5 Microbial Indicators for Monitoring Pollution and Bioremediation

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5.1 Introduction

Microorganisms metabolically rely upon chemicals scavenged from their environment and correspondingly defend themselves against toxic chemicals to ensure their growth and survival. These essential needs can be genetically exploited to create bioreporter organisms that can rapidly and specifically indicate the presence and bioavailability of target environmental contaminants. Using the toolbox of genetic engineering, genes or their operon units that are activated upon exposure to a target chemical agent or chemical class can be isolated and linked to reporter genes to serve as operational switches that bioindicate chemical presence with sensitivity and reproducibility that oftentimes rivals conventional analytical methods. Reporter genes most often consist of light signaling elements such as the bioluminescent bacterial (*lux*) and firefly (*luc*) luciferases, green fluorescent protein (*gfp* as well as its palette of multicolored fluorescent derivatives), and colorimetric indicators like β-galactosidase (*lacZ*) (Close et al., 2009). These reporter genes are widely available within numerous types of cloning vectors for straightforward manipulation and have been successfully applied toward the creation of a large number of bioreporter organisms. The challenge in bioreporter development, however, lies in discovering and isolating the appropriate promoter gene switch that activates in response to the desired chemical target or targets. Although often provided via the evolutionary adaptation of microbial populations to new chemical bioavailability, particular promoter elements still must be isolated and characterized, which requires significant time, effort, and cost. Additionally, what nature supplies is not always optimal: sensitivity and specificity may be lacking. Synthetic biology addresses some of these issues by providing the tools necessary to modify and strengthen promoters for improved and more tailored sensing capacities and effectively complements directed evolution methods to create wholly new chemical-responsive genetic architectures

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that nature has not yet designed (van der Meer and Belkin, 2010; Checa et al., 2012; Cobb et al., 2013). Notwithstanding this ability to create a new generation of upgraded bioreporters, decades of past bioreporter development and unique applications testify to their utility as successful monitors for numerous environmental contaminants (Trogl et al., 2012; Xu et al., 2013).

5.2 Choosing a Whole Cell Bioreporter

Bioreporters are typically designed with either a "lights-off" or "lights-on" signaling mechanism. A lights-off bioreporter is built by linking the reporter gene to a constitutive genetic element that remains activated as long as the cell is healthy, and thus this type of bioreporter emits its bioluminescent, fluorescent, or colorimetric light signal continuously. Upon exposure to a chemical or chemical mixture that harms the cell or disrupts cellular metabolism, an analogous reduction in reporter signal intensity, or a lights-off response, occurs that correlates to the degree of toxic interaction. Although these bioreporters do not specifically identify the chemical(s) affecting the cell, they clearly indicate that there is a toxic, biologically relevant interaction occurring, and pre-alarm to the need for further downstream analyses to identify the chemical(s) of concern. The classic lights-off bioreporter is exemplified in the Microtox® assay, where bioluminescence from *Vibrio fischeri* now reclassified as *Aliivibrio fischeri* (Urbanczyk et al., 2007) bacteria is quantified after exposure to a dilution series of an allegedly toxic sample (Hermens et al., 1985). A resulting decrease in bioluminescence denotes toxicity, with subsequent bioluminescent measurements being correlated to relative levels of toxicity expressed as an EC_{50} value (a concentration at which a 50% reduction in light is produced).

In a lights-on bioreporter, the microorganism is designed to remain "dark" until it encounters its designated target(s), upon which it emits a light signal. These bioreporters rely upon a fusion between the reporter gene and an inducible promoter that activates after interacting with the designated target(s). Activation can be designed to occur either specifically or nonspecifically. Under specific activation, the promoter/reporter gene fusion directly associates with a particular chemical or class of chemicals, with the intensity of subsequent signal generation exhibiting proportionality to the bioavailable portion of the chemical. This ability to measure bioavailability rather than strictly the total amount of chemical present in the sample is a key characteristic of bioreporters. Being living entities, bioreporters are capable of describing that portion of the chemical that is freely available to cross the cellular membrane—i.e., the bioavailable portion—and therefore denote the biological effect of a chemical rather than the mere presence or absence of a chemical as is defined using analytical measurement techniques like gas chromatography/mass spectrometry (GC/MS). However, when using bioreporters, one must be careful in the interpretation of bioavailability since its measurement is heavily influenced by the bioreporter's physiological status, growth rate, membrane

composition, membrane transport mechanisms, and many other factors that shift in tandem with myriad environmental influences (Harms et al., 2006).

A lights-on bioreporter designed to be nonspecific uses a promoter/reporter gene complex whose promoter is activated in the presence of a general class of chemicals that can be identified as affecting certain cytotoxic, genotoxic, mutagenic, or stress-related cellular pathways. Thus, although the chemical itself cannot be specifically identified, the chemical's effect on a living system can be described within certain specifications that assist in delineating and defining exposure risk profiles.

The conventional inventory of reporter genes and their advantages and disadvantages are discussed below. The reader is directed to several detailed reviews for more in-depth information (Girotti et al., 2008; Diplock et al., 2010; Ripp et al., 2011; Shin, 2011; Struss et al., 2010; Xu et al., 2013).

