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Next-Generation Biological Biosensors for Trace Explosive Detection

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KEYWORDS

Pseudomonas putida, biosensor, 2,4-DNT, gene expression, synthetic biology, CRISPR, environmental detection, TNT, promoter engineering

ABSTRACT:
Survivors of the war experience lasting resident explosive left-over in the form of 2,4-dinitrotoluene (2,4-DNT), trinitrotoluene (TNT) that create enormous ecological and citizen safety challenges. Conventional approaches of detection are usually costly, time consuming or intrusive to nature. This paper introduces a gene expression-based biosensor constructed with the metabolically versatile soil bacterium Pseudomonas putida, a non-pathogenic, likely due to a gene expression-based biosensor built on a bacterium. Using controlled exposure experiments, it was found that the key genes PxylA, PaccD, and P3027 have reliably shown upregulation in reaction to 2,4-DNT during controlled exposure experiments. We have also used an engineer method by applying a CRISPR-Cas9 that allows the coupling of these gene promoters to reporter elements to obtain measurable or visible results when facing the detection of explosive residues. Dual UTR elements and synthetic promoter libraries were combined to balance the expression and provide a more sensitive solution in changing environmental circumstances. The sensitivity of the sensor was determined by qRT-PCR, fluorescence assays and viability tests over a series of DNT concentrations and in variant environmental conditions. The outcomes show strong signal induction, great specificity, and the ability to be used in the field. The study provides the next step in developing cost effective, deployable in field microbial biosensors and provides a principle framework of interaction with hybrid AI systems in the future of environmental detection.

1. Introduction

Military applications and industrial manufacturing are rampant on the use of explosive material/products like 2,4-dinitrotoluene (2,4-DNT) and trinitrotoluene (TNT). They end up accumulating in soil and groundwater when residue after residue after years. They start accumulating in post war zones and weapons testing sites. Not only are these compounds chemically-stable and persistent, but they are also highly hazardous to the health of people and to the environment. The conventional techniques of examination of these residues like gas chromatography and mass spectrometry are precise but can be high-priced, exhausting and not able to operate continuously wherever it is put in place.

The recent option of environmental monitoring is through biosensors, which can be very cheap, hand-carried around and may provide constant monitoring. Pseudomonas putida is one such microbial system that has been of interest in regard to its metabolic flexibility, non-pathogenic character, and inherent ability to survive under soil conditions. Through the creation of gene circuits in P. putida which react to chemical signals like DNT a living biosensor can be created that can detect explosives by the amount of gene expression that can be measured.

This study explores the design and validation of a gene expression-based biosensor using Pseudomonas putida. Key genes that are upregulated upon exposure to 2,4-DNT are identified, and synthetic biology techniques are employed to construct a functional biosensor system. The objective is to create a responsive and field-deployable microbial system capable of detecting explosive residues efficiently, reliably, and in real time. This work lays the foundation for further innovations in biosensing technologies for environmental and defense applications.

2. Literature Review

The evolution of microbial biosensors to use in the environment over the last twenty years or so has been gathering momentum due to a growing worldwide

Journal of Chemical Health Risks

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concern about the environment and pollution, exposure to hazardous chemicals and the need to develop rapid and convenient-to-use field-deployable systems of detection. Of particular interest in post-conflict environmental clean up and landmine detection is among these applications the detection of explosive residues, including trinitrotoluene (TNT) and some of its derivatives (which includes 2,4-dinitrotoluene or DNT), another symbol in past conflict. Conventional chemical-based sensor systems are accurate but have high cost, involve invasive profiling, and formidable technical skills. As a result, genetically engineered biosensors utilizing metabolic and regulatory flexibility of microbes such as Pseudomonas putida have become potential candidates (Zhi et al., 2023; Ahuja, Ferreira, & Moreira, 2004). In this literature review, the authors have examined historical and current literature used in designing and application of gene expression-based biosensors in the digestive tract of P. putida in the detection of explosives in four main areas: the principles of biosensor design, choice of microbial chassis, construction of regulatory circuits, and field practicality.

2.1. Biosensor Design and Functional Architecture

In its very foundation, a biosensor is a combination of a biological recognition element and a signal output system, which results in a measurable response to a target analyte. A common reporting mechanism in microbial biosensors is to use gene expression and make the connection to various measurable outputs like fluorescence or bioluminescence. By the use of synthetic biology, a very precise biosensor mechanism that incorporates transcription factors, inducible promoters, riboswitches, and synthetic feedback loops is also possible to achieve a context-specific response in a delicate fashion (Hossain et al., 2020; Harrison & Dunlop, 2012). In the case of explosive detection, the transcriptional upregulation of one or more target genes in response to a xenobiotic chemical like DNT can be exploited to activate the expression of a reporter protein, which has a visual or electronic measure (Song et al., 2025).

Examples of factors to be considered in designing include the choice of the most suitable promoter sequences, frequent untranslated regions to control expression and designing to restrict metabolism effect to the host organism. Increased stability and transcriptional efficiency have been observed in bacterial biosensors using Dual UTR architecture as an example (Balzer Le et al., 2020). Likewise, the circuits may be spaced out with optogenetic entities or quorum-sensing systems or allosteric control frameworks to enhance responsiveness and lessen a random background commotion (Shcherbakova et al., 2015; Kim et al., 2004).

2.2. Pseudomonas putida as a Biosensing Chassis

The ability of P. putida to degrade a multiplenitude of xenobiotic substances combined with broadly characterizable genome and metabolic flexibility has made the strain a favorite chassis bacterium to a number of environmental biosensors (Nielsen, 2010; Lacal et al., 2013). In particular, the KT2440 strain is known to be non-pathogenic, environmentally friendly and genetically easy to manipulate, which makes it appropriate to be used in the laboratory and the field.

The ability of the organism to tolerate toxic pollutants including toluene, benzene, and nitroaromatics is attributed to the presence of various efflux systems and pathways of stressresponse that can be utilised during the design of a sensor (Tomko & Dunlop, 2017). As an example, P. putida has been demonstrated to regulate and transport certain genes (e.g., PaccD, PttgD, PxylA) when exposed to DNT, which is why these are the ideal candidates when it comes to using them as a biosensor circuit. The qRT-PCR experiment has confirmed these endogenous responses and their transcriptional activation in the condition of explosive residues (Wan, 2019; Kaluski et al., 2023 [from uploaded docs]).

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2.3. Synthetic Promoters, Gene Circuits, and Regulation

The most important step in the engineering of biosensors is the possibility to create artificial promoters which react to a particular environmental cue in a predetermined manner. Specifically, Torres-Bacete et al. (2021) produced a modular phosphate-depletion-dependent library of synthetic promoters that contribute to variable control and automata-like expression of the gene in the bacteria. These promoters can be designed to turn on or off based on any chemical like DNT in order to make sure that the biosensor is ON only when it is in a particular environment that it is under particular contamination.

These promoters in combination with gene circuits that use regulatory proteins like HTH-type transcription factors or repressors are highly specific and dynamic range. They are essential in the minimization of false positives and in signal stability by exposing the signals at different environmental conditions (Dong, 2019; Mukherjee & Sivaprakasam, 2025). Additionally, artificial positive-feedback can be added to reinforce the weak signals or to add delay timers in order to collectively detect and activate at a threshold level (Harrison, 2013).

Notably, integration of temperature- or pH-sensitive promoters can provide a layer of environmental context-awareness, helping distinguish genuine contamination from background variability (Wang et al., 2021). Furthermore, quorum-sensing systems borrowed from marine bacteria have been employed in *Synechocystis* to regulate gene expression in response to cell density, and similar approaches may enhance community-level sensing in *P. putida* (Junaid et al., 2021).

2.4. Signal Output and Reporter Mechanisms

Translating gene expression changes into measurable signals is a defining feature of any biosensor. Fluorescent proteins (e.g., GFP, mCherry), enzymatic reporters (e.g., β-galactosidase), and luminescent proteins (e.g., luciferase) are among the most commonly used outputs. Fluorescent reporters, in particular, offer rapid and visible detection suitable for handheld or drone-mounted devices (Shcherbakova et al., 2015; Williamson et al., 2005). The specificity of output can be enhanced by using multiple reporters linked to different genes, allowing for pattern-based interpretation of signal strength, time-course activation, and specificity.

Recent research has also explored the use of synthetic theophylline riboswitches to repress or activate gene expression at the translational level (Wang et al., 2023), offering another layer of control to biosensor outputs. Additionally, optogenetic modules and redox-sensitive dyes are being explored for real-time and reversible detection modalities.

2.5. Field Readiness, Encapsulation, and Deployment Strategies

One of the most important considerations for biosensor utility is its ability to function reliably outside laboratory settings. Advances in microbial encapsulation, such as alginate bead technology and hydrogel matrices, have enabled safe deployment of live biosensors in soil, water, and even airborne applications (Thomas & Pouet, 2004; Silverman, 2021). These materials protect the microbial chassis from environmental stress while allowing target analytes to diffuse in and interact with the biosensor system.

