Andrea van Langen Rosón

Bacteriophages as agents against locust swarming

A Safe-by-Design study on the application of gene and microbiome therapies.

TU Delft

Image: (IEMA, 2017)

Bacteriophages as agents against locust swarming

A Safe-by-Design study on the application of gene and microbiome therapies.

By

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Contents

Abstract

The rapid advancements in synthetic biology have allowed for the development of revolutionary technologies such as bacteriophage-mediated gene and microbiome therapies. These technologies have created the need for management of the upcoming risks and uncertainties. Safe-by-Design is a strategy to build in safety measures to mitigate and anticipate risks in synthetic biology applications. This study aims to portray a nuanced review of the opportunities and challenges posed by Safe-by-Design through a case study on this year's iGEM project, the application of gene and microbiome therapy principles to mitigate the issue of Desert Locust swarms.

To gather an all-round perspective on the current state of synthetic biology, bacteriophage-based therapies, and Safe-by-Design, these aspects are assessed in terms of governance. Synthetic biology regulations have long ensured safe research practices, but the lack of adequate regulations for novel phage-based technologies hampers the development of this field of research. In further detail is discussed how the use of the precautionary principle guarantees biosafety but also limits the discovery of risks and uncertainties. Downstream Safe-by-Design is proposed as a method to facilitate policy-making and to tackle concerning issues such as transnational regulations and public perception of biosafety.

In this report, the risks associated with the application of bacteriophages for non-therapeutic purposes are determined and multiple Safe-by-Design approaches are analyzed to mitigate these risks and uncertainties. This resulted in the collection of quorum sensing, kill switches, encapsulation, anti-CRISPRs, and auxotrophy as suitable Safe-by-Design measures for this project.

Furthermore, the challenges and limitations regarding the application of Safe-by-Design are reviewed. This analysis concludes that uncertainty and a lack of knowledge of the intricate synthetic biology systems are the main constrains on Safe-by-Design for synthetic biology. Lastly, it is concluded that Safe-by-Design is a promising strategy to ensure biosafety because of its dynamic and proactive character, and recommendations are made to stimulate future progress in this area of research.

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1 Introduction

In the last two decennia, the world has seen a fast development of novel technologies in the emerging field of synthetic biology (Shapira, Kwon & Youtie, 2017). Synthetic biology technologies have a vast range of applicability in a variety of sectors, for example as environmental cleanup, in the generation of environmentally friendly biofuels, for treatment of human medical conditions through novel biological delivery systems, or the use of standardized GMOs in agriculture (Saukshmya & Chugh, 2009; Bhutkar, 2005). It has served as a fundamental tool to tackle global challenges, but with each innovation comes a potential for harm (Singer, 2006).

The rapid development of synthetic biology has created the need for management of the related uncertainties and risks (Breitling, Takano & Gardner, 2015; UN Environment, 2019) such as irreparable damage to the natural ecosystems (Schmidt & de Lorenzo, 2012), e.g. by introducing toxic metabolites into natural habitats or by disrupting ecosystems by competing with native species (Dana, Kuiken, Rejeski, & Snow, 2012). The development of safety strategies is therefore necessary to ensure responsible research practices and maximize the safety of biotechnological applications. A recent study shows that there is still insufficient information about responsible research and innovation in synthetic biology (Shapira, Kwon & Youtie, 2017). There is thus a need to analyze and develop biosafety measures for the long-term performance of synthetic biology applications and to consider all the potential implications before deployment (Schmidt, 2008; Cheng & Lu, 2012). An approach to do so is Responsible Research and Innovation (RRI), which is promoted by the European Commission through the European Union's Horizon 2020 Framework Programme (European Comission, 2020). It aims to integrate desired societal values in the development of technological innovations and research (RRI Tools, n.d.). One of the pillars of RRI is safety, which can be addressed through multiple approaches that deal with risks and the acquisition of safety in terms of design choices. In this report, the attainment of safety will be discussed by implementing built-in biosafety measures through the concept of 'Safe-by-Design'. Safe-by-Design (SbD) strategies are developed to mitigate harmful effects and avert potential risks on human health, animals, and the environment by engineering out hazards (Schwarz-Plaschg, Kallhoff & Eisenberger, 2017).

An emerging technology are bacteriophages, or phages in short, which account for one of the most innovative and important bodies of research in the field of synthetic biology. They are the most prolific microorganisms on earth and are widely known as bacteria-killing viruses (Nikolich & Filippov, 2020). Since their discovery, bacteriophages have been essential for the development of molecular biology, from understanding the structure of DNA to the development of CRISPR/Cas9 technology (Hosseinidoust, 2017). In recent decades there has been a wide interest in the development of bacteriophages for multiple applications such as for phage therapy, as drug delivery vehicles, to build new nanostructured materials, and for gene therapy (Pires et al., 2016). Also in the Netherlands there have been new developments and the implementation of new policies regarding phage research. Increased media attention by i.a. the TV broadcast called 'Dokters van Morgen', which aired in 2017, has led to a greater interest of the mainstream public towards research into bacteriophages and highlighted the importance of research into bacteriophages for phage therapy (AVROTROS, 2019). In 2019 the first phage bank in the Netherlands was opened at the Delft University of Technology (TU Delft Communication TNW, 2019).

In addition to phage therapy, bacteriophages are also widely being developed for their use in gene therapy as gene delivery vectors. Gene therapy is a technique that transfers and integrates therapeutic genes into a target cell and modifies its genome in order to treat a disease (NLM, n.b.-b). In the last decades, gene therapy has made major advancements and clinical trials for the application of gene therapy to treat diseases has been approved in 28 countries (Gillet et al., 2009). Furthermore, gene therapy is also used for microbiome therapy. This technology entails the alteration of an organisms' microbiome to treat pathogenic bacteria and is considered, as stated by Hosseinidoust, a "new frontier for bacteriophage-based gene therapy" (2017, p. 6).

The fast developments in synthetic biology have provided scientists with the tools to create new designer bacteriophages with desired therapeutic features (Barbu, Cady & Hubby, 2016). However, the genetic modification of bacteriophages also meets a range of challenges in terms of safety. Examples of safety concerns in the use of phages to modify other organisms include the transfer of virulence factors through generalized transduction or the induction of an inflammatory response by the host's immune system (Nobrega et al., 2015). This emphasizes the importance of developing and managing the concept of safety for gene therapy and microbiome therapy with bacteriophages.

This raises the question: How can Safe-by-Design help address and anticipate safety concerns in bacteriophage applications?

The following sub-questions will be discussed:

I. How does the concept of Safe-by-Design apply to the field of synthetic biology?
II. What risks and uncertainties are related to the use of bacteriophages in the non-therapeutic application of gene and microbiome therapies?
III. What Safe-by-Design measures could be applied to maintain safety in the use of gene and microbiome therapy principles for non-therapeutic applications?

This issue will be examined through a case study regarding the implementation of principles from gene and microbiome therapy to solve the issue of Desert Locust swarms. iGEM, short for International Genetically Engineered Machine Competition, is an international competition that aims to inspire students to use synthetic biology to solve societal issues (iGEM, n.d.). The iGEM team at the TU Delft has devoted this year's project to help solve the problem of Desert Locust swarms by implementing mechanisms from bacteriophage-mediated gene and microbiome therapies. According to the Food and Agriculture Organization of the United Nations, the swarms formed by the Desert Locust (*Schistocerca gregaria*) are the most destructive migratory pest in the world (FAO, 2020 -a). Due to optimal environmental conditions such as heavy rainfall, large swarms of locusts have recently been forming. The changes in climate stimulate the formation of swarms of otherwise solitary locusts, which can contain up to billions of individuals (Meynard, Lecoq, Chapuis & Piou, 2020; World Meteorological Organization & Food and Agriculture Organization of the United Nations, 2016). These migrating swarms have taken over parts of East Africa, Southwest Asia and around the Red Sea since the beginning of the year (FAO, 2020 -a). The locusts consume and ravage food crops and vegetation, therefore posing serious dangers to food security. The iGEM team has come up with several strategies to mitigate this issue. They want to reach their objective by preventing the locust swarming phase or by killing the locusts (iGEM TU Delft, n.d. -a).

In this report, we will take a closer look at the concept of safety and specifically regarding the development of bacteriophages for gene therapy and microbiome therapy in non-therapeutic

applications. The acquisition of safety through SbD will be reviewed by performing a literature study of the key principles of SbD and an analysis of the current state of SbD in terms of synthetic biology. Specific measures to manage safety through SbD strategies will be assessed. This knowledge will be applied to a case study about bacteriophage development to solve the problem of Desert Locust swarms. The aim is to discuss and set forth SbD strategies to manage the concerning issues and to direct the research towards a safe and ethical implementation.

