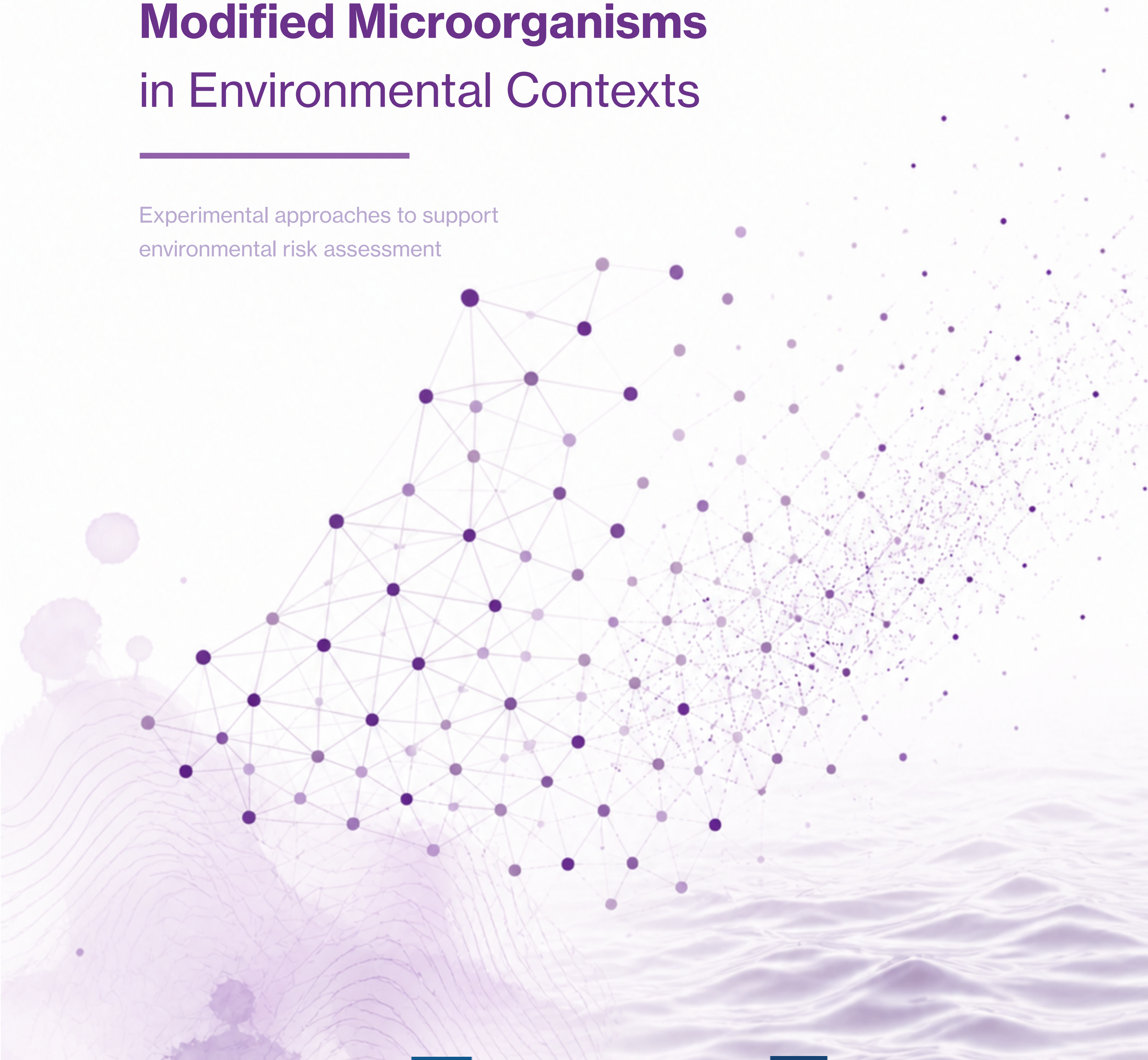
Assessing Survival and Competition of Genetically Modified Microorganisms in Environmental Contexts

Experimental approaches to support environmental risk assessment



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This report was prepared by Sonia Mecacci and Enrique Asin-Garcia, on behalf of the Ministry of Infrastructure and Water Management in the Netherlands (I&W), and the National Institute for Public Health and the Environment of the Netherlands (RIVM). The information in this report is based on sources deemed reliable and on the best knowledge and insights of the authors at the time of writing. The views expressed in this report are the professional opinion of the authors and do not necessarily reflect those of I&W, RIVM, members of the advisory Committee, the Competent Authorities, or the European Commission. It is acknowledged that only the European Court of Justice can give a binding decision on the interpretation of EU law.

Project period

1st May 2025 to 1st May 2026

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Public summary

Genetically modified microorganisms (GMMs) could help tackle environmental challenges, such as cleaning polluted water or monitoring harmful substances. However, their use outside the laboratory is still very limited. One of the main reasons is that we do not yet fully understand what happens to these organisms once they are released into natural environments.

In this report, we explore different ways to study how GMMs behave in real-world conditions. Do they survive? Do they spread? Do they affect other microorganisms? By combining existing scientific knowledge with new laboratory experiments, we identify which methods work well and where important gaps remain.

Our results show that there is no single test that can answer all these questions. Instead, a combination of approaches is needed to build a complete picture. This work helps improve how we study and evaluate GMMs, supporting safer and more responsible use of these technologies in the future.

Publiekssamenvatting

Genetisch gemodificeerde micro-organismen (GGMs) kunnen helpen bij het oplossen van milieuproblemen, zoals het schoonmaken van vervuild water of het opsporen van schadelijke stoffen. Toch worden ze buiten het laboratorium nog nauwelijks gebruikt. Een belangrijke reden hiervoor is dat we nog niet goed weten wat er met deze organismen gebeurt in de natuur.

In dit rapport onderzoeken we verschillende manieren om te bestuderen hoe GGMs zich gedragen onder realistische omstandigheden. Overleven ze? Verspreiden ze zich? Hebben ze invloed op andere micro-organismen? Door bestaande kennis te combineren met nieuwe experimenten in het lab, brengen we in kaart welke methoden goed werken en waar nog kennis ontbreekt.

De resultaten laten zien dat er geen eenvoudige test bestaat die alle antwoorden geeft. Er is een combinatie van methoden nodig om een goed beeld te krijgen. Dit onderzoek helpt om GGMs beter te beoordelen en draagt bij aan een veilige en verantwoorde toepassing van deze technologie in de toekomst.

Executive summary

Genetically modified microorganisms (GMMs) hold considerable promise for applications beyond the laboratory, including pollutant monitoring, bioremediation, agriculture, soil restoration, and environmental health. Yet their broader deployment remains limited. One major reason is not the absence of regulatory tools, but the limited availability of experimental data describing how GMMs behave once they enter complex natural environments: whether they survive, persist, compete, disappear, or affect native microbial communities.

This report addresses that gap by evaluating methodologies that can support the assessment of GMM survival and competition under environmentally relevant conditions. It combines a literature-based analysis with experimental work using *Pseudomonas putida* EM42 as a model chassis. The aim is not to define universal risk, but to identify practical approaches that can generate the evidence needed for case-by-case environmental risk assessment.

The literature search shows that much of the foundational work on GMM survival was carried out before the 2000s and relied mainly on classical microbiology methods, particularly colony-forming unit (CFU) counting. These studies remain valuable, but they often used GMMs as tracking tools rather than application-specific engineered strains. As a result, relatively little is known about how modern, function-driven genetic modifications influence survival, fitness, stability, and interaction with native microbiota.

Experimentally, CFU counting proved useful as a first-line method because it is accessible, inexpensive, and directly measures culturable cells. However, it also showed important limitations, including high variability between biological replicates, dependence on strain-specific selective media, and limited capacity to detect viable but non-culturable cells. Molecular tracking through PCR/qPCR can complement CFU counting, but only if the assay is highly specific to the GMM. In this study, the tested SYBR Green-based qPCR approach was not sufficiently robust, highlighting the need for improved primer design, probe-based assays, or dedicated genetic barcodes.

Shotgun metagenomics provided a broader view of microbial community composition and helped explore whether the presence of *P. putida* EM42 affected native microbial populations. While highly informative, this method remains technically demanding, costly, and sensitive to biases, especially in low-biomass water samples. It is therefore best viewed as a complementary approach rather than a routine early-stage assay.

Overall, the report concludes that no single method can adequately assess GMM fate upon escape or release. A robust assessment requires a stepwise and iterative framework: selecting

and characterizing the chassis, evaluating relevant phenotypic traits, designing traceable genetic modifications, testing survival and competition under simulated conditions, and implementing containment strategies when needed. Laboratory assays are essential for early screening, but realistic evaluation will ultimately require application-specific strains tested in settings that better reflect the intended environment.

The central recommendation is therefore methodological: to enable responsible deployment of GMMs, the field needs standardized experimental logic, not standardized risk conclusions. Each GMM must still be assessed according to its organism, genetic modification, application, and receiving environment. However, the way evidence is generated can and should become more systematic, transparent, and comparable. This will require close collaboration between synthetic biologists, microbial ecologists, risk assessors, modelers, regulators, and technology developers.

Managementsamenvatting

Genetisch gemodificeerde micro-organismen (GGM's) bieden veelbelovende mogelijkheden voor toepassingen buiten het laboratorium, zoals monitoring van verontreinigingen, bioremediatie, landbouw, bodemherstel en milieugezondheid. Toch blijft bredere toepassing beperkt. Een belangrijke reden is niet het ontbreken van regelgeving, maar het gebrek aan experimentele gegevens over hoe GGM's zich gedragen in complexe natuurlijke omgevingen: of ze overleven, blijven bestaan, concurreren, verdwijnen of invloed hebben op inheemse microbiële gemeenschappen.

Dit rapport richt zich op die kennislacune door methoden te evalueren die kunnen bijdragen aan de beoordeling van overleving en competitie van GGM's onder milieurelevante omstandigheden. Het combineert een literatuurstudie met experimenteel werk met *Pseudomonas putida* EM42 als modelchassis. Het doel is niet om universeel risico te definiëren, maar om praktische benaderingen te identificeren die bewijs kunnen leveren voor casus-specifieke milieurisicobeoordeling.

De literatuurstudie laat zien dat veel fundamenteel werk naar GGM-overleving vóór de jaren 2000 is uitgevoerd en vooral gebruikmaakte van klassieke microbiologische methoden, met name telling van kolonievormende eenheden (CFU). Deze studies blijven waardevol, maar gebruikten GGM's vaak als volgmodel in plaats van als toepassingsgerichte, genetisch aangepaste stammen. Daardoor is nog relatief weinig bekend over hoe moderne, functiegerichte genetische modificaties invloed hebben op overleving, fitness, stabiliteit en interactie met inheemse microbiota.

Experimenteel bleek CFU-telling bruikbaar als eerste methode, omdat deze toegankelijk en goedkoop is en direct kweekbare cellen meet. Tegelijkertijd kent de methode duidelijke beperkingen, zoals grote variatie tussen biologische replicaten, afhankelijkheid van stam-specifieke selectieve media en beperkte detectie van levensvatbare maar niet-kweekbare cellen. Moleculaire detectie via PCR/qPCR kan CFU-telling aanvullen, maar alleen wanneer de assay zeer specifiek is voor de GGM. In deze studie bleek de geteste SYBR Green-gebaseerde qPCR-aanpak onvoldoende robuust, wat het belang onderstreept van beter primerontwerp, probe-gebaseerde assays of specifieke genetische barcodes.

Shotgunmetagenomica bood een breder beeld van de samenstelling van microbiële gemeenschappen en hielp verkennen of de aanwezigheid van *P. putida* EM42 invloed had op inheemse microbiële populaties. Hoewel deze methode zeer informatief is, blijft zij technisch veeleisend, kostbaar en gevoelig voor bias, vooral in watermonsters met lage biomassa.

Daarom is shotgunmetagenomica vooral geschikt als aanvullende methode, niet als routinematige vroege test.

De hoofdconclusie van dit rapport is dat geen enkele methode op zichzelf voldoende is om het lot van GGM's na ontsnapping of introductie goed te beoordelen. Een robuuste beoordeling vraagt om een stapsgewijs en iteratief kader: selectie en karakterisering van het chassis, evaluatie van relevante fenotypische eigenschappen, ontwerp van traceerbare genetische modificaties, testen van overleving en competitie onder gesimuleerde omstandigheden, en toepassing van inperkingsstrategieën waar nodig. Laboratoriumtesten zijn essentieel voor vroege screening, maar realistische beoordeling vraagt uiteindelijk om toepassingsspecifieke stammen die worden getest onder omstandigheden die de beoogde omgeving beter benaderen.

De centrale aanbeveling is daarom methodologisch: om verantwoorde toepassing van GGM's mogelijk te maken, heeft het veld gestandaardiseerde experimentele logica nodig, niet gestandaardiseerde risicoconclusies. Elke GGM moet worden beoordeeld op basis van het organisme, de genetische modificatie, de toepassing en de ontvangende omgeving. De manier waarop bewijs wordt verzameld kan en moet echter systematischer, transparanter en beter vergelijkbaar worden. Dit vraagt om nauwe samenwerking tussen synthetisch biologen, microbiële ecologen, risicobeoordelaars, modelleurs, regelgevers en technologieontwikkelaars.

