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Detection of buried explosives with immobilized bacterial bioreporters

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Summary

The unchecked dispersal of antipersonnel landmines since the late 19th century has resulted in large areas contaminated with these explosive devices, creating a substantial worldwide humanitarian safety risk. The main obstacle to safe and effective landmine removal is the identification of their exact location, an activity that currently requires entry of personnel into the minefields; to date, there is no commercialized technology for an efficient stand-off detection of buried landmines. In this article, we describe the optimization of a microbial sensor strain, genetically engineered for the remote detection of 2,4,6-trinitrotoloune (TNT)-based mines. This bioreporter, designed to bioluminescence in response to minute concentrations of either TNT or 2,4-dinitotoluene (DNT), was immobilized in hydrogel beads and optimized for dispersion over the minefield. Following modifications of the hydrogel matrix in which the sensor bacteria are encapsulated, as well as their genetic reporting elements, these sensor bacteria sensitively detected buried 2,4-dinitrotoluene in laboratory experiments. Encapsulated in 1.5 mm 2% alginate beads containing 1% polyacrylic acid, they also detected the location of a real metallic antipersonnel landmine under field conditions. To the best of our knowledge, this is the first report demonstrating the detection of a buried landmine with a luminescent microbial bioreporter.

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Introduction

The main rate-limiting factor holding back global demining efforts is not the actual removal of buried landmines, but rather pinpointing their exact locations. Current landmine detection methodologies mostly require the risky presence of personnel on the minefield, as there is no technology currently in use that allows the stand-off detection of buried explosive devices. A possible alternative has been put forward over two decades ago by Burlage *et al.* (1999), who proposed to genetically modify microorganisms to emit an optical signal in the presence of explosives' vapours, and spread them over the minefield. The optical response of these bioreporters to the presence of landmines in the soil below them would then be remotely imaged and analysed. Several microbial sensor strains have since been described (Looger *et al.*, 2003; Altamirano *et al.*, 2004; Radhika *et al.*, 2007; Garmendia *et al.*, 2008; Davidson *et al.*, 2012; Yagur-Kroll *et al.*, 2014; Shemer *et al.*, 2015, 2017, 2018, 2020) that emit a dose-dependent signal in the presence of the main explosive landmine constituent (Jenkins *et al.*, 2001), 2,4,6-trinitrotoluene (TNT), and/or of its manufacturing by-product 2,4-dinitrotoluene (DNT). The latter compound, due to its relatively high volatility, is considered a useful 'signature chemical' for the presence of TNT-based landmines.

Yagur-Kroll *et al.* (2014) reported an *Escherichia coli* sensor strain, harbouring a plasmid-borne fusion of the green fluorescent protein GFPmut2 to the *E. coli yqjF* gene promoter. The latter promoter was shown to be activated by both TNT and DNT (Yagur-Kroll *et al.*, 2014), and directly induced by their degradation product 2,4,5-trihydroxytoluene (Shemer *et al.*, 2018). The *yqjF* promoter was further optimized by directed evolution (Yagur-Kroll *et al.*, 2015), and bacteria hosting the enhanced plasmid, encapsulated in Ca–alginate beads, successfully detected real antipersonnel landmines over which they were spread (Belkin *et al.*, 2017). Excitation and imaging of the fluorescent signal were performed by computer-controlled screening of the target area with a

Table 1. Luminescent response of immobilized BS02 bioreporters to DNT (3 mg l^{-1} in LB–agar) as a function of bead size (top, 1.51×10^5 cells per bead) and cell concentration (bottom, bead diameter 1.55 mm). Errors represent the standard deviation over at least three repeats.

Nozzle diameter (μm)	Bead diameter (mm)	Response intensity[a] (RLU[c])	Response ratio[b]
1000	2.95 ± 0.14	$1.16 \times 10^7 \pm 1.04 \times 10^6$	59.2 ± 5.02
750	1.55 ± 0.09	$2.07 \times 10^7 \pm 1.05 \times 10^6$	79.2 ± 7.83
300	1.03 ± 0.21	$9.87 \times 10^7 \pm 1.36 \times 10^6$	38.6 ± 5.12
Bacterial concentration in alginate (% w/v)	**Bacterial density in beads (CFU per bead)**	**Response intensity[a] (RLU[c])**	**Response ratio[b]**
0.05	$7.92 \times 10^4 \pm 8.13 \times 10^3$	$1.62 \times 10^7 \pm 2.28 \times 10^6$	22.3 ± 5.1
0.1	$1.51 \times 10^5 \pm 3.10 \times 10^4$	$2.28 \times 10^7 \pm 2.78 \times 10^6$	35.3 ± 6.4
0.5	$3.35 \times 10^5 \pm 3.77 \times 10^4$	$7.42 \times 10^6 \pm 7.7 \times 10^5$	24.6 ± 7.6
1	$6.16 \times 10^5 \pm 1.83 \times 10^5$	$5.48 \times 10^6 \pm 1.34 \times 10^5$	30.7 ± 7.4

a. Luminescence in the presence of DNT minus that in its absence.
b. Luminescence in the presence of DNT divided by that in its absence.
c. Plate reader's arbitrary relative light units.

dedicated optical system. One of the lessons learned from that experiment was the difficulty inherent in sustaining the viability of the alginate-immobilized bacteria for the relatively long time (22 h in that specific case) required to obtain a significant fluorescent signal.

In the present report, we describe two approaches undertaken to overcome this difficulty: optimization of the bacterial alginate encapsulation formulation and procedure, in order to increase field survival time, and the introduction of enzyme-driven bacterial bioluminescence as the reporter entity, instead of a fluorescent protein. In the framework of the latter effort, aimed at enhancing sensitivity as well as shortening response times, we have substituted the GFPmut2 gene by three different bacterial *lux* gene cassettes, originating from the terrestrial bacterium *Photorhabdus luminescens* and from the marine species *Aliivibrio fischeri* and *Photobacterium leiognathi*, each with different environmental requirements for optimal performance.

Results

Effects of bead diameter and cell density on DNT detection

Alginate beads in three diameters, encapsulating bioluminescent DNT sensor strain BS02 at four cell densities, were tested for their effect on DNT detection (Table 1). The different bead sizes were achieved by employing different nozzle diameters (300, 750 and 1000 μm) of the encapsulation device, as described under Experimental Procedures below. The different cell concentrations were obtained by suspending different amounts of a centrifuged cell pellet in a 2% alginate/1% polyacrylamide (PAA) solution, added dropwise into a $CaCl_2$ solution. Batches of at least ten beads were placed as a monolayer on the surface of LB–agar supplemented with DNT (3 mg l^{-1}) in an opaque white 24-well microtitre plate, and luminescence was monitored over a period of 17 h. The results, summarized in Table 1, indicated that total luminescence intensity in response to DNT decreased with increasing bead size, but that the 1.5 mm bead diameter yielded the highest response ratio.

