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Safety research in biotechnology

Analysis of 10 NWO projects with results for research, policy, and policy implementation

RIVM letter report 2024-0198

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Synopsis

Safety research in biotechnology

Analysis of 10 NWO projects with results for research, policy and policy implementation

Modern biotechnology enables the modification of the genes of organisms, offering possibilities to address societal challenges. For instance, it can facilitate cancer treatments (gene therapy) or contribute to food security (cultured meat). However, not everyone supports genetic modifications. In the Netherlands, there are therefore strict regulations for working with biotechnology.

Knowledge about the safety of this technology is essential to developing innovations safely. However, researchers tend to focus more on achieving innovation and less on thoroughly investigating safety concerns. The Dutch government therefore aims to enhance scientific understanding of the risks associated with biotechnology. Under the guidance of the Dutch Research Council (NWO), 10 research projects were carried out to explore these issues.

The National Institute for Public Health and the Environment (RIVM) has now analysed the safety questions addressed in these projects. It also looked at how they were investigated and what knowledge this has generated about safety.

It is certainly worthwhile to conduct safety research during the development of technologies and products. Such research provides valuable safety knowledge and methods for investigating safety. It is also important to consider societal questions and opinions about safety and to think ahead about safe production and use (Safe-by-Design).

However, there are barriers to conducting this type of research more frequently. These challenges relate to the organisation and funding of scientific research in the Netherlands. For instance, safety research has a low status, meaning there is little funding available. Furthermore, researchers and risk assessors do not collaborate sufficiently on safety questions, and there is often not enough time and money to study long-term effects. RIVM has made recommendations to ensure this type of research can be carried out, such as requiring safety research as a condition for research funding.

Keywords: safety research, Safe-by-Design, biotechnology, safety, research programme.

Publiekssamenvatting

Veiligheidsonderzoek binnen de biotechnologie

Analyse van tien NWO projecten met resultaten voor onderzoek, beleid en beleidsuitvoering

Met moderne biotechnologie kunnen genen van organismen worden aangepast. Dat geeft mogelijkheden om maatschappelijke problemen op te lossen. Het maakt bijvoorbeeld medicijnen tegen kanker mogelijk (gentherapie) of draagt bij aan voedselzekerheid (kweekvlees). Maar niet iedereen vindt aanpassingen aan genen gewenst. In Nederland zijn daarom strenge eisen om te mogen werken met biotechnologie.

Kennis over de veiligheid van de techniek helpt om innovaties veilig te ontwikkelen. Maar onderzoekers zijn vooral bezig met vernieuwing en minder met grondig onderzoek naar veiligheidsvragen. De Nederlandse overheid wil daarom meer wetenschappelijke kennis hebben over risico's van biotechnologie. Onder leiding van het Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO) is dat in 10 onderzoeksprojecten uitgezocht.

Het RIVM heeft nu geanalyseerd welke vragen over veiligheid in deze projecten zijn gesteld. Ook is gekeken hoe ze zijn onderzocht en wat dit voor kennis over veiligheid oplevert.

Hieruit blijkt dat het zeker de moeite waard te zijn om bij de ontwikkeling van technieken en producten onderzoek te doen naar veiligheid. Het levert kennis over veiligheid op en methoden om dit te onderzoeken. Het is belangrijk om daarbij ook oog te hebben voor de vragen en opvattingen over veiligheid in de samenleving en vroegtijdig na te denken over een veilige productie en veilig gebruik (Safe-by-design).

Wel zijn er hindernissen te nemen om dit soort onderzoek te doen. Die hebben bijvoorbeeld te maken met de organisatie en financiering van wetenschappelijk onderzoek in Nederland. Zo heeft onderzoek naar veiligheid weinig status, waardoor er nauwelijks geld voor beschikbaar is. Verder werken onderzoekers en risicobeoordelaars nog te weinig samen aan veiligheidsvragen. Ook is er vaak niet genoeg tijd en geld om lange termijneffecten te onderzoeken. Het RIVM geeft daarom aanbevelingen om de belemmeringen te verminderen. Bijvoorbeeld om bij de subsidie van onderzoek een verplichting op te nemen om veiligheidsonderzoek te doen.

Kernwoorden: veiligheidsonderzoek, safe-by-design, biotechnologie, veiligheid, onderzoeksprogramma.

Contents

Summary

To obtain scientific knowledge on the risks associated with modern biotechnology, the Dutch Research Council (NWO) conducted the 'Biotechnology and Safety 2017-2023' research programme on behalf of the Ministry of Infrastructure and Water Management. This programme encourages integrating risk research with studies into modern biotechnology innovations.

To assess the knowledge produced by this programme, the National Institute for Public Health and the Environment (RIVM) analysed the results of the research projects. The analysis focused on the risks explored, the safety questions posed and the approaches researchers used to address these questions. We took a broad view in our analysis by including the Safe-by-Design concept alongside environmental risk assessment perspectives. Safe-by-Design (SbD) is a proactive approach for identifying and addressing safety issues. Including SbD in this analysis made it possible to examine how to shape a comprehensive approach to safety and to safety research. Finally, the analysis considered the structure of the research programme, particularly the role of the user committee established for each project.

Themes of the research programme

The NWO research programme comprised 10 research projects, which we divided into four themes:

1. Medically related research.
2. CRISPR applications.
3. Industrial environmental safety of biotechnology.
4. Stakeholders and public engagement in biotechnology.

The first three themes address technical safety questions. The fourth theme involves safety research in a broader sense. Together with the technical research, this approach provides insight into how aspects of the Safe-by-Design concept are elaborated.

Approach and outcomes of research into technical safety questions

In most cases, researchers had identified technical safety questions in their project proposals. In medically related research involving the development of genetically modified viruses for cancer therapy or as vaccines, questions centred on the risks of unintended virus spread and the potential for genetic information exchange with circulating viruses (recombination). Researchers designed experiments to examine these risks and were able to show how virus spread could be limited, while experimentally demonstrating that recombination is a rare phenomenon. The rationale behind the experiments and their design could also be used for research into the spread and recombination of other viruses.

Researchers involved in a third project within Theme 1 developed a machine-learning (ML) model to predict protein aggregation, a characteristic of diseases such as Alzheimer's and Parkinson's. Insights

gained from the development of this ML model contribute to exploring how quantitative data might enhance an environmental risk assessment, which has so far relied on qualitative data.

CRISPR technology is a groundbreaking tool with applications across virtually all organisms. Therefore, four research projects within Theme 2 focused on CRISPR technology safety issues in various organisms: fungi, plants, and animal and human cells. The primary safety aspect in these projects was preventing off-target effects – mutations that occur in unintended locations within an organism's genetic material. The research results indicated that by making technical adjustments to the CRISPR system, these mutations can be reduced, and identified the appropriate techniques to detect these mutations.

Theme 3 concerns the safety of industrial biotechnology. This involves large-scale applications where substantial volumes of genetically modified micro-organisms (GMOs) are used to produce products such as enzymes, vitamins or insulin. Preventing leaks and discharges is essential to avoid the survival and spread of GMOs in the environment. In addition to robust installations and safety protocols, genetic safety switches can be incorporated into GMOs to minimise survival outside the reactor. The SafeChassis project developed various genetic switches for this purpose. A second project, TargetBio, focused on developing methods and experiments to research GMO survival and DNA stability. The outcomes of both projects provide a foundation for further research in this area.

Conducting technical safety research – a guideline

The technical research projects demonstrated that technical research can be combined effectively with studies into safety questions. Analysis of these projects subsequently enabled the development of a guideline for researchers, helping to set up this type of safety research. This guideline (see Box 3) is part of a Safe-by-Design strategy, emphasising cooperation between researchers from various disciplines and risk assessors.

Approach to safety research in a broader framework

In a broader framework, safety research refers to studies that address public safety and ethical concerns related to an application and that involve relevant stakeholders, including the general public. This is the focus of Theme 4. Based on the findings from the (sub-)projects within this theme, we developed a process-oriented approach for SbD safety research involving a biotechnological innovation (Figure 1). Here, we distinguish safety in three contexts: (1) safety of the technology or application; (2) safety within the development chain; and (3) safety from a societal perspective.

One of the research projects provided essential elements for advancing a biotechnological innovation responsibly (ethics parallel research), taking into account the societal perspective on safety. This involves research into the benefits of the innovation and exploring alternative solutions to the societal issue it addresses.

A second sub-project emphasised the importance of carefully considering which stakeholders should be involved in safety research and the need to contextualise and specify the application.

A third project highlighted the importance of the safety system, which comprises the regulatory framework and actors responsible for biotechnology safety. This system includes an external framework (policy implementation) and an internal framework (biosafety officer within an institution). The research project underscores the importance of these actors' roles in the early stages of an innovation. Researchers are recognised as key players in the biosafety system, occupying dual roles as 'providers of research data' and 'responsible designers'. Strengthening these roles is crucial for developing the safety system and positioning researchers within an SbD strategy.

Towards a responsible development of biotechnology

From the research programme's results, we derive a process required for a responsible and safe development of a biotechnological innovation (Figure 2).

Safety encompasses not only technical safety knowledge, but also knowledge of the innovation's benefits; on comparison with alternative solutions to the problem; on safety across the development and usage chain; and on the societal perspective on safety, including regulatory frameworks that may or may not enforce safety. Developing this knowledge requires biotechnological research, as well as social science and/or multidisciplinary research. In conjunction with all these areas of expertise and the interaction between researchers, policymakers and policy implementers, safety policy can be developed responsibly.

Recommendations

Safety research is essential for the responsible development of innovations in biotechnology. To facilitate and shape this safety research, we recommend testing and elaborating the process for responsible innovation development in biotechnology proposed in Figure 2 as a guideline.

This entails, broadly:

- determining with stakeholders a development/innovation to be developed further using the guideline;
- establishing a research programme to generate input for policy development on technical content as well as on the societal perspective, benefits and alternatives;
- shaping the interaction among researchers, policymakers, policy implementers and biosafety officers;
- engaging in an iterative process where decisions are made based on research data;
- determining how the roles and responsibilities of the parties can be developed further and embedded within the system.

For policymakers and research programme developers:

The outcomes of this RIVM analysis demonstrate the value of a research programme focused on safety. Given that knowledge on biotechnological innovations will continue to be needed to support the safety system's evolution, we recommend:

- establishing further research into the safety of developments in biotechnology, with attention to:
  - continuity of research, including examination of appropriate funding systems;
  - sufficient incentives and appreciation for safety research;
  - conducting not only technical research, but also studies into the societal perspective, benefits and alternatives.

For policy implementers:
Policy implementation can help guide the development of knowledge needed for environmental risk assessments:

- Investigate how safety research could play a more prominent role within the permitting process, providing an incentive for conducting safety research.
- Explore options for using quantitative research data, with a focus on:
  - developing and making quantitative data and models available;
  - investigating what might encourage researchers to contribute to this knowledge base;
  - supporting risk assessors' acquisition of necessary knowledge.
- Develop, expand and make available a knowledge base containing research results, experimental set-ups and tools for safety research. This could support risk assessments and safety research efforts.
- Assess whether, and if so, how a reference genome or baseline could be developed to aid in evaluations, such as for microbiomes.
- Incorporate recommendations made in this programme into guidelines and explanatory materials for environmental risk assessments.

For those who conduct safety research:

- Develop the guideline (6.2.3) for conducting SbD safety research further in a broad sense.
- Develop tools (e.g. training) to strengthen the identification of safety issues.
- Facilitate interaction between researchers, policymakers, policy advisers and risk assessors, aimed at:
  - translating scientific knowledge into policy and vice versa;
  - defining the researcher's role as a provider of knowledge.

1 Introduction

1.1 Background and objective

Background

The NWO research programme 'Biotechnology and Safety 2017–2023' was commissioned by the Ministry of Infrastructure and Water Management (IenW) (NOW, 2016). Its aim was to advance scientific understanding of the risks associated with modern biotechnology developments and applications, while also exploring ways to minimise and manage these risks. Aligned with the findings and recommendations in the 2016 Trend Analysis (COGEM, 2016), the programme's results were presented to the House of Representatives in June 2023 in an accessible magazine format (Rijksoverheid, 2023).

Objective

To better understand the knowledge generated, an analysis of the project results is essential. In this letter report, we analyse the results of the research projects. We focus on the safety questions that have been investigated and the significance of the research results for the safety assessment. This analysis takes a broad approach to identifying safety questions and knowledge, considering not only environmental risk assessments but also the concept of Safe-by-Design, as explained in Section 1.3. Including SbD allows us to explore how a comprehensive approach to safety can be implemented and to make recommendations for future safety research.

We also examine the methods researchers developed to investigate safety questions and how the structure of the research programme contributed to this. Our aim is to provide recommendations for the design of future research programmes.

1.2 The NWO research programme

The programme encourages integrating risk research with studies into modern biotechnology innovations. This is an unusual combination of knowledge development; it ensures the simultaneous development of knowledge about an innovation with knowledge about the risk assessment and risk management of the innovation. Knowledge exchange between researchers, risk assessors and policymakers is important for developing the latter. Each project had a guidance committee, the user committee, consisting of representatives from RIVM and the Ministry of Infrastructure and Water Management, in addition to societal parties and the business sector, and met twice a year. The researchers reported their results to the user committee in progress reports, which were discussed in the context of biotechnology safety.

The research programme comprised 10 projects (see box 1). We then organised these projects into groups according to similar biotechnological focus areas.

Box 1. *The 10 projects from the Dutch Research Council's Biotechnology and Safety research programme, grouped by theme.*

1. **Medically related research**, the projects:
   a. Safety of oncolytic viruses.
   b. Safety of replicon vaccines.
   c. Risks of exposure to amyloids.
2. **CRISPR applications**, the projects:
   a. CRISPR in eukaryotic cells.
   b. CRISPR in fungi.
   c. CRISPR specificity in tomato.
   d. CRISPR in plant breeding.
3. **Industrial environmental safety of biotechnology**, the projects:
   a. Technical development of safe industrial microorganisms.
   b. Transferral of antimicrobial resistance of (AMR) genes.
4. **Stakeholders and public engagement in biotechnology,** the projects:
   a. Improving knowledge exchange between research and policy (T-TRIPP).
   b. Subproject Safety of gene drives from project 2a.
   c. Subproject SbD stakeholder engagement from project 3a.

1.3 Biotechnology safety and Safe-by-Design

In modern biotechnology,[1] interventions in the DNA of organisms are subject to stringent safety regulations. Activities involving genetically modified organisms (GMOs) fall under the Genetically Modified Organisms (Environmental Management) Decree 2013 and the Genetically Modified Organisms (Environmental Management) Regulation 2013. This legislation, aligning with European legislation, assumes that activities can proceed under conditions where risks are negligibly small.

The premise of this legislation is to minimise the 'risks' or adverse effects of working with GMOs. Understanding these 'risks', their severity and the likelihood of occurrence forms the basis for risk assessment. As such, to gain insight into the risks of new biotechnological techniques and applications, it is crucial to develop knowledge about these potential hazards. The focus of research in this programme is on posing and answering safety questions: 'What are the potential hazards of a technique and/or application, and can we investigate to what extent these occur under which circumstances?'

Safety research can also focus on prevention, measures to minimise risks. This area of research looks into the reliability and stability of such measures.

Innovations in technology may not always be fully safeguarded by regulations, as these often lag behind technological advancements (Hogervorst et al., 2018). However, safety can be proactively incorporated into the development of a technology and/or application by using a Safe-by-Design (SbD) approach. Researchers and developers

[1] Hereinafter, any reference to 'biotechnology' should be taken to mean 'modern biotechnology'

then incorporate safety aspects[2] as criteria in the development process, while also considering future steps towards production, (new) use and waste streams. To effectively implement this, researchers and developers need additional knowledge about the SbD approach.

A proactive SbD approach also considers the relative safety of a development: how safe and healthy is it compared to possible alternatives? This entails fundamental questions relevant from a societal perspective, such as the problem the development intends to solve, who bears the costs and benefits, and what are potential future consequences. This broader perspective is based on the Responsible Research and Innovation (RRI) perspective (Stilgoe et al., 2013), which asserts that researchers and developers must take responsibility for ensuring their research will positively contribute to society. Safety and health are core values within RRI, and SbD can be aligned with the RRI perspective (Van Gelder et al., 2021).

Safe-by-design as a proactive approach encompasses several safety aspects. Depending on the type of research, whether fundamental or applied, the emphasis and implementation may vary. For fundamental research, the safety of the technical development itself and the safety of the technical innovation compared to alternatives is crucial. As the innovation progresses towards application(s), the safety of the chain(s) towards production and usage becomes more important, as does considering the societal perspective. However, this does not mean the other aspects are not important. Even in fundamental research, considering future usage or societal perspectives can be helpful, and safety of the technology itself must be ensured in applied research as well.