5.2.1 Bacterial Luciferase (lux)

Bioluminescence is defined as light generated and emitted by a living organism using luciferin and a luciferase enzyme. Luciferase has been found in a number of species including bacteria, jellyfish, fungi, algae, insects, shrimp, and squid. The bacterial luciferase (*lux)* gene cassette contains a series of five genes—*luxA*, *luxB*, *luxC*, *luxD*, and *luxE*—that bioluminesce by producing a blue-green 490 nm light signal when expressed (Meighen, 1994). The gene system is commonly referred to as *luxCDABE*, which corresponds to the order of the genes in the operon. Expression of *luxA* and *luxB* results in a heterodimeric luciferase enzyme that catalyzes the light-emitting reaction by converting the long-chain aldehyde synthesized by the *luxC*, *luxD*, and *luxE* gene products to a carboxylic acid. The other substrate for this reaction is a reduced flavin mononucleotide ($FMNH_2$), which becomes oxidized to flavin mononucleotide (FMN) by the luciferase enzyme as the reaction proceeds. The bioluminescent reaction cannot progress in the absence of oxygen and, therefore, bioreporters that incorporate the gene cassette *luxCDABE* are to date restricted to aerobic conditions. Depending on the desired application, it is possible to engineer a bioreporter to contain either the *luxAB* or the *luxCDABE* gene cassettes. The *lux* genes have additionally undergone a codon optimization process that allows for their expression under eukaryotic genetic controls. This permits broader application of bioluminescent bioreporters beyond bacteria and more toward yeast and human cells that ultimately serve as better proxies for describing a chemical's true effect on higher animal life forms.

5.2.1.1 luxAB

A *luxAB* bioreporter harbors only the *luxA* and *luxB* genes from the *luxCDABE* gene cassette. This allows the bioreporter to generate the luciferase enzyme but, due to lack of *luxCDE*, requires that the aldehyde, commonly *n*-decanal, be added exogenously. One advantage of this method of integration is that it allows the saturation of the reaction with substrate, which results in a stronger, brighter bioluminescent signal. However, this makes time point data contingent upon the addition

of aldehyde substrate, which could cause bioluminescent signals to be missed due to early or late addition of the aldehyde. Some studies have incorporated the aldehyde substrate into a growth medium to allow for continuous availability. Working with the fairly small 2.1 kb *luxAB* genes also simplifies the cloning process and numerous such bioreporters have been created in bacterial, yeast, and mammalian cells and used for various bioassay applications in the environment such as the detection of inorganic and organic compounds (Ripp et al., 2011; Xu et al., 2013).

5.2.1.2 *luxCDABE*

The *luxCDABE* bioreporter contains all elements of the *luxCDABE* gene cassette, thus permitting the organism to bioluminesce autonomously, independent of substrate or cofactor addition. This type of monitoring allows for real-time detection and has become popular in water quality and other environmental applications. *luxCDABE*-based bioreporters are particularly useful in biosensor applications where they are mated to transducers designed to monitor the light signal (Su et al., 2011). With no user interaction required, remote and deployable long-term biosensor surveillance of desired environments becomes feasible. Synthetic optimization of the *luxCDABE* gene cassette has permitted it to move away from its native AT-rich requirements towards GC-rich microorganisms, creating a wider availability of microbial hosts and allowing for a better complement of bioreporter hosts to test environments (Craney et al., 2007). At 7 kb, the size of the *luxCDABE* cassette does impede some cloning procedures but commercial cloning kits designed for larger size gene integrations have for the most part alleviated these problems. A unique application of *luxCDABE* biosensing has been its assimilation into the DuPont LuxArray, which represents a genome-wide collection of 689 *Escherichia coli* bioreporters that contain unique promoter regions linked to the *luxCDABE* (Van Dyk et al., 2001). The LuxArray allows for analysis of the transcriptional response of a cell to its environment and has the ability to collect kinetic data by measuring bioluminescence continuously over time.

5.2.1.3 *Eukaryotic Optimized* luxCDABE

The *luxCDABE* reporter gene cassette, although it originated from and has been used predominately in bacterial hosts, has also been optimized for expression in eukaryotic organisms to provide a bioreporter system that more accurately mimics human bioavailability with respect to human toxicity concerns. The major challenges for functional bioluminescent expression in mammalian cells were the low translational efficiency of bacterial gene sequences and the inability to polycistronically express multiple genes from a single promoter in the eukaryotic background. Thanks to the process of codon optimization and the utilization of unique genetic elements such as the internal ribosomal entry site (IRES) element (Baird et al., 2006) and the viral 2A element (Szymczak and Vignali, 2005), the *luxCDABE* cassette has been successfully expressed in the lower eukaryotic organism *Saccharomyces cerevisiae* (Gupta et al., 2003) and human cell lines (Close et al., 2010) for autonomous bioluminescent

emission. It has also been discovered during the development process that co-expression of a sixth gene, *frp*, which encodes a flavin mononucleotide oxidoreductase, is required for optimized light production in eukaryotic host cells (Gupta et al., 2003; Close et al., 2010). The Frp protein recycles and regenerates $FMNH_2$, which appears to be limited in the eukaryotic cellular background. Autonomously bioluminescent yeast bioreporters for estrogenic and androgenic compounds have been developed using the *luxCDABEfrp* genes with demonstrated dose-responsive bioluminescent signaling occurring within 3−4 h (Sanseverino et al., 2005, 2009; Eldridge et al., 2007; Eldridge et al., 2011).

5.2.2 Firefly Luciferase (luc)

The *luc* genes were originally identified and isolated from the firefly *Photinus pyralis.* This reporter gene has become popularized due to the high output of yellow/green light (550−575 nm) upon oxidation of the reduced luciferin substrate in the presence of ATP-Mg^{2+} and oxygen. The *luc* genes also harbor the greatest quantum yield of current bioluminescent systems (Fraga, 2008). Since posttranslational modifications are not needed for the Luc protein, it is made accessible immediately following translation; however, exogenous addition of the luciferin substrate is required for the ensuing light reaction. Thus, similar to the *luxAB* reporter system, *luc*-based bioreporters emit light discontinuously and are impeded from functioning autonomously. Nonetheless, with their robust signal strength, small size (1.7 kb), and wide availability of cloning vectors, a large variety of *luc*-based bioreporters have been constructed for the detection of environmentally relevant organic compounds, heavy metals, and estrogenic and endocrine disrupter agents (Xu et al., 2013).