1. Introduction

1.1. Genetically modified microorganisms for health and environment

Advancements in sequencing and genetic engineering technologies have revolutionized the bio-based sciences, offering a plethora of new possibilities. Microorganisms, as some of the smallest and comparatively less complex forms of life, have been employed since the early days of synthetic biology for a wide range of applications. Today, innovative uses of microorganisms span biomanufacturing and CO_2 sequestration to *in situ* bioremediation, biomining, and even *in vivo* therapeutics and diagnostics for both health and environmental applications (Bala et al., 2022; Dong et al., 2025; Mahajan et al., 2017; Özer Bergman et al., 2025).

While certain applications, such as the biomanufacturing of chemicals and CO_2 removal, typically rely on genetically modified microorganisms (GMMs) in confined or semi-confined environments, others involve their deliberate use in open environments. Although existing regulatory frameworks and guidelines provide tools to assess the risks associated with environmental introduction, their effective application relies on a robust understanding of how engineered organisms behave in natural ecosystems. In practice, this knowledge remains limited, which has contributed to the relatively low number of GMMs deployed in agriculture and bioremediation (Chelma et al., 2025). To date, only one example of a genetically modified probiotic strain, *Bacillus subtilis* ZB183 (ZBiotics), has reached the commercial stage (Appala Naidu et al., 2019).

Concerns about whether bacteria can escape, survive, and transfer engineered genetic material date back more than 30 years, emerging alongside the early development of bioproduction (Kane, 1993). Since then, questions surrounding the environmental fate of GMMs, associated risks, and mitigation strategies have become central topics in the literature and at scientific conferences. These discussions have also contributed to the establishment of dedicated sub-disciplines, such as biosafety and biosecurity.

1.2. Lack of experimental data on escape and survival of GMMs as a bottleneck for the advancement of deliberate release applications

The use and commercialization of GMMs in open environments is strictly regulated through environmental risk assessment (ERA). ERA is a case-by-case evaluation of the potential risks that a genetically modified organism (GMO) may pose to human and environmental health. It is grounded in the precautionary principle, reflecting the uncertainties associated with the release of GMMs. The main risks identified include: i) the emergence of resistant strains through horizontal gene transfer (HGT) of heterologous and synthetic genes; ii) the persistence of GMMs in non-target environments, iii) the disruption of native microbial communities. While plasmid transfer has been demonstrated by Genthner et al. (1988) and Kolenc et al. (1988) and the persistence of GMMs has been studied, to our knowledge limited research has actively addressed the extent of GMM escape and survival, and consequently the design of appropriate mitigation strategies.

In Europe, only a few field trials have been reported: 1) the work of Molina et al. (1998), which performed one of the first environmental field release trials with *P. putida* EEZ32 over 112 days; and 2) the study by Corich et al., (2007), which monitored for ten years the release of the GMM *Rhizobium leguminosarum* bv. *Viciae*. In Canada, Jamieson et al. (2004) assessed the persistence of the release of the GMM *E. coli* NAR in alluvial streams.

The focus on field-based assessment of GMM, which peaked in early 2000s, has not been substantially followed up. In contrast, over the last two decades, significant efforts have been directed toward the development of biocontainment strategies (Moon et al., 2025). These strategies aim to provide safer tools and increase the likelihood of real-world deployment. However, by predominantly relying on containment approaches, three major bottlenecks can be identified: 1) the limited feasibility of deploying GMMs in open environments without physical or biological containment; 2) the burden imposed by biosafety circuits; and 3) the lack of quantitative data on GMM survival and acceptable escape rates.

For the latter, we identify a clear gap in the literature. While substantial effort has been devoted to biocontainment, to our knowledge no recent efforts have focused on survival and competitive dynamics of released GMMs. This report therefore focuses on analyzing both established (“gold standard”) and emerging methods to better understand the environmental fate of GMMs intended for applications outside of the laboratory.

1.3. A step-by-step guideline to assess survival of GMMs in support of technology transfer

We propose a set of experimental approaches for the assessment of GMMs under simulated conditions. The focus of this work is on methodologies to determine survival, persistence, and competition with native microbial communities following intended or accidental release. Various approaches will be explored to enable both qualitative and quantitative assessment of GMM behavior in environments that approximate real-world conditions. While not exhaustive of all possible applications of GMMs, this report aims to gather and evaluate methodologies that can be used to assess the survival of GMMs envisioned for deliberate release applications.

The data generated through these approaches can inform scientists, regulatory bodies, and commercial entities about the environmental behavior and safety of GMMs, thereby supporting the ERA process. Furthermore, we envision this work as a starting point for the development of experimentally grounded procedures that can be followed when evaluating GMM survival in projects targeting applications in open environments.

With this project, we aim to answer following research questions:

- To which extent are currently available methodologies suitable for assessing survival and competitive dynamics of GMMs in real-world applications?
- Can existing methodologies (e.g., metagenomics-based approaches) be adapted to specifically evaluate GMM survival and their interactions with native microbial populations?

We aim to collect non-exhaustive experimental practices (**Section 2**) that can inform such procedures, and to provide experimental data (**Section 3**) using, as a case study, the microbial chassis *Pseudomonas putida* EM42. This strain is envisioned as model GMM for various applications, ranging from microbial whole-cell biosensors (MWCBs) to the bioremediation of environmental pollutants.

2. Material and methods

2.1. Literature search

The literature research, summarized in the **Appendix** (**Supplementary Table 1**), was conducted to identify methodologies used to assess the survival and competition of genetically modified bacteria. We compiled a literature set using the keywords “escape”, “survival”, “competition”, and “genetically modified microorganisms”, and complemented this by tracing citations from key papers published between 1980 and 1990. This resulted in an extensive, although not exhaustive, overview of methodologies. Data were extracted from the selected articles and subsequently analyzed.

2.2. Strains, media, and water samples

The strains used in this study are *Pseudomonas putida* KT2440 and *Pseudomonas putida* EM42 (Bagdasarian et al., 1981; Martínez-García et al., 2014).

The media used in this study include lysogeny broth (LB) (Difco™ LB borth,, Becton, Dickinson and Company (BD Franklin Lakes, New Jersey, USA) and M9 minimal medium (1.63 g/L NaH_2PO_4, 3.88 g/L K_2HPO_4, 2 g/L$(NH_4)_2SO_4$, 10 mg/L EDTA, 100 mg/L $MgCl_2{\cdot}6H_2O$, 2 mg/L $ZnSO_4{\cdot}7H_2O$, 1 mg/L $CaCl_2{\cdot}2H_2O$, 5 mg/L $FeSO_4{\cdot}7H_2O$, 0.2 mg/L $Na_2MoO_4{\cdot}2H_2O$, 0.2 mg/L $CuSO_4{\cdot}5H_2O$, 0.4 mg/L $CoCl_2{\cdot}6H_2O$, and 1 mg/L $MnCl_2{\cdot}2H_2O$) , supplemented with either 0.9% glucose or 0.2% sodium citrate. Chloramphenicol was used at a concentration of 15-30 mg/mL.

Water samples were collected at different locations and on different dates for the various experiments. Samples were either processed immediately for survival assays and metagenomic analyses or stored at 4°C for up to four months for other assays.

Sample	**Date**	**Characteristics**	**Location**
Sample 1	19/11/25	-	-
Sampe 2	10/12/25	Temperature 9.6°C; Ec 1707 µS/cm; pH 7.07; NO_3 20.95; N-NO_3 4.73; turbidity 0.0	-
Sample 3	17/12/25	-	Campus Wageningen University

2.3. Motility assay

This assay was carried out to assess the swimming motility of *P. putida* EM42 using a soft agar approach. The protocol was adapted from previous studies (Ha et al., 2014; Palma et al., 2022; Pfeifer et al., 2022).

M9 minimal medium supplemented with 0.3% (w/v) bacteriological agar was prepared with different glucose concentrations (0.1%, and 0.5% w/v) as carbon sources. *P. putida* KT2440 and EM42 were grown in biological triplicates overnight in M9 medium supplemented with 0.9% glucose at 30°C and 250 rpm.

A 5 µL aliquot of the overnight culture, adjusted to an OD_{600} of 0.05-0.1, was spotted onto the center of the soft agar plates. Plates were incubated at 30°C for several days. The diameter of the motility halo was measured in centimeters at different time points.

2.4. Cell inoculum

Sample 1: *P. putida* EM42 cells were grown overnight in M9 supplemented with 0.9% glucose, washed twice with MilliQ water, and inoculated into water samples at different final concentrations. Flasks were incubated in controlled climate room with day:night ratio of 16:8 at 20°C and 200 rpm.

Sample 2: *P. putida* EM42 cells were prepared as above and diluted into water samples at final OD_{600} of 0.2.

Sample 3: *P. putida* EM42 cells were first inoculated in 3 mL LB from glycerol stock and incubated for 5-6 h. A 1:1000 dilution was then transferred into 25 mL M9 supplemented with 0.9% glucose and incubated overnight. Cells were washed three times with sterile demi-water and inoculated into 500 mL water samples at different final concentrations. Flasks were incubated at 12°C and 100 rpm, and sampled at day 0, day 20, and day 40.

For both survival and competition assays, three spiking conditions were used. Throughout the report, these are referred to as: A, corresponding to a starting OD_{600} of 0.01; B, corresponding to a starting OD_{600} of 0.1; and a negative control, consisting of non-spiked water samples.

2.5. Growth assay with microplate reader

Growth assay is carried out with water sample 2. Cells are diluted to final OD_{600} of 0.2.in 200 µL of water sample in the 96-well plate. The experiment is carried out in microplate reader Synergy H1 (Agilent Biotek, Winooski, Vermont, USA) and measurements of optical density

(OD) at a wavelength of 600 nm were carried out every 30 minutes for 48 hours at the optimal temperature of 30 °C. Data analysis is performed with excel and Graphpad version 10.4.2.

2.6. Survival assay

Survival assays were performed using water samples 1 and 3. At each sampling time point, 200 µL was collected and serially diluted (1:10). Dilution factor used for plating range from 10^1 to 10^4. For the dilution chosen, 50 µL was plated onto selective medium (M9 supplemented with 0.2% sodium citrate and 15-30 mg/mL chloramphenicol) and incubated at 30°C for 24-72 h. Plates were then stored at 4°C until colony counting.

CFU/mL was calculated using the following formula:

$$\frac{dilution\ factor \times CFU}{volume\ (mL)}$$

2.7. DNA extraction

DNA extraction was performed using water sample 1. A total of 5 mL of sample was filtered through 0.22 µm mixed cellulose ester (MCE) membrane filters (Merk Millipore, Tullagreen, Co. Cork, Ireland). DNA was extracted using the DNeasy PowerWater kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions, with the following modification: a 1.5 mL tube was used instead of the provided 5 mL tube, and vortexing was performed at 5500 rpm for 1 min, repeated three times.

2.8. PCR and qPCR

PCR was performed using Phire Green Hot Start II polymerase (Thermo Scientific, Vilnius, Lithuania) and *endA2* primers (Integrated DNA Technologies, Inc. (IDT), Leuven, Belgium). Reactions were prepared in 20 µL volumes containing 10 µL Phire mastermix (2×), 2.5 µL forward primer, 2.5 µL reverse primer, 4 µL MilliQ water, and 1 µL of colony resuspended in MilliQ water.

Amplification was carried out in the Mastercycler (Eppendorf SE, Hamburg, Germany) with the following protocol: initial denaturation at 98°C for 2 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 67.7°C for 10 s and elongation at 72°C for 15 s, and a final elongation step at 72°C. Amplicons were analyzed on a 1% (w/v) agarose gel.

qPCR was performed using PerfeCTa SYBR Green (QuantaBio, Beverly, MA, USA) on a QuantStudio system (Themo Fisher, Applied Biosystem, Foster City, California, USA) using the same primer set. Reactions (10 µL) contained 5 µL mastermix (2×), 0.5 µL forward primer (500 nM), 0.5 µL reverse primer (500 nM), 2 µL nuclease-free water, and 2 µL template DNA. The protocol included denaturation at 95°C for 30 s followed by 40 cycles of 95°C for 1 s and 65°C for 15 s, followed with melting curve analysis with standard settings.

Name	**Sequence (5'->3')**	**Tm (°C) Phire polymerase**	**Tm (°C) Taq polymerase**
endA2 FW	aggaaacccgccaggccat	67.7	65
endA2 RV	cagccccggggaaaccatta	67.7	65

2.9. Shotgun metagenomics

Metagenomic processing was performed using water sample 3. First, a 11 µm Whatman filter was used to remove larger particles from the full 500 mL sample. This pre-filtration step is commonly applied to increase representative sampling volume (Davis et al., 2023).

Subsequently, 250 mL was filtered through a 0.22 µm MCE membrane filters, and an additional 250 mL was filtered as backup. To avoid cross-contamination, samples were processed in the following order: negative control, sample B, and sample A. Filters were stored at −80°C until further processing.

DNA extraction and shotgun sequencing (Illumina NovaSeq X plus, pair-end 150 bp, 6 Gb) were performed by Novogene (Novogene Europe).

Data analysis was conducted using the Galaxy platform (The Galaxy Community, 2024). Relative abundance analysis included trimming with TrimGalore! (removal of reads <50 bp, Phred score cutoff 20), followed by taxonomic profiling using MetaPhlAn with standard settings and Bowtie2 set to "very-sensitive". Reference database used for MetaPhlAn is CHOCOPhlAn, January 2025.