In this programme, the focus is on gaining insight into the safety of new biotechnological developments. We apply a Safe-by-Design approach in our analysis precisely because of its proactive stance. In line with this approach we will examine the implementation of three perspectives on safety:

1. safety *of the technology or application;*
2. safety *of the chain*, from development to production, usage, waste streams and potential utilisation;
3. safety *from a societal perspective.* What ethical, societal and sustainability questions arise and influence the desirability of the development.

Within the current regulations and risk assessment, the emphasis mainly lies on fulfilling the first perspective on safety. For this, knowledge exchange between risk assessors and policymakers is crucial. Additionally, implementing the other two perspectives requires also input from chain stakeholders and, in the case of the last perspective, representatives who can provide input on the societal perspective. Therefore, in our analysis, we also consider the process approach that involves engaging other stakeholders.

[2] Sustainability criteria may also be included in the approach. This is referred to as 'safe-and-sustainable-by-design'.

1.4 Approach to the project analysis

To gather information, we relied on progress reports for each project; minutes from user committee meetings; management reports from the research programme steering committee; and the project's final report. To extract targeted information from these documents, a set of questions was formulated as guidance (see Box 2). Where necessary, clarification was sought from RIVM colleagues who were part of the user committee concerned.

The collected data was discussed with colleagues from the 'Gene Technology and Biological Safety' (*Gentechnologie en Biologische Veiligheid*, GBV) department at RIVM, contributing to further refinement of the results. The draft report was presented to researchers according to the theme of their project and discussed with them in (hybrid) meetings. Based on these discussions, the report was revised where necessary. The draft was then discussed one final time with the commissioning body (Ministry of Infrastructure and Water Management, Biotechnology Unit) and RIVM-GBV, after which the report was finalised.

Box 2. *Guidance for information gathering.*
To collect targeted information from project documentation, the following questions were formulated:

- What development was investigated?
- How was the identification and formulation of safety questions established?
- Which safety issues were addressed? Also beyond environmental risks?
- How were the safety questions investigated?
- What results were obtained?
- What questions remain unanswered? Have any potential risks been overlooked?
- Are there connections to SbD? How is SbD implemented?
- What recommendations do the researchers give for further study?
- What are the implications of the results for policy and/or policy implementation? How can these results be utilised?
- What role did the user committee play in the approach and execution of the safety research?
- What is needed to make the research results useful for policy and/or policy implementation?

1.5 Reading guide

The outcomes gathered based on the questions from Box 2 are elaborated on in the following Chapters 2 to 5, organised by theme (see Box 1). Chapters 2 to 4 focus on specific use of biotechnology, while Chapter 5 addresses stakeholder and public involvement, particularly emphasising ‘safety from a societal perspective’.

For each project, we first provide general information, followed by a discussion of the results in the context of risk assessment and their usability for policy and policy implementation. With this focus, we do not provide a complete picture of a project's results.

Under the title 'Safe-by-Design', we address safety in a broad sense, examining the extent to which elements of SbD have been addressed and how this can be used for the implementation of SbD by researchers in biotechnology.

In Chapter 6, we consolidate the findings of this analysis. In Section 6.1, we focus on the safety questions related to environmental risk assessment. Section 6.2 summarises the lessons learned regarding the conduct of safety research, and in Section 6.3, we propose a process for developing a biosafety system that facilitates interaction between policy and research in the context of emerging innovations. The report concludes with a discussion in Chapter 7 and recommendations in Chapter 8.

When referring to 'the researchers' in this brief report, we mean the researchers working on the relevant project. When we refer to the researchers of this analysis, we specify ‘RIVM’. This also means that the conclusions and recommendations described per project result from this project analysis by RIVM, unless otherwise indicated by referring to ‘the researchers’.

2 Safety in medical biotechnology

2.1 Introduction to the projects

Viruses are primarily known as pathogens that cause diseases in humans, animals and plants, but they also lend themselves to various medical applications. One such example is gene therapy, where a virus is modified to serve as a vector to transfer a therapeutic gene into diseased (human) cells. A successful therapy for a certain type of skin cancer has been developed and brought to market in this way (Conry et al., 2018). Another application involves modifying a virus to create a vaccine to prevent diseases, such as the viral vector vaccine used against COVID-19 (Mendonca et al., 2019). Both of these applications were featured in the NWO research programme.

Of course, the use of viruses raises several safety questions. The modified virus must not lead to unwanted spread or cause diseases. It must be safe to apply in a clinical setting, and the modifications must not be lost due to mutation or recombination. These factors present not only opportunities but also challenges for safely employing viruses for medical purposes.

A third project included in this chapter focuses on disease-causing proteins. Examples of these include prions (Zerr et al., 2024), which cause diseases such as Creutzfeldt-Jakob, and β-amyloid protein (Hampt et al., 2021), which forms plaques in the brain and is associated with Alzheimer’s disease. The protein alpha-synuclein, the subject of this project’s research, appears to be linked to multiple diseases such as Parkinson’s and Alzheimer’s. There is still much uncertainty about the role of this protein in these diseases. Interestingly, the protein is present in healthy individuals but only becomes harmful when it is induced to fold in a particular way. The research in this project focuses on investigating this folding process and its effect on the protein's function.

2.2 Safety of oncolytic viruses

Project 15414
[Environmental safety evaluation of host range-modified oncolytic viruses: pivotal studies on reovirus and Newcastle disease virus]
Title: Evaluation of the safety of host range-modified oncolytic viruses for humans and their environment: pivotal studies on reovirus and Newcastle disease virus
Project description:
Oncolytic viruses (OVs) can be used for tumour treatment. This project focuses on an animal and a human OV, namely the Newcastle disease virus (NDV) and Reovirus (RV). The host range of these viruses has been altered using genetic techniques to optimise both safety (preventing spread and survival) and efficacy (oncolytic potential and genetic stability). To determine the environmental safety of these genetically modified OVs, tests are being developed to gather data required for an environmental risk assessment.
Safety questions:
Which risk questions need to be addressed to assess the environmental safety of a genetically modified OV used for treating cancer patients? And which tests will provide the necessary data to answer these questions?

2.2.1 Outcomes of this research project
The use of oncolytic viruses (OVs) in cancer therapy has been known for some time. In this project, researchers used mutant Reovirus (RV) and developed a set of genetically modified Newcastle disease viruses (NDV). Many safety questions needed to be answered regarding these viruses, and this research provides data to determine their environmental safety.

For human use, the virulence, host range and pathogenic potential of the oncolytic NDV and RV were modified and selected. However, if these viruses were to be released into the environment, various risk-related questions would arise. The researchers utilised the methodology of an environmental risk assessment to identify relevant elements to be studied (Koppers-Lalic and Hoeben, 2011), such as the stability of the virus. These elements were examined by developing scenarios in which OVs could potentially pose environmental risks. To gather the necessary data, tests were developed. Notably, during the development phase in the laboratory, research was already being conducted into safety questions that would emerge in later stages of the virus application process. The key areas investigated were:

- *stability* of the viruses on surfaces. In the event of accidental spillage during a clinical trial, it is important to know how long the viruses can survive on a surface. Both viruses were found to become quickly inactivated and could be completely inactivated using chloride and ethanol. This information helps in developing virus inactivation protocols, ensuring safe procedures during clinical application;
- *genetic stability.* This was studied by repeatedly passaging the RV and genetically modified NDV in cell cultures. It is crucial that the mutations reducing the pathogenic potential of the OVs (thus

making them suitable for clinical use) are retained, after all. After transferring the viruses 7 times (NDV) and 10 times (RV) in human cells, sequence analysis confirmed that they remained identical to the original variants;
- *virulence in chickens.* Poultry is susceptible to NDV and possibly also to the adapted RV, whose host range is altered. To assess the risk of infection and disease in poultry, the researchers conducted infection trials with chickens and eggs. The results showed that RV did not cause excretion or illness, and the modified NDV variants also did not cause disease. However, tests based on the intracerebral pathogenicity index (ICPI) – in accordance with European regulations (EC Directive 92/66) – indicated disease symptoms. The test outcomes for the modified NDV variants varied between experiments. During discussions within the user committee, different interpretations of the tests and their results emerged. There was no unanimous consensus on whether the authorities could be sufficiently convinced of the viruses' safety for clinical use based on these results. This discussion highlighted that merely providing test data as evidence of safety may not always be enough. It is also essential to justify the choice of the specific test, the question the test addresses and the relevance of the outcome.

2.2.2 Safe-by-Design working method

a) Identifying and addressing safety questions

In collaboration with the user committees, the researchers identified safety questions and the research design to investigate these issues. They reviewed the theoretical and other assumptions and the reasoning behind the different scenarios. Since only a limited number of scenarios could be tested, it was essential to make well-considered choices. The user committee had a strong composition, including experts in (oncolytic) viruses, biological safety, recombinant virus regulations, veterinary virology and medical specialists interested in OV therapy. As part of the discussions, the committee discussed the most suitable animal model and cell culture and decided that chickens were the most suitable animal model to establish the pathogenic potential of the mutant RV and genetically modified NDV. In animal experiments, specified pathogen-free (SPF) chickens are commonly used. This raised the follow-up question of whether poultry and hobby breeds should also be tested, which the experts argued was not obvious and required.

The diversity of experts in the user committee led to varying perspectives. For instance, a proposed animal test could not be conducted because the animal testing committee did not grant approval. Additionally, as previously mentioned, the ICPI tests and infection trials with young chickens yielded results that had different relevance for different experts. These discussions highlighted the importance of reviewing the research design and test justifications with multiple stakeholders. Such dialogue can help avoid the need for unnecessary tests.

Although the projects within the programme focused on environmental risk assessment, the user committee also raised questions about patient safety, such as the risk of certain alterations in the OVs affecting the patient's immune response. The environmental focus meant that these questions could not be fully addressed. This is regrettable, as it would have allowed for a more holistic view of safety, involving all relevant stakeholders and treating various safety aspects in a more integrated manner.

Safety assessments also have a regional component, which was evident in this project regarding NDV use. In the US, virulent NDV is listed as a ‘select agent’,[3] indicating that it poses serious risks to public, animal or plant health. This classification impacts the use of genetically modified NDV as a therapy in the US. While this does not currently apply in the Netherlands or the EU, it cannot be ruled out that it may influence future use in Europe as well.

b) Chain thinking
A crucial next step in the chain towards clinical use is obtaining permits. In the final phase of this project, the researchers initiated consultations with members of the Netherlands Commission on Genetic Modification (COGEM) and the GMO office (Bureau GGO) regarding their permit application for a future clinical trial. The draft application included all experimental data supporting the safety of the OVs. However, it lacked an explanation of why specific tests were conducted, as well as an interpretation of the results in terms of safety. The application form does not seem to encourage this, and the differing opinions within the user committee about the significance of the test results made providing a written justification challenging. The consultation highlighted the importance of both the applicant and the regulatory body gaining insight into the complex of required and available data for the safety assessment. It also revealed that experts within COGEM and GMO office might have different viewpoints.

In this project, the required OVs were developed in the laboratory. Simultaneously, the researchers considered the future use of these OVs. Clinical use requires large quantities of the virus, which cannot be produced in the laboratory and necessitate larger production facilities. The Netherlands lacks sufficient facilities for this purpose. The researchers therefore proposed constructing a production facility within their own research institute during the development phase. This proposal was accepted, and the facility is now operational.

Since 2012, the researchers have participated in the research consortium *Oncolytic Viro Immuno Therapy* (Kemp et al., 2020). This consortium was established to advance the development and application of OVs for cancer therapy. In addition to the researchers from this project, the consortium includes immunologists, physicians, oncologists and surgeons. This diverse group of representatives involved in both development and application simplifies the transition from research to clinical use.

For clinical use, a clear understanding of the risk of virus shedding by patients and the potential spread of the virus in the environment is

[3] A ‘select agent’ is, for example, a virus that can have severe consequences for public, animal or plant health. NDV is a class 3 pathogen, meaning it must be handled in a high containment laboratory. However, the genetically modified NDV variants used in this project can be handled at a lower safety level.

essential. This could lead to outbreaks in chickens, causing animal suffering and financial losses for the poultry sector. Discussions within the user committee showed that reaching a consensus on test design, test setup and interpretation of results is not always straightforward. Through indirect representation via experts who interact frequently with these groups, the voices of patients and the poultry industry were considered in these discussions.

c) The broader (societal) context
The researchers shared information about the project with the wider public through various channels, including contributions to a newspaper article, a website (supportcasper.nl) and a documentary by the *Tegenlicht* television programme. Additionally, a separate documentary was produced to inform the public about the careful development of new therapies and the involvement of various organisations in the process. This documentary highlighted the preliminary discussions surrounding the permit application for clinical use of genetically modified NDV. The researchers hope that this information will contribute to a better understanding of, and stimulate discussions on, genetic modification in medical research. It may also encourage further research in this area.

With regard to the Safe-by-Design approach, it is worth noting that the societal perspective in this research was primarily focused on informing the public. In addition, future studies could investigate the perspectives of stakeholders. This might include exploring how safety is perceived in this context, what information citizens or groups would like to receive and how people weigh the potential health benefits against risks, such as those for the poultry industry.

2.2.3 Conclusions and recommendations
The safety concerns that may occur during the clinical application of mutant RV and genetically modified NDV were already identified and investigated during the stages of developing these virus variants. As such, safety research necessary for the production phase was carried out alongside the laboratory work for creating the OVs.

The identification of safety questions followed the environmental risk assessment method, which is more familiar to risk assessors than researchers. In this project, its usefulness was well demonstrated, and it is recommended to make this method more accessible and widely available for safety research.

The user committee included experts from diverse fields. The researchers took advantage of this by involving them in identifying safety issues, designing experiments and interpreting the results. This project serves as a successful example of the added value of different expert perspectives when discussing safety issues.

2.3 Safety of replicon vaccines

Project 15791
[Environmental safety of synthetic replicon particle vaccines – risk for RNA recombination with wildtype viruses (RepliSAFE)]
Title: Environmental safety of synthetic replicon particle vaccines – risk for RNA recombination with wildtype viruses.
Project description:
A new biotechnological development involves the use of 'replicon particles' (RP) as vaccines. These particles are derived from a virus and are capable of self-replication, but they lack the genetic information necessary for transmission or causing disease. By adding genetic information (insertion) from, for example, a pathogenic virus, the RP can be used as a vaccine. The ability to apply different insertions allows the RP to be developed as a vaccine platform.
For safe use as a vaccine, it must be clearly demonstrated that recombination between the RPs and related wildtype viruses is not possible. Recombination could potentially restore the transmissible and pathogenic properties of the RP. In this project, an RP-based vaccine was developed from the TC-83 vaccine strain of *Venezuelan equine encephalitis virus* (VEEV).
Safety questions:
Can recombination occur between the wildtype virus and its derived RP vaccine? Could recombination occur between the insertion and the virus from which the insertion originated?

2.3.1 Outcomes of this research project

The researchers developed a cell system to assess the potential for recombination between the replicon particle (RP), based on the VEEV TC-38 vaccine strain, and various wildtype alphaviruses, including the Getah virus (GETV). They tested a variety of conditions, such as the sequence and timing of infection, the relative amounts of RP and wildtype virus, and the length of identical sequences between the virus and RP. The hypothesis was that recombination could occur if there were identical sequences between the RP and wildtype virus and both were replicating in a cell.

The results showed that recombination occurred very rarely: only one experiment out of many demonstrated recombination between the RP and GETV. Interestingly, the recombination occurred between sequences with *little* homology, indicating that sequence homology is not the only factor influencing recombination. Secondary RNA structures in the viral genome might also play a role. This suggests that, although recombination can occur, the likelihood is low.

Other findings from the cell system experiments include:

- The recombinant virus did not appear to be 'fitter' than the wildtype virus, meaning it did not replicate more quickly.
- Infection of the cell culture with the RP followed by the wildtype virus showed that the wildtype virus was suppressed in its replication, with the degree of suppression depending on how quickly the wildtype virus entered the cell.

- Co-replication of both the RP and wildtype virus occurred when both were present, and the RP could be packaged into the same viral particle as the VEEV genetic material.

These last two observations are crucial to consider in the safety assessment of an RP as a vaccine.