5.2.3 Green Fluorescent Protein

Green fluorescent protein (GFP) is part of a family of natural and recombinant photoproteins isolated and cloned from the jellyfish *Aequorea victoria.* The activation of GFP does not require a substrate; instead, bioreporters that contain GFP emit a fluorescent signal at 508 nm upon exposure to ultraviolet or blue light. Consequently, GFP-based bioreporters must always be tied to instrumentation capable of delivering the excitation wavelength and measuring the resulting emission wavelength. Nonetheless, the ability to autofluoresce allows for near real-time sensing, which makes GFP bioreporters an effective method of assessment for monitoring environmental conditions. The small size of GFP (∼750 bp) also facilitates straightforward cloning. Besides GFP, there are other fluorescent variants that emit in the cyan, red, yellow, and orange wavelengths (Shaner et al., 2005). With this selectable palette of colors to choose from, bioreporters capable of sensing multiple analytes can be developed by linking individual promoters to differently colored fluorescent reporter gene outputs. For example, Hever and Belkin (2006) created fusions of the stress-responsive *recA* and *grpE* promoters to green and red fluorescent reporter protein genes, respectively, to create a dual reporter for simultaneous monitoring of both toxic and genotoxic events. Dual bioreporter systems that

combine fluorescence with bioluminescence can also be created, as demonstrated by Mitchell and Gu (2004) in their similar work linking the stress-responsive *recA* and *katG* promoters to GFP and *luxCDABE*, respectively.

5.2.4 lacZ

The *lacZ* gene originates from *E. coli* and encodes β-galactosidase (β-gal), which hydrolyzes β-galactoside disaccharides into monosaccharides. In the presence of o-nitrophenyl-β-D-galactoside (ONPG), *lacZ* bioreporters yield a yellow by-product that can be measured colorimetrically. Color intensity corresponds to the β-gal activity and provides an estimate of the chemical concentration. For example, the SOS Chromotest is a commercially available kit that utilizes *lacZ*-based fusions to DNA damage-sensitive reporters to detect genotoxic compounds (Quillardet et al., 1982). One disadvantage to colorimetric analysis is low sensitivity, which has resulted in the development of new detection methods, such as the incorporation of *lacZ* bioreporters with different β-galactoside substrates that allow for fluorescent, luminescent, or chemiluminescent endpoints. Another drawback to *lacZ* bioreporters is the required lysis of reporter cells to allow quantification of β-gal activity, which causes delayed and discontinuous accumulation of results. This issue has been overcome with the introduction of electrochemical and amperometric interfaces to bypass permeabilization steps (Ron and Rishpon, 2010). For environmental analyses, it is important to account for the endogenous presence of β-gal in sample matrices and its high background activity potentially interfering with endpoint measurements.

5.3 Applying the Bioreporter as a Pollution Monitoring and Bioremediation Tool

The environmental application of bioreporters is a challenging prospect due to their genetically engineered nature and the consequent regulatory boundaries that limit their use outside the confines of a laboratory. Most applications of bioreporters, therefore, rely upon transport of the sample to the laboratory where they can be appropriately processed and then integrated into the bioreporter assay. However, bioreporters can be environmentally applied if properly safeguarded against uncontrolled release. This typically requires encapsulation or immobilization of the bioreporter into an interface device that receives the sample *ex situ* or, alternatively, *in situ* if used in a flow-through manner directly from the source sample. These biosensor interfaces ultimately form an all-in-one sensing unit that measures and monitors for signal emission by the integrated bioreporter organisms.

5.3.1 Keeping the Bioreporters Alive and Healthy

For sustained, long-term environmental biosensing, the bioreporters must be packaged in a format that maintains their viability such that they are continuously available to

report on their surroundings or can do so as rapidly as possible following a triggered resuscitation event. Encapsulants designed to maintain cells in a viable state for extended durations typically consist of liquid matrices to which the bioreporter culture is added; these then solidify to form sheets, layers, blocks, or discrete beads of cells. Agar, agarose, polydimethylsiloxane (PDMS), polyvinyl alcohol/polyvinyl pyridine copolymer, latex copolymer, carrageenan, polyacrylamide, alginate, polyurethane/polycarbonyl sulfonate, polyvinyl alcohol, and sol−gel silica glass are only a few of the matrices applied as bioreporter encapsulants (Date et al., 2010; Michelini and Roda, 2012). Encapsulation can also be as simple as drying the bioreporters onto a filter surface (Toba and Hay, 2005) or packaging lyophilized (freeze-dried) bioreporters into single-use test vials (Siegfried et al., 2012). The challenge is to retain the bioreporter cells within a microenvironment that contributes to long-term survival while maintaining compatibility with the measuring scheme in terms of optical clarity and minimizing signal interference, since these parameters ultimately affect assay sensitivity. Considering the vast number of biotic and abiotic factors that can impinge upon cellular bioreporter growth, survival, and longevity, effective encapsulation is a daunting challenge that has yet to be adequately solved; there likely will never be a universal matrix for doing so. Nonetheless, many research groups have demonstrated reasonably adequate microorganismal stability from days to months but, unfortunately, seldom under the authentic extreme conditions inherent to real-world environmental exposures. Other researchers have alternatively exploited what nature has already provided and used the innate ability of certain microorganisms to form spores as vehicles for long-term bioreporter storage (Knecht et al., 2011), or cultivated bioreporters in robust biofilm assemblages to enable resilient surface attachment on biocompatible materials followed by encapsulation (Ben-Yoav et al., 2011). Another well-applied option is to couple the bioreporter cells with surface-attached linkers, which typically entails chemical methods to facilitate covalent binding between the surface and functional groups on the cell (Fleming, 2010); nanomaterials have found a unique niche in this process that allows for high cell loading capacities with improved stability and viability (Dai and Choi, 2013). Surface-to-cell linkages can also be fabricated using antibodies targeted to cell surface antigens (Premkumar et al., 2001) as well as bacteriophages (bacterial viruses) that bind to specific cell surface receptors (Tolba et al., 2010).

