see, for example, Hansen, 2020). Four calves were examined, one of which was not genetically engineered, probably because the gene scissors had failed to work as expected. Another calf died unexpectedly, but the FDA assumes that this incident was not related to the genetic intervention. It is remarkable that neither of the 'successfully' genetically engineered animals show the intended changes consistently in all the cells of their body. This phenomenon is known as genetic mosaicism or chimeric formation. Unintended genetic changes were also found in the cattle, these were, however, considered to be less severe. At the same time, the data provided by the FDA includes no proof of whether the animals will stay healthy over their lifetime. If the male animals are used for further breeding, their intended and unintended genetic changes could rapidly spread throughout larger cattle populations. The animals will be marketed by Recombinetics and its affiliated company, Acceligen, also has filed patents (WO2017053315).

3.2.2 Hornless Synbio cattle

This is the first Synbio animal for which application was withdrawn from US market and also Brazil. In 2019, the US FDA scrutinized rejected the approval of hornless cattle engineered with TALEN gene scissors (SDN-2). At that time, it was shown that the processes of genetic engineering had caused genes from bacteria to be unintentionally integrated into the genome of the cattle and passed on to the next generation (Norris et al., 2020). The cattle had been genetically engineered before 2016 (Carlson et al., 2016), but it was only in 2019 that scientists noticed that genetic material of the bacteria used in the process had also been introduced into the genome of the cattle (Norris et al., 2020). Amongst other things, they found complete DNA-fragments able to confer resistance to antibiotics in the genomes. If the genetically engineered cattle had been used for breeding as planned, the unwanted genes could have spread rapidly through dairy herds. Consequently, the Synbio cattle were not approved for the market and had to be slaughtered. In Brazil, the cattle already passed deregulation, however, were withdrawn after the findings of Norris et al. (2020).[12] Hornless cattle were also generated using CRISPR/Cas (SDN-2). The process of Synbio similarly caused many unintended effects (Schuster et al., 2020). In 2022, another study was published (Hennig et al., 2022) in which researchers tried to apply CRISPR/Cas to delete a targeted

10 http://euginius.eu/euginius/pages/home.jsf
11 https://cacmap.fda.gov/media/155706/download
12 http://ctnbio.mctic.gov.br/tecnologias-inovadoras-de-melhoramento-genetico-rn16-

region in the genome instead of inserting a new gene function. While the deletion was partially successful, all calves still developed horn buds.

3.2.3 Synbio seabream with a change in growth

Japan allowed the first Synbio fish to be marketed in 2021. They were produced with the help of CRISPR/Cas (SDN-1).[13] Gene functions which regulate muscle growth were blocked in the genome of red seabream (*Pagrus major*). In response, the fish had more muscle growth, a larger body size, a reduction in body length and an abnormal position of the vertebra (Kishimoto et al., 2018). In comparison to the wild type, the fish gain weight faster and seem to move slower. No data are available to show how the genetic alteration affects their life span or health in general. There are also apparently no data available on animal welfare. There are similarly no data on changes in the composition of flesh in the fish or any potential impact on consumers. On a technical level, this shows that the genetic intervention was not precise: starting with hundreds of GE fish, the researchers selected those deemed suitable for further breeding. The targeted gene sites showed differing alterations. Furthermore, in many cases, genes were altered in some organs, but not in all cells of the body. It is assumed that the cost of feeding GE fish reared in special containers could be reduced (Kishimoto et al., 2018).

3.2.4 Synbio pufferfish with a change in growth

Japan approved another CRISPR/Cas (SDN-1) fish for the market in 2021.[14] Gene functions were blocked in the genome of pufferfish (*Takifugu rubripes*) that control the appetite of the fish: the leptin receptor gene in the fish was disrupted, which may be associated with weight gain and diabetes-like symptoms (Kurokawa & Murashita, 2009). Until now, fish species such as zebra fish (*Danio rerio*) inheriting similar genetic defects have been used as disease models to explore complex metabolic disorders in mammals (Audira et al., 2018). There are further studies on medaka fish (*Oryzias latipes*) which showed large deposits of visceral fat in the adult fish (Chisada et al., 2014). However, it is not possible to compare these data with the pufferfish since peer reviewed publications seem to be missing. It seems that the cost of feeding of the Synbio fish reared in special containers may be reduced. At least, this is the rationale behind the filed patent applications (such as WO2019066052) for the industrial usage of the fish.

3.2.5 CRISPR hens

Researchers in Israel have used CRISPR/Cas to alter hens so that no male offspring are able to hatch. A deadly gene is passed on to any male offspring with the intention of killing the male embryos in the egg before they hatch. At the same time, the female offspring will supposedly develop normally so that they can be used as laying hens for egg production. This Synbio application aims to solve the problem of male offspring in the process of breeding hens, as these are killed after hatching because they are of no economic benefit to the food producers. Patents for the process and the resulting hens have already been filed (such as WO2020178822) and could in due course be marketed in cooperation with a US company. The patent applicants claim that their technology is safe and there are no transgenes in the genome of the laying hens. However, no peer reviewed data could be identified on the intended and unintended effects in Synbio poultry and their eggs.[15]

13 http://euginius.eu/euginius/pages/home.jsf
14 https://euginius.eu/euginius/pages/gmo_detail.jsf?gmoname=GE-lepr+tiger+pufferfish
15 For further information also see: https://www.testbiotech.org/en/news/new-ge-deregulated-through-backdoor

4. Issues with relevance to the risk assessment of Synbio-LMOs

The following section provides an overview of some categories of environmental hazards and risks associated with Synbio processes. In addition, selected applications exemplify hazards and risks. These show that the technical potential of the technical processes and their risks and hazards are closely interrelated.

4.1 Specific risks associated with Synbio plants

As shown, Synbio can be used to achieve genomic changes extending beyond what is known from conventional breeding, even if no additional genes are inserted. Compared to methods of conventional breeding (including random mutagenesis), Synbio can overcome the boundaries of natural genome organization that have emerged over the course of evolution. CRISPR/Cas 'gene scissors' make it possible to alter the genome to a much greater extent than with any previous breeding.