3. Exploring methodologies for survival upon release: a literature search

Because of the previously mentioned risks associated with the use of GMMs in non-contained applications, the precautionary principle, a risk management approach that includes mitigation strategies to prevent potential harm, has been often applied. In practice, this has been translated into strict regulatory frameworks and permitting processes for the deliberate release of GMMs, along with strong emphasis on the implementation of physical and biological containment strategies.

As a result, research efforts have largely focused on the development of biocontainment strategies and genetic safeguards for medical and environmental applications (Moon, 2025). However, comparatively little attention has been given to systematically evaluating experimental methodologies that can support informed decision-making during ERA, particularly with respect to long-term survival and competition with native communities.

To address this gap, we conducted a literature search to identify methodologies used to assess GMM behavior in terms of survival, persistence, and interaction with native species. Specifically, we evaluated whether commonly used approaches can be considered "gold standard" methods and to what extent they are suitable for designing experimental strategies tailored to GMMs intended for deliberate release. This search focused primarily on GMMs and environmental microbiology studies. Reviews on bacterial pathogen surveillance and tracking are available elsewhere (Sosah et al., 2025). Several key observations emerged from this analysis.

First, a substantial proportion of studies have been conducted using model organisms such as *Escherichia coli* and environmental soil bacteria including *Pseudomonas fluorescens* and *Pseudomonas putida* (**Figure 1A**), which together account for approximately half of the studies collected (**Figure 1B**). This suggests that current methodological approaches are largely derived from a limited set of model organisms, which may not fully capture the diversity of behaviors expected from GMMs in different environmental contexts.

Second, in most of the studies identified, GMMs are used primarily for fundamental microbiology research. One of the few example that considers a GMM in the context of its intended application is the work of McClure et al. (1989), which investigated survival, genetic stability, and degradation potential using a laboratory-scale activated sludge system. While many studies address the research question of whether GMMs survive, they often do not incorporate the perspective of application-specific functionality. In many cases, genetic modifications, such as the introduction of fluorescent markers or antibiotic resistance genes, are used solely for tracking purposes. These modifications do not reflect the functional traits required for real-world applications, nor do they account for metabolic burden, selective pressures, or potential fitness advantages associated with the heterologous gene expression. While such studies remain valuable, there is still a lack of research addressing escape, survival, and competition of GMMs designed for specific applications. In particular, limited attention has been given to how application-relevant genetic modifications, often involving one or multiple heterologous genes, affect the overall fitness, stability, and ecological behavior of the organism, including its interaction with native microbial communities under realistic environmental conditions.

Third, although some studies consider the effects of genetic modifications on traits such as carbon source utilization compared to wild-type strains as well as competition (Kozdrój, 1996; Vahjen et al., 1995, 1997), most focus primarily on survival in simulated environments. In these cases, survival often reaches a steady state without complete extinction, yet it is rarely assessed whether cells remain metabolically active or continue to produce metabolites that could influence native microbial communities. Moreover, while colony-forming unit (CFU) counting is widely identified as a “gold standard” method for assessing survival (**Figure 1C**), relatively few studies have been designed to quantitatively evaluate competitive interactions between GMMs and native populations (Vahjen et al., 1997).

Fourth, many studies published before the 2000s rely predominantly on classical microbiological techniques, such as colony counting, to assess the survival of both wild-type and genetically modified strains. More recent studies have incorporated techniques such as microscopy and quantitative PCR (qPCR) (Banihashemi et al., 2017; Qin et al., 2023) (**Figure 1D**). However, several advanced methodologies, including fluorescence in situ hybridization (FISH), high-resolution microscopy, and metagenomics, remain underutilized in this context, despite their potential to provide deeper insights into the environmental fate and activity of GMMs.

The studies collected in the **Appendix** provide a valuable foundation and baseline knowledge. However, there is a clear need to update and expand these approaches using modern techniques, with a stronger focus on GMMs designed for specific applications and deployment scenarios.

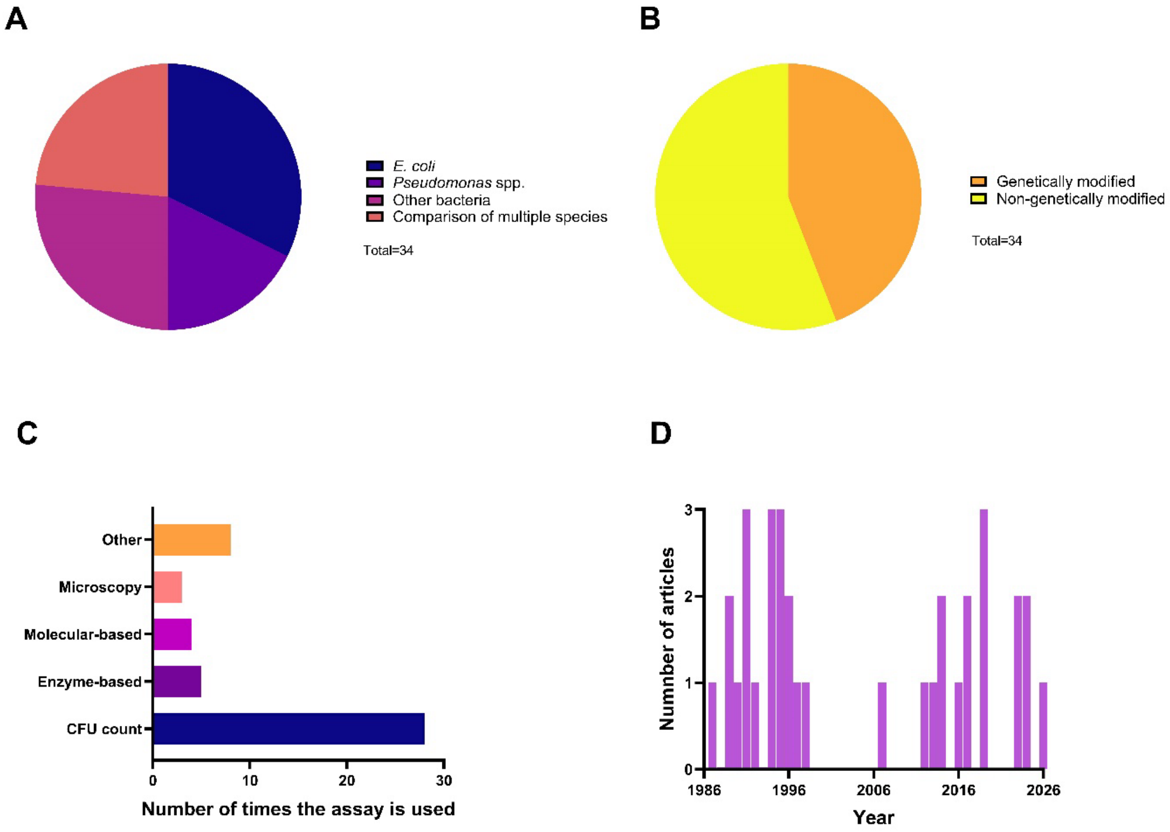


Figure 1. Overview of the literature analysis (see Appendix, Supplementary Table 1 for extended data). (A) Distribution of microbial strains used across the reviewed studies. When multiple species are reported within a single study, these are grouped under "Comparison of multiple species", indicating studies explicitly designed to assess differences between microorganisms. (B) Proportion of studies employing genetically modified microorganisms (GMMs) versus non-genetically modified organisms. (C) Frequency of assay types used across the literature; individual studies may contribute to multiple assay categories. (D) Temporal distribution of publications identified using the selected keywords (**Section 2.1.**) over the past 40 years.

4. Experimental results

4.1. Choice and study of the chassis

The selection of an appropriate microbial chassis is a critical step when assessing the environmental behavior of GMMs, particularly for applications envisioned outside controlled laboratory conditions. The chassis defines not only the engineering potential of the system, but also its intrinsic ecological properties, which ultimately determine its survival, persistence, and interaction with native microbial communities. Key characteristics such as genetic accessibility, physiological robustness, and environmental adaptability influence both the feasibility of introducing specific genetic modifications and the resulting behavior of the organism under realistic conditions.

In the context of this report, where the focus is on evaluating methodologies to assess survival and competition, the choice of chassis is especially relevant. The baseline properties of the host organism, such as its ability to tolerate environmental stress, utilize diverse substrates, or respond to nutrient limitations, directly affect the outcome and interpretation of experimental assays. Moreover, genetic modifications introduced for biotechnological purposes may alter these traits, impacting fitness, stability, and ecological interactions in ways that are not always predictable. Therefore, selecting a well-characterized and environmentally relevant chassis enables a more robust and interpretable assessment of survival, while also allowing the systematic evaluation of how specific genetic features influence behavior upon release. In this sense, the chosen chassis serves both as a model organism and as a test platform to benchmark methodologies aimed at understanding the fate of GMMs in complex environments

4.1.1. *Pseudomonas putida* EM42

P. putida was selected as the microbial chassis to study environmental release due to its genetic accessibility and its natural occurrence in water and soil environments (dos Santos et al., 2004). More generally, *P. putida* is known for its high tolerance to stress, versatile metabolism, and the availability of extensive molecular biology and genetic engineering toolkits (de Lorenzo et al., 2024; Martin-Pascual et al., 2021), making it a suitable GMM candidate for a variety of applications.

In this study, we used *P. putida* EM42, a genetically streamlined derivative of the well-characterized *P. putida* KT2440 strain. EM42 was developed through targeted genome reductions aimed at improving its performance for biotechnological applications (Martínez-García et al., 2014).

4.1.2. Motility

One of the genetic modifications introduced in *P. putida* KT2440 is the deletion of the entire flagellar machinery present in the parental *P. putida* KT2440. In static or diffusion-limited environments, this modification may represent a competitive disadvantage, as the GMM has a reduced capacity to actively move toward nutrients and compete for resources.

Our results show that the absence of the flagellum significantly reduces motility, as evidenced by the limited ability of EM42 to form a halo in soft agar assays compared to the motile KT2440 strain. This effect becomes apparent after 5 days of incubation and is more pronounced at higher carbon source concentrations (**Figure 2**).

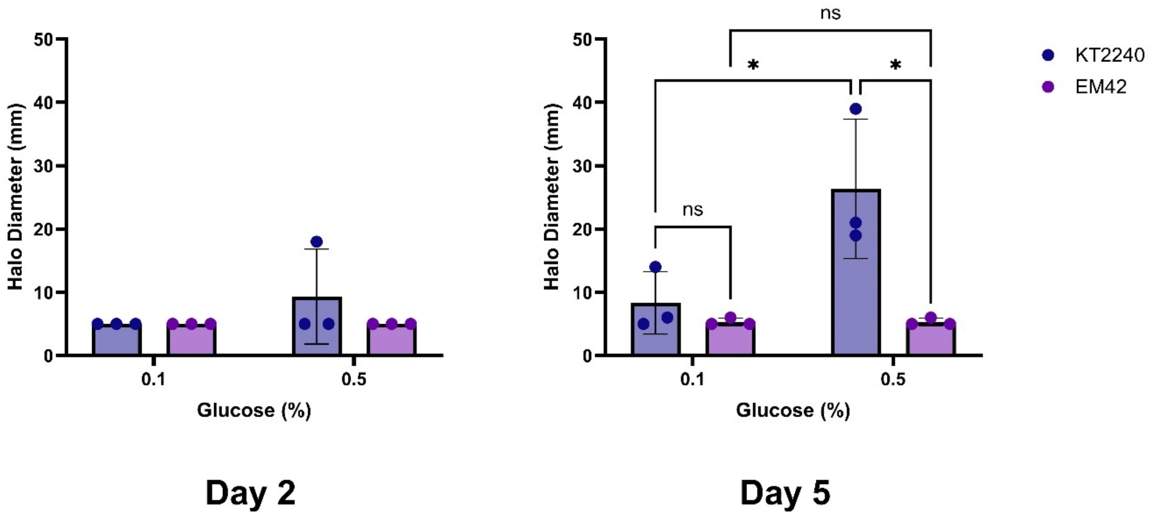


Figure *2*. **Motility assay.** Diameter of halo (mm) as a function of a glucose concentration (%w/v) over time (day 2 and day 5). Results for KT2440 (control) and EM42 were represented based on the mean of each biological triplicate (n=3 biological, mean +/- SEM). Statistical analysis performed: Two-way ANOVA with Tukey's multiple comparisons test, where * is adjusted p-value <0.027.

4.1.3. Growth assessment in non-optimal medium conditions

To evaluate the ability of the selected chassis to grow under nutrient-limited conditions representative of environmental water samples, a growth assay was performed using water sample 2. In contrast to nutrient-rich conditions (e.g., glucose at 0.18% and 0.9%), *P. putida* EM42 did not show growth in river water alone and instead exhibited a decline in cell density over time (Figure 3).

These results indicate that EM42 requires an external carbon source for active growth. However, this experimental setup does not directly reflect cell viability, as lack of growth does not necessarily imply cell death. Therefore, to better assess survival under these conditions, CFU-based survival assays were performed (Section 4.2.).