Testing the luminescent response of beads containing different bacterial densities revealed that higher bacterial content is not linearly linked to stronger luminescent signals (Table 1). A concentration of 0.1% bacteria in the alginate solution, equivalent to 1.5×10^5 cells per bead, yielded the strongest response in terms of both signal intensity and response ratio. While higher bacterial densities resulted in somewhat more rapid signal development (Fig. 1), reaching a maximum after ca. 270 min, the overall signal intensity at these concentrations suffered a significant decrease. The stronger signals produced by the lower bacterial densities peaked after 550–700 min. Based on these results, a bead diameter of 1.55 mm and a cell load of ca. 1.5×10^5 per bead were selected for the continuation of the study.

Polyacrylic acid as an alginate additive

The effects of PAA, a biocompatible and biodegradable (Larson *et al*., 1997; Chiellini *et al*., 2003) hygroscopic compound, on water loss from alginate beads was investigated by including different PAA concentrations (0%–1% w/v) in the alginate formulation. Higher concentrations (2–4%) proved to be mechanically unsuitable due to the high viscosity and enhanced stickiness of the beads, probably due to high levels of syneresis. As shown in Figure 2A, water maintenance in the two PAA concentrations tested was significantly higher than in the PAA-free control. Following 4 h of incubation at room temperature, close to 50% of the original water content

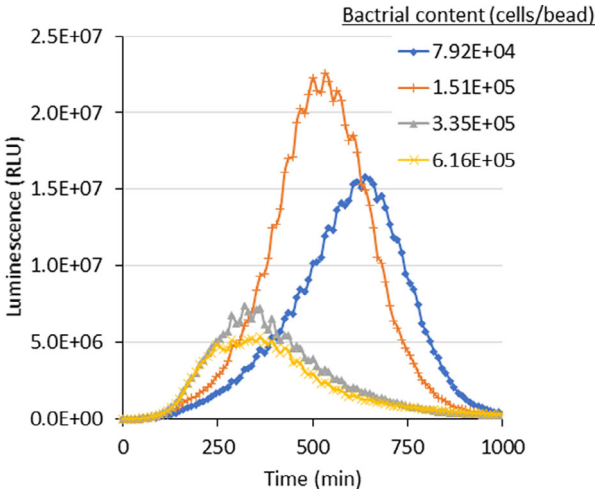

Fig. 1. Luminescent response of alginate–PAA beads (2% w/v alginate, 1% w/v PAA) when placed on LB–agar containing 5 mg l^{-1} DNT. The beads were prepared with various concentrations of the BS02 bioreporter strain. The response is presented as the difference in luminescent signal intensity between the sample and the control, in the plate reader's arbitrary relative light units (RLU).

was preserved in the presence of 1 % PAA, in comparison with only 20% in the control.

As may be observed in Figure 2B, the inclusion of PAA as a bead component favourably affected not only water loss, but also the efficiency of DNT detection. Addition of PAA at a concentration of 0.5–1% resulted in a 2- to 4-fold increased response when compared to alginate beads with no PAA, whereas 1.5% PAA exerted a minor negative effect. The latter effect may have been due to the very high polymer density, which could have adversely affected both the bacterial well-being and DNT diffusion into the bead.

A possible explanation of the positive effect of PAA on the intensity of the bioreporter's response is adsorption

Table 2. Effect of PAA on DNT concentration in alginate beads.

	DNT concentration (mg l^{-1})	
PAA (1%) in beads	In beads	In medium
+	3.56 ± 0.09	3.69 ± 0.09
−	1.68 ± 0.23	3.95 ± 0.23

of DNT to PAA, which may increase the inter-bead DNT levels and hence the DNT concentrations 'seen' by the cells. To test this hypothesis, 2% alginate beads with or without added PAA (1%) were incubated overnight in a 5 mg l^{-1} DNT solution. DNT concentrations were then determined by LC/MS in methanol extracts of the beads and of the medium, and the results are presented in Table 2.

As can be seen in Table 2, the presence of PAA has increased the DNT concentration in the beads by over twofold, indicating an excess uptake of DNT by these beads; this observation confirms the explanation proposed above for the positive effect of PAA addition on the bacterial response to DNT.

Switching to different reporter lux *gene cassettes*

The luminescent activity of the *Photorhabdus luminescens luxCDABE* cassette, employed as the reporter element in the original *yqjF*-based bioreporter (Strain BS01; Yagur-Kroll *et al*., 2014), significantly deteriorated at temperatures below 25°C. To allow trace DNT detection at lower temperatures, the *lux* genes in plasmid C55-*luxPI* were replaced by two other *lux* variants, originating from *Aliivibrio fischeri* and *Photobacterium leiognathi*, to generate plasmids *C55_luxAf* and *C55_luxPleio* respectively. Each of these plasmids was transformed into an *E. coli* strain with a mutated *pykF* gene, a

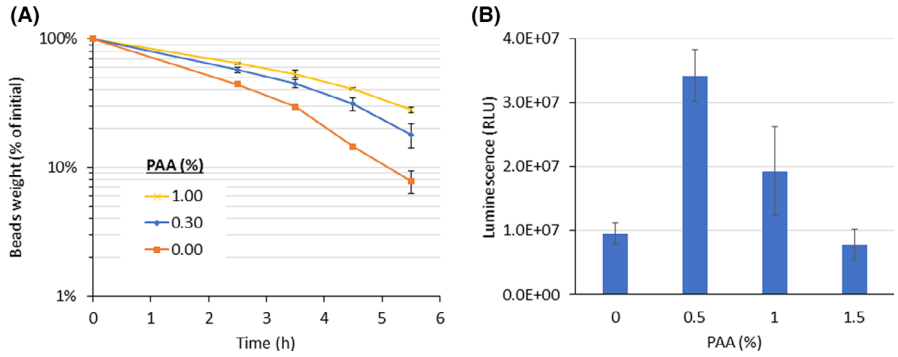

Fig. 2. (A) Effect of PAA content on the rate of water loss from alginate–PAA beads. Beads were allowed to desiccate on an open Petri dish at room temperature. Error bars represent the standard deviation of three samples, each containing 10 beads of ca. 3 mm diameter. (B) Effect of PAA on the response to DNT. A monolayer of alginate beads (including 0%–1.5% PAA), encapsulating reporter strain BS02 (1.5×10^5 cells per bead, 1.55 mm diameter), was placed on LB–agar with DNT (5 mg l^{-1}) in a 24-well plate. The response is presented as the maximal difference (in the plate reader's arbitrary relative light units) between the sample and the control over a period of 10 h. Error bars represent the standard deviation over three different samples.

