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An autonomous bioluminescent bacterial biosensor module for outdoor sensor networks, and its application for the detection of buried explosives

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ABSTRACT

We describe a miniaturized field-deployable biosensor module, designed to function as an element in a sensor network for standoff monitoring and mapping of environmental hazards. The module harbors live bacterial sensor cells, genetically engineered to emit a bioluminescent signal in the presence of preselected target materials, which act as its core sensing elements. The module, which detects and processes the biological signal, composes a digital record that describes its findings, and can be transmitted to a remote receiver. The module is an autonomous self-contained unit that can function either as a standalone sensor, or as a node in a sensor network. The biosensor module can potentially be used for detecting any target material to which the sensor cells were engineered to respond. The module described herein was constructed to detect the presence of buried landmines underneath its footprint. The demonstrated detection sensitivity was 0.25 mg 2,4-dinitrotoluene per Kg soil.

1. Introduction

We describe herein a biosensor module (BSM) designed primarily for implementing a sensor network that monitors and maps the presence of target materials on the ground in open environments. The BSM employs sensing bacteria that serve as its core sensing elements. These bacteria are genetically engineered to respond by producing bioluminescence to the presence of a specific target material (TM), and act as minuscule biochemical laboratories that emit light in response to their exposure to the TM. The BSM is an autonomous self-contained unit designed for either standalone operation, or as a node in a sensor network. The module detects and processes the biological signal, and transmits a digital record to a remote receiver. The BSM described herein has been configured to detect the presence of buried landmines underneath its footprint.

For many decades, the main and most prevalent approach for monitoring the presence of deleterious compounds in the environment has been chemical analysis. Indeed, modern analytical techniques and instrumentation allow extremely sensitive and highly precise analysis of practically any toxic molecule that may be present in an environmental sample, be it water, air, or soil. This approach, however, while essential for regulatory purposes as well as for understanding pollution sources and processes, is almost universally limited by the need to collect a sample in the field, and transport it to a fully (and expensively) equipped central laboratory, where trained and experienced technical personnel employ an array of sophisticated analytical instrumentation (Belkin, 2003). The most problematic issue inherent in this approach is not necessarily its high cost and complexity, but rather the temporal discontinuity between sample collection and availability of the results; when the target chemicals are unknown, days and weeks may pass before reliable information is available to field personnel and regulators alike (US EPA, 2015). There is thus an acute need to close this spatial and temporal gap, and implement complementary in situ technologies that will report, ideally in real time, on the wellbeing of the studied environment (Claxton et al., 2004; Ohe et al., 2004; Umbuzeiro et al., 2017).

As sensing elements, bacteria can be regarded as minuscule sensing devices that produce an optical signal as their output, in response to

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their exposure to a specific TM through their input. The fact that bacteria may be genetically engineered to respond to practically any specific TM, makes them highly versatile. This bears significance to both the plethora of application areas where they can be employed for sensing different materials, and - from the perspective of system engineering - to the wide range of configurations in which they can be employed.

We describe in this article an autonomous biosensor module that employs live bacteria as its core sensing elements. These bacteria were genetically engineered to respond to the presence of a specific TM by producing bioluminescence. The module is designed to be deployed outdoors, and report the presence of the TM in the soil underneath its footprint. We address the challenges put forward by the need to incorporate live biological entities in an optoelectronic (OE) circuit, in which they function in unison with the circuit components as a unified sensing system. The biosensing module is designed both for standalone operation, as well as a node in a sensor network deployed outdoors across large areas, reporting the presence of TMs at the respective locations of its elements. The sensor network may be used for early warning purposes as well as for risk mapping of large areas.

The present article describes the design, construction, and testing of the basic unit module of such a network, at the heart of which are the microbial bioreporters as the sensing entities. Advances in synthetic molecular biology allow the precise "tailoring" of such sensors towards the detection of specific compounds, groups of chemicals, or diverse global parameters such as toxicity or genotoxicity (van der Meer and Belkin, 2010).

The application that serves herein as a benchmark for the concept is the remote detection of buried landmines, employing a genetically engineered *E. coli* strain that emits a luminescent signal in the presence of 2,4-dinitrotoluene (DNT). Traces of this compound emanate from 2,4,6-trinitrotoluene (TNT)-based mines and infiltrate to the surface of the ground, serving as a chemical "signature" of the buried landmine (Dubey et al., 1999; Sylvia et al., 2000).

Deactivation of explosive devices and buried landmines, remnants of past wars and regional conflicts worldwide, constitute a severe and pressing humanitarian need. It is estimated that 110 million mines are spread around the world, causing thousands of deaths and countless casualties every year ("Demining | United Nations," 2017). Landmine deactivation is currently primarily conducted by manual removal, at a cost of US$ 300 – US$ 1000 per mine. Moreover, it is estimated that for every 5000 mines cleared by employing existing technologies, one disposal operator is killed and two are injured by accidental explosions ("Demining | United Nations," 2017). This necessitates the development of an efficient and reliable standoff mine detection methodology. To that end, many techniques are being considered and are under development. Current explosives detection methodologies include: (i) The use of animals (e.g. dogs (Hall and Wynne, 2018; Lazarowski et al., 2018; Lazarowski and Dorman, 2014; Sargisson et al., 2012), rats (Poling et al., 2011), elephants (Miller et al., 2015), and pigs (Habib, 2007)); (ii) Chemical methods: colorimetric chemo-sensors (Salinas et al., 2012), electrochemical assays (Yu et al., 2017), and other chemical sensors (Wu et al., 2015); (iii) Physical methods: the traditional metal detection by a magnetometer that remains the most common method, induction devices (Dula et al., 2013), acoustic waves (Van Neste et al., 2009), x-ray/neutron backscatter (van den Heuvel and Fiore, 2012), IR spectrometry (Wen et al., 2018), mass spectrometry (Forbes and Sisco, 2018), fluorescence spectrometry (Sun et al., 2015), ion mobility spectrometry (Caygill et al., 2012), gas chromatography (Grate et al., 2012), and surface enhanced Raman scattering (Gillibert et al., 2018).