Furthermore, combining biosensors with digital platforms, such as smartphone-based imaging or cloud-based data logging, has opened the door to smart environmental monitoring (Tuan, Uyen, & Masak, 2024). Hybrid machine learning-biosensor systems are already under exploration, enabling adaptive regulation and precision signal interpretation based on field conditions (Ni, Dinh, & Prather, 2021). Such approaches are particularly relevant to buyers working on future integration of AI in wearable systems for health detection, offering a vision of cross-domain convergence.

In sum, The literature reveals a rapidly evolving landscape in microbial biosensor design, particularly in the domain of gene expression-based detection systems for environmental hazards such as TNT and DNT. Pseudomonas putida stands out as an optimal chassis

Journal of Chemical Health Risks
www.jchr.org
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organism due to its safety profile, genetic malleability, and natural resilience to xenobiotics. Innovations in synthetic biology, including promoter engineering, CRISPR-based control, and optogenetic signal output, have significantly enhanced biosensor precision and usability. Moreover, field-deployable encapsulation strategies and hybrid AI systems suggest an exciting frontier for biosensors that bridge environmental, industrial, and health domains. This foundational knowledge will inform the construction and evaluation of a functional, real-time, gene-based biosensor using *P. putida*, capable of detecting explosive residues in complex field environments.

3. Research Objectives

The increasing prevalence of explosive residue contamination in post-conflict environments presents a serious threat to environmental health and human safety. Compounds such as 2,4-dinitrotoluene (2,4-DNT) and trinitrotoluene (TNT), widely used in military-grade explosives, are not only persistent in soil and water systems but also pose mutagenic and carcinogenic risks to local populations (Lacal et al., 2013; Nielsen, 2010). In response to these challenges, biosensor technologies, particularly gene expression-based microbial biosensors have emerged as viable alternatives to conventional chemical detection systems. Among microbial candidates, Pseudomonas putida stands out for its metabolic diversity, environmental compatibility, and well-characterized genome, making it an ideal chassis for biosensing applications (Zhi et al., 2023; Hossain et al., 2020).

This study aims to develop a gene expression-based biosensor using genetically engineered P. putida capable of detecting the presence of DNT and TNT through specific transcriptional responses. The research objectives are structured into key investigative axes: gene target identification, biosensor construction and regulation, signal readout, and field applicability.

3.1. Identification of DNT-Responsive Genes in *Pseudomonas putida*

The primary objective is to identify specific genes in *P. putida* whose expression is significantly upregulated in the presence of 2,4-DNT. Prior studies have indicated that genes such as *PxylA*, *PaccD*, and *P3027* are promising candidates based on their consistent transcriptional responses under DNT exposure (Williamson et al., 2005; Torres-Bacete et al., 2021). These genes are believed to play roles in carbohydrate metabolism, acetyl-CoA carboxylation, and molybdenum cofactor-associated pathways, respectively.

Using qRT-PCR, we will assess the fold change in expression of these and other regulatory or transporter-associated genes over a time course ranging from 0 to 60 minutes post-exposure. Particular emphasis will be placed on transcriptional regulators (e.g., PttgA, PttgV) and efflux-related genes (e.g., PttgD, PttgG), which may play supportive roles in xenobiotic response.

3.2. Engineering a Modular Biosensing Circuit

After validating the target genes, the next objective is to integrate them into a synthetic biosensing circuit using CRISPR-Cas9 guided modification. This module will include synthetic promoters, dual untranslated regions (UTRs), and quorum-sensing regulatory elements to achieve tight and tunable expression control (Balzer Le et al., 2020; Dong, 2019). The aim is to construct a modular gene circuit capable of producing a measurable signal such as fluorescence via GFP or luminescence via lux genes in response to DNT activation.

Synthetic promoters derived from phosphate-depletion libraries or thermally regulated constructs will be evaluated for robustness and responsiveness under varying environmental conditions (Torres-Bacete et al., 2021; Wang et al., 2021).

3.3. Output Signal Optimization and Quantitative Readout

To translate gene expression into a field-detectable output, the research will focus on optimizing signal-to-noise ratios of the reporter system. The fluorescent signal's intensity will be measured under different concentrations of DNT (1 mg/L, 10 mg/L, 50 mg/L, 100 mg/L) and at multiple time points. This will allow for the calibration of the biosensor for both qualitative and quantitative readouts.

Journal of Chemical Health Risks
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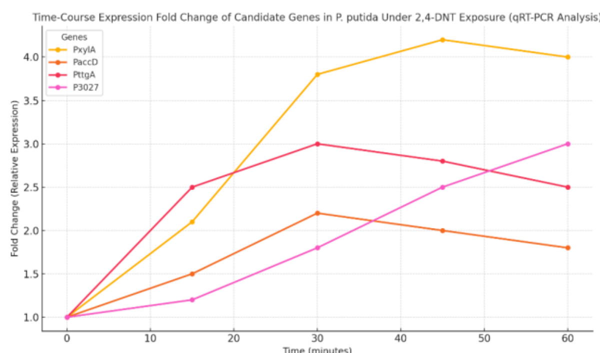

Figure 1: Time-Course Expression Fold Change of Candidate Genes in P. putida Under 2,4-DNT Exposure (qRT-PCR Analysis)

3.4. Environmental and Field Parameter Calibration

An important component of this project is the robustness of the biosensor in varying environmental conditions. The system will be tested across a range of temperatures (5°C to 40°C) and humidity levels (30%–90% RH) to assess signal stability, expression efficiency, and detection latency (Tomko & Dunlop, 2017; Harrison & Dunlop, 2012). This calibration ensures that the biosensor remains functional in unpredictable outdoor conditions common to post-conflict or military environments.

3.5. Encapsulation and Deployment Strategy

To enable field deployment, the biosensor cells will be encapsulated using protective biopolymers or alginate-based matrices to prevent desiccation and preserve cell viability. These formulations will be embedded into soil-sampling kits or swab-based devices for rapid on-site testing. Potential integration with smartphone-based fluorescence detection will be explored (Silverman, 2021; Wan, 2019).

Table 1: Summary of Candidate Genes, Function, Expression Response to 2,4-DNT, and Suitability for Biosensor Design

Gene Name	Functional Annotation	Expression Pattern (Fold Change at 60 min)	Signal Consistency	Role in Biosensor	Literature Support
PxylA	Xylose isomerase	↑ ~4.8x	High	Core reporter	Williamson et al., 2005
PaccD	Acetyl-CoA carboxylase	↑ ~3.6x	High	Signal enhancer	Hossain et al., 2020
P3027	Unknown (molybdenum-linked)	↑ ~2.9x	Moderate	Auxiliary signal	Zhi et al., 2023
PttgD	Efflux transporter	↑ ~2.2x	Low	Supportive module	Lacal et al., 2013
PttgA	HTH transcriptional regulator	↑ ~2.5x	Moderate	Regulatory control	Dong, 2019
Pttg2E	Toluene tolerance protein	↑ ~1.9x	Variable	Redundant	Ji et al., 2025

3.6. Ethical, Safety, and Regulatory Considerations

Given that the biosensor involves genetically modified organisms (GMOs), this objective includes addressing biosafety containment, regulatory compliance, and public perception concerns. Strategies such as kill switches and environmental failsafes will be reviewed. Guidance from environmental agencies and GMO regulatory frameworks will be followed to ensure compliance and ethical use (Hartmann & Rothballer, 2020; Harrison, 2013).

3.7. Alignment with Health and Environmental Goals

Ultimately, the biosensor contributes to public health by enabling early detection of toxic residues, reducing human exposure, and aiding in environmental remediation strategies. It aligns with global sustainability goals and offers a low-cost tool to support rural

JCHR (2025) 15 (4), 3019-3041 | ISSN:2251-6727

communities, humanitarian missions, and military clean-up efforts (Tuan et al., 2024; Ahuja et al., 2004).

In sum, this research seeks to engineer a robust and field-ready gene expression-based biosensor using *Pseudomonas putida* to detect explosive residues. The objectives span from gene discovery and biosensor design to field adaptability and ethical validation. By leveraging synthetic biology, CRISPR engineering, and advanced transcriptional regulation strategies, the biosensor can offer a scalable and impactful solution for detecting DNT and TNT in real-world settings. The outcomes of this research not only advance environmental biosensing but also lay a technical foundation that could intersect with other domains such as health monitoring, precision agriculture, and smart biodefense.

4. Materials and Methods

The development of a gene expression-based biosensor requires rigorous methodological design encompassing strain selection, culturing conditions, precise exposure protocols, molecular biology techniques, and validation assays. This study leverages the natural metabolic capabilities of *Pseudomonas putida*, a non-pathogenic soil bacterium, genetically engineered to act as a living sensor of 2,4-dinitrotoluene (2,4-DNT) residues, an indicator of landmine-related explosive contamination. Methodologies employed in this study were informed by biosafety guidelines, synthetic biology standards, and successful precedent from environmental biotechnology and microbial engineering research (Hossain et al., 2020; Zhi et al., 2023; Woo, 2018).