2 Bacteriophages for Therapy

2.1. Characteristics of Bacteriophages

Bacteriophages are viruses that can kill bacteria and were first discovered at the beginning of the 20th century by microbiologists Frederick Twort (1915) and Félix d'Herelle (1917) (Fruciano & Bourne, 2007). They are "nature's most prevalent living entities" and can be found in the harshest of environments (Kwiatek, Parasion & Nakonieczna, 2019, p. 985).

The bacteriophage structure is made up of a protein capsid that contains the phage's genetic material. They are able to target specific bacteria through binding proteins on the surface of the phages. As in other viruses, bacteriophages do not contain a complete replisome and must infect a host to replicate. Phages can be classified into two categories, virulent phages and temperate phages. Figure 1 illustrates the two viral replication mechanisms. Virulent phages use the lytic cycle as replication mechanism. They bind and inject their genetic material into the bacterial host cell, break down the host genome and shut down the bacterial defense mechanisms. Subsequently, they hijack the bacterial replication mechanisms to synthesize bacteriophage progeny (Ofir & Sorek, 2018). The phages are assembled inside the bacterial cell which leads to lysis and death of the cell, and release of the newly synthesized particles. A second category are temperate phages, which use both the lysogenic and the lytic replication cycles. After injection, the phages' genetic material is integrated into the bacterial host genome. This bacteriophage genome, now called a prophage, is in a dormant state and is replicated along with the bacterial genome when the bacteria multiply. Induction of the prophage by physical or chemical stressors such as UV light or a change in pH levels leads the phage genome to enter the lytic cycle where it again produces new phages and kills the host bacteria (Kwiatek, Parasion & Nakonieczna, 2019; Choi, Kotay & Goel, 2010).

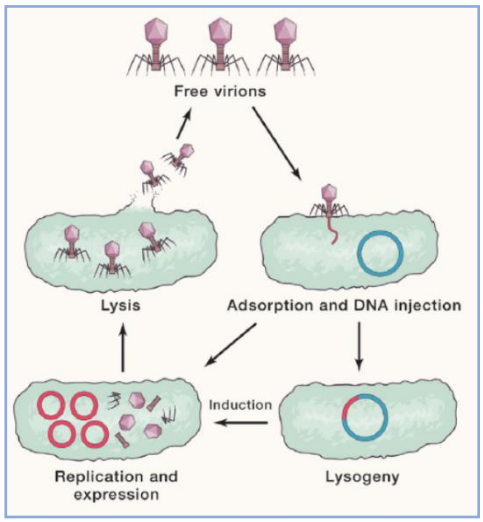

Figure 1. Phage life cycle. (Ofir & Sorek, 2018)

2.2. Phages as Therapeutic Agents

One of the most valued aspects of bacteriophages is their antibacterial capacity. They are deemed essential as natural therapeutic agents for phage therapy, an alternative method to treat pathogenic bacterial infections (Haq et al, 2012). As the threat of antibiotic-resistant bacteria increases (WHO, 2018), the interest of research into bacteriophages for phage therapy has risen. Despite very successful results in the former Soviet Union, the sudden discovery of antibiotics replaced most of the research into phage therapeutics in the West (Nikolich & Filippov, 2020). Since then, the wide-scale research into phage therapy has made a comeback in Western medical science (Fruciano & Bourne, 2007). While bacteriophages now know a wide range of applications, phage therapy marks the first introduction of research into phages as a method to treat and prevent human diseases (Verbeken, 2015).

The choice of bacteriophages as therapeutic agents proceeds from the fact that bacteriophages are considered inherently effective and safe organisms. In a phage, the protein capsid guards the genetic material and protects it from outside threats for an efficient gene transfer into the host bacteria. Bacteriophages can carry a large amount of genetic material. This feature allows phages to have a high cloning capacity and enables them to do multigenic transfer (Hosseinidoust, 2017; Paillard, 1998). Furthermore, phages are regarded as safe due to the fact that bacteriophages are bacteria-specific and each phage infects a very limited range of bacteria. This specificity is a result of the interaction between bacterial receptors and viral receptors, and thus the phages keep the host and other non-target bacteria intact (Lopez Cuevas, Medrano Felix, Castro del Campo & Chaidez, 2019; Kwiatek, Parasion & Nakonieczna, 2019; Keen, 2012). This implies that phages are safe for human use as they do not possess the mechanisms to infect eukaryotic cells (Pranjol & Hajitou, 2015; Paillard, 1998). In the West, the development and application of phages as pharmaceutical and food products has been accepted by institutions such as the United States Food and Drug Administration (FDA) or the European Medicines Agency (Hosseinidoust, 2017; Pranjol & Hajitou, 2015; Lopez Cuevas, Medrano Felix, Castro del Campo & Chaidez, 2019). For example, in 2013 the European Commission launched Phagoburn, the first large multicentre clinical trial into the potential of bacteriophages for human infections (PhagoBurn, n.d.). This study reinforced the notion of safety for bacteriophages but mostly emphasized that further applied research is required into bacteriophage applications (Jault et al., 2019). The recurring use of phages in e.g. phage therapy, the food we consume and its ubiquitous presence confirms that bacteriophages are safe for use in the application of phage vectors (Hosseinidoust, 2017).

2.3. Gene Therapy

A remarkable characteristic of phages is their ability to transfer foreign DNA into a host genome through a process called transduction (NLM, n.d.-a). Phages are capable of leaving parts of phage DNA in the bacterial genome or introducing parts of the bacterial genome into the newly synthesized phage genomes. Under natural circumstances, this can result in to e.g. the transfer of antibiotic resistance genes between bacteria (Steward, 2018).

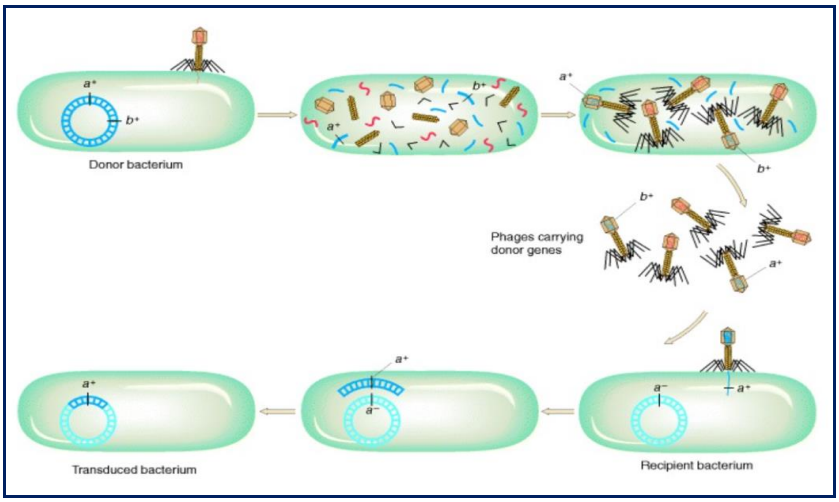

Figure 2. The mechanism of generalized transduction. (Griffiths et al., 2000)

In generalized transduction, a piece of bacterial DNA is incorporated into the bacteriophage genome and thus transferred to a new infected bacteria (Figure 2). Here, the phage DNA with the fragment of bacterial DNA is released and can undergo homologous recombination with the new host DNA (Griffiths et al., 2000). This way horizontal gene transfer is achieved. Both virulent and temperate

phages perform generalized transduction (Paul & Jiang, 2001). In the lab, transduction can be exploited to genetically modify microorganisms such as is done in gene therapy. Gene therapy uses the phages' intrinsic mechanisms to transfer the desired gene that is engineered into the phages' genome into a target bacteria. The mechanism of transferring genetic material with phages as gene vectors was first used to genetically modify bacteria in the 1950s (Steward, 2018). In the following decade, Joshua Lederberg developed the idea of modifying the nucleotide sequences in human chromosomes and conceived the concept of gene therapy (Pranjol & Hajitou 2015**;** Gillet et al., 2009). Gene therapy was developed as a way to genetically modify the genome of a host cell for it to manifest desirable traits. The modification of cell expression can be accomplished by knocking down the expression of a molecule, over-expressing a specific protein, inducing cell death, or replacing a defective or mutant gene that causes disease (Pranjol & Hajitou 2015; NLM, 2020). Viral and non-viral vectors are used in gene therapy, with eukaryotic viruses e.g. adenoviruses, adeno-associated viruses, retroviruses, and lentiviruses being the most commonly used (Ruan, Guse & Lee, 2013). Bacteriophages are regarded as a safer alternative to eukaryotic viruses for applications as vectors in gene therapy (Pranjol & Hajitou 2015).