5.3.2 Integrating Bioreporter Organisms with Biosensor Devices

The biosensor device consists of a merger between the bioreporter organisms and a transducer capable of measuring the endpoint signal emanating from the bioreporters. A simple embodiment of such a biosensor device is a fiber optic cable terminated on one end with encapsulated bioreporters and on the other end with an appropriate signal measurement instrument (Eltzov and Marks, 2010). For example, Ivask et al. (2007) combined *luxCDABE*-based heavy metal-specific bioluminescent bioreporters with a sodium alginate encapsulant that was solidified onto the tip of a fiber optic cable and measured resulting bioluminescent responses with a photomultiplier interface. The primary benefit of fiber optic interfaces is that they can terminate far from the measurement device, thus allowing infiltration of bioreporters into remote areas without

requiring human presence. However, being tied to such an interface raises logistical concerns dealing with power requirements and traversal and subsequent degradation of the signal over extended distances. More compact, all-in-one biosensors can more ideally be fabricated using lab-on-chip technologies where the reporter organisms are directly interfaced with miniaturized transducer units. The bioluminescent bioreporter integrated circuit (BBIC) was an early example wherein *luxCDABE*-based bioreporters were adhered to a 1.5 mm × 1.5 mm complementary metal oxide semiconductor (CMOS) microluminometer for remote sensing of water and airborne chemical contaminants (Ripp et al., 2003; Nivens et al., 2004). The more recent addition of more sensitive silicon photomultipliers and avalanche photodiodes as sensing platforms has increased the utility of these chip-based biosensors (Daniel et al., 2008; Lopes et al., 2012). When combined with microfluidics, true lab-on-chip biosensors can be fabricated to meet a wide variety of sensing needs. This typically entails the formation of on-chip channels fabricated from PDMS with a combination of pumps and valves directing the sample to the bioreporter cells and the transducer interface. Elad et al. (2011), for example, positioned single-photon avalanche diodes (SPADs) paired with *luxCDABE*-based bioluminescent bioreporters above a network of PDMS channels to enable on-line, flow-through surveillance and early warning of water contamination events. A similar on-line system referred to as Lumisens was developed by the Thouand research group wherein a charge-coupled device (CCD) camera was used to capture bioluminescent signals emanating from immobilized or lyophilized *luxCDABE*-based bioreporters localized in multiwell cards or plates after exposure to contaminated water delivered via pumps or microinjectors (Charrier et al., 2011a,b; Jouanneau et al., 2012). With a large number of wells being available within the Lumisens biosensor, multiple bioreporters could be accommodated for replicate and multiplexed sensing in a fairly easy-to-use plug-and-play format. Moving toward even higher level multiplexing are the bacterial sensor arrays consisting of whole genome bioreporters that incorporate hundreds to thousands of reporter gene fusions to globally indicate chem/bio effects across the entire living cell (Elad et al., 2010). Elad and Belkin (2013), for example, linked approximately 2000 *E. coli* K12 promoters to GFP to create a library of reporter cells that, when spotted into microtiter plate wells, were capable of collectively bioindicating a specific "fingerprint" based on each type of chemical exposure event. The previously mentioned LuxArray developed by Van Dyk et al. (2001) provides a similar array interface based on *luxCDABE*-based bioluminescent fingerprint endpoints. The challenge remains in accommodating these thousands of bioreporters on a single miniaturized detector interface, although the proof-of-concept for smaller numbers of bioreporters has been demonstrated using a variety of optical and electrical biochip platforms (Shacham-Diamand et al., 2010).

5.4 Examples of *In Situ* Field Applications

Despite the fact that prokaryotic and eukaryotic whole cell bioreporters for a wide range of pollutants are being continuously developed and refined in the laboratory,

very few bioreporters actually achieve practical application toward conventional on-site monitoring and testing due to logistical challenges of long-term maintenance and viability and mandatory restrictions on the release of genetically engineered organisms. Although advances in cell immobilization and preservation techniques as well as progress made in biosensor interface and device design (see discussions in the previous section) have significantly accelerated the technical development of field-operable biosensor systems, the lack of patent protection of the bioreporters is still a significant obstacle for biosensor devices to reach the market. Nevertheless, biosensor devices intended for on-site applications have been developed in the laboratory as proof-of-concept prototypes, some of which have been tested with real environmental samples either in the field or in laboratory-simulated settings that mimic potential filed applications (Table 5.1). Such devices incorporate various separation or immobilization technologies to comply with mandatory regulations in order to avoid uncontrolled release of recombinant bioreporter cells into the environment. Most on-site surveillance devices are designed for water quality monitoring due to the relative simplicity of prebioassay sample manipulations, while applications of soil testing are limited largely because soil samples often require complicated pretreatments or extractions prior to the bioassays, which have yet to be effectively integrated into the on-site biosensing pipeline.