mutation previously demonstrated to be beneficial for *yqjF* induction (Shemer *et al.*, 2020). These bioreporters also harboured plasmid *pACYC_yhaJ_G2* (Shemer *et al.*, 2020), expressing a modified version of YhaJ, the positive *yqjF* regulator (Palevsky *et al.*, 2016). The two new reporter strains thus generated were termed BS02 (carrying *C55_luxAf*) and BS03 (*C55_luxPleio*). Activity in liquid culture revealed a superior response of both alternative reporter systems at temperatures below 37°C (Fig. S1). When immobilized in PAA–alginate beads, both BS02 and BS03 displayed a significantly lower background luminescence in comparison to BS01, resulting in superior response ratios. Luminescence intensity of strain BS03 in response to DNT buried in sand (5 mg kg^{-1}) at 25°C–30°C was much higher than that of the other two strains; at 25°C, the integration of the *P. leiognathi lux* genes resulted in a 4-fold increase in signal intensity (Fig. 3) and a 21-fold increase in the response ratio (not shown). The activity of the BS02 reporter was superior at 15°C–20°C, but fell dramatically at temperatures of 25°C and above. The BS01 reporter proved slightly advantageous only when tested at 37°C, rendering it less suitable for most field applications. The decline in the luminescent response of BS01 at the lower temperatures may be attributed to the impaired activity of the *lux* enzymes, compounded by a more general slower cellular metabolic rate, which also led to a slower degradation of DNT.

Long-term storage of immobilized bioreporters

A parameter that would greatly affect any future adoption of microbial bioreporters for landmine detection, as well as other potential applications, is their amenability to long-term storage. To test viability and activity maintenance of the alginate/PAA-encapsulated sensor strains, identical batches of beads containing bioreporter strain BS03 were suspended in glycerol as a cryoprotectant and freeze-dried. The beads' morphology was maintained throughout this process; in the absence of glycerol, bead structure was lost and activity after resuscitation was below the detection limit (data not shown).

Over a period of three months, bead aliquots were removed from the temperatures at which they were maintained, and their response to DNT (2 mg l^{-1} DNT in LB–agar) was assayed. When maintained at temperatures above 0°C, deterioration of activity was very rapid, resulting in ca. 90% lower signal intensities after 30 days of storage (not shown). The beads maintained at both –20°C and −80°C, however, exhibited complete recovery of activity; in fact, activity after resuscitation was higher than that of the beads immediately after freezing (Fig. 4). The beads frozen at −20°C required a longer time than those kept at −80°C to reach the same activity levels; after 50 days of storage, the average time to reach a response ratio of 5, for example, was 367 min and 131 min for the −20 and −80 stored cells respectively.

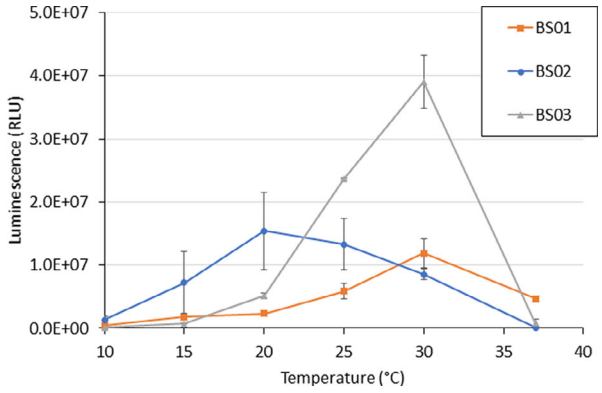

Fig. 3. Bioluminescent response of bead-encapsulated sensor strains BS01, BS02 and BS03 to DNT buried in sand (15 μg per well, 5 mg kg_{sand}^{-1}). All three strains carry a ΔpykF deletion and the pACYC_yhaJ_G2 plasmid, along with one of three *lux* variants; C55_Pl (BS01), C55_Af (BS02), and C55_Pleio (BS03). A single layer of 1.55 mm beads (1.51 × 10^5 cells per bead, 2% w/v alginate, 1% PAA) was placed on each well of an opaque white 24-well microtitre plate. Error bars represent the standard deviation over at least 3 measurements.

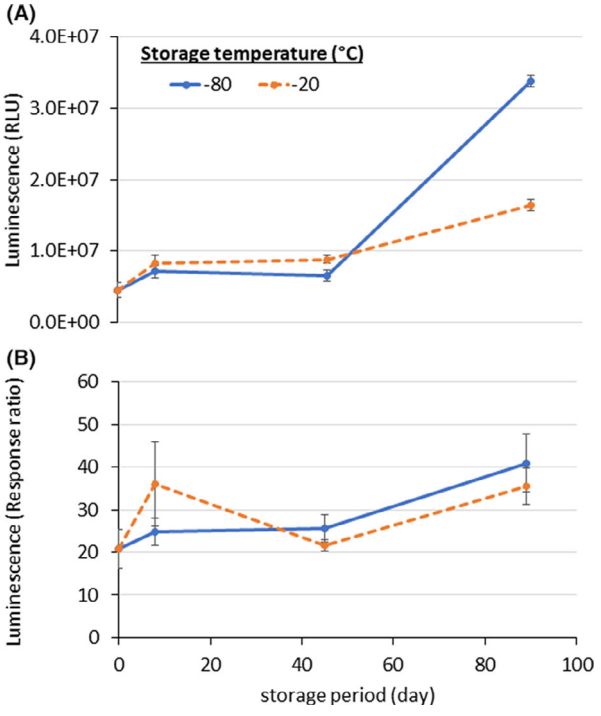

Fig. 4. Bioluminescent response to DNT (2 mg l^{-1} in LB–agar) of immobilized bioreporters (strain BS03), subjected to lyophilization and stored for three months at −80°C and −20°C. The signal intensity (A) and signal to background ratio (B) were monitored every 2–3 weeks. Error bars represent the standard deviation of 3 independent measurements.