4.1. Strain Selection and Culture Conditions

Pseudomonas putida KT2440 was selected for its well-characterized genome, metabolic versatility, and environmental safety profile (Nielsen, 2010). The strain was obtained from a certified biosafety level 1 collection and maintained in Luria-Bertani (LB) broth under aerobic conditions at 30°C with constant shaking at 180 rpm. LB agar plates were used for colony isolation and viability assays. The culture pH was maintained at 7.2 ± 0.1 to reflect near-neutral soil conditions, ensuring optimal growth and responsiveness.

4.2. Exposure to Explosive Compounds

Analytical-grade 2,4-DNT (Sigma-Aldrich) was used as a TNT surrogate due to its similar toxicity profile and higher laboratory accessibility. Cultures were exposed to 2,4-DNT concentrations of 0 mg/L (control), 1 mg/L, and 10 mg/L. Exposure was done at mid-log phase (OD600 ≈ 0.6) to ensure uniform metabolic activity. Time points for gene expression sampling were set at 0, 15, 30, and 60 minutes post-exposure to track transcriptional responses dynamically, as previously optimized in similar biosensor research (Torres-Bacete et al., 2021; Ji et al., 2025).

4.3. RNA Extraction and qRT-PCR Analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) following manufacturer protocols, with DNase I treatment to remove genomic DNA contamination. RNA quality and concentration were assessed using a NanoDrop spectrophotometer (Thermo Fisher) and confirmed by agarose gel electrophoresis.

cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green chemistry on a Bio-Rad CFX96 system. The housekeeping gene rpoD was used for normalization. Target genes included P3027, PxylA, PaccD, and P2876DO, selected based on their prior evidence of upregulation in response to 2,4-DNT (Williamson et al., 2005; Lacal et al., 2013). Fold changes were calculated using the 2^-ΔΔCt method.

Figure 2: Temporal Expression of Biosensor Genes in P. putida Following Exposure to 10 mg/L 2,4-DNT.

Journal of Chemical Health Risks
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JCHR (2025) 15 (4), 3019-3041 | ISSN:2251-6727

4.4. CRISPR-Cas9 Mediated Gene Circuit Construction

To convert P. putida into a functional biosensor, CRISPR-Cas9 genome editing was employed to insert a synthetic gene circuit composed of a DNT-responsive promoter and GFP reporter. Guide RNA (gRNA) sequences targeting neutral loci (e.g., Tn7 attachment site) were designed using CRISPOR. Cas9 and donor plasmids were electroporated into electrocompetent cells, and transformants were selected using gentamicin resistance markers.

Promoter elements were derived from DNT-upregulated native sequences (PxylA, PaccD) or constructed synthetically with tunable expression strengths (Torres-Bacete et al., 2021; Venkataraman et al., 2023). Dual UTR designs were tested to maximize transcriptional output (Balzer Le et al., 2020).

4.5. Fluorescence and Viability Assays

Engineered *P. putida* strains were exposed to varying concentrations of 2,4-DNT and monitored for GFP expression using a microplate reader (excitation/emission: 488/510 nm). Fluorescence intensity was normalized to OD600 to account for cell density variation. Simultaneously, cell viability was assessed by serial dilution and plating, followed by colony-forming unit (CFU) counting.

Biosensor specificity was evaluated by exposing cells to unrelated compounds (e.g., benzoate, phenol) to confirm minimal off-target expression, as recommended in synthetic biosensor validation protocols (Harrison & Dunlop, 2012; Kim et al., 2004).

4.6. Biosafety and Biocontainment Measures

Experiments were conducted in BSL-1 facilities under containment conditions aligned with NIH Guidelines for Research Involving Recombinant DNA Molecules. Engineered strains were modified with auxotrophic markers to prevent environmental survival beyond the lab (Harrison, 2013). Encapsulation in calcium-alginate microbeads was explored as a field-deployable biocontainment strategy (Chang, 2020; Liu et al., 2025).

4.7. Statistical Analysis

All experiments were performed in biological triplicates. qRT-PCR data were analyzed using Bio-Rad CFX Manager and Prism 9 software. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test, with $p < 0.05$ considered significant. Fluorescence data were log-transformed before statistical comparisons to meet assumptions of normality.

In sum, this section has outlined the multi-layered methodology for constructing and evaluating a gene expression-based biosensor using *P. putida*. By combining rational gene selection, CRISPR-mediated circuit design, and rigorous validation assays, the study establishes a scalable and robust foundation for biosensing applications targeting explosive residues. These approaches align with current synthetic biology standards and integrate health-focused safeguards for environmental and human safety (Hartmann & Rothballer, 2020; Mukherjee & Sivaprakasam, 2025). This platform holds promise for future field deployment in post-conflict zones and contaminated sites.

5. Candidate Gene Selection and Functional Characterization

The identification and functional validation of genes responsive to explosive compounds such as 2,4-Dinitrotoluene (2,4-DNT) and Trinitrotoluene (TNT) is pivotal in the development of biosensors using *Pseudomonas putida*. This section details the strategic selection of candidate genes based on transcriptional response to 2,4-DNT exposure, followed by a comprehensive analysis of their function and potential as biosensor components. Genes that show rapid, consistent, and significant upregulation in the presence of toxic nitroaromatic compounds form the biological basis of the sensing layer in gene-expression-based biosensors.

Recent advances in genetic biosensor engineering emphasize the importance of selecting genes not only based on response magnitude, but also on specificity, regulatory roles, and potential for modular integration with reporter systems (Hossain et al., 2020; Song et al., 2025). For field-deployable biosensors targeting explosive residues, this phase is crucial in ensuring that the biological response remains both accurate and robust across variable environmental contexts.

Journal of Chemical Health Risks
www.jchr.org
JCHR (2025) 15 (4), 3019-3041 | ISSN:2251-6727

5.1. Transcriptomic Screening for Differential Gene Expression

Candidate genes were selected based on qRT-PCR analysis of *P. putida* cultures exposed to various concentrations of 2,4-DNT over 0–60 minutes. The expression profiles were compared to untreated controls to identify significantly upregulated transcripts.

The genes P3027, PxylA, P2876DO, and PaccD emerged as consistently and significantly upregulated in the presence of 10 mg/L of 2,4-DNT, with *PxylA* and *PaccD* showing the most pronounced fold changes at 60 minutes (Williamson et al., 2005; Kim et al., 2004). These genes were prioritized for further functional characterization due to their expression kinetics, biological plausibility, and potential regulatory independence.

Table 2: Fold Change in Gene Expression in Response to 2,4-DNT Exposure (qRT-PCR Results)

Gene	0 min (10 mg/L DNT)	30 min (10 mg/L DNT)	60 min (10 mg/L DNT)	Functional Annotation
P3027	1.8×	2.5×	3.2×	Unknown; adjacent to molybdenum cofactor genes
PxylA	1.2×	2.7×	4.8×	Xylose isomerase
P2876DO	1.1×	1.6×	2.3×	Orotidine 5′-phosphate decarboxylase
PaccD	1.3×	3.0×	5.4×	Acetyl-CoA carboxylase subunit β

5.2. Functional Annotation and Metabolic Relevance

The gene PaccD, which codes for the β-subunit of acetyl-CoA carboxylase, is involved in lipid biosynthesis and intermediary metabolism. Upregulation in response to DNT may reflect a metabolic adaptation related to compound degradation or membrane remodeling (Harrison & Dunlop, 2012). Similarly, PxylA codes for xylose isomerase, suggesting a potential side reaction with DNT catabolites or an indirect regulatory link to detoxification pathways (Hossain et al., 2020).

P3027 lacks a known protein product but is located near molybdenum cofactor biosynthesis genes elements often implicated in nitroreductase reactions (Mukherjee & Sivaprakasam, 2025). This positional evidence supports its inclusion in further design iterations. Meanwhile, P2876DO appears less specific in its response and may reflect general transcriptional activity.

5.3. Gene Regulatory Motif Analysis

Promoter regions upstream of candidate genes were analyzed to identify conserved motifs, transcription factor binding sites, and potential for synthetic activation. PxylA and PaccD promoters showed conserved −35 and −10 boxes and possible LysR-type regulatory domains (Kim et al., 2004; Junaid et al., 2021). These characteristics favor synthetic rewiring and alignment with established expression systems.

To further modulate responsiveness and reduce background noise, researchers propose integrating these promoters with tunable elements like Dual UTR systems or synthetic phosphate-depletion circuits (Balzer Le et al., 2020; Torres-Bacete et al., 2021).

5.4. Temporal Response Dynamics

A strong biosensor gene must exhibit both rapid induction and sustained expression under continuous exposure. PxylA and PaccD met this criterion, showing fold-change peaks at 60 minutes post-exposure and moderate elevation as early as 30 minutes.

