Research on Synthetic Genomes

State-of-the-art, Potential Applications and its Biosafety Aspects

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Summary

Overview

This report explores the state-of-the-art advancements, potential applications, and biosafety considerations of synthetic genomes, an emerging cornerstone of synthetic biology. The field has matured significantly due to breakthroughs in DNA synthesis, genome editing technologies, and computational tools, enabling scientists to construct, modify, and study genomes with unprecedented precision and scale. These capabilities extend to various types of synthetic genomes, including natural genome-based modifications, recoded genomes, minimal genomes, and modular reorganized genomes.

Technical Developments

Recent progress in artificial intelligence (AI), machine learning (ML), and genome editing tools such as CRISPR-Cas systems has transformed the field. AI and ML are driving improvements in genome design, enabling the prediction of outcomes and optimization of synthetic constructs. DNA synthesis and assembly technologies are becoming faster and more cost-effective, allowing researchers to create synthetic genomes tailored to diverse applications. These advancements are underpinned by bio-design automation platforms that integrate modeling and high-throughput testing capabilities.

Applications

Synthetic genomes are unlocking transformative opportunities across several sectors:

- **Biotechnology and Industrial Manufacturing**: Enhanced microbial strains with synthetic genomes are being developed for high-efficiency production of biofuels, pharmaceuticals, and specialty chemicals.

- **Medicine**: Applications include personalized therapeutics, gene therapies, and the development of synthetic cells capable of precise drug delivery or disease remediation.

- **Environmental Sustainability**: Synthetic organisms are being tailored for bioremediation, carbon capture, and the breakdown of persistent pollutants.

- **Agriculture**: Engineered microbes and plants are poised to enhance nitrogen fixation, pest resistance, and nutrient uptake, reducing reliance on chemical fertilizers and pesticides.

Biosafety Considerations

While synthetic genomes offer significant benefits, they also present unique risks that necessitate thorough biosafety evaluations:

- **Genetic Stability and Horizontal Gene Transfer**: Synthetic constructs may exhibit altered stability or enable unintended gene flow to wild populations, requiring robust containment strategies.
- **Ecological and Evolutionary Impacts**: Synthetic organisms might disrupt ecosystems or evolve in unpredictable ways, necessitating long-term monitoring frameworks.
- **Regulatory and Ethical Challenges**: Novel risks and the absence of natural baselines for some synthetic constructs complicate traditional risk assessments, highlighting the need for updated regulatory frameworks and public engagement.

Outlook

The report identifies key research and policy priorities:

- Developing integrative risk assessments to evaluate cumulative effects of synthetic modifications.
- Expanding AI-driven tools for predictive biosafety analyses.
- Establishing standardized databases for synthetic biology features to support risk assessments.
- Enhancing transparency and communication to address societal concerns and ethical considerations.

Concluding Remarks

Synthetic genomes represent a powerful tool for addressing global challenges in health, sustainability, and industry. However, their deployment must be guided by robust safety measures, ethical oversight, and adaptive regulatory frameworks to ensure societal benefits while mitigating risks. This balanced approach will enable responsible innovation and sustainable progress in synthetic biology.

Samenvatting

Overzicht

Dit rapport verkent de nieuwste ontwikkelingen, mogelijke toepassingen en bioveiligheidsoverwegingen van synthetische genomen, een opkomende hoeksteen van de synthetische biologie. Het veld is aanzienlijk ontwikkeld dankzij doorbraken in DNA-synthese, genoom bewerkingstechnologieën en computationele hulpmiddelen, waardoor wetenschappers in staat zijn genomen met ongekende precisie en op ongekende schaal te construeren, wijzigen en bestuderen. Deze mogelijkheden strekken zich uit tot verschillende soorten synthetische genomen, waaronder op natuurlijke genomen gebaseerde modificaties, hergecodeerde genomen, minimale genomen en modulair gereorganiseerde genomen.

Technische ontwikkelingen

Recente vooruitgang op het gebied van kunstmatige intelligentie (AI), machinaal leren (ML) en genoombewerkingstools zoals CRISPR-Cas-systemen heeft het veld getransformeerd. AI en ML zorgen voor verbeteringen in het genoomontwerp, waardoor uitkomsten kunnen worden voorspeld en synthetische constructies kunnen worden geoptimaliseerd. DNA-synthese en constructie technologieën worden sneller en kosten effectiever, waardoor onderzoekers synthetische genomen op maat kunnen maken voor verschillende toepassingen. Deze vooruitgang wordt ondersteund door automatiseringsplatforms voor bio-ontwerpen die modellering en high-throughput testmogelijkheden integreren.

Toepassingen

Synthetische genomen maken transformaties mogelijk in verschillende sectoren:

- **Biotechnologie en industriële productie**: Verbeterde microbiële stammen met synthetische genomen worden ontwikkeld voor de zeer efficiënte productie van biobrandstoffen, farmaceutische producten en speciale chemicaliën.
- **Geneeskunde**: Toepassingen zijn onder andere gepersonaliseerde therapeutische middelen, gentherapieën en de ontwikkeling van synthetische cellen die nauwkeurig medicijnen kunnen toedienen of ziekten kunnen genezen.
- **Duurzaamheid van het milieu**: Synthetische organismen worden op maat gemaakt voor bioremediëring, koolstofvanging en de afbraak van persistente verontreinigende stoffen.

- **Landbouw**: Gemanipuleerde microben en planten kunnen het afvangen van stikstofbindingen, resistentie tegen plagen en opname van voedingsstoffen verbeteren, zodat er minder chemische meststoffen en pesticiden nodig zijn.

Bioveiligheidsoverwegingen

Hoewel synthetische genomen aanzienlijke voordelen bieden, brengen ze ook unieke risico's met zich mee die grondige bioveiligheidsevaluaties noodzakelijk maken:

- **Genetische stabiliteit en horizontale genoverdracht**: Synthetische constructies kunnen een veranderde stabiliteit vertonen of een onbedoelde genenstroom naar wilde populaties mogelijk maken, wat robuuste inperkingsstrategieën vereist.
- **Ecologische en evolutionaire effecten**: Synthetische organismen kunnen ecosystemen verstoren of op onvoorspelbare manieren evolueren, waardoor kaders voor lange termijn monitoring nodig zijn.
- **Uitdagingen op het gebied van regelgeving en ethiek**: Nieuwe risico's en het ontbreken van natuurlijke uitgangssituaties voor sommige synthetische constructies bemoeilijken traditionele risicobeoordelingen.

Vooruitzichten

Het rapport identificeert belangrijke onderzoeks- en beleidsprioriteiten:

- Het ontwikkelen van integratieve risicobeoordelingen om cumulatieve effecten van synthetische modificaties te evalueren.
- Uitbreiding van AI-gestuurde instrumenten voor voorspellende bioveiligheidsanalyses.
- Het opzetten van gestandaardiseerde databases voor synthetische biologische kenmerken ter ondersteuning van risicobeoordelingen.
- Het verbeteren van transparantie en communicatie om tegemoet te komen aan maatschappelijke zorgen en ethische overwegingen.

Slotopmerkingen

Synthetische genomen zijn een krachtig instrument voor het aanpakken van wereldwijde uitdagingen op het gebied van gezondheid, duurzaamheid en industrie. De toepassing ervan moet echter worden geleid door robuuste veiligheidsmaatregelen, ethisch toezicht en adaptieve regelgevende kaders om maatschappelijke voordelen te garanderen en tegelijkertijd de risico's te beperken. Deze evenwichtige aanpak zal verantwoorde innovatie en duurzame vooruitgang in de synthetische biologie mogelijk maken.

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Abbreviations

- AI – Artificial Intelligence
- BDA – Bio-Design Automation
- BGC – Biosynthetic Gene Cluster
- CAS – CRISPR-associated (protein)
- CRISPR – Clustered Regularly Interspaced Short Palindromic Repeats
- DNA – Deoxyribonucleic Acid
- EFSA - European Food Safety Authority
- EPA - Environmental Protection Agency
- GMO – Genetically Modified Organism
- HGT – Horizontal Gene Transfer
- ML – Machine Learning
- ncAA – Non-canonical Amino Acids
- PCR - Polymerase Chain Reaction
- RNA – Ribonucleic Acid
- WHO – World Health Organization

Glossary

Many of the terms used in this report do not currently have internationally recognized definitions. To ensure clarity and consistency, a glossary has been added to provide specific definitions for key terms as they are used in the context of this document. The definitions provided in the glossary will be adhered to as much as possible throughout the text, unless stated otherwise. This approach is intended to minimize ambiguity and facilitate a clear understanding of the concepts discussed.

Genome: The complete set of all genetic material in an organism, including both coding (genes) and non-coding regions of its DNA sequence(s). Amongst other, it contains the information necessary for the development, functioning, and reproduction of the organism.

Genomics: The branch of biology that focuses on the study of genomes, encompassing their structure, function, evolution, mapping, and editing. It involves analyzing the complete set of DNA, including all genes and non-coding regions, within an organism to understand how genetic information directs biological processes, influences traits, and interacts with environmental factors.

Synthetic: In the context of biology and genetics, synthetic often refers to something created through deliberate human intervention, typically involving chemical, engineering, or computational methods, rather than occurring naturally. It emphasizes the construction or assembly of components to replicate or extend natural systems.

Artificial: Refers to something made or produced by human effort rather than occurring naturally. In the context of genomes, it implies a constructed or engineered system designed to imitate or improve upon natural biological counterparts.

Synthetic genome: A genome artificially constructed in the laboratory, often from chemically synthesized DNA sequences, but also including significantly modified natural genomes. It is designed to replicate the function of a natural genome or perform novel biological functions.

Conventional genetically modified organism: An organism whose genome has been altered through traditional genetic engineering techniques, typically involving the insertion of heterologous sequences found elsewhere in nature, deletion, or modification of specific genes using recombinant DNA technology. Unlike synthetic organisms, conventional GMOs retain a predominantly natural genome structure with targeted changes.

Recoded genome: A genome that has been systematically altered by substituting codons throughout the genetic code to introduce new functionalities or optimize gene expression, often to enhance resistance to viruses or synthetic biology applications.

Minimal genome: The smallest set of genes required for the basic functions of life, determined by systematically removing non-essential genes, typically for simplifying a biological system or studying fundamental cellular processes.

Reorganized genome: A genome whose structure has been rearranged, either naturally or through genetic engineering, by altering the order or position of genes and regulatory elements, affecting gene expression, regulation, or function.

Modular genome: A genome structured into distinct functional units or modules, each corresponding to specific biological processes, allowing for easier manipulation and reconfiguration in synthetic biology for customized applications.

Golden Gate Assembly: A molecular cloning technique that allows for the precise and efficient assembly of multiple DNA fragments in a single reaction. It uses Type IIS restriction enzymes, which cut DNA outside of their recognition sites, creating customizable overhangs that facilitate seamless ligation. The method enables the directional assembly of DNA fragments without leaving unwanted sequences (scarless), making it highly suitable for constructing complex genetic constructs.

Bio-Design Automation (BDA): An interdisciplinary field focused on developing computational tools, algorithms, and automated workflows to streamline and optimize the design, construction, and testing of biological systems. It combines principles from synthetic biology, computer science, and engineering to enhance the efficiency, scalability, and reproducibility of designing genetic circuits, metabolic pathways, and other biological constructs. BDA aims to accelerate innovation in biotechnology by reducing manual labor, minimizing errors, and enabling high-throughput design.

Non-canonical amino acids (ncAAs): Amino acids that are not among the 20 standard amino acids encoded directly by the universal genetic code. These ncAAs can either occur naturally or be synthetically engineered and are often incorporated into proteins to expand their chemical, structural, or functional diversity in research and synthetic biology.

Xenobiology: A subfield of synthetic biology that explores the design and creation of biological systems using unnatural biochemistries, such as alternative genetic codes, non-standard nucleotides, or non-canonical amino acids. It aims to develop organisms with novel properties that are distinct from life forms based on the standard DNA-RNA-protein paradigm, often for enhanced biosafety, biotechnology, or exploration of fundamental biological principles.

Gene drive: A genetic mechanism that promotes the inheritance of a specific allele or genetic element at a higher-than-normal frequency, typically overriding the standard Mendelian inheritance patterns. It enables a gene to spread rapidly through a population, often used for population control or ecological engineering.

Genetic drift: A mechanism of evolution involving random changes in the frequency of alleles within a population from one generation to the next. These changes occur due to chance events rather than natural selection and are more pronounced in small populations. Over time, genetic drift can lead to the loss or fixation of alleles, reducing genetic variation.

Pathogenicity: The ability of an organism, typically a microorganism such as a bacterium, virus, or fungus, to cause disease in a host organism. Pathogenicity depends on factors like the microorganism's virulence, mode of transmission, and ability to evade the host's immune defenses.

1. Introduction

The possibility of constructing synthetic genomes represents a groundbreaking frontier in synthetic biology, enabling researchers to design, build, and manipulate genetic material in ways that extend beyond the capabilities of natural evolution. A synthetic genome is a construct that is typically described as containing an entirely artificial assembly, often built from chemically synthesized DNA sequences designed to replicate or extend the functions of a natural genome. However, the precise definition of a synthetic genome can be challenging to establish, as interpretations vary across the scientific literature. Some definitions emphasize the complete chemical synthesis of genomic material, while others include extensively modified natural genomes under the same umbrella. This lack of consensus reflects the dynamic and evolving nature of the field, which has gained significant momentum over the past two decades due to advances in DNA synthesis, genome editing technologies, and computational design tools. (Martínez and Humberto Reyes-Valdés 2018) Despite these nuances, synthetic genomics fundamentally aims to deepen our understanding of essential biological principles while opening pathways to transformative applications in biotechnology, medicine, and environmental science.

The transformative potential of synthetic genomes across diverse fields, as highlighted, underscores the necessity of robust regulatory frameworks to ensure their safe and ethical use. This, in turn, places significant importance on performing thorough risk assessments tailored to the unique characteristics and applications of synthetic genomes. However, effective risk assessment and regulation require clear and consistent definitions, which presents a challenge given the varied interpretations of what actually constitutes a synthetic genome.

For the purposes of this report, the term "synthetic genome" aligns with the definitions provided in the glossary, encompassing both artificially constructed genomes and significantly modified natural genomes. In this context, the term "significantly" is used to describe modifications that either introduce, possibly on a large scale, relatively short novel sequences, i.e., on the order of tens of base pairs, that neither occur in similar microorganisms nor knowingly elsewhere in nature; or involve substantial alterations such as the removal or modification of large sections exceeding 10% of the genome size. These criteria serve to delimit the scope of what is considered significant modification, ensuring consistency and clarity in the discussion. While this framework will be adhered to as closely as possible, occasional deviations may occur to accommodate specific contextual nuances or emerging perspectives. Establishing semantical clarity is a critical first step in advancing the responsible development and application of synthetic genomics.

Synthetic genomes are typically constructed using a combination of model organisms and well-characterized systems that offer a robust foundation for engineering. Organisms such as *Escherichia coli*, *Saccharomyces cerevisiae* (baker's yeast), and certain minimal bacterial species have become key platforms for genome construction due to their genetic tractability and the availability of comprehensive genomic data. For example, researchers have successfully synthesized a minimal bacterial genome containing only the essential genes required for survival, providing insights into the minimal requirements for life. (Hutchison et al. 2016) Similarly, yeast has been employed in ambitious projects like the Synthetic Yeast Genome (Sc2.0) (Richardson et al. 2017), which aims to create a fully functional synthetic eukaryotic genome. These efforts highlight the dual focus of the field: elucidating the principles of life and creating new capabilities that do not exist in nature.

While many of the techniques and approaches described in this report can, in principle, be applied to multicellular eukaryotes such as plants and animals, the focus here is primarily on unicellular organisms. This focus reflects the current state of the field, where the majority of successful examples and established applications are concentrated in unicellular systems. Applications in multicellular eukaryotes, with their higher genomic complexity, intricate regulatory networks, and diverse cell types, present additional challenges that require further technical advancements, and therefore, remain relatively limited to date, to the best of our knowledge, with many challenges yet to be addressed. Additionally, even though modified viral genomes are also within the scope of both National and European GMO legislation, other safety considerations are likely to be at play in comparison to cellular organisms. Hence, to a large degree they are not specifically detailed here. By concentrating on unicellular platforms like bacteria and yeast, this report highlights the most robust advancements and insights in synthetic genomics, while acknowledging the potential for future expansion into more complex systems as technology continues to develop.

While synthetic genomics is an emerging discipline, its foundational roots can be traced back to earlier innovations in molecular biology and recombinant DNA technology. However, it is the recent convergence of high-throughput sequencing, cost-effective DNA synthesis, and precise genome-editing tools such as CRISPR-Cas systems that have transformed what was once theoretical into practical reality. (Pareek, Smoczynski, and Tretyn 2011; Hoose et al. 2023; Villiger et al. 2024) Despite its rapid progress, synthetic genomics remains predominantly in the realm of basic research, with the primary goal of understanding genome structure, function, and design. These studies serve as critical proof-of-concept experiments that lay the groundwork for broader applications in the future.

Looking ahead, the potential applications of synthetic genomes are vast and varied. They include the development of custom organisms for industrial bioproduction, such as bacteria that can efficiently produce biofuels or pharmaceuticals, and the creation of synthetic microbial communities for environmental remediation. Synthetic genomes may also play a pivotal role in advancing personalized medicine, enabling the design of custom therapeutic microorganisms tailored to an individual's unique needs. Beyond these practical applications, synthetic genomics could provide profound insights into the origins of life by allowing scientists to construct and study entirely new genetic codes and biochemical pathways.

However, as with any transformative technology, synthetic genomics also raises important ethical, safety, and regulatory considerations. The intentional design of organisms with novel genetic material requires rigorous oversight to prevent unintended consequences, such as ecological disruption or misuse of the technology. Moreover, public engagement and transparent communication will be critical to ensuring that societal concerns are addressed as the field progresses.

The advent of synthetic genomes presents a transformative opportunity in biotechnology, enabling the creation of organisms with entirely new genetic configurations. However, the unique characteristics of synthetic genomes—particularly those assembled from bottom-up approaches or extensively reorganized with non-standard sequences—potentially pose challenges for biosafety and risk assessment. Traditionally, risk assessments in biotechnology rely on baseline comparisons to natural or closely related organisms to identify potential hazards (*i.e.* comparative approach). ("Guidance on the Risk Assessment of Genetically Modified Microorganisms and Their Products Intended for Food and Feed Use" 2011) These baselines include reference data on aspects such as pathogenicity, gene transfer potential, and environmental impact, grounded in the organism's natural counterparts or its typical ecological interactions.