Some of the early efforts to realize on-site monitoring have utilized bioreporter cell suspensions trapped in sealed containers to ensure that no recombinant microorganisms are freely discharged to the environment. Gu et al. (1996) originally designed a single miniature bioreactor with a 58 mL working volume to house *luxCDABE*-expressing recombinant bioluminescent *E. coli* bioreporter cells for online toxicity monitoring, which was later upgraded to a two-stage bioreactor system in which the first mini-bioreactor functioned to provide a stable and continuous supply of the bioreporter cells for the second mini-bioreactor where exposure to test sample and collection of signal occurred (Gu et al., 1999). Bioluminescent signals emitted from the bioreporter cells were transmitted via a fiber optic probe to a luminometer interface for data acquisition and analysis. Gu and Gil (2001) further exploited the modular feature of this design and developed a multichannel monitoring system for continuous multiplexed toxicant profiling to satisfy the demand for real-world water quality monitoring since environmental water samples are rarely contaminated by a single (type of) pollutant. The parallel setup of four two-stage mini-bioreactor channels with each channel employing *E. coli* bioreporter cells whose bioluminescent emission was linked to a target-specific promoter allowed for simultaneous detection of toxic contaminants causing DNA (strain DPD2794 with the *recA* promoter), membrane (DPD2540 with the *fabA* promoter), and protein (TV1061 with the *grpE* promoter) damage, as well as general cellular toxicity (GC2 with a constitutive *lac* promoter), in a water sample artificially contaminated with 50 ppb mitomycin C, 5 ppm cerulenin, and 100 ppm phenol. A perhaps more interesting aspect of the multichannel system for field applications is that it provides a means of reporting unexpected additive, antagonistic, or synergistic effects of mixture samples to produce a unique toxicity signature of the test sample (Gu and Gil, 2001). In addition, since the test sample can be pumped or injected into

Table 5.1 Examples of *In Situ* Field Applications of Biosensor Devices

Biosensor Design		Sample Matrix	Bioreporter Strain	Target Analyte
Bioreporter Phase	Signal Detection			
Continuous culture in bioreactor (Gu and Gil, 2001; Gu et al., 2001; Kim and Gu, 2005)	Fiber optic cable and luminometer	Surface water and wastewater	*E. coli recA-luxCDABE*	DNA damage
			E. coli fabA-luxCDABE	Membrane damage
			E. coli grpE-luxCDABE	Protein damage
			E. coli katG-luxCDABE	Oxidative stress
			E. coli lac-luxCDABE	General toxicity
Immobilized on a transducer (Ivask et al., 2007)	Fiber optic cable and luminometer	Soil and sediment suspension	*E. coli merR-luxCDABE*	Mercury
			E. coli ars-luxCDABE	Arsenic
Immobilized and freely released *in situ* (Ripp et al., 2000)	Fiber optic cable and photomultiplier	Soil	*P. fluorescens HK44 nahG-luxCDABE*	PAH
Immobilized on a transducer cable (Eltzov and Marks, 2010)	Fiber optic cable and photomultiplier tube	Tap water and wastewater	*E. coli recA-luxCDABE*	DNA damage
			E. coli grpE-luxCDABE	Protein damage
Immobilized on a multiwell cartridge (Roda et al., 2011)	Fiber optic taper and CCD camera	Chemical mixture	*S. cerevisiae* human estrogen receptor regulated wild-type firefly luciferase	Estrogenic compounds
			S. cerevisiae human androgen receptor regulated red-shifted firefly luciferase	Androgenic compounds

Immobilized on a PDMS chip (Elad et al., 2011)	Single-photon avalanche diode	Wastewater	*E. coli recA-luxCDABE* *E. coli micF-luxCDABE* *E. coli arsR-luxCDABE* *E. coli CP38-luxCDABE*	DNA damage Oxidative stress Arsenic General toxicity
Immobilized on a multiwell card (Jouanneau et al., 2012)	CCD camera	Wastewater	*E. coli* pBMerlux	Mercury
Lyophilized in a 96-well plate (Siegfried et al., 2012)	CCD camera	Wastewater	*E. coli* pBMerlux *E. coli* pBZntlux *E. coli* pBArslux *E. coli* pBCoplux	Mercury Zinc Arsenic Copper
Lyophilized in a sealed vial (Siegfried et al., 2012)	Luminometer	Groundwater	*E. coli arsR-luxCDABE*	Arsenic
Lyophilized on a paper strip (Stocker et al., 2003)	Color change	Wastewater	*E. coli arsR-lacZ*	Arsenic
Lyophilized in a sealed vial (Shin et al., 2005)	Color change	Wastewater	*E. coli capR-lacZ*	Phenolic compounds

the bioreactor in an adjustable fashion (i.e., flow rate, step-wise, or continuous flow) and with the bioluminescent signal being continuously transmitted to and processed on the luminometer/computer interface, such a system can be potentially placed at sites near drinking water facilities or wastewater treatment plants for on-line monitoring to serve as an early warning checkpoint. The feasibility of this concept has been tested with surface water (Gu et al., 2001) and wastewater discharged from power plants (Kim and Gu, 2005). In the surface water test, samples collected at different times and locations displayed distinct toxicity signatures, highlighting the importance of integrating multiple channels into the water quality monitoring scheme. Kim and Gu (2005) also demonstrated in their test with power plant wastewater that a feedback-dependent dilution loop might be essential for samples with high toxicity to minimize false negative results.