The greater accessibility of the genome enables pervasive changes in the biological characteristics of the organisms, even without the insertion of additional genes. It also enables more extreme versions of already known traits or the generation of new traits which are often associated with 'trade-off' responses (side effects).

Furthermore, unintended genetic changes have been observed (on-target and off-target) that are specific to the processes of Synbio and unlikely to occur due to random processes or conventional breeding. These genetic irregularities must be considered as risks inherent to the technology.

Risk assessment needs to consider both the indirect effects caused by the intended traits and the unintended genetic alterations.

4.1.1 Risks associated with the intentionally introduced traits

Many of the intended Synbio traits that can be generated without the insertion of any new gene functions (SDN-1 processes), such as changes in oil content (Morineau et al., 2017), protein composition (Sanchez-Leon et al., 2018), sugar concentration (Kannan et al., 2018), plant architecture (Shen et al., 2017), yield (Roldan et al., 2017) or biologically active plant constituents such as GABA (Nonaka, et al., 2017), reach beyond what is likely to be achieved by conventional breeding (for overview, also see Kawall, 2021b). These new intended GE traits are the result of specific patterns of genetic changes introduced by gene scissors such as CRISPR/Cas. In a similar way to that by transgenic plants produce insecticidal proteins originating from bacteria, such genotypes are unlikely to result from random mutations and other conventional breeding methods. The depth of interventions may unavoidably cause 'trade-off' responses (metabolic side effects) in the organisms which are associated with the unintended biological effects. The following section describes the risks that can emerge from these genotypes.

a) Case study – Synbio camelina

A first detailed risk scenario for Synbio plants was provided by Kawall (2021a). This scenario examined Synbio camelina with intended changes in oil content that are unlikely to be achievable with conventional breeding (Morineau et al., 2017, see also example 3.1.4). Kawall (2021a) shows that if the composition of the fatty acids is changed, unintended effects on various processes can occur in addition to the desired properties. This may be related to effects on the formation of certain

messenger substances with which plants communicate and with which they, for example, 'warn' of a pest infestation. A change in the composition of fatty acids can affect and influence existing food webs. In addition, there is also the possibility that genome-edited plants will hybridize with wild species leading to unintended effects in subsequent generations. At the same time, the genome-edited camelina has the potential to persist in the environment and spread uncontrollably. Therefore, the risks identified concern the food web, the defense mechanisms of the plants and uncontrolled gene flow. Kawall (2021a) concludes: "*There are also special concerns regarding interventions in well-balanced signalling pathways that regulate communication and interactions between plants, animals, associated microbiomes, beneficial predators and pollinators potentially affecting ecoservices. In addition, next-generation effects can occur in case genome-edited plants have the potential to persist and propagate in the environment.*"

b) Case study – Synbio wheat

As shown in example 3.1.6, there are several Synbio applications in wheat which result in genotypes that are unlikely to result from the use of previous breeding methods. EFSA analyzed one of these examples EFSA (2021) when discussing new challenges for risk assessment (Sanchez-Leon 2018, see also trait 1 of example 3.1.6). EFSA (2021) states in its case study: "*(…) the large number of mutations required to achieve gluten-free wheat is far beyond any plant previously assessed. This is likely to require SynBio approaches to correctly identify all gliadins and glutenins in the hexaploid genome of bread wheat and to identify an engineering strategy that introduced mutations of the correct nature and positions in each gene to prevent the accumulation of any peptide fragments associated with initiation of the inflammatory cascade*". Kawall (2021b) summarized these findings: "*One example to illustrate the generic risks of CRISPR/Cas is a wheat generated by Sanchez-Leon et al. (…). The same study was also listed as an example by the European Food Safety Authority (EFSA) in its recent scientific opinion (…) According to EFSA, their case study shows that a strategy is needed to identify the type of alteration and position in each individual gene to prevent the accumulation of any unintended peptide fragments. Such analyses are of major importance for risk assessment, especially when considering SDN-1 applications with a higher level of complexity and/or depth of intervention.*"

In conclusion, this case shows that even if changes are successfully introduced into the target genes, complex questions with regard to the safety of the plants need to be considered: each targeted genetic site needs to undergo a detailed examination to determine whether the alpha-gliadin proteins are still being produced, or if new proteins are produced unintentionally, or if any other unintended effects may occur.

c) Case study – Synbio *de-novo* domesticated tomato

Zsögön et al., (2018), Kawall (2021b) and EFSA (2022a) appear to come to similar conclusions for *de novo* domesticated tomatoes (see also example 3.1.5). As Kawall (2021b), states: "*(…) plants altered with SDN-1 which contain traits that are known from cultivated varieties, but are expressed in a new genetic background, cannot be equated to their conventional or natural counterparts, as the corresponding target gene(s) might have divergent functions or interactions in different species. De novo domesticated plants generated using CRISPR/Cas9 are interesting examples in that regard. (…) Comprehensive environmental and health risk assessments will be needed to ensure that no effects with negative impacts have occurred.*"

EFSA (2022a) comes to the conclusion that current EU guidance, which is based on comparative risk assessment, would not be sufficient to assess these risks: "*This case study highlighted potential issues for the applicability of the existing comparative analysis guidelines with respect to the*

availability of the conventional counterpart and non-GM reference varieties. The parental line used to obtain this SynBio product (S. pimpinellifolium) is not commonly consumed (…). The selection of reference varieties would also be challenging: wild tomato varieties of commercial use as food and feed might not be available. Tomatoes cultivated for food and feed purposes could be of interest for comparison, considering the intended use of the SynBio tomato, but would be genetically far from the SynBio plant. As a consequence of the lack of an appropriate comparator (…), the comparative analysis for this SynBio case may not be carried out as described in the existing guidelines."