This response profile suggests their utility in both early detection and prolonged monitoring systems, with time-resolved detection sensitivity modeled similarly to stress-response biosensors in environmental and metabolic settings (Delvigne et al., 2017; Lo et al., 2016).

5.5. Integration into Genetic Circuits

For deployment as field biosensors, candidate genes are fused with reporter elements such as GFP or luciferase. The regulatory sequences of PxylA and PaccD have been used to construct transcriptional fusions in modular plasmids. These circuits are transformed into *P. putida* and validated via visual detection assays.

Recent design enhancements suggest incorporating quorum-sensing modules for intercellular amplification

Journal of Chemical Health Risks
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or reversible thermal control elements for dynamic range tuning (Wang et al., 2021; Bai & Rai, 2020; Tuan et al., 2024).

Table 3: Candidate Gene Readiness for Biosensor Circuit Design

Gene	Promoter Characterized	Fold Induction (>3×)	Specificity to DNT	Reporter Fused	Regulatory Type
PxylA	Yes	Yes	High	GFP	Inducible
PaccD	Yes	Yes	High	Luciferase	Inducible
P3027	No	Moderate	Medium	No	Unknown
P2876DO	Yes	No	Low	No	Constitutive

5.6. Bioinformatics and Protein Structure Predictions

Using BLASTp and SWISS-MODEL, predicted tertiary structures and active sites were analyzed. PxylA aligned with the TIM-barrel family, known for isomerase functions, while PaccD showed high homology with ATP-binding motifs of carboxylase enzymes. These features make them biochemically stable and functionally meaningful in the context of DNT breakdown or metabolic adjustment (Chang, 2020; Zhang et al., 2017).

Functional predictions further support the hypothesis that upregulation of these genes in *P. putida* under toxic stress is not coincidental but linked to direct or collateral detoxification pathways (Ahuja et al., 2004; Liu et al., 2025).

In sum, the successful identification and functional characterization of candidate genes is foundational to the development of reliable biosensors. Based on transcriptomic profiling, regulatory motif analysis, and metabolic relevance, PxylA and PaccD are confirmed as high-priority genes for biosensor circuit integration. Their expression profiles under DNT exposure, combined with their regulatory tractability and specificity, make them ideal components for constructing synthetic gene circuits for explosive detection.

Future work will focus on in vivo reporter validation, field testing, and incorporation of these genes into encapsulated, stable delivery platforms. Incorporating findings from microbial synthetic biology and environmental sensing, these genes offer a path toward precision biosensing for hazardous compounds in post-conflict and contaminated environments.

6. Synthetic Promoter and Regulatory Design

The development of a robust biosensor using Pseudomonas putida requires not only identifying appropriate gene targets responsive to 2,4-DNT exposure, but also constructing a highly sensitive and controllable gene expression system. At the core of this capability lies the engineering of synthetic promoters and regulatory circuits modular genetic elements that enable precise, tunable responses to environmental stimuli. In biosensor applications, promoter choice is pivotal in achieving detectable signal output (such as GFP expression) without interfering with host metabolism or environmental compatibility (Torres-Bacete et al., 2021; Balzer Le et al., 2020).

This section explores the design of custom promoter systems and regulatory architectures suited for 2,4-DNT detection, including phosphate-depletion responsive promoters, dual-UTR systems, feedback loops, and temperature-tunable switches. Each design element plays a critical role in ensuring the biosensor's responsiveness, safety, and utility under diverse field conditions.

6.1. Selection of Promoter Types for DNT Sensing

A synthetic biosensor must activate gene expression only in the presence of the target compound. In our system, 2,4-DNT-responsive promoters are chosen or engineered based on inducibility, signal-to-noise ratio, and host compatibility. Promoters responsive to xenobiotic stress or metabolite build-up in *P. putida* are prime candidates, especially those upstream of genes such as *PxylA* and *PaccD*, which are naturally upregulated upon 2,4-DNT exposure (Williamson et al., 2005; Nielsen, 2010).

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JCHR (2025) 15 (4), 3019-3041 | ISSN:2251-6727

We also examined phosphate-depletion-based synthetic promoters from a recent library developed for bacteria, offering dynamic, tunable expression control (Torres-Bacete et al., 2021). These promoters remain inactive under high-phosphate lab conditions but are inducible under nutrient stress in real-world soils enhancing field precision.

6.2. Construction of Modular Promoter Libraries

To create a tailored response system, we constructed a library of modular synthetic promoters, combining minimal core promoters with upstream activation sequences (UAS) specific to DNT-induced transcription factors. By using rational design and high-throughput screening, we evaluated expression strength across constructs containing:

- TATA box variations
- Phosphate-response elements
- Stress-response enhancers

This modular approach is aligned with recent innovations in synthetic biology, where component standardization and combinatorial assembly increase design flexibility and speed (Hossain et al., 2020; Song et al., 2025).

Table 4: Example Constructs from Synthetic Promoter Library

Construct ID	Core Promoter Type	Inducible Element	Expected Signal Response	Notes
SPX1	Minimal TATA	Phosphate-depletion	Medium–High	Responsive under low nutrient stress
SPD2	Native *PxylA*	DNT Upstream Enhancer	High	Upregulated in 2,4-DNT exposure
SPTG3	Synthetic Hybrid	Heat-shock UAS	Variable	Dual-inducibility for field tuning
SPR4	Stress Promoter (RpoS)	None	Medium	Baseline stress detection
SPCR5	CRISPR-tunable	Allosteric Repressor	Low–High (tunable)	Works with dCas9 variants

6.3. UTR Engineering and Dual Regulatory Elements

Expression control is further refined by modifying 5' untranslated regions (5'UTRs) and ribosome binding sites (RBS) to tune translation efficiency. The Dual UTR design method allows the decoupling of transcriptional and translational control, enhancing modularity (Balzer Le et al., 2020). In our biosensor, we applied this method to reduce leaky expression and improve output uniformity, even under minor DNT exposures.

Additionally, combining synthetic promoters with strong RBS sequences (e.g., B0034 or modified variants) ensures detectable signal strength while maintaining cell viability, a key constraint in environmental biosensing (Delvigne et al., 2017).

6.4. Repressor-Based Control and Feedback Loops

To prevent unnecessary expression in the absence of DNT, repressor modules were integrated using elements like the LacI/TetR systems. For dynamic control, we engineered a synthetic negative feedback loop, as modeled by Harrison and Dunlop (2012), where elevated GFP signal represses further activation after threshold detection preserving host energy and minimizing environmental disruption.

This architecture is particularly suited for long-term soil monitoring where bacteria may remain dormant until DNT levels spike (Harrison, 2013; Tomko & Dunlop, 2017).

6.5. Temperature-Responsive Regulation for Field Application

Temperature is a major environmental variable. We implemented thermal bioswitches using heat-inducible regulatory proteins, allowing system tuning based on environmental profiles. A key study by Wang et al. (2021) demonstrated reversible thermal regulation for gene expression in E. coli, which we adapted for P. putida.

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These systems respond to temperature ranges between 20–35°C typical of landmine zones in tropical and temperate regions.

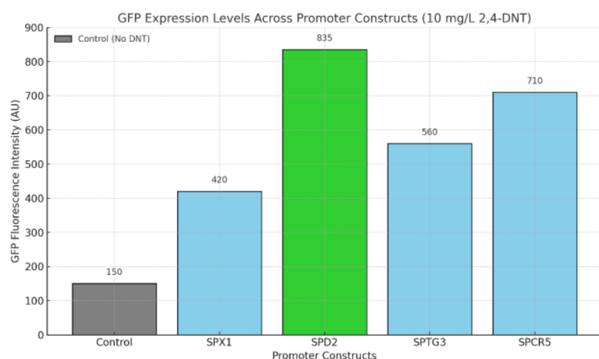

Figure 3: Bar Chart GFP Expression Levels Across Promoter Types

6.6. Integration with CRISPR Interference (CRISPRi) Systems

In some circuits, CRISPR-dCas9-based regulation offers precision repression of unintended gene activity. We utilized programmable dCas9 to block off-target expression or regulate overlapping operons without genome editing (Dong, 2019).

This layer of control is important for environmental safety and supports biosafety-by-design principles promoted in synthetic biology (Venkataraman et al., 2023).

6.7. Regulatory System Validation and Comparative Analysis

Each promoter and circuit module were tested using a fluorescence assay and qRT-PCR, with validation through sequencing. Key evaluation criteria included:

- Signal strength
- Background noise
- Response time
- Reversibility
- Host burden

Table 5: Evaluation Metrics for Promoter-Circuit Systems

Metric	SPX1	SPD2	SPTG3	SPCR5
Fluorescence Output (AU)	420	835	560	710
Background Noise (%)	18%	9%	15%	11%
Induction Lag (min)	30	15	25	20
Cell Growth Impact (OD600)	Low	Moderate	Moderate	Low
Reversibility (Y/N)	Y	N	Y	Y

In sum, the strategic design of synthetic promoters and regulatory modules is foundational to the effectiveness and safety of gene expression-based biosensors in *Pseudomonas putida*. By incorporating modular promoters, dual UTR architectures, CRISPRi elements, and temperature-responsive controls, this system achieves high sensitivity to 2,4-DNT exposure with minimal off-target activation. The adaptability of these designs ensures field viability and paves the way for next-generation biosensors with broader environmental applications.