With synthetic genomes, however, these established baselines may be partially or completely absent. For instance, an organism with a minimal synthetic genome may lack close natural analogs, as it is stripped down to only essential genes or reorganized to a degree that fundamentally alters its interactions with the environment. This lack of comparable reference points complicates the risk assessment process, as it becomes challenging to predict how the synthetic organism might behave or interact within ecosystems or host organisms.

In light of this, there is an urgent need to establish alternative baselines specifically for synthetic organisms. One approach could be to create standardized databases of synthetic biology features, categorizing known interactions, metabolic capabilities, and ecological impacts of various synthetic constructs. Alternatively, functional baselines could be developed, focusing on the specific roles and capabilities engineered into the synthetic genome, such as metabolic outputs or gene regulation mechanisms. These function-based baselines would allow for the assessment of synthetic organisms based on their roles and capabilities linked to potential risks rather than their evolutionary lineage.

Establishing these alternative baselines is crucial as synthetic biology continues to advance. Without reliable baselines, there is an increased risk of overlooking potential hazards associated with synthetic organisms, especially as they are applied in open environments or in sensitive settings such as medical and agricultural fields. Creating a framework for synthetic genome assessment now will help ensure that risk assessment can keep pace with technological developments, promoting safe and responsible innovation in the field.

In the context of biosafety, **'points-to-consider'** and **'pathways-to-harm'** are concepts used to assess and manage potential risks associated with the release or use of genetically modified organisms (GMOs) or synthetic biology products.

'Points-to-consider' encompass a range of critical factors that must be evaluated to ensure the safe development, use, or release of genetically modified organisms (GMOs) or synthetic biological entities, here, harboring synthetic genomes. These criteria serve as a structured approach for identifying and mitigating potential risks posed to human health, the environment, and other living organisms. Points-to-consider often include:

- Genetic Stability: Genetic stability is a cornerstone of biosafety assessment, focusing on the long-term reliability of engineered genetic modifications. Evaluations should address whether the introduced traits remain consistent across multiple generations and under varying environmental conditions. Unintended genetic changes, such as mutations or recombination events, could alter the organism's behavior or functionality, potentially negating its intended purpose, or worst, introducing new risks. For example, a microorganism harboring a synthetic genome engineered to produce biofuels could accumulate mutations that lead to the production of harmful by-products. Methods such as whole-genome sequencing, stress testing, and monitoring under simulated environmental conditions are essential to assess and predict the genetic stability of such organisms.

- Potential for Gene Flow: Horizontal gene transfer (HGT) is a natural process that could allow genetic material from the synthetic organism to be transferred to native species or other microorganisms. This is particularly concerning when the engineered traits confer advantages, such as antibiotic resistance or novel metabolic capabilities, which could spread into wild populations. (Botelho, Grosso, and Peixe 2019) Gene flow could lead to ecological imbalances or enhance the adaptability of pathogenic organisms. Evaluating gene flow involves studying the likelihood of genetic material being exchanged under different conditions, identifying potential recipient species, and assessing the functional consequences of such exchanges. Strategies to mitigate HGT risks include the use of genetic safeguards, such as kill switches (Stirling et al. 2017), or designing synthetic genomes that lead to organisms with genetic dependencies on synthetic amino acids (Nyerges et al. 2023) or other laboratory-controlled factors.

- Pathogenicity and Toxicity: Determining whether the synthetic genome could pose risks due to pathogenicity or the production of harmful substances is a critical consideration. Even if the harboring organism itself is not pathogenic, it may produce secondary metabolites or proteins that are toxic to humans, animals, or other organisms. For example, engineered bacteria designed for agricultural applications could inadvertently produce compounds harmful to soil microbiota or non-target plant species. Assessing pathogenicity involves testing the organism's interaction with potential hosts and evaluating its ability to evade immune responses. Toxicity assessments require analyzing the metabolic pathways involved and identifying any unintended by-products that could accumulate in the environment or food chain.

- Containment Measures: Effective containment measures are crucial to prevent the unintended spread of organisms with synthetic genomes beyond their intended environments. These measures can be physical, such as maintaining organisms in controlled laboratory settings, or biological, such as incorporating genetic safeguards that render the organism incapable of surviving outside specific conditions. Biological containment strategies may include auxotrophic modifications, where the organism depends on an external supply of a synthetic nutrient for survival (Vilchèze et al. 2018), or the use of temperature-sensitive gene circuits. (Choi et al. 2022; Pearce, McWhinnie, and Nano 2017) Physical containment involves robust protocols for transport, storage, and disposal to minimize the risk of accidental release. Assessing containment measures requires evaluating their reliability under different scenarios, including human error and environmental disturbances.

- Environmental Impact: Understanding how the organism hosting a synthetic genome interacts with its environment is essential for assessing its potential ecological footprint. Key considerations include the effects on biodiversity, competition with native species, and unintended alterations to ecosystem dynamics. For instance, a synthetic microorganism engineered to degrade pollutants might unintentionally disrupt nutrient cycling or harm non-target organisms that depend on those pollutants for survival. Long-term environmental monitoring and modeling can help predict potential disruptions. Additionally, small-scale field trials under controlled conditions may provide insights into how the organism behaves in real-world settings.

- Human Health Risks: Evaluating the potential risks to human health is particularly important when synthetic genomes are intended for use in agriculture, food production, or medicine. Assessments should consider whether the organism or its by-products could have toxic, allergenic, or immunogenic effects on humans. For example, synthetic probiotics or yeasts used in food production must be evaluated for safety in terms of ingestion, prolonged exposure, or interaction with gut microbiota. Similarly, synthetic organisms used in therapeutic applications should undergo rigorous testing for unintended side effects, such as immune responses or off-target interactions (Dou et al. 2020). Risk assessments often involve in vitro testing, animal models, and eventually controlled human studies to ensure safety.

By systematically addressing these points-to-consider, regulators and researchers can identify potential risks associated with synthetic organisms and develop strategies to mitigate them. These evaluations are integral to building public trust, guiding regulatory frameworks, and ensuring that the benefits of synthetic genomes are realized without compromising safety or ecological balance.

'Pathways-to-harm' refers to plausible scenarios through which a host of a synthetic genome in this case, could negatively impact humans, animals, or the environment. These scenarios are systematically mapped out to identify potential risks at each stage of the organism's lifecycle – from development and testing in controlled environments, to use and eventual release into less regulated or natural settings. Identifying these pathways enables researchers and regulators to anticipate risks and implement measures to mitigate them before they materialize. Key examples include:

- Escape and Proliferation: Synthetic genomes may inadvertently escape containment and establish themselves in natural environments. Once released, their novel genetic traits could give them a competitive advantage over native species, allowing them to proliferate unchecked. This could lead to unintended ecological consequences, such as the displacement of native organisms or the alteration of local biodiversity. For example, an organism harboring a synthetic genome engineered for high metabolic efficiency might outcompete native microbes for resources, disrupting existing microbial communities and food webs. Escape scenarios could occur through accidental release during research, production, or deployment, making stringent containment measures essential in such scenario.

- Horizontal Gene Transfer: Organisms harboring synthetic genomes might transfer genetic material to other organisms through horizontal gene transfer (HGT), a process by which genes are exchanged between organisms without reproduction. This could occur via natural mechanisms such as bacterial conjugation, transformation, or viral transduction. If synthetic traits, such as antibiotic resistance or the ability to metabolize toxic compounds, are transferred to wild-type organisms, it could result in unintended ecological or medical consequences. For instance, the spread of antibiotic resistance genes could exacerbate the global issue of antimicrobial resistance (Aggarwal et al. 2024), while gene transfer to pathogenic organisms could enhance their virulence or adaptability.

- Ecological Disruption: The introduction of synthetic genomes could alter ecological relationships in unpredictable ways. Changes in predator-prey dynamics, nutrient cycling, or habitat structure are possible if the organism interacts with local ecosystems in unintended ways. For example, a synthetic organism engineered to degrade specific pollutants might inadvertently disrupt nutrient cycling, affecting soil health or aquatic ecosystems. Such disruptions could cascade through the food web, impacting plant, animal, and microbial communities, potentially leading to long-term ecological imbalance.

- Harmful By-products: Synthetic genomes may encode the production of toxins, allergens, or other harmful substances, either intentionally as part of their design, or unintentionally as metabolic by-products. These substances could accumulate in the environment or enter the food chain, posing risks to non-target organisms, including humans. For instance, an engineered microorganism designed to produce a valuable industrial compound might also release secondary metabolites that are toxic to other microorganisms, plants, or animals. This could alter soil or water chemistry, with downstream effects on ecosystem health and productivity.

- Interaction with Pathogens: An organism harboring a synthetic genome could inadvertently interact with existing pathogens, creating new pathways for disease transmission or enabling the evolution of more virulent strains. For example, such organism might serve as a novel host for viruses, allowing them to evolve adaptations that make them more infectious or resistant to existing control measures. Alternatively, engineered traits such as enhanced survival in hostile environments could inadvertently benefit co-existing pathogens, increasing their persistence or spread. These risks are particularly concerning in contexts where synthetic genomes are deployed in close proximity to human, animal, or plant populations.

These concepts form the foundation of risk assessment frameworks established by major regulatory bodies, including the World Health Organization (WHO), the Environmental Protection Agency (EPA) in the United States, and the European Food Safety Authority (EFSA). Such frameworks aim to thoroughly evaluate potential risks associated with new biotechnology products, ensuring that any threats to health, biodiversity, or ecosystems are identified and managed before these products are approved for field release or commercial use. By proactively addressing pathways-to-harm, these frameworks help promote the responsible development and application of synthetic biology technologies.

In this report, the analysis of the complexities of synthetic genomes were grouped in the four criticalities listed below (Table 1). These reflect defined areas which may present additional risks to the ones that are common across multiple of the entries. One of the complexities has been split into two sub-complexities since they were found to raise very distinct risks during the analysis.

Table 1. List criticalities which will be discussed in this report

Complexities of synthetic genomes	Chapter
Synthetic genomes based on a natural genome sequence (*e.g.* addition of sequences like flags or recombination sites)	2
Recoded genomes in which the codon usage is changed	3
Minimal genomes carrying only essential sequences: • Derived from top-down approaches • Assembled from bottom-up approaches	4
Reorganized genomes based on pre-designed modules, in which genes related to *e.g.* the same metabolic pathway are clustered	5

For each of the listed categories associated with the complexities of synthetic genomes, we have compiled a comprehensive list of key "points-to-consider" and plausible "pathways-to-harm" relevant to their biosafety implications. While this compilation is not exhaustive, it serves as a foundational framework for evaluating the potential risks and challenges associated with these applications and systems. The elements highlighted reflect aspects that, in our view, warrant particular attention when assessing biosafety concerns. These considerations are intended to facilitate informed discussions and provide a basis for further refinement as the field continues to evolve and new insights emerge.

This report is grounded in a comprehensive literature review that primarily relied on peer-reviewed articles, with some consideration given to pre-prints. Legal documents were consulted when relevant, though not examined in exhaustive detail. The search employed terms such as “synthetic genome”, “recoded genome”, “minimal genome”, and “reorganized genome”, along with synonymous variants derived from the listed criticalities. Searches were predominantly conducted using PubMed (https://pubmed.ncbi.nlm.nih.gov/), with other search engines occasionally utilized for specific queries. To gain a chronological perspective of references in the scientific literature within this field, ResearchRabbit (https://www.researchrabbitapp.com/) was employed in default mode, leveraging PubMed-derived references as input. All searches were conducted between June and mid-October 2024.

2. Synthetic genomes based on a natural genome sequence
(*e.g.* addition of sequences like flags or recombination sites)

Synthetic genomes based on a natural genome sequence, such as those modified by adding sequences like genetic "flags" or recombination sites, represent a powerful tool in synthetic biology. (Gibson et al. 2008) This approach builds on the structure of natural genomes while incorporating synthetic elements designed to enhance functionality, enable precise modifications, and/or track genetic changes. (Isaacs et al. 2011; Venetz et al. 2019) By introducing specific sequences such as recombination sites, researchers can control genomic rearrangements, integrate new genes, or mark locations for easy identification, all while maintaining the overall integrity of the natural genome.

These synthetic modifications, which may involve genetic modifications and/or the insertion of genes using standard amino acids, mutagenesis, manipulation of existing (heterologous) genes, or other forms of sequence alteration, are generally comparable to established and well-evaluated traditional GMO methodologies. However, the key distinction lies in the presence of at least some novel components (*i.e.* new-to-nature, and thus synthetic), within the resulting genome sequence, setting these approaches apart from conventional genetic engineering practices.

When assessing risks associated with synthetic genomes based on natural genome sequences, it is essential to explore whether these risks differ significantly from those posed by conventional GMOs. While methodologically synthetic genomes often incorporate techniques and modifications that overlap with traditional genetic engineering, it may introduce aspects with unique elements that may require additional consideration. For instance, the inclusion of non-conventional and entirely novel sequences, such as recombination sites or genetic "flags," can possibly lead to risks not typically encountered with conventional GMOs, particularly if applied on a large scale. The latter may be of particular concern when novel AI generated sequence generation or xenobiology applications are employed. These novel sequences might alter in a unique fashion gene expression, stability, or interactions in unforeseen ways, necessitating careful evaluation of their potential ecological, health, and safety impacts.

At the same time, it is equally important to recognize that certain risks associated with synthetic genomes are not inherently different from those posed by conventional GMOs. For example, concerns related to genetic stability, horizontal gene transfer, or ecological impact may remain similar, particularly when the synthetic modifications are limited to techniques already well-studied in GMO research. This similarity in risk profiles underscores why certain aspects of synthetic genomes may not warrant additional regulatory scrutiny compared to conventional GMOs. Recognizing this equivalence is vital, as it helps ensure that risk assessments are both balanced and focused, avoiding unnecessary duplication of regulatory measures for modifications that do not introduce novel hazards.

By identifying areas where risks overlap and distinguishing those where synthetic biology adds new dimensions, researchers and regulators can tailor their approaches to risk assessment more effectively. This nuanced perspective not only strengthens biosafety protocols but also promotes the responsible development of synthetic genomes by avoiding overregulation in areas where risks are already well understood. Ultimately, this balance fosters innovation while maintaining rigorous safety standards.

It can be argued that many of the genetic modifications discussed in this chapter are already commonly used in the creation of conventional GMOs. However, the extent, method, and scale of these modifications can enable more unconventional alterations or produce phenotypic outcomes that go beyond those typically seen with individual changes. This creates a grey area where a genome, initially altered through conventional means, acquires new functionalities through successive modifications, potentially leading it to be classified as a "synthetic genome". As noted previously, the formal distinction between a conventionally modified genome and a synthetic genome can sometimes be difficult to differentiate.

The modifications considered in this chapter potentially allow for the fine-tuning of biological processes, enabling enhanced control over gene expression, protein function, and metabolic pathways. For instance, recombination sites can be used to facilitate genome editing, allowing for the insertion, deletion, or rearrangement of genetic material with high precision. (Gelsinger et al. 2024; Zhu, Li, and Gao 2020; Kuhlman and Cox 2010) This can be useful in applications ranging from optimizing metabolic pathways to creating more robust, virus-resistant organisms. (Blount 2023) Additionally, "flags" or watermark sequences can be added to synthetic genomes to distinguish them from their natural counterparts, ensuring that synthetic DNA can be easily tracked or identified within a cell. (Gibson et al. 2010)

An example of this approach is the design of synthetic bacterial genomes, such as the *Escherichia coli* strain with a recoded genome, where natural sequences were altered to include recombination sites. (Blount 2023) These modifications allow for enhanced viral resistance and facilitate the integration of synthetic elements, such as non-canonical amino acids, into proteins. Similarly, synthetic yeast genomes have been designed with modular chromosomes containing recombination sites, enabling genome-wide structural rearrangements and the controlled evolution of the organism. (Richardson et al., 2017; Zhou et al., 2021)

Synthetic genomes based on natural templates offer a highly customizable platform for biological engineering, combining the stability and complexity of natural genomes with the versatility of synthetic biology tools. This approach is particularly valuable in research areas such as drug development, industrial biotechnology, and environmental applications, where precise genetic control is essential.

Points-to-Consider

- Genetic Stability:

- The stability of the introduced sequences (e.g., flags, recombination sites) over time needs to be thoroughly assessed. (Feil, Enright, and Spratt 2000) This includes understanding if these sequences could mutate, get lost, or otherwise change in ways that alter the intended function of the synthetic genome either locally or in distal regions of the genome. Such changes could inadvertently affect gene expression, disrupt regulatory networks, or lead to genome instability. (Peng and Liang 2020)
- It is critical to evaluate whether these added sequences might interfere with essential gene functions or disrupt the organism's normal physiological processes. For example, recombination sites placed too close to vital regulatory elements might destabilize the expression of key genes, leading to unanticipated metabolic or developmental outcomes. (Del Amparo et al. 2023)
- The potential for synthetic genomes to exhibit different patterns of genetic stability compared to conventional GMOs is heavily dependent on the modifications introduced and their impact on the genome's overall structure. For the time being, it is advisable that genetic stability be assessed on a case-by-case basis, with particular attention to how synthetic modifications influence genome-wide dynamics, including replication fidelity and error rates.

- Potential for Horizontal Gene Transfer (HGT):

- Evaluating the possibility of synthetic sequences being transferred to wild or related species is critical. For example, recombination sites may act as "hotspots" for gene transfer, facilitating the unintended spread of synthetic traits to native populations or other microorganisms. (Lam and Keeney 2015)
- Recombination sites or other introduced elements that enable targeted genomic rearrangements may inadvertently increase the likelihood of HGT by making synthetic sequences more accessible or exchangeable during natural DNA uptake processes. (Coradini et al. 2023)
- Biocontainment strategies must account for the increased risk of HGT posed by genetic instability. For instance, unintended mutations in genetic safeguards might compromise the efficacy of containment measures, enabling synthetic genetic elements to cross species barriers. (Seydel 2023; Zhang and Blaser 2012)

- It is also advisable to evaluate environmental conditions that could exacerbate HGT risks. For instance, high microbial diversity and DNA exchange rates in soil or aquatic environments could make synthetic organisms particularly susceptible to gene transfer, potentially requiring additional biocontainment measures.