Most of these techniques require actual presence in the minefield, with obvious risks to personnel and equipment. Other limitations include high costs, a large ratio of false positives, time-consuming handling, and a very limited ability to detect non-metallic landmines. There is thus an ongoing search for alternative methodologies that would combine high efficiency, low cost, and standoff detection. We have previously reported the use of a fluorescent bacterial bioreporter for this purpose (Belkin et al., 2017). The sensor cells were encapsulated in 3 mm alginate beads and spread over the target area. Twenty-two hours later, the field was remotely scanned by a dedicated optoelectronic system, and the locations of the positively responding fluorescent beads were mapped, indicating potential sites of buried landmines. While this system was, as far as we are aware, the first successful demonstration of a standoff detection of buried landmines, it was nevertheless limited by several factors: (i) to reduce optical interference, the scanning had to be carried out at night, thus limiting operational options; (ii) a relatively large and costly standoff scanning and detection apparatus was required; (iii) lifetime of the ground-spread bacteria is limited, eventually terminated by desiccation; (iv) atmospheric conditions may hinder fluorescence excitation and measurement; (v) fluorescence of the proteins synthesized by the sensor bacteria may be quenched by ambient light; and (vi) ground conditions (vegetation, surface unevenness etc.) may physically mask the bacterial signal from the detection apparatus.

The scheme presented in this paper is implemented by a miniaturized, autonomous biosensor module for outdoor ground deployment, as a node in a sensor network that overcomes these drawbacks. The reporting mechanism employed by the bacteria, the BSM core sensing elements, is bioluminescence. This obviated the need for a fluorescence excitation source. Bioluminescence has been shown to allow faster and more sensitive target detection than fluorescent proteins (Sagi et al., 2003). Furthermore, the spontaneous signal emitted by the bioluminescent bacteria was significantly weaker than the background fluorescence produced by the sensor bacteria used by (Belkin et al., 2017).

Studies of the environmental fate of explosives and their transport through the soil indicate that a detectable amount of explosives reaches the surface (Dubey et al., 1999). TNT (2,4,6-trinitrotoluene) is the most common landmine explosive, found in approximately 80 percent of all mines (Cumming et al., 2001). However, TNT-based mines also contain impurities and degradation products such as dinitrobenzene (DNB) and dinitrotoluene (DNT). TNT and its manufacturing impurities DNB and DNT infiltrate through the landmine components, as well as cracks or pores in the mine casing, and migrate to the surface (Sylvia et al., 2000). These vapors are richer in DNT than in TNT, due to the higher volatility of the former. This phenomenon has been taken advantage of in the past for the detection of buried explosives, either in the field (Belkin et al., 2017) or – as in the present study - in the laboratory (Shemer et al, 2018, 2020, 2021). Thus, the bacterial sensors presented in this paper were genetically engineered to respond to the presence of trace amounts of DNT in their microenvironment.

2. Material and Methods

2.1. The biosensor module (BSM)

The BSM is a small autonomous unit designed to report the presence of a target chemical underneath its footprint. It is constructed of three units: (i) A sensing unit that harbors a bioluminescent bacterial sensor strain that enhances its light emission in the presence of its target compounds underneath the module, along with an optoelectronic (OE) circuit that detects and processes the bioluminescent signal; (ii) A digital unit that digitizes the analog signals received from the sensing unit, and produces a digital record ready for transmission; (iii) A communication unit that transmits the information produced by the module to the outside world, and receives control instructions from the network's manager. All three units, and the device's batteries, are installed in a specially designed casing, surrounded by a dark 'skirt' that is pressed onto the ground surface by flexible metallic fingers. The skirt, which prevents ambient illumination from affecting the bioluminescence measurements, is attached to the BSM bottom part and consists of two elements: (i) a flexible, light, and opaque black fabric; and (ii) auxiliary elastic arms that ensure a proper seal. The electronic circuit is insulated from water vapors released by the humid environment of the bacteria,

allowing it to convey and process very weak analog signals. A schematic illustration of the BSM, and an image of its inner components, are presented in Fig. 1a and Fig. 1c, respectively.

To compensate for background bioluminescence emitted by the bacteria, the sensing unit is constructed of two identical channels, an "Induced channel", and a "Reference channel". The former measures the light emitted by the bacteria in contact with the ground underneath the BSM, whereas the latter, in which the bacteria are insulated from the ground, measures the spontaneous uninduced bioluminescence. The signal that expresses the concentration of the target is defined as the difference between the outputs of the two channels. The two channels detection scheme minimizes potential inaccuracies driven by variations in environmental conditions, bacterial batch differences, and manufacturing irregularities of the detection circuits (Vasilescu, 2005). A schematic illustration of this scheme is presented in Fig. 1b. The complete BSM, showing its 'dark compartment' is depicted in Fig. 1d.