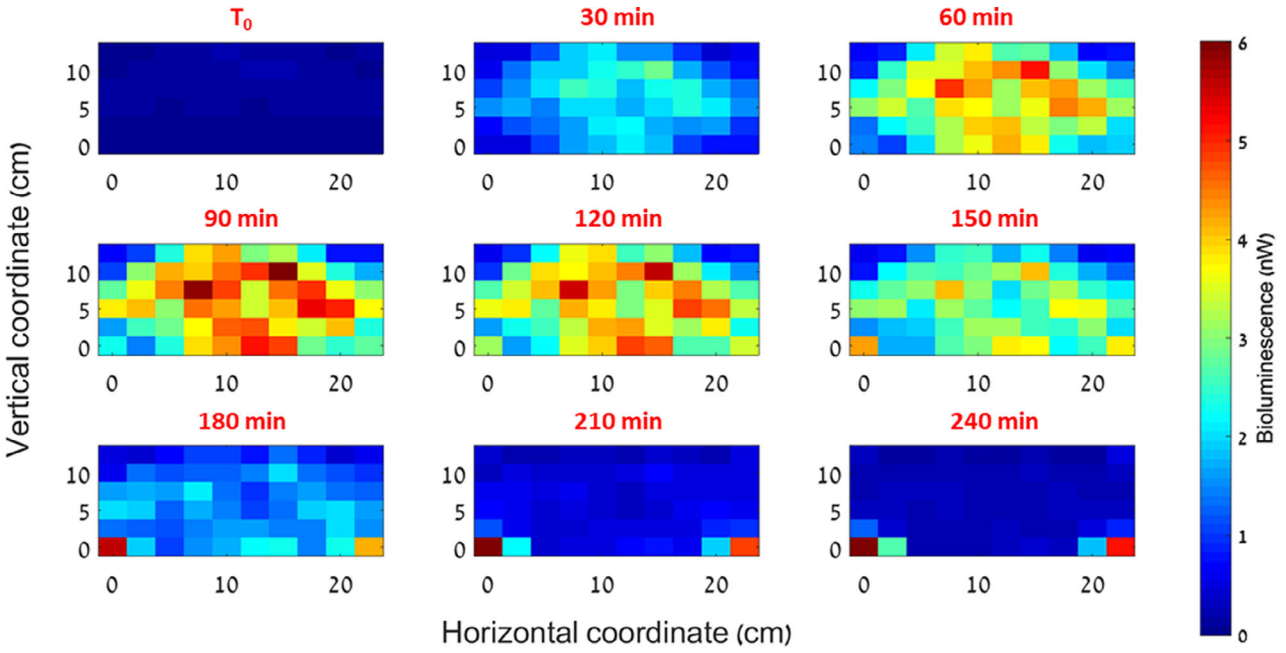

Fig. 5. Response of the immobilized BS01 reporter strain to buried explosives. DNT (50 g) was buried under 8 cm of sand in a 15 cm × 25 cm container, and incubated for 1 year at room temperature. Luminescent response of the biosensors was monitored in an emulator chamber (70% humidity, 25°C) by a custom-made detection system, consisting of a photomultiplier device that traversed the target area at constant time intervals.

Response of immobilized bioreporters to buried explosives

A laboratory-scale experiment was conducted to demonstrate the detection capabilities of buried explosives by the immobilized bioreporters. Strain BS01, immobilized in 1.5 mm alginate/PAA beads, was spread over a sand-filled container harbouring DNT (50 g), buried 8 cm below the surface ca. 12 months before the experiment. A strong, rapid but transient response was observed, mostly around the centre of the field, peaking after 1.5 h and ending 2.5 h later (Fig. 5). Four reference samples were measured in parallel: two negative control samples of DNT-free sand (top corners of the container) and two positive control samples of 5 mg kg^{-1} of DNT mixed in sand (bottom corners). Peak luminescence intensity measured was 4-fold higher than that observed in a control sample filled with DNT-free sand (Fig. S2), and developed much faster than that of the positive controls at the bottom corners.

Detection of a buried landmine with immobilized bioreporters

Actual concentrations of DNT and TNT above buried landmines are highly variable, depending upon mine type and age, soil type and composition, soil water content, local vegetation and climatic conditions (Jenkins *et al.*, 1999, 2001; Webb and Phelan, 2000; Leggett *et al.*, 2001). In this experiment, a small (100 g explosive content) metallic antipersonnel landmine (model PRB M3; von Tresckow, 1978) was buried a few cm below the surface of a sand-filled cylindrical container ($H = 50$ cm, $R = 25$ cm), and left in the open for 15 months. While the porous sand matrix normally promotes the permeation of explosive vapours, after 15 months of exposure to the elements, including significant rainfall, its topmost layer turned into a solid crust. Imaging of the container 5.5 h after spreading the immobilized BS03 sensor strain revealed a clear increase in the luminescent signal at the centre, directly above the spot where the landmine was buried (Fig. 6).

Discussion

While detection of explosives with microbial bioreporters has been studied for two decades, the actual implementation of such a solution for the detection of real landmines has scarcely been reported, with the exception of the initial design reported by Burlage (MacDonald *et al.*, 2003) and the more recent report by Belkin *et al.* (2017). In the present report, we demonstrate for the first time the detection of an actual buried landmine in an outdoor set-up, by a bioluminescent bioreporter. To reach this milestone, significant enhancement of the luminescent signal from the original bioreporter design (Yagur-Kroll

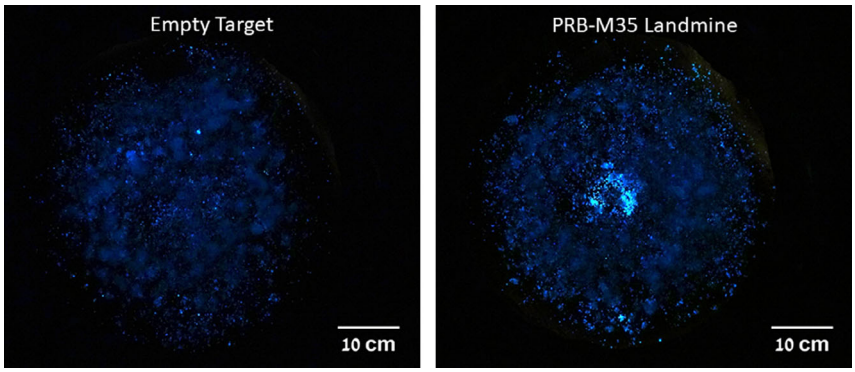

Fig. 6. Luminescent response of BS03 reporter strain, immobilized in 1.55 mm alginate–PAA beads (1.51×10^5 cells/bead, 2% w/v alginate, 1% PAA), 5.5 h following exposure to an empty sand target (left) and to an aged target containing a PRB-M35 antipersonnel landmine buried in sand (right).

et al., 2014, 2015) was achieved (Shemer *et al.*, 2020), as well as optimization of the formulation in which the microbes are encapsulated for dispersal in the field.

In previous studies (Belkin *et al.*, 2017), the Ca–alginate beads employed to immobilize the sensor bacteria and provide them with a suitable microenvironment in which to function, were extremely prone to desiccation; in view of the long exposure time required for signal development, the beads had to be intermittently moisturized in the course of the experiment. To circumvent bead desiccation under field conditions, thus allowing sufficient time for bacterial signal development, we have explored two separate strategies: limiting bead water loss, and enhancing bacterial signal development in terms of both intensification of the signal and shortening of the response time. As is demonstrated in Figure 6, the combination of the two strategies has allowed the clear detection of a real antipersonnel landmine within a few hours, with no evident deterioration of bead morphology and function, in spite of the low humidity of the test environment.