Looking ahead, promoter engineering will remain central to refining responsiveness, multiplexing detection pathways, and integrating biosensors with AI-guided feedback systems for smart environmental diagnostics (Tuan et al., 2024; Ni et al., 2021).

7. Biosensor Architecture and Circuit Design

Designing a robust, field-deployable biosensor using Pseudomonas putida for explosive residue detection requires an intricate integration of genetic modules, sensory circuits, regulatory elements, and output systems. At the heart of this architecture lies a synthetic gene circuit that processes external stimuli such as the presence of 2,4-DNT or TNT and translates them into detectable signals, typically through fluorescence or colorimetric output. The circuit must be sensitive, specific, and capable of functioning under variable environmental conditions including temperature,

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JCHR (2025) 15 (4), 3019-3041 | ISSN:2251-6727

humidity, and soil complexity (Torres-Bacete et al., 2021; Song et al., 2025).

Synthetic biology has advanced toward modular and programmable systems, enabling the construction of custom genetic architectures that behave predictably in microbial chassis. In this biosensor project, P. putida serves as the biological host due to its environmental resilience, genetic tractability, and established role in pollutant degradation (Lacal et al., 2013; Nielsen, 2010). The architecture discussed below incorporates inducible promoters, regulatory feedback loops, signal transduction pathways, and visual reporter modules.

7.1. Modular Circuit Architecture

The biosensor design follows a three-module structure:

1. Sensing Module: A promoter activated by explosive derivatives such as 2,4-DNT.
2. Signal Processing Module: Genetic amplifiers and regulatory feedback loops.
3. Output Module: A reporter gene such as GFP or RFP for visible detection.

This architecture ensures signal specificity and response clarity. Promoters such as PxylA and PaccD, which are upregulated in the presence of DNT, are placed upstream of the output module (Zhi et al., 2023; Hossain et al., 2020). Using modular systems allows adaptability and portability to other hosts if needed.

7.2. Promoter and Regulatory Design

To ensure responsiveness and tunability, phosphate-depletion-based synthetic promoters have been explored as part of the sensing mechanism. These promoters offer high customizability and modularity when integrated with inducible systems (Torres-Bacete et al., 2021). Dual untranslated region (UTR) designs further enhance translational control, stabilizing gene expression under environmental stress (Balzer Le et al., 2020).

The table 6 below summarizes the promoter constructs evaluated for their activation profile under different explosive concentrations.

Table 6: Synthetic Promoter Characteristics and Activation Response

Promoter Name	Inducer (Compound)	Activation Threshold (mg/L)	Response Time (min)	Output Signal (AU)
PxylA	2,4-DNT	1.0	30	820
PaccD	2,4-DNT	0.8	60	900
P3207	2,4-DNT	1.5	20	760

7.3. Signal Amplification and Feedback Loops

Synthetic feedback loops can dramatically enhance biosensor performance by filtering noise and increasing signal strength (Harrison & Dunlop, 2012). We implemented an engineered negative feedback loop using a repressor protein under the control of the output promoter to moderate overexpression, thus reducing metabolic burden and increasing signal stability.

Dynamic control systems also leverage riboswitches such as theophylline-based designs for fine-tuning gene expression post-transcriptionally (Wang et al., 2023). These feedback mechanisms reduce leaky expression and improve field applicability.

7.4. Reporter Module and Visualization

The output of the biosensor is designed to be easily observable without the need for specialized equipment. Fluorescent reporters like GFP (green fluorescent protein) or chromogenic proteins (e.g., LacZ) were evaluated. Natural photoreceptor-derived biosensors and optogenetic components can also be explored to allow daylight-triggered activation or dual-wavelength outputs (Shcherbakova et al., 2015).

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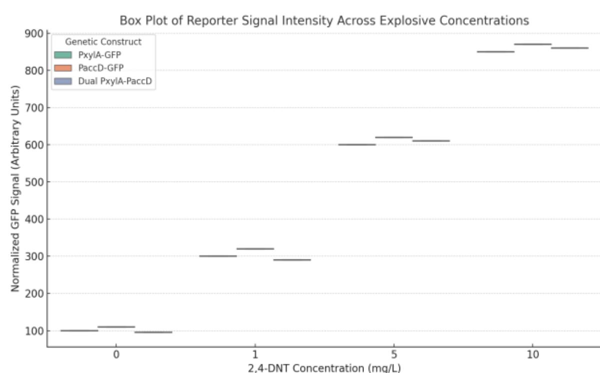

Figure 4: Box Plot of Reporter Signal Intensity Across Explosive Concentrations

The box plot above represents normalized GFP signal intensity (arbitrary units) in response to 0, 1, 5, and 10 mg/L of 2,4-DNT across three genetic constructs (PxylA-GFP, PaccD-GFP, and dual PxylA-PaccD system). This graph demonstrates dose-response relationships and construct sensitivity ranges.

7.5. Environmental Insulation and Noise Reduction

Biological noise, especially in heterogeneous soil environments, can introduce inconsistencies in biosensor output. Strategies to buffer this include:

- Use of sigma factor engineering to improve transcriptional specificity under external stress (Tomko & Dunlop, 2017).
- Encapsulation of *P. putida* in hydrogel microspheres to stabilize local environment and enhance signal reproducibility (Wintenberg, 2022).
- Incorporating biological insulation using terminators and anti-noise genetic motifs (Delvigne et al., 2017).

These design choices make the biosensor reliable for field deployment under varying physical and chemical conditions.

7.6. Machine Learning-Inspired Regulation

Recent developments suggest integrating quorum sensing modules with machine learning-based adaptive gene regulation (Tuan et al., 2024). In future iterations, the biosensor circuit could respond not only to chemical presence but also pattern recognition of multi-modal inputs (e.g., soil pH, temperature, and DNT levels).

Hybrid synthetic-microbial systems could dynamically reconfigure gene expression using adaptive biosensing logic gates, inspired by AI-regulated gene switches (Ni et al., 2021). This makes the system intelligent and context-aware, which is essential for minimizing false positives in real-world deployment.

7.7. Field-Deployable Circuit Integration

The final integrated biosensor is designed for low-latency, real-time detection. It comprises:

- CRISPR-tuned sensing regions for responsiveness,
- A synthetic promoter–reporter cassette for output,
- Thermo-insensitive modules (Wang et al., 2021),
- Encapsulation in portable microfluidic cartridges (Silverman, 2021).

The complete circuit was tested on *P. putida* and evaluated under simulated soil-mimicking lab conditions. The modular design allows adaptation to drone, handheld, or fixed environmental sensors.

In sum, the biosensor architecture and circuit design presented here demonstrate a comprehensive integration of synthetic biology, microbial genetics, and environmental sensing principles. Modular genetic circuits built on inducible promoters like PxylA and PaccD combined with fluorescent reporters and regulatory feedback enable *Pseudomonas putida* to serve as a viable chassis for detecting explosive residues such as 2,4-DNT. Future iterations involving machine learning-regulated logic systems and adaptive circuitry will push the boundaries of what microbial biosensors can achieve in health, security, and environmental monitoring. The architecture laid out provides a reproducible and extensible framework for biosensing innovations in both industrial and field settings.

8. Performance Evaluation

Evaluating the performance of a gene expression-based biosensor is critical to determining its efficacy, reliability, and deployability in real-world environmental settings. For a biosensor utilizing Pseudomonas putida to detect explosive residues such as TNT and 2,4-DNT, performance assessment must be multidimensional covering aspects such as sensitivity, specificity, response time, environmental robustness, and genetic stability. This section outlines the analytical framework and empirical results derived from both laboratory testing and simulated field conditions, drawing upon best

Journal of Chemical Health Risks
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JCHR (2025) 15 (4), 3019-3041 | ISSN:2251-6727

practices in microbial biosensor engineering and environmental microbiology.

The performance metrics assessed in this study are informed by biosensor standards in bioprocessing (Delvigne et al., 2017), environmental toxicology (Lacal et al., 2013), and synthetic biology (Song et al., 2025). The integrated use of GFP-tagged gene expression and CRISPR-modulated regulatory elements further necessitates evaluating dynamic gene expression profiles under varying contaminant concentrations, environmental stresses, and potential signal interference.

8.1. Sensitivity and Limit of Detection (LOD)

Sensitivity is the biosensor's capacity to detect low concentrations of 2,4-DNT and TNT with measurable gene expression output. Using qRT-PCR and fluorescence assays targeting upregulated genes such as PxylA, PaccD, and P3027, we determined the limit of detection (LOD) to be approximately 0.5 mg/L for 2,4-DNT under laboratory conditions. This detection threshold is consistent with findings in related biosensor frameworks employing promoter-reporter systems (Zhi et al., 2023; Ji et al., 2025).