In conclusion, this case seems to exemplify a specific aspect of the unique technical potential of CRISPR/Cas: until now, traditional breeding has developed new varieties step-by-step over many years. Now, however, CRISPR/Cas can change multiple copies of a gene as well as change several different genes at the same time in just one step, an approach known as 'multiplexing' (Kawall et al., 2020; Raitskin and Patron, 2016). Even though no additional genes are inserted, the impact is extraordinary: the number of fruits, their size, form and compounds as well as the architecture of the plants can be changed in just a few working steps and within a short period of time. However, the resulting risks are complex. Whether these tomatoes, which look just like normal tomatoes, are actually safe to eat can only be clarified by thorough investigations.

d) Overview: Unintended effects linked to intended changes

In general, direct and indirect effects can be caused by the intentionally generated traits. The traits derived from Synbio can cause extreme variants of biological characteristics and also generate new traits which are unlikely to be achieved with conventional breeding. The depth of intervention may unavoidably cause 'trade-off' responses (metabolic side effects) in the organisms. The traits derived from Synbio can likewise generate extreme variants of biological characteristics and new traits which are unlikely to be achieved with conventional breeding. The unintended direct and indirect effects associated with the intended traits may, for example, have serious adverse impacts on the environment, plant or animal health, agricultural yield, pesticide use and food safety. If released into the environment, the interactions with other Synbio-LMOs and with the environment, including pests, pathogens, climatic conditions etc., adds further complexity to these risk scenarios.

In many cases, the desired advantages are linked to trade-offs caused by the pervasive changes in biological characteristics. As the summary of examples in Table 1 shows, such unintended effects were identified as relevant to several Synbio plants. Similarly, as is the case with the intended traits, these unintended effects are likely to go beyond what was caused by previous methods of breeding (see Kawall, 2021a and 2021b).

Table 1: Selected examples of unintended effects associated with the intended traits and relevant to the risk assessment of Synbio plants.

Species	Intended trait	Unintended metabolic and physiological effects and hypothesized risks
Wheat	Powdery mildew resistance (example 3.1.6, trait 3)	Growth aberration, accelerated senescence, induced necrosis, increased susceptibility to other fungal pathogens. (Spanu, 2022)
Wheat	Decreased acrylamide content (example 3.1.6, trait 2)	Reduced growth and germination rate, potentially increased susceptibility to fungal plant pathogens. (Raffan et al., 2021)
Camelina	Altered oil quality (example 3.1.4)	Weakened defense mechanisms against biotic (pathogens) or abiotic (climate change) stressors. (Kawall, 2021)
Tomato	Enhanced GABA content (example 3.1.3)	The changes in plant composition may also cause unintended health effects at the stage of consumption. Furthermore, unexpected reactions of the plants to environmental stress conditions are not unlikely. (Nonaka et al., 2017)
Tomato	Accelerated domestication (example 3.1.5)	Differences in plant composition are observed in comparison to previously bred tomatoes. These differences may also impact health at the stage of consumption. (Zsögön et al., 2018)
Rice	Improved salinity tolerance	Enhanced invasiveness might occur in weedy rice after hybridization. (Zhang et al., 2019)

Unintended effects associated with the intended traits listed in Table 1 may have serious adverse impacts on the environment, plant health, agricultural yield, pesticide use, and/or food safety. If grown in fields, the interactions between Synbio-LMOs and the environment, including pests, pathogens, climatic conditions etc., adds further complexity to these risks. These unintended direct or indirect effects associated with the intended trait are the result of interactions in the complex networks of genes, proteins and other biologically active molecules. Such unintended effects can also emerge in cases where the genetic intervention is targeted and precise.

4.1.2 Specific, unintended effects caused by the processes of Synbio

In a similar way to the intended traits, unintended effects can also cause patterns of genetic change that go beyond what can be achieved with conventional breeding and result in specific risks. The unintended genetic changes include off-target DNA cleavage, repetitive unit deletion, indels of various sizes, larger structural changes in the targeted genomic region and the unintended insertion of transgenes. While some of these ‘types’ of genetic alteration might also be observed in conventional breeding (EFSA, 2022c), the probability for these changes to occur on a specific site in the genome and the resulting genotype can be very different (for overview see Kawall, 2021). If these unintended effects are overlooked, they may quickly spread within large populations. Moreover, if the seeds are used for further propagation and breeding, potentially hazardous genetic alterations can remain undetected for a longer period of time and may also accumulate.

Findings relating to a broad range of unintended effects caused by CRISPR/Cas have already been published. Several publications describe how CRISPR/Cas causes unintended changes, including off-target effects, on-target effects and chromosomal rearrangements (Adikusuma et al., 2018; Biswas et al., 2020; Burgio et al., 2020; Cho et al., 2014; Grunewald et al., 2019; Haapaniemi et al., 2018; Kapahnke et al., 2016, Kosicki et al., 2018; Kosicki et al., 2022; Lalonde et al., 2017; Leibowitz et al., 2020; Liu et al., 2021; Michno et al., 2020; Ono et al., 2019; Sharpe, 2017;

Skryabin et al., 2020; Tuladhar et al., 2019; Weisheit et al. 2020; Wolt et al., 2016; Chu & Agapito-Tenfen, 2022).

In several cases, unintended genetic alterations in the target region (on-target) or in other genomic regions (off-target) specific to gene scissors, such as CRISPR/Cas, have been described. For example, larger structural genomic changes, such as translocations, deletions, duplications, inversions and scrambling of chromosomal sequences, can occur near the SDN target site (as well as at the SDN target site) which would otherwise be unlikely to occur (see e.g., Hahn & Nekrasov 2019). In addition, specific unintended on-target effects often include the integration of DNA from vector DNA derived from transformation processes, where, for example, bacterial DNA was unexpectedly integrated (e.g., Andersson et al., 2017; Li et al., 2015; Zhang et al., 2018). Overall, the CRISPR/Cas9 system has been confirmed to have a high frequency of integration into the target site, resulting in large deletions at the target sites (Lee et al., 2019; Yang et al., 2022).

In general, the CRISPR/Cas machinery is known for its potential to confuse target regions with specific off-target regions, in addition to causing the unintended insertion of additional genes, decoupling of genes and other specific genomic alterations (of categories such as inversions, deletions or rearrangements) that are unlikely to emerge from spontaneous mutations or physical and chemical mutagenesis (see, for example, Biswas et al., 2020; Braatz et al., 2017; Hahn & Nekrasov 2019). In some cases, unusual patterns of inheritance have also been observed, thus escaping the Mendelian rules (Yang, et al., 2022).