2.4. Microbiome Therapy

The microbiome of an organism is defined as "the entire habitat, including the microorganisms (bacteria, archaea, lower and higher eukaryotes, and viruses), their genomes, and the surrounding environmental conditions" (Marchesi & Ravel, 2015, p. 31). The microbiome is indispensable for the performance of the host organism, e.g. humans. Phages are found to be prolific in the human gut microbiome and are responsible for healthy or pathogenic conditions (Ofir & Sorek, 2018). By eliminating pathogens or enhancing beneficial bacteria in the microbiome, the health of the host organism can be improved (Grady, Petrof & Claud, 2016). Thus, control of one's microbiome has effects on the entire organism. The alteration of the microbiome is called microbiome therapy and can be achieved through gene therapy. Bacteriophages are excellent vehicles to introduce foreign DNA and modify microorganisms from the inside-out. The phages can cause targeted knockdown of specific bacteria (Hsu et al., 2019) or induce the production of metabolites by the bacteria.

In this report, principles of gene therapy and microbiome therapy are reviewed to synthetically introduce new genes into a target bacteria's genome in a non-clinical application of bacteriophage therapies.

3 Designing for Safety

3.1. Risk Governance

Synthetic biology is defined as “the application of science, technology, and engineering to facilitate and accelerate the design, manufacture and/or modification of genetic materials in living organisms” by the European Commission Scientific Committees (Breitling, Takano & Gardner, 2015). Advancements in i.e. DNA and RNA synthesis, bio-engineering design and modeling techniques, enhanced tools for biological assembly and the development of standardized biological parts have magnified the possibilities of synthetic biology (Shapira, Kwon & Youtie, 2017). It has created a way to control complex facets of living systems such as inheritance, genetics, and evolution and to produce customized organisms (Saukshmya & Chugh, 2009).

Since the emergence of synthetic biology numerous policies and guidelines have been set up in an effort to ensure safe research practices. The establishment of these guidelines has lifted some of the constraints that hindered the development of synthetic biology. The topic of safety for synthetic biology has remained a dynamic debate over the course of decades. The Asilomar Conference in 1975 marks the first attempt to regulate biotechnological innovations and address the concerns regarding their potential risks (Berg et al., 1975). Other important protocols have since been set up such as the Biosafety in Microbiological and Biomedical Laboratories in 1984 (CDC, 2018), the Cartagena Protocol on Biosafety in 2000 (CBD, 2020), or more recently in 2019 the NIH Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules (NIH, n.d.-b). The precautionary principle, which is highly embedded in the Cartagena Protocol on Biosafety, is an approach to protect the environment and human health from plausible hazards that may arise from novel genetically modified organisms even when scientific certainty on the risks is insufficient (Juma, 2014). These protocols aim to preserve biological diversity and human health from the potential risks posed by synthetic biology. The current state of biosafety practices concerning synthetic biology differs between countries. The EU implements risk assessment through the precautionary principle and the perception of the high risks of synthetic biology, while in the US it is focused on risk minimization and cost-benefit analysis (US DoD of OTI, 2015). Therefore, policies may vary between jurisdiction (e.g. the Biosafety, Biosecurity, and Emerging Biotechnology Policies in the United States (NIH, n.d.-a) or the three Scientific Opinions on synthetic biology of the Scientific Committees of the European Union in the EU (Epstein & Vermeire, 2016)), but their overall objective remains the same. These policies are crucial to help guide the development of synthetic biology applications towards safe and responsible deployment.

3.2. Regulations on Bacteriophage Therapeutics

As for the development of bacteriophages, the absence of bacteriophage-specific regulatory measures have long formed the bottleneck of its research. This pattern can be traced back to the beginning of bacteriophage developments and still continues to be relevant in today’s society (Nobrega, Costa, Kluskens & Azeredo, 2015; Verbeken, 2015; Keen, 2012). As no bacteriophage-specific regulation exists (RIVM, David et al., 2018) bacteriophages fall under the regulations for conventional medicinal products.

Worldwide, the current legislation for human medicinal products hampers the development of bacteriophages. Two main issues are identified when looking at these regulatory pathways for bacteriophage development for therapeutic purposes. Firstly, these regulations are set up for the production of standardized large-scale pharmaceuticals which are extremely costly and are not reconcilable for bacteriophages as these are patient- and situation-specific. A second hindrance is the issue of intellectual property. As phages are living and evolving organisms they are hard to define under patent laws and have limited intellectual property protection. Both these issues obstruct the economic viability of bacteriophage therapies (Verbeken, 2015). Regardless, a few patents have been granted and clinical trials have started in Europe and the U.S., which have so far not led to any licensing (Fauconnier, 2019).

In the EU, the European Commission has shown a resistant stance to regulatory change for bacteriophage therapy and has rejected the appeal of new legislature as they argue that the existing regulation would be suitable for bacteriophage therapy. It is therefore the Member States' responsibility to draw up policies that conform to bacteriophage therapeutics (Fauconnier, 2019; Dalli & European Commission, 2011). A possible solution is the use of a biological master file (BMF) for the regulation of therapeutic phage products. A BMF is defined as "the submission for regulatory approval of a stand-alone package covering only part of a dossier, independent of the eventual approval of the full marketing authorization application for a medicinal product" (Fauconnier, 2017, p. 199). Therefore, the production and safety of individual phages or a group of homologous phages could be approved through a BMF, and personalized phage products from this BMF would not need regulative approval as it regards an individual medicine (Fauconnier, 2017; RIVM, David et al., 2018). In other sectors such as agriculture and food production, regulations involving bacteriophages do exist and have already been applied (RIVM, David et al., 2018).

3.3. Safe-by-Design for Biosafety

Multiple approaches have been developed to assess and minimize risks e.g., adaptive risk management, technology assessment, or SbD. The latter originated from the research area of chemical engineering where inherent safety principles are strongly embedded. The concept of inherently safer designs was first applied in the chemical, oil and nuclear industries but has meanwhile found applications in a vast range of fields including synthetic biology (Kletz, 2003). Inherent safety entails the elimination or diminution of hazards instead of developing ways to manage the hazards. This implies that the application is fundamentally safer of its nature and not because of features that have been added to it. Through inherently safer designs, safety is adopted as an intrinsic property of the innovation. Nonetheless, it is important to keep in mind that inherently safer applications cannot remove all possible hazards (Kletz, 1996). Associated constraints that limit safety acquisition through SbD will be discussed later in the report.

The growth of SbD has been enhanced by the rapid emergence of innovative technologies in synthetic biology. In this field, SbD is an indispensable tool to eliminate or reduce potential future hazards of novel technologies. The concept of SbD encompasses the development of technologies with safety as the main value by identifying and integrating risk management strategies in design choices, from the production to the final employment of the technology (Robaey, 2018; NanoReg2, n.d.). Diverse SbD approaches have been explored to produce inherently safer applications, an example of such a SbD measure is synthetic auxotrophy for biocontainment (Kato, 2015). In recent years, the implementation of SbD has been further promoted by official institutions, e.g. the European Union through the PROSAFE Project and the NanoReg2 Project, that emphasize its

importance and salience for current biotechnological innovations (NanoReg2, n.d. ; CORDIS, 2019). The aim of this report is to set forth the scope of opportunities and challenges created by SbD strategies for synthetic biology applications.

3.4. Identifying Risks for Synthetic Biology

As stated by Robaey, “Safe-by-Design requires to identify potential risks during the design phase, identify a strategy to deal with the risk, develop measures, and formulate design options for safety” (2018, p. 37). The first step in developing a safe technology is anticipating the possible risks it poses to human health and the environment (van de Poel & Robaey, 2017; NIH, 2019). Risks are defined as the hazard multiplied with the probability of that hazard happening (Groot Kormelink, 2019). A risk entails a known negative consequence, which is quantifiable. By identifying scenarios created by the new technology that could lead to negative outcomes, these risks can be addressed. The harmful effects can originate from the applied modifications and lead to organisms that “affect non-target organisms, cause unintended effects on target organisms, become persistent or invasive or develop a fitness advantage in ecosystems with limited or no management, transfer genes to other organisms/populations, and become genotypically or phenotypically unstable” (CBD, 2016, p. 29-30).

For biotechnological applications the spectrum of risks is broader than in traditional risk assessment, which includes risks such as virulence, pathogenicity, environmental stability or toxicity (NIH, 2019). As we deal with self-replicating and living systems, these processes have far more intricate effects such as reversibility, manageability, and uncertainty (Cummings & Kuzma, 2017). Uncertainties are risks that are not yet identified and are thus more difficult to estimate. Synthetic biology concerns the creation of new rewired organisms and thus the consequences of such technologies are not yet fully understood and remain unpredictable. Even when all components and functions of a synthetic organism are known, the microorganism could have unforeseen features (Zhang, Marris & Rose, 2011). Uncertainty is thus a paramount aspect of risk assessment for synthetic biology applications. Adequate methods to deal with uncertainty will be discussed in more detail later in the report.