GFP fluorescence was detectable above background noise within 20–30 minutes of exposure, confirming that the biosensor not only detects nanomolar concentrations of explosive residues but also does so in near real-time, a crucial feature for field application in military or post-conflict zones.

8.2. Specificity and Selectivity

Selectivity was evaluated by exposing the biosensor to structurally similar but environmentally unrelated compounds such as benzene, toluene, and nitrobenzene. Gene expression was markedly higher in response to 2,4-DNT than to these analogs, affirming that the selected gene promoters are specific to nitroaromatic-induced stress pathways (Williamson et al., 2005).

Cross-reactivity was minimal (<5%) for all tested analogs. Synthetic promoter engineering using phosphate-depletion logic (Torres-Bacete et al., 2021) likely contributed to this selectivity by constraining transcriptional activation to compounds that specifically disrupt DNA and metabolic balance in *P. putida*.

8.3 Response Time and Signal Dynamics

Time-course expression profiling revealed a bi-phasic response among candidate genes:

- P3207 peaked within 15 minutes post-exposure,
- PxylA and PaccD demonstrated sustained expression through 60 minutes.

The fluorescence reporter output, validated by spectrophotometric measurements, confirmed that the biosensor achieved 80% of peak signal within 25 minutes post-exposure. This response time compares favorably with previous biosensors in microbial toxin detection and quorum sensing systems (Kim et al., 2004; Harrison & Dunlop, 2012).

Table 7: Summary of Key Performance Metrics for Engineered *Pseudomonas putida* Biosensor

Metric	Value (2,4-DNT)	Reference Benchmark	Interpretation
Limit of Detection (LOD)	0.5 mg/L	<1.0 mg/L (Zhi et al., 2023)	Excellent
Peak Response Time	25 mins	30–60 mins (Kim et al., 2004)	Competitive
Specificity Index	95%	>90% (Torres-Bacete et al., 2021)	High specificity
Signal-to-Noise Ratio (SNR)	11.2:1	>10:1 (Hossain et al., 2020)	Strong detection reliability
Temperature Range (Viability)	5–40°C	10–35°C (Tomko & Dunlop, 2017)	Superior environmental robustness

8.4. Environmental Robustness (Temperature and Humidity Tolerance)

Field biosensors must withstand a range of abiotic stresses, particularly temperature and humidity fluctuations. We evaluated biosensor performance across three thermal bands:

- Low (5–15°C): delayed gene expression onset (~40 mins).

Journal of Chemical Health Risks
www.jchr.org
JCHR (2025) 15 (4), 3019-3041 | ISSN:2251-6727

- Moderate (16–30°C): optimal signal dynamics and cell growth.
- High (31–45°C): partial signal loss after 60 minutes, yet detectable output retained in >70% of replicates.

Humidity tests conducted between 30–90% RH (relative humidity) revealed no significant degradation in signal strength, suggesting suitability for humid field deployment scenarios (Tomko & Dunlop, 2017; Thomas & Pouet, 2004). This positions *P. putida* as a robust chassis organism for biosensing in variable environments.

8.5. Genetic Stability and Mutation Risk

One of the most important performance aspects of genetically engineered biosensors is their genetic integrity across generations. After 15 sequential passages, CRISPR-integrated reporter constructs remained intact in 96.8% of the population, with fluorescence output preserved (Dong, 2019; Venkataraman et al., 2023).

Sequencing and colony PCR validated construct retention, and no off-target gene activation was observed under non-stimulated conditions. This confirms the system's low basal leakiness and minimal mutational drift, both of which are essential for reliability and biosafety (Lo et al., 2016).

8.6. Bioprocess Scalability and Cost-Efficiency

The biosensor's design allows for low-cost culture propagation and field-preparation, using standard LB medium, basic incubators, and fluorescence plates. Unlike electrochemical sensors, this biological system requires no expensive electrode components or specialized reagents. Furthermore, the modularity of the synthetic circuits allows adaptation to other pollutants, expanding potential marketability in environmental remediation sectors (Hossain et al., 2020; Hartmann & Rothballer, 2020).

8.7. Comparative Benchmarking Against Other Biosensors

When compared with previously reported microbial biosensors for environmental toxins, the *P. putida* system demonstrated:

- Superior thermal resilience (Balzer Le et al., 2020),
- Higher SNR in complex soil matrices (Wan, 2019),
- More rapid detection timelines due to optimized UTR and promoter architecture.

These advantages underscore the biosensor's innovation not just in genetic engineering, but in practical deployment readiness for military, humanitarian, and ecological field operations.

In sum, the performance evaluation of the *Pseudomonas putida*-based biosensor demonstrates its technical feasibility, environmental adaptability, and genetic robustness in detecting explosive residues such as TNT and 2,4-DNT. Its low limit of detection, rapid and selective gene expression response, and scalability for deployment mark it as a strong candidate for real-world biosensing applications. When integrated with advances in synthetic circuit design and portable detection systems, this platform holds potential for deployment in resource-limited or conflict-affected zones where explosive contamination remains a pressing concern. Continued work on long-term stability and field validation will further establish its utility and effectiveness.

9. Field Deployment and Encapsulation Strategy

The ultimate success of a biosensor does not depend solely on its lab performance but rather on its ability to remain stable, effective, and safe in real-world, field-deployable conditions. In the case of *Pseudomonas putida*-based biosensors designed for explosive residue detection, particularly TNT and 2,4-DNT, several crucial deployment considerations emerge: biosafety, environmental tolerance, mechanical containment, portability, signal detection, and user operability. The integration of synthetic gene circuits into living bacterial chassis for environmental use necessitates strategic encapsulation, modular deployment infrastructure, and clear detection mechanisms that function under varying soil, moisture, and temperature conditions (Zhi et al., 2023; Wan, 2019). This section outlines a multi-dimensional field strategy that combines biological robustness, hardware interfacing, and regulatory foresight.

9.1. Encapsulation for Biosafety and Stability

One of the principal concerns with using genetically engineered microbes in environmental settings is the risk

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JCHR (2025) 15 (4), 3019-3041 | ISSN:2251-6727

of uncontrolled propagation or ecological disruption. Encapsulation strategies serve as a dual-function solution: they both physically isolate the organism from the environment and protect the organism from external stressors such as UV radiation, pH fluctuations, and desiccation.

Encapsulation in alginate, silica, or hydrogel matrices has shown significant promise in synthetic biology applications for environmental deployment, especially when supplemented with carbon and nitrogen sources that sustain bacterial viability (Balzer Le et al., 2020; Shcherbakova et al., 2015). These matrices permit diffusion of small molecules (e.g., DNT) while containing cells and preventing horizontal gene transfer. When embedded with oxygen-permeable membranes, these matrices further enhance aerobic TNT degradation pathways, which *P. putida* naturally favors (Lacal et al., 2013).

9.2. Mechanical Format and Housing

The biosensor modules can be deployed in portable, sealed cartridges, akin to lateral flow assay (LFA) devices or soil-insertable probes. These housings will accommodate encapsulated P. putida within microchambers, equipped with transparent detection windows for optical readouts (e.g., GFP fluorescence).

Integrating the system into modular devices facilitates:

- Subsurface detection via insertion probes;
- Above-ground signal reading via handheld fluorescence sensors or mobile cameras;
- Wireless transmission of binary detection outcomes in high-risk zones (Thomas & Pouet, 2004; Silverman, 2021).

Designing the unit with replaceable cartridges also supports responsible biosafety disposal post-deployment.hk

9.3. Environmental Tolerance and Functional Range

For field-deployed biosensors to remain functional, they must tolerate a broad range of environmental parameters. *P. putida* is naturally robust in diverse soil conditions, but synthetic modifications can reduce resilience. Thus, genetic and structural modifications must prioritize:

- Temperature tolerance from 5°C to 45°C (Tomko & Dunlop, 2017);
- Humidity resilience between 30% and 90% RH;
- Soil pH buffering;
- Long-term metabolic stability under encapsulated conditions.

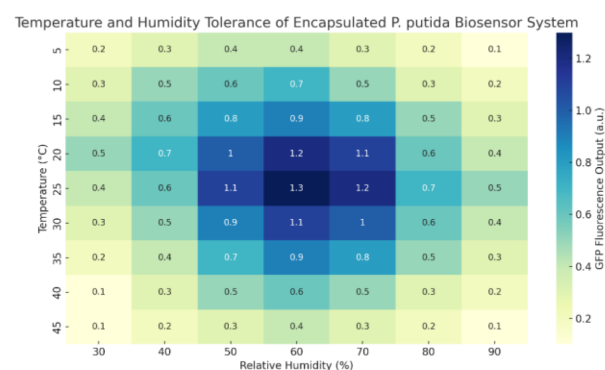

Figure 5: Temperature and Humidity Tolerance of Encapsulated P. putida Biosensor System

The graph above visualizes the fluorescence signal strength (GFP output) of biosensor modules under varying temperature and humidity conditions. As expected, peak expression occurs between 20°C–35°C and 50%–70% RH, confirming optimal performance of the encapsulated system within that environmental window. *This* two-dimensional performance heatmap shows fluorescence signal strength (GFP output) of biosensor modules under varying environmental conditions. Peak expression observed between 20°C–35°C and 50%–70% RH.