The development of an RP as a vaccine platform involves adding genetic information (an insertion) from another virus, raising further questions about recombination and co-replication for this combination of viral sequences. To test this, the researchers used an insertion from the porcine epidemic diarrhoea virus (PEDV), a coronavirus known for frequent recombination, into the RP from VEEV TC-83 (an alphavirus). Experiments with this RP and wildtype PEDV in cell systems demonstrated that, while both particles could replicate, no recombination occurred between the insertion and the wildtype virus. A possible explanation for this is that the two viruses naturally replicate in different cellular compartments, preventing them from ‘meeting’ to exchange genetic material.

Finally, the possibility of recombination in mice was examined. Both RP and GETV were injected into various tissues, and replication of both particles was detectable. However, using the cell system to detect recombination, no recombinant virus was found. Sequence analysis of RNA from the infected tissue showed the presence of chimeric viral RNAs, likely artefacts from the RNA preparation process for sequencing. Therefore, this analysis did not definitively prove that recombinant viruses capable of replication were generated.

Based on these results, the researchers identified three key questions that should be investigated to determine the occurrence and potential harmful effect of recombination:

a. Do the replicon and wildtype virus co-occur in the same cell?
b. Do the replicon and wildtype virus replicate in the same cellular compartment, allowing for recombination?
c. Could recombination result in a replicating virus, leading to the pathogenic spread of the recombinant?

Answering these questions will provide crucial information for the environmental risk assessment of replicon vaccines.

2.3.2 *Safe-by-Design working method*

a) Identifying and addressing safety questions

During discussions with the user committee about identifying recombinant viruses, the option of using next-generation sequencing (NGS) analysis was raised because this method is more sensitive for detecting recombination. Due to the high costs of this analysis, additional research funding was requested and granted.

The user committee also discussed the significance of the very low occurrence of recombination. If the likelihood of recombination is extremely low, can the potential risks associated with recombination be considered negligible? This question cannot be answered universally, and even the experts in the user committee had differing views on what constitutes a negligible risk. The environmental risk assessment assumes that, if there is a possibility of recombination, even a very small one, it must be assumed that recombination can occur. The next step is to assess the potential harm (e.g. virus spread) that might result. Such an assessment can only be done on a case-by-case basis for each RP application, as the environmental risk assessment process requires.

The researchers considered the design of an RP vaccine based on a virus as Safe-by-Design. The RP can be regarded as a safe design because it can only replicate within the cell it enters and cannot spread to other cells or cause disease. This focus on safe design considers only the technical aspects of the Safe-by-Design approach.

b) Chain thinking
This project provided insights into the occurrence of recombination between an RP and a wild-type virus. Such knowledge is needed at all stages of RP application. On a laboratory scale, this may imply that working with an RP is safer than working with the wild-type virus, and therefore less stringent safety measures may be required. Additionally, this knowledge about recombination is essential for the environmental risk assessment of large-scale vaccine production (industrial production) and the eventual use of the vaccine in humans or animals.

c) The broader (societal) context
No stakeholders were directly involved in this project, nor was a subproject formulated to address this aspect of the Safe-by-Design approach. However, given the concerns that certain groups amongst the public nowadays have about vaccines, further research into public perceptions of RP vaccine development could provide valuable insights into how different groups view such developments and the information they would like to receive.

During the course of the project, the COVID-19 pandemic highlighted the need for a versatile and rapidly deployable vaccine platform. The researchers' decision to demonstrate the RP’s potential as a vaccine platform using the porcine coronavirus PEDV reflects an awareness of this societal relevance.

2.3.3 Conclusions and recommendations
The theoretical risk of recombination between replicons and related viruses has been substantiated by the experimental data from this project, which demonstrate that this risk is not zero. Regulations concerning genetically modified organisms (GMOs) aim to achieve a ‘negligibly small risk’.

The researchers provide recommendations on the elements that need to be studied and assessed to determine the occurrence of recombination.

This project, or its continuation, offers a good opportunity to further develop the Safe-by-Design concept along the vaccine development chain. The safety concerns relevant to production and clinical studies should be identified and investigated, covering environmental safety, patient safety and animal health in the case of veterinary vaccines.

Using the results, information could be developed, for example in the form of an information sheet, with key safety research points for vaccine development. A similar information sheet has already been developed for industrial biotechnology (RIVM, 2023).

2.4 Risks of Amyloid Exposure

Project 15784
Protein DOMINO: Amyloid exposure and associated risks
Title: Protein DOMINO: Amyloid exposure and associated risks
Project description:
The clumping of proteins into so-called amyloid fibrils is characteristic of neurodegenerative diseases such as Alzheimer's and Parkinson's. The proteins associated with these diseases are also present in healthy individuals, but they only become harmful when they form amyloid fibrils. Research shows that amyloid fibrils formed in one cell can prompt proteins in neighbouring cells to also form fibrils, allowing the disease to spread from cell to cell.
Amyloid fibrils are also present in food, although they are formed from different proteins. It is unclear whether food-related amyloid fibrils can also induce the aggregation of disease-related proteins. Understanding how proteins trigger each other to form fibrils provides insight into predicting and preventing this process.
This research project focuses on the protein alpha-synuclein, which plays a role in Parkinson's disease. The data generated contributes to developing a predictive model for protein clumping and determining the harmfulness of protein fibrils.
Safety questions:
What factors determine whether protein fibrils can prompt other proteins to aggregate? Is it possible for protein fibrils to cross the blood-brain barrier? Can this knowledge be used to develop a model that can predict fibril formation?

2.4.1 Outcomes of this research project
The researchers considered two potential mechanisms for how amyloid fibril formation is transmitted from, for example, food proteins to disease-related proteins. One possibility was that an amyloid fibril would allow identical proteins to adhere to it, causing it to grow. The other was that the amyloid fibril would induce clumping of a different protein (e.g. alpha-synuclein) without the fibril itself being part of the clumping process. In vitro experiments revealed that the latter mechanism was at play. Furthermore, the researchers discovered that there were fibrils that could trigger alpha-synuclein clumping and those that could not. Both categories included proteins found in food: amyloid fibrils from the food protein lysozyme (found in eggs) were able to induce aggregation of alpha-synuclein, while amyloid fibrils from the food protein β-lactoglobulin (found in milk) were not.

This raised the question of which protein properties lead to fibril formation. A (bio)informatics analysis showed that the fibrils which induced alpha-synuclein aggregation shared a 13-amino-acid sequence with an identical charge distribution. This sequence was absent in fibrils that could not induce aggregation. Surface charge of the fibrils and the negatively charged end of alpha-synuclein were also important factors.

The researchers also examined whether protein fibrils from food could cross biological barriers such as the intestinal wall and the blood-brain barrier. In model systems, lysozyme fibrils were able to pass these barriers and induce alpha-synuclein fibril formation. However, whether this can also occur in the brain is not yet clear. Moreover, most protein fibrils from food were not able to cross these barriers.

To predict which proteins might trigger alpha-synuclein fibril formation, the researchers developed an algorithm based on Support Vector Machine, an artificial intelligence model. By determining the ability of around 50 proteins to induce alpha-synuclein fibril formation and feeding the experimental data into the algorithm, it was possible to make fairly accurate predictions about which proteins could pose a problem. This tool could be useful for setting safety measures when working with certain proteins, for example, or when using protein fibrils in new materials, such as for tissue regeneration or heavy metal filtration.

2.4.2 *Safe-by-Design working method*

In some cases, meetings with the user committee did not take place, or the committee was not fully represented, partly due to the COVID-19 pandemic. Additionally, the composition of the user committee could have been more diverse. For instance, experts in occupational health and safety were absent. These experts could have contributed to discussions on safety measures needed for workers involved in the development and production of new materials based on proteins that could also induce fibril formation. As a result, the discussions primarily focused on fundamental research, which was the main emphasis of this project, rather than on product development. Consequently, the various aspects of Safe-by-Design were less prominent in this project.

a) Identifying and addressing safety questions

The research questions in the project also defined the safety concerns: what mechanisms and protein properties lead to amyloid plaques? This knowledge is also relevant for environmental risk assessments. For example, if a GMO produces a newly designed protein, it may be necessary to exclude the possibility that this protein induces fibril formation, as this could have adverse effects on other organisms in the environment.

For the development of the algorithm, the researchers consulted specialists from KU Leuven, who had published extensively on protein aggregation. The advice to test several proteins with extreme properties was incorporated into the testing process.

However, to optimise the algorithm, data from even more proteins are needed. This would require more time and research funding than was available in this project. Further development of the tool awaits additional research funding.

b) Chain thinking
During discussions with the user committee, a question arose related to concerns about pesticide use and a potential link to Parkinson's disease. Could the model also be used to predict a potential relationship between pesticides and protein aggregation? The researchers indicated that this is not currently possible. The model was built around protein properties, not pesticide properties. However, they noted that, if experimental data were available, such a model could be developed for pesticides. The researchers also mentioned that the developed algorithm could be useful for research groups working on new materials based on proteins.

c) The broader (societal) context
The researchers noted that diseases caused by neurodegenerative protein aggregation are becoming more prevalent in our ageing society. Exposure to amyloid proteins and amyloid aggregates may increase the risk of developing these diseases. The project results contribute to knowledge about protein properties and the mechanism of plaque formation. Unfortunately, it is still not possible to predict whether exposure to amyloid proteins will actually lead to disease.

2.4.3 Conclusions and recommendations
The development of the algorithm described here could be useful for creating other algorithms with predictive value for specific harmful properties of proteins. Identifying which harmful properties and which groups of proteins are of interest would be the first step in this process.

This project demonstrates that a tool based on artificial intelligence can be useful for risk assessment.

As the ability to design new proteins using artificial intelligence continues to advance, predictive models for harmfulness will become increasingly valuable. These models can help determine whether newly designed proteins need further investigation for specific properties.

3 CRISPR application in animal and plant cells

3.1 Introduction to the projects

Since 2012, methods for making targeted changes in the DNA of organisms (gene editing) have advanced significantly. CRISPR technology is the prime example of this advancement. This technology has numerous applications and has led to many positive, sometimes groundbreaking developments, such as the treatment of sickle cell disease (FDA, 2023). Precisely because of the many opportunities presented by application of CRISPR, understanding its risks is important.

Broadly speaking, two types of risks can be distinguished. Firstly, there is the risk of CRISPR editing occurring at other locations in the DNA, known as off-target effects. This type of off-target mutations occurs with a certain frequency, depending on the level of optimisation of the CRISPR technique. Additionally, there may be risks associated with the application itself. An example of a risky application is gene drive (Carbellar et al., 2020). In gene drive, CRISPR is applied to an organism's DNA in such a way that it remains active and the intended change occurs repeatedly, even after sexual reproduction in offspring. This results in a change in DNA spreading throughout an entire (natural) population.

Within the NWO research programme, there were four CRISPR projects. In these projects, CRISPR techniques were refined and applied in human cells, plants and fungi, respectively. The research focused on the efficiency and specificity of the CRISPR techniques being applied. In this way, the projects shed light on safety from the perspective of ‘technology safety’ and examine the reliability of the CRISPR technique. The assumption here is that, as the technique becomes more specific, it becomes more reliable and gene editing will be safer. This approach to safety overlooks the fact that the use of a precise technique can still result in organisms or applications with risks, as described above.

Only the subproject ‘Safety of gene drives’, which is part of the project focused on human cells (no. 15804), addressed the ethical and societal aspects of gene drives, a specific application of CRISPR, in addition to the safety of the technique. Given this primary perspective, we discuss this subproject not here but in Chapter 5.

3.2 CRISPR in eukaryotic cells

Project 15804
[Biosafety of novel gene editing systems]
Title: Opportunities and safety of new gene modification techniques
Project description:
This project explores (1) ethical aspects surrounding the use and regulation of CRISPR gene drives and (2) the CRISPR/Cas13 system as a tool for killing tumour cells. The first subproject takes a broad view of gene drive safety, analysing not only the risks but also ethical considerations, societal impacts and risk mitigation measures. It also considers the perspectives of scientists and stakeholders on gene drives. Subproject 2 explores the usability and precision of the CRISPR/Cas13 system in animal and human cells, focusing on its potential application in cancer therapy.
Safety questions:
1. What ethical challenges are posed by gene drive technology, and can this technology be developed and regulated in an ethically responsible manner?
2. Is the CRISPR/Cas13 system sufficiently usable and specific for cancer therapy development?

3.2.1 Outcomes of this research project

This project delves into two CRISPR applications: gene editing and gene drive. The research explores safety from two angles: subproject 2 examines the technique's safety, while subproject 1 scrutinises safety from a societal perspective. Since our focus here is on the safety of the CRISPR technique itself, most results from subproject 1 will be discussed in Chapter 5.

In the second subproject, the researchers first evaluated the functional application and specificity of the CRISPR/Cas13 technique. Cas13 has the ability to cleave RNA instead of DNA. With Cas13 and a correctly chosen guide RNA, complete RNA degradation in cells, and thereby the death of those cells, can be achieved. For instance, the guide RNA can be targeted to the RNA of a tumour gene, causing selective destruction of tumour cells without harming other cells. As such, Cas13 could potentially be used in the treatment of cancer.

Researchers employed various software programs to design the guide RNA sequence as specifically as possible for recognising the tumour gene. Subsequently, it was observed that Cas13 indeed demonstrated a certain specificity for the tumour gene. However, the expected outcome of selectively killing tumour cells was not fully realised. Therefore, the envisioned use as cancer therapy is deemed unattainable.

The researchers noted conflicting results about Cas13 activity in the scientific literature: on one hand, high specificity for cleaving the specific RNA, and on the other hand, the so-called 'collateral damage' effect, meaning that all RNA in a cell is cleaved alongside the specific RNA. These varying mechanisms of action were further explored through analysis of published results. It was found that collateral damage only occurred when Cas13 targeted RNA that was present in large quantities.

This occurred, for example, in Cas13 application in diagnostic assays. Furthermore, the researchers identified the toxicity of Cas13. This toxicity depends on the cell type used and the RNA chosen as the target. As such, the biological context determines the type of Cas13 activity.

3.2.2 *Safe-by-Design working method*

a) Identifying and addressing safety questions

The research on Cas13 did not yield the expected results. After completing the experimental work, the researchers analysed recent publications on Cas13 and mapped out the conflicting results they gathered regarding Cas13's function. This provides several interesting findings that, with current knowledge, can help address safety questions in future CRISPR technologies:

- Various protocols were employed to determine the specific or nonspecific degradation of RNA by Cas13. The researchers noted that not all publications clearly indicated whether the extensive RNA degradation observed was determined by the specific (target) RNA present in large quantities or by the degradation of nonspecific (non-target) RNA (the aforementioned 'collateral damage'). This ambiguity may have contributed to the conflicting descriptions of Cas13's function.
- In some cell lines, Cas13 behaves as a cytotoxic protein leading to cell death. This means that the application of Cas13 cannot be assumed to be universally successful for other cell lines or organisms.
- CRISPR technology is based on a natural system present in prokaryotes (bacteria) but is also successfully applied in eukaryotes (fungi, plants, animals, humans). However, at the cellular level, prokaryotes and eukaryotes are very different, including differences in cell size and content, compartmentalisation and genetic architecture. This difference in biological context is crucial in determining whether CRISPR can be successfully applied.

3.2.3 *Conclusions and recommendations*

The literature review on various Cas13 activities illustrates that, for the development of a CRISPR application, it is not only essential to carefully consider the design of the guide RNA beforehand but also worthwhile to consider and investigate to what extent the biological context of the cell used allows CRISPR use.

While Cas13 activity was initially conceived to have potential for cancer therapy, that application quickly proved unrealistic. The knowledge about Cas13 activity developed during this research project and the researchers' approach to comparing the different applications of Cas13 provides generic knowledge to assess new CRISPR technologies.

3.3 CRISPR in fungi

Project 15807
[Towards safe applications of CRISPR/Cas9 technology in fungi]
Title: Towards safe applications of CRISPR/Cas9 technology in fungi
Project description:
This research focuses on developing methods for applying CRISPR/Cas9 gene editing in fungi. It involves two groups of fungi: 1) *Aspergillus niger*, which has applications in industrial biotechnology, where CRISPR/Cas9 is used to study genes involved in plant cell wall degradation. Understanding this is crucial for utilising this fungus in plant biomass degradation in a bioeconomy. 2) The genus *Phyllosticta*, comprising pathogenic and non-pathogenic species. CRISPR/Cas9 is used to modulate the expression of plant cell wall-degrading genes to find indicators of pathogenicity levels. Safety evaluation of CRISPR/Cas9 focuses on the extent of off-target mutations. Finally, the potential of barcoding is explored to enable the traceability of CRISPR/Cas9-modified strains.
Safety questions:
What is the extent of off-target mutations resulting from CRISPR-Cas9 technology in fungi? Can genetic barcoding aid in the traceability of genetically modified fungi?