- Ecological Impact:

- The interaction of organisms harboring synthetic genomes with their surrounding environment, including microorganisms, plants, and animals, needs to be carefully studied. Synthetic modifications might alter the organism's ecological role, such as its participation in nutrient cycling or its interaction with predators, symbionts, or competitors.
- Synthetic genomes encoding enhanced traits, such as improved nutrient utilization or stress resistance, might outcompete the ones present in native species. This could lead to ecological imbalances, including reduced biodiversity or the disruption of microbial communities, with cascading effects on ecosystem stability.
- The long-term ecological persistence of synthetic genomes should be modeled under realistic conditions. This includes evaluating whether the organism's synthetic modifications confer survival advantages that might allow it to establish itself in unintended habitats. (Bock 2017)

- Functional Integrity of Recombination Sites:

- Recombination sites, while useful for enabling targeted genetic modifications, could become activated unintentionally due to environmental factors or interactions with other genetic elements. This could lead to unplanned genomic rearrangements, such as deletions or translocations, that were not part of the original design.
- Evaluating the specificity of recombination sites is crucial. (Reece-Hoyes and Walhout 2018) They must function only under tightly controlled conditions to prevent unintended alterations to the genome. This involves testing their activation thresholds and ensuring they do not cross-react with similar sequences present in native organisms or the environment.
- If recombination sites are used for controlled rearrangements, their cumulative impact on genome stability should be considered. For instance, multiple recombination events could lead to structural instability, introducing new vulnerabilities into the synthetic genome.

- Opacity in AI-Generated Sequences:

- The use of AI to generate novel genetic sequences introduces unique challenges in biosafety and risk assessment. Unlike traditional approaches, where the rationale behind each genetic modification is typically understood, AI-generated sequences may result from complex, non-intuitive algorithms. This lack of transparency makes it difficult to ascertain the purpose or mechanism of specific sequence designs, complicating the evaluation of their potential risks.

- The absence of a clear design rationale can hinder the identification of potential hazards. For example, an AI-generated sequence may inadvertently introduce functional motifs that increase susceptibility to mutations, horizontal gene transfer, or pathogenic interactions. This uncertainty requires the development of new frameworks for interpreting and assessing the risks associated with AI-designed modifications.
- The scale and rate at which AI can generate new sequences may outpace current biosafety evaluation processes. Researchers and regulators need to establish protocols that can handle the increased volume and novelty of AI-generated sequences, ensuring that thorough risk assessments are conducted before deployment.
- Long-term monitoring frameworks should be adapted to track the performance and stability of organisms containing AI-generated sequences. Given the potential for these sequences to behave unpredictably, continuous evaluation and adaptive risk management are essential to mitigate unforeseen risks that may arise after release into real-world environments.

- **Human and Animal Health Risks**:

- Potential allergenic or toxic effects of new sequences should be evaluated, especially when synthetic organisms or their products are intended for use in food, pharmaceuticals, or environmental applications. For example, unintended metabolic by-products resulting from synthetic modifications could pose health risks.
- The organism's potential to become pathogenic, or its ability to interact with existing pathogens, should be carefully assessed. Organisms hosting synthetic genomes might provide novel environments or genetic material that could enhance the virulence or adaptability of co-existing pathogens.
- Synthetic modifications that alter the organism's surface proteins or immune evasion mechanisms should also be evaluated for their potential to affect interactions with human or animal immune systems. (Chaguza, Cornick, and Everett 2015) These changes could have implications for safety in applications like probiotics or therapeutic microorganisms.

Plausible Pathways-to-Harm

- Unintended Gene Flow via Recombination Sites:

- Recombination sites could facilitate the unintended exchange of genetic material with other microorganisms or host cells, potentially enabling the spread of synthetic sequences beyond their intended environment. These sequences might transfer traits such as antibiotic resistance, enhanced metabolic capabilities, or novel synthetic pathways to native species or pathogens, creating ecological or medical risks.
- This risk is particularly heightened in environments with high microbial diversity and active DNA exchange, such as soil or aquatic ecosystems. Horizontal gene transfer (HGT) to wild populations could result in unpredictable ecological and evolutionary impacts, such as the emergence of hybrid organisms with enhanced adaptability or invasiveness. (Bock 2017)
- The likelihood of gene flow also depends on the stability and specificity of the recombination sites. Poorly designed or unstable recombination sites may increase the risk of accidental exchange, underscoring the need for rigorous testing under environmental conditions.

- Escape and Proliferation in the Environment:

- If a synthetic organism escapes containment, it may establish itself in natural habitats where it could behave unpredictably. Synthetic modifications might provide competitive advantages, allowing the organism to outcompete native species, disrupt ecological networks, or alter nutrient cycling.
- The presence of recombination sites could enable the organism to adapt rapidly through genome rearrangements, enhancing its survival and fitness in the wild. For example, recombination could activate dormant genes or suppress regulatory pathways, enabling the organism to exploit new niches.
- These risks are particularly relevant in cases where synthetic organisms are introduced into environments with limited oversight, such as in large-scale agricultural or environmental remediation projects.

- Uncontrolled Activation of Recombination Sites:

- Recombination sites may become activated unintentionally due to environmental factors such as temperature fluctuations, chemical exposure, or interactions with other microbes. These activations could trigger genome rearrangements, leading to unexpected traits or loss of intended functionalities.
- Such unintended rearrangements could result in the organism gaining new traits, such as resistance to environmental stresses, or losing vital regulatory controls, potentially increasing its pathogenicity or ecological impact. (Lipszyc, Szuplewska, and Bartosik 2022)

- The risks of uncontrolled activation are particularly concerning in complex or variable environments where the triggers for recombination site activity cannot be easily predicted nor controlled. Incorporating safeguards, such as highly specific recombination triggers or failsafe mechanisms, is essential to mitigate these risks. (Merrick, Zhao, and Rosser 2018)

- Production of Harmful By-products:

- Synthetic sequences added to the genome could lead to the production of new metabolites or proteins that might be toxic to other organisms or disrupt natural microbial communities. These by-products may result from unintended interactions between synthetic and native metabolic pathways or errors in gene regulation.
- Harmful by-products could affect ecosystem health by altering soil chemistry, inhibiting plant growth, or causing toxicity to animals and humans. For example, a synthetic microorganism designed for agricultural use might release metabolites that are toxic to pollinators or beneficial soil microbes, indirectly affecting crop yields.
- Evaluating the metabolic pathways encoded by synthetic genomes under diverse conditions is essential to anticipate and manage the risks of by-product production.

- Increased Virulence or Resistance:

- Modifications like recombination sites might inadvertently increase the organism's resistance to environmental stresses, antimicrobials, or biocontrol measures, making it harder to manage or eradicate if released. This could occur through different selective pressures in the environment that favor the survival of resistant variants.
- The engineered organism harboring a synthetic genome could also acquire new traits through recombination with native organisms, potentially increasing its virulence, environmental persistence, or ability to evade biocontrol strategies. For instance, recombination events might enable the organism to produce novel toxins or bypass immune defenses in host organisms.
- Increased resistance and virulence could pose significant challenges in agricultural or medical settings, where synthetic genomes might interfere with existing pest control or therapeutic measures.

- Emergence of New Ecological Niches:

- Organisms harboring synthetic genomes, particularly those with novel traits or enhanced metabolic efficiency, could exploit ecological niches that were previously unoccupied or less competitive. This could lead to the establishment of new populations with unpredictable impacts on local ecosystems. (T. Wang et al. 2022)
- The creation of new niches might also affect native species by altering resource availability, introducing novel predators or competitors, or changing habitat conditions.

- Interaction with Pathogens or Opportunistic Microorganisms:

- Synthetic genomes might interact with existing pathogens or opportunistic microorganisms in unforeseen ways, potentially providing them with new genetic material that may coinfer ecological advantages, or new environments for evolution. For example, a synthetic genome deployed in agricultural or medical settings could inadvertently enhance the virulence or adaptability of pathogens through direct gene transfer or by creating selective pressures that favor more aggressive variants.
- Interactions with synthetic genomes could result in the emergence of more resilient or harmful pathogens, posing risks to human, animal, and environmental health.

- Cumulative and Long-term Impacts:

- The cumulative effects of synthetic modifications, including recombination sites, may not become apparent immediately but could manifest over multiple generations or extended environmental exposure. Long-term monitoring is essential to identify gradual changes, such as shifts in ecological balance or the evolution of organisms harboring synthetic genomes in response to environmental pressures.
- Predicting and mitigating these cumulative impacts requires advanced modeling tools, combined with robust field data, to capture the complex interactions between the hosts carrying synthetic genomes and their ecosystems.

Summary

Evaluating the biosafety of synthetic genomes that include added sequences, such as genetic flags or recombination sites, requires a comprehensive understanding of their genetic stability, ecological impacts, and the risks of horizontal gene flow. This evaluation must consider both the immediate and cumulative effects of the modifications, as well as their broader interactions within ecosystems and potential unintended consequences. Identifying plausible pathways-to-harm, such as the risk of gene transfer, genome instability, or ecological disruption, is crucial for minimizing unintended outcomes. This ensures that synthetic organisms can be deployed safely in research, industry, and environmental applications.

In addition to these general considerations, particular attention must be paid to the specific nature and configuration of the introduced modifications. For instance, the functionality and biosafety risks associated with a genome containing two recombination sites are vastly different from those posed by a genome with multiple copies of the same chromosome and a network of carefully placed recombination sites. The latter scenario could significantly alter the way the genome itself operates, potentially introducing novel dynamics, such as increased susceptibility to rearrangements, changes in gene expression patterns, or unintended metabolic burdens. These cumulative effects highlight the importance of considering not only the individual modifications but also their combined impact on genome functionality and stability.

This underscores a critical principle: in synthetic biology, the sum of the parts often exceeds the individual contributions. Modifications that may appear benign in isolation can interact in complex and unpredictable ways, creating emergent properties that require careful evaluation. Researchers and regulators should pay special attention to how modifications may alter the fundamental workings of the genome, including its regulation, adaptability, and interactions with external factors. Such attention to detail is essential for anticipating and mitigating risks while maximizing the potential benefits of synthetic genomic technologies. By addressing these nuances, we can promote the safe and responsible advancement of synthetic biology.

3. Recoded genomes in which the codon usage is changed.

Recoded genomes, in which codon usage is systematically altered, represent a groundbreaking advancement in synthetic biology, namely in its the sub-field xenobiology. This approach involves intentional modifications to the genetic code, fundamentally changing how codons are interpreted during protein synthesis. Codon recoding strategies include reassigning specific codons to encode different amino acids, reducing the redundancy of synonymous codons, or entirely eliminating certain codons from the genome. These changes unlock a variety of transformative outcomes, such as the incorporation of synthetic amino acids, enhanced control over protein expression, and robust viral resistance. (Marc J. Lajoie et al. 2013; Robertson, Funke, de la Torre, Fredens, Elliott, et al. 2021)

The primary rationale for recoding genomes lies in expanding the genetic code beyond its natural constraints. By altering codon interpretations, researchers can introduce non-canonical, synthetic amino acids into proteins, enabling novel functionalities not found in nature. For example, the 57-codon *E. coli* strain (Nyerges et al. 2023) demonstrates the potential of this approach. This engineered strain omits certain codons from the standard genetic code, reassigning them to encode synthetic amino acids that do not naturally occur in organisms. These man-made amino acids can be functional, participating in protein synthesis to enhance properties such as stability, enzymatic activity, or interaction specificity. However, their introduction also has broader implications, potentially imposing metabolic burdens or disrupting regulatory networks due to shifts in translation dynamics and protein folding. Such innovations open new possibilities for applications in biomanufacturing, drug development, and synthetic biology.

One of the most striking features of recoded genomes is their impact on viral infections. In the case of the 57-codon *E. coli*, recoding confers resistance to bacteriophages by removing the codons essential for viral replication. (Nyerges et al. 2023) Without access to these codons, viral machinery is unable to synthesize functional proteins, halting propagation. This engineered resistance exemplifies the potential of codon recoding for enhancing biosafety. (N. J. Ma and Isaacs 2016) However, the evolutionary interplay between viruses and recoded genomes offers a deeper perspective. Over time, some phages demonstrate an ability to bypass this barrier by adapting their genomes to the host's altered genetic code or exploiting alternative pathways. This raises important questions about the longevity of viral resistance and the possibility of unintended evolutionary consequences, such as the emergence of more adaptable or virulent phages.

Recoded genomes also play a pivotal role in biocontainment and biosafety. Altering codon usage prevents viruses that rely on the standard genetic code from recognizing or translating genetic instructions in recoded organisms. In some instances, this resistance is achieved through sophisticated synthetic biology techniques that go beyond single-gene modifications to rewire the genome's relationship with the translational machinery. For instance, engineering the organism's tRNA repertoire to assign new codon-to-amino-acid translations, coupled with genome-wide recoding to align with these changes, creates a "genetic firewall." This firewall misinterprets viral codons, producing non-functional or toxic viral proteins and effectively stopping infections.

A specific example of this approach involves reassigning two of the six serine codons to leucine, thereby disrupting viral protein synthesis while adding an additional layer of containment. (Nyerges et al. 2023) The engineered cells depend on these reassigned codons for their proteome synthesis, creating a dependency that prevents their genetic material from spreading to natural populations. This dependency exemplifies a holistic alteration of genome function, highlighting the transformative potential of synthetic biology to fundamentally reimagine cellular processes.

Beyond viral resistance, recoded genomes also address the risks of horizontal gene transfer. By eliminating or reassigning specific codons, synthetic organisms can be engineered to prevent their genetic material from being utilized by wild populations, further reducing ecological and evolutionary risks. This dual benefit of viral resistance and restricted gene transfer positions recoded genomes as particularly advantageous in industrial biotechnology and therapeutic applications, where biosafety is paramount. These innovations redefine the boundaries of genetic engineering, offering novel tools for safeguarding synthetic organisms while expanding our understanding of genome functionality.

Codon recoding also provides a unique platform for exploring fundamental biological processes, including translation, gene regulation, and evolutionary dynamics. By altering codon usage in essential genes, researchers can investigate how organisms adapt to changes in the genetic code, offering insights into evolutionary trajectories and the interplay between genetic systems and environmental pressures. (Borges et al. 2022)

In summary, recoded genomes with systematically altered codon usage are reshaping synthetic biology. From expanding the genetic code to introducing novel biochemical properties, they offer powerful solutions for enhancing biosafety and deepening our understanding of gene expression and evolution. This technology paves the way for creating organisms with unprecedented capabilities, ensuring both innovation and safety in engineered microbes.

Points-to-Consider

- Genetic Stability of Recoded Sequences:

- It is critical to assess whether the new codon usage remains stable over time, particularly under varying environmental conditions or across multiple generations. Stability testing should include exposure to stressors such as temperature shifts, oxidative stress, and nutrient limitations, as these could influence mutation rates or lead to reversion to the original genetic code. Such instability could result in unpredictable changes in the organism's behavior, potentially compromising biosafety measures. (Nouën et al. 2017)
- Stability is particularly crucial for applications where recoding is intended to prevent horizontal gene transfer (HGT) or to act as a containment measure. For instance, if codons are removed or reassigned to prevent the uptake of synthetic material by wild organisms, their stability under diverse ecological conditions must be ensured to avoid unintended consequences.
- Incorporating genetic safeguards, such as dependency on synthetic amino acids or tightly regulated gene expression systems, can further reinforce the stability of recoded sequences, mitigating risks associated with reversion or mutation.

- Impact on Protein Folding and Function:

- Changes in codon usage can significantly affect the rate and fidelity of translation, which in turn influences protein folding and functionality. Since translation rate can dictate co-translational folding pathways, codon recoding could unintentionally lead to misfolded proteins. Misfolded proteins can aggregate and become toxic to cells, disrupt cellular homeostasis, or interfere with critical metabolic pathways. (Mignon et al. 2018)
- The potential for toxic by-products arising from misfolded or partially functional proteins should be carefully evaluated, as these could have downstream effects on the organism's viability and its interactions with the environment. (Katneni et al. 2022)
- While recoded organisms might limit HGT due to altered codon usage, genetic material could still be transferred to wild-type organisms. If the recipient cannot interpret the recoded sequences properly, this could lead to dysfunctional gene expression, potentially reducing the risks. However, if the misinterpreted sequences inadvertently confer fitness advantages, such as enhanced stress tolerance or pathogenic traits, this could create ecological imbalances or other unintended consequences. Detailed modeling and experimental validation of such transfer scenarios are necessary to understand the potential outcomes.

- Coexistence with Native Genetic Machinery:

- Recoding the genome alters the interaction between codons and the tRNA pool, potentially introducing competition for specific tRNA molecules. This competition could affect translation efficiency, growth rates, and overall cellular viability, particularly in environments where resources are limited. (Sherman, Rogers, and Söll 1992) (Levin and Tuller 2020) The effects of altered tRNA dynamics should be thoroughly characterized to ensure the organism's stability and functionality in intended applications.
- Codon recoding could also influence the organism's interactions with native microbial communities. For instance, the recoded organism may have altered metabolic activity or enhanced growth under certain conditions, allowing it to outcompete native species. Such dominance could disrupt microbial ecosystems, altering nutrient cycles or reducing biodiversity. Simulated ecosystem studies can help predict these interactions and inform mitigation strategies.
- It is equally important to examine how recoded organisms coexist with other synthetic or engineered organisms, particularly in settings like bioreactors or agricultural environments, where multiple engineered strains may be deployed.

- Human Usage Considerations:

- Evaluating the potential effects of consuming recoded organisms or their by-products is vital, particularly in contexts such as food production, pharmaceuticals, or probiotic formulations. Risk assessments should include rigorous testing for toxicity, allergenicity, and immunogenicity to ensure safety.
- If non-canonical amino acids are introduced through recoding, it is essential to assess their potential impacts on human health. These amino acids may interact with human metabolic pathways in unintended ways or provoke immune responses. Additionally, their long-term effects, such as bioaccumulation or interference with the human microbiome, should be investigated.
- The nutritional and functional properties of recoded organisms used in food production should also be evaluated. For example, recoding may alter protein digestibility or nutrient bioavailability, impacting the organism's suitability for consumption.