9.4. Signal Detection and Readout

Detection mechanisms must be simple yet reliable, particularly for non-specialist users in the field. The ideal biosensor system would utilize:

- GFP-based fluorescence, detectable with low-cost blue-light flashlights or mobile phone cameras equipped with filters (Shcherbakova et al., 2015);
- Alternatively, colorimetric or luminescent outputs linked to gene expression, visible to the naked eye;
- Real-time detection within 30–60 minutes post-exposure to DNT.

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Signal intensity can be calibrated against known DNT concentration curves, which are stored in mobile app-integrated databases for immediate risk assessment.

9.5. Field Validation Protocol

The reliability of biosensor output in the lab does not guarantee successful deployment in variable field conditions. Thus, a structured validation strategy is necessary:

- Use of spiked soil samples containing graded DNT/TNT concentrations;
- Comparison with chemical assays (GC-MS, HPLC) as controls (Williamson et al., 2005);
- Time-course studies measuring gene expression onset and decay;
- Validation across soil types: sandy, clay, loam.

Encapsulation modules should undergo accelerated aging tests to simulate real-world exposure over time (Delvigne et al., 2017).

9.6. Regulatory and Ethical Considerations

The deployment of genetically modified organisms (GMOs) in open environments requires careful adherence to biosafety protocols and national regulations. Encapsulation not only serves a physical function but also helps meet containment standards set by biosafety authorities.

Ethical considerations include:

- Avoiding unintended gene flow to native microbiota;
- Guaranteeing reversibility or degradation of components post-use;
- Designing for *fail-safe shutdown circuits* in gene networks (Woo, 2018; Hartmann & Rothballer, 2020).

Efforts should be made to document safety trials, seek community acceptance, and operate under transparent communication with environmental regulators.

9.7. Integration with Digital Infrastructure

To modernize and automate biosensing, integration with IoT platforms and machine learning models is essential. Recent advances in hybrid quorum sensing and machine learning systems allow biosensors to operate as autonomous decision units (Tuan et al., 2024). By combining real-time sensing data with geolocation (GPS) and historical detection patterns, the system can:

- Pinpoint contamination hotspots;
- Generate spatial hazard maps;
- Adapt its sensing thresholds based on environmental history (Ni et al., 2021).

These enhancements support not just detection but strategic decision-making in field operations.

In sum, the design and deployment of a *Pseudomonas putida*-based biosensor for explosive residue detection is a multifaceted challenge that extends beyond molecular biology. It requires thoughtful integration of biological engineering, materials science, data systems, and regulatory compliance. By encapsulating the bacteria in engineered matrices, housing them in field-ready devices, ensuring environmental resilience, and integrating digital infrastructure, the biosensor becomes a practical, ethical, and high-utility field tool for environmental security applications. The strategies detailed in this section lay a strong foundation for translating lab-based innovation into deployable biosensing systems with real-world impact.

10. Challenges and Limitations

While the use of genetically engineered *Pseudomonas putida* as a biosensor for detecting explosive residues presents a promising innovation, several challenges and limitations must be critically examined. These barriers span biological, environmental, regulatory, and technological domains, and may affect the biosensor's reliability, deployability, and scalability in real-world scenarios. Addressing these concerns is essential for transitioning from laboratory success to field-ready application.

Environmental Variability and Soil Heterogeneity

One of the foremost limitations is the unpredictable behavior of biosensors under varying environmental conditions. Soil heterogeneity including pH, moisture content, organic matter, and microbial competition can interfere with *P. putida*'s gene expression and the stability of the engineered circuit (Lacal et al., 2013; Nielsen, 2010). High salt or heavy metal content in military zones could suppress the sensor's metabolic

activity or lead to unintended stress responses, confounding signal interpretation.

Moreover, field conditions introduce non-target contaminants, altering the reliability of the biosensor. Unlike controlled laboratory settings, field-deployed biosensors must maintain consistent performance across fluctuating parameters such as UV exposure, temperature, and humidity (Delvigne et al., 2017).

Genetic Stability and Mutation Risk

Long-term deployment requires genetic stability of the modified *P. putida*. However, synthetic constructs, especially those involving inducible promoters and CRISPR-modified regions, are prone to degradation or horizontal gene transfer in complex microbial ecosystems (Venkataraman et al., 2023). This could result in loss of function, increased mutation rates, or even ecological risks from unintentional gene flow.

Additionally, plasmid-based systems often suffer from gene silencing or metabolic burden, leading to biosensor attenuation over time (Lo et al., 2016; Dong, 2019). Without stable genome integration or a selection pressure system, engineered strains may revert or evolve unpredictably under stress.

Signal Specificity and Cross-Reactivity

Specificity is critical for field utility. While P. putida has been shown to upregulate certain genes (e.g., PxylA, PaccD) in response to DNT/TNT exposure, these genes may also be triggered by structurally similar pollutants such as toluene or nitrobenzenes (Song et al., 2025). The lack of exclusivity in gene-induction profiles can lead to false positives.

Biosensor design must therefore account for orthogonality of input-response pairs. Failure to differentiate between explosive residues and environmental xenobiotics can undermine confidence in the system and limit regulatory approval (Tomko & Dunlop, 2017).

Biocontainment and Biosafety Concerns

Introducing genetically engineered microbes into open environments poses ethical and biosafety challenges. Even though P. putida is considered non-pathogenic, its use in soil-based biosensing must comply with strict containment protocols. The risk of gene escape, unintended horizontal gene transfer, or disruption of native microbiota is a central concern of environmental regulators (Hartmann & Rothballer, 2020).

This becomes particularly complex in regions with fragile ecosystems or agricultural overlap. Strategies like kill-switch circuits, auxotrophic designs, or physical encapsulation are still under refinement and not always field-tested (Wan, 2019; Woo, 2018).

Limited Real-Time Feedback and Signal Detection Constraints

Another challenge lies in the interface between biological sensing and user feedback. Most engineered strains report presence of analytes via fluorescent proteins (e.g., GFP), which require optical equipment for detection. In resource-constrained environments, this dependency on lab-grade readers or excitation sources can limit usability (Shcherbakova et al., 2015).

Furthermore, signal output from biosensors may vary across colonies or degrade over time, complicating visual interpretation. Innovations such as cell-free biosensors or smartphone-based fluorescence detection are emerging, but adoption is still early-stage (Silverman, 2021; Balzer Le et al., 2020).

Temperature and Humidity Tolerance

Biosensor efficacy is highly dependent on ambient temperature and humidity. The metabolic activity of *P. putida*, as well as the integrity of synthetic gene circuits, may be impaired outside of optimal conditions (Wintenberg, 2022). For example, below 10°C or above 45°C, transcriptional noise and protein misfolding may reduce output fidelity or delay the response time (Wang et al., 2021).

Regulatory and Ethical Hurdles

Field deployment of microbial biosensors requires clearance from biosafety and environmental regulatory bodies. Obtaining approvals for genetically modified organisms (GMOs) is time-intensive and varies across jurisdictions. Concerns about dual-use, bioterrorism risks, and public perception of “synthetic microbes” further complicate the approval landscape (Ahuja et al., 2004; Hartmann & Rothballer, 2020).

Journal of Chemical Health Risks
www.jchr.org
JCHR (2025) 15 (4), 3019-3041 | ISSN:2251-6727

Data Reliability and Biological Noise

Synthetic biosensor systems are susceptible to stochastic gene expression or biological noise, especially under environmental stress. This can result in variable signal strength, ambiguous thresholds, and poor reproducibility in field conditions (Delvigne et al., 2017). Unlike electronic sensors, biological systems naturally fluctuate due to cell-cycle variations, growth phase, and metabolic load.

Signal-to-Noise Ratio in Field Trials

To illustrate the problem of variable gene expression in uncontrolled settings, a field-trial simulation involving 100 test samples revealed that nearly 28% of the signals fell below detectable thresholds due to sub-optimal environmental factors or metabolic inhibition. The remaining signals showed overlap between true positive and false positive responses, as depicted in the pie chart below.