These unintended changes can cause a variety of unwanted effects. For example, the integrity of a non-target gene may be compromised if its coding region is cleaved by CRISPR/Cas (e.g. cleavage at off-target-sites). This could lead to changes in the metabolism of the organism that could affect its safety for human health and the environment. Such effects are highly dependent on the genomic context within which such unintended alterations occur (e.g. within a gene, loss of function mutations; outside of genes, unintended alterations in promoters could alter gene expression).

As a result, in similar way to the case with the intended effects, unintended effects can also cause patterns of genetic change that go beyond what can be achieved with conventional breeding and result in specific risks. Yang et al. (2022) give an overview of irregular genetic changes and specific unintended effects caused by intrinsic factors of the CRISPR/Cas systems in plants. These include off-target DNA cleavage, repetitive unit deletion, and indels of various sizes (Chakarbarti et al., 2019; Kapusi et al., 2017; Manghwar et al. 2020; Molla and Yang, 2020; Zhang et al., 2014). In this context, the dosage of CRISPR/Cas complexes expressed in cells can also result in a significant increase of off-target mutation frequency (Ordon et al., 2017; Zhang et al., 2018).

In addition, it should be taken into account that Sybnio is a multi-step process, with inherent and specific risks independent of the purposed traits. For example, the application of CRISPR/Cas in plants, typically make use of older genetic engineering methods, i.e. non-targeted methods to deliver the DNA coding for the nuclease into the cells. Thus, in most cases, the result of the first step of the CRISPR/Cas application is a transgenic plant which may show a broad range of unintended genetic changes that are unlikely to emerge from conventional breeding. Conventional breeding is only used at the end of the multistep process to remove the transgenic elements from the plant genome (segregation breeding). However, without adequate standards of risk assessment in place, the unintended genetic changes may remain undetected in the genome, spread quickly and widely within the populations, and may also accumulate.

The mechanisms and outcomes of these technical processes for the insertion of genes, such as biolistic methods and usage of *Agrobacterium tumefaciens*, cannot be equated to effects occurring naturally or in previous methods of breeding. For example, Yue et al. (2022) identified larger and smaller insertions as well as deletions caused by the biolistic method of gene insertion into papaya. The larger insertion consisted of 77 rearranged and translocated fragments; the larger deletion included 44 genes. More than 600 genes were changed in their activity. The changes caused by the method of genetic engineering could be clearly distinguished from other genomic changes, which had occurred during the (around) 4000 years of the domestication of papayas. In conclusion, the processes used for the technical insertion of DNA can cause effects which are different in their scale, in the sites and in the patterns of the genetic change as well as their biological characteristics when compared to those of non-regulated breeding methods or natural processes. This is also true even if no additional genetic information is added to the gene pool of a species. Such effects may be related to epigenetic regulation, the disruption of genes, position effects, open reading frames, the unintended introduction of additional genes, changes in gene expression and genomic interactions which can involve plant constituents, plant composition and agronomic characteristics (Forsbach et al., 2003; Gelvin et al., 2017; Jupe et al., 2019; Makarevitch et al., 2003; Liu et al., 2019; Rang et al., 2005; Windels et al., 2003; Yue et al., 2022; Chu & Agapito-Tenfen, 2022; Heinemann et al., 2022).

In summary, at each stage of the process - including (i) insertion of the gene scissor DNA into the cells, (ii) target gene recognition and cutting and (iii) cellular repair of the genes - specific unintended alterations can occur along with risks. Some of the relevant specificities (on-target and off-target) are summarized in Figure 4.

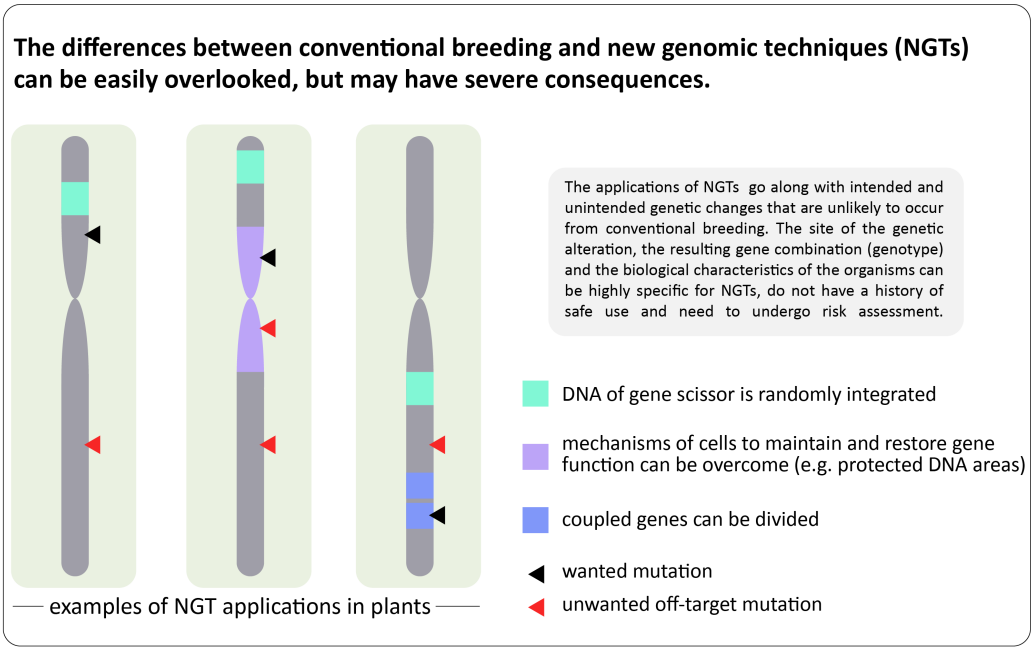

Figure 4: Unintended genetic changes (mutations) can also occur in conventional breeding. However, Synbio methods (in here called: NGT methods) are accompanied by changes that are not to be expected with conventional breeding and random mutations: both, the site of mutation and the resulting gene combination, can be significantly different from the results of conventional breeding. This is true not only for intentional, but also for unintentional genetic changes. Some reasons are: Synbio processes can overcome constraints on natural genome organization used by cells to maintain gene function (such as repair mechanisms, gene duplications, or epigenetic mechanisms). In addition, several different gene loci can be altered simultaneously (multiplexing).