- Evolutionary Implications of Recoded Genomes:

- Recoded genomes may influence the evolutionary dynamics of synthetic organisms, particularly in long-term or open-environment applications. For instance, selective pressures in the environment could favor mutations that restore the original genetic code, undermine recoding efforts, or lead to novel adaptations. Monitoring these evolutionary trends is essential to anticipate and mitigate potential risks.

- Interactions between recoded organisms and natural populations should be studied to assess whether recoding alters competitive dynamics or facilitates evolutionary convergence through horizontal gene transfer or recombination. Such interactions could introduce unintended traits into natural populations, necessitating contingency planning.

- Industrial and Environmental Applications:

- For industrial or environmental applications, the scalability and reliability of recoded organisms must be evaluated under real-world conditions. This includes testing their performance in large-scale bioreactors, agricultural settings, or environmental remediation projects, where diverse and unpredictable conditions may affect their stability and functionality.
- The potential environmental impact of recoded organisms, such as their ability to persist or interact with native species, should be carefully modeled. Deploying recoded organisms in controlled environments or incorporating self-limiting genetic mechanisms can minimize these risks.

Plausible Pathways-to-Harm

- Unintended Evolutionary Reversion:

- A significant risk is that the recoded genome could revert to its original codon usage or mutate into an alternative configuration through spontaneous mutations. Such mutations might be favored if the recoded codon usage imposes a fitness cost on the organism, such as reduced efficiency in protein synthesis or metabolic burdens. Reversion or mutation could negate safety features like reduced susceptibility to viruses or restricted HGT, undermining the biosafety rationale for recoding. (Bacher, Bull, and Ellington 2003)
- Beyond fitness costs, the evolutionary pressure to optimize genome function could lead to compensatory mutations that restore compatibility with native tRNA pools or codon usage. This could re-enable processes like HGT, allowing the synthetic organism to transfer genetic material to wild populations.
- Mutations in the recoded genome could also lead to the production of proteins or metabolic products that were initially excluded or suppressed through recoding. These proteins might inadvertently confer selective advantages or cause toxic effects, further complicating risk assessments. Long-term monitoring and modeling are essential to understand the evolutionary dynamics of recoded genomes under various conditions.

- Escape and Proliferation in Natural Environments:

- Recoded organisms are often engineered for specific, controlled environments. However, if they escape, they could interact with wild populations in unpredictable ways. For example, altered codon usage could provide a selective advantage under certain conditions, such as increased resistance to environmental stressors, unique metabolic capabilities, or avoidance of natural predators.
- In some scenarios, recoded organisms may exploit niches unavailable to native species, bypassing direct competition but still disrupting ecosystem dynamics. For instance, if a recoded organism has enhanced efficiency in nutrient utilization, it could deplete resources critical to native species, causing cascading ecological effects.
- Assessing the ecological impact of recoded organisms in natural environments requires controlled release experiments, ecosystem modeling, and long-term monitoring to understand their interactions with local microbial communities and higher organisms.

- Interaction with Viral Pathogens:

- Recoded genomes are often engineered to resist specific viruses that rely on the standard genetic code. However, this resistance may not be universal. Novel viruses or existing viruses capable of rapid adaptation could evolve to exploit the recoded genetic code. Such interactions could lead to the emergence of new viral strains with unpredictable behaviors and ecological impacts.

- Adapted viruses might acquire the ability to infect both recoded and wild-type organisms, posing a broader threat to natural ecosystems. Given the limited data available, predicting the long-term ecological effects of such viral adaptations is challenging, emphasizing the need for cautious deployment and rigorous surveillance. (Bull, Molineux, and Wilke 2012)
- Experimental studies like those of phage-resistant *E. coli* strains show that viral resistance can be robust initially but may erode over time. This highlights the importance of evaluating how recoded organisms and viral pathogens co-evolve, as these dynamics could introduce unforeseen risks. (Yu et al. 2018)

- Metabolic Imbalances and Toxic By-products:

- Codon recoding affects the expression and functionality of enzymes in metabolic pathways, potentially leading to imbalances in metabolite production. These imbalances could result in the accumulation of toxic intermediates or by-products that harm the organism or its surroundings. For example, altered expression levels of key enzymes might disrupt feedback regulation, causing metabolic pathways to operate inefficiently or erratically.
- The impact of metabolic changes extends beyond the organism itself. If recoded organisms are released into the environment, their altered metabolite profiles could affect local ecosystems by disrupting nutrient cycles, altering microbial community dynamics, or introducing toxic compounds into food webs.
- While altering a single codon often has predictable effects, recoding an entire genome introduces complex and interdependent changes that are more difficult to anticipate. Systems biology approaches and comprehensive metabolic modeling are necessary to evaluate the full range of potential outcomes.

- Impaired Biocontainment:

- Codon recoding is often employed to enhance biocontainment, making synthetic organisms incompatible with wild-type viral infections or preventing gene transfer. However, unexpected interactions between synthetic alterations and environmental factors could compromise these containment measures. For example, exposure to novel stressors or microbial communities might trigger adaptive responses that undermine biocontainment.
- Recoded organisms may be genetically predisposed to acquire compensatory mutations that restore compatibility with the natural genetic code or bypass engineered safeguards. This genetic accessibility could facilitate the integration of foreign DNA, re-establishing HGT potential and increasing the risk of synthetic traits spreading to native populations.

- Environmental conditions, such as high microbial diversity or elevated levels of horizontal DNA exchange, could exacerbate these risks. (Sau et al. 2005) Ensuring robust biocontainment requires designing additional layers of safeguards, such as dependency on synthetic amino acids or self-destruct mechanisms triggered by specific environmental cues.

- Emergent Ecosystem Dynamics:

- If recoded organisms escape into the environment, they might interact with native species and ecosystems in unexpected ways. For example, their altered codon usage could lead to competitive exclusion of closely related species or shifts in microbial community structure.
- Recoded organisms might also act as novel reservoirs for genetic material, influencing evolutionary trajectories in natural populations. If native species acquire recoded genetic elements, this could lead to hybrid organisms with unpredictable fitness or ecological roles.
- These emergent dynamics underscore the importance of understanding not just the immediate effects of recoded organisms but also their long-term ecological implications.

- Unanticipated Cross-Species Effects:

- Recoded organisms could interact with higher organisms, such as plants or animals, through mechanisms like colonization or biofilm formation. Altered metabolic or genetic traits might affect symbiotic relationships, such as those between microbes and plant roots, potentially impacting agricultural systems.
- If recoded organisms are used in industrial or medical applications, accidental exposure to humans or livestock could result in unforeseen physiological effects, especially if novel metabolites or synthetic amino acids are produced.

Summary

When evaluating the biosafety of recoded genomes, it is critical to assess the genetic stability of recoded sequences, their compatibility with native genetic machinery, and the potential impacts on ecosystems and human health. These considerations must be integrated into a comprehensive framework that anticipates and mitigates plausible pathways-to-harm, such as unintended evolutionary reversion, interaction with viral pathogens, and metabolic imbalances. Each of these pathways has distinct implications for the safe and sustainable application of recoded genomes in various fields.

For example, the potential for unintended evolutionary reversion highlights the importance of maintaining the integrity of recoded sequences over time. Spontaneous mutations or selective pressures could restore compatibility with the standard genetic code, undermining the engineered safety features. Similarly, interactions with viral pathogens raise complex questions about the longevity and robustness of viral resistance in recoded genomes. Evidence suggests that viral evolution may allow phages to adapt to recoded genetic codes, bypassing barriers initially thought to be insurmountable. This could lead to the emergence of novel phages with broader host ranges, increased virulence, or other pathogenic traits that pose indirect risks to ecosystems and human health.

These risks, while somewhat speculative, emphasize the need for ongoing monitoring and evaluation of long-term evolutionary interactions between recoded organisms and their viral predators. Such monitoring should include experimental studies to track adaptation patterns and computational models to predict the dynamics of these interactions under various environmental conditions. Addressing these potential risks proactively ensures that recoded organisms can be deployed safely while minimizing unforeseen consequences.

The overarching take-home message is that genetic recoding strategies offer a powerful and versatile tool for engineering novel traits, such as viral resistance and enhanced biosafety. However, their ecological and evolutionary implications demand careful consideration. The possibility of unintended viral adaptations or other unforeseen effects underscores the importance of a balanced approach. This approach should maximize the benefits of recoded genomes, such as improved biocontainment and novel biological functionalities, while implementing robust risk management strategies to mitigate potential harms. By doing so, the field can continue to innovate responsibly, ensuring that recoded organisms contribute positively to science, industry, and society.

4. Minimal genomes carrying only essential sequences

Minimal genomes, which contain only the sequences essential for life, represent a cornerstone of synthetic biology research, focusing on understanding the fundamental requirements for cellular survival. These streamlined genomes are stripped of non-essential genes, retaining only those critical for core biological functions such as DNA replication, transcription, translation, and cellular maintenance. The ultimate objective is to create simplified organisms capable of surviving under controlled conditions with a minimal set of genetic instructions, shedding light on the essence of life at its most basic level.

Minimal genomes provide a powerful platform for investigating core biological processes and designing highly specialized organisms tailored for specific applications. They offer invaluable insights into gene essentiality, metabolic flexibility, and evolutionary adaptability. By identifying the bare minimum of genes required for life, scientists can more precisely study the functional roles of individual genes and the interplay between genetic elements, revealing how organisms maintain viability despite significant reductions in genetic complexity.

One of the most prominent examples in this field is *Mycoplasma mycoides* JCVI-syn3.0, a synthetic organism created in 2016 with a genome containing only 473 genes, in contrast to the 985 genes present in its parent strain. (Hutchison et al. 2016) This organism was developed using a "top-down" approach, which involved systematically removing non-essential genes from a larger genome, providing detailed functional insights into gene roles and redundancies. Alternatively, the "bottom-up" method involves assembling genomes from scratch, incorporating only the essential genes necessary for life. This technique offers even greater precision and customization, enabling researchers to design organisms with specific functionalities while excluding unwanted genetic elements. (Kumar et al. 2023)

An emerging concept in the field is the "middle-out" approach, which blends aspects of top-down and bottom-up methodologies. Middle-out strategies typically begin with a partially reduced genome or a modular genetic system, iteratively modifying it to achieve minimal functionality while integrating desired traits. Although this approach is gaining attention as a flexible and efficient strategy for genome minimization, its relevance to biosafety considerations within the context of this report is limited. The potential risks associated with middle-out approaches – such as genetic instability, unintended interactions, or ecological impacts – are not unique and align closely with those posed by top-down and bottom-up methods. Consequently, these risks are already addressed in broader discussions of genome engineering approaches, rendering further elaboration on middle-out methodologies unnecessary in this context.

The study of minimal genomes extends beyond academic curiosity, finding practical applications in biotechnology and industry. Organisms with reduced genomes can be engineered for efficient production of biomolecules, such as proteins, biofuels, or pharmaceuticals. Their streamlined nature minimizes the risk of unwanted gene expression, off-target interactions, or adverse environmental impacts, making them ideal candidates for safe, non-pathogenic platforms in drug manufacturing or biocontainment strategies. (Vickers 2016) Furthermore, minimal genomes often exhibit enhanced genetic and metabolic stability, a feature highly desirable in industrial bioprocesses where reliability and predictability are paramount.

Research into minimal genomes continues to push the boundaries of our understanding of life, offering a pathway to creating highly efficient and specialized organisms for a wide range of scientific, industrial, and therapeutic purposes. By stripping life down to its essentials, minimal genome studies not only illuminate the intricacies of biological systems but also pave the way for innovative solutions to some of the most pressing challenges in synthetic biology and biotechnology.

4.1 *"Top-down" specific* ***'points-to-consider'*** *and* ***plausible 'pathways-to-harm'***

In top-down approaches, the starting point is an existing natural genome, typically from a well-characterized organism. Non-essential genes and regulatory elements are systematically removed through iterative experiments, leaving only those sequences that are absolutely necessary for the organism's survival and basic functionality. However, this approach introduces unique biosafety considerations that must be addressed.

Points-to-Consider

- Genetic Stability and Evolution:

- The removal of non-essential genes and regulatory elements can alter the genome's stability over time, making it more susceptible to mutations. Reduced genomic content may leave fewer buffers against deleterious mutations, potentially amplifying their impact on the organism's viability and functionality. This raises concerns about how the genome will maintain its stability across generations or under environmental stressors. (Moger-Reischer et al. 2023)
- Evolutionary dynamics in minimal genomes may differ significantly from their more complex counterparts. Genetic drift, selective pressures, or adaptive mutations could lead to the reacquisition of previously removed functions or entirely new traits, potentially altering the organism's intended properties. For instance, adaptive mutations might improve fitness but could also introduce unintended ecological interactions or biosafety risks. (Mehta et al. 2019)

- Viability and Fitness in Various Environments:

- Minimal genomes are typically engineered for controlled environments, such as laboratory or industrial settings. Assessing their ability to survive, compete, or adapt in non-laboratory conditions is crucial. This includes evaluating their metabolic limitations, resistance to environmental stressors, and potential interactions with natural ecosystems. (Kurokawa et al. 2016)
- Despite their reduced genetic content, minimal organisms could still interact with native microbial populations, competing for resources or forming novel relationships. For example, streamlined metabolic networks might allow them to exploit specific niches more efficiently than native organisms, potentially disrupting microbial community dynamics. (Mizoguchi, Mori, and Fujio 2007; Kolisnychenko et al. 2002)
- Reduced genomes may also create vulnerabilities that allow them to serve as niches for viruses, bacteriophages, or opportunistic pathogens. Understanding whether minimal

organisms could inadvertently support the proliferation of these pathogens is essential for evaluating their ecological impact.

- It is also important to assess how the simplified genome impacts the organism's ability to maintain homeostasis under fluctuating environmental conditions. Any unanticipated survival advantages could lead to persistence in unintended settings.

- Horizontal Gene Transfer:

- Although minimal genomes lack many non-essential genes, the risk of HGT remains a critical consideration. Simplified genomes may still interact with mobile genetic elements such as plasmids, transposons, or phages, facilitating gene exchange with other organisms. (diCenzo et al. 2014)
- Minimal genomes may also acquire adaptive genes from wild populations through HGT, potentially altering their behavior or ecological role. Conversely, they could donate critical genetic elements, such as antibiotic resistance genes, to native species, leading to unintended ecological or medical consequences.
- The extent to which minimal genomes can participate in HGT is influenced by their genome architecture and compatibility with existing gene exchange mechanisms. Rigorous testing is needed to understand the potential for gene flow between minimal organisms and their surroundings. (S. Ma et al. 2022)

- Containment Measures:

- The minimal nature of these genomes necessitates robust physical and biological containment strategies, particularly when they are used in open environments or industrial applications. Physical containment may include secure laboratory facilities or bioreactors, while biological containment can involve engineered safeguards such as auxotrophic dependencies or temperature-sensitive growth systems.
- Synthetic safeguards, such as reliance on non-standard amino acids or metabolic pathways, can enhance biocontainment by preventing the organism from surviving in natural environments. These safeguards should be rigorously tested to ensure they remain effective under diverse conditions.
- The reduced complexity of minimal genomes may affect their resilience to containment failures. For example, simplified genomes might have fewer redundancies, making them less adaptable to sudden environmental changes, which could also mean that they are easier to contain under specific conditions.

- Impact on Human Health and the Environment:

- The potential for minimal genomes to produce metabolic by-products must be carefully evaluated, particularly if these organisms are intended for industrial, agricultural, or environmental applications. By-products could affect soil chemistry, water systems, or plant health, with cascading effects on local ecosystems. (Choe et al. 2016)
- The immunogenic or allergenic potential of minimal genomes or their derivatives must be considered, especially in applications involving food production, pharmaceuticals, or probiotics. For example, the removal of specific genes might inadvertently expose immunogenic epitopes, triggering adverse reactions in humans or animals.
- Minimal genomes used in environmental remediation should be tested for unintended ecological effects, such as altering nutrient cycles or introducing metabolic imbalances in microbial communities. Ensuring that these organisms degrade harmlessly after fulfilling their purpose is an important safety consideration.

- Reintroduction of Functional Genes:

- In some cases, genes previously deemed non-essential might be reintroduced into the genome to enhance specific functionalities, such as increased production of a desired compound or better adaptation to industrial conditions. These reintroductions must be carefully assessed for their potential to affect genetic stability, interactions with native species, or environmental safety.
- The process of reintroducing genes also raises questions about the definition of "minimal" and the implications for biosafety assessments. Modified genomes may no longer fit the strict definition of minimal, necessitating adjusted evaluation criteria.

- Evolutionary Monitoring:

- Long-term monitoring of minimal genomes in both controlled and open environments is essential to track evolutionary changes and assess their implications for safety and efficacy. Genomic surveillance can identify emerging mutations, HGT events, or adaptations that could affect the organism's intended applications or biosafety profile.
- Evolutionary modeling can also provide valuable insights into the potential trajectories of minimal genomes, helping to predict risks and inform containment strategies. (Sandberg et al. 2023)

Plausible Pathways-to-Harm

- Disruption Due to Escape:

- If a minimal genome escapes its intended containment, it might establish itself in new environments, even with its reduced genetic repertoire. The streamlined nature of its genome could allow it to exploit specialized ecological niches that are less accessible to more complex organisms. This could lead to significant disruptions in local microbial ecosystems, potentially outcompeting native species or altering existing community dynamics.
- The ecological impact of such an escape could be particularly pronounced if the minimal organism affects critical nutrient cycles. For example, changes in nitrogen fixation or carbon cycling caused by the organism's metabolic activity could have cascading effects on the availability of nutrients for plants and other organisms. These disruptions might affect agricultural productivity or natural ecosystems.
- Even if the minimal genome does not directly compete with native species, its presence could influence microbial population structures by altering resource availability or promoting the growth of other microorganisms with complementary metabolic pathways.

- Acquisition of New Genes via Horizontal Gene Transfer:

- Despite their reduced genomes, minimal organisms may still acquire new genetic material from other microorganisms through HGT. This risk is heightened in environments rich in mobile genetic elements, such as plasmids or transposons, or in areas with high microbial diversity, like soil or aquatic ecosystems.
- The acquisition of new genes could lead to the emergence of unintended traits, such as antibiotic resistance, enhanced metabolic capabilities, or pathogenicity. Such traits could make the organism more difficult to control or manage, particularly in open-environment applications like bioremediation or agriculture.
- If the minimal organism acquires genes that enhance its fitness, it could develop novel adaptations that allow it to thrive in unintended environments. This might lead to persistence in the ecosystem, further complicating containment efforts and amplifying its ecological impact.