Alginate was selected as the main polymeric component for sensor strain encapsulation, based on numerous previous studies attesting to its transparency, facility of preparation, excellent biocompatibility and high permeability (Leth *et al.*, 2002; Michelini and Roda, 2012; Axelrod *et al.*, 2016). In the present study, three parameters were optimized in the encapsulation of the bacteria in the alginate matrix: bead size, cell density and PAA concentration, leading to a formulation (2% alginate, 1% PAA, 1.55 mm diameter, 1.51×10^5 cells per bead) that proved to be superior to formerly used beads (Belkin *et al.*, 2017) in terms of both signal intensity and resistance to desiccation. The concentration of bacteria inside the beads proved to be a vital factor in optimizing their response. Interestingly, the overall signal was not linearly linked to bacterial population density, as several conflicting factors appear to play a role here: while higher cell numbers are expected to provide a stronger signal, they are nevertheless hindered by physical proximity of other cells, light emission is masked, and activity may be limited by availability of nutrients, oxygen and DNT. The addition of PAA, originally intended to improve desiccation resistance, turned out to also enhance the bioreporter's signal intensity, apparently by absorbing DNT and thus increasing the concentration of this compound in the immediate environment of the cells. While it is expected that same may be true also for traces of TNT, this has not been examined in the present study.

Another significant environmental factor that must be considered when applying these sensor microbes under field conditions, is the need to function at temperatures that may be sub-optimal for the host bacteria. This obstacle was at least partially surmounted by constructing a set of bioreporters with different *lux* cassettes, originating from three different luminescent bacteria, thereby broadening the operational temperature spectrum. Across the entire panel, satisfactory results were obtained at temperatures ranging from below 15°C to above 30°C. In addition, the *lux* systems originating from *A. fischeri* and *P. leiognathi* not only allowed faster signal development, but also exhibited a much lower background luminescence, resulting in lower detection thresholds at all temperatures.

While *E. coli* may not be an ideal species in terms of survival and functionality in natural environments, it was nevertheless selected as the host bacterium due to the accessibility of relevant molecular, biochemical and physiological data and knowhow, combined with amenability to genetic manipulations and the availability of the required molecular tools. The successful detection of a buried antipersonnel landmine by strain BS03 serves as an obvious justification to this decision. Nevertheless, future studies will be devoted to the

development of an equivalent detection system in different microbial hosts, including soil bacteria.

The results presented in this study demonstrate that the current sensitivity and signal intensity of the BS03 reporter strain are already sufficient to produce a significant optical signature not only in response to buried explosives dispersed in soil, but also to actual landmines, for which the explosive signature is minute. Nevertheless, efforts directed at additional lowering of the detection thresholds are currently underway, aiming to generate improved bioreporter generations. Future large-scale field applications will also require the development of practical solutions, among others, for mechanized bead dispersion and image processing, both of which are currently under development. Additional issues that will need to be addressed relate to the release of engineered microbes to the environment, including the risks of lateral gene transfer along with antibiotic resistance. While a chromosomal integration of the sensor–reporter fusion may be impractical due to a significantly lower signal, other solutions may be implemented, including selection characteristics which are not antibiotics-based (Vandermeulen *et al*., 2011), auxotrophic mutations that will prevent bacterial growth in the absence of specific nutritional elements, or toxin-based interdependency of the host/plasmid pair (Wright *et al*., 2015).

Experimental procedures

Chemicals and growth media

All chemicals used in this work were of the highest analytical grade and were purchased from Sigma-Aldrich (Israel). DNT stock solution was prepared by dissolving DNT crystals in ethanol to a concentration of 27 g l^{-1}. This solution was then diluted in water or in lysogeny broth (LB) at least 50-fold, so that final ethanol concentration in the reaction mixture did not exceed 2% (v/v). DNase–RNase-free water (Sigma-Aldrich) was used for all molecular biology procedures. Restriction enzymes were purchased from New England Biolabs, and DNA purification kits from Qiagen. Sodium–alginate (CAS 9005-38-3) and polyacrylic acid (PAA, CAS 9003-01-4, MW ~ 250 000, 35% in H_2O) were purchased from Sigma-Aldrich (Israel).

All bacteria were grown in LB and stored on LB–agar plates. Antibiotics (100 mg l^{-1} ampicillin, 50 mg l^{-1} kanamycin or 30 mg l^{-1} chloramphenicol) were added to the media depending on the bacterial hosts and plasmids employed.

Encapsulation of bacteria in alginate beads

Optimal sodium–alginate working solution concentration was 2% (w/v); higher concentrations were too viscous, whereas lower concentrations were more prone to desiccation and supported sub-optimal activity (Fig. S3). The solution was stirred until all the alginate were dissolved, and allowed to settle overnight at room temperature (RT) to remove air bubbles and ensure full dissolution. A 10% PAA solution was prepared by diluting PAA in deionized water. The solution was neutralized to pH = 7.0 with 10 M NaOH, following which it was filter-sterilized (0.22 μm Biofil™ PVDF filter, Porvair Filtration Group, Fareham, UK) and stored at room temperature. PAA was added to the fully dissolved alginate to a final concentration of 0%–1.5% and stirred to full homogenization. The volume of the added 10% PAA solution was subtracted from the total volume of H_2O in which the alginate was dissolved.

Bacteria were grown overnight at 37°C with shaking (200 r.p.m.) in LB supplemented with the appropriate antibiotics to ensure plasmid maintenance. The culture was then diluted ×1/50 in 1 l of fresh LB supplemented with the same antibiotics and grown under the same conditions to an optical density at 600 nm (OD_{600}) of 0.8–1.0. The culture was then centrifuged (Sorvall Lynx 6000, Thermo Scientific, 7000 r.p.m., 4°C) for 40 min, and the supernatant was discarded. The pellet was re-suspended in 5 ml sterile saline (0.9% w/v NaCl) and re-centrifuged at 4000 r.p.m. for 10 min. The supernatant was discarded, and the pellet was weighed and re-suspended in 5 ml saline. An appropriate volume of the re-suspended bacteria was added to a previously prepared sodium–alginate solution, with or without PAA, and stirred gently to ensure homogeneity. The alginate–PAA–bacteria solution was stored at 4°C and immobilized the following day.