2.2. The bioreporters

The bioreporter bacteria employed in this study as the core sensing elements of the BSM, are a genetically modified *E. coli* strain, transformed with genetic elements which enable it to emit visible light in response to the target explosives (Fig. 2). A basic design, previously described by Yagur-Kroll et al. (2014) consists of a fusion between the promoter region of the *yqjF* gene and the *luxCDABE* gene cassette originating from the terrestrial bacterium *Photorhabdus luminescens*. While this design displayed a high specificity towards TNT and DNT, its detection sensitivity was inadequate to perceive the minute vapor

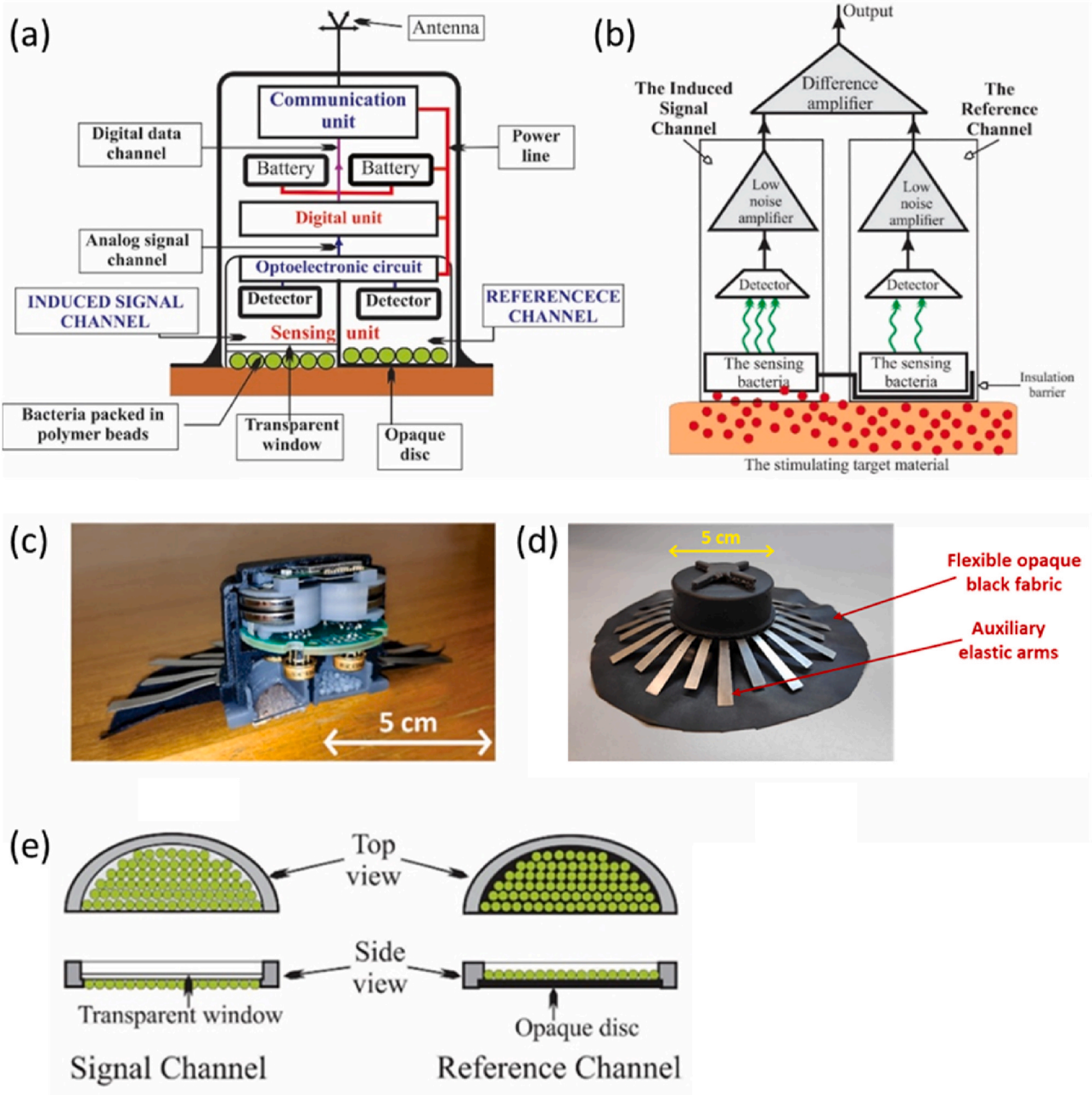

Fig. 1. The biosensor module (BSM). (a) A schematic illustration of the BSM. **(b)** A schematic illustration of the "two channels" detection scheme. **(c)** A BSM cut into two, allowing a view of the internal components, including the printed circuit board (PCB) containing the OE circuit with the photodetectors and the differential amplifiers. **(d)** The complete BSM, showing its 'dark compartment'. **(e)** A schematic illustration of the two half circles of the bead cassette.