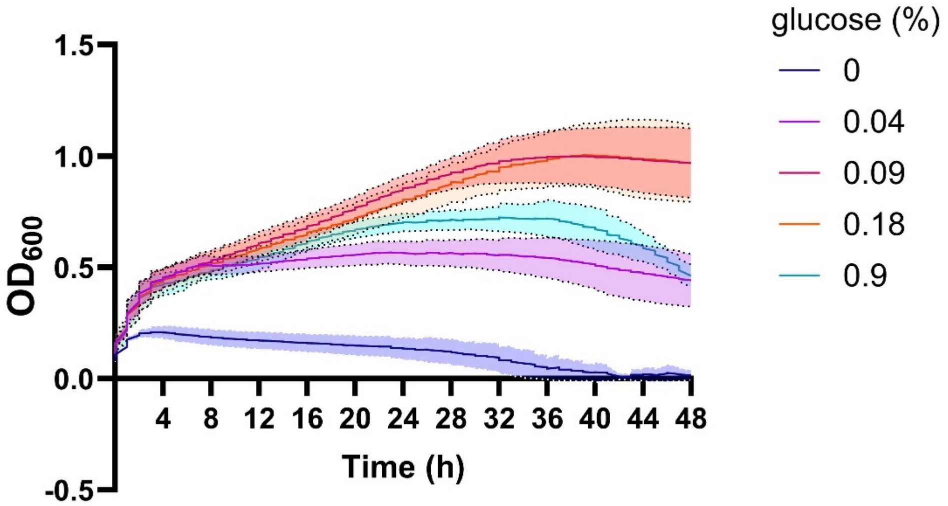


Figure 3. Growth curve of *P. putida* EM42 with water samples supplemented with increasing concentrations of glucose (0-0.9%). Curves represent the mean of three biological replicates, each measured in two technical replicates.

4.2. Survival

Assessing the survival of GMMs is of central importance when considering their use outside controlled laboratory conditions, regardless of whether physical or biological containment strategies are implemented, and particularly in scenarios where full environmental release is envisioned.

To evaluate survival, we applied what is commonly considered the “gold standard” approach: sampling environmental water matrices, plating on selective media, and enumerating CFUs.

4.2.1. Survival assay with CFU count

Survival assays were performed using two different water samples (samples 1 and 3) under two experimental conditions.

In the first setup, experiments were conducted in a controlled climate chamber at 20°C, closer to the optimal growth temperature of *P. putida*, with a defined day:night cycle. Under these conditions, all samples showed a decline in CFU counts over time (**Figure 4**).

In the second setup, performed in parallel using the same water samples later analyzed by metagenomics, experiments were conducted at a lower temperature (12°C) to better approximate environmental conditions during the sampling period. A similar overall trend was observed, with declining CFU counts over time. However, an unexpected increase in CFU counts at later time points (day 40) was observed in both the negative control and the lowest inoculum condition (sample B) (**Figure 5A**).

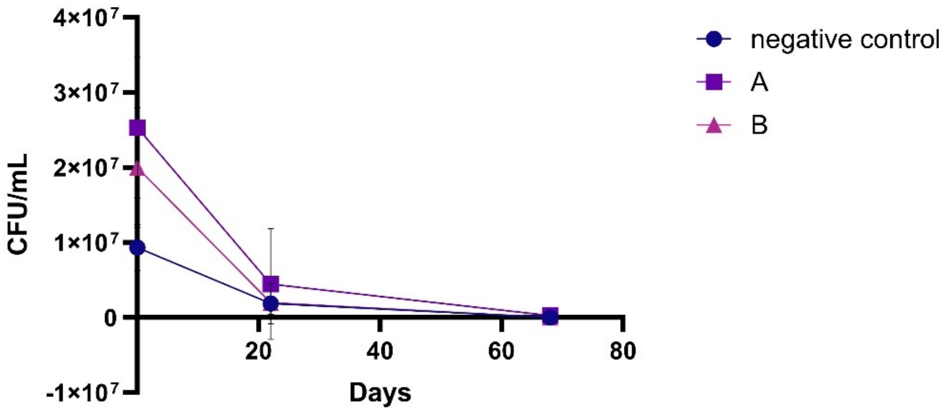
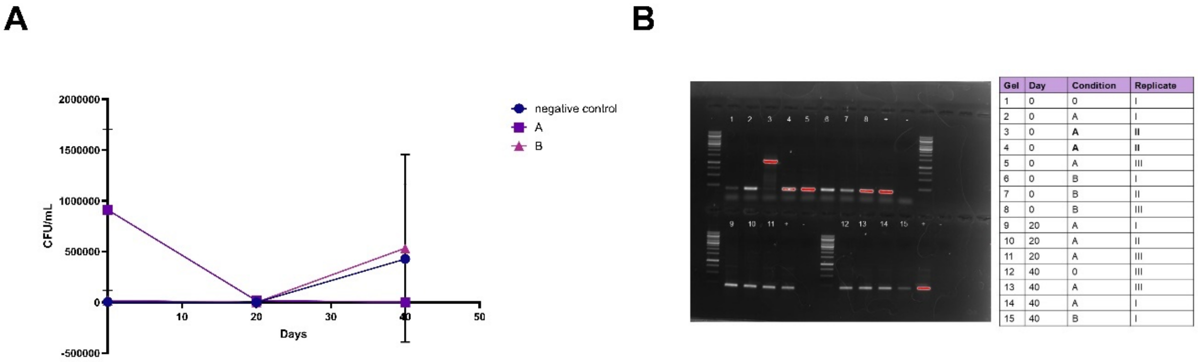


Figure *4*. Survival assay of *P. putida* EM42 over 68 days in sample 1. CFU count on selective media of samples A, B and negative control at day 0, 20 and 40. CFU/mL is represented as the mean of three biological replicates.

To investigate whether these colonies originated from the GMM or from native *Pseudomonas* species, we designed a primer set targeting regions flanking the deleted *endA2* locus, which is absent in EM42 and therefore provides specificity for the engineered strain. The expected amplicon size (~250 bp) confirmed the presence of *P. putida* EM42 in most samples. However, one colony from sample A (replicate II, day 0) showed a larger amplicon (~1.3 kb), corresponding to the intact *endA2* gene and indicating the presence of wild-type *P. putida* (**Figure 5B**).

At the same time, bands corresponding to the EM42 amplicon size were also detected in the negative control (day 0, replicate I; day 40, replicate III). This suggests either non-specific amplification by the primer set or potential cross-contamination during sample handling.



Gel	Day	Condition	Replicate
1	0	0	I
2	0	A	I
3	0	**A**	**II**
4	0	**A**	**II**
5	0	A	III
6	0	B	I
7	0	B	II
8	0	B	III
9	20	A	I
10	20	A	II
11	20	A	III
12	40	0	III
13	40	A	III
14	40	A	I
15	40	B	I

Figure *5*. Survival assay of *P. putida* EM42 over 40 days in sample 2. (A) CFU counts on selective media for samples A, B, and the negative control at days 0, 20, and 40. Values represent the mean of three biological replicates. (B) Colony PCR using Phire polymerase on selected colonies. Lane annotations correspond to individual colonies isolated from different conditions, with metadata (sampling day, condition, and biological replicate) provided in the accompanying table.

4.2.2. Tracking presence with PCR and qPCR

To complement CFU-based measurements, we evaluated whether molecular methods could be used to specifically detect and quantify the GMM in environmental samples. Although established primer sets exist for *Pseudomonas* species (Widmer et al., 1998), we used the same endA2-based primer design to target sequences specific to EM42.

PCR and quantitative PCR (qPCR, SYBR Green chemistry) were performed on DNA extracted from the negative control and sample A of the survival assay conducted under day:night controlled conditions (sample 1, day 60). Genomic DNA from *P. putida* EM42 was used as a positive control, while *E. coli* BW25113 and nuclease-free water served as negative controls.

The primer set successfully amplified the expected fragment in conventional PCR (**Figure 6A**), indicating functionality at the qualitative level. However, qPCR analysis revealed abnormally high amplification efficiency (**Appendix, Supplementary Figure 1**), compromising quantitative reliability. As a result, the assay was unable to clearly distinguish between spiked and non-spiked water samples when using the negative control as a threshold (**Figure 6B**).

These results indicate that, while the primer design is suitable for qualitative detection, it is not sufficiently robust for quantitative tracking of the GMM in complex environmental samples under the tested conditions.

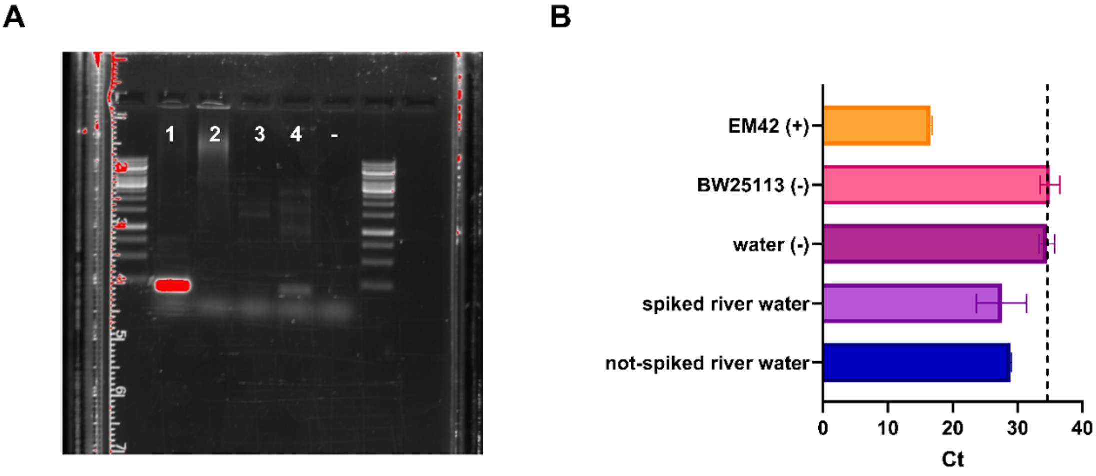


Figure 6. Detection of *P. putida* EM42 using PCR and qPCR. (A) PCR with Phire polymerase and *endA2* primers of different samples, in order: 1 = *P. putida* EM42 genome; 2 = *E. coli* BW25113 genome (negative control for the primers); 3 = DNA extraction of not-spiked river water (negative control, replicate I); 4=DNA extraction of spiked water (sample A, replicate I); - = nuclease free water (negative control for the primers). (B) qPCR with PerfeCTa SYBR Green, Ct values are reported as average of three technical replicates. In order, EM42 (+) = genomic DNA of *P. putida* EM42; BW25113 (-) = genomic DNA of *E. coli* BW25113; water (-) =

nuclease free water; spiked river water = DNA extraction of sample A, replicate I; not-spiked water = DNA extraction of negative control sample, replicate I.

4.3. Competition

Understanding whether a GMM can not only survive but also compete with native microbial communities is essential to assess its potential ecological impact upon release. Shotgun metagenomics, based on next-generation sequencing (NGS) of environmental DNA (eDNA), provides a culture-independent approach to characterize both the composition and dynamics of microbial communities, offering insight into which organisms are present and how they may be affected.

At the phylum level (**Figure 7**), the dominant groups (Pseudomonadota, Actinomycetota, and Verrucomicrobiota) remain relatively stable over the 40-day period and do not appear to be significantly affected by the presence of EM42. In contrast, Bacteroidota shows a decrease at day 40 compared to the negative control. Additionally, some low-abundance phyla (e.g., Acidobacteriota and Myxococcota) appear in samples A and B at later time points (day 20 or 40) but are not detected in the negative control.

At the species level, no major shifts in community composition are observed. The complete disappearance of GGB25672_SGB187276 at day 40 across all conditions suggests that environmental or experimental factors (e.g., temperature, absence of a stable day:night cycle, or shaking conditions) may influence its persistence. Other species show condition-dependent trends: GGB26028_SGB38031 decreases in the negative control but remains more stable in samples A and B, while GGB38373_SGB52357 declines across all conditions. *Planktophila vernalis* increases in sample A but decreases in the negative control and sample B. One species, GGB26951_SGB39152, appears over time in all samples, suggesting that experimental conditions may favor its growth independently of the presence of the GMM.

Overall, these observations indicate that, under the tested conditions, the introduction of *P. putida* EM42 does not lead to major detectable shifts in the microbial community structure at the phylum or species level.