3.3.1 *Outcomes of this research project*

The researchers conducted various types of experiments with CRISPR/Cas9 to observe its effects on the genetics of a fungus. Using CRISPR/Cas9 to introduce *point mutations* in genes resulted in more unintended deletions and insertions than when CRISPR/Cas9 was used to induce *deletions* in genes. Additionally, other experiments showed little difference in the number of unintended mutations in Cas9-treated and untreated fungal protoplasts. This indicates that the unintended mutations are not solely due to the application of CRISPR/Cas9 but also result from protoplast generation and the genetic instability inherent in fungi. As such, the researchers noted that the genetic instability of the fungi was greater than expected.

Another phenomenon studied was the Non-Homologous End Joining (NHEJ) complex, naturally present in cells. This complex repairs DNA breaks. In the absence of this NHEJ complex, in *A. niger*, fewer unintended mutations occurred when CRISPR/Cas9 was applied. However, this does not hold true for every species. For example, one dataset, which was admittedly more limited than the one available for *A. niger*, indicated that *Dichomitus squalens* exhibited more genomic instability. In this case, the absence of NHEJ was less important in reducing unwanted mutations.

These results provide insight into the variables relevant to different applications of CRISPR/Cas9 in fungi. Due to variability across species, a universal protocol for CRISPR/Cas9 application in fungi could not be developed.

In one subproject, CRISPR/Cas9 was used to investigate indicators that could distinguish between plant-pathogenic and non-pathogenic

Phyllosticta species. By disabling certain genes using CRISPR/CAS9, it showed that pathogenicity could *not* be linked to the absence of specific genes. The researchers concluded that pathogenicity, measured as growth difference, may be related to the expression level of genes (phenotype) rather than their presence or absence (genotype). This example underscores the importance of considering both genotype and phenotype in risk assessment.

The focus on barcoding in this project aimed to determine if this coding could have any undesired effects. The barcode is a predetermined piece of unique DNA sequence (20 nucleotides) inserted into the genome during CRISPR/Cas9 treatment. Subsequently, the genetically modified fungus can be traced and identified using the barcode. Such a barcode can also be helpful for patent protection. Analysis of CRISPR-treated strains with and without barcodes showed no measurable difference in growth rate and protein production of these strains, indicating that the barcode does not affect the phenotype of the fungi.

3.3.2 *Safe-by-Design working method*

a) Identifying and addressing safety questions

The research into the safety of CRISPR/Cas9 application in fungi focused on the technical safety of the technique. The researchers argued that, if the use of CRISPR/Cas9 results in few off-target effects, this method is safer than chemical mutagenesis. Chemical mutagenesis leads to numerous unwanted mutations but is exempt from GMO regulations. Developers and users of CRISPR techniques do not think this is justified. This research contributes to this discussion: the results show that the mutations found when using CRISPR/Cas9 can also be attributed to the transformation process and the genetic instability of fungi.

The knowledge gained about CRISPR/Cas in fungi and the development of barcoding was seen as part of a Safe-by-Design strategy. It is important to emphasise that Safe-by-Design encompasses multiple safety perspectives, not just technical safety. The technical safety perspective adopted here is therefore not the same as Safe-by-Design. Awareness of other perspectives, such as the desirability and acceptance of products from gene-edited fungi, should also be considered in future research.

Raising awareness and exploring different safety perspectives are not always straightforward. During a mid-project NWO programme day, the researchers were challenged, along with other project teams, to consider their projects within a broader context by identifying safety questions and how their research results could address them. This was then discussed in the user committee. The following reflects the outcomes of this discussion.

b) Chain thinking

During a user committee meeting, the researchers noted that unintended mutations from mutagenesis sometimes only become apparent during large-scale use. After all, when the best-performing strain is selected under laboratory conditions, for example, its performance may significantly decrease in large volumes. This underscores the importance of conducting analyses under (possible) future production conditions early in the development of production strains. Any minor performance issues of the strain during future use can thus be detected and rectified early in the development of suitable production strains. This example illustrates a Safe-by-Design strategy.

Based on their findings, the researchers suggested that industrial biotechnology could benefit from developing a production strain based on an NHEJ-deficient parent strain. Using such a strain could reduce the number of unintended mutations. However, the NHEJ deficiency would need to be reversed in the final production strain to maintain optimal phenotypic performance under fermentation conditions.

Lastly, concerning barcoding, integrating an extra piece of genetic information (the barcode) enables the identification of the GMO. In industrial applications of a genetically modified microorganism, this provides the opportunity for identification and patent protection. This type of barcode can also contribute to monitoring and traceability in genetically modified plants, which can be beneficial, for example, where GMO plants cannot be distinguished from non-GMO plants, such as through the use of CRISPR. However, the disadvantage is that the extra piece of foreign DNA causes these plants to continue to fall under GMO regulations, even if the rules for CRISPR-treated plants were to be relaxed in the future.

3.3.3 Conclusions and recommendations

Unwanted mutations can result not only from a technical intervention but also from natural genetic instability. This raises the question of which reference genome should be used to identify unwanted mutations, as well as the question of what the baseline should be for the number and location of the mutations found. This means that sequence determination is not automatically the suitable method for identifying unwanted mutations. It only provides an indication of the mutations that have occurred. An indication of the extent to which the mutations are unwanted can be obtained through research on changes in phenotype as a result of the mutations.

In the research on the use of barcoding, the phenotype of strains with and without barcodes was analysed. An important point that the researchers mention but were not able to investigate with the current scope of the project is the extent to which the barcode used remains genetically stable when the strains are propagated. This requires further research, taking into account the position of the barcode in the genome and the selection pressure on that position. This analysis may also yield a different outcome in a different biological context, such as in an organism with different mechanisms for genetic exchange, leading to no generic conclusions.

Research into the safety of a technique generally focuses on potential immediate effects of the applied technique. There is little room for research into the occurrence of possible longer-term effects. This is important for the eventual application, for the choice of suitable production strains and for risk assessment. As such, research into this topic is of great importance and should be an integral part of a research and development process. Financial resources should be allocated for this purpose, which may be 'recouped' by identifying more suitable strains early in a development trajectory.

3.4 CRISPR specificity in tomato

Project 15792
[Specificity and side-effects of mutagenesis by nuclease-induced breaks and Cas9-mediated epigenome editing in plants; identifying hazards, analyzing risks and creating inherent safety]

Title: Specificity and side-effects of mutagenesis by nuclease-induced breaks and Cas9-mediated epigenome editing in plants; identifying hazards, analyzing risks and creating inherent safety

Project description:
The project investigates the specificity and efficacy of CRISPR/Cas mutagenesis in tomato plants. Due to the time-consuming nature of plant research, the researchers are devising methods to study CRISPR effects in plant cell cultures. CRISPR/Cas induces breaks in the DNA, which can lead to desired mutations during DNA repair. However, these breaks may also occur at unintended sites, resulting in undesired mutations. Various Cas enzymes and guide RNAs are compared to assess the occurrence of undesired mutations. The collective findings offer scientific insights beneficial for both risk assessors and plant breeders.

Safety questions:
What is the extent of off-target mutations and chromosomal instability caused by CRISPR/Cas? What types of mutations occur, and can they be prevented?

3.4.1 Outcomes of this research project

The researchers conducted a literature review on off-target mutations resulting from CRISPR gene editing in plants. A significant finding was that the frequency of off-target mutations due to CRISPR editing in plants is low compared to mutation frequencies from tissue culture, radiation and chemical mutagenesis.

The literature review supported the decision to focus the experimental research on small off-target mutations resulting from CRISPR. For this purpose, the researchers developed a plant cell system to conduct the necessary analyses: they generated protoplasts (plant cells) and treated them with Cas, combined with different guide RNAs. The identification of mutation sites was done in a biased manner: these were predicted using software and then examined for actual mutations using next-generation sequencing.

Of many predicted off-target sites, only a small number were actually affected by Cas9 and an even smaller number by Cas12a. These were off-target sites with only one mismatch compared to the target site. For Cas9, some off-target sites with two to three mismatches compared to the target site were also found. The type of mutation encountered also differed for both enzymes: Cas9 mainly induces deletions and insertions, whereas Cas12a almost exclusively causes larger deletions. It also

emerged that some predicted off-target sites were not edited by CRISPR.
Additionally, the mutations were analysed using unbiased detection methods such as GUIDEseq and whole-genome sequencing. This involves examining the entire plant genome for the occurrence of off-target effects. The results indicated that unpredicted off-target effects occurred rarely, if at all, in tomato protoplasts.

Furthermore, the researchers also studied side effects such as potential chromosome loss due to CRISPR. The research results initially suggested deletion of the reporter gene in the F1 offspring, but further investigation in the F2 offspring suggested that there was no deletion but rather 'silencing' of the gene in question.

Finally, a strategy was developed for a step-by-step approach for the identification, detection and safety assessment of off-target mutations. This strategy sparked a discussion in the user committee about the risk assessment of off-target effects. For example, about conducting extensive off-target research in gene-edited CRISPR crops, while it is not necessary for more traditional methods. The strategy and the report have not been published pending the ongoing discussion in Europe regarding New Genomic Techniques.

3.4.2 Safe-by-Design working method

a) Identifying and addressing safety questions
The researchers exemplify a systematic approach to examining safety questions: beginning with a literature review, then determining which mutations are relevant for investigation in tomatoes and finally designing experiments to explore this. In doing so, they were able to utilise existing crossing lines. This approach illustrates how safety inquiries can be operationalised in research setups.

Furthermore, the researchers observed a lack of information about potential follow-up effects of off-target mutations, such as any impact on protein or phenotypic levels. For instance, an off-target mutation may exist without altering a protein or phenotype, and it may not affect product safety. This observation is important for further research into the relationship between genotype and phenotype, but it is also relevant for plant lines obtained through chemical mutagenesis.

b) Chain thinking
The researchers propose that, if the number of off-target mutations in a CRISPR-generated plant line is very limited, these unwanted mutations can be bred out. This approach allows for the development of a commercial plant line containing only the desired 'on-target' mutation. However, discussions with the user committee revealed that this approach may not be applicable to crops that are vegetatively propagated.

The findings from this project are valuable for both plant breeding and policy formulation. In plant breeding, they can help ensure crop development with minimal risk of off-target effects. From a policy perspective, these results assist in shaping national and European biotechnology policies.

There have been discussions within the user committee regarding the steps of risk assessment and the necessary data for evaluating a CRISPR-treated crop. The researchers mentioned that a considerable amount of sequence data on off-target effects and mutations can be collected. However, the risk assessor pointed out that the required data may need to focus on information that directly addresses questions in risk assessment. For example, sequence data may show mutations, but it is not always clear whether they are of a natural origin or due to CRISPR. Requesting such data does not necessarily help in ruling out unwanted mutations or determining their impact on the phenotype.

c) The broader (societal) context
A participant in the user committee stressed the need to avoid overstating the specificity of CRISPR technology in public discussions. The term 'specific' is often misconstrued as 'absolutely specific' by the public, when it actually refers to a relative level of specificity. Moreover, it is important to clarify that genetic changes occur naturally during the breeding process, even without CRISPR. Therefore, he emphasised that a more nuanced communication approach is necessary in public debates about CRISPR technology.

Furthermore, there was a discussion among plant breeders, policymakers and risk assessors within the user committee regarding regulations. The focus was on whether new techniques should fall under regulatory oversight. These discussions facilitate the exchange of perspectives, leading to a better understanding of each other's viewpoints.

Lastly, this project did not explore gathering perspectives from other stakeholders or the public. Doing so could have shed light on the information citizens or groups of citizens want regarding gene editing techniques and their off-target effects. The question remains whether more specific techniques change the public perception of genetic technologies.

3.4.3 Conclusions and recommendations
This study enhances our understanding of the occurrence of undesired effects resulting from CRISPR application in tomatoes. The insights into the extent of off-target mutations are valuable for researchers and plant breeders as they prepare for crop breeding. They also provide policymakers with valuable input for discussions regarding potential legislative adjustments. Lastly, they offer risk assessors insights into detecting off-target mutations and the mutations themselves, which may not necessarily be CRISPR-related.

This project did not allow for an investigation into the downstream effects of CRISPR mutations on protein expression and phenotype. Further research into these effects is recommended. It is crucial to pay attention to the potential implications of off-target mutations during the eventual commercialisation of the plant line and its societal acceptance.

3.5 CRISPR in plant breeding

Project 15815
[Assessing safety-by-design in novel plant breeding techniques by comparing native gene based modification with classical breeding]

Title: Assessing safety-by-design in novel plant breeding techniques by comparing native gene-based modification with classical breeding

Project description:
This project investigates the safety of cisgenesis and CRISP using potato resistance genes against the plant pathogen *Phythophthora infestans*. Mutations in the resistance genes were chosen based on known natural resistance genes in wild relatives and plant lines where mutations were obtained through classical breeding. Mutants generated via CRISPR and cisgenesis are compared with the plant lines obtained via classical breeding. Transcriptome and metabolome data from potato tubers of the various lines are collected. Additionally, existing statistical methods for comparing metabolite composition differences between the lines are refined.

Safety questions:
Are plants obtained through cisgenesis or CRISPR as safe as the respective parental organism, the unmodified plant and the plant varieties obtained through classical breeding? Can statistical methods based on changes in metabolite composition effectively demonstrate safety?

3.5.1 Outcomes of this research project
The previous project focused on assessing the safety of CRISPR in terms of unwanted DNA mutations. However, the presence of a mutation in DNA does not necessarily mean an immediate change in protein or phenotype expression. This research project delves into phenotypic changes. The researchers examined potato lines with and without mutated resistance genes against *Phytophthora infestans* and looked for unexpected changes in the metabolite composition of these potato lines resulting from the genetic changes. The researchers compared plant lines obtained through genetic modification techniques (CRISPR and cisgenesis[4]) with varieties obtained through classical breeding, which have a history of safe use. The aim of the comparison was to determine whether differences could be detected at the phenotypic level between the various plant lines, which are classified under regulatory frameworks as genetically modified organisms (GMOs) or non-GMOs.

For their research into metabolite composition, the researchers developed statistical analyses. A univariate statistical method previously developed in some EU projects was found to be unsuitable. This method does not work well when comparing large datasets with significant variability. Therefore, the researchers developed a multivariate equivalence test, which allows for the comparison of a large number of variables simultaneously instead of just one.

To perform the statistical analyses, the researchers collected transcriptome and metabolome data from potato tubers of the

[4] Cisgenesis is the insertion of a gene sequence from a wild relative. The sequence is thus not considered species-foreign, but part of the natural gene pool of the species.

aforementioned plant lines and varieties obtained through classical breeding. If the -omics profiles were equivalent, it would be possible to assume equal safety. In cases where equivalence could not be proven, further research into the toxicity or allergenicity of the new potato line would be advised to investigate potential adverse health effects.

The researchers indicated that the statistically enhanced methodologies they developed could contribute to improving the methodologies used by the EFSA for compositional data analysis since 2011. They discussed these methodologies in a workshop with the EFSA. They stated that these methodologies are applicable to compositional data analysis but are not yet optimal for use with metabolomics data.

Additionally, the project produced fundamental knowledge for the development of disease-resistant potato varieties. By comparing inactive resistance alleles from disease-susceptible varieties with active resistance genes from disease-resistant wild species, the researchers identified genetic differences. Subsequently, they used CRISPR technology to modify the inactive allele into an active one. This change made the susceptible potato plant resistant to *Phytophthora* and genetically comparable to a classically bred plant. By designing a new transformation method, the repair of inactive alleles was carried out without leaving CRISPR tools in the plant's genome. This approach contributes to safety-by-design, according to the researchers.

3.5.2 Safe-by-Design working method

a) Identifying and addressing safety questions

In this research, the focus was on technical safety aspects of the Safe-by-Design concept, with an emphasis on using the safest possible technique. By utilising CRISPR technology and cisgenesis, a genetic modification is applied resulting in plants deemed as safe as those obtained through classical breeding techniques. The researchers view CRISPR and cisgenesis as more precise than classical breeding because the modifications are more targeted. Other aspects of SbD were thus not addressed in this study. The researchers referred to their approach as 'safety-by-design', a term closely related to SbD. This could cause semantic confusion, as it may lead to the technical approach being equated with SbD.

The methodology and data used by the researchers are typically employed in determining food safety. However, the NWO research programme focused on environmental safety. Consequently, at the start of the project, there was considerable resistance within the user committee to this approach, which focused on effects on the metabolite composition of the different plant lines. The committee would have preferred an approach addressing questions from environmental risk assessment, such as targeting a specific risk question from environmental risk assessment. This illustrates that combining safety realms is not yet straightforward.

b) Chain thinking

The user committee engaged in numerous discussions regarding regulations, revealing diverse perspectives. The risk assessor on the committee clarified the regulatory framework, helping to resolve potential misunderstandings. This also provided greater insight into the underlying issues and highlighted that ensuring safety through regulations often involves political decisions.