The assessment of risks in synthetic biology should encompass the engineered organism and all processes involved such as the employment of the application, its impact on native ecosystems and the eventual discarding of the used organism. Two key concepts in risk assessment for genetically modified organisms are comparative analysis and a case-by-case approach (SYNENERGENE, 2017). Comparative analysis entails risk assessment through the comparison to similar organisms with known characteristics and the identification of differences caused by applied modifications (SYNENERGENE, 2017). Acquiring safety in synthetic biology does not have a ‘one-size-fits-all’ solution and separate customizable problem-solving methods should be assessed for each application and its context (Kuzma & Tanji, 2010). It is therefore important to perform individual risk assessments on a case-by-case basis as the specific context and therefore risks vary for each application (CBD, 2016; Robaey, 2018). Knowledge of the likelihood and consequences of the reviewed applications’ risks are essential to identify the right measures and design options for safety.

As mentioned by Bhutkar, the risks associated with the intended or unintended release of synthetically modified microorganisms can roughly be classified into three categories (Bhutkar, 2005; De Vriend, 2006; Epstein & Vermeire, 2016; CBD, 2012; CBD, 2016; iGEM Wageningen, n.d.):

- Risk of negative environmental impact: The microorganisms could interact with environmental substances and develop negative side-effects. This includes adverse impacts on non-target species and ecosystems and unintended effects on target organisms such as virulence, toxicity or allergenicity. The aspect of transmissibility, which is the ability to infect another organism, is also an important factor to take into account with these hazards. The host range and specificity of the organism are thus of importance for the safety of the application.
- Risk of natural genome pool contamination: Any genetic exchange between a synthetic biological entity and a naturally-occurring biological entity would result in natural genome pool contamination. This includes the transfer of genetic material through vertical gene flow or horizontal gene transfer. This is moreover dangerous in the case of the transfer of antibiotic resistance genes or pathogenic genes.
- Run-off risk: Organisms could compete with the natural ecosystem, become persistent or invasive and disrupt the native biodiversity. Therefore, the synthesized microorganisms should have a controlled lifespan outside the lab.

After assessing the impact of an application and determining all the potential risks, the initial framework is established. These risks need to be translated into concrete strategies and measures for implementation in design choices. Two procedures that are helpful to formulate specific measures for SbD are iteration and experimentation. Through experimentation one can assimilate and formulate hypotheses about the technology and how to mitigate its risks. The use of these methods reveals the risks and corresponding design options early on in the design process (Robaey, 2018).

Several SbD approaches have been developed to address these biosafety issues. As stated earlier, the proposed solutions must be specific for each application**.** Existing SbD strategies are e.g. building organisms that are less competitive than the native species by changing their metabolic pathways or designing organisms that have an alternative biochemical structure to avoid gene flow to naturally occurring species (Epstein & Vermeire, 2016). Later in this report, viral risk mitigation for gene and microbiome therapy through SbD will be reviewed.

4 iGEM Case Study

4.1. Project Setup

The iGEM team at the TU Delft wants to exploit the valuable bacteriophage properties to design a technology to mitigate the problem of Desert Locust swarms. Currently used methods to kill the locusts such as pesticides are not as efficient and impact the ecosystem negatively. Therefore, two techniques were developed to specifically target the locusts and keep the environment intact. Their goal is to use principles from gene therapy and microbiome therapy to modify the bacteria inside the locusts' gut in such a way as to lessen the formation of swarms (Figure 3).

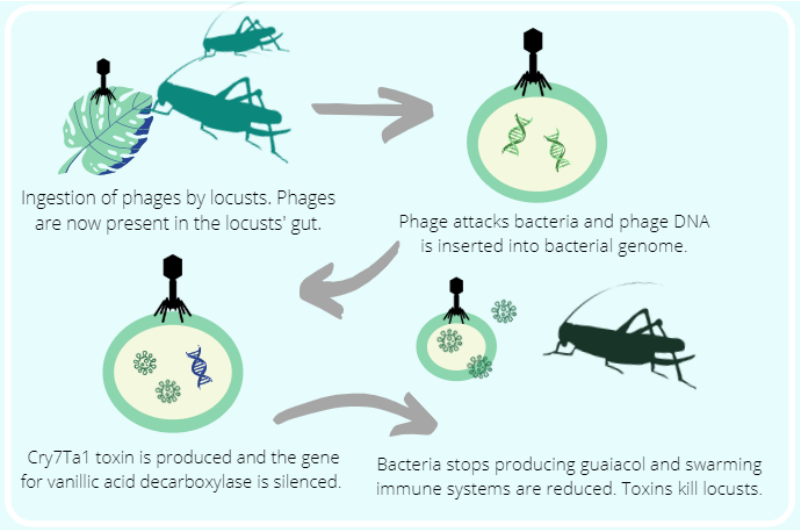

Figure 3. iGEM project design to reduce swarming of Desert Locusts.

A first proposal is to diminish the aggregation of locusts by infecting the locusts' gut bacteria with phages and inhibiting or degrading guaiacol production. Guaiacol is produced inside the gut of locusts and together with phenol they constitute two key components of a pheromone that stimulates the locusts' gregarious behavior (Dillon, Vennard & Charnley, 2000). Guaiacol is synthesized by converting vanillic acid through non-oxidative decarboxylation (Crawford & Perkins Olson, 1978). Therefore, by silencing the gene for vanillic acid decarboxylase, this compound is no longer produced and locusts are less eager to form swarms. The methods used to silence the gene are CRISPR interference system (CRISPRi) and antisense RNA (iGEM TU Delft, 2020). CRISPRi requires a customizable single guide RNA (sgRNA) that is complementary to the target DNA and a catalytically inactive Cas9 protein. Together they form a Cas9-sgRNA complex that cleaves to the target gene, thereby creating a steric block that hinders the transcription of the gene (Larson et al., 2013). Antisense RNA is used to silence gene expression by using a complementary ssRNA that binds to the vanillic acid decarboxylase mRNA, obstructing the translation of the gene (Xu, Zhang & Zhang, 2018). An alternative to this approach is breaking down guaiacol and phenol with enzymes. The enzymes that can be used to simultaneously degrade both compounds are 4-hydroxybenzoate decarboxylase, cytochrome P450 monooxygenase, peroxidase and laccase. This leads to lower pheromone levels and therefore a decrease of locust swarm formation.

A second proposal is inducing the excretion of a toxic metabolite Cry7Ta1 in the locusts' gut to kill the locusts. The Cry7Ta1 protein is an insecticidal crystal protein and is regarded as an

‘environmentally friendly insecticide’ as it does minimal damage to the natural ecosystem (Jing et al., 2018). The gene for production and secretion of Cry7Ta1 is again introduced into the bacterial genome through a viral vector.

4.2. Bacteriophage Modification

In this chapter, the procedure for acquiring the phages with desired characteristics will be reviewed. The purpose of this study is to set up a model to demonstrate the use of gene and microbiome therapies to mitigate the issue of Desert Locust swarms. The developed model system can be used for future applications and can be adapted to each separate species of locusts. The recombinant bacteriophages are obtained by re-engineering and modifying existing phages. To acquire highly specific and efficient vectors, potential phages are selected by sequencing the viriome and microbiome of the locusts and determining the most competent phages. This enables the alteration of the bacteriophages for each new situation. A cocktail of multiple phages must be used in the application since the microbiome of locusts varies in each type of locusts and even between locust of the same species.

As the choice of phages is specific for each situation, this study will be performed by using the well-studied bacteria *Escherichia coli* and the corresponding phage that is known to infect it, the lytic bacteriophage T7 (Ofir & Sorek, 2018). As one of the first phages to be completely sequenced in 1983, the characterization of the T7 genome and the function of its genes has made significant progress (Kutter & Guttman, 2001). Furthermore, the T7 phage is often used for genome engineering because of its phage-encoded RNA and DNA polymerases which allow rapid transcription of the phage genome (Chan, Kosuri & Endy, 2005) and the high-yield expression systems capable of producing half of the cellular protein as product (Kutter & Guttman, 2001). This makes the T7 phage an excellent candidate as a prototype phage for the iGEM study. The T7 genome is classified into three categories: class I, class II and class III genes. Class I genes or the ‘early’ region is expressed in the early stages upon infection and contains e.g. gene 1, which encodes for the T7 RNA polymerase. Class II or ‘middle’ genes and class III or ‘late’ genes are expressed later upon infection (ViralZone, n.d.).

After selection, the phages are re-engineered to perform optimally and to ensure their safety for application. The phages’ genome is synthesized with Bacteriophage Recombineering of Electroporated DNA (BRED). BRED is a simple and efficient method to manipulate and modify a bacteriophage genome. It uses electroporated phage DNA to reengineer the genome by introducing defined mutations in the bacteriophage DNA (Marinelli et al., 2008).