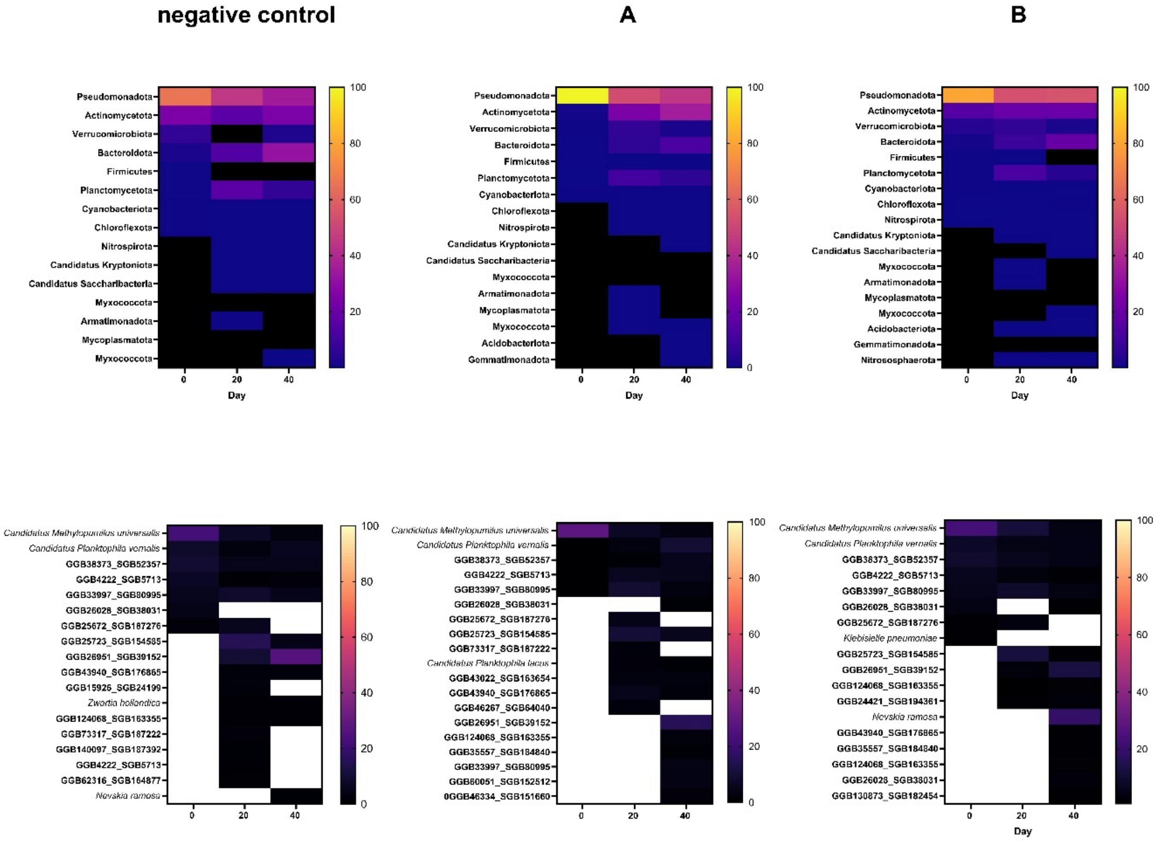


Figure 7. Relative abundance from MetaPhlAn analysis of the different samples at day 0, 20 and 40. (Top panels) Relative abundance normalized to the number of classified taxa, showing phylum-level composition. Color gradients represent the average of three biological replicates; black indicates absence of the taxon. (Bottom panels) Relative abundance at the species level, excluding P. putida, normalized to classified taxa. Color gradients represent the average of three biological replicates; white indicates absence of the taxon. Species labeled as species-level genome bins (SGBs) correspond to distinct taxa lacking formal taxonomic classification.

From MetaPhlAn analysis we are able to appreciate the spike of *P. putida* at different concetrations, as can be seen from the higher relative abundance of sample A and B compared to the negative control at day 0. Decrease of relative abundance of the two spiked samples can also be apprciated overtime, reaching similar level to the negative control (**Figure 8**).

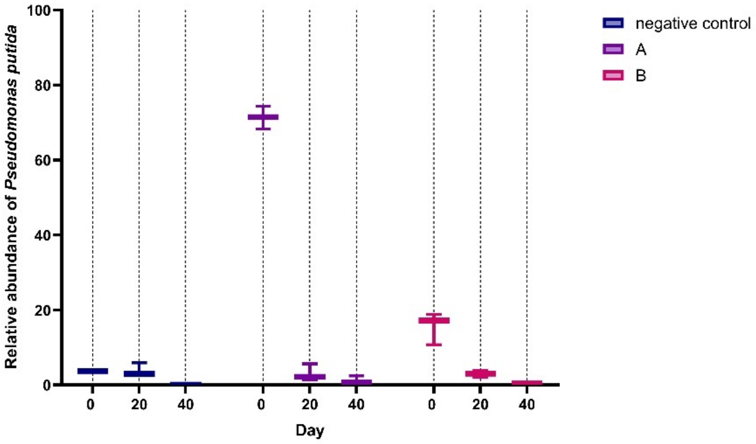


Figure 8. Relative abundance from MetaPhlAn analysis of *P. putida* of the different samples at day 0, 20, and 40. Relative abundance is plotted as average of three biological replicates and normalized considering classified taxa only.

5. Discussion

5.1. Literature search and methodology choice

Our literature search highlights that GMM survival and its impact on native microbial communities were prominent research topics in the late 1980s and 1990s, coinciding with the rapid expansion of recombinant DNA technologies. Interest in this field then stabilized over the past two decades, and has recently resurged (Bala et al., 2022; Chemla et al., 2025; Docter & Mansfeldt, 2025), reflecting the growing need to translate synthetic biology innovations into real-world applications.

The methodologies selected in this study combine established approaches identified through this literature search (e.g., CFU counting) with more recent techniques that have not been widely applied to the study of GMM environmental fate (e.g., shotgun metagenomics). It should be noted, however, that our search was limited to specific keywords (Section 2.1.). As a result, relevant studies and methodologies from adjacent fields may not have been captured, particularly where different terminology is used.

5.2. Survival of GMMs

To study the survival of the genome-reduced laboratory strain *P. putida* EM42, we applied what is widely consider the “gold standard”: enumeration of viable cells through plating and CFU counting from non-treated environmental water over time. Our results show that higher initial cell densities (i.e., sample A) decline over time but do not reach complete extinction.

However, this approach presents several limitations. First, the selectivity of the medium used was insufficient to fully discriminate the GMM from naturally occurring *P. putida* populations present in water samples. This likely explains the observed increase in CFU counts at later time points (day 40) in both the negative control and the lowest inoculum condition (i.e., sample B) (**Figure 5A**). Second, the assay is inherently sensitive to variability, as reflected by the spread across biological replicates. This variability is largely attributable to the heterogeneity of environmental water samples and represents a recurring limitation across assays in this study (see **Section 5.5**).

Although this trend is less apparent when data are represented on a linear scale, the persistence of detectable CFU over time is consistent with previous studies. For example, wild-type *P. putida* has been shown to persist for extended periods (up to 80 days) and to form resilient biofilms under nutrient-limited conditions (Ramezanian et al., 2026). Other studies have demonstrated that treated water samples (e.g., autoclaved or filtered) result in more stable populations and reduced decline compared to untreated samples (Flint, 1987; Kersters, Huys, Duffel, et al., 1996), suggesting that competition with native microbial communities plays a role in limiting persistence. While the use of untreated water better reflects environmental conditions, the inclusion of sterilized controls would have provided a clearer baseline for assessing intrinsic GMM fitness.

An additional limitation of CFU-based approaches is the potential presence of viable but non-culturable (VBNC) cells. In this state, bacteria remain metabolically active but are not detected by standard cultivation methods, leading to underestimation of survival. Although several assays exist to assess metabolic activity, their application in mixed microbial communities is challenging. Approaches such as cell division inhibition assays (e.g., 3-MBA) offer specificity but limited applicability across species (Gray et al., 2019), while broader assays such as catalase activity (Hadwan et al., 2024) or live/dead staining lack specificity for the GMM.

On the one hand, the 3-MBA assay offers high specificity but is limited to a narrow range of organisms, such as *B. subtilis*. On the other hand, broader approaches such as catalase assays or live/dead staining lack the specificity required in mixed communities, as they cannot distinguish the GMM from native microorganisms. A potential way to overcome this limitation is the use of propidium monoazide (PMA) as a pretreatment prior to PCR, which selectively inhibits amplification of DNA from dead cells or extracellular sources (Banihashemi et al., 2017). When combined with the higher specificity of probe-based qPCR, this approach could enable more accurate detection and tracking of the GMM within complex environmental samples.

5.3. Competition of GMMs with native microbiota

5.3.1. Shotgun metagenomic sequencing

Shotgun metagenomic sequencing is a powerful, culture-independent approach to characterize microbial communities. Compared to techniques such as 16S amplicon sequencing, it offers improved taxonomic resolution, enables detection of a broader diversity of organisms, and provides insights into functional potential through gene prediction (Ranjan et al., 2016). It has been widely applied for surveillance of antimicrobial resistance and characterization of the resistome in environmental samples (Davis et al., 2023; Munk et al., 2022)), , as well as for studying microbial dynamics in complex ecosystems like lakes (Oh et al., 2011) and synthetic communities (Morgan et al., 2010). To our knowledge, its application to GMM studies remains limited, with previous work mainly focused on contamination in industrial processes (D'aes et al., 2025). Here, we applied this approach to assess the impact of a GMM on native microbial communities in water samples.

Our results indicate that the introduction of *P. putida* EM42 does not lead to major shifts in community composition at the phylum or species level (**Figure 7**), in line with previous studies reporting limited ecological impact of introduced microorganisms (McClure et al., 1989). While some low-abundance taxa appear or disappear over time, it is not possible to determine whether these changes reflect biological effects or technical variability arising from DNA extraction, sequencing, or data processing. Further insights would require statistical analysis and functional annotation. At this stage, despite the inclusion of biological replicates, our analysis remains primarily qualitative.

We show that this approach can provide useful insights into the fate of the GMM (**Figure 8**). While the detection of genetic material does not necessarily reflect the presence of viable cells, the metagenomic signal observed for sample A follows the same trend as the CFU-based survival assay, suggesting consistency between methodologies. This also indicates that the elevated CFU counts observed for sample B at day 40 (**Figure 5A**) may reflect a technical artifact rather than a biological effect.

A key limitation, however, is that metagenomic analysis cannot distinguish between wild-type *P. putida*, naturally present in environmental samples, and the engineered strain, as also observed in the negative control (**Figure 8**). This highlights the need to complement metagenomic profiling with strain-specific molecular tracking approaches, such as qPCR (see **Section 5.3.2.**).

Several other technical limitations must be considered. DNA extraction from low-biomass samples such as water remains challenging and is prone to bias introduced by sample handling, extraction protocols, and downstream processing (Demkina et al., 2023). Contamination is a particular concern in such samples, as it can occur during multiple steps including sampling, extraction, and sequencing (Fierer et al., 2025), disproportionately affecting low-abundance taxa and potentially generating artifacts. Appropriate controls (e.g., blank filters) are therefore essential.

In addition, variability between biological replicates is a well-recognized limitation in metagenomic studies. Biological replication helps account for spatial and temporal heterogeneity, while technical replication addresses sequencing-related variation (Davis et al., 2023). However, due to cost constraints, many studies prioritize broader sampling over replication, often leading to increased variability across datasets (Filazzola & Cahill Jr, 2021; Jia et al., 2021). This trend is consistent with our observations, where variability between biological replicates was also evident.

Lastly, the costs and expertise required to plan, design, perform, and analyze the complex data, as well as the capacity needed for data storage, should not be underestimated. These aspects are usually not considered during the early stages of development, highlighting how this can become a bottleneck for the implementation of such tests in the development of novel GMMs. While overall sequencing costs are decreasing, shotgun metagenomics may still be far from becoming a routine assay.

Finally, with this methodology we are able to focus on the possibility of tracking the disruption or disturbance of native microorganism populations. However, some GMMs could become opportunistic pathogens or disrupt higher organisms such as animals and plants. For this, in vivo models that allow the study of bacterial–host interactions would be an interesting complement to metagenomic data (Virgo et al., 2025). Therefore, the data collected can serve as a solid basis for further analysis; for example, functional annotation can be performed to reconstruct metabolic pathways in the samples, as well as to support models that help predict the behavior of GMMs.

For the assays involving water samples, a high variability within biological replicates was observed for both survival and metagenomic analyses. One possible way to overcome this would be the use of synthetic communities. While this is not fully representative of open environments, it can provide valuable insights to understand mechanisms of communication, adaptation, and possible interferences within a bacterial community.

5.3.2. Complementing metagenomics with GMM tracking

In our study, we used SYBR Green chemistry to evaluate whether this approach could be used to track the specific GMM and complement the metagenomics analysis. While this methodology allows for a cost-effective assay (Sarkar et al., 2022) and has been applied to water samples (Khan et al., 2007; Song et al., 2021), we encountered several challenges.

We identified the major bottleneck as the design of primers that are both specific to our GMM and optimal for qPCR performance. The region selected for this assay, approximately 100 bases upstream and downstream of the gene *endA2*, resulted in non-specific amplification in both PCR and qPCR assays (**Figure 5B**, **Figure 6B**). Because we aimed to distinguish our GMM from P. putida strains naturally present in the environment, we targeted regions corresponding to deleted genes. However, this approach restricts the number of suitable target regions, limiting flexibility in primer design and subsequent qPCR optimization.

This limitation could be overcome by integrating a synthetic barcode specifically designed for qPCR detection, enabling reliable tracking of the GMM. In addition, the use of dye-based chemistry instead of probe-based assays further reduces specificity, which is particularly critical when combined with metagenomic approaches for strain-level detection. The implementation of probe-based qPCR (e.g., TaqMan) would allow for more specific and quantitative detection of the target organism (Liu et al., 2012) and we therefore recommend this approach for future tracking of the GMM.

5.3.3. Modeling approaches to support prediction of GMM survival and competition

Omics data, such as genomics and metagenomics, can be valuable to support the construction of predictive models. Computer simulations have already been used to study changes in bacterial communities. By integrating different types of information (e.g., metabolic pathways, cellular behavior, and community composition), models can be developed to better understand the impact of factors such as cell density on interbacterial competition, allowing for initial screening of conditions prior to experimental validation.

An interesting approach to study the impact of GMMs is the use of agent-based modeling (ABM), which enables the simulation of bacterial populations at the level of individual cells (Virgo et al., 2025). Other modelling frameworks, such as the one developed by Drummond et al., (2018) o track pathogen transport in water systems, may also be relevant. Given the complexity of open environments, simulations that incorporate multiple variables and environmental factors could help predict outcomes related to survival, competition, and potential changes in behavior.