Several plant breeders from companies were represented in the user committee, reflecting an effort to involve key players from across the production chain. The chain participants chosen for this project had a specific interest in regulations and technical development.

c) The broader (societal) context
This study did not include the perspectives of other stakeholders beyond those represented in the user committee. Future research could consider these perspectives. For example, it could explore the perception that citizens and groups of citizens have of genetic technologies, particularly CRISPR, in the context of societal challenges such as food shortages or climate change. It could also investigate the views of organic farmers and what they need to ensure the safety of their products.

3.5.3 Conclusions and recommendations
The methodologies developed by the researchers were initially suggested as an alternative to the current environmental risk assessment system, which marks a significant advancement. These methods could potentially be refined for evaluating environmental safety. For example, utilising -omics could help assess the impacts of mutations on non-target organisms or soil microbiota. However, this requires acquiring more knowledge about the relationship between changes in -omics data that are indicative of these effects.

4 Industrial environmental safety of biotechnology

4.1 Introduction to the projects

Industrial biotechnology has been producing various substances and materials for decades using genetically modified microorganisms. This production occurs in closed facilities, ensuring that the microorganisms remain isolated from the surrounding environment, a practice known as contained use.

In recent years, there has been a growing interest in production within semi-open setups. This interest is primarily driven by the use of genetically modified algae, which require sunlight. Additionally, developments stemming from the circular economy have led to the utilisation of (semi-)open systems. In these setups, microorganisms are no longer isolated from their surroundings, raising potential risks associated with their spread into the environment. To assess these risks, a more comprehensive risk assessment is needed that explicitly addresses them. The safety questions that need to be answered pertain to the possibilities of genetic exchange with wild-type microorganisms (horizontal gene transfer), the survival rate of GMOs, and the potential effects on other organisms in the surrounding environment. To mitigate risks related to environmental spread, preventive safety mechanisms could be integrated into the GMOs themselves, contingent upon the reliability and stability of such mechanisms.

Two NWO research projects have investigated safety concerns arising from these developments. The findings have the potential to contribute to the safe application of GMOs in industrial settings and open or semi-open installations.

The sub-project ‘SbD Stakeholder Engagement’, part of the SafeChassis project (no. 15814), focused not only on the safety of the technology but also on how safety is determined and perceived. They organised workshops with experts and public meetings. As this sub-project takes a different approach, it will be discussed not here but in Chapter 5.

4.2 Technical development of safe industrial microorganisms

Project 15814
[SafeChassis: Implementing and Assessing Safeguards for Lifestyle Engineering of a Versatile Industrial Chassis]
Title: Implementing and Assessing Safeguards for Lifestyle Engineering of a Versatile Industrial Chassis
Project description:
The project involved research on (1) genetic safety mechanisms for the safe use of genetically modified microorganisms (GMOs) in industrial installations and (2) organising workshops and public discussions on the safety of biotechnology. The first sub-project focused on the industrially significant microorganism *Pseudomonas putida.* The researchers based the genetic safety mechanisms on limiting survival via riboregulation, synthetic auxotrophy and genetic recoding. Through experiments and modelling, the researchers sought to establish safety thresholds. The second sub-project involved discussions with experts in workshops about how to determine safety and what data is necessary for that. Public forums addressed the perception of biotechnology safety and the criteria for deeming it safe.
Safety questions:
1. Are the genetic safety mechanisms robust enough to prevent the survival of GMOs in the environment?
2. How can we assess the effectiveness of these mechanisms, and what criteria define a mechanism as safe?

4.2.1 *Outcomes of this research project*

For the industrially relevant microorganism *Pseudomonas putida* (*P. putida*), the researchers developed three different genetic safety mechanisms. These mechanisms ensure that the microorganism cannot survive outside the production environment. When applied in large-scale bioreactors or even in a semi-open production system, from which escape to the environment is conceivable, the safety mechanisms prevent the microorganism from surviving after escape. The safety mechanisms developed in this project are based on:

- *Riboregulation*, where a promoter is activated by a specific molecule, leading to a gene product that is essential for growth;
- *Synthetic auxotrophy*, in which genes involved in phosphate metabolism in *P. putida* are modified so that the microorganism cannot utilise phosphate but requires phosphite to survive. Phosphite is not typically found in the environment;
- *Cross-feeding*, utilising *E. coli* and *P. putida* strains modified to be dependent on each other for nutrient exchange and unable to survive without each other. The genetic information of *P. putida* has been extensively modified (recoded) using a technique developed by the researchers. The recoded microorganism depends on synthetic amino acids, and its genetic information is unreadable by other organisms, thus eliminating the risk of genetic exchange via horizontal gene transfer.

The robustness of the genetic safety mechanisms is crucial for successful application. The researchers investigated this through experimental setups and modelling, proceeding as follows for the 'synthetic auxotrophy' method:

- *Experimental research:* the researchers cultured the GMO for 21 days under laboratory conditions using a growth medium similar to soil composition. They observed a mutation frequency of 10^{-13}, which provides an indication of the stability of the safety mechanism. However, to gain more insight into mutation frequency (stability) under semi-open system conditions, further research is necessary according to the researchers;
- *Modelling:* using experimentally obtained culture data in a model, the researchers predicted the survival of the GMO. However, the data proved too limited for reliable predictions. More data on various culture conditions is required.

In addition to this technical-scientific approach to safety, the researchers organised an expert workshop, conducted interviews with stakeholders and held public meetings. These project components are discussed in Chapter 5.

4.2.2 Safe-by-Design working method

a) Identifying and addressing safety questions

During meetings of the user committee, broader safety questions were frequently raised, such as: 'Who is responsible for conducting safety research?' and 'Who is funding this research?' The researchers noted that the current research environment is not conducive to conducting (additional) safety research. This is because projects are typically funded for only a few years, and results must be publishable quickly. Within this framework, implementing long-term routine experiments necessary to study evolutionary processes and gain insight into the stability of genetic mechanisms seems challenging.

b) Chain thinking

A practical application of 'Safe-by-Design chain thinking' emerged in the user committee of this project. A representative from the industry pointed out that the safety mechanism based on riboregulation and dependent on a chemical substance is not commercially viable. The substance is too expensive and environmentally polluting. These are criteria that can be considered at an early stage. Conversely, another example with an opposite effect was also highlighted. The safety mechanism based on synthetic auxotrophy demonstrated that it generated NADH, a cellular energy source crucial for metabolism and organism growth. This is noteworthy because typically a genetic mechanism requires energy from an organism. In response, the industry emphasised the importance of assessing the amount of energy a genetically (safe) mechanism requires from an organism, as energy used by the mechanism is unavailable for production. Thus, when designing a GMO for production, the criterion of energy consumption must also be considered.

4.2.3 Conclusions and recommendations

The experimental research on the safety of genetic safety mechanisms in *P. putida* demonstrates the initial steps towards investigating stability. However, the results are still too limited to conclude that these mechanisms can ensure the safe application of a GMO in a semi-open installation. Additional experiments on their stability and reliability remain necessary.

The implementation of the SbD elements by the researchers in this project (see also Chapter 5.4) and their outcomes highlight the complexity of applying the SbD concept and the need for tools such as guidelines. Based on this research, guidelines could be developed for the safe development of genetic safety mechanisms. These guidelines could address the laboratory experiments to be conducted, as well as criteria related to sustainability and costs.

Furthermore, we recommend additional research into the robustness of genetic safety mechanisms, including investigations into:

- the functioning of the safety mechanism under the environmental conditions present during future use;
- long-term studies on the occurrence of spontaneous mutations that could render the genetic mechanism ineffective;
- collecting data on the safety mechanism by employing the GMO in a semi-open setup where safety is ensured by, for example, additional physical barriers. This would allow for data collection during the operation of the installation on aspects such as survival, mutation frequency and exchange of genetic material with other microorganisms.

Research into the desirability of deploying safety mechanisms for industry should also be added. The public could be surveyed on aspects such as the use of semi-open systems and the safety requirements they deem necessary.

4.3 Transmission of antimicrobial resistance genes

Project 15812
[Transmission of Antimicrobial Resistance Genes and Engineered DNA from Transgenic Biosystems in Nature]

Title: Transmission of Antimicrobial Resistance Genes and Engineered DNA from Transgenic Biosystems in Nature.

Project description:
This research aimed to identify the genetic and physiological characteristics of microorganisms that facilitate DNA transfer. Researchers utilised DNA from antibiotic resistance genes (AMR) sourced from antibiotic-producing bacteria (Actinobacteria) to investigate this transfer. Experiments were conducted under conditions mimicking those found in wastewater, with an additional focus on studying the formation and characterisation of cell-wall-deficient cells that readily absorb DNA and likely exist in nature.

Safety questions:
What role does horizontal gene transfer (HGT) play in the dissemination of 'foreign' DNA from environments such as wastewater treatment plants to wild-type microorganisms? What safety measures are necessary to potentially mitigate this?

4.3.1 *Outcomes of this research project*

For the investigation into HGT, researchers utilised AMR genes and antibiotic-resistant bacteria found in wastewater. This was a strategically sound choice for research purposes. After all, microorganisms with these genes are easily identifiable, and selective pressure can be applied to promote HGT and study the mechanism. Additionally, research on AMR is pertinent due to widespread concern in healthcare about the emergence of antibiotic-resistant bacteria. Excessive antibiotic use leads to wastewater pollution and the development of resistant, pathogenic microorganisms in wastewater treatment plants.

This project yielded valuable insights and data for environmental safety considerations. Inactivation of microorganisms is typically done through a process known as autoclaving, which is designed to kill them. Researchers demonstrated that autoclaving breaks down microbial DNA into small fragments but does not completely eliminate it. Further research is needed to assess the extent to which these fragments may still pose a risk, including whether microbial uptake of this DNA in the environment is possible and whether the sequence information of the DNA presents a risk.

To address this, the researchers investigated which DNA is present in wastewater. They developed a protocol to purify freely occurring DNA from wastewater by distinguishing between this DNA fraction and the fraction present in living organisms. Sequence analysis of the freely occurring DNA revealed the presence of both AMR genes and mobile genetic elements (such as plasmids and transposons) in the water. Based on this presence, microbial uptake of the DNA cannot be ruled out. Further follow-up research was conducted to gain insight into this.

Through a bioreactor experiment, the researchers aimed to understand DNA uptake by microorganisms in environmental conditions. They used activated sludge: a byproduct of wastewater treatment plants containing a high density of various microorganisms. By adding known DNA to the activated sludge and replicating the cultivation conditions in the bioreactor to mimic those of the purification plant, sequencing analysis techniques were used to determine whether DNA uptake occurred. DNA uptake could not be demonstrated in this setting. However, DNA uptake was evident when the experiment was repeated under high antibiotic concentrations, such as those found in the gut. The developed protocol for this bioreactor experiment is valuable for future research into DNA uptake and microbial survival under environmental conditions.

Furthermore, this research provides insights for a method to purify wastewater from DNA contamination. The researchers demonstrate the use of ‘biochar’ and iron-oxide coated sand, which have DNA-binding properties and are thus useful for wastewater purification. Remarkably, the iron-oxide coated sand is a byproduct of drinking water treatment, contributing to reuse and a circular economy.

In a parallel study, knowledge was gained about Actinobacteria, some of which can form cell-wall-deficient cells under stressful conditions. This raises the question of whether the absence of a cell wall can facilitate HGT. In that case, such conditions would need to be prevented in a wastewater treatment plant to limit HGT. Research on these cells and the potentially involved genes in HGT indicates that HGT is an active process requiring intact cells of related bacterial species. Further research is needed to fully understand the precise mechanism of HGT.

The results of this project contribute to expanding knowledge that is useful for developing safe (semi-)open systems, circular use of waste streams, and risk assessment of these applications and future developments.

4.3.2 *Safe-by-Design working method*

a) Identifying and addressing safety questions

The project proposal outlined the safety questions to be investigated. During the research, the nature of follow-up experiments was determined based on the achieved results and discussions within the user committee. Additionally, the researchers consulted with wastewater management experts to design the bioreactor experiment using activated sludge, aiming to determine the necessary parameters (temperature, pH, etc.) for conducting an accurate environmental experiment.

To conduct the bioreactor experiments, approval under the GMO Decree (*Besluit ggo*) was required. Part of the application for approval included a risk assessment to determine the expected risks of 1) potential presence of class 3 pathogens in the activated sludge and 2) the use of a plasmid that could promote HGT, posing a higher risk than one that does not. To support the risk assessment, the researchers conducted additional experiments, such as identifying microorganisms present in activated sludge through whole genome sequence analysis. Based on these results, the risk assessment, which was reviewed by COGEM, concluded that safety measures equivalent to a level 2 laboratory and a few additional safety measures were sufficient to ensure the safety of both humans and the environment.

During user committee meetings, there was repeated mention of the desirability of a broader meeting involving researchers and risk assessors than those represented in the committee. The aim was to discuss how to translate research findings into practical applications for regulation and risk assessment. In the end, however, a scheduled date for this meeting did not materialise, partly due to the COVID-19 pandemic. Priority was given to carrying out the research lines within the PhD projects for publications, indicating that PhD research projects allow relatively little room for adjustment.

Even without the direct exchange between researchers and risk assessors, discussions of risks during user committee meetings yielded valuable outcomes:

- In a final progress report, the researchers noted that the release of DNA after autoclaving may not immediately be identified as a risk. Whether it poses a risk depends, among other factors, on whether the DNA encodes something harmful such as antibiotic resistance and whether this DNA is transferred and expressed in another organism. Based on these observations, follow-up experiments can be designed. One of the researchers makes

recommendations for this in his dissertation (Calderon Franco, 2023).
- Upon completion of the research project, the researchers observed insufficient alignment between research and risk assessment practices regarding the determination of necessary data for risk assessment. For example, initially, the researchers stated in their project proposal that their research would lead to a better understanding of potential DNA transfer between organisms. However, this understanding, such as insights into the occurrence of cell wall-deficient bacteria, does not directly provide the data needed for risk assessments or for improving regulations as the researchers had anticipated. The translation of research data into usability for risk assessment still requires a translation or additional research.

b) Chain thinking
In this project, researchers consulted with knowledge partners from wastewater treatment about, among other things, the bioreactor experiment. This discussion was valuable because it provided the researchers with insights into the environmental conditions of a wastewater treatment plant, which they could replicate in the bioreactor experiment.

c) The broader (societal) context
During the final meeting of the user committee, the discussion centred on how research findings could be disseminated to a wider audience. This question arose from concerns about potential repercussions. For example, revelations regarding DNA presence in wastewater could spark alarm and possibly lead to economic repercussions for industrial biotechnology. The reporting on DNA release has not gone unnoticed: the researchers have opted for careful communication in the magazine 'Biotechnology and Safety (Rijksoverheid, 2023) under the section 'From Lab to Practice'. Here, they argue that incomplete DNA degradation does not necessarily imply immediate concerns.

4.3.3 Conclusions and recommendations
The researchers point out several areas that warrant further investigation. For instance, there is a need to better characterise the various types of microorganisms present in the bioreactor experiment, sourced from samples taken from different types of wastewater treatment plants. They aim to determine, for example, whether common types of microorganisms in these samples can also form cell-wall-deficient cells. These cells take up DNA more readily, potentially heightening the risk of antibiotic resistance.
Conducting genomic analysis of these cell-wall-deficient mutants would be valuable for understanding the diversity of mutations in these cells and their implications.

Further research on DNA uptake by microorganisms in the environment is essential for better assessing associated risks in risk assessment and determining appropriate safety measures. Currently, when using antimicrobial resistance (AMR) genes, especially those crucial for patient care as a last resort, it is assumed that preventing their spread in the environment will also prevent their uptake. Therefore, appropriate safety measures are mandated for research. Knowledge about the possibility of DNA uptake by microorganisms in the environment could potentially reduce the need for safety measures if it is clear that DNA uptake does not occur. The follow-up experiments proposed by the researchers (as mentioned above) to study DNA uptake serve as a promising starting point.

Further research is also crucial for the use of waste streams in a Circular Economy. It is important, for example, to determine whether the presence of AMR genes in a waste stream affects reuse. If microorganisms intended for reuse are employed, it must be established whether there is a risk if they come into contact with AMR genes from the waste stream. This knowledge is also vital for wastewater treatment plants and can help prevent the spread of AMR genes.