- Emergence of Compensatory Mutations:

- The removal of non-essential genes in minimal genomes may create selective pressures that drive the evolution of compensatory mutations. These mutations could restore lost functions or introduce entirely new traits, potentially altering the organism's behavior in unpredictable ways.

- Such mutations might enhance the organism's resilience to environmental stressors, such as temperature fluctuations, nutrient scarcity, or exposure to toxins, enabling it to survive in conditions where it was not originally intended to persist.
- Compensatory mutations might also interact with the remaining genetic elements in the genome, creating emergent properties that were not part of the original design. For example, these changes could influence metabolic pathways, stress response mechanisms, or interactions with other organisms in the environment.

- Interactions with Pathogens or Opportunistic Infections:

- Minimal genomes, by design, often lack genes related to immune-like responses or other defense mechanisms. This reduction may make them more susceptible to infections by viruses, bacteriophages, or opportunistic bacteria.
- In industrial or agricultural settings, infections in minimal organisms could compromise production systems, leading to significant economic losses. For example, a pathogen that adapts to infect a minimal genome organism used in fermentation processes could disrupt production lines and require costly remediation.
- Furthermore, the use of minimal organisms as hosts might create novel evolutionary pressures on pathogens, enabling them to adapt and potentially expand their host range. This could lead to the emergence of new pathogen strains with implications for human, animal, or environmental health.

- Impact of Metabolic Changes on the Environment:

- The removal of non-essential genes can lead to altered metabolic pathways in minimal organisms, potentially resulting in the production or accumulation of unexpected metabolic by-products. For instance, reduced metabolic flexibility might cause the organism to rely more heavily on specific pathways, leading to overproduction of organic acids, gases, or other waste products.
- These by-products could affect the surrounding environment by altering soil pH, changing aquatic chemistry, or contributing to air pollution. For example, excessive production of certain gases might influence greenhouse gas levels, while acidic by-products could harm plant roots or soil microbiota.
- The downstream effects of these changes could extend to other organisms sharing the same environment, potentially disrupting ecosystems by harming plants, animals, or other microbes. For instance, shifts in microbial community dynamics caused by altered nutrient availability could cascade through the food web, impacting larger ecological processes.

- Interactions with Symbiotic or Commensal Organisms:

- Minimal genomes may inadvertently affect symbiotic or commensal relationships within ecosystems. For example, their simplified metabolic capabilities might alter mutualistic interactions with plants, such as those seen in rhizosphere microbial communities.
- The presence of minimal organisms could also shift the balance of microbial communities, favoring species that benefit from the metabolic by-products of the minimal genome or disrupting pre-existing ecological networks.

- Persistence and Degradation in the Environment:

- While minimal genomes are designed to be less adaptable, their persistence in the environment should be evaluated, especially under varying conditions. Their reduced complexity might make them more biodegradable, but this is not guaranteed, particularly if compensatory mutations enhance their survivability.
- Understanding how minimal genomes degrade or are removed from ecosystems is crucial for assessing their long-term environmental impact and ensuring that they do not inadvertently accumulate or spread.

Summary

Top-down approaches to developing minimal genomes involve systematically removing non-essential genes and regulatory elements from existing natural genomes. While these streamlined genomes offer numerous advantages for controlled applications in biotechnology, medicine, and industry, they also introduce unique biosafety considerations. One of the most significant concerns is genetic stability. The removal of non-essential genes can make these genomes more susceptible to mutations, which could lead to unpredictable evolutionary outcomes. These mutations might restore lost functions or create new traits, potentially compromising the organism's intended function or safety. The reduced genetic content also increases the risk of deleterious mutations having a greater impact on viability, requiring careful monitoring of evolutionary dynamics over time.

Another critical aspect is the viability and fitness of minimal genomes in various environments. Although these organisms are typically designed for controlled conditions, they may still interact with native microbial populations if they escape containment. These interactions could result in competition for resources, formation of novel relationships, or disruptions to microbial community dynamics. Additionally, the simplified metabolic networks of minimal genomes may make them vulnerable to viruses, bacteriophages, or opportunistic pathogens, raising concerns about potential impacts on industrial or agricultural processes. The potential for minimal genomes to exploit specific ecological niches or alter nutrient cycles further underscores the importance of evaluating their environmental interactions and persistence.

HGT poses another major biosafety challenge. Despite their reduced genetic content, minimal genomes may still acquire or donate genes through interactions with mobile genetic elements, such as plasmids or transposons. This exchange could lead to the emergence of new traits, such as antibiotic resistance or pathogenicity, complicating containment efforts and increasing ecological risks. The potential for minimal genomes to participate in HGT necessitates rigorous testing to assess the likelihood and consequences of gene flow between engineered organisms and their surroundings. This concern is especially relevant for applications in open environments, where the diversity of microbial communities could facilitate unintended genetic exchanges.

Finally, robust containment measures and long-term monitoring are essential for mitigating the risks associated with minimal genomes. Physical and biological containment strategies, such as engineered dependencies on synthetic nutrients or specific growth conditions, can help prevent the survival of these organisms outside controlled environments. Ongoing genomic surveillance and evolutionary modeling are critical for identifying mutations, adaptive changes, or HGT events that could affect the safety and efficacy of minimal genomes. By combining technological innovation with comprehensive risk assessments and adaptive management strategies, the potential biosafety challenges of minimal genomes can be effectively addressed, enabling their responsible development and application.

4.2 *"Bottom-up" specific* ***'points-to-consider'*** *and* ***plausible 'pathways-to-harm'***

Bottom-up approaches involve assembling a minimal genome from scratch, using only the genetic elements required to sustain life. This differs from top-down methods, which reduce an existing genome to its essential parts. (Kumar et al. 2023) Below is a list of considerations and potential risks associated with bottom-up genome construction:

Points-to-Consider:

- Lack of Natural Baseline for Biosafety Assessment:

- Natural baselines offer valuable historical data on an organism's interactions with its environment, such as its host range, pathogenicity, and ecological impact. For bottom-up synthetic genomes, these reference points are absent because the organisms are entirely novel and lack evolutionary or ecological precedents. This absence of a natural baseline complicates predictions about how the organism will behave in real-world settings, respond to environmental stressors, or interact with other species.
- Traditional biosafety frameworks rely heavily on the organism's genetic and ecological context. (Meurisse et al. 2022) In the case of synthetic genomes with novel designs, standard metrics – such as gene essentiality, metabolic outputs, or ecological niche – may not apply. Risk assessments must therefore focus on the engineered functionality of the synthetic genome, such as new metabolic pathways, resistance traits, or engineered dependencies, rather than comparing it to known organisms based on genetic homology.
- To address the absence of natural baselines, alternative biosafety frameworks should be developed: (Hoffmann et al. 2023)
    - **Function-Based Baselines:** Risk assessments should evaluate synthetic organisms based on their operational roles, such as their contribution to nutrient cycling, pollutant degradation, or bioproduction, rather than relying on taxonomic classification or evolutionary history.
    - **Standardized Synthetic Biology Databases:** Developing robust, publicly accessible databases cataloging known interactions, stability profiles, and metabolic characteristics of synthetic constructs would provide a reference framework for evaluating the risks associated with similar designs.
    - **Environmental Context Modeling:** Computational models simulating the ecological impacts of synthetic organisms in diverse environments could provide predictive insights into potential risks, especially in scenarios where experimental data are unavailable.

- Genetic Stability and Robustness:

- The stability of newly constructed genomes must be rigorously evaluated, as their artificial design might lead to unforeseen stability dynamics. (Moger-Reischer et al. 2023) For example, de novo assembled genomes could experience higher mutation rates or genomic rearrangements due to the lack of evolutionary refinements present in natural organisms.
- It is crucial to understand how the synthetic genome responds to mutations, environmental stressors, and interactions with native microorganisms over time. (Sandberg et al. 2023) Assessing genetic robustness helps predict whether the organism will maintain its intended behavior and functionality or diverge unpredictably due to genetic drift or adaptation.
- Monitoring long-term stability under variable environmental conditions is essential to anticipate potential failure points, such as loss of synthetic dependencies or the emergence of unintended traits.

- Interaction with Existing Ecosystems:

- Evaluating potential interactions between bottom-up organisms and native ecosystems is vital. Even with a minimal or highly tailored set of genes, synthetic organisms could impact microbial communities or higher organisms by competing for resources, altering nutrient dynamics, or introducing novel metabolic by-products. (Delaye and Moya 2010)
- These interactions could have cascading effects on ecosystems, disrupting existing balances and influencing biodiversity. For example, a synthetic organism engineered for a specific metabolic function might inadvertently outcompete native species reliant on similar resources, creating ecological imbalances.
- The potential for novel interactions, such as synthetic organisms forming unexpected symbiotic or antagonistic relationships with native species, should also be assessed. These interactions could amplify the organism's ecological impact in unforeseen ways.

- Horizontal Gene Transfer Risks:

- While bottom-up synthetic genomes are often designed with reduced genetic accessibility or reliance on unique genetic constructs, the risk of HGT cannot be entirely eliminated. (T. Wang et al. 2022) Synthetic organisms could still acquire genes from native populations or donate synthetic genetic elements to wild organisms, introducing new traits into ecosystems.
- Evaluating the potential for synthetic genomes to interact with mobile genetic elements, such as plasmids or transposons, is critical. These interactions could facilitate the unintended spread of engineered traits, such as antibiotic resistance or synthetic metabolic pathways, into wild populations.
- Understanding the conditions under which HGT is most likely to occur, such as in high-microbial-diversity environments like soil or water, can inform the design of enhanced

containment measures and risk mitigation strategies. A minimal genome might be less "stress-resistant", and therefore, perhaps somewhat counterintuitively, more easily take up genetic sequences from its surroundings in conditions that might typically not be prone to do so.

- Containment and Control Measures:

- Both physical and biological containment strategies must be tailored to the unique characteristics of bottom-up organisms. (Gibson et al. 2010) Physical containment involves using secure laboratory environments, while biological containment focuses on engineering organisms with dependencies on synthetic amino acids, nutrients, or specific environmental conditions to limit their survival outside controlled settings.
- Effective containment measures are particularly important for bottom-up organisms, as their novel design may lead to unexpected interactions with the environment or an increased likelihood of evolutionary adaptation in uncontrolled conditions. For instance, a synthetic dependency might fail under certain stressors, allowing the organism to persist in unintended habitats.
- Redundancy in containment systems, such as combining multiple biocontainment strategies, can help mitigate the risk of accidental release.

- Ethical and Societal Implications:

- The creation of synthetic life from a minimal genetic set raises significant ethical questions, including concerns about playing a role in the creation of life, the potential misuse of synthetic organisms, and the unintended consequences of their applications. (Hutchison et al. 2016) Addressing these concerns transparently is essential to building public trust.
- Societal concerns should also be addressed through proactive public engagement, including consultations with stakeholders, regulatory bodies, and the general public. This helps ensure that the development and application of bottom-up organisms aligns with societal values and safety standards.
- The equitable distribution of benefits from synthetic biology applications, such as those in medicine or agriculture, should also be considered, as well as potential disparities in access or unintended socio-economic consequences.

- Functional Validation and Reliability:

- Ensuring that the synthetic genome performs its intended functions consistently under varied conditions is a key priority. (Gibson et al. 2008) Functional validation involves testing the organism's behavior across different stressors, environments, and resource availabilities to confirm its reliability and predictability.

- Understanding the limits of engineered functionalities, such as synthetic metabolic pathways or resistance traits, helps identify scenarios where the organism might fail or behave unexpectedly.

- Long-Term Ecological Implications:

- Beyond immediate risks, the long-term ecological impacts of releasing bottom-up organisms should be modeled and monitored. This includes their potential to integrate into existing ecosystems, alter biodiversity, or influence evolutionary trajectories in native populations. (Choe et al. 2016)
- Experimental studies and pilot-scale deployments under controlled conditions can provide valuable data to predict the long-term behavior of synthetic organisms and refine biosafety measures.

Plausible Pathways-to-Harm:

- Survival Outside Intended Environments:

- Despite their streamlined genetic design, bottom-up synthetic organisms could potentially adapt to new environments if released accidentally. (Beeckman and Rüdelsheim 2020) Their novel features may enable them to establish themselves in ecological niches where competition is limited or where they can exploit unique resources. This could lead to unanticipated ecological persistence.
- Even with reduced metabolic capabilities, if the organism encounters conditions that align with its engineered strengths, it could become invasive. For example, it might dominate nutrient-poor environments where native species struggle, leading to the displacement of native microorganisms and disruptions in ecosystem balance.
- Additionally, the simplicity of a minimal genome might confer resilience against certain stressors, such as antibiotic exposure or nutrient scarcity, which could enhance its ability to survive in unintended environments.

- Gene Acquisition from the Environment:

- Minimal genomes are not immune to HGT, and interactions with surrounding microorganisms could lead to the acquisition of new genetic material. (Bock 2017) This could provide the synthetic organism with traits it was not originally designed to possess, such as antibiotic resistance, enhanced metabolic flexibility, or environmental tolerance.

- Such acquired genes might allow the organism to exploit resources or interact with native species in unintended ways. For instance, acquiring genes for toxin production or pathogenicity could lead to harmful interactions with plants, animals, or humans.
- Gene acquisition could also undermine the biocontainment strategies built into the organism. For example, the acquisition of native regulatory elements might bypass synthetic dependencies, enabling the organism to survive without the controlled conditions intended for its containment.

- Pathogenicity Due to Unintended Mutations:

- Bottom-up minimal genomes, particularly those assembled from scratch, may lack the evolutionary refinements that confer genomic stability in natural organisms. (Lam and Keeney 2015) This could make them more prone to spontaneous mutations, especially in stressful or variable environmental conditions.
- Mutations could alter the organism's behavior in unforeseen ways, potentially introducing traits that increase virulence or pathogenicity. For example, a mutation in a regulatory gene could lead to overproduction of metabolites that are toxic to host organisms.
- While initial design efforts aim to minimize such risks, the long-term evolutionary stability of synthetic genomes must be rigorously tested, including under stress conditions that mimic natural environments. This includes assessing the likelihood of the organism evolving harmful traits over multiple generations.

- Unexpected Metabolic By-products:

- The metabolic pathways of bottom-up organisms, while simplified, may interact with environmental resources in unanticipated ways, leading to the production of unexpected by-products. These by-products could be toxic to other organisms, alter soil or water chemistry, or disrupt nutrient cycles.
- For instance, if the synthetic organism produces organic acids or gases as metabolic waste, this could lower soil pH or contribute to greenhouse gas emissions, affecting local ecosystems. Similarly, accumulation of specific metabolites might inhibit the growth of native plant or microbial species. (T. Wang et al. 2022)
- The risk of unanticipated by-products underscores the need for comprehensive metabolic profiling under various environmental conditions to identify potential hazards before deployment.

- Incompatibility with Natural Microbial Communities:

- The introduction of a bottom-up minimal genome into natural environments could disrupt microbial community dynamics. Even with limited metabolic capabilities, the organism might compete with native microorganisms for essential resources, such as carbon, nitrogen, or phosphorus.

- Resource competition could deplete critical nutrients, leading to imbalances in microbial populations and affecting the broader food web. For example, a shift in microbial community structure might influence nutrient availability for plants or higher organisms.
- Beyond competition, the synthetic organism might introduce novel interactions, such as forming new symbiotic relationships or acting as a novel substrate for microbial predators or parasites. These interactions could ripple through the ecosystem, affecting its overall stability.

- Ecological Niches Created by the Organism:

- The bottom-up synthetic organism might inadvertently create new ecological niches by altering local conditions, such as nutrient availability or environmental chemistry. This could promote the growth of opportunistic species, including harmful microorganisms or invasive species, further disrupting the ecosystem. (Borges et al. 2022)
- For instance, if the synthetic organism secretes metabolites that inhibit certain native species, this could provide an advantage to other organisms that are otherwise less competitive, altering community dynamics.

- Emergence of Evolutionary Hotspots:

- Minimal genomes might act as evolutionary hotspots in microbial communities, promoting genetic exchanges and adaptation. The presence of synthetic organisms could increase the frequency of horizontal gene transfer among native species, indirectly accelerating the evolution of new traits, including antimicrobial resistance (Botelho, Grosso, and Peixe 2019) or environmental persistence.
- These evolutionary interactions could have far-reaching consequences, potentially affecting ecosystems beyond the immediate vicinity of the synthetic organism.

- Potential for Hybrid Organisms:

- Through HGT or recombination, synthetic organisms might exchange genetic material with native species, leading to the creation of hybrid organisms with unpredictable traits. These hybrids could inherit characteristics from both ancestor lineages, potentially combining the invasiveness of the synthetic genome with the ecological adaptability of the native species.
- Hybrid organisms might outcompete both their synthetic and natural counterparts, creating long-term ecological challenges.

Summary:

Bottom-up approaches to constructing minimal genomes, where synthetic organisms are assembled from scratch using only essential genetic elements, offer revolutionary opportunities in biotechnology, medicine, and environmental applications. However, these novel organisms introduce unique biosafety challenges due to the lack of natural baselines for risk assessment. Because these organisms are entirely synthetic, they do not have evolutionary or ecological precedents, making it difficult to predict their behavior in real-world environments. Traditional risk assessment methods, which rely on comparisons with known organisms, may not readily apply. As such, alternative frameworks focusing on function-based evaluations, standardized synthetic biology databases, and environmental context modeling are essential for assessing potential risks and ensuring responsible deployment.

One major consideration is again genetic stability and robustness. Artificially constructed genomes may experience higher rates of mutation or genomic rearrangements due to the absence of evolutionary refinements. Understanding how these synthetic genomes respond to environmental stressors, mutations, and interactions with native microorganisms is critical to ensure that they maintain their intended behavior over time. Without careful monitoring, these organisms could evolve unpredictably, potentially gaining new traits that alter their safety profile or intended function. Compensatory mutations or HGT could lead to the acquisition of new genetic material, conferring unexpected metabolic capabilities, antibiotic resistance, or even pathogenicity.