If seeds with hazardous unintended genetic alterations remain undetected over longer periods of time, and are then used for further propagation, breeding and crossings, these genetic conditions may spread quickly and widely within the plant populations. Therefore, in each case, intended and unintended changes have to be assessed as to whether they can have either direct or indirect, immediate or delayed, or cumulative effects on human health and on the environment.

4.2 Specific risks associated with Synbio animals

The technical applications of Synbio in animals, at least in vertebrates, are associated with the risks and hazards of intended and unintended genetic changes. In a similar way to plants, there are examples of traits that are new or extreme variations of already known traits or new traits that are unlikely to result from random mutations and conventional breeding methods. Such traits can be associated with direct and indirect unintended effects that are relevant for risk assessment. In addition, there are also unintended genetic alterations in the target region (on-target effects) or in other genomic regions (off-target effects) that are specific to gene scissors, such as CRISPR/Cas, and are unlikely to occur from methods of conventional breeding.

4.2.1 Risks associated with intentionally introduced traits

It is obvious that traits such as those introduced into fish, e.g. seabream and pufferfish, can cause unintended effects that are triggered by the intended traits. As observed in GE pufferfish (Example 3.2.4), the blocked gene may be involved in metabolic functions. For example, the composition of the fish tissues can be altered and the susceptibility of the fish to diseases and infections may be increased. However, it is difficult to explore these questions since there are no peer reviewed publications or specific data. In addition to the risks, further questions need to be asked about health and animal welfare. In the case of the seabream, the animals have more muscle, they also have changes in body size and the vertebra are in an abnormal position (Kishimoto et al., 2018). Behavior also appears to have altered compared to the wild type since the Synbio fish seem to move more slowly.

Synbio pigs altered to increase their muscle mass are further examples of the many detrimental effects with regard to animal health. However, it cannot be finally concluded from the published data (Wang et al., 2015) whether these effects were caused by the intended traits or by unintended effects caused by the process, which also involved cloning at some stage. In this context it should be mentioned that the gene defect induced by CRISPR/Cas in the seabream and the pigs, is also known to occur in the conventional breeding of cattle. However, the extreme effects observed in Synbio animals seem to be absent in conventionally bred species. One reason may be because the fitness of the animals is impacted. For example, in cattle, the conventional trait can only be established via additional technical measures, such as cesarean intervention at birth. It is astonishing that such a dubious trait is now being introduced with the help of Synbio in fish, pigs, sheep, goats and dogs (see, for example, Cohen 2019).

4.2.2 Unintended effects caused by the processes of Synbio

With regard to unintended effects, there are many publications reporting potential medical applications (in animal cells or animals used in the laboratory). In this backgrounder, however, we can only include selected examples: experiments on human cell lines showed that cuts, also called

double-strand breaks, caused by CRISPR/Cas gene scissors in the genome can lead to large, unwanted DNA rearrangements (see, for example, Geng et al., 2022; Leibowitz et al., 2021; Weisheit et al., 2020; Zuccaro et al., 2020) which may have detrimental effects during the early embryonic development of mammalian embryos (Papathanasiou et al., 2021).

In experiments with zebrafish, researchers have shown that unintended effects of CRISPR/Cas applications are inherited in subsequent generations (Höijer et al., 2022). The publication describes large structural changes at off-target sites. This shows that the gene scissors cut genomic regions outside of the target site, and thus cause specific unintended mutations. Many of the unintended genetic alterations have also been observed in the following generation. In some cases, the researchers found non-Mendelian patterns of inheritance, with some alterations being homozygous while others were heterozygous. The findings show that unintended effects caused by the gene scissors can lead to specific effects and risks.

Consequently, the offspring of animals manipulated with CRISPR/Cas for use in agriculture need to be examined in greater detail to detect unintended genetic alterations. This issue also seems to be relevant for genetically engineered hens: researchers in Israel used CRISPR/Cas to alter hens so that they do not produce male offspring (see Example 3.2.5). A deadly gene is passed on to any male offspring, which is meant to kill the male chicks (at early stage of development) before they hatch from the egg. As the research on zebrafish shows, surviving offspring may suffer from unintended genetic changes that can be associated with specific risks.

The most prominent example of unintended effects caused by Synbio are the hornless cattle in which the processes of genetic engineering caused genes from bacteria to be unintentionally integrated into the genome of the cattle (Example 3.2.2). In animal cells, it was found that unintentionally inserted foreign DNA fragments may not only come from the vector construct (Norris et al. 2020), but may also come from the genome of the bacteria used to multiply the vector DNA (e.g. *Escherichia coli*) or, surprisingly, taken up from the source of the growth medium, e.g. bovine or goat DNA, or retrotransposons (Ono et al., 2015, 2019).

Another study published in 2020 (Schuster et al., 2020) described the use of CRISPR/Cas to introduce the hornless trait in cattle. Being associated with many unintended effects, the publication shows just how complicated the processes of Synbio are: in this study, the scientists used CRISPR/Cas12a which is a variant of the “classic” CRISPR/Cas9 gene scissors. They took some skin cells for cloning from the ear of a Holstein-Friesian cow, a breed that is often used in milk production. They cultivated these cells in a cell culture and introduced the gene scissors into the cells together with a guide RNA to target the region in the cow genome coupled with a DNA template for the hornless trait (SDN-2). A total of 70 positive clones were produced in which the additional piece of DNA was inserted into the genome to convey the desired trait. The nuclei of the altered cells were then injected into previously denucleated (i.e. emptied of the nucleus) egg cells, which were then meant to develop into embryos. A total of nine embryos were transferred to surrogate cows. Three of the embryos did not induce pregnancy and died in the uterus. Four of the cows suffered serious complications in the course of their pregnancy and lost their calves. Another calf was killed prematurely for experimental purposes. Only one calf was born alive by caesarean section but then died the same day. It had malformations in several organs. It also increased its body weight. The causes of the serious damage to health were not examined in depth. It is likely that the cloning process played a major role in the undesirable outcome of the experiments, as cloning is known to result in birth defects. The study examined the genome of the genome-edited calf only to a limited extent with regard to unintended changes in the genome: PCR methods were used to search for off-target effects at three regions in the genome. Off-target effects are unwanted changes