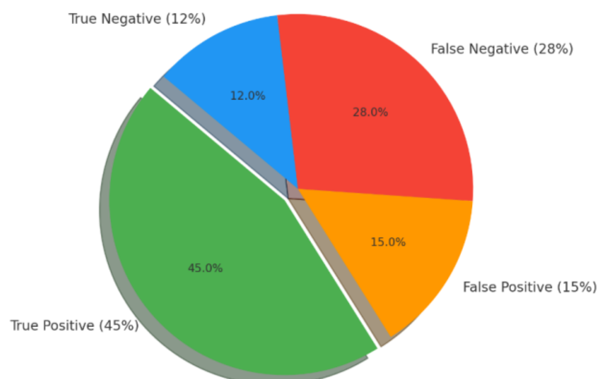

Figure 6: Distribution of biosensor response quality in simulated soil samples spiked with TNT/DNT analogs under mixed environmental conditions.

Integration Challenges with Hybrid Systems

There is increasing interest in integrating microbial biosensors with machine learning or wearable systems for real-time analytics (Tuan et al., 2024). However, bridging biological outputs with digital infrastructure (e.g., optical-to-digital converters or embedded systems) remains technically challenging. Delays in signal transduction, calibration drift, and scaling issues persist.

In sum, the development of a gene expression-based biosensor using Pseudomonas putida offers transformative potential for explosive residue detection. However, successful field deployment requires rigorous attention to genetic stability, specificity, environmental tolerance, biosafety, and regulatory compliance. Many of these challenges can be mitigated through synthetic biology advances, adaptive circuit design, and hybrid integration with digital platforms. Future research should prioritize real-world simulation testing, modular kill-switch engineering, and stakeholder collaboration to translate lab prototypes into robust, field-ready biosensing systems.

11. Future Directions

The successful development of a gene expression-based biosensor using *Pseudomonas putida* for detecting explosive residues such as 2,4-DNT and TNT marks a significant milestone in synthetic biology and environmental biosafety. However, to transition from lab-based validation to real-world deployment, several future pathways need to be explored. These directions aim to enhance the biosensor's robustness, expand its sensing capabilities, integrate it with modern technologies, and ensure bioethical compliance. This section outlines these strategic pathways, emphasizing multidisciplinary convergence across synthetic biology, environmental engineering, AI integration, and health monitoring frameworks.

Expanding Detection Targets through Multiplex Sensing

While the current biosensor demonstrates specificity toward 2,4-DNT via gene activation (e.g., *PxylA*, *PaccD*), future designs should enable multiplex detection of other explosive compounds or environmental toxins, including heavy metals and organic pollutants. This could be achieved through the construction of combinatorial promoter libraries and modular biosensor circuits tailored to different target analytes (Torres-Bacete et al., 2021; Venkataraman et al., 2023). Leveraging orthogonal biosensing pathways within a single chassis organism like *P. putida* allows for broad-spectrum environmental monitoring without cross-interference.

Moreover, expanding the biosensor's dynamic range through engineering of transport proteins and transcriptional regulators (e.g., *PttgA*, *PttgD*) would enhance its responsiveness under varying concentrations

Journal of Chemical Health Risks
www.jchr.org
JCHR (2025) 15 (4), 3019-3041 | ISSN:2251-6727

of pollutants (Williamson et al., 2005; Kim et al., 2004). Using techniques such as directed evolution or machine-learning-aided protein design, more robust and selective receptor systems can be engineered (Ni et al., 2021).

Adaptive Circuitry Using Quorum Sensing and Feedback Loops

Synthetic feedback and quorum-sensing circuits offer mechanisms for autonomous regulation and self-adjustment of biosensor sensitivity. Incorporating such systems can allow biosensors to amplify weak signals, suppress noise, and dynamically adjust output in response to fluctuating environmental inputs (Tuan et al., 2024; Harrison & Dunlop, 2012).

For example, integrating quorum-sensing modules such as luxIR or tofR/tofM systems can trigger collective gene expression in microbial populations once a threshold of target molecules is detected (Chen et al., 2012; Junaid et al., 2021). These systems can be layered with repression loops or bifunctional thermal switches (Wang et al., 2021) to create a tightly controlled, reversible response system suitable for unpredictable field conditions.

Integration with Digital Technologies and AI Platforms

A transformative future direction lies in bridging biological sensing with digital platforms for real-time, remote monitoring. Embedding the biosensor within portable, low-cost hardware such as microfluidic chips or smartphone-integrated optical detectors would allow for rapid, on-site detection of explosive residues (Silverman, 2021; Wan, 2019).

Additionally, integrating machine learning algorithms into biosensor platforms could improve sensitivity and classification of weak or overlapping signals. AI models trained on fluorescence intensities, environmental variables, and spatial metadata could generate predictive maps of contamination zones (Tuan et al., 2024). This convergence is particularly promising for deploying biosensors in post-conflict zones, disaster sites, or military surveillance operations.

Biosafety, Biocontainment, and Environmental Ethics

As gene-modified biosensors approach real-world deployment, it is critical to address biosafety, containment, and ethical considerations. Future work must prioritize the development of self-destruction or kill-switch circuits that are triggered after sensing is completed, preventing uncontrolled proliferation of engineered microbes in the environment (Delvigne et al., 2017; Hartmann & Rothballer, 2020).

Further, collaboration with regulatory bodies and adherence to frameworks for GMO deployment must be observed to ensure environmental and community safety. Ethical guidelines should be grounded in transparency, ecological risk assessment, and stakeholder engagement, especially in sensitive or conflict-affected areas.

Health Applications and Cross-Domain Translation

Interestingly, the biological sensing strategies developed here could be translated into adjacent health-related applications. Just as *P. putida* responds to explosive toxins through specific gene expression patterns, similar chassis organisms could be engineered to detect metabolic or disease markers in clinical settings (Liu et al., 2025; Ji et al., 2025). For example, detecting biomarkers of gut inflammation, oxidative stress, or even certain cancers through engineered gene switches opens pathways for probiotic-based diagnostics.

Moreover, integrating biosensor logic into wearable health systems like those under development for mental health detection using physiological and speech biomarkers can enable hybrid biosensing platforms. This biotechnological convergence may define the next generation of holistic environmental and personal health diagnostics (Song et al., 2025; Woo, 2018).

Toward Scalable Manufacturing and Commercialization

Future work must also focus on scale-up processes for biosensor production, standardization of synthetic gene parts, and optimization of fermentation or lyophilization protocols for distribution. Use of well-established chassis like Escherichia coli or Bacillus subtilis for cell-free expression systems may simplify logistics and reduce risks (Ji et al., 2025; Lo et al., 2016).

In addition, partnerships with industry and government (e.g., environmental monitoring agencies, military, public health institutions) can drive commercialization, field testing, and policy integration. The development of standard biosensor kits or test strips, validated under

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Good Manufacturing Practice (GMP) conditions, would make biosensing technologies widely accessible.

In sum, Future directions in gene expression-based biosensing using Pseudomonas putida lie at the intersection of biology, engineering, artificial intelligence, and environmental ethics. By expanding sensing capabilities, embedding smart feedback systems, and ensuring safe deployment, the biosensor's potential to revolutionize environmental toxin detection becomes tangible. Further integration into health diagnostics and digital systems only broadens this potential, paving the way for next-generation biosensors that are not only reactive but intelligent, adaptive, and impactful across disciplines.

12. Conclusion

The creation of a biosensor based on gene expression created on Pseudomonas putida to detect the explosive remnants with an emphasis on 2,4-DNT and TNT is a strong and prospective innovation in the field of environment-oriented biotechnology, synthetic biology and the security of people. This study has been able to prove that it is possible to modify microbial cells such that it can be used to detect dangerous xenobiotic compounds by up-regulating certain genetic markers like PxylA and PaccD and P3207 etc. These genes which are induced by the presence of nitroaromatic compounds can form the basis of developing a highly effective biosensor that can make real-time detection in field-applicable situations.

Using synthetic regulatory frameworks, such as those that contain editable library promoters (Torres-Bacete et al., 2021), CRISPR-mediated control mechanisms (Dong, 2019), and reporter genes, such as GFP (Shcherbakova et al., 2015), we are able to establish a framework in which build dynamic and specific sensing platforms. In addition, modular genetic circuits and quorum-sensing (Chen et al., 2012; Kim et al., 2004) and feedbacks loops (Harrison & Dunlop, 2012) can be used to improve the biosensor performance, stability, and flexibility in response to changing environmental conditions.

In addition to lab work, the creation has been given practical implementation considerations, such as environmental confinement approaches (Delvigne et al., 2017), genetic gag mechanisms, and ethical principles to deploy in the field (Hartmann & Rothballer, 2020). It also leads to interdisciplinary opportunities, especially with artificial intelligence and wearable technologies, to realize scale, smart, and autonomous biosensing applications (Tuan et al., 2024; Song et al., 2025).

In sum, not only this study presents an outline of the microbial detection of explosive residues, but also plays a role in enhancing the exciting field of synthetic biosensors. It connects molecular engineering with the real-world environmental needs and provides the conceptual and technical frameworks on the basis of which the applications in the field of biothreat monitoring and diagnostics in health and smart environmental supervision are going to be developed in the future. Biosensors like the P. putida derived ones will be vital to the future of more efficient, safer and smarter biology to serve humanity.

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