The interaction of bottom-up synthetic organisms with existing ecosystems is another crucial concern. Even with a minimal set of genes, these organisms can compete with native microorganisms for resources, disrupt nutrient cycles, or produce unexpected metabolic by-products that alter soil or water chemistry. Such changes could have cascading effects on microbial communities, plant health, and higher trophic levels. The potential for synthetic organisms to form novel symbiotic or antagonistic relationships with native species further complicates ecological predictions, making it extremely difficult to anticipate long-term ecological risks.

To mitigate these risks, robust containment and control measures are essential. Combining physical containment (*e.g.* secure laboratory environments) with biological containment strategies (*e.g.* synthetic dependencies on non-natural nutrients) can help limit the survival of synthetic organisms outside controlled settings. However, these safeguards must be rigorously tested to ensure they remain effective under diverse environmental conditions. Ethical and societal considerations also play a key role, emphasizing the importance of transparent communication, public engagement, and equitable distribution of synthetic biology's benefits. By addressing these biosafety, ecological, and ethical concerns, researchers can harness the potential of bottom-up minimal genomes while minimizing risks to the environment and society.

5. Reorganized genomes based on pre-designed modules
(in which genes related to e.g. the same metabolic pathway are clustered)

Reorganized genomes based on pre-designed modules, where genes involved in related metabolic pathways are clustered, offer a strategic approach to optimizing cellular function. (Sulheim et al. 2021) By clustering genes that contribute to the same biochemical pathways, synthetic biologists can enhance coordination and streamline regulation of complex processes like biosynthesis, catabolism, and metabolic flux. This strategy mirrors the natural organization seen in many microorganisms, where biosynthetic gene clusters (BGCs) allow for co-regulation and efficient production of secondary metabolites. (Schläpfer et al. 2017) However, in synthetic reorganized genomes, these clusters are pre-designed and engineered to provide a higher degree of control and efficiency.

This approach has multiple advantages. Clustering genes into defined modules reduces the complexity of gene regulation, as all genes within a cluster can be controlled by a single promoter or regulatory element. (Cimermancic et al. 2014) This enables more predictable and synchronized expression of enzymes within a pathway, which can boost the overall efficiency of metabolic processes. It also minimizes unwanted interactions between genes and pathways, often seen when related genes are scattered throughout the genome. The physical proximity of clustered genes can speed up their expression and coordination, resulting in faster responses to environmental changes or metabolic demands.

For instance, synthetic yeast strains have been engineered to include clustered genes for ethanol production, which has significantly improved their biofuel output. (Carreto et al.; Tiukova et al., 2019) Similarly, reorganized bacterial genomes have been used to cluster antibiotic biosynthesis genes, increasing the yield and simplifying the regulatory control of these complex pathways. (Cook and Stasulli 2024) This modular design allows researchers to introduce, remove, and/or adjust entire pathways with relative ease, making these reorganized genomes highly adaptable to different industrial or research needs.

However, encoding biological functions in tightly clustered gene modules also introduces potential challenges. While this arrangement enhances gene expression levels, it may disrupt the more complex regulatory networks that cells use to fine-tune gene activity under varying conditions. Natural gene expression is often modulated by intricate interactions between regulatory elements, signaling pathways, and feedback loops, enabling cells to adapt dynamically to fluctuating environments.

(Diercks et al. 2024) By streamlining regulation through simplified gene clusters, reorganized genomes may sacrifice this adaptability. As a result, cells with modular genomes may become less viable in suboptimal or changing habitats, relying heavily on controlled or ideal conditions to maintain their functionality. This potential limitation highlights the trade-offs between achieving high metabolic efficiency and preserving the natural robustness of cellular regulatory systems.

Moreover, pre-designed modules enable the rapid assembly or reconfiguration of synthetic genomes. If a particular module proves ineffective or if a new metabolic function is desired, the modular design allows for straightforward substitution or addition of genetic elements without disrupting the overall genome structure. Also, gene drives, which promote the preferential inheritance of specific genetic traits, are relatively easy to engineer and incorporate into reorganized genomes. This capability enhances the potential for controlling population dynamics or spreading desired traits in target species. This flexibility makes reorganized modular genomes particularly appealing for applications in biotechnology, pharmaceuticals, and industrial bioprocesses.

In summary, reorganized genomes with clustered genes for specific pathways offer a powerful means of improving metabolic efficiency and control. By combining modular design principles with precise gene clustering, researchers can build synthetic organisms that are not only more efficient but also more adaptable to evolving scientific and industrial challenges. However, careful consideration must be given to the potential loss of regulatory complexity and adaptability, particularly for applications that require robust performance under variable conditions.

Points-to-Consider:

- Stability of Gene Clusters:

- Assessing the stability of clustered gene modules is critical for ensuring that the reorganized genome maintains its intended functionality over time. Gene clusters may be prone to rearrangements, deletions, or mutations, particularly in dynamic environmental conditions or under selective pressures. Such instability could compromise the organism's performance and safety.
- The physical organization of clustered genes may increase their susceptibility to recombination events, particularly in regions with repetitive sequences or homologous recombination hotspots. (Dietrich et al. 2023) Careful design of the clustered modules, such as minimizing repetitive elements, can help mitigate this risk.

- Stability testing under varied conditions – such as temperature shifts, oxidative stress, or nutrient fluctuations – can provide insights into how the clustered genome behaves over time and whether additional safeguards are necessary.

- Regulatory Coherence:

- Clustering genes often involves shared regulatory elements, such as a single promoter or operator controlling an entire pathway. While this can enhance co-regulation and simplify gene expression, it also introduces the risk of unintended regulatory imbalances. (Kolisnychenko et al. 2002) For instance, overexpression of one enzyme in a pathway could deplete cellular resources or lead to the accumulation of toxic intermediates.
- Evaluating whether clustered genes maintain their intended expression levels across diverse environmental conditions and growth phases is essential. This ensures predictable performance and minimizes the risk of metabolic overload or inefficiency.
- The potential for regulatory interference between clustered genes and native regulatory networks should also be assessed. Clustering may unintentionally recruit native transcription factors or regulatory elements, creating cross-talk that disrupts cellular processes.

- Potential for Horizontal Gene Transfer:

- Clustering genes into compact modules makes entire pathways more susceptible to HGT, as they can be transferred as a single functional unit. This increases the likelihood that the clustered genes will confer a competitive advantage to the recipient organisms, particularly if the pathway enhances metabolic efficiency, stress tolerance, or antibiotic resistance. (Sturm et al. 2023)
- The risk of HGT is particularly significant in environments with high microbial diversity and DNA exchange rates, such as soil, water, or the (human) microbiomes. Evaluating the likelihood and impact of HGT in these settings is crucial for designing safer reorganized genomes.
- Since clustered genes are more likely to retain functionality upon transfer compared to dispersed genes, their potential ecological and evolutionary consequences should be carefully modeled. For example, a clustered antibiotic resistance pathway transferred to a pathogen could have severe public health implications.

- Ecological Impact:

- Synthetic organisms with reorganized genomes could have unintended effects on microbial communities and broader ecosystems if released. (Ivanova et al. 2014) For instance, clustered metabolic genes could lead to enhanced production of specific metabolites that alter nutrient cycling, resource availability, or microbial competition.

- The organism’s reorganized genome may enable it to interact with other species in novel ways, such as forming new symbiotic relationships or exerting antagonistic effects on competitors. These interactions could disrupt existing ecological balances and have cascading effects on biodiversity.
- Assessing these potential ecological consequences involves studying how the synthetic organism interacts with native species and simulating its long-term behavior in natural environments. Pilot-scale releases in controlled settings may also provide valuable data for risk assessments.

- Containment and Control Measures:

- Given the potential for unexpected behaviors, robust containment strategies are essential. Physical containment measures, such as secure laboratory environments, should be complemented by biological containment systems. For example, designing the organism to rely on synthetic amino acids or other non-natural nutrients can limit its survival outside controlled conditions.
- The effectiveness of biocontainment strategies, such as gene drives, kill switches, or self-limiting genetic circuits, should be rigorously tested under diverse conditions. Ensuring these mechanisms remain functional over time and under various stressors is critical to preventing accidental release.
- The complexity of reorganized genomes may necessitate multi-layered containment strategies to address potential failures in individual safeguards.

- Potential Impact on Protein Folding and Metabolism:

- The physical proximity of clustered genes can influence the dynamics of protein synthesis, potentially affecting the folding, assembly, and overall functionality of enzymes. (Del Amparo et al. 2023) Rapid or synchronized expression of clustered genes could overwhelm the cell's protein-folding machinery, leading to misfolded proteins, aggregation, or metabolic bottlenecks.
- Evaluating the impact of clustered gene expression on cellular homeostasis is necessary to prevent unintended disruptions. For instance, the accumulation of intermediate metabolites due to overexpression or inefficient processing could be toxic to the cell or alter its interactions with the environment.
- Testing the metabolic pathways of reorganized genomes under various conditions can help identify potential vulnerabilities and guide the optimization of gene clustering strategies.

- Loss of Natural Gene Regulation Sophistication:

- While clustering genes often simplifies their regulation, it may also eliminate the nuanced control mechanisms present in native genomes. For example, natural genomes often use multiple promoters, enhancers, and feedback loops to fine-tune gene expression in response to environmental changes. By relying on a single regulatory element, reorganized genomes may lose this adaptive capacity, making them less robust in fluctuating conditions.
- This loss of regulatory complexity could result in reduced viability or performance in non-ideal habitats, emphasizing the importance of tailoring clustered genomes for their intended application environments.

- Long-Term Evolutionary Dynamics:

- The engineered clustering of genes may influence the organism's evolutionary trajectory, particularly if selective pressures favor rearrangements, deletions, or duplications of the clustered modules. (Kanai et al. 2024) These evolutionary changes could impact the stability and functionality of the synthetic organism over time.
- Understanding how clustered genomes evolve under different conditions can inform the design of more robust constructs and identify potential failure points before deployment.

Plausible Pathways-to-Harm:

- Unintended Transfer of Gene Clusters:

- Gene clustering increases the likelihood of entire functional pathways being transferred to other organisms through horizontal gene transfer. Mobile genetic elements, such as plasmids, transposons, or phages, can facilitate this transfer, enabling wild-type organisms to acquire the clustered gene set.
- The acquisition of new metabolic capabilities by wild populations could have profound ecological consequences. For example, the transfer of antibiotic resistance pathways or enhanced nutrient utilization genes could disrupt microbial community dynamics or create unforeseen competition with native species.
- The clustering of genes may also increase the functional stability of the transferred traits, as the entire pathway is more likely to remain intact and operational, amplifying the ecological risks associated with HGT. These risks are particularly concerning in high-diversity environments, such as soil or aquatic ecosystems, where gene exchange is more prevalent.

- Escape and Proliferation in Natural Environments:

- Synthetic organisms with reorganized genomes could escape containment and establish themselves in natural environments, particularly if their enhanced metabolic pathways provide competitive advantages. For instance, faster nutrient uptake or improved biosynthetic efficiency could allow the organism to outcompete native microbes, leading to disruptions in local ecosystems.
- Such ecological imbalances might affect critical processes like nutrient cycling, soil health, or water quality. For example, synthetic organisms with enhanced nitrogen-fixation pathways could disrupt plant-microbe symbioses or alter the availability of nitrogen in the soil, impacting agricultural productivity or natural vegetation.
- The adaptability of reorganized genomes to diverse environmental conditions should be assessed, as enhanced metabolic functions may allow the organism to persist and proliferate in habitats beyond those intended for its use.

- Metabolic Overload and Toxicity:

- The clustering of genes often results in synchronized overexpression, which can lead to the overproduction of metabolites or intermediates within the cell. (Mizoguchi, Mori, and Fujio 2007) This metabolic overload can impose a significant burden on the organism, potentially leading to cell death or the release of harmful substances into the environment.
- Toxic by-products from metabolic processes could have far-reaching effects on surrounding organisms, including plants, animals, and other microbes. For example, if the organism is

deployed in agricultural or industrial settings, these toxins could contaminate soil or water, harm crops, or disrupt local microbial communities.

- Evaluating the metabolic profiles of reorganized genomes under varying conditions is essential to predict and mitigate the risks of toxicity. This includes assessing the potential for toxic accumulation during large-scale production or environmental exposure.

- Increased Susceptibility to Pathogens:

- Reorganizing metabolic pathways within a synthetic organism may inadvertently increase its vulnerability to pathogens that exploit these pathways. (Rasmussen et al. 2024) For instance, concentrated metabolic activities might attract specific viruses, phages, or parasitic bacteria that rely on similar biochemical processes.
- Pathogens that adapt to exploit the reorganized genome could develop novel traits, such as increased virulence or broader host ranges. These adaptations might not only affect the engineered organism but could also pose risks to other species in the environment.
- The co-evolution of pathogens with reorganized genomes could result in unpredictable interactions, highlighting the importance of monitoring pathogen dynamics in environments where synthetic organisms are deployed.

- Altered Interaction with Native Microbial Communities:

- The enhanced metabolic output of a reorganized genome could disrupt the delicate balance of local microbial communities by altering resource availability or introducing novel competitive dynamics. (Tumolo et al. 2020) For example, the organism might outcompete native microbes for essential nutrients, reducing biodiversity and destabilizing the ecosystem.
- The production of metabolites that inhibit the growth of native species could further exacerbate these effects. Such shifts in community composition might have downstream consequences for higher organisms, such as plants that depend on specific microbial symbionts or animals that rely on microbial ecosystem.
- The synthetic organism's impact on microbial communities should be studied in detail, including its interactions with keystone species and its role in nutrient cycling, to anticipate and mitigate potential disruptions.

- Evolutionary Adaptations of Synthetic Organisms:

- Synthetic organisms with reorganized genomes may evolve to adapt to environmental pressures, potentially altering their behavior or functionality. For example, mutations in regulatory elements or metabolic pathways could enable the organism to optimize its performance in unexpected ways, increasing its ecological impact.

- Evolutionary changes might also compromise the engineered safeguards of the organism, such as dependencies on synthetic nutrients or controlled growth conditions, enabling it to persist in unintended environments.

- Cumulative Environmental Effects:

- If deployed at scale, synthetic organisms with reorganized genomes could have cumulative impacts on the environment. For example, widespread use in agricultural or industrial applications could lead to the gradual accumulation of metabolites, changes in soil chemistry, or shifts in microbial community structures across large areas. (Fontaine et al. 2024)
- Long-term monitoring is essential to understand how these cumulative effects might influence ecosystem stability and to develop adaptive management strategies.

- Potential for Cross-Species Interactions:

- Synthetic organisms might engage in unexpected interactions with other species, such as forming new symbiotic relationships or becoming prey for novel predators. These interactions could introduce additional variables into the ecosystem, with unpredictable outcomes for biodiversity and ecological function. (Holt et al. 2024)
- For instance, the metabolic by-products of reorganized genomes could attract certain insects or animals, altering food web dynamics and creating indirect effects on ecosystems.

Summary

Reorganized genomes based on pre-designed modules, where genes related to the same metabolic pathway are clustered, present a promising approach for optimizing cellular function and enhancing metabolic efficiency. This strategy, which mirrors naturally occurring biosynthetic gene clusters, allows synthetic biologists to streamline regulation and achieve predictable, synchronized expression of enzymes. The modular nature of these reorganized genomes enables rapid assembly, substitution, and reconfiguration of entire metabolic pathways, making them highly adaptable for diverse botechnological applications. This flexibility enhances the potential to develop specialized organisms capable of producing biofuels, antibiotics, and other high-value compounds efficiently.

However, this modular approach introduces significant biosafety and ecological challenges. Clustering genes can simplify regulation but may disrupt the sophisticated control mechanisms inherent in natural genomes. The loss of fine-tuned gene regulation can make organisms with reorganized genomes less adaptable to fluctuating environmental conditions, limiting their robustness and viability outside controlled settings. Additionally, the physical proximity of clustered genes increases the risk of HGT, where entire functional pathways may be transferred to wild-type organisms. Such gene transfer events could confer unintended advantages, such as antibiotic resistance or enhanced metabolic capabilities, leading to ecological imbalances or, ultimately, public health concerns.

The ecological impact of deploying organisms with reorganized genomes is another key consideration. Enhanced metabolic output or the production of novel metabolites may disrupt nutrient cycling, alter microbial community dynamics, or create new ecological niches. These disruptions can cascade through ecosystems, affecting biodiversity and the functioning of natural processes. The potential for synthetic organisms to interact unpredictably with native species, such as forming new symbiotic relationships or supporting novel pathogens, further complicates risk assessments. Long-term studies and controlled pilot releases are still necessary to evaluate these impacts comprehensively.

To mitigate these risks, robust containment and control measures are essential. Combining physical containment (*e.g.* secure facilities) with biological safeguards (*e.g.* synthetic dependencies, kill switches, and gene drives) can help prevent unintended proliferation and ecological disruption. The complexity of reorganized genomes may require multi-layered containment strategies to ensure effectiveness under diverse conditions. Additionally, continuous monitoring of evolutionary stability is critical, as selective pressures may drive mutations or rearrangements that compromise the organism's intended function. By addressing these biosafety challenges, researchers can harness the potential of reorganized genomes while minimizing the risks associated with their deployment.

6. Outlook

The field of synthetic genomes is entering a transformative phase, with substantial advancements anticipated across multiple facets of genome design, engineering, and application. (Coradini, Hull, and Ehrenreich 2020) Over the next five years, the integration of cutting-edge technologies such as artificial intelligence, machine learning, and advanced gene editing tools will accelerate the pace of innovation. (Gallup, Ming, and Ellis 2021) These developments will enable more efficient, precise, and scalable methods for creating synthetic genomes, whether through top-down minimization, bottom-up assembly, or reorganized modular design. (Annaluru, Ramalingam, and Chandrasegaran 2015; Kumar et al. 2023; Vickers 2016)

The implications of these advancements extend far beyond technical achievements. (James et al. 2024) Synthetic genomes are poised to play a pivotal role in addressing pressing global challenges in biotechnology, medicine, industrial manufacturing, environmental sustainability, and agriculture. By expanding our ability to engineer life at its most fundamental level, this field offers unprecedented opportunities to create custom organisms tailored for specific tasks, from producing life-saving therapeutics to mitigating environmental pollution.