In addition, tools such as the Python package *EnCen* have been developed to model the impact of introducing a GMM into a microbial community and have been tested across different organisms and metagenomic datasets (Docter & Mansfeldt, 2025).

A remaining challenge is the integration of genotype-phenotype relationships into such models. Linking specific genetic modifications to phenotypic traits, including fitness, survival, and interaction with the community, would significantly improve predictive capacity. In particular, understanding how engineered genetic elements may influence traits related to competitiveness or unintended effects remains an open question and an area for future development.

5.4. Genetic modifications: affecting or providing fitness?

On one hand, several studies show that the presence of native microbiota can lead to a decline of GMM populations (Flint, 1987; Kersters, Huys, Duffel, et al., 1996), suggesting that genetic modification does not necessarily confer a competitive advantage in complex environments. On the other hand, it has also been shown that *Pseudomonas* species, if released in an uncontrolled manner, can be detrimental to other organisms (Purtschert-Montenegro et al., 2022; Tsipa et al., 2025).

The impact of genetic modifications on fitness is therefore not straightforward. For example, plasmid carriage has been shown to reduce bacterial growth rates and competitiveness under nutrient-rich conditions (Godwin & Slater, 1979; Zünd & Lebek, 1980). However, under starvation conditions, plasmid-bearing strains can persist similarly to plasmid-free counterparts, suggesting that fitness costs may be context-dependent (Flint, 1987). In addition, while plasmid instability may occur in the absence of selective pressure, some studies report maintenance of plasmid-associated functions even during prolonged nutrient limitation.

Therefore, the nature of the genetic modification should be carefully evaluated through rigorous experimental design to assess whether it confers a fitness advantage or alters ecological behavior. This is highly context-dependent and influenced by multiple factors, including the function of the heterologous gene, its stability, the resulting fitness of the host, and the composition of the native microbiota in the receiving environment.

Targeted genetic modifications aimed at removing potentially harmful traits. For example, genes involved in biofilm formation or the type IVB secretion system (T4BSS), which are associated with competitive interactions (Purtschert-Montenegro et al., 2022), may be beneficial. However, such design strategies should always be complemented with robust experimental assessment to evaluate their impact under relevant conditions.

5.5. Simulated-laboratory conditions and multi-factor considerations in open environment

These experiments, together with other types of assays such as the use of synthetic communities or simplified environmental model systems (e.g., sludge or microcosm setups), provide a solid basis to explore what could happen in open environments. However, this knowledge relies on assumptions that are necessary to enable controlled experimentation. In laboratory settings, many factors are not considered, such as natural elements like wind, rain, (drastic) changes in temperature, and radiation, but also more complex phenomena such as climate change and pollution.

While this might seem far-fetched, recent studies have shown how, for example, plastic can favour the exchange of genetic material, particularly antimicrobial resistance genes (Ferheen et al., 2024; Ram & Kumar, 2020). If a GMM is designed with antimicrobial resistance genes, genes related to pathogenicity, or traits that confer a competitive advantage, these factors should also be taken into account.

Because of the multi-factor nature of open environments, results obtained under simulated laboratory conditions cannot be directly translated. For this reason, open field trials are fundamental. At the same time, we consider pre-screening approaches (using synthetic communities, controlled laboratory simulations, and environmental model systems) complemented with predictive models, to be key steps before moving towards real-world applications.

6. Conclusion and final recommendations

The experiments presented in this report provide a first set of practical approaches to assess survival and competition of GMMs upon escape or release. Several key insights emerge.

First, CFU counting remains the “gold standard” for survival assessment due to its simplicity and accessibility. However, the literature specifically addressing GMM survival is largely outdated. In addition, there is no consensus on experimental protocols, which vary depending on the environmental matrix. Our results align with previous studies, showing that populations tend to reach a steady state rather than complete extinction. At the same time, high variability between biological replicates, especially in water samples, limits robust interpretation. Moreover, the need for strain-specific selective media reduces modularity and reproducibility across studies.

Second, the choice of chassis and its early characterization are critical. Basic phenotypic traits, such as motility, chemotaxis, biofilm formation, and potential pathogenicity, should be systematically assessed under simulated application conditions. Our motility assay illustrates how even a single engineered trait can influence environmental behavior, and such characterization should be considered a standard step in GMM development.

Third, molecular tracking approaches require careful design. The PCR/qPCR strategy used here highlights how specificity becomes a major bottleneck when the chassis is closely related to naturally occurring species. Reliable detection of GMMs in complex environments will require improved primer design or alternative strategies, such as dedicated genetic barcodes or probe-based assays.

Fourth, shotgun metagenomics provides valuable insights into community-level effects and potential ecological impact. However, it remains technically demanding, costly, and sensitive to biases, particularly in low-biomass samples. At present, it should be considered a complementary method rather than a routine assay.

Finally, predictive models are currently underutilized but have clear potential to support experimental design and interpretation, especially in complex and variable environmental settings.

Taken together, these findings highlight that assessing GMM survival and competition requires a combination of methods rather than a single approach. Laboratory-based assays are essential for initial screening, but they cannot fully capture the complexity of open environments. Ultimately, validation in application-relevant settings, using the final engineered strain, will be necessary to obtain realistic insights into escape, survival, and ecological impact.

At the same time, the field of synthetic biology has reached a stage where many GMM-based technologies are technically mature but remain difficult to deploy. This gap is not primarily technological, but methodological. While risk assessment must remain case-specific, depending on the organism, the genetic modification, and the intended application, there is a clear need to standardize how we generate the data that inform these decisions.

For this reason, we propose a stepwise experimental framework (**Table 1**) to guide GMM development. This framework is not intended as a fixed protocol, but as a practical and iterative structure that integrates chassis selection, characterization, genetic design, tracking, survival and competition assessment, and containment strategies. Importantly, it allows feedback between steps, enabling refinement of both the organism and the experimental approach as new data are generated.

Ultimately, advancing the safe and effective use of GMMs in open environments will require closer integration between experimental microbiology, molecular biology, ecology, modeling, and regulatory science. Interdisciplinary collaboration will be key to move from proof-of-concept studies to robust, deployable, and trustworthy applications.

Table 1. Proposed stepwise guideline to support the development and environmental risk assessment of GMMs. It integrates experimental methodologies for characterization, tracking, survival, competition, and containment.

Step	Methodology	
1	**Choice of the chassis**	Literature search, database search, decision making matrix/tree.
2	**Study of the chassis for the envisioned application**	Experimental design for the assessment of viability of the chassis in the condition of the final application.
3	**Genetic modifications**	3.1. Genetic engineering for genome reduction through knock-out of specific pathways. Assessment of reduced fitness compared to wild-type strain. 3.2. Insertion or characterization of a unique identifier with genetic engineering. Experimental validation through PCR/qPCR and literature search.

		3.3. Safety assessment of heterologous genes with literature search. Further safety assessment: genetic engineering toolbox, antibiotic resistance removal. Possibly, experimental validation depending on origin of the genes (when from a pathogenic organism or if they can infer pathogenicity).
4	**Physical containment**	Assessment of escape of the GMM from physical containment with CFU count assay with selective media, PCR and qPCR.
5	**Survival assessment**	5.1. Survival assay of the GMM, based on escape. 5.2. Competition assay of the GMM, based on escape and survival from physical containment. Assessment of competition and interaction of the GMM over native microbial population.
6	**Biological containment**	Based on physical containment assessment, biocontainment strategies can be implemented.
	Final considerations	Overview and final recommendations. Possibility of going back to previous steps and implementing acquired knowledge.

7. References

Álvarez, B., López, M. M., & Biosca, E. G. (2007). Influence of Native Microbiota on Survival of Ralstonia solanacearum Phylotype II in River Water Microcosms. *Applied and Environmental Microbiology*, *73*(22), 7210–7217. https://doi.org/10.1128/AEM.00960-07

Appala Naidu, B., Kannan, K., Santhosh Kumar, D. P., Oliver, J. W. K., & Abbott, Z. D. (2019). Lyophilized B. subtilis ZB183 Spores: 90-Day Repeat Dose Oral (Gavage) Toxicity Study in Wistar Rats. *Journal of Toxicology*, *2019*, 3042108. https://doi.org/10.1155/2019/3042108

Arias, C. R., LaFrentz, S., Cai, W., & Olivares-Fuster, O. (2012). Adaptive response to starvation in the fish pathogen Flavobacterium columnare: Cell viability and ultrastructural changes. *BMC Microbiology*, *12*(1), 266. https://doi.org/10.1186/1471-2180-12-266

Bagdasarian, M., Lurz, R., Rückert, B., Franklin, F. C., Bagdasarian, M. M., Frey, J., & Timmis, K. N. (1981). Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in Pseudomonas. *Gene*, *16*(1–3), 237–247. https://doi.org/10.1016/0378-1119(81)90080-9

Bailey, E. S., Casanova, L. M., & Sobsey, M. D. (2019). Effects of environmental storage conditions on survival of indicator organisms in a blend of surface water and dual disinfected reclaimed water. *Journal of Applied Microbiology*, *126*(3), 985–994. https://doi.org/10.1111/jam.14186

Bala, S., Garg, D., Thirumalesh, B. V., Sharma, M., Sridhar, K., Inbaraj, B. S., & Tripathi, M. (2022). Recent Strategies for Bioremediation of Emerging Pollutants: A Review for a Green and Sustainable Environment. *Toxics*, *10*(8), 484. https://doi.org/10.3390/toxics10080484

Banihashemi, A., Van Dyke, M. I., & Huck, P. M. (2017). Application of long amplicon propidium monoazide-PCR to assess the effects of temperature and background microbiota on pathogens in river water. *Journal of Water and Health*, *15*(3), 418–428. https://doi.org/10.2166/wh.2017.161

Bashan, Y., Puente, M. E., Rodriguez-Mendoza, M. N., Toledo, G., Holguin, G., Ferrera-Cerrato, R., & Pedrin, S. (1995). Survival of Azospirillum brasilense in the Bulk Soil and Rhizosphere of 23 Soil Types. *Applied and Environmental Microbiology*, *61*(5), 1938–1945. https://doi.org/10.1128/aem.61.5.1938-1945.1995

Blaustein, R. A., Pachepsky, Y., Hill, R. L., Shelton, D. R., & Whelan, G. (2013). *Escherichia coli* survival in waters: Temperature dependence. *Water Research*, *47*(2), 569–578. https://doi.org/10.1016/j.watres.2012.10.027

Bogosian, G., & Kane, J. F. (1991). Fate of recombinant Escherichia coli K-12 strains in the environment. *Advances in Applied Microbiology*, *36*, 87–131. https://doi.org/10.1016/s0065-2164(08)70452-0

Bogosian, G., Morris, P. J. L., Hale, M. D., & Kane, J. F. (1992). Fate in water of a recombinantEscherichia coli K-12 strain used in the commercial production of bovine somatotropin. *Journal of Industrial Microbiology*, *9*(1), 27–36. https://doi.org/10.1007/BF01576365

Corich, V., Giacomini, A., Vendramin, E., Vian, P., Carlot, M., Concheri, G., Polone, E., Casella, S., Nuti, M. P., & Squartini, A. (2007). Long term evaluation of field-released genetically modified rhizobia. *Environmental Biosafety Research*, *6*(3), 167–181. https://doi.org/10.1051/ebr:2007006

D’aes, J., Fraiture, M.-A., Bogaerts, B., Van Laere, Y., De Keersmaecker, S. C. J., Roosens, N. H. C., & Vanneste, K. (2025). Metagenomics-based tracing of genetically modified microorganism contaminations in commercial fermentation products. *Food Chemistry: Molecular Sciences*, *10*, 100236. https://doi.org/10.1016/j.fochms.2024.100236

Davis, B. C., Brown, C., Gupta, S., Calarco, J., Liguori, K., Milligan, E., Harwood, V. J., Pruden, A., & Keenum, I. (2023). Recommendations for the use of metagenomics for routine monitoring of antibiotic resistance in wastewater and impacted aquatic environments. *Critical Reviews in Environmental Science and Technology*, *53*(19), 1731–1756. https://doi.org/10.1080/10643389.2023.2181620

de Lorenzo, V., Pérez-Pantoja, D., & Nikel, P. I. (2024). Pseudomonas putida KT2440: The long journey of a soil-dweller to become a synthetic biology chassis. *Journal of Bacteriology*, *206*(7), e00136-24. https://doi.org/10.1128/jb.00136-24

Demkina, A., Slonova, D., Mamontov, V., Konovalova, O., Yurikova, D., Rogozhin, V., Belova, V., Korostin, D., Sutormin, D., Severinov, K., & Isaev, A. (2023). Benchmarking DNA isolation methods for marine metagenomics. *Scientific Reports*, *13*(1), 22138. https://doi.org/10.1038/s41598-023-48804-z

Docter, J., & Mansfeldt, C. (2025). Environmental Census: Modeling Synthetic Biology Ecological Risk with Metagenomic Enzymatic Data and High-Performance Computing. *ACS Synthetic Biology*, *14*(12), 4846–4856. https://doi.org/10.1021/acssynbio.5c00618