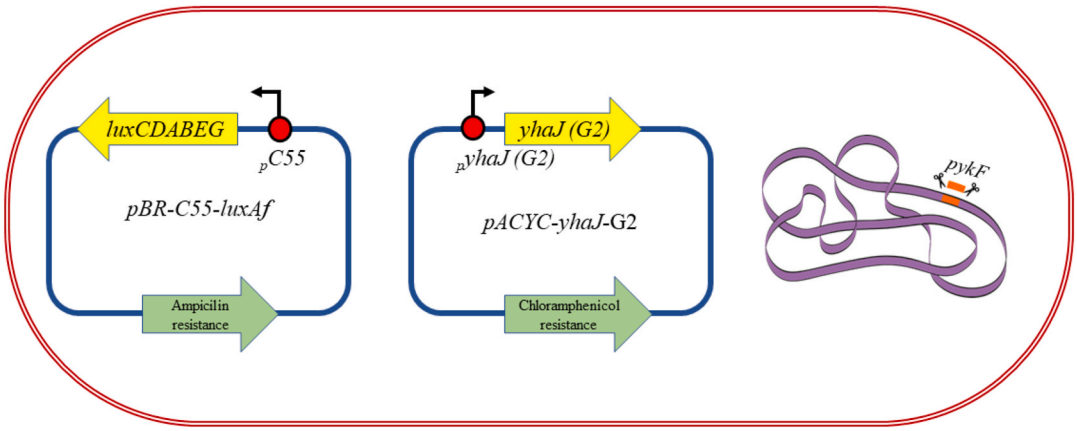

Fig. 2. Schematic design of the biological sensing-reporting element. The design consists of an *Escherichia coli* strain harboring a chromosomal deletion of the *pykF* gene, an activator plasmid containing an enhanced version of the *yhaJ* activator, and a sensor-reporter plasmid harboring a fusion between the *yqjF* DNT-sensitive gene promoter (version C55; Shemer et al., 2020) and the *luxCDABEG* cassette of *A. fischeri*.

concentrations present above buried landmines. The *E. coli* sensor strain thus employed in the present study was strain BS02, described in detail by (Shemer et al., 2021). In this strain, several components in the detection scheme were enhanced compared to the sensor described by (Yagur-Kroll et al., 2014): (i) the promoter region was replaced with an improved version, obtained by repeating cycles of random mutagenesis (Yagur-Kroll et al., 2015); (ii) the host strain was replaced with an *E. coli* strain harboring a deletion of the *pykF* gene (Shemer et al., 2020); (iii) a plasmid carrying an enhanced version of the *yhaJ* gene and promoter, obtained by repeating cycles of random mutagenesis and encoding an activator of the native *yqjF* promoter (Palevsky et al., 2016) was transformed into the host cell in parallel to the plasmid carrying the promoter-reporter elements (Elad et al., in preparation); (iv) the *P. luminescens luxCDABE* reporter cassette was replaced with the *luxCDABEG* cassette of *Aliivibrio fischeri*, enabling superior responses at temperatures relevant for environmental monitoring (15 °C – 25 °C) (Shemer et al., 2020). These modifications have significantly enhanced the bioreporter's detection capabilities in terms of both signal intensity and detection threshold.

To supply the reporter bacteria with a suitable environment for supporting their viability and luminescent activity, the reporter strain was immobilized in an alginate-based matrix, enabling rapid diffusion of tracer explosives, nutrients, and oxygen, and maintaining a moist environment required for optimal activity. Alginate is transparent to light in the wavelength range of the emitted bioluminescence (490*nm*± 100*nm*), enabling high bioluminescence transmittance. The alginate was amended with polyacrylic acid (PAA), previously shown (Shemer et al., 2021) to improve bead water retention and to lower DNT detection threshold.

Bacterial growth conditions and immobilization procedures were as described by (Shemer et al., 2021). A sodium alginate (CAS 9005-38-3, Sigma-Aldrich, Israel) solution was stirred until all the alginate was dissolved, and allowed to settle overnight at room temperature to remove air bubbles and ensure full dissolution. A filter-sterilized (0.22 μm BiofilTM PVDF filter, Porvair Filtration Group Ltd, UK) 10%, pH 7, PAA solution (CAS 9003-01-4, MW ~250,000, Sigma-Aldrich, Israel) was added to the fully dissolved alginate to a final concentration of 1% and stirred to full homogenization. BS02 bacteria were grown overnight at 37 °C with shaking (200 rpm) in lysogeny broth (LB), diluted x1/50 in 1 L of fresh LB, and grown under the same conditions to an optical density at 600 nm (OD600) of 0.8–1.0. The culture was then centrifuged (Sorvall Lynx 6000, Thermo Scientific, 7000 rpm, 4 °C) for 40 min, the supernatant was discarded, and the pellet was washed and resuspended in sterile saline (0.9% w/v NaCl). An appropriate volume of the resuspended bacteria was added to the sodium-alginate-PAA solution, and stirred gently to ensure homogeneity. Immobilization of the bacteria in alginate beads was performed by dripping the bacteria-alginate-PAA solution into a gently stirred CaCl2 (0.1 M) solution, using an automated Büchi B-390 encapsulator (Büchi Labortechnik AG, Switzerland). The beads formed upon contact with the Ca2+ 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. The alginate beads used in this study were ca. 1.5 mm in diameter and harbored $1.5 \times 10^5 \pm 0.3 \times 10^5$ cells per bead, a cell density previously shown to be optimal (Shemer et al., 2021). Larger diameters beads were found to be suboptimal, probably due to lower surface-to-volume ratios that lead to reduced levels of oxygen, an essential component of the bioluminescent reaction (Brodl et al., 2018). Beads with diameters lower than 1.5 mm proved difficult to handle due to increased cohesiveness.

2.3. The beads cassette

The 'Induction' cassette is loaded at the bottom of the BSM and, upon deployment, brings the bioluminescent sensor bacteria into a direct contact with the ground, maximizing the light reaching the detectors. The beads are glued to the bottom face of a thin transparent film, so that upon deployment of the BSM the beads are in direct contact with the ground and the light they emit pass through the transparent backing film. A special purpose pressure-sensitive adhesive, with high adhesiveness to hydrophilic surfaces (Feldstein et al., 2015), was prepared with three requirements in mind: (i) Strong adherence of the beads to their substrate in a liquid environment; (ii) Optical transparency; (iii) Full biocompatibility. The preparation protocol is based on a mixture of high molecular weight poly (N-vinyl pyrrolidone) (PVP) with polyethylene glycol (PEG), at a 64:36 wt% ratio in ethanol. A 50 μm transparent adhesive layer was prepared on the bottom of the 'Induction' cassette by casting-drying. Its adherence to the beads was found to be very good, and the bioluminescent activity of the bacterial sensors was not affected by the adhesive.