Immobilization of the bacteria in alginate beads was performed by dripping the bacteria–alginate (+/-PAA) solution into a gently stirred $CaCl_2$ (0.1 M) solution, using an automated Büchi B-390 encapsulator (Büchi Labortechnik AG, Flawil, Switzerland). The nozzle diameters used in this work ranged from 300 to 1000 μm. The pressures employed were 200–400 mbar (N_2) and the applied vibration frequency was 40–200 Hz, depending on the selected nozzle, to allow the separation of droplets. The beads formed upon contact with the Ca^{2+} ions were stirred for 30 additional minutes to ensure complete gelation. The beads were then strained, washed with saline supplemented with TWEEN® 80 (0.5% w/v) to reduce stickiness, strained again and kept at 4°C until used.

To enumerate bacterial density inside the beads, batches of 10 beads were placed in a 1.5 ml microcentrifuge tube (Eppendorf), to which sodium citrate (2%) was added until the Ca–alginate beads were fully dissolved. The cells thus released to the surrounding solution were enumerated by plating serial dilutions on LB–

agar plates; the number of colony-forming units (CFU) was determined following overnight incubation at 37°C.

Assessing alginate bead water loss

The effect of PAA content on the rate of water loss was measured by placing batches of 10 beads, 3 mm in diameter, on an open Petri dish at room temperature, and following their weight over a period of 5 h.

Assessing DNT adsorption by alginate beads

Alginate beads (2% w/v) with or without PAA (1% w/v) were prepared as previously described and washed with double-distilled H_2O. Batches containing 90–150 beads of each matrix type were selected, bead diameter was measured, and total bead volume was calculated (ca. 1.7–3.1 ml per batch). Next, each batch was incubated in a 5 ml DNT (5 mg l^{-1}) solution for 3 h at 30°C. The medium was filtered (0.45 μm syringe filter) and stored at −20°C until analysed. The beads were lightly dried from excess liquid, transferred to methanol (3 ml) and shaken at 30°C for 3 h. The methanol extract was filtered (0.45 μm syringe filter) and stored at −20°C until analysed. DNT concentrations in the medium and bead extracts were determined by liquid chromatography/mass spectrometry (LC/MS) using an Agilent 1200 system composed of a ZORBAX Eclipse XDB-C18 (2.1 mm × 50 mm) column and an Agilent 6520 Accurate-Mass Q-TOF LC/MS apparatus, equipped with an electrospray ionization source (ESI) and a UV detector (Agilent Technologies, Santa Clara, CA, USA) as previously described (Shemer *et al.*, 2018). Two sets of calibrants were used to quantify DNT in the media surrounding the beads and the bead extract, containing DNT dissolved in H_2O or methanol, respectively.

Construction of novel reporter plasmids

Three reporter plasmids were used in this study, containing three different *lux* gene cassettes as bioluminescent reporters: *luxCDABE* genes of *Photorhabdus luminescens* (C55_luxPl), the *luxCDABEG* of *Aliivibrio fischeri* (C55_luxAf) or the *luxCDABEG* genes of *Photobacterium leiognathi* (C55_luxPleio). In all three variants, the *lux* cassette was fused downstream of the C55 promoter, a variant of the *E. coli* native *yqjF* promoter, the DNT-detection capability of which was enhanced by directed evolution (Shemer *et al.*, 2020). Replacement of the *lux* cassette in the C55-luxPl plasmid with that of *A. fischeri* or *P. leiognathi* was performed by digestion of the plasmid with *Sac*I and *Eco*RI restriction enzymes, followed by insertion of the alternative *lux* cassette using the Gibson assembly technique (Gibson *et al.*, 2009). The *luxCDABEG* cassette of *A. fischeri* was PCR-amplified (primers 94_luxV_SacI_F and 93_luxV_RI_R, Table 2) from plasmid *pUCD615* (Rogowsky *et al.*, 1987), and the *luxCDABEG* cassette of *P. leiognathi* (Leibniz Institute DSMZ strain 21260, Germany) was PCR-amplified directly from this organism's genomic DNA (primers 125_luxPL_F and 126_luxPL_R, Table 2). A *Sac*I restriction site, located in

Table 3. List of bacterial strains, plasmids and primers used in this study.

Bacterial strain	Genomic mutations	Reference
Photobacterium leiognathi subsp. *leiognathi*		Boisvert *et al.* (1967)
Escherichia coli BW25113	*ΔpykF*	Baba *et al.* (2006)

Plasmid	Backbone	Source
C55_luxPl	pBR2TSS	Shemer *et al.* (2020)
C55_luxAf	pBR2TSS	This work
C55_luxPleio	pBR2TSS	This work
pACYC_yhaJ_G2	pACYC	Elad *et al.*, unpublished

Bioreporter strain	Host, genetic background, plasmid	Plasmids
BS01	*E. coli BW25113, ΔpykF, C55_luxPl*	C55_luxPl, pACYC_yhaJ_G2
BS02	*E. coli BW25113, ΔpykF, C55_luxAf*	C55_luxAf, pACYC_yhaJ_G2
BS03	*E. coli BW25113, ΔpykF, C55_luxPleio*	C55_luxPleio, pACYC_yhaJ_G2

Primer	DNA sequence
93_luxV_RI_R	GTGAATTGTAATACGACTCACTATAGGGCGAATTCCTTTATACGTATGCAAAAGCATCG
94_luxV_SacI_F	CAATCAGCAGCCTGAGTGGCGAGCTCAGGAGGGGCAAATATGAATAAATGTATTCCAATG
125_luxPL_F	CAGCAGCCTGAGTGGCGAGCTCAGGAGGGGCAAATATGATTAAGAAGATCCCAATGATTATTG
126_luxPL_R	CGACTCACTATAGGGCGAATTCAATATCCCTATAAATAGAACTATCTATTGGGTAC

the *luxD* gene of the inserted *lux* cassette was silenced by adding a point mutation (GAGCTC → GAGTTC), to allow further manipulations using the *Sac*I site flanking the reporter genes. All constructed plasmids were verified by colony PCR and by sequencing. All plasmids were chemically transformed to an *E. coli* strain (BW25113) carrying a deletion of the *pykF* gene (Baba *et al*., 2006), a mutation beneficial for DNT detection in terms of signal intensity and detection threshold (Shemer *et al*., 2020). Plasmid pACYC_yhaJ_G2 (T. Elad, B. Shemer, E. Shpigel and S. Belkin, unpublished data), encoding an enhanced version of *yqjF*'s positive regulator YhaJ, was also transformed into the bacterial hosts, along with the *yqjF-lux* plasmids, to further improve their detection capabilities. All strains, plasmids and primers used in this work are listed in Table 3.