5 Ethical and societal considerations in biotechnology

5.1 Introduction to the projects

The previous nine research projects primarily focused on technical safety issues in biotechnological developments. The tenth project, T-TRIPP (see 5.2), examines *the system that ensures biosafety*, alongside the roles and performance of the actors within this system. This project also explores how this system can be made more future-proof in light of biotechnological advancements.

Ethical and societal questions are addressed in a sub-project of the CRISPR in eukaryotic cells project (3.2). This sub-project examines the ethical and societal questions associated with emerging technologies like gene drive. These questions are essential for shaping the development of a technology, including safety aspects, in an ethically responsible manner.

The SafeChassis sub-project (4.2) explores how diverse perspectives can be involved in identifying risks. It also investigates how insights can be gained into perceptions of biosafety and how to shape public engagement.

These (sub-)projects mainly focus on processes that contribute to achieving safety and on the societal perspective on safety. Here, stakeholders and their roles, values and perceptions play a crucial role. Core questions include ‘how do you do it?’ – how to develop a technology responsibly and engage stakeholders – and ‘what does it yield?’. In this way, these projects address all three perspectives on safety – technical safety, chain safety and safety from a societal perspective – that we identified in 1.3 as elements of a Safe-by-Design strategy.

Our findings from these three (sub-)projects are summarised in the concluding section of this chapter.

5.2 Improving knowledge exchange between research and policy

Project 15809
[T-TRIPP: Tools for Translation of Risk research into Policies & Practices | NWO]
Title: T-TRIPP: Tools for Translation of Risk research into Policies & Practices
Project description:
This project aims to enhance interactions between the science & technology domain and the policy & governance domain. Effective interaction and a shared understanding of biotechnology safety contribute to the responsible development of safety policies. Such policies seek a balance between legally required measures and ongoing research and development, necessitating active exploration and organisation of this interaction.
Research questions:
How can actors involved in implementing biosafety policy communicate and learn from each other effectively regarding biosafety? What is required to future-proof the governance ecosystem surrounding biosafety?

5.2.1 Outcomes of this research project
Researchers focused on actors from two domains involved in the implementation and development of biotechnology safety policies: the ‘science and technology’ domain and the ‘policy and governance’ domain. The premise was that good interaction between actors in these domains ensures effective safety policy implementation and enables timely anticipation of developments. To support this, researchers used a model from the International Risk Governance Council (see reference IRGC) that addresses risk situations marked by a mix of complexity, uncertainty and/or normative ambiguity. Within this model, effective communication is central to establishing a future-proof safety policy.

The researchers examined the structure and functioning of current biosafety policies. This involved a literature review, interviews, iterative learning workshops and a stakeholder meeting. The findings were evaluated against five characteristics of strong communication: clarity of roles; a clear division of responsibilities and accountability; effective communication; inclusive two-way communication; and learning capacity and flexibility. Based on these criteria, areas for improvement were identified.

Biotechnology safety policy involves an external permitting system, in which the government must approve activities involving GMOs. Various government agencies, each with its own role, participate in this process: the RIVM GMO Office (as risk assessor), the Ministry of Infrastructure and Water Management (as the legal authority responsible for permitting decisions), the Netherlands Commission on Genetic Modification (COGEM, as independent scientific adviser) and the Human Environment and Transport Inspectorate (as supervisor). Additionally, an internal care system ensures compliance with regulations and procedures for permit applications and safe biotechnology use within organisations.

The researchers note a historical shift in responsibility for safe use of biotechnology. Initially, researchers bore this responsibility. Today, the role of the biosafety officer (BSO), specifically appointed for biosafety, is central within the internal care system. BSOs, alongside RIVM, are key intermediaries between the external permitting system and the internal care system. However, there is a significant gap between researchers and the external permitting system, which does not encourage researchers' safety awareness or their taking of responsibility for safe and responsible design. Moreover, there is little attention for and recognition of biosafety knowledge within innovation-oriented research. Consequently, the interaction between researchers and policy developers and implementers is inadequate.

5.2.2 *Recommendations from the research*

To manage challenges in maintaining up-to-date knowledge and ensuring safe and responsible biotechnology use, the role of researchers and the interaction between the 'science and technology' and 'policy and governance' domains must be strengthened further. Specifically, the researchers recommend the following:

- Sustained government support for biosafety research. It should be noted in this respect that the costs and benefits for researchers should also be considered. Attitude differences between medical biotechnology (red biotechnology) and industrial and agricultural biotechnology (white and green biotechnology) should be taken into account, as biosafety awareness in red biotechnology receives a considerable boost from adjacent regulatory frameworks that require, for instance, patient safety research. This is not the case for white and green biotechnology.
- Encouraging researchers to integrate biosafety research. Interdisciplinary cooperation could be part of this integration.
- Enhancing a safety culture among researchers and safety awareness during training. This would better equip researchers to fulfil their role as safe and responsible designers.
- Further knowledge development and knowledge exchange.
    - In terms of *knowledge development*, researchers' knowledge of the system must be strengthened, as well as BSOs' knowledge of biotechnological research (and its potential risks). Also the parties involved in the external permitting system must have more knowledge about new developments. Researchers play a key role as knowledge providers.
    - *Knowledge exchange* is necessary between BSOs, between BSOs and RIVM, and between researchers and the external permitting system (policy developers and implementers). Strengthening this final interaction is especially important for complex new developments marked by significant uncertainty. On the one hand, this exchange helps policymakers and implementers anticipate potential risks and uncertainties while, on the other, it also encourages researchers to consider safety, including potential applications outside the laboratory.

To address the gap between the two domains, 'science and technology' and 'policy and governance,' the T-TRIPP project developed a number of tools: three serious games and a protocol. Two of the games

(MachiaCelli Switch and MachiaCelli Teams) focus on the interaction between researchers, policymakers and risk assessors. The third game (Cards for Biosafety) is designed to educate researchers and raise awareness of biosafety and biosecurity.

The social learning protocol offers a framework for a workshop that researchers can use to identify a range of risks together with stakeholders. This will support researchers in shaping the societal perspective on safety and is in line with the Safe-by-Design concept.

5.2.3 Safe-by-Design working method

The researchers' findings and recommendations contribute to developing the SbD concept further.

The SbD concept

In this project, the relationship between SbD and 'inherent safety' was examined. Inherent safety is predominantly product-focused, aimed primarily at producing a safe end product. By contrast, SbD is interpreted as a safe and responsible innovation process, which encompasses not only the safety of the product, but also the entire process from design to market launch. In discussing SbD, it is essential to clarify the specific angle being addressed. Additionally, the researchers note that the terms SbD and inherent safety may lead to unrealistic expectations, as they suggest an ideal of 100% safety that is unattainable in practice. Therefore, they advocate for the term *Safer*-by-Design.

Implementing SbD

The researchers note that in biotechnology development, broader public values and ethical considerations may play a role in addition to safety. They recommend involving the societal domain at an early stage, particularly when uncertain or ambiguous aspects of specific developments emerge. They also stress the importance of integrating this process with the biosafety system, including both the external permitting system and the internal care system, to foster mutual learning and dialogue towards new actionable perspectives.

Researchers play a central role in SbD as designers committed to safe and responsible design, but also as providers of knowledge in uncertain or ambiguous situations to assess risks. The T-TRIPP project offers important recommendations for strengthening the role of researchers, as outlined earlier.

New developments may involve risk situations that are complex, uncertain and ambiguous. The researchers suggest that, depending on the risk situation, diverse groups of experts and members of the public should be involved in the development and design stages to facilitate knowledge exchange and provide a comprehensive perspective on all the angles. This approach enables collective assessment of design choices, determination of acceptable uncertainty levels and identification of areas requiring additional risk research. An official moment of broad biosafety knowledge exchange is advised within such a process.

5.2.4 *Conclusions*

This research provides insight into the history and performance of the current biosafety system, offering an analytical framework that clarifies system performance and areas of improvement. These recommendations also provide guidelines for SbD process development in general and for enhancing researchers' roles within the biosafety system (as safe and responsible designers and as knowledge providers). The recommendations should be implemented cohesively, with one responsible party acting as the lead, in cooperation with all parties mentioned here.

5.3 The ethical landscape of gene drive technology

Project 15804
Biosafety of novel gene editing systems
Title: Opportunities and safety of new gene modification techniques
Project description:
This project explores (1) ethical aspects surrounding the use and regulation of CRISPR gene drives and (2) the CRISPR/Cas13 system as a tool for killing tumour cells. The first sub-project takes a broad view of gene drive safety, analysing not only the risks but also ethical considerations, societal impacts and risk mitigation measures. It also considers the perspectives of scientists and stakeholders on gene drives. Sub-project 2 explores the usability and precision of the CRISPR/Cas13 system in animal and human cells, focusing on its potential application in cancer therapy.
Safety questions:
1. What ethical challenges are posed by gene drive technology, and how can this technology be developed and regulated in an ethically responsible manner?
2. Is the CRISPR/Cas13 system sufficiently usable and specific for cancer therapy development?

In 3.2, we analysed sub-project 2. Here, we focus on sub-project 1, which investigates the ethical questions surrounding the application of gene drive technology. This technology enables genetic modifications to be passed to all offspring, impacting future generations. It can potentially reduce or eliminate populations of organisms or replace a trait with another trait within a population. This type of technology raises ethical questions, such as when its use is justified. This sub-project demonstrates how research into ethical questions around new technologies, known as ethics parallel research, can assist both the content and process needed to shape the development of these technologies.

5.3.1 *Outcomes of this sub-project*

Understanding the issues to be addressed
A literature review was conducted to identify ethical and societal questions in technology development. This sub-project shows that the debate on gene editing in animals is primarily led by technical experts, which is logical as they are at the forefront of development. However, this focus often sidelines other perspectives, such as animal welfare and public concerns over – among other things – the accessibility and

commercialisation of the technology. For a broader view on these issues during technology development, a variety of stakeholders, including the public, should be involved. Additionally, distinctions should be made regarding different applications, as the questions may vary depending on the context.

The sub-project also reveals that scientists generally agree on *which* knowledge gaps on gene drive technology there are. However, they often disagree on the implications of these gaps for further development of the technology, as well as on which alternatives the technology should be compared to. The researchers believe that these differences stem from conflicting underlying values, which they argue should be explicitly acknowledged and addressed within the context of alternatives and societal interests. The researchers did not specify how this process should be structured. This process became more clear when discussed in view of patent-related issues and the potential roles of private governance and procedural justice.

In summary, this sub-project identifies key areas that should be addressed when using a new technology like gene drives: benefits, risks and uncertainties. It also suggests the need to compare the technology with alternatives. The researchers advocate for a broader perspective that considers the context in which the issue arose and the determinants. Additionally, the underlying values must also be addressed, meaning the role of humans in nature – a recurring theme in gene drive debates – should be considered.

What are the key considerations for structuring the technology’s development process?
Three factors are crucial in structuring the process of further development: 1) the involvement of stakeholders, communities and the public; 2) decision-making, including whether to follow the conventional decision-making process and making explicit whether this process is or is not sufficient; and 3) power dynamics, such as the deployment of technology by wealthy countries in developing nations and the historical (e.g. imperialistic) power imbalances that may influence these interactions. Each factor requires further elaboration and involves numerous questions. When it comes to factor 1, for instance, it is necessary to determine the purpose of involving stakeholders, who should be included, the timing, the methods of engagement, the information and framing that is selected and the independence and oversight of the process.

5.3.2 *Safe-by-Design working method*
This sub-project illustrates that adopting a broad perspective in an SbD approach requires ethics parallel research, which identifies questions arising from technology from various perspectives and addresses them in a well-considered and structured process that involves technical safety research.
Although the technical sub-project focuses on gene editing rather than gene drive, the two parts collectively demonstrate what a comprehensive SbD approach might entail. As this project indicates, this does require researchers to be aware of non-technical risks from the

outset. A complete evaluation of a technology should encompass both its benefits and its risks and uncertainties, including alternatives.

5.3.3 *Conclusions and recommendations*

This research sheds light on how the process of responsible further development following emergence of a new biotechnology can be shaped consciously and responsibly. This process requires careful design and oversight, with the understanding that any decision on next steps involves a political judgment based on input from this process. This sub-project demonstrates not only how to shape technological developments, but also emphasises that this is not solely the biomedical researchers' responsibility. Given the ethical and societal questions raised by new biotechnologies, the researchers advocate for concurrent ethical research alongside technological development.

5.4 Safety perception in industrial biotechnology

Project 15814
SafeChassis: Implementing and Assessing Safeguards for Lifestyle Engineering of a Versatile Industrial Chassis

Title: Implementing and Assessing Safeguards for Lifestyle Engineering of a Versatile Industrial Chassis

Project description:
The project involved research into (1) genetic safety mechanisms for the safe use of genetically modified micro-organisms (GMOs) in industrial installations, and (2) organising workshops and public discussions on the safety of biotechnology. The first sub-project focused on the industrially significant micro-organism *Pseudomonas putida.* The researchers based the genetic safety mechanisms on limiting survival via riboregulation, synthetic auxotrophy and genetic recoding. Through experiments and modelling, the researchers sought to establish safety thresholds. The second sub-project involved discussions with experts in workshops about how to determine safety and the necessary data. Public forums addressed the perception of biotechnology safety and the criteria for deeming it safe.

Safety questions:
1. Are the genetic safety mechanisms robust enough to prevent the survival of GMOs in the environment?
2. How can we assess the effectiveness of these mechanisms, and what criteria define a mechanism as safe?

In the sub-project outlined in 4.2, researchers developed genetic safety mechanisms that could be applied to micro-organisms, designed to prevent these organisms from surviving outside their cultivation environment. Technical research into safety mechanisms is a core part of that sub-project.

However, there is still limited experience in assessing the safety of these mechanisms. What technical requirements are needed? Also, at what point is 'safe' truly 'safe enough'? To gather input for addressing these questions, the researchers organised a pilot workshop for policymakers, risk assessors and researchers. They also held workshops with interested members of the public to explore perspectives on safety, including the question of when ‘safe enough’ is achieved.

5.4.1 *Outcomes of this research project*

Pilot workshop on technical safety assessment

The researchers hosted a pilot workshop with policymakers, risk assessors and researchers to determine as a group which technical data is needed to assess whether a genetic safety mechanism is indeed safe. This proved challenging, as the topic was overly abstract. To enable meaningful discussions, a highly concrete and specific case study is essential, detailing both the application and the genetically modified organism (GMO) involved. Only then can the necessary data and research requirements for safety questions be considered and discussed thoughtfully. This requires technical expertise on the part of the participants.

The pilot workshop intentionally brought together knowledge from various risk assessment domains. As such, food safety and chemical safety experts were also invited. However, due to limited biotechnology safety knowledge among these experts, the discussion remained at an overly high level of abstraction. The researchers concluded that specialists in molecular genetics, microbial ecology and biotechnology risk assessment would be better suited to gather input on the environmental safety of genetically modified micro-organisms. Unfortunately, a follow-up workshop with such experts could not take place.

Stakeholder research in industrial biotechnology

The researchers based their investigation on their technical work of creating genetic safety mechanisms in the micro-organism *P. putida* for commercial applications. They were curious to know the perspectives of researchers, industry and policymakers on the realisation of safety.

The stakeholder research showed that researchers prioritise the technical safety of GMOs, taking a scientific perspective. Industrial developers focus on well-established safety measures they have relied on for years – primarily physical containment through closed processing installations. As such, they are less interested in genetic safety mechanisms unless they require semi-open production systems. Policymakers, on the other hand, approach safety from an ‘assessment’ perspective, focusing less on how safety is achieved and more on proving it. Policymakers require evidence to support safety, but do not specify what data is necessary for safety assessments.

These stakeholder research findings indicate that developing and potentially using genetic mechanisms for GMOs will require dialogue among developers, future users and risk assessors. Such dialogue will provide insight into each other’s perspectives and the implications for safety questions and the research needed to answer them.

Public events

Throughout the project, several public events took place on ‘engineering life’ and managing risk when zero risk is unrealistic. In these workshops, researchers discussed broad safety themes, such as how biotechnological developments are ethically perceived as safe. A key outcome was the recognition that 'safe enough' cannot be determined without understanding the application context. Questions such as whether a genetic safety mechanism offers adequate safety or whether the genetically modified *P. putida* is safe enough cannot be answered universally. Stakeholders need to understand the specific application and circumstances involved. Only then can they indicate whether they consider the application to be safe. Furthermore, participants considered safety to extend beyond environmental aspects, viewing it as a concept that should reflect their values regarding such issues as naturalness, freedom of choice and rights of use.