2.4. The optoelectronic circuit

The optoelectronic (OE) circuit in the sensing unit detects the optical signals emitted by the bacteria in the induced and reference channels, and produces an analog electronic signal that is proportional to the difference between them. The OE circuit is designed to exhibit a high signal-to-noise ratio at low input light levels (i.e. in the pW range), centered around the wavelength at which the bioluminescence emission is maximal (~490 nm). Its design adopts the concept of "differential

architecture", sometimes referred to as "balanced architecture" (Vasilescu, 2005) (Fig. 3a). The circuit detects simultaneously but separately the optical signals generated by the bacteria in each channel ($I_{induced}$ and $I_{Reference}$ in Fig. 3a), amplifies them, and produces an analog electric signal that is proportional to the difference between the two (I_{Output} in Fig. 3a). The detectors used were silicon photodiodes integrated with a front-end operational amplifier (model OPT301, *Texas Instruments*). These detectors were chosen due to their low dark current, high sensitivity, and good responsivity at the bioluminescence emission wavelength range. The differential amplifier (DA in Fig. 3a) is a low-noise amplifier designed for amplifying very weak currents (model INA106, *Texas Instruments*). Each module was equipped with a printed circuit board (PCB) containing two OE circuits, so that each module harbored two identical sensors (A and B) operating in parallel. This enabled the simultaneous monitoring of the induced and reference signals as well as the output signal in both sensors. The signals $I_{Induced}$, $I_{Reference}$, and I_{Output} of the BSMs were sampled simultaneously by a Keithley multichannel voltmeter (Model DAQ6510, Data Acquisition and Logging Multimeter System) enabling simultaneous measurement of 80 channels sensitively and with high accuracy. An image of the PCB with the two OE circuits (A and B) is presented in Fig. 3b, and the alignment of the PCB to the half circles of the cassette in Fig. 3c. As may be observed, the PCB is placed above the bead cassettes, so that photodiodes PDA1 and PDB1 detect the light emitted by the half circle of the induced channel, whereas photodiodes PDA2 and PDB2 detect the light emitted by the half circle of the reference channel. Thus, the two OE circuits (Circuits A and B in Fig. 3b) can perform simultaneously two independent measurements of $I^{A,B}_{Induced}$, $I^{A,B}_{Reference}$, and $I^{A,B}_{Output}$.

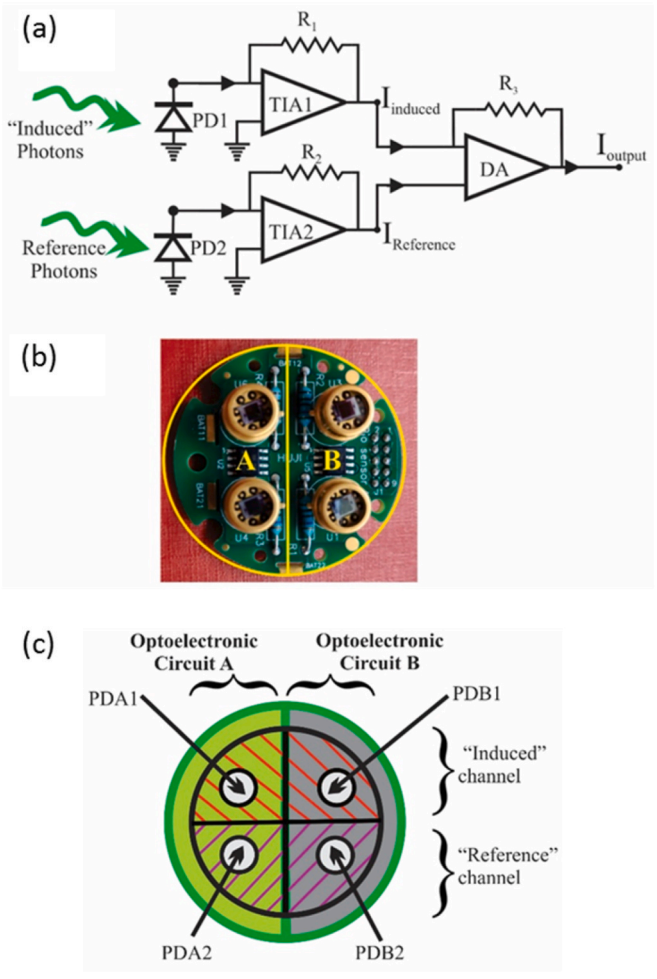

Fig. 3. The optoelectronic circuit of the BSM. (a) Schematic illustration of the optoelectronic circuit containing the photodiodes (PD1 and PD2), the transimpedance amplifiers (TIA1 and TIA2), the feedback resistors (R_1 and R_2), and the differential amplifier (DA) with its feedback resistor (R_3); **(b)** An image of the printed circuit board installed in each module. Each printed circuit board contains two OE circuits (A and B) operating in parallel; **(c)** Alignment of the printed circuit board with the two half-circle bacterial cassette.

3. Results and discussion

The BSM underwent a series of tests, carried out in a temperature-, humidity- and illumination-controlled chamber. First, the OE circuits were tested by placing the module on top of a tunable LED light source, exposing the OE circuit to light intensities similar to the typical bioluminescence emitted by the bacterial sensors, between 0.1 and 100 nW/cm^2. Preliminary experiments (not shown) yielded a very sensitive and reproducible performance of the BSM in detecting ultra-low intensity light in the spectrum of the bacteria bioluminescence (490 nm ± 100 nm). The respective sensitivity of the BSM was found to be better than sub-pW, an illumination level that is much lower than the typical bacterial bioluminescence.