4.3. Safe-by-Design Measures

As the intended use of this design project is in the natural ecosystem, the biosafety of its hypothetical release into the environment needs to be ensured. In this section, it is reviewed how safety can be achieved through established Safe-by-Design measures. By introducing new safety attributes into the phages’ genome the known as well as uncertain risks can be addressed.

A first safety measure is the use of lytic phages instead of lysogenic phages to reduce the phages’ ability for horizontal gene transfer (HGT). Lysogenic bacteriophages are more prone to transduction of genes as the viral genome is integrated into the bacterial genome during the lysogenic cycle (Nobrega et al., 2015; Lopez Cuevas, Medrano Felix, Castro del Campo & Chaidez, 2019). The transduced genes spread further as the bacteria is not immediately killed and the genes are passed

on to daughter cells when multiplying. By using strictly lytic phages, the risk of contamination of the natural gene pool is mitigated. Through quorum sensing, a method is developed to ensure that if any lysogenic phages are selected, they will obligately enter the lytic replication cycle and not the lysogenic cycle. This method is further discussed later in this chapter.

To ensure that no pathogenic genes are transferred, the lytic bacteriophages should not carry any harmful genes such as virulence, antibiotic resistance or allergenicity. These genes are eliminated from the bacteriophage genome during the phage production. This reduces the risk of the phages introducing pathogenic genes in the ecosystem and attenuates the risk of them harming the environment.

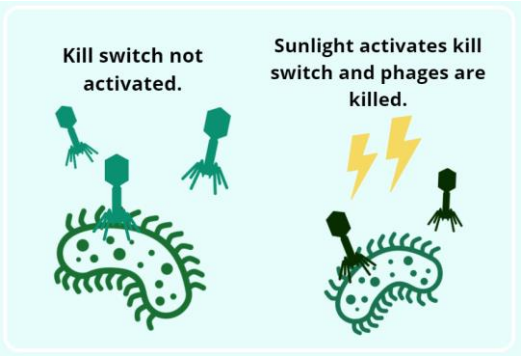

Figure 4. Illustration of a photosensitive kill switch.

A third built-in safety measure is the use of ‘kill switches’ (Figure 4). A much debated and prominent issue of genetically modified organisms is the run-off risk. As the complete functioning of a genome is a very intricate system to control and not every function is known, it is implausible to predict all the effects of the applied modifications. Newly added features could have unforeseen consequences and the organism could lead to an imbalance of the environment. It is therefore crucial to control the life span of the organism to ensure biosafety, this is attained through biocontainment. In this case study, the intended application is in the natural ecosystem. Therefore, biocontainment in this situation does not refer to the prevention of the unintended release of the organism, but rather to the containment of the organism outside the lab. Multiple approaches have been developed to achieve biocontainment such as auxotrophy (the requirement for a specific metabolite to be present for cellular function for the survival of the organism), recoding and kill switches (iGEM Wageningen, n.d.). Kill switches induce lethality of the organism through host–construct dependencies such as toxin-antitoxin pairs. An example is the use of a stimulus from the environment that represses the production of a toxin. Once the stimulus is removed, the toxin is no longer repressed and the toxin will kill the organism (iGEM Wageningen, n.d.). In this case study, the use of a photosensitive kill switch is explored. When an infected bacteria is exposed to daylight, the kill switch is activated and the phages’ functioning is neutralized. This prevents phages from infecting and spreading in bacteria outside the locusts, as those are exposed to daylight.

Another SbD measure is re-engineering the quorum sensing mechanism in the bacteriophages. Quorum sensing is “the ability to regulate gene expression according to population density” (Molin Høyland-Kroghsbo, Baadsgaard Mærkedahl & Lo Svenningsen, 2013, p. 1). This cell-to-cell signaling system is used by bacteria to adapt to high-density populations. Bacteria are known to produce autoinducers, which accumulate as the population of bacteria increases. When the autoinducers are present in abundance, the bacteria have the ability to control gene expression to

ensure a higher survival rate. Bacteriophages are able to perceive these signals through autoinducer receptors and adapt to the circumstances by regulating gene expression and by adopting lysis or lysogeny cycles (Silpe & Bassler, 2019).

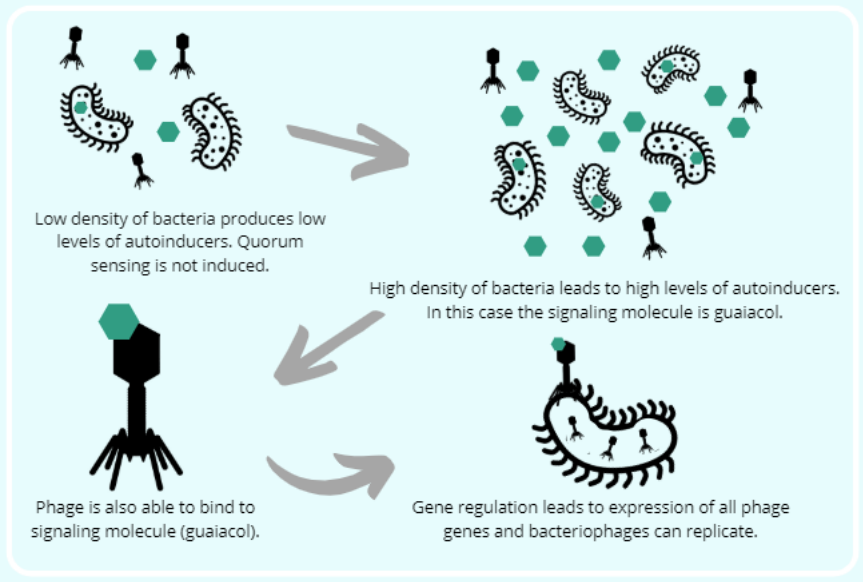

Figure 5. Illustration of QS mechanism developed by iGEM with guaiacol as the signaling molecule to induce expression of all bacteriophage genes.

This system can be hijacked and exploited to increase the safety of the application. In this case study, the use of quorum sensing is hypothesized for two purposes. A first idea, as shown in Figure 5, is modifying the phages' quorum sensing mechanism to intercept guaiacol as a signal. If guaiacol is not present, only the early genes are expressed. Therefore, the phage is not able to replicate as the indispensable genes for replication are not transcribed. When guaiacol is available it leads to regulation of the gene expression so that all the genes are expressed.

A second idea is to eliminate the quorum sensing system in the phages so that all phages enter the lytic replication cycle instead of the lysogenic cycle and kill the host bacteria. This would ensure that no synthetic DNA is preserved in the environment through lysogenic phages. Furthermore, as phages dependent on a host bacteria for reproduction, by inducing the lytic cycle most of the potential bacterial hosts are killed and the phages' reproduction is reduced. This would result in a decrease in recombinant phages in the environment. These measures are excellent candidates to mitigate the risks that the synthetic phages pose to the ecosystem, as the amount of synthetic bacteriophages is diminished or completely eliminated.

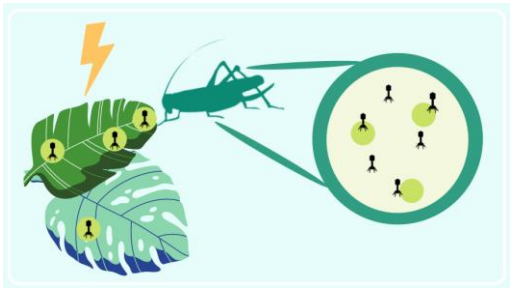

Figure 6. Illustration of encapsulation method designed by iGEM.

To make certain that the phages are only present inside the locust gut and are not introduced into the surrounding environment, a method of encapsulation can be applied as shown in Figure 6. Encapsulation is a procedure whereby the material, in this case the bacteriophages, is guarded in a capsule to minimize losses or lesions of the material and impede its release (Lopez Cuevas, Medrano Felix, Castro del Campo & Chaidez, 2019). Under the correct conditions the phages can be released again. The iGEM team is exploring the use of encapsulation with cellulose. The application of the phages takes place in a desert environment where it is subject to i.a. high temperatures and heavy UV-radiation. Through encapsulation the phages are protected from these stressors and are safely delivered to the locusts. The locusts' gut contains the cellulases needed to break down cellulose. This is another way to achieve biocontainment and ensure the phages pose no risks to the ecosystem. However, this method is not foolproof as other animals are also capable of breaking down cellulose, and should be combined with other methods to ensure a higher level of safety.

In this section the Safe-by-Design measures adopted by the iGEM team were discussed. The following chapter sets forth other plausible Safe-by-Design measures to mitigate the risks and address uncertainties created by the implementation of this technology.