that can be caused by the gene scissors in parts of the genome that are very similar to the target sequence. No off-target effects were found in the three areas examined. However, the rest of the genome was not investigated. In addition, the scientists examined the genome by applying further PCR methods for unintentionally integrated DNA fragments. Their findings show just how limited the informative value of such a biased detection method is: the scientists could not completely rule out that there was additional antibiotic resistance in the calf genome. This was used for the work in the laboratory and should have no longer been present in the calf genome. In addition, the scientists could not clearly prove with the PCR method whether the integrated piece of DNA that mediates the hornless trait had been integrated into the calf genome once or several times. Only with a genome-wide analysis using whole genome sequencing methods would the scientists have been able to provide meaningful findings relating to the unintended changes.

4.3 Specific risks associated with Synbio microorganisms

Synbio microorganisms that are released may be able to survive and persist in the receiving environment, or invade new environments where they can have multiple interactions with other organisms. Even microorganisms not intended for release and whose purpose is for contained use only, may spread in the environment: experience with genetically engineered microorganisms used in food production processes shows that such applications may result in large-scale contamination with the bacteria or bacterial DNA (Deckers et al., 2021). Therefore, risk management questions relating to contained usage also have to be considered.

In general, many microorganisms are closely associated with species from other domains (plants, fungi or animals). These organisms are considered to be symbiotic ‘hosts’ of the microorganism. The microbiome of plants, insects, mammals and humans are all made up of specific combinations of microorganisms. This means that the biological effects and potential adverse effects of Synbio microorganisms may emerge from these symbiotic interactions in a non-linear pattern. These biological systems cannot, therefore, be assessed simply by examining their individual parts and pieces in isolation, they all have to be considered as a larger assemblages known as holobionts (or hologenomes when considering the total DNA of all involved organisms). It should also be taken into account that all species in the same habitat interact and influence each other (see, for example, Arif et al., 2020; Richardson, 2017; Sanchez-Canizares, 2017). It is not only the Synbio microorganisms which may act upon target and non-target organisms, but also the host and the hologenome may impact the characteristics of the genetically engineered microbes. Furthermore, risk assessment of genetically engineered hosts, which may be combined with microorganisms by accident or on purpose, also needs to be considered.

These risks may have serious consequences for consumers. As EFSA (2022d) states in an opinion on what they consider to be SynBio microorganisms: “*Perturbation of the gut microbiome structure and microbial metabolism can also have consequences on the gastrointestinal (including metabolic, barrier defence and immune) function. Gut microbiome imbalances can impact epithelial integrity and, therefore, trigger adverse immune responses and inflammation. This can be of particular relevance in infants during the first months of life when severe disturbances of the gut microbiome balance and gut function may trigger chronic diseases at this point or later in life.*”

5. Synbio applications which concern self-propagating artificial genetic elements such as gene drives

There is a strong increase in Synbio application that concern LMOs with self-propagating artificial genetic elements (SPAGE) such as gene drives (see von Gleich & Schröder, 2020). These applications are intended to actively spread technically inserted genetic elements within domesticated or non-domesticated populations. They involve a move from the laboratory to the fields and go beyond the applications of gene drives (Adelmann, 2021; BfN, 2022).

5.1 Gene Drives

A report from the Federal Agency for Nature Conservation (BfN 2022) gives a short overview on some technical characteristics: Synbio gene drives involve genetic engineering tools (e.g. CRISPR/Cas) being incorporated as a part of the genetic modification. If organisms with synthetic gene drives are released, these genetic engineering tools are released too – one might say that the genetic engineering experiment is moved into the environment, a “lab in the field” (Simon et al., 2018). Gene drive organisms can interbreed with their wild relatives. The effect of the gene drive on inheritance is that the genetic modification (including the genetic engineering tools) is inherited by more than half, and up to all, of the offspring. Without the gene drive, according to Mendel’s principles of inheritance, only half the offspring would inherit the modification.

Gene drives are intended to enable the genetic modifications to persist more successfully in wild populations over time, and to also become prevalent under certain circumstances. The technique involved in synthetic gene drives thus theoretically permits LMOs to spread, even where they possess characteristics which are disadvantageous for the organism and/or for its reproduction (for instance only bringing forth male offspring) and having the potential to cause a population to collapse or even become extinct. This method is regarded, for instance, by the Genetic Biocontrol of Invasive Rodents consortium as a potential contribution to combating rodents which people have introduced to other continents and which have become a threat to other species in their new ecosystem. Doubts have, however, been raised as to whether the method is feasible (Dolezel et al., 2019; Champer et al., 2021).

Currently, there are two basic Synbio gene drive concepts: “Suppression drives” are meant to introduce genetic elements that reduce or eradicate natural populations, for example, by interfering with their capacity to reproduce (Kyrou et al., 2018; Hammond et al., 2021); “replacement drives” are meant to replace natural populations with persistent GE populations with altered biological characteristics, inheriting artificial genetic elements (Gantz et al., 2015; Carballar-Lejarazú et al., 2020; Green et al., 2022).

The target organisms involve mosquitoes (e.g. Gantz et al., 2015; Hammond et al., 2021; Kyrou et al., 2018), flies (Ni et al., 2021; Yan et al., 2021; Kaduskar et al., 2022), rodents (Grunwald et al., 2019; Bunting et al., 2022), mites (Faber et al., 2021), plants (Siddiqui et al., 2021; Zhnag et al., 2021; Barret et al., 2019; Tek & Budak 2021) and yeast (Di Carlo et al., 2015).

Some of the organisms involved in these applications are enabled to perform gene flow across the borders of single species (Taylor et al., 2001; Weetman et al., 2014; Wolf et al., 2023).

5.2 Synbio Viruses

There are further Synbio applications purposed to actively spreading technically inserted genetic elements within domesticated or non-domesticated populations. Again, these applications involve a move from the laboratory to the fields.