An essential feature of the OE unit in the BSM is that it should operate in complete darkness, even when deployed outdoors in full daylight. A special 'dark compartment' was thus designed, as described in Material and Methods. The effectiveness of the dark compartment in preventing ambient illumination from penetrating the BSM was tested by placing the module on top of different types of terrain, under ~1000 lux illuminance, equivalent to midday conditions in an overcast day. The tests were carried out on sea sand, as well as on three rough surfaces prepared by spreading three types of stones over sand, as shown in Fig. 4: (a) small (~1 cm) white stones; (b) medium (~3 cm) black stones; and (c) large (~6 cm) white stones. The performance of the BSM's dark compartment was measured at least 50 times for each surface, at different locations and orientations of the BSM.

The illumination levels measured inside the BSM are summarized in Table 1, and indicate that the dark skirt created sufficient darkness in the inner space of the module in all tested terrain types.

These results show that background illumination is effectively blocked from penetrating into the optoelectronic sensing unit of the BSM by the 'dark compartment'. The ratio between the light intensity measured inside the BSM to the outside background illumination was in the range of 10^{-11} to 10^{-7}. For the sand surface, and for the small and medium stone surfaces, the level of background luminescence that penetrated the BSM was negligible compared to the minimal intensity of the bacterial bioluminescence, which is at least 100 pW per cm^2. As for the large stones surface, the optical signal penetrating into the BSM was in the range of tens of pW/cm^2, close to the minimal bioluminescence intensity of the bacteria. However, as bacterial bioluminescent intensity increases over time to values that are significantly higher than that, the BSM may be used in daylight, even on relatively rough surfaces.

In the next series of measurements, a network of six BSMs was sampled in parallel to detect minute concentrations of DNT. Each BSM was placed on top of a sand layer containing a different quantity of DNT, ranging from 0.25 mg to 3 mg per Kg sand. The signals $I_{Induced}$, $I_{Reference}$, and I_{Output} of the BSMs were sampled simultaneously, and the results are presented in Fig. 5a–e. The measurements were carried out at 25 °C and a 30% humidity. Bioluminescence evolution in response to a single DNT concentration (3 mg per Kg sand) is presented in Fig. 5a, demonstrating that the module's two independent sensing circuits yielded almost identical results. Fig. 5b and c depict the response to lower DNT concentrations. A clear detection of 0.25 mg DNT per Kg soil is demonstrated. Fig. 5d and e plot the maximal responses as a function of DNT concentration for a broader DNT concentration range. The lower responses to DNT levels above 3 mg/kg indicate that these concentrations are probably deleterious to the sensor bacteria.

In the experiments the results of which are described in Fig. 5g, the

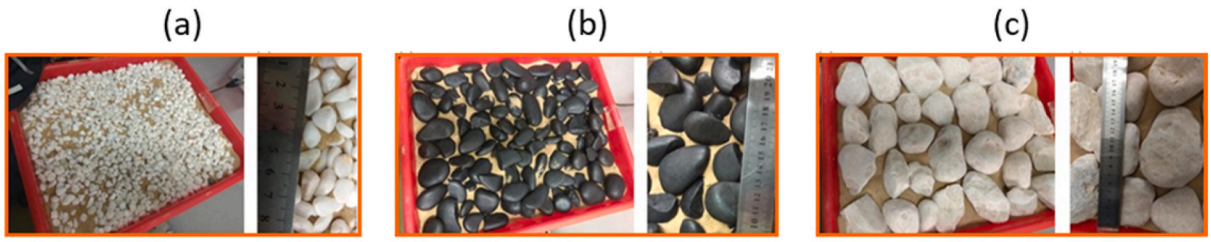

Fig. 4. Test surfaces for the effectiveness of the BSM 'dark compartment'. **(a)** small [~1 cm] white stones; **(b)** medium [~3 cm] black stones; **(c)** big [~6 cm] white stones.

Table 1
Evaluation of the effectiveness of the dark 'skirt' on different terrains. The table shows the illumination measured inside the BSM by the optoelectronic sensing unit.

Ground surface	Illumination recorded by the BSM [fW/cm^2]	Ratio between the detected signal and ambient illumination	STD [%]
Sea sand	3	10^{-11}	90
Small white stones (av. diam. ≈ 1cm)	300	10^{-9}	70
Medium black stones (av. diam. ≈ 3 cm)	1500	$5{\cdot}10^{-9}$	40
Large white stones (av. diam. ≈ 6 cm)	60,000	$2{\cdot}10^{-7}$	20

DNT was not mixed with the sand but buried under it, 12 months prior to the experiment, as a disc-shaped lump, with a diameter of either 5 cm (5 g DNT buried 2.5 cm or 5 cm below the surface) or 10 cm (50 g DNT buried 8 cm below the surface). These measurements were carried out at 20 °C, at a 30% humidity. In all cases of the buried DNT targets, a bioluminescent response became evident within 2–4 h, peaking after ca. 5–10 h. The results shown in Fig. 5 were selected from a large collection of experiments in which 10 prototypes were tested, using bacteria from different batches. All the measurements indicated that under similar conditions, different BSMs yield similar performance.

These results demonstrate the feasibility of monitoring the presence of chemicals on the surface of the ground by a network of autonomous biosensor modules, based on genetically engineered bacteria that luminesce in the presence of their target compounds. The modules are designed to be distributed over large areas, providing real time information as to the location of hazardous chemicals or other targets of interest, obviating the need for operating teams to enter the suspected areas. They can be easily retrieved from the examined area and loaded with fresh bacteria enabling repetitive deployment.