5 Alternative Safe-by-Design Approaches

The described SbD approaches thus far were developed by the iGEM team as part of their design project. In this chapter, we go beyond those proposals and analyze alternative plausible SbD measures that are available to achieve biosafety in the application of bacteriophages as agents against Desert Locusts. Furthermore, the concept of process-applied SbD is introduced and its application for the reviewed case study is shortly discussed.

5.1. Procedural Safety

Until now, the reviewed SbD measures can be labeled as 'upstream' approaches or 'product-applied' SbD. This terminology entails the application of safety strategies during the engineering and manufacturing phases of the product. It focusses on the technical aspects of SbD. An extension to this is 'downstream' or 'process-applied' SbD which concerns SbD on the policy level of the implementation of a technology. According to Bouchaut and Asveld, "the biggest difference between product- and process-applied SbD lies in the decision-making process on what is an acceptable level of risk" (2020, p. 4). In upstream SbD the risks are considered to be more straightforward as they concern the technical aspects of a technology such as the development process and the materials used. In downstream SbD the risks are more difficult to identify as it concerns the applicability and ramifications of the technology and therefore deals with higher levels of uncertainty (Bouchaut & Asveld, 2020). Risks might not be perceivable in the design phase but come into existence during implementation. As mentioned, in biotechnological applications unknown risks and uncertainty are significant and complex issues to take into consideration.

The iGEM competition entails a design project that could hypothetically be implemented in a real situation, but remains laboratory-confined research at the experimental level and is not deployed outside the lab (iGEM, 2019). It is however very important to think about the risks and consequences of these technologies should the project become a product. The implications of this application in the field go beyond the laboratory design process. Implementation of the engineered phages could e.g. lead to the colossal death of Desert locusts or accumulation and release of the produced toxic metabolite in the ecosystem, which creates the need for management of the remains and residue in affected areas. This brings new issues and uncertainties to the table that can be solved through process-applied SbD.

First and foremost the safety of the developed application and all concerning parts should be thoroughly evaluated, including the concerning organism, any modifications and the applied safety measures. Extensive research and risk assessment should be performed into the possible consequences of the technology in the natural ecosystem. As mentioned earlier, this involves experimentation and iteration. The acquired knowledge should be analyzed in iterative stakeholder communication where multiple relevant actors contribute knowledge, experience and different points of view to gather an all-round perspective of the associated risks and safety of the application. An examples of a small-scale stakeholder discussion is the use of a Group Decision Room, such as was done in The Food Warden project (Robaey, Spruit & van de Poel, 2017). Relevant stakeholders are e.g. scientists in the field of research, policymakers, industry professionals, experts from academia or professionals in consultancy (Bouchaut & Asveld, 2020). In this specific case that would involve scientists and professionals from academia with expertise on bacteriophages and

locust swarming, policymakers in the affected areas such as Kenya or Ethiopia (FAO, 2020 -b), professionals with experience on the application on insecticidal crystal proteins or local agriculturers and citizens. By engaging a wide range of stakeholders and the general public into the dialogue, the notion of safety of synthetic biology can be reinforced. This can help facilitate and positively influence resolution on the application of synthetic biology. Means of ensuring further engagement are, for example as was done in the SYNENERGENE project, by organizing public events, developing learning tools, establishing communication through interfaces of art and science, involving the media, et cetera (CORDIS, 2017). Decisions should be made on the level of safety of the application, on trade-offs between the risks and benefits for society and on responsibility allocation. Through collaborative stakeholder communication areas of concern can arise and new risk mitigation strategies can be devised and translated into concrete design choices.

To be able to apply this technology in a real-life scenario, the modified organism should comply with local, national and international legislation. In this case that involves policies regarding synthetic biology in e.g. Eastern Africa or Southwest Asia, where locusts swarms are becoming a problem. One of the affected countries is for example Kenya, where a progressive stance is adopted towards the use of genetically modified organisms (Agaba, 2019). A supportive view on the use of genetically modified organisms is promising for a future development of regulation that allows the use of bacteriophages for control of the locust swarms. Furthermore, there is the issue of the locusts crossing geographical and political borders where different regulations apply. As the locust swarms are known to be highly migratory organisms that cross countries and continents, the implementation of this technology could have continent-wide consequences. Therefore, these initiatives should go beyond one specific country and regulation cannot be limited by geopolitical boundries. These are all aspects that need to be taken into account in stakeholder discussions when performing downstream SbD. In this report, we will focus on the technical aspects of SbD and limit our analysis of safety to product-applied SbD measures.

5.2. Additional Safe-by-Design Measures

The main way to decrease uncertain risks and increase safety through product-applied SbD is by integrating multiple SbD approaches in one application, such as is done in the iGEM project. The combination of SbD measures helps anticipate and diminish potential risks and uncertainties. In this section, additional SbD measures are outlined and their compatibility with the recombinant bacteriophages is reviewed.

Besides kill switches, other biocontainment SbD measures are available to mitigate the run-off risk and make sure the organism does not take over the environment, such as e.g. auxotrophy. Auxotrophy is the requirement for a specific metabolite to be present for the survival of the organism. The organism is not able to produce this metabolite and the molecule has to be provided by the environment. Through auxotrophy biocontainment is achieved as the organism is not able to survive and replicate outside a set ecosystem where the metabolite is present (iGEM Wageningen, n.d.). A hypothetical application of auxotrophy for biocontainment of the phages could be the requirement for a specific metabolite that can only be found in the locust's gut to be present for the replication of the bacteriophages. This would limit the bacteriophage replication to only cells inside the locust gut and therefore phages could not spread outside the locust's gut. Compared to the photosensitive kill switch, which limits the phages' replication to locations where no light is available, this method would ensure that phages are further limited to the locusts' gut. The actual development of this safety measure for the iGEM project requires further research.

Another SbD approach that knows various applications is the use of orthogonal systems. Orthogonal biological systems create distance between synthetic and natural structures in an effort to hinder any interaction between the two (Kuldell et al., 2015). It “guarantees that modifying one component of a system does not propagate side effects to other components of the system” (Schmidt, 2010). Through orthogonal biological systems, the genetic setup of a synthetic organism is rearranged in such a way that natural systems are not able to understand or correctly reproduce it. Two examples of orthogonal methods used to ensure biosafety are recoding and xenobiology.

In recoding, the TAG stop codon is replaced with a TAA codon so that the TAG codon can be reassigned to a desired synthetic amino acid. The synthetic amino acid can be inserted into essential genes, thereby making the expression of these enzymes dependent on this synthetic molecule (Rovner et al., 2015; Kuldell et al., 2015). As the synthetic genetic material cannot be correctly translated by wild-type organisms, these genes are not spread to natural organisms and HGT is reduced (Wright, Stan & Ellis, 2013; Kuldell et al., 2015). Furthermore, auxotrophy is created as the synthetic amino acid is not available in nature and the synthetic organism is not able to produce the synthetic amino acid. Therefore, the organism cannot survive in the natural ecosystem and biocontainment is achieved (iGEM Wageningen, n.d.).

Xenobiology entails the use of a different engineered type of genetic material called xeno nucleic acids (XNA). The XNA is not recognized by natural organisms and synthetic XNA organisms cannot interpret DNA or RNA, thus creating a ‘genetic firewall’ (Wright, Stan & Ellis, 2013). This means that XNA organisms can interact with natural organisms on an ecological level but not on a genetic level (Schmidt, 2010). This again mitigates the risk of HGT and contamination of the natural genome pool. Furthermore, XNA organisms are not capable of producing their own nucleotides and therefore these organisms have an auxotrophic dependency on the xeno nucleotides. As these nucleotides are not present in the natural ecosystem either, the synthetic organisms cannot reproduce in nature and a safety tool is created to achieve biocontainment of XNA organisms. Xenobiology is regarded as “the safest biocontainment mechanism possible through the incorporation of both trophic and semantic containment” (Wright, Stan & Ellis, 2013).

With the acquired knowledge it can be concluded that orthogonal systems are not compatible with the engineered bacteriophage application. The phage genome needs to be translated by the host bacteria in order to express the desired genes. Synthetic XNA or recoded DNA are not read correctly by the bacterial replication mechanisms, thereby undermining the functionality of the application. The use of orthogonal systems is thus not suitable for this project, but can be very valuable for other SbD applications.