The report from BfN (2022) as quoted above also gives an overview on some applications: Genetically engineered viruses are currently being engineered once more for a number of different purposes, with increasing risks for health and the environment (Lentzos et al., 2022). In order to introduce the viruses to their target organisms, research is also underway on how to spread the viruses via insects so that they can transmit them to plants. The aim of these applications is to allow genetic modifications to be implemented on the plants of an existing population, independently of reproduction (“horizontally”), and quickly. This method, which is also referred to as Horizontal Environmental Genetic Alteration Agents (HEGAAs) (Reeves et al., 2018; Frieß et al., 2020; Pfeifer et al., 2022), is being developed as a crop protection strategy – funded by the US Ministry of Defense’s Defense Advanced Research Projects Agency (DARPA). There are specific viruses under development to be used for these purposes in plants (Gentzel et al., 2022; Nagalakshmi et al., 2022) This and other virus-based strategies are also being discussed in connection with environmental and nature conservation (Lentzos et al., 2022). Beyond that, there are also applications under development to use viruses to change the genome of gut bacteria (Lam et al., 2022).

5.3 Other Synbio LMOs with the potential to spread genetic information

Other application which are intended to spread genetic information within undomesticated populations include constructs to suppress or disrupt mosquito populations by introducing lethal gene constructs (Evans et al., 2019; Waltz, 2021) or flies (Ant et al., 2012). There are also publications showing interest in establishing genetic engineering mechanisms which are inherited to the next generations (Impens et al., 2022).

Applications are under development to engineer bacteria and fungi which are part of the plant rhizosphere (Shelake et al., 2019; Shulse et al., 2019; Temme et al., 2012; Ke et al., 2020; Shekhawat et al., 2022; Shanmugam et al., 2019) or animal microbiome (Bilgo et al., 2017; De Vooght et al., 2014; Fang et al., 2011; Gilbert et al., 2016; Leonard et al., 2018; Lovett et al., 2019; Leonard et al., 2020; Rangberg et al., 2012; Ren et al., 2008; Lam et al., 2021) or symbiotic in corals (Levin et al., 2017). These applications, after release, would allow Synbio LMOs to persist, spread and propagate over longer, maybe even unlimited periods of time.

In this context, also Synbio or transgenic application in trees (Ahuja, 2009; Bauer Panskus et al., 2020; GeneWatchUK, 2020; NAS, 2019; Wang, 2004; Zhang et al., 2013; Zeeman & Solhaug 2022; Wang et al., 2022; Tao et al., 2022) or fish (Moreau et al., 2011; Sundström et al., 2014; Devos et al., 2019; Vandersteen et al., 2019; Magalhães et al., 2022) should be considered, which have a potential for unintended geneflow into wild populations.

6. Cumulative risks

Many organisms created with Synbio processes, across all kinds of species and different traits, may soon be released into the environment. Indirect, delayed and cumulative adverse effects arising from the releases may be more or less likely, depending on their specific biological characteristics (intended or unintended). Large scale releases may increase the likelihood of such effects.

Given the specific characteristics of Synbio LMOs as listed above, the legal requirement for assessing cumulative and long-term effects, which may have a wide-ranging impact on ecosystems, is a much more pressing issue with regard to LMOs derived from Synbio in comparison to previous applications of genetic engineering (see also Heinemann et al., 2021). There are at least two categories that need to be taken into account:

(1) Cumulative effects of Synbio LMOs belonging to several species: environmental risk assessment that only takes single 'events' into account, may fail to predict or assess long-term cumulative effects, or possible interactions with the receiving environment and/or other Synbio-LMOs. Consequently, although releasing low numbers of a specific Synbio LMO for a short period of time may possibly not result in adverse effects on the ecosystem, the combination with other Synbio LMOs or the release of larger numbers of a specific Synbio LMOs over a longer time period, might lead to a tipping point that would trigger irreversible damage. These cumulative effects may, for example, also be caused by interactions between Synbio microorganisms and plants or animals, which raises challenges of potentially extreme complexity for risk assessment. For example, EFSA (2020b) in its draft opinion on the risk assessment of SynBio microorganisms states: "*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.*"

(2) Cumulative effects from traits of Synbio LMOs within the same species:
Synbio applications in one species or within a family of crossable species, which may also be susceptible to a specific range of pathogens, is a factor in potential cumulative effects, e.g. applications in wheat (*Triticum aestivum*) (see Example 3.1.6). The cumulative risk assessment in this case may face complex challenges. For example, cumulative effects of traits with (unintended) higher susceptibility to biotic stressors grown together with traits that have (unintended) reduced tolerance to abiotic stressors, may cause the collapse of plant populations which would otherwise have been successfully cultivated. Furthermore, different traits may be stacked via technical means, further breeding or also by spontaneous crossings, and thus result in offspring exhibiting biological characteristics absent in the parental plants. Under these circumstances, unintended genetic changes emerging from the processes of Synbio may become relevant. This could magnify uncertainties and unknowns with regard to environmental risk assessment as well as the food and feed safety of Synbio LMOs.

In general, effects occurring from interactions that may be additive, antagonistic or synergistic, are hard to predict. Due to the intended and/or unintended effects emerging from different Synbio traits established in one species, parallel cultivation, stacking or further crossing of the traits may cause unintended and even disruptive effects on plant health and response to biotic and abiotic stressors. These effects may be dependent on specific combinations of the traits and/or the exposure to stressful conditions. Even if each of the traits were to be considered 'safe', uncertainties or even unknowns will still emerge in the combination of the traits. Therefore, environmental risk

assessment of the single traits may fail to predict or assess short- or long-term cumulative effects, or possible interactions with the receiving environment, or several traits in combination.

Just as with environmental pollution from plastics and chemicals, it is not always an individual Synbio LMO which may create the real problems, but rather the sum of diverse effects on the environment. Environmental problems created by the release of Synbio LMOs may last as long as or longer than those caused by plastics and pesticides – thus impacting future generations.

7. Horizon scanning reveals a new dimension of hazards

This backgrounder describes several Synbio LMO and their characteristics that may contribute to potential pathways causing such harm. The likelihood of damage occurring will also be dependent on exposure in the environment and the potential of the Synbio LMOs to persist, spread and propagate. Hazards include the disturbance or disruption of ecosystems as well adverse health effects at the stage of consumption.