While the experiments conducted during this study represent relatively optimal conditions for bacterial luminescence, the effect of environmental conditions should be further addressed, in view of the fact that the BSMs are designed to be deployed outdoors. For that purpose, the BSM may contain advanced microbial sensor strains, enabling high activity across broader temperature and humidity ranges (Shemer et al., 2021). As the BSM has been designed for repeatable operation, interchangeable bead cassettes can be loaded, selected according to local conditions and to the target analyte(s). It should be noted that while all experiments presented in this article were conducted on sand, the activity of such bacterial sensors has been successfully demonstrated on more complex soils (Belkin et al., 2017).

The DNT detection sensitivity demonstrated in this article is 0.25 mg DNT per Kg sand. This value is in the range of the typical DNT soil concentrations above landmines (Cumming et al., 2001; Jenkins et al., 2001). To the best of our knowledge, this is the highest reported bacterial-based detection sensitivity of DNT in soil. Burlage (Burlage et al., 1999), the first to present the principle of detecting landmines using genetically engineered bacteria, claimed a proof of concept for TNT bio-detection, but no detection threshold was specified. Neither did all follow-ups since then, in which schemes for bio-detection of buried landmines have been suggested, quantify detectable concentrations of explosive materials in soil.

In a separate study (Shemer et al., 2021), we have also studied the bioluminescent response of the bacterial bioreporters in liquid assays, and have demonstrated a detection limit of ca. 40 nM DNT. For comparison, the first reported DNT bio-detection threshold using bioluminescent\fluorescent *Pseudomonas putida* sensors was 2 mM (De Las Heras et al., 2008; De Las Heras and de Lorenzo, 2011; Garmendia et al., 2008). The reported bio-sensitivity for other explosive materials was in the same order of magnitude. For example, the *E. coli* based bioreporter for nitrite or nitrate (as a proxy for N-based explosives) reported 12 mM or 2.5 mM values, respectively (Kim et al., 2008). Using *Saccharomyces cerevisiae* the DNT detection threshold was improved to 25 μM (Radhika et al., 2007), and 5 years later a similar sensitivity was achieved by using *E. coli*-based fluorescent biosensors: 50 μM (Davidson et al., 2013) and 10 μM (Lönneborg et al., 2012). Comparable fluorescent bioreporters for TNT or DNB manifested similar sensitivities of 21 μM or 28 μM, respectively (Tan et al., 2015). The most sensitive bioreporter reported to date exhibited a sensitivity of 2.2 μM TNT, using *Dictyosphaerium chlorelloides* (Altamirano et al., 2004). The detection threshold reported by our microbial bioreporters is thus ca. 50-fold lower than that of the most sensitive bacterial sensor for explosive materials reported earlier.

4. Conclusions

We provide in this paper a proof of concept for a versatile and generic optoelectronic BSM in which genetically engineered bioluminescent bacteria serve as the core sensing elements.

The BSM described above was developed for detecting buried landmines. For demonstrating this application, the bacteria were genetically engineered to respond to DNT, the presence of which in the soil surface indicates the presence of a buried TNT-based landmine. The performance of the BSM was demonstrated by detecting trace concentrations of DNT mixed with soil, or DNT lumps buried below the surface. The BSM was able to detect traces of DNT down to 0.25 mg DNT per Kg soil, and also by detecting the presence of DNT buried several centimeters deep into the ground.

It should be emphasized that beyond its importance in addressing the tremendous humanitarian problem of detecting landmines and other explosive devices, the BSM bears significance as a benchmark for the whole concept of bacterial-based optoelectronic sensors and sensor networks. As such, it serves as a proving ground for a chemical sensing methodology for outdoor operation. This relates in particular to the concept of employing live bacteria to operate as a minuscule chemical laboratory acting as the sensing entity in an optoelectronic circuit, where they operate in unison with standard optoelectronic devices. The fact that the bacteria can be genetically engineered to respond to practically any chemical target, renders this sensing methodology to be