5.4.2 *Safe-by-Design working method*

The three perspectives on safety within the SbD working method – technical safety, chain safety, and societal safety (see Chapter 1) – were all addressed in this project. Within each of these perspectives, specifying the application context is essential for assessment. For technical safety, it is crucial to consider which genetic safety mechanism is used for which application and under what conditions. This specific context is also necessary for the risk assessor, as the risk assessment depends on factors such as the genetic modification, the activity for which the GMO is used and the conditions under which the activity takes place. When engaging stakeholders and the public, specifying the application and context is equally important, as it helps determine the desirability of the application. This involves considering 'the purpose, actions and potential outcomes' and weighing these against possible alternatives.

5.4.3 *Conclusions and recommendations*

This research highlights the importance of defining the purpose of stakeholder engagement, which in turn guides the selection of stakeholders. Different stakeholders are necessary for determining safety questions and required research than for assessing safety in terms of societal acceptability. A well-developed case study that includes the context of use is also required, with the level of detail depending on the target audience and the question at hand.

Furthermore, it is important to consider how the results from stakeholder research and public dialogues can be used. How can these results feed back into the design and production phases? What adaptations are needed to ensure the information is useful for researchers and designers? Notably, not all 'issues' raised need to be resolved through technological development alone.

5.5 Guidelines for responsible innovation development

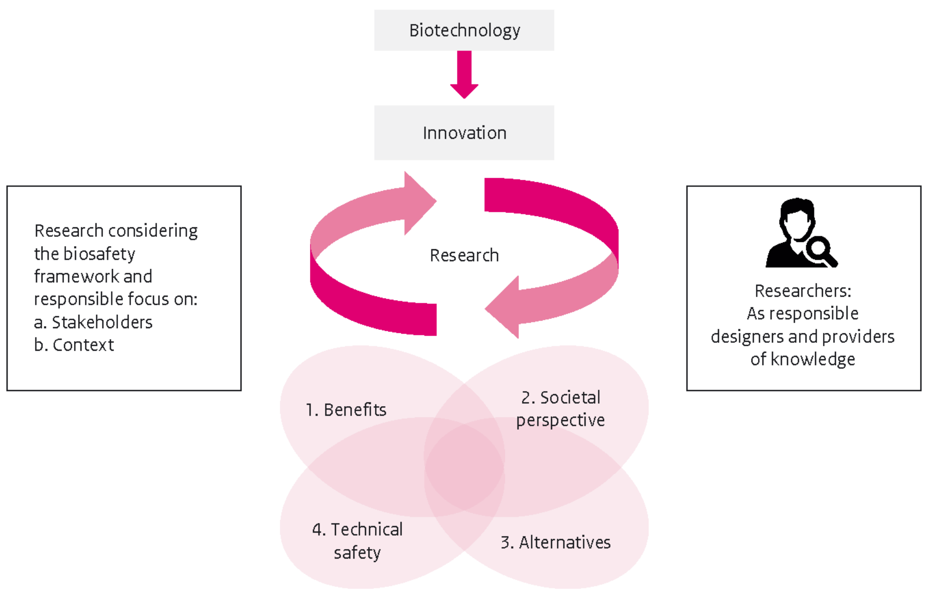
Each of the three research projects provides guidelines for elaborating the three safety perspectives as part of the SbD approach (1.3). In particular, the 'gene drives' sub-project (5.3) offers a foundation for a general framework, known as ethics parallel research, to guide the development of biotechnological innovation responsibly. This framework aligns with the three safety perspectives central to SbD and proposes ways to incorporate the societal perspective on safety. This is achieved primarily through exploratory research into the benefits of the innovation and exploring alternative solutions to the societal issue it addresses. Together, these projects contribute to the three safety perspectives of SbD and aid in determining subsequent development steps.

The T-TRIPP project examines the current biosafety system and the challenges introduced by emerging innovations. From this standpoint, research insights into risks and uncertainties are critical for helping the biosafety system to develop. Researchers are therefore recognised as key players in the biosafety system, occupying dual roles as ‘providers of research data’ and ‘responsible designers’. These roles need to be strengthened further to help develop the safety system effectively, assigning researchers specific responsibilities within a SbD strategy. The T-TRIPP project also underscores the importance of the societal perspective, advising that it be developed in relation to regulatory systems, rather than as an isolated aspect.

Finally, the SafeChassis sub-project highlights the importance of the careful selection of stakeholders to involve in safety research – a point also emphasised by the ‘gene drives’ sub-project. In addition, SafeChassis, like the ‘gene drives’ sub-project, points to the need for context-specific development and implementation of the application. This requires a tailored approach that applies equally to technical safety research and research concerning the societal perspective on the innovation.

The guidelines identified as part of these research projects support the shaping of the Safe-by-Design safety perspectives. The following figure brings these guidelines together, outlining a process-based approach for safety research from the researcher’s perspective.

Figure 1 Process-based approach for Safe-by-Design safety research of a biotechnological innovation.



6 Findings and conclusions of the research programme

The NWO research programme 'Biotechnology and Safety' was established in response to the emergence of new biotechnological advancements. The programme's goal is to enhance safety knowledge concerning these technologies, which in turn can strengthen biotechnology policy and biotechnology implementation.

This chapter revisits the question of the demand for knowledge about these technologies and its implications for policy and practice. We first explore the three core research themes: medically related research, CRISPR applications and industrial environmental safety projects, summarising the programme's contributions within each area (6.1). We then examine the outcomes regarding the execution and evaluation of this technical safety research (6.2). Finally, we consider the insights gained from the projects described in Chapter 5 for developing the biosafety system further (6.3). Particularly in these last two sections, we provide an analysis and reflection on these outcomes.

6.1 Research into the safety of an innovation: safety questions

6.1.1 Medical applications using viruses

Viruses provide a means to transfer genetic information into cells, enabling their development for use in cancer therapies (2.2) or vaccines (2.3). To ensure safe application, viruses must be modified so that they no longer cause disease. Key safety questions surrounding these modified viruses involve the potential for spreading and recombination. This latter concern is especially pertinent when a virus has been modified, for example by removing the genes linked to infection. Through genetic exchange with wild-type viruses, a modified virus could potentially reacquire its pathogenic characteristics, compromising its safety.

The virus projects within this research programme developed experiments to gather knowledge and data about these safety questions. Researchers working on oncolytic viruses (OVs) primarily focused on *the risk of a genetically modified OV spreading*. Their approach involved a phased assessment, beginning with identifying conditions under which spreading could occur during clinical use. They then devised experiments to address these questions, emphasising the need for a well-reasoned justification for each experimental choice. The experimental set-up and resulting data must address the safety question thoroughly. Conversations with a variety of experts and risk assessors offer valuable perspectives for refining experimental designs. However, differing views among experts can arise, with some categorising certain experiments as 'need-to-know' and others as 'nice-to-know'. The necessity for these potential experiments must always be well-supported, especially when involving animal testing, in which case alternative methods should be considered.

The RepliSafe researchers conducted an extensive investigation into the *occurrence of recombination* by designing experiments to determine if and when recombination might take place. Events like recombination between a genetically modified virus and a wild-type virus, which may be very rare or even nonexistent, are challenging to study and had not previously been examined in such depth. A range of cell and animal experiments revealed that recombination in the studied alphaviruses is indeed rare, but cannot be completely ruled out. Notably, the common hypothesis – requiring identical sequences in the replicon and wild-type virus for recombination – may not always apply, suggesting other mechanisms could be involved. This underscores the importance of empirical support for the prevention of recombination events. Based on the insights gained, the researchers proposed three safety questions to guide future studies on viral recombination (see 2.3).

In conclusion, both research projects demonstrated how experiments can be structured and executed to address safety questions when using genetically modified viruses. Although these experimental results cannot be extrapolated universally to other viruses, the approach – comprising both the rationale for selected experiments and the experimental design itself – could also be used for research into the spread and recombination of other viruses. Furthermore, knowledge could be developed for each virus used. This will require a carefully considered approach and methodology. Ensuring transparency and searchability of these findings is essential for utilising knowledge in environmental risk assessments.

6.1.2 CRISPR technology

CRISPR technology can be used for nearly all cell types across organisms, making safety-related research into this technology highly relevant.

The primary safety concern with CRISPR is off-target effects, where CRISPR activity impacts unintended areas of an organism's genetic material. Minimising off-target mutations is essential, as alterations in the genotype can potentially affect the phenotype. The CRISPR projects involving eukaryotic cells (3.2), fungi (3.3) and tomatoes (3.4) explored which guide RNA sequences determine specificity and how this knowledge can inform the design of guide RNAs to minimise off-target effects.

The fungi (3.3) and tomato (3.4) projects revealed that unwanted, unexpected mutations in the genome are generally not a direct result of the CRISPR technology, but are instead due to the entire modification process and/or natural genetic instability. Both projects also found that off-target mutations do not necessarily impact the phenotype or the product's safety. Not all mutations are therefore automatically undesirable. However, this does raise the question of when a mutation *should* be deemed undesirable. This was one of the focuses of the plant breeding project (3.5), which investigated the link between genotype and phenotype by comparing mutations in resistance genes with potential changes in potato metabolite composition.

While CRISPR technology is deemed widely applicable, the 'eukaryotic cells' CRISPR project highlighted the importance of biological context for the efficacy of CRISPR. The literature initially suggested that Cas13 could lead to complete RNA degradation, hinting at a potential for cancer therapy development. However, research showed that Cas13 behaves differently in prokaryotic versus eukaryotic cells, with complete RNA degradation unattainable in eukaryotic cells.

In conclusion, as a new technology becomes more widespread, knowledge development is progressing rapidly. CRISPR technology is a great example of this. These projects have provided advancing insights into the technology and developed methods to understand off-target effects, demonstrating the importance of biological context for CRISPR applications: results observed in one cell type or organism may not translate to others.

Statistical analyses are still infrequently applied in environmental safety assessments of GMOs. The plant breeding project suggests that these analyses could offer valuable insights, though significant work remains to be done to determine how statistical outcomes should be weighed against negligible risk.

Due to the relatively short project duration, no studies were conducted into the long-term stability of mutations produced using CRISPR technology. Additional data, particularly for gene drive applications, is needed to assess CRISPR's safety comprehensively.

6.1.3 Environmental safety in industrial biotechnology

Industrial biotechnology involves the large-scale use of genetically modified micro-organisms to produce various raw materials, chemicals, pharmaceuticals and food ingredients. Innovations also include using genetically modified micro-organisms in semi-open environments, such as biosensors, bioremediation (i.e. environmental pollution clean-up) and photobioreactors. For these applications, understanding the survival of GMOs outside the installation and their potential for DNA transfer is crucial. This includes understanding genetic exchange possibilities with wild-type micro-organisms (horizontal gene transfer) and the effects on other organisms. Research in an 'industrial setting' provides key insights.

To prevent genetically modified micro-organisms from surviving outside the installation, a technical approach involving a genetic safety mechanism can be implemented. The SafeChassis project demonstrates that various safety switches are possible, theoretically preventing organism survival in the environment. However, the robustness of such a safety switch must be substantiated, as it depends on contextual factors like the organism used, the application's nature and the environment in which the organism could end up. An initial assessment of one safety switch's robustness showed a low mutation frequency over several weeks in laboratory conditions. More growth data over an extended period and under different environmental conditions is needed to gain further insights into robustness.

The TargetBio project developed a small bioreactor for monitoring the microbial composition of sludge. This bioreactor can also aid in collecting growth data to test the robustness of genetic safety switches under environmental conditions.

Another critical safety question concerns horizontal gene transfer (HGT), an active process where DNA is transferred from one organism to another. Little is known about the conditions that facilitate HGT. Research into environmental conditions that could be stressful to actinobacteria found that cell wall-deficient cells can emerge. These cells are potentially more capable of HGT.

The TargetBio project also investigated DNA stability. It revealed that, while autoclaving kills micro-organisms, DNA does not completely degrade and persists in single-stranded form (ssDNA). This small fraction of DNA was also found to be present in wastewater. This suggests micro-organisms could potentially absorb it. Follow-up research could be conducted using the bioreactor developed in this project. Identifying ssDNA-binding materials provides a potential method for removing ssDNA from water, which could be useful when recycling waste streams.

In the fungi project, adding a short barcode enabled traceability of mutations without compromising fungal fitness. Although the barcode's long-term stability was not tested, a traceable barcode could be an attractive option for specific applications.

In conclusion, the research within this theme highlights the potential for using genetic safety switches as a risk management strategy to prevent survival. Additionally, tools, methods and experiments for studying survival and DNA stability have been developed, offering a foundation for follow-up research into the safety of developments towards larger-scale and semi-open systems. Ultimately, this could lead to better predictions of survival and DNA uptake, allowing for more targeted safety measures.

6.2 Conducting technical safety research and safety assessment

The research programme has provided insights into conducting safety research, with the identification of relevant safety questions being central. Based on an analysis of discussions held within the user committees, we offer guidelines and focal points in 6.2.1 for identifying and making technical safety questions researchable.

The research programme has also developed and refined methods and techniques that are applicable for data collection in environmental risk assessment. In 6.2.2, we delve into these methods and their impact on risk assessment methodology and the knowledge development of risk assessors.

6.2.1 Technical safety questions: from identification to making them researchable

As mentioned, technical safety research begins with identifying the relevant technical safety questions. The OV project (2.2) illustrates the steps required to formulate and make these questions researchable:

a. Inventory of potential risks within the specific development.
b. Examination of whether, how and under what conditions a potential risk may arise.

c. Development of experiments to make the questions arising from step b researchable.

Involvement of the user committee, composed of researchers from various disciplines and representatives from policy and implementation areas, enables a dialogue essential for discussions, analysis and knowledge acquisition among the involved parties. The following focal points are relevant for these discussions:

- Justifying and substantiating the selected experiments and the data derived from them to answer the safety questions.
- Differentiating between experiments that yield 'need-to-know' information and those that yield 'nice-to-know' information, with the latter referring to data that does not provide essential insights for answering the safety question.
- Allowing differences in interpretation, associated arguments and underlying assumptions among experts to be fully expressed, fostering an understanding of each other's approaches.

These discussions ultimately result in a shared selection of safety questions and experiments. Especially for the risk assessment process, it is crucial that the selection of safety questions and experiments is documented clearly, with reasoning and a logical sequence of arguments. This documentation provides risk assessors involved in the permitting process with insights into the reasoning followed.

For the identification of safety questions as outlined above, the focus is on the technical safety of a specific application. For this scope within the safety perspective, it is also essential to:

- consider carefully which stakeholders to involve and for what purpose (see the findings from the SafeChassis sub-project, 4.2);
- define the development as specifically as possible (see the gene drive sub-project, 5.3);
- avoid semantic confusion over the term 'safety', as it may be perceived and interpreted differently.

Identifying and discussing safety questions in a broader sense requires a multidisciplinary approach. It is essential to specify the disciplines and stakeholders needed to cover the entire chain from development to (new) usage. Moreover, it is crucial to clarify the context of use, as safety is context-dependent, varying according to the nature of the genetic modification, the type of use (e.g. in which organism), the method of application (e.g. experimental settings) and the end use (e.g. by the consumer).

For the societal perspective on safety questions related to a development, it is also important to elaborate on the application and context of use. This enables insights into the safety questions and concerns among the public and their reasons for these concerns. Especially for major developments, it is desirable to gain such insights, so that they can be incorporated into safety research and further development.

6.2.2 *Methods and techniques and their use in environmental risk assessment*

This programme has seen the use of whole genome sequencing (WGS), quantitative analysis techniques and machine learning, and has led to the development of a methodology for tracking developments within a microbiome. Based on the experiences and outcomes of this research programme, we discuss considerations and key points for using these methods in environmental risk assessment.

Whole genome sequencing (WGS)
Determining the genetic information of an organism, or even of a single cell, has seen tremendous advances in recent years. An increasing amount of WGS data is becoming available to support research, such as in the CRISPR tomato project (3.3) for designing the optimal guide RNA.

In environmental risk assessment, WGS data can also be useful for determining the precise location of a genetic modification within a genome and whether any significant genes have been disrupted as a result.

However, more genetic information also raises new questions. This programme demonstrates that, when examining an organism's entire genome using WGS data, mutations are identified of which not always can be established whether they result from the technique, genetic instability or natural variation. Additionally, this programme shows that mutations in the genetic sequence (the genotype) do not always manifest in a way that changes the organism (phenotype). This brings up the question of the relevance and biological significance of the mutations identified.

For risk assessment purposes, it would therefore be useful to determine the relevance of a mutation, potentially through a baseline or reference genome. Such a baseline or reference genome could possibly be derived from WGS data of multiple genomes of the same organism. However, questions remain on how this should be approached and what considerations are involved.