Dong, M.-M., Li, Y.-X., Xu, M., Ding, Y.-X., Song, L., Xiong, J., Wei, D.-Z., Xiong, L.-B., & Wang, F.-Q. (2025). An *Escherichia coli* Nissle 1917-based live therapeutics platform with integrated phage resistance and programmable temperature sensitivity. *Journal of Controlled Release*, *387*, 114188. https://doi.org/10.1016/j.jconrel.2025.114188

Drummond, J. D., Boano, F., Atwill, E. R., Li, X., Harter, T., & Packman, A. I. (2018). Cryptosporidium oocyst persistence in agricultural streams –a mobile-immobile model framework assessment. *Scientific Reports*, *8*(1), 4603. https://doi.org/10.1038/s41598-018-22784-x

Ferheen, I., Spurio, R., & Marcheggiani, S. (2024). Vehicle transmission of antibiotic-resistant pathogens mediated by plastic debris in aquatic ecosystems. *iScience*, *27*(6), 110026. https://doi.org/10.1016/j.isci.2024.110026

Flint, K. P. (1987). The long-term survival of Escherichia coli in river water. *Journal of Applied Bacteriology*, *63*(3), 261–270. https://doi.org/10.1111/j.1365-2672.1987.tb04945.x

Gray, D. A., Dugar, G., Gamba, P., Strahl, H., Jonker, M. J., & Hamoen, L. W. (2019). Extreme slow growth as alternative strategy to survive deep starvation in bacteria. *Nature Communications*, *10*(1), 890. https://doi.org/10.1038/s41467-019-08719-8

Ha, D.-G., Kuchma, S. L., & O'Toole, G. A. (2014). Plate-Based Assay for Swimming Motility in Pseudomonas aeruginosa. *Methods in Molecular Biology (Clifton, N.J.)*, *1149*, 59–65. https://doi.org/10.1007/978-1-4939-0473-0_7

Hadwan, M. H., Alta'ee, A. H., Mohammed, R. M., Hadwan, A. M., Al-Kawaz, H. S., & Al Talebi, Z. A. (2024). An efficient protocol for quantifying catalase activity in biological samples. *Bulletin of the National Research Centre*, *48*(1), 34. https://doi.org/10.1186/s42269-024-01189-z

Hartel, P. G., Fuhrmann, J. J., Johnson Jr., W. F., Wolf, D. C., Lawrence, E. G., Staley, T. E., Lopez, C. S., Wollum II, A. G., Mullen, M. D., Skipper, H. D., & Zuberer, D. A. (1994). Survival of a lacZY-Containing Pseudomonas putida Strain under Stressful Abiotic Soil Conditions. *Soil Science Society of America Journal*, *58*(3), 770–776. https://doi.org/10.2136/sssaj1994.03615995005800030019x

Hirose, S., Konishi, N., Sato, M., Suzumura, K., Obata, H., Ohtsuka, K., Doi, R., Goto, K., Kai, A., Arai, S., & Hara-Kudo, Y. (2024). Growth and Survival of *Escherichia albertii* in Food and Environmental Water at Various Temperatures. *Journal of Food Protection*, *87*(4), 100249. https://doi.org/10.1016/j.jfp.2024.100249

Ibrahim, E. M. E., El-Liethy, M. A., Abia, A. L. K., Hemdan, B. A., & Shaheen, M. N. (2019). Survival of E. coli O157:H7, Salmonella Typhimurium, HAdV2 and MNV-1 in river water under dark conditions and varying storage temperatures. *The Science of the Total Environment*, *648*, 1297–1304. https://doi.org/10.1016/j.scitotenv.2018.08.275

Jamieson, R. C., Joy, D. M., Lee, H., Kostaschuk, R., & Gordon, R. J. (2004). Persistence of enteric bacteria in alluvial streams. *Journal of Environmental Engineering and Science*, *3*(3), 203–212.

Kane, J. F. (1993). Environmental assessment of recombinant DNA fermentations. *Journal of Industrial Microbiology*, *11*(4), 205–208. https://doi.org/10.1007/BF01569592

Kersters, I., Huys, G., Duffel, H. V., Vancanneyt, M., Kersters, K., & Verstraete, W. (1996). Survival potential of Aeromonas hydrophila in freshwaters and nutrient-poor waters in comparison with other bacteria. *Journal of Applied Bacteriology*, *80*(3), 266–276. https://doi.org/10.1111/j.1365-2672.1996.tb03219.x

Kersters, I., Huys, G., Van Duffel, H., Vancanneyt, M., Kersters, K., & Verstraete, W. (1996).

Survival potential of Aeromonas hydrophila in freshwaters and nutrient-poor waters in comparison with other bacteria. *Journal of Applied Bacteriology*, *80*(3), 266–276. https://doi.org/10.1111/j.1365-2672.1996.tb03219.x

Khan, I. U. H., Gannon, V., Kent, R., Koning, W., Lapen, D. R., Miller, J., Neumann, N., Phillips, R., Robertson, W., Topp, E., van Bochove, E., & Edge, T. A. (2007). Development of a rapid quantitative PCR assay for direct detection and quantification of culturable and non-culturable *Escherichia coli* from agriculture watersheds. *Journal of Microbiological Methods*, *69*(3), 480–488. https://doi.org/10.1016/j.mimet.2007.02.016

Korhonen, L. K., & Martikainen, P. J. (1991). Survival of Escherichia coli and Campylobacter jejuni in untreated and filtered lake water. *The Journal of Applied Bacteriology*, *71*(4), 379–382. https://doi.org/10.1111/j.1365-2672.1991.tb03804.x

Kozdrój, J. (1996). Competition between different mutants of pseudomonas fluorescens introduced into soil. *Journal of Environmental Science and Health . Part A: Environmental Science and Engineering and Toxicology*, *31*(5), 1111–1125. https://doi.org/10.1080/10934529609376411

Lim, C.-H., & Flint, K. P. (1989). The effects of nutrients on the survival of Escherichia coli in lake water. *Journal of Applied Bacteriology*, *67*(6), 559–569. https://doi.org/10.1111/j.1365-2672.1989.tb02540.x

Liu, C. M., Aziz, M., Kachur, S., Hsueh, P.-R., Huang, Y.-T., Keim, P., & Price, L. B. (2012). BactQuant: An enhanced broad-coverage bacterial quantitative real-time PCR assay. *BMC Microbiology*, *12*(1), 56. https://doi.org/10.1186/1471-2180-12-56

Mahajan, S., Gupta, A., & Sharma, R. (2017). Bioleaching and Biomining. In R. L. Singh (Ed.), *Principles and Applications of Environmental Biotechnology for a Sustainable Future* (pp. 393–423). Springer. https://doi.org/10.1007/978-981-10-1866-4_13

Martínez-García, E., Nikel, P. I., Aparicio, T., & de Lorenzo, V. (2014). Pseudomonas 2.0: Genetic upgrading of P. putida KT2440 as an enhanced host for heterologous gene expression. *Microbial Cell Factories*, *13*(1), 159. https://doi.org/10.1186/s12934-014-0159-3

McClure, N. C., Weightman, A. J., & Fry, J. C. (1989). Survival of Pseudomonas putida UWC1 containing cloned catabolic genes in a model activated-sludge unit. *Applied and Environmental Microbiology*. (world). https://doi.org/10.1128/aem.55.10.2627-2634.1989

McFeters, G. A., & Terzieva, S. I. (1991). Survival ofEscherichia coli andYersinia enterocolitica in stream water: Comparison of field and laboratory exposure. *Microbial Ecology*, *22*(1), 65–74. https://doi.org/10.1007/BF02540213

Molina, L., Ramos, C., Ronchel, M.-C., Molin, S., & Ramos, J. L. (1998). Construction of an Efficient Biologically Contained Pseudomonas putida Strain and Its Survival in Outdoor Assays. *Applied and Environmental Microbiology*, *64*(6), 2072–2078. https://doi.org/10.1128/aem.64.6.2072-2078.1998

Moon, T. S. (2025). Be a GEM: Biocontained, environmentally applied, genetically engineered microbes. *Advanced Drug Delivery Reviews*, *221*, 115578. https://doi.org/10.1016/j.addr.2025.115578

Moreira, L., Agostinho, P., Morals, P. V., & Da Costa, M. S. (1994). Survival of allochthonous bacteria in still mineral water bottled in polyvinyl chloride (PVC) and glass. *Journal of Applied Bacteriology*, *77*(3), 334–339. https://doi.org/10.1111/j.1365-2672.1994.tb03082.x

Morgan, J. L., Darling, A. E., & Eisen, J. A. (2010). Metagenomic Sequencing of an In Vitro-Simulated Microbial Community. *PLOS ONE*, *5*(4), e10209. https://doi.org/10.1371/journal.pone.0010209

Mosimann, C., Oberhänsli, T., Ziegler, D., Nassal, D., Kandeler, E., Boller, T., Mäder, P., & Thonar, C. (2017). Tracing of Two Pseudomonas Strains in the Root and Rhizoplane of Maize, as Related to Their Plant Growth-Promoting Effect in Contrasting Soils. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.02150

Mouree, K. R., Fakruddin, Md., Shishir, Md. A., Azmuda, N., & Akhtar, H. (2023). Bacterial Persistence in Aquatic Environments: A Study of Green Fluorescent Protein (GFP)-Tagged Escherichia coli in Laboratory Microcosms. *Research Journal of Microbiology*, *18*(1), 63–71. https://doi.org/10.3923/rjm.2023.63.71

Muela, A., Pocino, M., Arana, I., Justo, J. I., Iriberri, J., & Barcina, I. (1994). Effect of growth phase and parental cell survival in river water on plasmid transfer between Escherichia coli strains. *Applied and Environmental Microbiology*, *60*(12), 4273–4278. https://doi.org/10.1128/aem.60.12.4273-4278.1994

Munk, P., Brinch, C., Møller, F. D., Petersen, T. N., Hendriksen, R. S., Seyfarth, A. M., Kjeldgaard, J. S., Svendsen, C. A., van Bunnik, B., Berglund, F., Larsson, D. G. J., Koopmans, M., Woolhouse, M., & Aarestrup, F. M. (2022). Genomic analysis of sewage from 101 countries reveals global landscape of antimicrobial resistance. *Nature Communications*, *13*(1), 7251. https://doi.org/10.1038/s41467-022-34312-7

Oh, S., Caro-Quintero, A., Tsementzi, D., DeLeon-Rodriguez, N., Luo, C., Poretsky, R., & Konstantinidis, K. T. (2011). Metagenomic Insights into the Evolution, Function, and Complexity of the Planktonic Microbial Community of Lake Lanier, a Temperate Freshwater Ecosystem. *Applied and Environmental Microbiology*, *77*(17), 6000–6011. https://doi.org/10.1128/AEM.00107-11

Özer Bergman, G., Mecacci, S., Martins dos Santos, V. A. P., & Asin-Garcia, E. (2025). Engineering chimeric signaling proteins for microbial whole-cell biosensors: From design to deployment. *Trends in Biotechnology*. https://doi.org/10.1016/j.tibtech.2025.08.002

Pachepsky, Y. a., Blaustein, R. a., Whelan, G., & Shelton, D. r. (2014). Comparing temperature effects on Escherichia coli, Salmonella, and Enterococcus survival in surface waters. *Letters in Applied Microbiology*, *59*(3), 278–283. https://doi.org/10.1111/lam.12272

Palma, V., Gutiérrez, M. S., Vargas, O., Parthasarathy, R., & Navarrete, P. (2022). Methods to Evaluate Bacterial Motility and Its Role in Bacterial–Host Interactions. *Microorganisms*, *10*(3). https://doi.org/10.3390/microorganisms10030563

Perrot, F., Jouenne, T., Feuilloley, M., Vaudry, H., & Junter, G.-A. (1998). Gel immobilization improves survival of *Escherichia coli* under temperature stress in nutrient-poor natural water. *Water Research*, *32*(12), 3521–3526. https://doi.org/10.1016/S0043-1354(98)00155-9

Pfeifer, V., Beier, S., Alirezaeizanjani, Z., & Beta, C. (2022). Role of the Two Flagellar Stators in Swimming Motility of Pseudomonas putida. *mBio*, *13*(6), e0218222. https://doi.org/10.1128/mbio.02182-22

Purtschert-Montenegro, G., Cárcamo-Oyarce, G., Pinto-Carbó, M., Agnoli, K., Bailly, A., & Eberl, L. (2022). Pseudomonas putida mediates bacterial killing, biofilm invasion and biocontrol with a type IVB secretion system. *Nature Microbiology*, *7*(10), 1547–1557. https://doi.org/10.1038/s41564-022-01209-6

Qin, Y., Qu, B., & Lee, B. (2023). Rapid Monitoring of Viable Genetically Modified Escherichia coli Using a Cell-Direct Quantitative PCR Method Combined with Propidium Monoazide Treatment. *Microorganisms*, *11*(5), 1128. https://doi.org/10.3390/microorganisms11051128

Ram, B., & Kumar, M. (2020). Correlation appraisal of antibiotic resistance with fecal, metal and microplastic contamination in a tropical Indian river, lakes and sewage. *Npj Clean Water*, *3*(1), 3. https://doi.org/10.1038/s41545-020-0050-1

Ramezanian, S., Kang, J., Eskhan, A., & Abu-Lail, N. (2026). Survival and Biofilm Formation of Pseudomonas Putida Under Glucose Starvation on Model Sand Surfaces. *European Journal of Soil Science*, *77*(1), e70285. https://doi.org/10.1111/ejss.70285