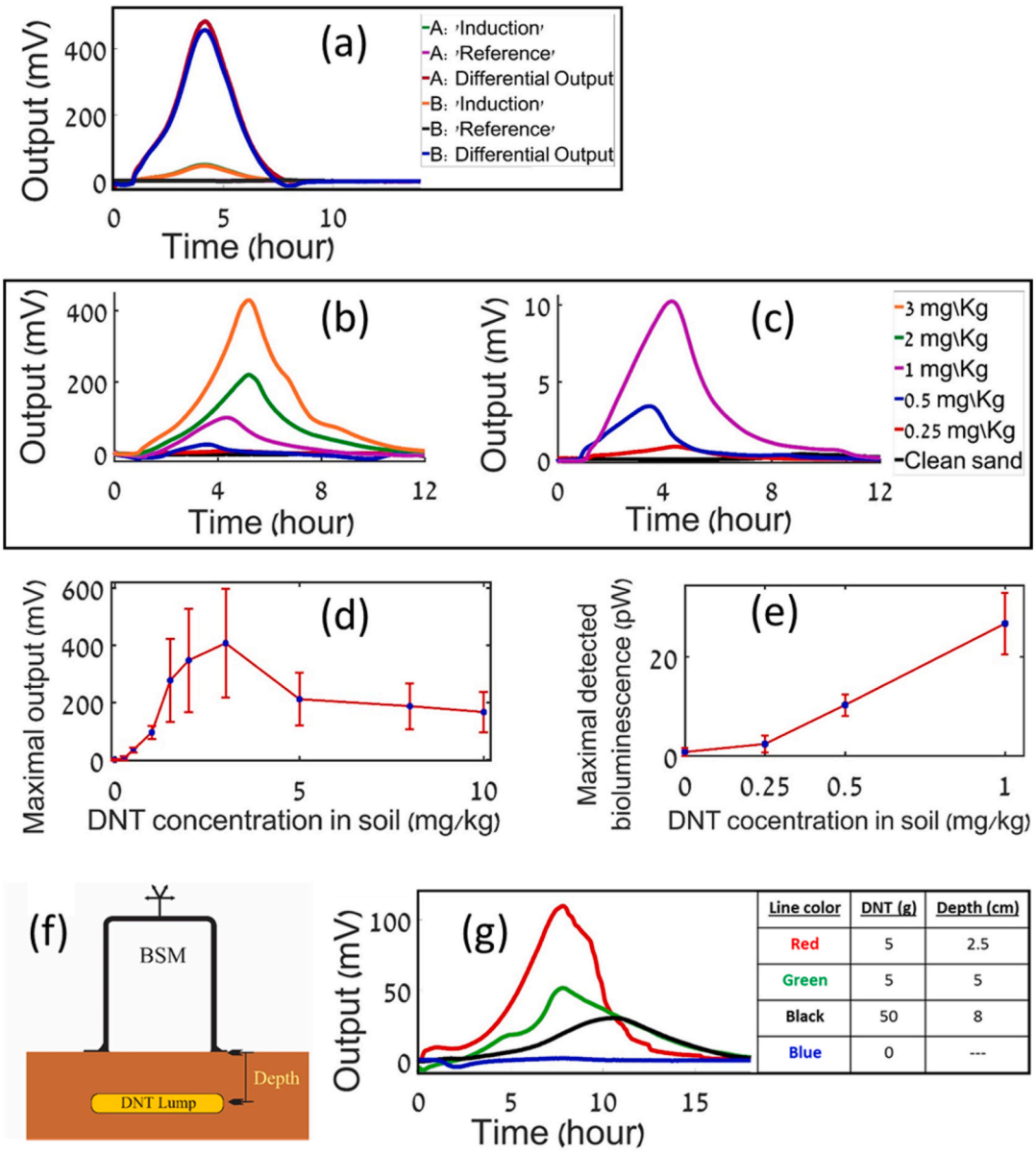

Fig. 5. BSM performance in detecting low concentrations of DNT mixed or buried in soil. (a) All six output channels of a single BSM unit positioned on a sand/DNT mixture (3 mg DNT per Kg sand). The output of sensing circuits A and B is almost identical. **(b)** Differential outputs for a network of BSMs positioned on mixtures of sand with DNT, ranging from 0 mg to 3 mg DNT per Kg sand. **(c)** Induced channel output for BSMs positioned on mixtures of sand with the low DNT concentrations. **(d)** Maximal differential output vs. concentration of DNT in soil. Error bars represent the deviation between different experiments conducted at different dates under the same conditions. **(e)** Maximal detected bioluminescence vs. DNT concentration (low range). **(f)** Illustration of the measurement setup of DNT buried in sand. **(g)** Differential outputs for BSMs positioned on lumps of DNT buried at different depths.

inherently versatile and generic. In essence the same optoelectronic system built for measuring bioluminescence can be adapted to detect the presence of any selected TM simply by loading it with a different type of bacteria. Moreover, the BSM possess a unique set of features which together enable its use in versatile configurations. These features include in particular: (i) Bacteria longevity is sustained by the water and nutrients stored in the hydrogel beads; (ii) The BSM can be operated repeatedly by repeated refreshing and reloading of the bead cassette; (iii) The BSM is built to be operated in the field in daylight illumination; and (iv) The BSM is built for autonomous operation due to a built-in power source and a communication unit.

The BSM can be used either as a standalone autonomous unit, or as a node in a sensor network. For the latter option, the BSM was designed to be compact and light, constructed of off-the-shelf standard electronic components, thus making it cost effective for mass production, ready for simultaneous deployment in large numbers using drones. In such a network, an ensemble of BSMs deployed outdoors and operating in concert, will map the presence of the TM across large inspected areas. In fact, the sensor ensemble can contain BSMs loaded with different types of bacteria, so that the sensor network will provide simultaneously information relating to the presence of a collection of TMs in the inspected area.

In summary, the combination of the variability of the core sensing operation of the bacteria with the unique features of the sensing module brings together three complementary elements: (i) A sensing methodology tailored for sensing the presence of target chemicals by genetically engineered bacterial bioreporters; (ii) A module configured for optimal operation outdoors; and (iii) The deployment of the module as a node in an outdoor sensor network. These render the BSM a highly versatile sensing tool that can be used for different purposes, be it monitoring

pollutants and other hazardous materials in the environment, monitoring the distribution of fertilizers in farmlands, or a fast 'deployment force' for providing real time mapping of accidental spillage events.

CRediT authorship contribution statement

Aharon J. Agranat: Conceptualization, Methodology, Writing – original draft, Supervision, Funding acquisition. **Yossef Kabessa:** Conceptualization, Methodology, Validation, Writing – original draft, Visualization, Supervision, Project administration. **Benjamin Shemer:** Methodology, Investigation, Writing – original draft. **Etai Shpigel:** Methodology, Investigation, Project administration. **Offer Schwartsglass:** Conceptualization, Methodology, Supervision. **Loay Atamneh:** Investigation, Validation, Visualization. **Yonatan Uziel:** Investigation, Validation. **Meir Ejzenberg:** Resources, Validation. **Yosef Mizrachi:** Validation. **Yehudit Garcia:** Resources. **Galina Perepelitsa:** Resources. **Shimshon Belkin:** Conceptualization, Methodology, Writing – original draft, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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