Luminescence assays, liquid cultures

An overnight culture (LB, 37°C with shaking, 200 r.p.m.) was diluted ×1/100 in fresh LB and grown to an early exponential growth phase ($OD_{600} \approx 0.2$). Culture aliquots (50 μl) were then added to 50 μl of DNT in 4% ethanol in individual wells of an opaque white 96-well microtitre plate (Greiner AG, Kremsmünster, Austria). The final ethanol concentration in all wells was 2%. The plate was covered with a SealPlate tape (Thermo Fisher Scientific, Waltham, MA, USA), and bioluminescence development was monitored with an Infinite 200 PRO plate reader (TECAN, Switzerland). When the desired incubation temperature was lower than 25°C, the plate reader was placed in a refrigerated room (4°C).

Luminescence assays, immobilized bioreporters on sand or agar

Activity assays for beads were performed either on sand or on LB–agar. For sand assays, 3 g of sand (Sigma-Aldrich, 50–70 mesh) was introduced into each well of a 24-well transparent microtitre plate (Nunclon) and was moistened by 700 μl DNT-containing LB. For LB–agar assays, 2 ml of DNT-containing warm LB–agar was transferred to each well of a similar 24-well plate. The agar was allowed to solidify at room temperature before use.

Ten ml of refrigerated bacterial beads was reconditioned prior to use by a 1 h incubation in 25 ml LB at 37°C with shaking (200 r.p.m.). The beads were then strained and spread in a single layer in the well of a sand- or agar-containing 24-well microtitre plate, prepared as described above. Bioluminescence was quantified in an Infinite 200 PRO plate reader (TECAN, Switzerland). When the desired incubation temperature was lower than 25°C, the plate reader was placed in a refrigerated room (4°C).

The luminescent response is presented in the plate reader's arbitrary relative luminescence units (RLU), either as the difference (ΔRLU) or the ratio (response ratio) between the DNT-exposed sample and the DNT-free control.

The response of the immobilized biosensors was also tested over a sand-filled container (27 cm × 16 cm) in which DNT (50 g) was buried under 8 cm of sand (50–70 mesh, Sigma-Aldrich), and kept at room temperature for ca. 1 year to allow permeation of the DNT vapours to the topmost sand layer. Bacterial bioreporters of strain SB02 (Table 3) were immobilized in 1.5 mm diameter alginate (2%)-PAA (1%) beads, at a bacterial concentration of 0.1% (ca. 1.5×10^5 cells per bead). The target was lightly sprayed with LB, following which the immobilized biosensors were spread evenly over the container's surface. The container was then placed in an environmentally controlled chamber (70% humidity, 25°C, Fig. S2) and was continuously scanned using a special purpose optoelectronic set-up. The luminescent signal emitted by the bacteria was sampled over a period of 20 h by a sensitive photodetector (photomultiplier tube, Model H10493, *HAMAMATSU*) and an optical system for imaging the emittance of the bacteria on the photodetector. Both the photosensor and the optical system were mounted on XYZ motorized stages for scanning the entire area of the target. The optoelectronic system, designed for detecting very weak optical signals (down to 3 femto-watt at $\lambda = 490$ nm, the peak wavelength of the bacterial bioluminescent emittance), was isolated from both optical and electronic background noise.

A detection test was also conducted with a real metallic antipersonnel landmine (model PRB M-35, von Tresckow, 1978) with the detonator removed, buried 4 cm below the surface of a cylindrical container ($R = 25$ cm, $H = 50$ cm) filled with Mediterranean coastal sand. The container was left in the open for ca. 15 months. Two weeks before the experiment, *E. coli* sensor strain BS03 (Table 1) was immobilized in 1.5 mm alginate–PAA beads containing 2% alginate, 1% PAA and 1.5×10^5 cells per bead, and kept at 4°C. Prior the experiment, 150 g of beads was reconditioned in LB for 1 h at 37°C, then strained and transferred to the experiment location. LB was lightly sprayed on the target's surface, and the beads were sprinkled evenly on the whole target area. A dark chamber was placed over the target, and a Sony α7S II camera equipped with a Sony FE 28 mm f/2 lens was used to image the luminescent response (25600 ISO, 30 s exposure, maximal aperture).

Long-term storage of immobilized sensor strains

To investigate the potential for long-term storage of the alginate/PAA immobilized bioreporters, *E. coli* sensor

strain BS03 was immobilized in 1.5 mm alginate–PAA beads containing 2% alginate and 1% PAA at a cell density of 1.5×10^5 cells per bead. The beads were washed with 0.5% TWEEN® 80 in saline, strained, placed in 5 ml glass vials, covered with glycerol (20% v/v) and frozen at −80°C. The frozen beads were lyophilized at −40°C in an Epsilon 1-4 LSCplus freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), under a pressure of 0.05 mbar, for 72 h. The lyophilized beads were stored at −80°C, −20°C, 4°C and at room temperature. At intervals, vials were shifted to room temperature for 20 min, following which 20 ml of LB were added to each of the vials, which were further incubated at 37°C for 2 h. A layer of beads was placed on the surface of each well of a transparent 24-well microtitre plate (Nunc) containing 1.8 ml LB–agar with or without DNT (2 mg l^{-1}). Luminescence was measured every 10 min in an Infinite 200 PRO plate reader (TECAN, Switzerland) at 28°C.

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Conflicts of interest

The authors have no conflict of interest to declare.

References

Altamirano, M., García-Villada, L., Agrelo, M., Sánchez-Martıín, L., Martıín-Otero, L., Flores-Moya, A., *et al.* (2004) A novel approach to improve specificity of algal biosensors using wild-type and resistant mutants: an application to detect TNT. *Biosens Bioelectron* **19:** 1319–1323.

Axelrod, T., Eltzov, E., and Marks, R.S. (2016) Bioluminescent bioreporter pad biosensor for monitoring water toxicity. *Talanta* **149:** 290–297.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., *et al.* (2006) Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2,** 2006.0008.

Belkin, S., Yagur-Kroll, S., Kabessa, Y., Korouma, V., Septon, T., Anati, Y., *et al.* (2017) Remote detection of buried landmines using a bacterial sensor. *Nat Biotechnol* **35:** 308–310.

Boisvert, H., Chatelain, R., and Bassot, J.-M. (1967) Étude d'un Photobacterium isolé de l'organe lumineux des poissons Leiognathidae. *Ann Inst Pasteur Paris* **112:** 520–524.

Burlage, R.S., Patek, D.R., Everman, K.R., andLockheed Martin Energy Research Corp (1999) Method for detection of buried explosives using a biosensor. U.S. Patent 5,972,638.

Chiellini, E., Corti, A., D'Antone, S., and Solaro, R. (2003) Biodegradation of poly (vinyl alcohol) based materials. *Prog Polym Sci* **28:** 963–1014.