While the use of continuous bioreporter culture in bioreactors most closely mimics the optimal laboratory bioassay conditions, it is somehow less ideally conceptualized as a field-deployable strategy due to its lack of long-term self-sustainability. The relatively large size of the system, the external input required to maintain continuous bioreactors for stable performance, as well as appropriate handling of the collected waste containing genetically engineered microorganisms prior to disposal represent some of the major hurdles for its implementation in the field. Alternatively, biosensor devices exploiting encapsulated bioreporter organisms immobilized on an easily sterilizable and disposable interface offer the advantages of being compact, low maintenance, and providing a simple postassay procedure. A common design of such a device is to utilize a fiber optic transducer cable coated with layers of encapsulated bioreporter cells on one end with the other end connected to signal detection and processing device (recently reviewed in Eltzov and Marks, 2010). As an example, Ivask et al. (2007) developed fiber optic cables layered with alginate-encapsulated *luxCDABE*-based *E. coli* bioreporter cells to detect mercury and arsenic. Although not as sensitive as nonimmobilized bioreporters, these fiber optic biosensors were shown to be able to detect as little as 0.0026 mg Hg/L, 0.012 mg As(III)/L, and 0.14 mg As(V)/L within a 3 h incubation time and successfully detect the presence of heavy metals in known contaminated soil and sediment samples. However, since multiple steps of pretreatment (drying, homogenization, resuspension, etc.) were necessary for the effective sensing-and-reporting reaction chain, such a biosensor is still restrained to on-site applications for solid matrices such as soil and sediment. On the other hand, samples already in an aqueous phase are more or less immediately ready for analysis as they can be directed to the fiber optic biosensor in a flow-through mode, as demonstrated in the flow-through on-line water toxicity biosensor developed by Eltzov et al. (2009). They immobilized the *E. coli* DPD2794 (*recA-luxCDABE*) genotoxicity bioreporter and the *E. coli* TV1061 (*grpE-luxCDABE*) protein-damaging agent bioreporter on a fiber optic transducer using alginate as an encapsulant and tested their ability to detect toxicant in flowing tap water and wastewater continuously over 24 h. Using mitomycin C (100 μg/L) and *p*-chlorophenol (50 μg/L) as respective positive controls for strains DPD2794 and TV1061, this flow-through system displayed a 1 h response time and an up to 10 h recovery time. The dose-dependent detection range

for the DPD2794 and TV1061 biosensor was determined to be 32−2000 μg mitomycin C/L and 10−5000 μg *p*-chlorophenol/L, respectively. Also addressed in this study was the importance of nutrient supplementation in the maintenance and viability of the immobilized bioreporter cells. It was found that a minimum Luria-Bertani media concentration of 7.5% was necessary to maintain the system while still minimizing the potential of biofouling. Although the system maintained functionality during the 24 h tap water test, its long-term use for more polluted wastewater remained challenging due to the loss of cell viability and sensitivity after 20 h.

Instead of directly immobilizing the bioreporter organisms on the surface of signal transducers, they can be encapsulated and incorporated into disposable chips or cartridges that are not in direct physical contact with the optical transducer or other signal capturing device, thereby improving field deployability. For example, Elad et al. (2011) applied a panel of recombinant *luxCDABE*-expressing *E. coli* bioreporters on a multiwell PDMS biochip coupled with SPAD detectors for simultaneous detection of arsenic and toxicants causing genotoxicity and oxidative stress. The PDMS chip featured multiple wells in which the bioreporter culture was immobilized using agar as an encapsulant and prefabricated channels to facilitate on-chip water sample movement. The multiwell platform not only allowed concurrent analysis of various contaminants but also enabled on-chip replications for robust statistical evaluation. In this study, the multichannel biochip was challenged with a 10-day continuous flow of tap water with five simulations of distinct contamination events which consisted of a 2 h pulse spiking of nalidixic acid (genotoxicity), paraquat (oxidative stress), arsenic, or a mixture of the three model compounds. With a surprisingly small volume of bioreporter cells (60 μL/well), this system remained functional during the 10-day exposure period and was able to detect each simulated contamination within 0.5−2.5 h. Similarly, Charrier et al. (2011a,b) developed the Lumisens 3 biosensor system, which consisted of a removable multichannel card housing the immobilized bioluminescent bioreporter cells, fluidics for sample transportation, and a CCD camera placed directly above the card for photon collection. Unlike the SPAD detectors that collect signal on a well-by-well basis (Elad et al., 2011), the CCD camera used in Lumisens 3 and other similar devices collects optical signal on a per-card (or cartridge) basis, thus allowing high-throughput analysis of all channels on the entire card surface. Jouanneau et al. (2012) recently tested the feasibility of the Lumisens 3 system housing a mercury bioreporter for environmental applications. It was demonstrated that the system was capable of responding to daily 100 min spikes of 500 nM mercury with a 6 h response cycle over a 10-day continuous flow setting. Unfortunately, contradictory to the Elad study (Elad et al., 2011), the Lumisens 3 produced unstable bioluminescence levels with an overall variation of approximately 40% during the 10-day period and also suffered from biofouling in long-term analysis.

An interesting conclusion from the Jouanneau et al. (2012) study was that the freeze-drying preservation approach was shown to improve the stability and reproducibility of the system. A Lumisens 4 biosensor device was subsequently developed, similar to that of Lumisens 3, with the exception of the use of lyophilized bioreporter cells entrapped in an easily exchangeable 96-well plate. Even with an

initial 30 min reconstitution step for the freeze-dried bioreporters, Lumisens 4 was able to respond to the target toxicant within 1 h as compared to the 6 h for Lumisens 3. In the same 10-day simulated mercury exposure experiment described above, Lumisens 4 exhibited consistent responses to each spike, displaying only a 3% variation in bioluminescence. To further evaluate its potential for field applications, Lumisens 4 with a panel of heavy metal bioreporters was applied to monitor a wastewater effluent spiked with several heavy metal contaminants over a 1-week period. With assistance from the data analysis software Metalsoft (Jouanneau et al., 2011), Lumisens 4 successfully detected each pollution event at the level of specific heavy metals and their concentrations, showing potential for future field applications. Another successful example of field application of lyophilized bioreporters is the portable ARSOlux biosensor arsenic test kit developed by Siegfried et al. (2012). Instead of using a flow-through system, this group packaged freeze-dried bioluminescent arsenic-sensitive bioreporters into single-use vials, which were subsequently transported to Bangladesh for a test campaign of local tube well water arsenic contamination. The full test kit consisted of 160 sealed vials of lyophilized *E. coli arsR-luxCDABE* bioreporter cells (enough for a 10-point calibration curve and 150 tests), a miniature battery-powered luminometer, and other supplies such as syringes and racks. Field testing was performed by injecting water samples into the bioreporter vials, followed by 2 h incubation before reading of the bioluminescent signal using the luminometer. Bioluminescent data were immediately analyzed using a prepared datasheet on a laptop computer and the results were communicated to local residents within the same day. Used vials were then heat inactivated in boiling water to ensure no genetically engineered microorganisms were released into the environment. It was shown that the bioreporter kit produced results comparable to other analytical chemical analyses, resulting in only two false positives and no false negatives with respect to the 10 μg/L total arsenic World Health Organization standard. This campaign highlighted some practical advantages of the bioreporter kit: compact and portable, fast and high-throughput (estimated ~150 tests including sample collection, incubation, and data analysis during an 8 h work day), minimal training for operators, easy waste handling, and low costs for manufacturing, storage, and transportation.