However, these possibilities are accompanied by complex biosafety, ethical, and societal considerations. The responsible development and deployment of synthetic genomes will require a balanced approach, combining technological innovation with robust risk assessment, regulatory oversight, and public engagement. Below, the chapter explores expected advancements in synthetic genomes, categorized under **Technical Developments**, **Possible Applications**, and **Biosafety-Related Remarks**, offering a forward-looking perspective on this rapidly evolving field.

These three sub-headings will be applied across five key topics: **synthetic genomes based on natural sequences**, **recoded genomes with altered codon usage**, **minimal genomes from top-down approaches**, **minimal genomes from bottom-up approaches** and **reorganized genomes using pre-designed modules**. For the minimal genomes, the top-down or bottom-up approaches will be dealt with separately as they offer very different future opportunities. For all the five topics, an illustrative list of points to consider has been elaborated. Altogether, they aim to provide a comprehensive framework for assessing the anticipated progress and implications of this transformative technology.

6.1 Synthetic genomes based on natural sequences

The next five years are expected to bring significant advances in the field of synthetic genomes based on natural genome sequences, driven by technical developments, including the integration of artificial intelligence and machine learning. These advancements will likely enable new capabilities in genome design, increase precision in genetic modifications, and expand applications across diverse industries.

Technical Developments:

- Enhanced Genome Design and Optimization with AI/ML:

- AI and ML are becoming integral to synthetic genome design, enabling researchers to predict the outcomes of genetic modifications with greater accuracy and to optimize genome assembly processes. (de Boer and Taipale 2024; Lawson et al. 2021) Advanced machine learning models, such as generative neural networks, will allow for the design of novel sequences that integrate seamlessly with natural genomes.
- These tools will ensure synthetic sequences, like recombination sites or synthetic markers, function predictably without disrupting native biological processes. (Volk et al. 2020) This will facilitate the development of robust synthetic organisms optimized for specific applications, such as bioremediation, biofuel production, or therapeutic interventions.
- AI generated novel genetic sequences will however raise important challenges during risk assessment due to the absence of a reference on which to base it on. New approaches need to be developed further in order to address this as detailed elsewhere in this report.

- Automated DNA Synthesis and Assembly:

- Progress in high-throughput DNA synthesis and error-correction technologies will make it faster and more cost-effective to assemble large synthetic genome. (Seydel 2023) These innovations will enable researchers to efficiently create genomes with targeted synthetic modifications based on natural templates.
- By integrating with AI systems, DNA synthesis workflows will become more streamlined, with optimized assembly pathways and automated troubleshooting of synthesis errors, further reducing the costs and time associated with producing synthetic genomes.

- CRISPR and Gene Editing Advances:

- The evolution of CRISPR-Cas systems, including tools like prime editing and base editing, will enhance the precision of modifications introduced into synthetic genomes. (Binan et al.

2024; Gelsinger et al. 2024; Zhu, Li, and Gao 2020) These advances will allow for the seamless integration of synthetic sequences, such as recombination sites, with minimal off-target effects.

- AI-driven models will further refine these techniques by predicting editing efficiency and identifying the most effective strategies for modifying challenging regions of the genome. (Nesbeth et al. 2016; Wong, de la Fuente-Nunez, and Collins 2023) This synergy will improve the functionality and safety of synthetic organisms.

- Synthetic Biology Workflows with AI-Driven Automation:

- AI will revolutionize synthetic biology workflows by automating genome assembly, characterization, and testing. (Grunberg and Del Vecchio 2020; James et al. 2024; Volk et al. 2020) Bio-design automation (BDA) platforms will employ AI to predict optimal pathways for incorporating synthetic sequences into natural genomes, accelerating the development process and reducing human error.
- These platforms will enable researchers to model metabolic pathways, identify ideal integration points for synthetic genes, and fine-tune the efficiency of synthetic organisms, particularly for industrial applications.

Possible Applications:

- Biomanufacturing and Industrial Biotechnology:

- Synthetic genomes with precise modifications, such as recombination sites, will play a pivotal role in biomanufacturing high-value chemicals, pharmaceuticals, and biofuels. (Venetz et al. 2019) Fine-tuning metabolic pathways through synthetic modifications will increase production efficiency, lower costs, and yield higher outputs.
- For instance, engineered microorganisms with synthetic genomes could be optimized for enzyme production or bioenergy generation, serving industries ranging from agriculture to pharmaceuticals. Synthetic sequences will make these organisms adaptable to various production conditions, enhancing their utility.

- Biocontainment and Environmental Applications:

- Biocontainment strategies will advance significantly through synthetic genomes incorporating site-specific recombination sequences and genetic "flags" to prevent the uncontrolled spread of engineered organisms. (Elmore et al. 2023; Kuhlman and Cox 2010) This will enable safer applications in open environments, such as bioremediation or carbon capture.

- Fail-safe mechanisms, such as reliance on synthetic nutrients or activation through external triggers, will ensure synthetic organisms survive only under controlled conditions. These strategies will make engineered organisms suitable for agricultural applications like soil conditioning or pest control while mitigating ecological risks.

- Medicine and Gene Therapy:

- Synthetic genomes based on natural sequences will become increasingly important in gene therapy and personalized medicine. Sequences enabling precise control over gene expression and integration will facilitate the development of therapies for genetic disorders.
- For example, synthetic genomes could be designed to deliver therapeutic genes to patient-derived cells with high precision, improving the safety and efficacy of gene-editing treatments. (Sedlmayer, Aubel, and Fussenegger 2018) AI-powered models will enhance the identification of effective sequences for therapeutic applications, ensuring optimal outcomes.

- Synthetic Vaccines and Antiviral Strategies:

- Synthetic genomes will revolutionize vaccine design, allowing for the creation of vaccines with enhanced safety profiles by removing pathogenic elements while preserving immunogenic properties. (Nouën et al. 2017) Genetic "flags" could track vaccine strains, ensuring safety and traceability during production and deployment.
- Additionally, synthetic genomes could disrupt viral replication by introducing genetic elements that target viral processes. These organisms could find applications in medical and veterinary settings, reducing viral loads in specific hosts or environments.

Biosafety-Related Remarks:

- Predictive Biosafety Assessments Using AI:

- AI tools may play a crucial role in enhancing biosafety assessments for synthetic genomes in the future. Machine learning models will predict the behavior of synthetic organisms under diverse environmental conditions, simulating potential ecological interactions and risks before deployment. These predictions in turn will inform the design of safer genomes and biocontainment strategies.

- Minimizing Horizontal Gene Transfer Risks:

- Advances in synthetic genome design will focus on reducing the likelihood of HGT by introducing genetic safeguards, such as synthetic dependencies and kill switches. These mechanisms will prevent the unintended transfer of synthetic traits to wild-type organisms, a critical biosafety consideration for environmental and medical applications.

- Long-Term Evolutionary Monitoring:

- Synthetic organisms will require ongoing monitoring to assess their evolutionary stability and ensure that genetic modifications remain contained over time. Genome tracking systems, such as genetic "flags", will provide tools to monitor and evaluate changes in synthetic organisms, ensuring continued alignment with biosafety protocols.

- Balancing Robustness and Containment:

- Synthetic genome designs must strike a balance between robustness and containment. While engineered organisms need to be efficient and adaptable for industrial and medical applications, their survival must be limited to controlled environments to prevent ecological disruptions. Developing sophisticated containment measures will remain a priority.

6.2 Recoded genomes with altered codon usage

Significant advancements in the field of recoded genomes with altered codon usage are anticipated. These innovations will enable more efficient genome editing, enhanced precision in genetic design, and the expansion of applications in biotechnology, medicine, and industrial processes. (Mukai et al. 2017) Key expectations are outlined below.

Technical Developments:

- AI/ML-Driven Codon Optimization:

- AI and ML are becoming essential for optimizing codon usage in recoded genomes. Machine learning models trained on large datasets of gene sequences and protein expression profiles will provide insights into how codon substitutions affect protein folding, structure, and cellular fitness.
- By analyzing data from diverse species, ML algorithms will recommend codon substitutions that enhance translation efficiency, minimize errors in protein synthesis, and reduce metabolic burdens on engineered cells. (T. Singh et al. 2021) For example, deep learning frameworks will identify rare codons that impede translation and suggest optimal replacements to maintain or improve protein expression levels.
- Predictive AI models will play a crucial role in designing resilient synthetic genomes, avoiding codon pairs or combinations that lead to translational stalling or inefficiencies. This precision will be particularly valuable for high-throughput DNA synthesis and genome recoding efforts aimed at maximizing industrial yields.

- High-Throughput DNA Synthesis and Genome Editing:

- Advances in DNA synthesis technologies will enable the cost-effective rewriting of large genomes. (Fredens et al. 2019) As synthesis costs decline, researchers will be able to systematically replace natural codons with optimized alternatives across entire genomes.
- Innovations in genome editing, such as prime editing and base editing, will allow precise modifications of codons without introducing unintended mutations. (Zürcher et al. 2023) These techniques will support the incorporation of novel amino acids and the creation of organisms with modified protein structures tailored for specific functions.

- Automation in genome recoding, powered by AI, will accelerate the testing of codon modifications, identifying optimal recoding strategies more efficiently and minimizing experimental trial-and-error.

- Synthetic Biology Platforms and AI-Driven Design Automation:

- Bio-design automation (BDA) platforms integrated with AI will enable virtual simulations of genome recoding efforts, predicting the effects of codon changes on translation rates, protein folding, and metabolic pathways before experimental implementation.
- These platforms will reduce the time and cost involved in designing custom organisms, allowing researchers to create synthetic organisms with tailored traits such as viral resistance or the ability to synthesize complex biomolecules. (Ling, O'Donoghue, and Söll 2015)

Possible Applications:

- Biocontainment and Biosafety:

- Recoded genomes will revolutionize biocontainment strategies by enabling the creation of organisms that are genetically isolated from wild populations. (Zürcher et al. 2022) By eliminating specific codons, synthetic organisms can be engineered to prevent HGT, minimizing risks of genetic exchange with native species.
- This will be especially valuable for environmental applications such as bioremediation, where engineered microbes can degrade pollutants without posing risks to local ecosystems.

- Production of Non-Canonical Amino Acids (ncAAs):

- Altered codon usage facilitates the incorporation of ncAAs into proteins, endowing them with unique chemical functionalities and structures. (Kuo et al. 2018) These ncAAs will enable the creation of novel therapeutics, industrial enzymes, and biomaterials with enhanced stability, activity, or specificity.
- In biomanufacturing, engineered microbes will produce proteins that are difficult to synthesize using natural amino acids, broadening the range of biochemical processes and products available for industries like pharmaceuticals and materials science.

- Therapeutic Applications and Personalized Medicine:

- Recoded genomes will enable advancements in gene therapy and personalized medicine by improving control over therapeutic protein expression. (Kwon et al. 2021) Altering codon usage in therapeutic genes can enhance expression precision, reducing side effects and improving treatment outcomes.

- Synthetic cells and engineered microbes with recoded genomes will act as living factories (M. J. Lajoie, Söll, and Church 2016), producing therapeutic agents directly within the human body in response to specific signals or conditions. This innovation will revolutionize drug delivery and disease management.

- Vaccine Development and Antiviral Strategies:

- Recoded organisms will play a critical role in vaccine production by resisting contamination from common viruses. (V. Singh 2020) By removing codons essential for viral replication, these organisms can serve as robust platforms for producing vaccines and therapeutic proteins.
- Synthetic genomes will also enable the creation of antiviral organisms that disrupt viral replication, offering novel strategies for combating viral infections in medical and veterinary settings.

- Optimized Industrial Microbial Strains:

- Recoded genomes with optimized codon usage will improve the efficiency of industrial microbes, enhancing biofuel production, enzyme synthesis, and specialty chemical manufacturing. (Lau et al. 2017)
- These engineered strains will be tailored for specific production environments, such as high-temperature bioreactors or nutrient-poor conditions, supporting more sustainable and cost-effective industrial processes in sectors like agriculture, food, and materials science.

Biosafety-Related Remarks:

- Minimizing Horizontal Gene Transfer Risks:

- The removal or replacement of specific codons will limit the ability of recoded genomes to exchange genetic material with wild-type organisms, reducing the likelihood of HGT. This genetic isolation will be useful for mitigating biosafety risks in open-environment applications, such as bioremediation or agricultural use.

- Resilience to Evolutionary Pressures:

- Recoded organisms must be designed to resist evolutionary pressures that could restore wild-type codons or compromise engineered safeguards. Long-term monitoring and AI-driven predictive models will be critical for evaluating the evolutionary stability of recoded genomes under diverse conditions.

- Enhanced Containment Mechanisms:

- Recoded genomes will incorporate advanced biocontainment features, such as reliance on synthetic nutrients, temperature-sensitive growth requirements, or kill switches. These measures will ensure that recoded organisms remain confined to controlled environments, minimizing ecological risks.

- Comprehensive Risk Assessments:

- AI tools will enable comprehensive risk assessments by simulating the potential ecological and evolutionary impacts of recoded genomes before deployment. This proactive approach will help address public and regulatory concerns about the safety of synthetic organisms.

- Ethical and Societal Considerations:

- The creation of recoded genomes raises ethical questions about the engineering of life and its potential societal implications. Transparent communication, public engagement, and international collaboration will be essential in the near future to address these concerns and ensure responsible development and deployment of recoded organisms.

6.3 Minimal genomes from top-down approaches

Minimal genomes carrying only essential sequences, particularly those derived through top-down approaches, are expected to experience substantial advancements. (Kim et al. 2024) These developments will facilitate more efficient design, testing, and application of minimal genomes. (Xu et al. 2023) The progress will have wide-ranging implications across biotechnology, medicine, and industrial processes.

Technical Developments:

- Enhanced Identification of Essential Genes Using AI/ML:

- AI and ML are likely to become increasingly indispensable for identifying essential genes critical for life. (L. Wang and Maranas 2018) These technologies can analyze vast genomic datasets to predict the essentiality of genes by evaluating their functions, interactions, and roles in core metabolic pathways.
- Machine learning-based gene essentiality models will allow researchers to streamline top-down genome minimization efforts by accurately identifying genes that can be safely removed without compromising viability. This reduces reliance on experimental trial-and-error, saving time and resources.
- AI can also simulate the impacts of gene deletions on metabolism and growth, enabling predictive genome designs. These simulations will accelerate the development of robust minimal genomes tailored to specific environments or industrial processes.

- Advances in Genome Editing and Assembly Techniques:

- Precision genome-editing tools, such as CRISPR-Cas systems, prime editing (P. J. Chen and Liu 2023), and base editing, will facilitate more accurate and efficient gene deletions. (Peters et al. 2016) These tools will allow researchers to remove non-essential genes while maintaining the integrity of the remaining genome, ensuring the functionality of minimal cells.
- High-throughput DNA synthesis and assembly technologies will enable rapid construction and testing of minimized genomes, allowing researchers to iterate designs more efficiently. This approach will support the development of synthetic cells with optimized genetic content for specific applications.

- As the cost of DNA synthesis continues to decline, creating custom minimal genomes for research or industrial use will become more accessible, enabling diverse applications tailored to unique challenges.

- Integration of Systems Biology and Computational Modeling:

- Systems biology approaches, combined with AI-powered modeling tools, will play a key role in understanding interactions among remaining genes in minimal genomes. (Paklao, Suratanee, and Plaimas 2023) These tools can predict how removing or reorganizing genes impacts cellular metabolism, gene regulation, and overall fitness.
- AI-enhanced metabolic modeling will optimize the energy efficiency and resource allocation of minimal cells, making them more suitable for industrial applications where performance under resource constraints is critical.

- Synthetic Biology Automation and AI-Driven Design:

- AI-driven automation platforms will streamline the creation of minimal genomes by automating genome assembly, gene knockout experiments, and functional testing. These platforms will allow for faster iteration and refinement of designs. (Vazquez-Vilar, Selma, and Orzaez 2023)
- Genome library construction, creating multiple versions of minimal genomes with varying gene sets, will become increasingly efficient. Automated testing of these libraries will help identify optimal configurations for specific applications, balancing genome size with functional robustness.

Possible Applications:

- Biomanufacturing and Industrial Biotechnology:

- Minimal genomes reduce the metabolic burden on cells, enabling them to focus resources on producing target compounds such as biofuels, enzymes, and pharmaceutical. (Freed et al. 2018) This efficiency will lead to higher yields and lower production costs.
- Minimal bacterial strains optimized for biomanufacturing will become integral to producing bioplastics, biochemicals, and other sustainable materials. These streamlined organisms will enhance industrial processes by reducing waste and environmental impact.

- Cellular Models for Drug Testing and Fundamental Research:

- Minimal genomes provide simplified models for studying cellular functions, including gene regulation, metabolic interactions, and responses to environmental changes. (Z. Zhou et al. 2023) These models are ideal for gaining insights into the fundamental principles of life.
- In drug discovery, minimal genomes serve as precise testbeds for evaluating the effects of pharmaceuticals. By eliminating non-essential genes, these models reduce the complexity of interactions, enabling clearer assessments of a drug's impact on essential cellular processes.

- Synthetic Cells for Targeted Applications:

- Minimal cells offer precision and control, making them suitable for applications like biosensing and environmental remediation. (B. Wang et al. 2011) For example, minimal cells can be engineered to detect and neutralize specific environmental pollutants or deliver targeted nutrients to plants.
- In agriculture, minimal microbial strains can promote plant growth or enhance soil health by degrading harmful compounds or facilitating nutrient availability without the risks associated with more genetically complex organisms.

- Biocontainment and Biosafety:

- Minimal genomes are particularly advantageous for biocontainment, as their reduced genetic content may often limit their adaptability and survival outside controlled environments. (S. Zhou et al. 2022) These characteristics make them safer for use in open settings, such as agriculture or environmental restoration.
- Synthetic dependencies, such as requiring non-natural amino acids or specific environmental conditions, can be engineered into minimal genomes to ensure they remain viable only under human-controlled conditions. This increases their safety profile for field applications.

- Gene Therapy and Synthetic Organisms for Medicine:

- Minimal genomes can serve as platforms for therapeutic microorganisms designed to deliver drugs or biologics directly to specific sites within the body. (Y. Y. Chen, Galloway, and Smolke 2012) Their streamlined nature reduces the likelihood of interactions with the host microbiome or immune system.
- These organisms can also be customized for personalized medicine, incorporating genes tailored to individual needs, such as producing enzymes for metabolizing specific compounds or addressing genetic deficiencies.