As aforementioned, the characteristics of the Synbio LMOs may contribute to potentially harmful pathways: for example, NGT camelina (Example 3.1.4) has a new genotype that is associated with a change in oil quantity and quality as well as other changes in plant metabolism. The food webs, the interaction with microorganisms and/or pollinators as well as natural defense mechanisms in the plants may all be disturbed (or even disrupted). Furthermore, any spread and propagation in the environment might lead to the offspring acquiring new characteristics absent in the original ‘event’ (see Bauer-Panskus et al., 2020). In addition, the degree of exposure in the environment will also be dependent on the potential of the Synbio plants to persist, spread and propagate. In this case, the hazards include the disturbance or disruption of ecosystems (including detrimental effects on ecosystem-services involving beneficial and pollinating insects) as well adverse health effects at the stage of consumption.

Overall, Synbio creates a new dimension of hazards: the introduction of tools, such as CRISPR/Cas, enables a new depth of technical intervention at the level of the genome that, for example, can result in extreme variations in the traits as well as unintended genetic changes that are unlikely to occur with conventional breeding methods. Many of these effects are happening within a rapidly developing field with an increasing number of applications. Applications are not just confined to domesticated plants or animals, an increasing number of projects are investigating wild populations and a broad range of organisms, e.g. microorganisms, insects, rodents and trees, all of which are embedded in their own complex ecosystems.

There is growing evidence of complex interactions between plants and animals as well as genomic mechanisms that allow for resilience, adaption and co-evolution of ecosystems, populations and species. The underlying mechanisms of these evolutionary dynamics are scarcely understood. It has to be ensured that releases of Synbio LMOs do not negatively impact these natural dynamics within biodiversity by, for example, causing evolutionary mismatch effects between the Synbio LMOs and their environment, or by causing destabilization or disturbance of the natural networks of co-evolution and resilience.

It has also been shown that, for example, honeybees and pollinated plants can evolve together and survive conditions arising from climate change in what could be called an orchestrated process of development (Bartomeus et al., 2011). Genetically engineered organisms may promote evolutionary mismatch-effects within such complex interactions, and may thus interrupt the finely-tuned interactions between the species and the dynamics of co-evolution.

We also take into account that Synbio LMOs, such as honeybees, corals, amphibians, trees or crops, might look promising as short-term solutions. However, in the long-term, once these genotypes are introduced into complex natural networks and interactions, they may disturb and destabilize existing mechanisms of resilience and climate adaption. These considerations also underline the need for prospective technology assessment (see below).

This has created a potentially new dimension of hazards which could be triggered by potential releases of Synbio LMOs capable of rapidly overwhelming the adaptability of ecosystems. It is possible that releases of Synbio LMO may, in addition to man-made effects such as climate change, contribute to the destabilization of ecosystems or intensify specific unfavorable effects. Given the high technical potential of the Synbio as described above, assessment of the overall hazards linked to Synbio is potentially vital for averting the next man-made technology crisis and safeguarding planetary health (Horton & Lo, 2015). For this reason, there may be a case for generally restricting the introduction of organisms derived from genetic engineering into the environment.

8. Requirements for Synbio regulation and decision-making against the backdrop of the precautionary principle

As shown, political decision-making on the future regulation of Synbio LMOs is faced with huge challenges. There is evidence that the intrinsic factors of Synbio processes deserve more attention from the regulators. For example, according to Yang et al. (2022), "*mutation locations and scales, potential off-target modifications, complexity of the introduced changes, and novelty of the developed traits*" make it necessary to apply "*rigorous research on genome-editing applications and reliable techniques for risk assessments of genome-edited plants*".

Kawall (2021a), in investigating the generic risks associated with the application of the CRISPR/Cas machinery, concludes, "*In summary, this review here shows that about half of the market-oriented plants developed by SDN-1 applications contain complex alterations in their genome (i.e., altering multiple gene variants or using multiplexing). It also illustrates that data on both the process- and the end-product are needed for a case-by-case risk assessment of genome edited plants. The broad range of genetic alterations and their corresponding traits reflects how diverse and complex the requirements are for such a risk assessment.*"

Eckerstorfer et al. (2021) come to a similar conclusion (using the terminology of the EU): "*To this end, we suggest that two sets of considerations are considered: (1) trait related-considerations to assess the effects associated with the newly developed trait(s); and (2) method-related considerations to assess unintended changes associated with the intended trait(s) or with other modifications in the GE plant (…) Based on these considerations, further guidance should be developed to ensure the high safety standards provided by the current regulatory framework for GMOs in the EU for GE plants in an adequate and efficient way, taking into account the existing knowledge and experience in a case-specific manner. This guidance should thus strengthen the case-specific approach that is recommended by numerous EU and Member States institutions.*"

Consequently, all Synbio LMOs need to undergo a mandatory approval process before being released into the environment or brought to market. Risk assessment should aim to identify the intended and unintended changes resulting from the Synbio processes and should evaluate their potential to cause adverse effects on health and the environment. The differences between and natural occurring processes (or conventional breeding) and NGTs may be easily overlooked, but

nevertheless can have serious consequences. In this context, direct and indirect effects which may be immediate, delayed or cumulative have to be taken into account. It is likely that in several cases, larger uncertainties will remain, and therefore that make it hard to come to reliable conclusions on the safety of NGT-GMOs. Therefore, 'cut-off' criteria might be needed if decision-making is required in the face of greater unknowns (see Then et al., 2020).

Furthermore, a comprehensive and prospective assessment is necessary to address systemic risks to biodiversity. As mentioned, it may not always be an individual Synbio LMO which creates the real problems, but rather the sum of diverse effects on the environment.

Therefore, there is the need (for the risk manager) to generally restrict the number and scale of releases of Synbio LMOs into the environment in order not to lose control in regard to potential cumulative adverse effects on health and the environment, and also to avoid passing potential tipping points for irreversible damage to ecosystems. The concepts of nature conservation and environmental protection are largely based on the principle of avoiding interventions. These must also be applied in the field of genetic engineering and Synbio LMOs.

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