In addition to the use of bioluminescent signal for detection, colorimetric-based biosensors are particularly attractive for deployable field applications since no specialized instrument is required to detect the color change in response to target contaminant exposure. However, because color comparison with the naked eye is somewhat arbitrary, such biosensors better serve as qualitative rather than quantitative sensing tools. Stocker et al. (2003) developed an arsenic test paper strip coated with freeze-dried *E. coli arsR-lacZ* bioreporters which were able to rapidly detect arsenic present at >10 μg/L following a 30-min incubation with the test water sample. Although not capable of distinguishing arsenite concentrations above 0.3 or 0.4 μM, the test trip correctly identified arsenic contamination at >10 μg/L when tested with known contaminated Bangladesh water samples. Similarly, Shin et al. (2005) packaged lyophilized *lacZ*-based phenolic compound-sensitive bioreporters in sealed vials which were directly tested with untreated wastewater samples. Color

development was triggered by incubation with 3 mM β-D-galactopyranoside for 5−7 h. To improve measurement precision, a regression curve using absorbance readings previously obtained in the laboratory was also introduced into the on-site testing scheme to enable semiquantitative determination of the measured concentration. Although not capable of producing the same concentration measurements compared to analytical methods, the biosensors successfully identified the presence of phenolic compounds in the wastewater samples, presenting a viable preliminary detection method.

5.5 Field Release of *Pseudomonas fluorescens* HK44 for Monitoring PAH Bioremediation in Subsurface Soils

Pseudomonas fluorescens strain HK44 is a genetically engineered whole cell bioreporter that emits visible light upon exposure to naphthalene, salicylate, or 4-methyl salicylate when physiologically active (King et al., 1990). This property is attributed to the presence of an engineered plasmid, pUTK21, which harbors a transposon-based bioluminescent *luxCDABE* gene cassette derived from *A. fischeri* inserted within its naphthalene degradation pathway (Figure 5.1). Successful applications of strain HK44 as a real-time bioreporter have been clearly established and documented in both on-line and *in situ* studies (King et al., 1990; Burlage et al., 1994; Heitzer et al., 1994; Trogl et al., 2007). To test its biosensing capabilities under actual field conditions, strain HK44 was released in 1996 into semi-contained lysimeter structures (4 m deep × 2.5 m diameter) containing PAH-contaminated soil to serve as a bioremediation process monitoring and control tool

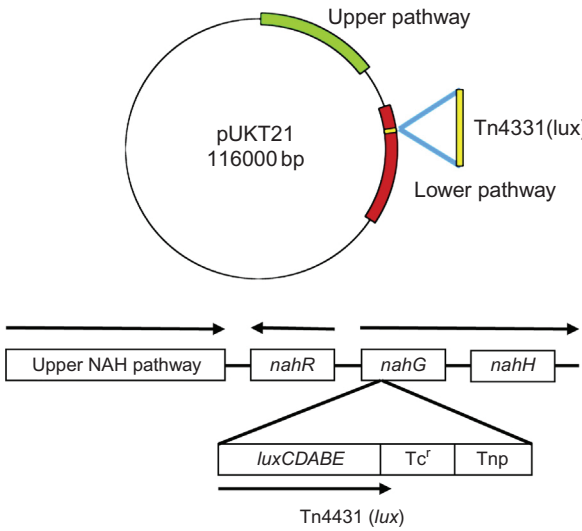

Figure 5.1 The microbial bioreporter *P. fluorescens* HK44 carries plasmid pUTK21 which contains a transposon-based *luxCDABE* gene cassette inserted into the degradation pathway for naphthalene. *Source*: Adapted with permission from Trogl et al., 2012.