A SbD measure that was discovered and has been further developed to control the engineered CRISPR mechanism in recombinant phages are anti-CRISPR proteins (Acr). Anti-CRISPRs are phage-derived proteins that block the CRISPR-Cas defense systems in bacteria. More than 50 Acr proteins have been discovered, which can be used as switches to control CRISPR activity in gene-editing applications (Dolgin, 2020). Acr proteins serve two functions for genetic engineering applications, they are capable of disrupting the CRISPR activity in various manners as well as limiting the off-target effects of the CRISPR mechanism. Different Acr proteins affect different parts of the CRISPR-Cas system (Ofir & Sorek, 2018). One of the ways that Acr proteins can hamper CRISPR activity is by inhibiting target-DNA binding of the CRISPR complexes. This could be a relevant mechanism to control the gene-editing activity of the engineered phages in the locust’s gut and ensure a higher level of biosafety. Also, research has been done into limiting the activity of anti-CRISPRs to specific cells or tissues by using microRNAs (Dolgin, 2020). This could serve the same

purpose as the auxotrophy measure by limiting the activity of the phages to the locust's gut. In summary, Acr proteins could be applied in this project to improve the CRISPR mechanism to be highly efficient, to control its gene-editing activity and to confine its activity to the locust's gut. The future use of Acr proteins is promising, but extensive research is needed before this method could be applied to the recombinant bacteriophages.

Another plausible SbD measure to ensure biosafety is the use of the self-exhausting daisy-chain gene drives (Noble et al., 2019). However, the use of gene drives requires a special exception from the iGEM Safety Committee and could have implications on the eligibility of the iGEM project (iGEM, 2019). Therefore, this application is only briefly mentioned and not discussed as a potential SbD measure for the iGEM case study.

Multiple interesting SbD measures have been identified that serve a wide range of purposes. The use of orthogonal systems through recoding and xenobiology and the use of daisy-chain gene drives are not deemed applicable for the iGEM project. They could however be significant for other synthetic biology applications to limit synthetic gene transfer into the environment and ensure biocontainment. Two additional SbD measures are found, the use of auxotrophy and the use of anti-CRISPR proteins. Both these measures seem achievable and could possibly be applied in the iGEM project. In the following chapter, the uncertainties and challenges will be discussed that complicate decision-making for different SbD measures.

6 Challenges for Safe-by-Design

SbD is a crucial strategy for the achievement of safety in synthetic biology applications. It is an effective approach to anticipate and mitigate risks, but SbD is not flawless and 100% safety cannot be achieved. In this chapter, the limitations associated with SbD are analyzed and the applicability and constraints of SbD as a biosafety tool for the development of the genetically engineered bacteriophages are evaluated. Furthermore, methods to manage these constraints are reviewed and it is discussed how SbD could also be helpful to manage some of these challenges.

The main constraint for SbD for synthetic biology applications is the lack of knowledge on the functioning and associated risks of the engineered system in the natural environment. The aspects of uncertainty and complexity are an inseparable part of risk assessment in synthetic biology systems. As the effects and inner workings of the biological systems that make up life are very intricate and largely undetermined, there will always be a level of uncertainty associated with a novel application. Risk identification for biotechnological applications is increasingly difficult as technologies are rapidly evolving in a manner that enables the complete creation of synthetic organisms from scratch. The chemical structure of these innovations differs from their natural counterparts, which complicates classical risk assessment through methods such as comparative analysis (Zhang, Marris & Rose, 2011). The modification and addition of synthetic pathways decrease the predictability of an organism thereby creating more uncertainties (iGEM Wageningen, n.d.).

For this project, the increment in uncertainty is created by introducing foreign genes in the bacteriophage genome. By changing the genetic setup of the bacteriophages, it is more difficult to predict how the organism will behave in the natural ecosystem. It increases the distance between the natural and synthetic bacteriophages, thereby complicating comparative risk assessment with e.g. the original T7 phage or other bacteriophages.

Partly due to regulatory bottlenecks such as the discussed lack of bacteriophage-specific regulations or the application of the precautionary principle, research into biosafety measures for synthetic biology applications is mostly conducted in a laboratory-confined, experimental manner. Therefore, the available knowledge on crucial factors such as rate of HGT, effects of the application on native communities, the performance of SbD measures, and other uncertainties remains minimal and only depicts a simplistic view of the actual application. The absence of sufficient scientific data not only limits the technical side of safety acquisition but also hampers decision-making on the safety of the application. Without concrete, real-life trials, these aspects will remain unknown and the lack of knowledge and experience will continue to limit the progress of synthetic biology applications.

In this case study, the lack of knowledge, experience and available time of the project restricts the choice of compatible and attainable SbD strategies. The limiting factors are that the project is performed by students who are not experts in the field of bacteriophages and have limited knowledge and expertise on the modification of bacteriophages. Another constraint is that the project has a restricted time frame of less than a year, which limits the attainable amount of research that can be conducted on the safety measures and thereby reduces the final acquired safety. An advantage is that the knowledge on the T7 bacteriophage genotype and phenotype is substantial and with help from experts in the field accurate and efficient genetic safeguards can be

developed. However, the iGEM competition demands that this project remains at an experimental phase and applied research in the natural ecosystem will not be performed. This withholds the application from acquiring the needed levels of research and safety to be applied in a real-life situation.

The level of safety that is achieved is also constrained by technical aspects such as e.g. the capsid capacity of the phages. The amount of genetic material carried by the phages is dependent on the size and shape of the phage. There is thus an upper limit on the number of foreign genes that can be inserted while maintaining the function of the phage. The more genes that are engineered into the bacteriophage, the more unstable and ineffective the bacteriophage becomes. That restricts the choice of safety measures depending on the size of the corresponding gene sequence. For example, the reviewed photosensitive kill-switch is encoded by a large genetic sequence and therefore raises concerns on the feasibility of this SbD. This leads to making compromises and trade-offs between the functionality and the safety of the application.

Another issue regarding the implementation of the bacteriophage application is the determination of an effective dosage. Phages reproduce when there is a high density of host bacteria, especially as the phages are engineered to be strictly lytic. Therefore, determining the correct dosage depends on the concentration of hosts and not solely on the amount of administered phages. This aspect makes phage kinetics difficult to predict, and an equilibrium needs to be established between applying an effective dosage of phages without releasing an excessive amount of phages into the environment and jeopardizing the natural ecosystem (Meaden & Koskella, 2013). A method used by the iGEM team to analyze possible consequences and effects of the application is the use of computer simulations. These mathematical models depict the real-world performance of the application and can conceptualize and anticipate possible consequences and uncertainties. For example, it helps illustrate the replication and spread of the phages inside the locusts' gut or the spread of phages within a locust population. This tool is used to determine the correct dosage of bacteriophages needed for successful application in the environment (iGEM TU Delft, n.d. -b).

The chosen approach also raises concerns about the preservation of the natural ecosystem. Related uncertainties could be the infection and production of Cry7Ta1 or degradation of guaiacol and phenol in non-target organisms or the induction of inflammatory defense systems in non-target organisms, thereby killing or inducing pathogenicity in non-target organisms. These possible unknown risks, and the induced lethality on the Desert Locusts, could disrupt native communities and ecological cycles (Meaden & Koskella, 2013). As mentioned, possible methods to determine these uncertainties and risks are concrete field-trials in the ecosystem or iterative stakeholder involvement.

A concern that relates to all living entities is that organisms are prone to gene silencing, which could undermine the engineered biosafety features. It could result in the SbD measures being mutated out through evolution, especially since these characteristics limit the viability of the organism and hinder its survival (Lee, Chan, Slomovic & Collins, 2018). For example, the frequency of mutations inactivating a kill switch lies around 10^{-3} to 10^{-7} (Simon & Ellington, 2016). This would lead to uncontrolled organisms on the loose that could take over the ecosystem and disrupt the native biodiversity. The risks of failure or elimination of the engineered safety features are minimized by combining multiple SbD measures in one application.

As the level of attainable safety is limited by the amount of knowledge and experience, developing an absolutely safe application is not feasible. Therefore, the aim is to attenuate the risks and uncertainties and minimize its negative effects. This also brings new questions to the discourse on biosafety such as ‘What is safe enough?’, ‘What risks are acceptable?’ and ‘How to discover uncertain risks in a responsible way?’. Furthermore, these discussions bring forward the aspect of responsibility allocation: ‘Who is responsible for ensuring the safety of an application?’ and ‘Who is responsible for the decision making on what can be considered safe enough?’

These are complex issues that can be addressed with downstream SbD through iterative communication between stakeholders (Bouchaut & Asveld, 2020). By engaging multiple stakeholders, an all-round perspective of the issues can be achieved, which can help expose and mitigate possible risks and facilitates risk assessment. In the previous chapter a hypothetical example of stakeholder engagement was given which shows its relevance and salience in acquiring safety for synthetic biology applications. This is especially valuable in a field such as synthetic biology where knowledge of the risk factors is still very limited.