Davidson, M.E., Harbaugh, S.V., Chushak, Y.G., Stone, M.O., and Kelley-Loughnane, N. (2012) Development of a 2, 4-dinitrotoluene-responsive synthetic riboswitch in E. coli cells. *ACS Chem Biol* **8:** 234–241.

Garmendia, J., De Las Heras, A., Galvão, T.C., and De Lorenzo, V. (2008) Tracing explosives in soil with transcriptional regulators of Pseudomonas putida evolved for responding to nitrotoluenes. *Microb Biotechnol* **1:** 236–246.

Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison, C.A., and Smith, H.O. (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods* **6:** 343–345.

Jenkins, T.F., Leggett, D.C., Miyares, P.H., Walsh, M.E., Ranney, T.A., Cragin, J.H., and George, V. (2001) Chemical signatures of TNT-filled land mines. *Talanta* **54:** 501–513.

Jenkins, T.F., Leggett, D.C., and Ranney, T.A. (1999) Vapor signatures from military explosives. Part 1. Vapor transport from buried military-grade TNT, DTIC Document.

Larson, R.J., Bookland, E.A., Williams, R.T., Yocom, K.M., Saucy, D.A., Freeman, M.B., and Swift, G. (1997) Biodegradation of acrylic acid polymers and oligomers by mixed microbial communities in activated sludge. *J Environ Polym Degrad* **5:** 41–48.

Leggett, D.C., Cragin, J.H., Jenkins, T.F., and Ranney, T. (2001) Release of explosive-related vapors from land mines, DTIC Document.

Leth, S., Maltoni, S., Simkus, R., Mattiasson, B., Corbisier, P., Klimant, I., *et al.* (2002) Engineered bacteria based biosensors for monitoring bioavailable heavy metals. *Electroanal Int J Devoted Fundam Pract Asp Electroanal* **14:** 35–42.

Looger, L.L., Dwyer, M.A., Smith, J.J., and Hellinga, H.W. (2003) Computational design of receptor and sensor proteins with novel functions. *Nature* **423:** 185–190.

MacDonald, J., Lockwood, J., McFee, J., Altshuler, T., and Broach, T. (2003) *Alternatives for Landmine Detection*. Arlington, VA: Rand Corporation.

Michelini, E., and Roda, A. (2012) Staying alive: new perspectives on cell immobilization for biosensing purposes. *Anal Bioanal Chem* **402:** 1785–1797.

Palevsky, N., Shemer, B., Connolly, J.P., and Belkin, S. (2016) The highly conserved Escherichia coli transcription

factor YhaJ regulates aromatic compound degradation. *Front Microbiol* **7:** 1490.
Radhika, V., Proikas-Cezanne, T., Jayaraman, M., Onesime, D., Ha, J.H., and Dhanasekaran, D.N. (2007) Chemical sensing of DNT by engineered olfactory yeast strain. *Nat Chem Biol* **3:** 325–330.
Rogowsky, P., Close, T., Chimera, J., Shaw, J., and Kado, C. (1987) Regulation of the vir genes of Agrobacterium tumefaciens plasmid pTiC58. *J Bacteriol* **169:** 5101–5112.
Shemer, B., Koshet, O., Yagur-Kroll, S., and Belkin, S. (2017) Microbial bioreporters of trace explosives. *Curr Opin Biotechnol* **45:** 113–119.
Shemer, B., Palevsky, N., Yagur-Kroll, S., and Belkin, S. (2015) Genetically engineered microorganisms for the detection of explosives' residues. *Front Microbiol* **6:** 1175.
Shemer, B., Shpigel, E., Glozman, A., Yagur-Kroll, S., Kabessa, Y., Agranat, A.J., and Belkin, S. (2020) Genome-wide gene-deletion screening identifies mutations that significantly enhance explosives' vapor detection by a microbial sensor. *New Biotechnol* **59:** 65–73.
Shemer, B., Yagur-Kroll, S., Hazan, C., and Belkin, S. (2018) Aerobic transformation of 2, 4-dinitrotoluene by *Escherichia coli* and its implications for the detection of trace explosives. *Appl Environ Microbiol* **84:** AEM-01729.
von Tresckow, A. (1978) *Land Mines (Landminen)*. Washington, DC: Naval Intelligence Support Center.
Vandermeulen, G., Marie, C., Scherman, D., and Préat, V. (2011) New generation of plasmid backbones devoid of antibiotic resistance marker for gene therapy trials. *Mol Ther* **19:** 1942–1949.
Webb, S.W., and Phelan, J.M. (2000) Effect of diurnal and seasonal weather variations on the chemical signatures from buried land mines/UXO. International Society for Optics and Photonics, pp. 474–488.
Wright, O., Delmans, M., Stan, G.-B., and Ellis, T. (2015) GeneGuard: A modular plasmid system designed for biosafety. *ACS Synth Biol* **4:** 307–316.
Yagur-Kroll, S., Amiel, E., Rosen, R., and Belkin, S. (2015) Detection of 2,4-Dinitrotoluene and 2,4,6-Trinitrotoluene by an Escherichia coli bioreporter: performance enhancement by directed evolution. *Appl Microbiol Biotechnol* **99:** 7177–7188.
Yagur-Kroll, S., Lalush, C., Rosen, R., Bachar, N., Moskovitz, Y., and Belkin, S. (2014) Escherichia coli bioreporters for the detection of 2, 4-dinitrotoluene and 2, 4, 6-trinitrotoluene. *Appl Microbiol Biotechnol* **98:** 885–895.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Bioluminescent response of three different lux variants to 0.25 mg/L DNT at various temperatures. All strains are pykF mutants and carry the pACYC_yhaJ_G2 plasmid. Strain BS01 carries the previously used lux cassette, originating from P. luminescens, while BS02 and BS03 carry the lux cassettes of A. fischeri or P. leiognathi, respectively. The response is demonstrated in terms of the maximal signal intensity over 1000 minutes of incubation (A, C) or the ratio between the maximal response and the maximal background (B, D). Panels C and D focus on the response at 15°C. Error bars represent the standard deviations over three repetitions.
Fig. S2. Luminescent response of immobilized BS01 biosensors to DNT buried in sand. The response was measured by a traversing photodetector placed in an environmentally controlled chamber (A) and is represented by the signal intensity at the most induced area across the target ('induced') and the signal intensity over two control targets, comprised of clean sand ('control') (B).
Fig. S3. Response of the BS03 reporter strain, immobilized in PAA-alginate beads, and placed on 1.8 mL of LB-agar supplemented with DNT (2 mg/L). The response is represented as the maximum difference between the sample and the control over a period of 10 hours. Error bars represent the standard deviation over 3 different samples.