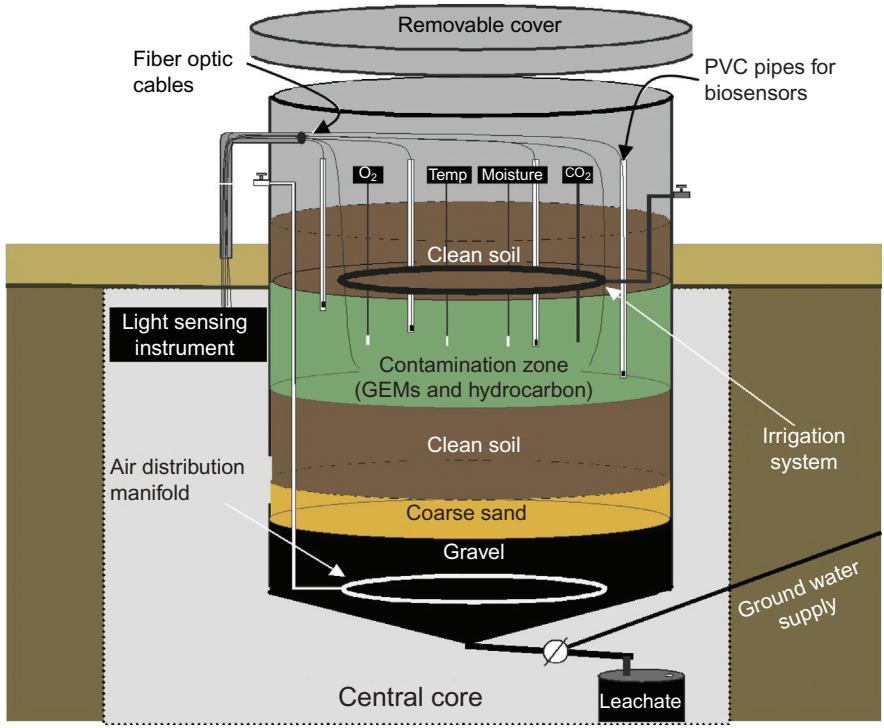

Figure 5.2 Schematic of the lysimeter structure used to contain the *P. fluorescens* HK44 microorganisms during their *in situ* testing. Fiber optic cables terminating at a photomultiplier tube penetrated the soil matrix to record HK44-derived bioluminescence in real time directly from the PAH-contaminated treatment zone of the soil.

(Ripp et al., 2000) (Figure 5.2). A secondary objective was to test the hypothesis that a genetically engineered microorganism could be successfully introduced and maintained for a significant time span under field release exposures. Thus, both the dynamics of bioluminescent light emission and the population status of the HK44 microorganisms were monitored over the initial 2 years of the field release. Bioluminescence from active HK44 cells at different depths of soil lysimeters was measured using a portable multiplex light detection system involving fiber optics and photomultiplier tubes, whereas biosensors, utilizing alginate-encapsulated HK44 cells, monitored for the presence of naphthalene in the vapor phase. Results showed that the field study successfully provided real-time data that reflected naphthalene bioavailability, degradative activity, and optimal degradation conditions for *P. fluorescens* HK44 (Ripp et al., 2000). Thus, the bioremediation field practitioner could potentially use the bioluminescent or other preferred signaling response from an appropriately engineered bioreporter to progressively track the effectiveness of a bioremediation event *in situ*.

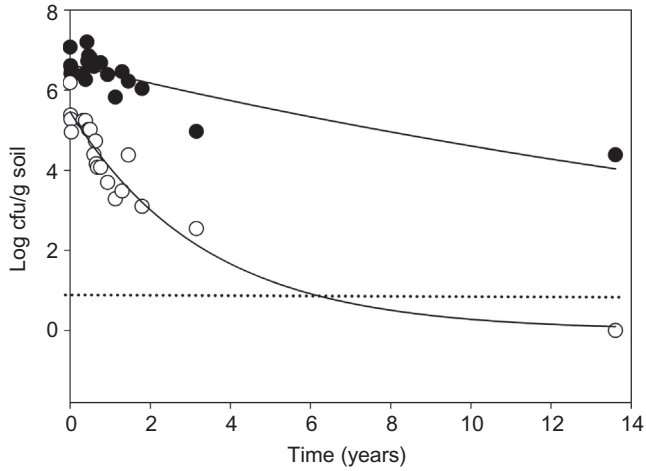

Figure 5.3 Population decay rates of tetracycline resistant microorganisms (○), inclusive of *P. fluorescens* HK44, and total heterotrophic microbial populations (●) over a 14-year duration of sampling of the lysimeter soils. The dotted line indicates the approximate culturable detection limit of 10 cfu/g soil.
Source: Adapted with permission from Layton et al., 2012.

A perhaps more important aspect of this field release study was gaining an enhanced understanding of the long-term survivability of the engineered HK44 microorganisms. It is often assumed that an organism created in a laboratory environment and designed to carry, express, and maintain nonnative genes has reduced fitness, and will therefore quickly perish when released into a highly competitive microbial ecosystem. Indeed, over the first 2 years of this study, HK44 populations were reduced from an initial inoculum of $\sim 1 \times 10^6$ colony-forming units (cfu)/g soil to a final concentration of $\sim 1 \times 10^3$ cfu/g soil. The lysimeter soils were again extensively sampled in 2000, approximately 4 years postrelease and, surprisingly, HK44 cells were successfully revived by selective plate counting even though the selective pressure of the hydrocarbon contaminants that it degraded were depleted at least 2 years prior. Bioluminescence was also detected, indicating that HK44 and the recombinant *luxCDABE* genes were still functionally active. Recovery of *lux* mRNA from the soil complemented these findings. In 2010, approximately 14 years postrelease, the lysimeter soils were again sampled (Figure 5.3). Interestingly, HK44 microorganisms could not be revived using traditional plate counting methods but *luxA*, HK44-specific tetracycline resistant (*tetA*), and naphthalene dioxygenase (*nahA*) genes were detected at low concentrations using more sensitive quantitative polymerase chain reaction (qPCR) methods ($\sim 100-2000$ copies/g soil). Thus, the inability to culture viable HK44 cells did not imply their absence from the microbial community, but rather pointed towards the limitation of culture-based techniques to capture less abundant microbial

populations. Therefore, more sensitive and robust culture-independent approaches, such as metagenomics, need to be considered and applied to better delineate microbial community structures. Metagenomics can additionally provide information on how the native microbial community changes over time after introduction of non-native engineered microbial populations as well as on the risks associated with horizontal gene transfer of the introduced recombinant DNA. In this work, 16S rDNA and shotgun libraries were made from the pooled DNA of one of the lysimeters and sequenced using a next-generation Roche454 platform (Layton et al., 2012). Although the results obtained from the metagenomic analysis did not confirm the presence of HK44-derived genes after >14 years residency in the lysimeter soils, genetic markers related to HK44 such as the *nah* and *tet* (tetracycline) genes were identified but were more closely related to other strains because of their widespread distribution across different genera. These findings clearly point towards the scientific need for the development of more robust strain-specific molecular markers and/or techniques and development of new methods for confident analysis of data generated from the community analysis. However, accumulation of such information from field studies is necessary and provides a better understanding of the impact of recombinant gene introduction into environmental ecosystems.

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