On a policy level, these questions are often addressed by comparing the risks of the application to the benefits to society. This approach is strongly applied in the US where trade-offs between risk and benefits are decisive in regulation concerning synthetic biology. In some cases, one might argue that the consequences of not intervening are worse than the conveyed risks, therefore being it safer to implement the application than not to. In the EU, the precautionary principle encourages the assemblage of knowledge and scientific certainty before deploying an application. Better risk assessment requires more knowledge on cause and effect relationships of the building blocks of life, how the systems interact with each other, and their surrounding environment (SYNENERGENE, 2017). Therefore, as is embedded in the precautionary principle, by increasing the amount of knowledge on the risks and uncertainties they can be assessed and regulated better (Zhang, Marris & Rose, 2011). This approach can however be counterproductive for the development of synthetic biology, as precautionary action or the reservation from taking any action can also hamper the discovery of potential risks and uncertainties and how to deal with them (Ricci & Zhang, 2011). Others call for a new prudent approach where a certain amount of risks are allowed to be taken in order to discover and learn from unknown risks (Gilbert, 2017). This way a compromise can found by taking calculated risks and thereby actively furthering the development of synthetic biology technologies.

Lastly, there is the concern that, if unwanted effects would arise, no technologies have yet been developed to selectively remove the recombinant bacteriophages from the environment (Meaden & Koskella, 2013). In the near future, technologies might be developed to achieve this as a last resort.

In closing, SbD for synthetic biology is still met by a significant amount of constraints. Some solutions to these challenges are proposed and can be tackled with current tools and technologies. As discussed, helpful approaches to diminish uncertainties are the implementation of concrete field studies or iterative stakeholder communication through downstream SbD. Other constraints can be mitigated by making trade-offs between different SbD values, by using technologies such as computational models to predict future behavior or by diminishing the probability of risks occurring by combining multiple SbD measures into one application. However, new innovative ways to deal with these constraints and uncertainties should be developed to further progress the field of synthetic biology and SbD.

7 Conclusions & recommendations

In the past decade, synthetic biology has seen the fast progress of revolutionary technologies to alter the building blocks of life. These technologies have rendered the use of SbD inevitable to manage the associated risks and concerns. This study attempts to contribute to the knowledge base by exploring the concept of SbD in the context of synthetic biology. The objective is to bring forth an assessment of the opportunities and constraints associated with SbD through a case study on the application of bacteriophages as agents to mitigate the issue of Desert Locust swarms. Finally, recommendations are made for successful future research into Safe-by-Design for synthetic biology.

In conclusion, SbD is a promising strategy to increase the biosafety of novel applications. Because of its proactive nature, it is a powerful tool to mitigate known and anticipate future uncertain risks of an application. A vast range of options are available as built-in safety measures for bacteriophage development. Each measure can be adjusted to meet specific needs, which makes SbD a highly dynamic approach to deal with risks. Furthermore, it is a versatile strategy that can be applied to different facets of safety acquisition through upstream and downstream SbD. It allows for safety to be reviewed further than just the technical aspects of the application as it can influence policy-making and public perception of safety for synthetic biology. The main constraint on risk assessment for synthetic biology and SbD is the aspect of uncertainty. It is a paramount feature of synthetic biology and stems from the complexity and lack of knowledge of these systems. Subsequently, it is concluded that SbD is not able to provide absolute safety and the overall aim is to attenuate risks and uncover uncertainties to the highest extent.

To acquire an all-round perspective on the current situation of synthetic biology and Safe-by-Design, these concepts are assessed in terms of governance. In summary, synthetic biology regulations have taken big leaps forward since the establishment of the Asilomar Conference in 1975 but bacteriophage-specific regulation is still lacking. Current regulations for bacteriophage-based therapies hamper the development of gene and microbiome therapies and obstructs them from reaching their full potential. Existing policies should be revised and adequate regulations should be established to fit the specific needs of these technologies. Alternative regulations such as the use of BIMs are possible solutions for this regulatory bottleneck.

Differences in regulatory approaches between countries are discussed and a new prudent approach is suggested to help uncover uncertainties by taking calculated risks. It is also concluded that biosafety is not limited by geopolitical boundries and should be addressed at an international level. In Europe, the precautionary principle narrates that when the knowledge on possible risks is insufficient, the application should not be deployed. This impedes the discovery of risks and uncertainties and thereby hinders the progress of genetically modified applications. Therefore, research efforts should navigate towards concrete real-life scenarios and deployment of applications in controlled field trials to unveil unknown risks and diminish the uncertainties of synthetic biology.

SbD can be helpful to deal with the constraints of uncertainty and lack of knowledge in synthetic biology governance. When knowledge of the risk factors is insufficient, it is difficult to make an informed decision on aspects such as i.a. the accepted level of safety of an application or responsibility allocation. Downstream SbD is proposed as a method to assess risks and uncertainties of synthetic biology applications on a policy level. Through iterative stakeholder

communication and by using risk to benefit comparison assessments, decision-making can be facilitated. Process-based SbD can help engage international stakeholders in the conversation on biosafety thereby allowing a broader transnational debate. Moreover, by opening up the dialogue to a wider audience, process-based SbD can help positively influence the public perception of safety for synthetic biology. By establishing the notion of safety, decision-making can be directed towards more progressive regulations on synthetic biology.

Overall, the risks associated with engineered organisms can be categorized into three groups: risk of negative impact on the environment, risk of natural genome pool contamination, and the run-off risk. A comparative analysis is identified as a key method to approach risk assessment for synthetic biology and it is concluded that this should be conducted on a case-by-case basis for each specific application. To ensure the safety of their bacteriophage application, the iGEM team has developed a broad range of SbD measures. These risks and associated safety measures are summarized in Figure 7.

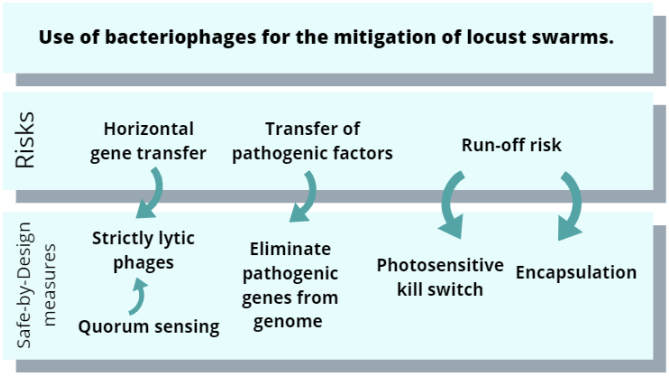

Figure 7. Summary of the mitigated risks and corresponding SbD measures in the iGEM project.

To attenuate the risk of HGT and spread of synthetic genes to the environment, a reviewed way of performing quorum sensing is developed that produces strictly lytic phages. The run-off risk is mitigated by introducing a photosensitive kill switch and applying a method of encapsulation with cellulose. The transfer of pathogenic genes can be minimized by eliminating these genes from the bacteriophage genome. These can all be considered effective SbD measures, but as this is an ongoing project, the design of these measures is still in its nascent stage and further research is required to fully develop and examine their working in the recombinant bacteriophages.

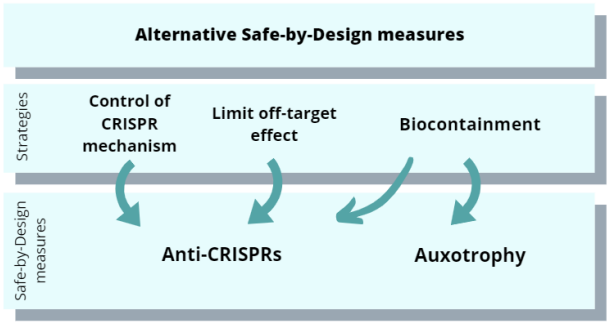

Figure 8. Summary of alternative SbD measures for iGEM project and the associated mitigated risks.

Two additional plausible SbD measures are identified that are also compatible with the iGEM project, namely auxotrophy and anti-CRISPRs. The SbD measures and corresponding risks are shown in Figure 8. Combined with other measures they could provide a higher level of safety of the

bacteriophage application. The use of orthogonal systems and daisy gene-drives are not deemed applicable for this innovation.

The reviewed case study is an outstanding example of current research into the possibilities of SbD to guarantee the safety of an application. iGEM encourages its participants to think about hypothetical real-life implications of their technologies and stimulates the inclusion of biosafety measures in the project design. As SbD is a highly customizable safety strategy, it has high compatibility with the iGEM project. It is a testament to how SbD measures can be implemented in the development of an innovative technology with limited knowledge and research. It also shows how SbD is hampered by the aspects of uncertainty and complexity and reveals the issues, limitations, and other areas of concern of SbD where progress needs to be made. Whilst there is room for improvement safety-wise as well as regulatory-wise, the outlooks are optimistic and a real-life application of the engineered bacteriophages in the near future is not ruled out.

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