Using WGS data to gain insight into mutations is therefore not straightforward. The relationship between genotype and phenotype, along with insights into a baseline or reference genome, are avenues of research that could allow for better interpretation of WGS data.

Use of quantitative data and AI/ML models
Environmental risk assessment in biotechnology is qualitative: environmental risks are assessed based on reasoned scientific knowledge and empirical data. Quantitative data tailored to environmental risk assessment is available only to a limited extent.

However, biotechnological researchers collect a substantial amount of quantitative data to develop statistical models aimed, for example, at predicting functionality and, in recent years, for AI/ML models. As shown in the CRISPR plant breeding project, such models can support risk assessment. For the DOMINO project, researchers even developed an AI/ML model.

Quantitative data could therefore be useful for environmental risk assessment. When conducting functionality research, it should be directly examined whether this research could also contribute to insights into safety. In doing so, it would be necessary to determine what additions or modifications to the research are required to make the data applicable to a safety question. This data could then be used in the permitting process for risk assessment. For example, additions might include sufficient diverse experimental data on environmental conditions.

To facilitate multipurpose use of research, the involvement of statisticians and/or methodologists is essential. They can consider in advance whether the research can be used in this way or what additions or changes would enable this. This approach should be developed further to collect relevant data that can generate reliable models for answering safety questions. Such a development would require risk assessors to increase their knowledge and understanding of aspects such as the data collection upon which the model is based and the model's limitations.

Bioreactor experiments
The use of genetically modified (cyano)bacteria as biosensors, environmental clean-up agents (bioremediation) or for sustainable production (using sunlight) of basic products is widely documented in scientific literature. These bacteria will eventually be used in semi-open systems, outside the laboratory. In environmental risk assessment, an important question is what is known about the survival of these GMOs. Obtaining data to answer this question is complex. Simulating environmental conditions in the lab is challenging, and it is difficult to determine the time required to establish that there is no longer any survival. Additionally, the method for confirming this remains in question.

For the TargetBio project, an environmental study was set up by simulating conditions in a bioreactor. The knowledge gained from this experimental set-up is highly valuable and can be developed further for environmental impact research or to monitor changes within microbiomes. It can also be used to study evolutionary effects, horizontal gene transfer (HGT) or the robustness of a genetic switch, as developed in the SafeChassis project (4.2).

Setting up a bioreactor experiment and the time required for data collection and analysis makes this type of research relatively costly. However, such experiments can yield a wealth of valuable data. This data can be used as input for quantitative (AI/ML) predictive models that can support risk assessment.

The programme provides insights into how qualitative risk assessment can incorporate quantitative data. This integration will not happen automatically and requires considerable thought, knowledge acquisition and time and resources, including from the risk assessor. Nevertheless, it can contribute to the necessary advancement of risk assessment.

6.2.3 *Guideline for SbD safety research*

The preceding sections 6.2.1 and 6.2.2 provide the elements for a guideline for conducting safety research (see Box 3). This guideline makes it possible to develop the technical safety of Safe-by-Design, as described in 1.3, further. This approach allows for proactive incorporation of safety in cases where regulations have not yet caught up with innovation.

Box 3. Guideline for technical safety research in a Safe-by-Design approach.

1. *Establish a common conceptual framework.* Safety can be interpreted in different ways and may relate to one or more of the following areas, which can be interrelated:
   a. Specific technical actions.
   b. The process within which these actions occur.
   c. The immediate result/product and its application.

   By considering the concept of safety ahead of anything else, it can be clarified in advance which aspect of safety (‘safety with respect to what’) is being discussed, helping to avoid miscommunication.
2. Identify *which safety questions* are relevant to points a, b and c mentioned above. This can be done in cooperation with researchers from different disciplines and risk assessors.
3. Review existing literature to establish *what is already known* and then determine which experimental studies are needed to answer these research questions. Careful consideration of the experimental design, the type and quantity of data needed and how the results contribute to answer the safety question is essential.
4. *Translate the safety questions into their implications for the research.* Safety questions can be translated into specific research questions regarding safety aspects. This can also be done in cooperation with researchers and risk assessors. It should be noted that safety questions broader than technical ones may lead to fundamental changes in the research.
5. *Determine which experimental studies are needed* to answer the research questions. Careful planning of the research design and determining the necessary type and amount of data is essential.
6. *Assess whether modelling the research results is feasible* and consult with methodologists and statisticians to establish the conditions for models to yield reliable results. This allows for targeted collection of relevant data to develop reliable models.

6.3 Towards responsible innovation development

The results of the research programme provide building blocks for a process description for the responsible development of innovation within the safety system. This process builds upon Fig. 1 in Chapter 5. Below, we discuss the elements of this process as shown in Fig. 2 and how these elements are derived from the programme.

As innovation progresses, safety knowledge is required to continue developing the innovation responsibly and safely (see I and II in Fig. 2). While the research programme primarily focused on technical safety, it has shown that safety encompasses more than just technical aspects (1–4 in Fig. 2). Knowledge about the benefits of an innovation is also important, including comparisons with alternative solutions (from broad to specific[5]) and insights into the societal perspective. This complete set of knowledge influences choices and decisions for a responsible and safe innovation.

We also add knowledge about safety across the development chain through to (new) applications (2b in Fig. 2). This knowledge can be considered a more specific aspect of technical safety knowledge. In the research into oncolytic viruses (2.2), this was addressed by incorporating a safety question that would emerge later in the chain, during potential clinical use, into the fundamental research phase. We make special mention of this safety aspect of Safe-by-Design, because it could otherwise be easily overlooked. Additionally, we add the aspect of knowledge about alternative regulatory frameworks (3b in Fig. 2). This knowledge is also necessary to develop the safety framework in coordination with other regulatory frameworks.

From the research programme results, it can be concluded that, in order for safety policy to adapt responsibly to innovations in biotechnology and safety research, knowledge in all of the above areas is essential (1 to 4 in Fig. 2). Developing this knowledge requires not only biotechnology research, but also social science and/or multidisciplinary research, and should evolve iteratively over time.

To achieve the desired knowledge development, interaction between researchers and actors in policy development and implementation is necessary (III in Fig. 2). This interaction leads to safety research questions and research outcomes (IV in Fig. 2).

This knowledge informs both policy development and implementation within the biosafety system (V and VI in Fig. 2). In Fig. 2, we indicate policy development and implementation in the form of the Big 8 (see IPLO), which also illustrates the interaction between policy and implementation. Within this interaction, policy provides input to implementation, and implementation provides feedback to policy.

Safety knowledge enables all parties involved in the biosafety framework – researchers, policymakers and policy implementers[6] – to make responsible and safe choices, thereby contributing to the responsible development of an innovation.

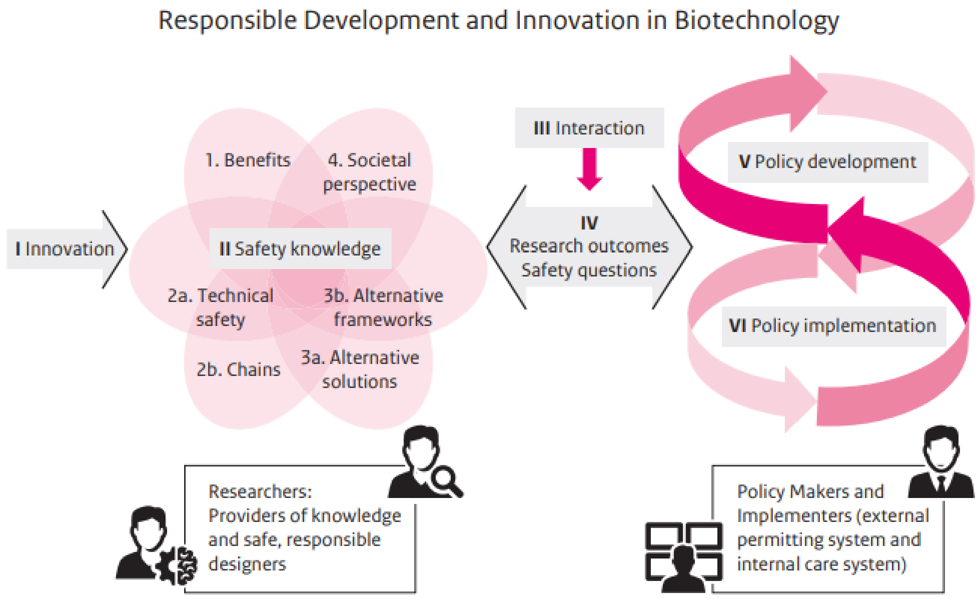
With these additions to the process approach outlined in Fig. 1 for the responsible development of innovation in biotechnology, Fig. 2 not only presents a process for safely and responsibly shaping innovations, but also serves as a foundation for further development of the biosafety system. As T-TRIPP indicates:

- Interaction between researchers and policy development and implementation will need to be strengthened further.

[5] The gene drive sub-project indicates that there is a choice in this matter. The issue can be described at various levels, from as narrow as an alternative technique to as broad as a societal problem.
[6] In relation to the T-TRIPP research, the external permitting system and the internal care system.

- The structure and implementation of safety research will need to be ensured. This will also require strengthening the roles of researchers as 'safe and responsible designers' and 'knowledge providers'.

The research projects discussed in Chapter 5 emphasise the importance and the role of actors within the safety system and the consideration of process elements. These elements, as noted in Chapter 5, include stakeholder engagement and the design of the decision-making process. Recommendations on these points should therefore be incorporated in both the set-up of the aforementioned system and in the design and implementation of the various elements of safety research.
In conclusion, the research programme provides the building blocks for the responsible development of innovation and a foundation for the further development of the biosafety system.



7 Discussion

Scope limitations of the programme and thus this research

The Biotechnology Trend Analyses of 2016 and 2023 (COGEM, 2016 and 2023) provide an overview of developments in biotechnology. The ten projects in the 'Biotechnology and Safety' programme cover only a fraction of these developments. Researchers were free to choose the development they wished to study. In this sense, the programme offers a limited contribution toward the goal of building scientific knowledge about the risks and uncertainties of 'state-of-the-art' and future biotechnological developments. However, it is the first biotechnology research programme specifically focused on gathering knowledge about risks and uncertainties. As such, it can be viewed as a pilot study to assess whether this type of programme yields the desired knowledge. If there is a need to gather knowledge about a specific development, it should be explicitly stated in the research call.

Attractiveness of safety research

This research has demonstrated that a programme like this can indeed contribute to the desired knowledge development. However, this does not necessarily mean there is widespread enthusiasm among (biotechnology) researchers for such studies. Safety research is time-consuming and offers limited appreciation or recognition. In this programme, it also proved challenging to adjust a research project. Research objectives are typically fixed in the project proposal, and underlying PhD research projects often have limited flexibility due to tight timelines and the urgency of achieving publishable results.
Safety research can be integrated with functional research, but requires significant incentives to foster a culture in which this becomes second nature.

Research into 'safety': scope and interpretation

- Although the concept of safety may appear straightforward, its interpretation can vary significantly in terms of content and scope. As we discussed in Chapter 1 on SbD, safety ranges from the safety of the technology itself to safety within a societal perspective. It can also concern absolute or relative safety compared to alternatives. Furthermore, the concept of safety may also be defined by regulatory frameworks, as is the case in biotechnology. Here, the dominant interpretation is the one prioritised in regulations: a focus on technology and environmental safety. This emphasis may lead stakeholders – from policymakers to risk assessors and researchers – to overlook the broader aim of safety: taking a comprehensive view of the potential hazards of what is created and done.
- Safety and society. Ideally, biotechnology safety research should be a step-by-step process that considers the entire chain. Given the societal sensitivity surrounding biotechnology applications, it is crucial to take the societal perspective, as well as the necessity and purpose, into account. This does not mean that every fundamental study must be framed within a broad safety

perspective. Nor does it mean that every technically-oriented research group should always add social science research to their technical research. However, such research does need to be conducted, and the potential implications of fundamental research for eventual applications should be considered explicitly. Moreover, technically-oriented researchers should have some insight into public perceptions and the questions they may have on the topic. This enables these researchers to identify any missing research outcomes and to gather data that can help address public concerns.
- *Elements of safety research* (see Fig. 1). Research into the societal perspective, benefits and alternatives is relatively rare (and systematic research even more so). This was also the case in this programme. However, such research is important for responsibly advancing an innovation. Policy decisions needed during further development can benefit from systematic research in these areas. Conducting this type of safety research requires numerous choices that need to be substantiated. For example, research into alternatives might examine alternative solutions to the societal problem or alternative techniques for the solution itself.
- *Assessing safety.* Safety assessments and safety research do not depend solely on the type of safety in question. They also depend on the following factors:
  - *What is considered sufficiently safe.* Is an extremely low probability of mutations in a genetic safety switch low enough to consider the switch safe? Or is a slight chance of off-target effects in CRISPR safe enough?
  - *The assessor.* Communication between researchers and risk assessors is necessary to clarify the risk question and determine the required data to assess that risk. Small differences may exist between risk assessors with different expertise. Additionally, the role of the risk assessor, whether as an adviser or a permit authority, can also lead to potential (small) differences. Consequently, interaction with a risk assessor during research does not guarantee a smooth approval process later.

Responsibility

Who is responsible for what? This question arose in meetings of the user committees and was addressed in the (sub)projects in Chapter 5. Many parties, each with their own role, share partial responsibility. There is not always clarity among the parties about their roles, and differing expectations about roles and responsibilities may arise between them. For example, the research programme shows that there are varying perspectives on how to implement the SbD approach. Furthermore, it is unclear who holds responsibility for researching other aspects of safety, such as the societal perspective, benefits and alternatives. Within an RRI perspective, there is a general assumption that technically-focused researchers should also adopt or at least consider a broader perspective within their research. However, the question remains as to whether this is necessary for every study and when it should or should not be required. As such, discussions on responsibility must be held frequently to establish clarity and consensus on who is responsible for what within the safety system and what this entails. In this regard, there are a few points for consideration:

- How does a researcher's significant responsibility as a knowledge provider and safe designer align with the resources and recognition available for safety research?
- The government designates a different responsible agency for each theme. This applies not only to safety but more broadly. Who, then, is responsible for ensuring coherence and consistency across policies?
- How far does responsibility for safety extend, and when is 'safe' considered safe enough?
- The programme reveals that interaction between researchers and risk assessors is seen as valuable. This, however, raises the question of the separation of responsibilities within the risk assessor's roles. This concerns the risk assessor's responsibility in the role of permit authority versus their responsibility as an adviser.

8 Recommendations

This research provides a process description (Fig. 2) for the responsible development of innovations in biotechnology. We recommend testing this and developing it further as a guideline.

This entails, broadly:

- determining with stakeholders a development/innovation to be developed further using the guideline;
- establishing a research programme to generate input for policy development on technical content as well as on the societal perspective, benefits and alternatives;
- shaping the interaction among researchers, policymakers, policy implementers and biosafety officers;
- engaging in an iterative process where decisions are made based on research data;
- determining how the roles and responsibilities of the parties can be developed further and embedded within the system.

For policymakers and research programme developers:

This research demonstrates that a research programme focused on safety is worthwhile. Given that knowledge on biotechnological innovations will continue to be needed to support the safety system's evolution, we recommend:

- establishing further research into the safety of developments in biotechnology, with attention to:
  - continuity of research, including examination of appropriate funding systems;
  - sufficient incentives and appreciation for safety research;
  - conducting not only technical research, but also studies into the societal perspective, benefits and alternatives.

For policy implementers:

Policy implementation can help guide the development of knowledge needed for environmental risk assessments:

- Investigate how safety research could play a more prominent role within the permitting process, providing an incentive for conducting safety research.
- Explore options for using quantitative research data, with a focus on:
  - developing and making quantitative data and models available;
  - investigating what might encourage researchers to contribute to this knowledge base;
  - supporting risk assessors' acquisition of necessary knowledge.
- Develop, expand and make available a knowledge base containing research results, experimental set-ups and tools for safety research. This could support risk assessments and safety research efforts.

- Assess whether, and if so, how a reference genome or baseline could be developed to aid in evaluations, such as for microbiomes.
- Incorporate recommendations made in this programme into guidelines and explanatory materials for environmental risk assessments.

For those who conduct safety research:

- Develop the guideline (6.2.3) for conducting SbD safety research further in a broad sense.
- Develop tools (e.g. training) to strengthen the identification of safety issues.
- Facilitate interaction between researchers, policymakers, policy advisers and risk assessors, aimed at:
    - translating scientific knowledge into policy and vice versa;
    - defining the researcher’s role as a provider of knowledge.

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