Sarkar, S. L., Alam, A. S. M. R. U., Das, P. K., Pramanik, Md. H. A., Al-Emran, H. M., Jahid, I. K., & Hossain, M. A. (2022). Development and validation of cost-effective one-step multiplex RT-PCR assay for detecting the SARS-CoV-2 infection using SYBR Green melting curve analysis. *Scientific Reports*, *12*(1), 6501. https://doi.org/10.1038/s41598-022-10413-7

Schulz, J., Ruddat, I., Hartung, J., Hamscher, G., Kemper, N., & Ewers, C. (2016). Antimicrobial-Resistant Escherichia coli Survived in Dust Samples for More than 20 Years. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.00866

Sessitsch, A., Wilson, K. J., Akkermans, A. D., & de Vos, W. M. (1996). Simultaneous detection of different Rhizobium strains marked with either the Escherichia coli gusA gene or the Pyrococcus furiosus celB gene. *Applied and Environmental Microbiology*, *62*(11), 4191–4194. https://doi.org/10.1128/aem.62.11.4191-4194.1996

Song, F., Kuehl, J. V., Chandran, A., & Arkin, A. P. (2021). A Simple, Cost-Effective, and Automation-Friendly Direct PCR Approach for Bacterial Community Analysis. *mSystems*, *6*(5), 10.1128/msystems.00224-21. https://doi.org/10.1128/msystems.00224-21

Sosah, F. K., Odoom, A., Anim-Baidoo, I., & Donkor, E. S. (2025). How long do pathogens persist and survive in water? A systematic review. *Frontiers in Microbiology*, *16*. https://doi.org/10.3389/fmicb.2025.1654785

Stubbs, T. L., Kennedy, A. C., & Skipper, H. D. (2014). Survival of a Rifampicin-Resistant Pseudomonas fluorescens Strain in Nine Mollisols. *Applied and Environmental Soil Science*, *2014*(1), 306348. https://doi.org/10.1155/2014/306348

Tsipa, A., Pettemereidi, M., Varnava, C. K., Ungor, I., Fragkou, E., & Apidianakis, Y. (2025). Drosophila attraction, colonization, contagion, and mortality by Pseudomonas spp. And toxicity of their biosurfactants. *Applied Microbiology and Biotechnology*, *109*(1), 135. https://doi.org/10.1007/s00253-025-13518-x

Vahjen, W., Munch, J. C., & Tebbe, C. C. (1997). Fate of three genetically engineered, biotechnologically important microorganism species in soil: Impact of soil properties and intraspecies competition with nonengineered strains. *Canadian Journal of Microbiology*, *43*(9), 827–834. https://doi.org/10.1139/m97-120

Vahjen, W., Munch, J.-C., & Tebbe, C. C. (1995). Carbon source utilization of soil extracted microorganisms as a tool to detect the effects of soil supplemented with genetically engineered and non-engineered Corynebacterium glutamicum and a recombinant peptide at the community level. *FEMS Microbiology Ecology*, *18*(4), 317–328. https://doi.org/10.1111/j.1574-6941.1995.tb00188.x

van Overbeek, L. S., van Elsas, J. D., Trevors, J. T., & Starodub, M. E. (1990). Long-term survival of and plasmid stability inPseudomonas andKlebsiella species and appearance of nonculturable cells in agricultural drainage water. *Microbial Ecology*, *19*(3), 239–249. https://doi.org/10.1007/BF02017168

Virgo, M., Mostowy, S., & Ho, B. T. (2025). Emerging models to study competitive interactions within bacterial communities. *Trends in Microbiology*, *33*(6), 688–700. https://doi.org/10.1016/j.tim.2024.12.009

Widmer, F., Seidler, R. J., Gillevet, P. M., Watrud, L. S., & Di Giovanni, G. D. (1998). A Highly Selective PCR Protocol for Detecting 16S rRNA Genes of the Genus Pseudomonas (Sensu Stricto) in Environmental Samples. *Applied and Environmental Microbiology*, *64*(7), 2545–2553. https://doi.org/10.1128/aem.64.7.2545-2553.1998

Yoneda, I., Nishiyama, M., & Watanabe, T. (2024). Significant Factors for Modelling Survival of Escherichia coli in Lake Sediments. *Microorganisms*, *12*(6). https://doi.org/10.3390/microorganisms12061192

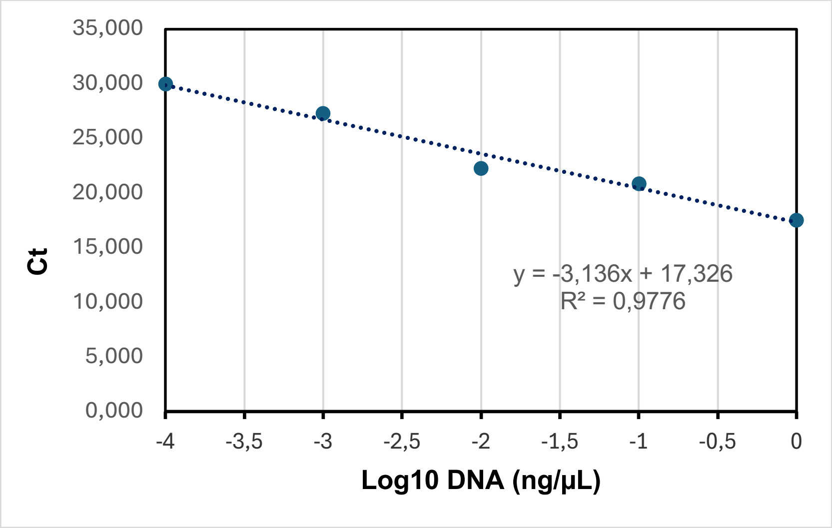
Appendix

Supplementary Table 1. Methodologies used to assess survival in non-optimal conditions. Non-exhaustive collection of methodologies to assess survival of microorganisms in non-optimal conditions, along with year of publication and strain used. It is also indicated whether the microorganism is a GMM not. In this table GMM is considered as such with any small modification, such as knock-out (KO).

Aim of the study	Methodologies	Strain	GMM	Year	Reference
Long-term survival of *E. coli* in river water.	CFU count (Log viable count).	*E. coli* strains: R1-19; R144-3 and nal-R	Yes	1987	(Flint, 1987)
Survival of *E. coli* in lake water.	Colony counting (Log viable count).	*E. coli* ML10	No	1989	(Lim & Flint, 1989)
Survival of *P. putida* UWC1 containing genes for the breakdown of 3-chlorobenzoate in activated-sludge unit.	CFU count (Log count).	*P. putida* UWC1	Yes	1989	(McClure et al., 1989)
Survival and plasmid stability in agricultural drainage water of different organisms.	CFU count (CFU/mL).	*P. fluorescens* R2f, *P. putida* CYM318, *Klebsiella aerogenes* NCTC418	Yes	1990	(van Overbeek et al., 1990)
Fate of recombinant *E. coli* K-12 strains in the environment.	Not accessible	Not accessible	Yes	1991	(Bogosian & Kane, 1991)
Persistence of pathogenic and nonpathogenic strains in water: field and laboratory conditions.	CFU count (Log count), violet binding assay.	*E. coli* strain E7, *Yersinla enterocolitica strains E661 and E77*	No	1991	(McFeters & Terzieva, 1991)
Survival of different microorganisms in untreated and filtered lake water.	CFU count (Relative survival)	*E. coli* ATCC25922 and *Campylobacter jejuni* biotype 1	No	1991	(Korhonen & Martikainen, 1991)
Fate in water of the recombinant *E. coli* K-12 used for the bioproduction of bovine somatotropin.	CFU count (Viable count)	*E. coli* K-12 strain LBB269 pBGH 1	Yes	1992	(Bogosian et al., 1992)
Effect of growth phase on plasmid transfer between *E. coli* strains in river water.	CFU count, bacterial size measurements, heterotrophic bacterial activity estimations, and conjugal transfer assay.	*E. coli* strains EC, 416S, J62, 416S, J62	Yes	1994	(Muela et al., 1994)
Survival of a *lacZY*-containing *P. putida* under abiotic soil conditions.	CFU count (Log colony forming unit).	*P. putida*	Yes	1994	(Hartel et al., 1994)
Survival of *Allochthonous* bacteria in bottled water.	CFU count (Log colony forming unit).	*E. coli* (ATCC 8677), *Ent. cloacae* (ATCC 13047), *Kl. pneumoniae* (ATCC 13833) and *P. aeruginosa* (ATCC 27853)	No	1994	(Moreira et al., 1994)
Survival of *Azospirillum brasilense* in different soil types.	CFU count (Log CFU/g), ELISA, time-limited liquid enrichment technique.	*A. brasilense* Cd (ATCC 29710) and Sp-245	No	1995	(Bashan et al., 1995)
Effect on carbon source utilization in soil supplemented with genetically engineered and non-engineered bacteria.	Substrate utilization, CFU count.	*Corynebacterium glutamicum ATCC 13032*	Yes	1995	(Vahjen et al., 1995)
Competition between different mutants of *P. fluorescens* introduced into soil.	CFU count (Log colony forming unit).	*P. fluorescencens*	Yes	1995	(Kozdrój, 1996)

Survival of *Aeromonas hydrophila* in different water microcosms.	CFU count (Log colony forming unit).	*A. hydrophila,*	Yes	1996	(Kersters, Huys, Van Duffel, et al., 1996)
Detection of different *Rhizobium* strains marked with *gusA* gene or the *celB* gene.	Histochemical substrate.	*Rhizobium* spp.	Yes	1996	(Sessitsch et al., 1996)
Fate of genetically engineered species in different soil types and competition with nonengineered strains.	CFU count (Log CFU/g soil).	*Corynebacterium glutamicum* ATCC 13032 and *C. glutamicum* pUN1	Yes	1997	(Vahjen et al., 1997)
Survival of immobilized *E. coli* under temperature stress and nutrient-poor natural water.	CFU count (Log CFU/ml), viability with staining and microscope.	*E. coli* 1044552	No	1998	(Perrot et al., 1998)
Impact of native microbiota on survival of *Ralstonia solanacearum* in river water microcosms.	CFU count (Log CFU/mL).	*Ralstonia solanacearum* IVIA-1602.1	No	2007	(Álvarez et al., 2007)
Adaptation of *Flavobacterium columnare* in starving conditions.	CFU counts, light microscopy, scanning electron microscopy	*F. columnare*	No	2012	(Arias et al., 2012)
Survival of *E. coli*: temperature dependence.	Mathematical model	*E. coli*	No	2013	(Blaustein et al., 2013)
Survival of *Pseudomonas fluorescens* strain in different soil types.	CFU count (Log CFU/mL).	*P. fluorescens* D7rif	No	2014	(Stubbs et al., 2014)
Comparing temperature effects on different species survival in surface waters.	Mathematical model.	*E. coli*, *Salmonella* and *Enterococcus*	No	2014	(Pachepsky et al., 2014)
Survival of *E. coli* in dust samples over 20 years.	CFU count (CFU/g).	*E. coli*	No	2016	(Schulz et al., 2016)
Assessment the effect of temperature and microbiota on pathogens in river water.	qPCR	*Yersinia enterocolitica, Salmonella enterica, Campylobacter jejuni*	No	2017	(Banihashemi et al., 2017)
Persistence of *Pseudomonas* species in roots and rhizoplane.	CFU count (Log10 CFU/g).	*Pseudomonas fluorescens* Pf153 and *Pseudomonas* sp. DSMZ 13134	No	2017	(Mosimann et al., 2017)
Survival and stability of these pathogens in the Nile River water.	CFU count (CFU/mL).	*E. coli* O157:H7, *Salmonella Typhimurium* ATCC 14028	No	2019	(Ibrahim et al., 2019)
Survival of *Bacillus subtilis* cells in deep starvation conditions for many months.	Colony counting (Log10 CFU/mL), membrane potential, transcriptomics	*Bacillus subtilis*	Yes	2019	(Gray et al., 2019)
Effects of storage conditions on survival of indicator organisms in different water types.	Spot-plate titre assay, CFU count (CFU/mL)	*E. coli*, F+/male-specific coliphages, somatic coliphages and *C. perfringens* spores and *Enterococcus* sp.	No	2019	(Bailey et al., 2019)
Persistence and survival of *E. coli* DH5α with GFP marker.	CFU count (Log CFU/mL).	*E. coli*	Yes	2023	(Mouree et al., 2023)
Monitoring of viable *E. coli*.	qPCR.	*E. coli*	Yes	2023	(Qin et al., 2023)
Survival of *Escherichia albertii* in food and water.	CFU count (Log CFU/mL), qPCR.	*E. albertii*	No	2024	(Hirose et al., 2024)
Survival of *E. coli* in lake sediments.	CFU count (Population change rate).	*E. coli*	No	2024	(Yoneda et al., 2024)

Study of survival and biofilm formation of *P. putida* in sand surfaces.	CFU count (Log CFU/mL), extraction of extracellular polymeric substances, atomic force microscopy for biofilm adhesion.	*P. putida*	No	2026	(Ramezanian et al., 2026)



Supplementary Figure 1. qPCR standard curve generated using primers endA2 FW and RV. The slope (-3.136) and intercept (17.326) are indicated. PCR efficiency was calculated as $E = 10^{(-1/\text{slope})}$, and expressed as percentage efficiency using %E = (E-1) × 100.