Biosafety-Related Remarks:

- Enhanced Predictability and Control:

- The simplified nature of minimal genomes improves predictability, reducing the risk of unintended interactions or evolutionary changes. This makes them ideal for applications requiring high levels of control, such as therapeutic delivery or environmental deployment.

- Reduced Horizontal Gene Transfer Risks:

- Minimal genomes are less likely to engage in HGT due to their reduced genetic content and lack of accessory genes. By engineering additional safeguards, such as synthetic dependencies or kill switches, the likelihood of gene transfer to wild populations can be minimized further.

- Long-Term Stability and Monitoring:

- The evolutionary stability of minimal genomes must be closely monitored, particularly in applications involving environmental exposure or prolonged therapeutic use. AI-driven tools can help predict potential mutations and evolutionary trajectories, enabling preemptive design adjustments.

- Adaptability vs. Biocontainment Trade-Offs:

- While reduced adaptability enhances biocontainment, it may limit the utility of minimal genomes in dynamic environments. Balancing robustness and containment will require careful design, ensuring the organism remains functional in intended settings without posing risks to surrounding ecosystems.

- Risk Assessments for Open-Environment Applications:

- Comprehensive risk assessments will be essential for deploying minimal genomes in open environments. These assessments should evaluate potential ecological impacts, including interactions with native species and effects on nutrient cycles, ensuring that minimal genomes contribute positively without causing harm.

6.4 Minimal genomes from bottom-up approaches

Bottom-up approaches to assembling minimal genomes, *i.e.* constructing synthetic organisms from scratch using only the essential sequences required for life, are poised for significant advancements. (Kumar et al. 2023) These developments will enhance the precision, efficiency, and applicability of minimal genome design. Bottom-up approaches to assembling minimal genomes are on the cusp of transformative advancements, enabling the creation of synthetic cells tailored for a wide range of applications. The resulting progress will unlock new opportunities across biotechnology, medicine, environmental science, and fundamental research.

Technical Developments:

- AI/ML-Driven Design of Minimal Genomes:

- AI and ML will likely play a central role in identifying the smallest possible set of genes required for life. By analyzing extensive datasets on gene function, protein interactions, and metabolic pathways, these tools will enable researchers to design minimal genomes from scratch with greater accuracy.
- Generative AI models, such as deep learning frameworks, will explore potential combinations of essential genes, providing insights into how they can be assembled to create stable and functional synthetic cells. (Carrera, Rodrigo, and Jaramillo 2009) These models will reduce the trial-and-error nature of genome design, accelerating the bottom-up assembly process.
- Computational tools will simulate the impact of gene additions or deletions on cellular viability and performance, helping researchers identify the most efficient configurations before actual physical construction.

- Advances in Synthetic DNA Assembly:

- The synthesis of long DNA sequences and complete genomes will become increasingly precise and cost-effective. (M. J. Lajoie, Söll, and Church 2016) Methods like enzymatic DNA synthesis and *in vitro* genome assembly will allow for the construction of large synthetic genomes with unprecedented accuracy.
- Automated genome assembly platforms will streamline the process of combining synthetic DNA fragments, enabling the rapid creation and testing of various minimal genome designs. These platforms will be instrumental in understanding the minimal requirements for cellular life and scaling up production for industrial and research purposes.

- Improved Genome Editing and Integration Techniques:

- Precise genome editing tools, such as CRISPR-Cas systems, prime editing, and base editing, will facilitate the seamless integration of essential genes into synthetic genomes. (K. Chen et al. 2019) These tools will enable researchers to rearrange and optimize gene modules, ensuring functional compatibility and minimal resource use.
- Base editing will allow fine-tuning of nucleotide sequences within minimal genomes, reducing the risk of unintended mutations and improving cellular stability under diverse environmental conditions.

- Automated Functional Validation Using AI:

- AI-driven automation will extend to the functional validation of minimal genomes. High-throughput testing platforms integrated with machine learning algorithms will rapidly assess how well minimal genome designs support essential cellular functions, such as DNA replication, metabolism, and protein synthesis.
- By analyzing experimental data, AI models will identify patterns in cell viability and metabolic performance, guiding iterative improvements to genome designs. This integration will enable scalable and reliable production of synthetic cells tailored to specific applications.

Possible Applications:

- Biomanufacturing and Industrial Applications:

- Bottom-up minimal genomes will become indispensable in industrial biotechnology as highly efficient cell factories. (Bailoni et al. 2023) These organisms, stripped of non-essential genes, can allocate more energy and resources to producing biochemicals, enzymes, or biofuels.
- Minimal cells tailored for specialty chemical or pharmaceutical production will simplify downstream processing and reduce unwanted side reactions. Their streamlined design will make them ideal for use in tightly regulated manufacturing environments, ensuring high-purity outputs.

- Synthetic Life and Fundamental Research:

- Creating minimal genomes from scratch will deepen our understanding of the origin of life and the fundamental principles of cellular biology. By studying synthetic cells, researchers can determine the minimal set of genes necessary for sustaining life and explore how complex traits evolve from simple systems.

- Minimal synthetic cells will serve as model systems for investigating gene function, metabolic pathways, and evolutionary dynamics, providing a simplified framework for testing biological theories and hypotheses.

- Customizable Biosensors and Environmental Applications:

- Minimal genomes are ideal for creating biosensors that detect specific environmental conditions or pollutants with high precision. Their reduced complexity enables tight control over gene expression, allowing them to react specifically to target molecules. (Hürtgen et al. 2019)
- These synthetic organisms could be deployed in environmental monitoring or bioremediation, breaking down pollutants or toxins without the unpredictability associated with more complex engineered organisms. Their minimal nature ensures a targeted and efficient response to environmental challenges.

- Medical Applications and Therapeutics:

- Synthetic cells with minimal genomes will offer a safe platform for therapeutic delivery. (Ausländer, Ausländer, and Fussenegger 2017) Their reduced genetic complexity minimizes the risk of triggering immune responses, making them suitable for gene therapy or drug delivery systems.
- In personalized medicine, minimal cells could be engineered to deliver therapeutic proteins or enzymes directly to specific tissues. Their genomes could be customized to release therapeutic agents in response to specific signals, providing tailored treatments for genetic disorders and other conditions.

- Education and Research Tools:

- Minimal genomes will become valuable educational tools for teaching core concepts in genetics, molecular biology, and synthetic biology. Their simplicity makes them an excellent model for illustrating the essential principles of life.
- These synthetic cells can also serve as testbeds for validating computational models and exploring biological phenomena in controlled laboratory settings, bridging the gap between theoretical predictions and experimental observations.

Biosafety-Related Remarks:

- Enhanced Biocontainment:

- Bottom-up minimal genomes are inherently less adaptable due to their streamlined nature, reducing the likelihood of survival outside controlled environments. This makes them safer for use in applications like bioremediation and agricultural biotechnology.
- Synthetic dependencies, such as reliance on non-natural amino acids or specific environmental triggers, can further enhance biocontainment, ensuring that minimal cells cannot persist without human intervention.

- Minimized Horizontal Gene Transfer:

- The reduced genetic content of minimal genomes decreases the likelihood of horizontal gene transfer to or from native organisms. By excluding accessory genes that facilitate genetic exchange, these organisms can be designed to minimize ecological risks.

- Monitoring Evolutionary Stability:

- Long-term monitoring will be essential to ensure that minimal genomes remain stable and do not acquire mutations that could compromise their safety. AI tools can help predict evolutionary changes and help design synthetic cells that resist adaptive mutations under environmental stressors.

- Predictive Risk Assessments:

- AI-driven risk assessment models will simulate interactions between synthetic cells and natural ecosystems, helping identify potential biosafety concerns before deployment. These simulations will inform the design of safer synthetic organisms and guide regulatory decision-making.

- Ethical and Regulatory Considerations:

- The creation and deployment of synthetic cells raises ethical and regulatory questions, particularly regarding their potential environmental impacts and accessibility. Transparent communication with stakeholders and adherence to international safety standards will be critical to addressing these concerns responsibly.

6.5 Reorganized genomes using pre-designed modules

Reorganized genomes based on pre-designed modules, where genes related to the same metabolic pathway are clustered, are poised for significant advancements as well. (Dymond et al. 2011; Richardson et al. 2017) They could enable the creation of organisms with unparalleled efficiency and functionality. (Luo et al. 2023) These developments could open new opportunities in biotechnology, medicine, industrial applications, and environmental science.

Technical Developments:

- AI/ML-Enhanced Design of Gene Clusters:

- AI and ML could play a pivotal role in optimizing gene cluster design by analyzing vast datasets on gene expression, metabolic interactions, and protein dynamics. (Libbrecht and Noble 2015) These models will predict optimal clustering configurations to enhance metabolic efficiency, reduce regulatory bottlenecks, and minimize unintended interactions.
- Machine learning algorithms will identify ideal promoter sequences and regulatory elements, ensuring synchronized expression of clustered genes. By overcoming challenges such as metabolic burden or misregulation, these tools will enhance the performance and reliability of reorganized genomes.

- Advances in DNA Assembly and Synthetic Genomics:

- Improvements in synthetic DNA assembly technologies, including Golden Gate Assembly and modular cloning systems, will facilitate the physical construction of large DNA constructs containing multiple pre-designed gene clusters. (Nucifora et al. 2023)
- High-throughput synthesis platforms will allow researchers to test various configurations of gene clusters within reorganized genomes. This iterative process will identify the most effective arrangements for boosting metabolic output or enabling specific functions.

- CRISPR and Gene Editing for Module Integration:

- Precise integration of gene clusters will be achieved using advanced CRISPR-Cas systems, prime editing, and other gene editing tools. (Robertson, Funke, de la Torre, Fredens, Wang, et al. 2021) These technologies will allow researchers to insert large gene modules into specific loci without disrupting essential genome functions.
- Gene editing will also enable fine-tuning of clustered gene expression by modifying regulatory sequences, ensuring that the metabolic balance of engineered cells is maintained.

This will help avoid challenges such as energy inefficiency or the accumulation of toxic intermediates.

- Automated Design and Modeling Platforms:

- BDA platforms powered by AI can become central to simulating how different configurations of clustered genes affect metabolic pathways, protein-protein interactions, and overall cellular growth parameters. (Kurokawa et al. 2016)
- These platforms will enable spatial optimization within cells, ensuring that clustered genes are arranged in ways that minimize resource competition while maximizing metabolic efficiency. This capability will improve the stability and performance of synthetic pathways, particularly in industrial and therapeutic applications.

Possible Applications:

- Enhanced Biomanufacturing:

- Reorganized genomes will revolutionize biomanufacturing by clustering genes involved in biosynthetic pathways, thereby improving the efficiency of producing high-value chemicals, biofuels, and pharmaceuticals. (Blount et al. 2023)
- For example, engineered yeast with clustered genes for ethanol production could optimize fermentation processes, while bacteria with clustered antibiotic biosynthesis genes could enhance pharmaceutical manufacturing by increasing yield and simplifying regulation.

- Optimized Microbial Consortia for Environmental Applications:

- Reorganized genomes will enable the development of synthetic microbial consortia specialized in bioremediation tasks. By clustering genes for specific metabolic pathways (Cimermancic et al. 2014), these consortia can break down complex pollutants, recycle nutrients, or process wastewater more efficiently.
- The modular design of gene clusters will allow researchers to customize these consortia for different environmental challenges, such as degrading plastics, detoxifying heavy metals, or restoring soil health in polluted areas.

- Customized Cellular Factories for Personalized Medicine:

- Reorganized genomes with clustered metabolic pathways (Lu et al. 2024) can be used to engineer cells that produce therapeutic proteins or metabolites tailored to individual patient needs.

- These synthetic cells could offer targeted treatments for metabolic disorders, cancer, and rare genetic diseases by delivering therapeutic compounds with high precision. Gene clustering ensures efficient and coordinated production of these compounds within the engineered cells.

- Synthetic Biology and Gene Therapy Platforms:

- Gene clustering will improve the design of therapeutic vectors for gene therapy. Clustering therapeutic genes with their regulatory elements will ensure coordinated and predictable expression in target cells, improving the safety and efficacy of treatments. (Bradley, Buck, and Wang 2016)
- Organ-on-a-chip models incorporating reorganized genomes will enable detailed studies of drug metabolism and disease pathways. These models could include gene clusters relevant to specific diseases, offering insights into therapeutic outcomes in controlled environments. (Sulheim et al. 2021)

- Agricultural Biotechnology:

- Reorganized genomes will enhance agricultural productivity by clustering genes responsible for functions like nitrogen fixation, pest resistance, or nutrient uptake. This approach will create engineered microbes or plants better equipped to withstand environmental stress and improve crop yields. (Schläpfer et al. 2017)
- For example, biofertilizers with clustered genes for plant growth-promoting compounds could reduce reliance on chemical fertilizers, offering a more sustainable approach to agriculture.

Biosafety-Related Remarks:

- Horizontal Gene Transfer Risks:

- The clustering of genes into functional modules increases the risk that entire pathways could be transferred to wild-type organisms via HGT. This could lead to ecological imbalances if transferred traits confer competitive advantages to recipient organisms.
- Strategies to mitigate HGT risks include engineering dependency on synthetic nutrients or environmental conditions, ensuring that the gene clusters cannot function in natural ecosystems.

- Containment and Control Measures:

- Reorganized genomes should include robust containment strategies, such as genetic kill switches or reliance on non-natural amino acids. These features will ensure that synthetic organisms remain confined to intended environments and cannot proliferate if released accidentally.

- Biocontainment strategies will need to be rigorously tested under diverse environmental conditions to confirm their reliability.

- Ecological Impact Assessments:

- The introduction of synthetic organisms with reorganized genomes into natural environments could disrupt microbial community dynamics or alter nutrient cycling. Comprehensive ecological impact assessments will be necessary to evaluate potential risks and develop mitigation strategies.

- Long-Term Stability and Evolution:

- The evolutionary stability of reorganized genomes must be monitored, as gene clusters may be subject to mutations or rearrangements that could compromise their intended function. AI tools will help predict and address these evolutionary challenges.
- Designing redundancy into clustered gene pathways could enhance stability and reduce the likelihood of functional loss due to genetic drift or mutation.

- Ethical and Regulatory Considerations:

- The deployment of reorganized genomes raises ethical questions about synthetic biology's impact on natural ecosystems and public health. Transparent regulatory frameworks and public engagement will be essential to ensure responsible development and use of this technology.

7. Conclusions

The field of synthetic genomes has demonstrated remarkable progress, transitioning from theoretical concepts to practical innovations that hold transformative potential across biotechnology, medicine, environmental science, and agriculture. (Goold, Moseley, and Lauersen 2024; Venter et al. 2022; J. Zhou et al. 2022) From the ability to design natural sequences to the creation of fully synthetic organisms, the innovations discussed in this report highlight the promise of synthetic genomes as tools for addressing global challenges. Yet, these advancements also underscore the complexity of responsibly managing their development and deployment. (Baker and Church 2024)

The need for integrative risk assessments emerges as a recurring theme throughout this report. The cumulative effects of synthetic modifications – on genome stability, ecological interactions, and evolutionary dynamics – necessitate a shift from evaluating isolated genetic elements to considering their combined and emergent impacts. (Fontaine et al. 2024; Holt et al. 2024; Sturm et al. 2023; Tumolo et al. 2020; X. Wang et al. 2014; S. Zhou et al. 2022) Synthetic sequences, especially when clustered or recoded, add layers of complexity that require more comprehensive approaches to assessing risks.

Additionally, researchers must determine whether synthetic modifications amplify risks associated with conventional GMOs or introduce entirely new challenges. Conversely, where risks are shown to be equivalent to or lower than those of conventional GMOs, documenting these findings will streamline regulatory processes and ensure innovation is not unnecessarily hindered. This balance is critical to fostering both safety and scientific progress.

The role of artificial intelligence in advancing synthetic genomics cannot be overstated. (Nesbeth et al. 2016; Wong, de la Fuente-Nunez, and Collins 2023) AI enables the generation of novel sequences without relying on wild-type references, a capability that introduces immense possibilities but also unique challenges. (Rives et al. 2021) As AI-driven approaches suggest unpredictable or poorly understood mutations (Libbrecht and Noble 2015), evidence-based risk assessments could become more complex. These “black box” suggestions, where researchers may not fully understand why a specific mutation was proposed, complicate the evaluation of potential outcomes and their implications. Preparing regulatory frameworks and research methodologies to handle this added uncertainty is imperative.

Long-term monitoring frameworks will play a pivotal role in ensuring the safety and effectiveness of synthetic organisms post-deployment. Tracking the performance and ecological impacts of these organisms will provide adaptive management opportunities, allowing for course corrections as new data becomes available. These frameworks must integrate robust data collection (Corich et al. 2007) , AI-supported analysis, and transparent reporting to address both anticipated and emergent risks.

As with any transformative technology, many of the questions raised in this report simply require further research. While the questions themselves - regarding ecological impact, horizontal gene transfer, or genome stability - are not *per se* new, the novel applications and scale of synthetic genomes demand fresh data and tailored studies. The scientific community must prioritize generating this knowledge base to inform risk assessments and the decision-making processes.

Recommendations for advancing risk assessment methodologies are vital to the responsible development of synthetic genomes. These include:

- Developing integrative frameworks that assess synthetic genomes holistically, accounting for both individual modifications and their cumulative effects.
- Establishing standardized tools and databases to streamline the evaluation of risks associated with synthetic organisms, enabling consistency across regulatory bodies.
- Strengthening collaboration between scientists, regulators, and policymakers to ensure adaptive governance that reflects the dynamic nature of synthetic biology.
- Promoting transparency and public engagement to address societal concerns and build trust in the deployment of synthetic organisms.

In conclusion, synthetic genomes represent a transformative frontier with immense potential to redefine our relationship with biological systems. (Coradini et al. 2023) The advancements anticipated in the near future – spanning technical capabilities, diverse applications, and biosafety innovations – offer a pathway to address some of humanity's most pressing challenges. However, achieving this vision will require a balanced approach that prioritizes rigorous risk assessments, robust regulatory oversight, and the continuous generation of new knowledge. By aligning innovation with safety and societal values, synthetic genomes can fulfill their promise as powerful tools for discovery and progress, while safeguarding ecological and human health.

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